Université de Liège
Faculté de Médecine Vétérinaire
Département des Maladies Infectieuses et Parasitaires
Epidémiologie et Analyse de risques appliquées aux sciences vétérinaires

Lessons from experimental infections with Bluetongue and Schmallenberg viruses in ruminants

Enseignements tirés d'infections expérimentales avec les virus de la Fièvre Catarrhale Ovine et Schmallenberg chez les ruminants

Ludovic MARTINELLE

Thèse présentée en vue de l'obtention du grade de Docteur en Sciences Vétérinaires

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THESE PRESENTEE EN VUE DE L'OBTENTION DU GRADE DE
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“We are like dwarfs sitting on the shoulders of giants. We see more, and things that are more distant, than they did, not because our sight is superior or because we are taller than they, but because they raise us up, and by their great stature add to ours.”

— John of Salisbury, Metalogicon

« *Revenons à nos moutons* »

— Anon., *La Farce de Maître Pathelin*

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Liège, le 20 novembre 2018

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« Il faut savoir
que rien n'est sûr,
que rien n'est facile,
que rien n'est donné,
que rien n'est gratuit.
Tout se conquiert, tout se mérite.
Si rien n'est sacrifié, rien n'est obtenu. »

Hélie de Saint Marc

Abbreviations

ADNS	Animal Disease Notification System
AHSV	*African horse sickness virus*
ANOVA	Analysis of variance
ARSIA	*Association Régionale de Santé et d'Identification Animales*
BFAE	Bovine fetal arterial endothelial
BHK-21	Baby hamster kidney (cells)
BSA	Bovine serum albumin
BSL3	Biosafety level 3 facility
BSR	BHK-21 subclone
BT	Bluetongue
BTV	Bluetongue virus
c/rDNA	Complementary/Ribosomal deoxyribonucleic acid
CCID50	50% cell culture infectious dose
CI	Confidence interval
CID	Collision-induced dissociation
CLPs	Core-like particles
CODA-CERVA	Veterinary and Agrochemical Research Centre (currently Sciensano)
CPE	Cytopathic effects
Cq	Quantification cycle
CNS	Central nervous system
CPT-Tert	Choroid plexus cells from sheep, immortalized with SV40 T antigen and hTERT
Ct	Threshold cycle
CTL	Cytotoxic T-lymphocyte/ -cell
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin
ddH2O	Distilled deionized water
DEFRA	Department for Environment, Food and Rural Affairs
DGZ	*Dierengezondheidszorg Vlaanderen*
DIC	Disseminated intravascular coagulation
DISC	Disabled infectious single cycle
DIVA	Differentiation of infected from vaccinated animals
DNA	Deoxyribonucleic acid
dpc	Days post-coitus

dpi	Days post-infection
dpp	Days post-partum
ds	Double-stranded
EC	European Commission
ECE	Embryonated chicken eggs
ECs	Endothelial cells
EDTA	Ethylene diamintetraacetic acid
EFSA	European Food Safety Authority
EHDV	*Epizootic hemorrhagic disease virus*
EID	Emerging infectious disease
EIP	Extrinsic incubation period
ELISAs	Enzyme-linked immunosorbent assays
cELISA	Competitive enzyme-linked immunosorbent assay
iELISA	Indirect enzyme-linked immunosorbent assay
sELISA	Sandwich enzyme-linked immunosorbent assay
EMA	European Medicines Agency
EU	European Union
FAO	Food and Agricultural Organization
FARAH	Fundamental and Applied Research for Animals and Health
FASFC	Belgian Federal Agency for the Safety of the Food Chain
FLI	Friedrich-Loeffler-Institut
GST	Glutathione S-transferase
HCAb	Heavy-chain antibodies
His	Histidine
hTERT	Human telomerase reverse transcriptase
IAH	Institute for Animal Health
ID	Intradermic
IFN	Interferon
IFNAR(-/-)	Interferon alpha/beta receptor deficient
IFN-γ	Interferon gamma
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHC	Immunohistochemistry
IM	Intramuscular
IN	Intranasal
INRA	French National Institute of Agricultural Research

IRF-3	Interferon Regulatory Factor 3
ITM	Institute of Tropical Medicine
IV	Intravenous
KC	Cultured cells derived from *Culicoides sonorensis* larvae
kDa	Kilodalton
LPS	Lipopolysaccharide
MEM	Minimal essential medium
MHC	Major histocompatibility complex
MIR	Minimum infection rate
MLVs	Modified live virus
mRNA	Messenger RNA
MS	Member State
MW	Molecular weight
NRL	National Reference Laboratory
NS	Non-structural
OD	Optical density
OIE	World Organisation for Animal Health (*Office International des Epizooties*)
OROV	Oropouche virus
Pan-BTV/S5 RT-qPCR	Non-serotype specific RT-qPCR targeting segment 5
PBMC	Peripheral blood mononuclear cells
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PN	Percentage negativity
RBC	Red blood cells
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
ROC	Receiver Operating Characteristic
RPB1	Mammalian RNA polymerase II subunit RPB1
RT	Room temperature
RT-PCR	Reverse transcription-polymerase chain reaction
RTqPCR	Real time quantitative RT-PCR
SANITEL	Belgian animal identification and registration system
SBV	Schmallenberg virus
SC	Subcutaneous
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SFTSV	Severe fever with thrombocytopenia syndrome virus
SK-6	Swine kidney
SNT	Seroneutralisation test
SPF	Specific pathogen free
spp.	*Species pluralis*, several species
ss	Single-stranded
SV40	Simian virus 40
TCID50	50% tissue culture infective dose
TE	Tris-EDTA (buffer)
three Rs	Replacement, refinement, reduction
TLR	Toll-like receptor
TMB	3,3',5,5-tetramethylbenxidine
TOV	Toggenburg orbivirus
UV	Ultraviolet radiation
VIBs	Viral inclusion bodies
ViP	Viral inclusion body matrix protein
VLPs	Virus-like particle vaccines
VP	Viral (structural) protein

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Résumé - Summary

Résumé

En août 2006, le sérotype 8 (BTV8) du virus de la fièvre catarrhale ovine (FCO) ou maladie de la langue bleue a été confirmé pour la première fois aux Pays-Bas. Celle-ci était caractérisée par une sévérité inattendue chez les bovins, une expansion géographique sans précédent et une propagation épizootique efficace malgré l'absence du vecteur classique *Culicoides imicola* dans les zones touchées. En outre, le BTV8 européen a été le premier BTV de type sauvage dont la capacité à traverser la barrière placentaire et le potentiel tératogène ont pu être prouvés, ces caractéristiques étant jusque-là l'apanage des souches adaptées au laboratoire, comme les virus vaccinaux vivants modifiés.

En novembre 2011, c'est un nouvel *Orthobunyavirus* (famille *Peribunyaviridae*) qui a été identifié. Le nouveau virus a été appelé virus de Schmallenberg (SBV), du nom de la ville allemande où il a été isolé la première fois. Outre des manifestations cliniques – très rares et modérées - observées occasionnellement chez les bovins adultes, le SBV a été surtout associé à des avortements, des mortinatalités et des malformations congénitales chez les agneaux, les veaux et les chevreaux. Le virus de la FCO et le SBV ont un pouvoir tératogène, partagent une zone d'émergence commune ainsi qu'un spectre d'hôtes et des espèces communes de vecteurs. Ils sont tous deux des arbovirus à ARN segmenté à l'origine non identifiée et ayant un impact sérieux sur le bétail. Ces aspects convergents justifient les travaux actuels visant à clarifier et à discuter plus spécifiquement la pathogenèse de ces deux maladies.

Pour ce faire, six infections expérimentales de bovins ou de moutons ont été mises en place. La thèse présente est ainsi séparée en trois parties :

1) Contribution à la mise au point d'un modèle infectieux animal fiable et normalisé pour étudier la pathogenèse du virus de la FCO et du SBV chez les espèces cibles de ruminants (études 1 et 2)
2) Etudes de la pathogenèse et du potentiel tératogène du virus de FCO et du SBV chez des animaux gestants (études 3 et 4);
3) Évaluation de la réactivité croisée, de la super-infection et comparaison des sérotypes de BTV, avec un accent particulier sur les sérotypes du virus de la FCO historiquement présents en Europe continentale (études 5 et 6).

L'étude 1 nous a permis de mettre en évidence qu'un virus de la FCO issu d'une culture cellulaire passée un nombre limité de fois constitue un inoculum tout à fait valable pour une reproduction adéquate de la maladie en conditions expérimentales. Dans l'étude 2, nous avons conclu que les

voies d'inoculation sous-cutanée et intradermique étaient adaptées pour reproduire correctement une infection par le SBV similaire à une infection naturelle.

Nous avons rapporté dans l'étude 3 des hémorragies de l'artère pulmonaire - considérée comme presque pathognomonique de l'infection par BTV - chez des veaux nés de mères infectées avant la mi-gestation, malgré l'absence d'ARNémie à la naissance. De même, dans l'étude 4, après l'infection de brebis à 45 ou 60 jours de gestation, aucun agneau n'a présenté de malformation évoquant une infection à SBV et aucun d'entre eux n'avait d'anticorps anti-SBV avant la prise de colostrum. Cependant, la plupart d'entre eux avaient au moins une structure placentaire ou extra-embryonnaire avec une détection positive de l'ARN du SBV.

L'objectif principal de l'étude 5 était d'évaluer l'effet d'une surinfection par BTV1 chez des veaux naïfs ou vaccinés préalablement contre BTV8. L'inoculum s'est révélé être contaminé par BTV15. Nous avons ensuite caractérisé la co-infection BTV1-BTV15 en plus d'une comparaison BTV1 et BTV15 au cours de challenges infectieux à sérotype unique. Cette étude a fourni de nouvelles informations sur la protection hétérologue entre BTV1, BTV8 et BTV15 et sur leur adaptation au bétail. L'étude 6 décrit les paramètres cliniques, sérologiques et virologiques après les infections expérimentales de veaux avec 5 sérotypes européens de BTV (1, 2, 4, 9 et 16). Les expériences ont montré (i) la virulence limitée des souches virales sélectionnées pour les bovins, (ii) une réactivité croisée partielle, (iii) une faible protection croisée induite par la vaccination et/ou l'infection préalable contre BTV8. De plus, nous avons pu confirmer que les sérotypes de BTV sont adaptés différemment aux jeunes bovins (BTV1, 16 et 9 plus adaptés que 2 et 4).

Les travaux présentés ici ont apporté de nouvelles données utiles à la conception des infections expérimentales avec BTV et SBV, à la pathogenèse des anomalies reproductives et aux relations sérologiques entre les sérotypes européens de BTV. Ces aspects sont finalement discutés à la lumière des données expérimentales les plus récentes. En dépit des contraintes de biosécurité, d'éthique animale et de cout, la valeur des données fournies et l'absence de modèles alternatifs reproduisant de manière adéquate la pathogenèse de BTV et de SBV prouvent que les infections expérimentales de ruminants restent incontournables dans ce contexte.

Summary

In august 2006, bluetongue virus serotype 8 (BTV8) was for the first time confirmed in Netherlands, in the core of Western Europe. The bluetongue disease caused by BTV8 in Europe was characterized by its unexpected severity in cattle, an unprecedented geographic expansion, and an effective epizootic propagation despite the absence of *Culicoides imicola* in the areas of interest. In addition, European BTV8 was the first wild-type BTV whose ability to cross the placental barrier and its related teratogenic potential has been proven, as these features were previously only described in laboratory adapted strains, like modified live vaccine viruses.

In November 2011, a novel *Orthobunyavirus* (family *Peribunyaviridae*) was identified. The new virus was named Schmallenberg virus (SBV) out of the name of the German city of its first isolation. In addition to the clinical manifestations – very rare and mild - seen in adult cattle, SBV has been mostly associated with abortions, stillbirths and congenital affection in lambs, calves and kids, characterized by arthrogryposis/hydranencephaly syndrom.

BTV and SBV share a teratogenic potential, with a common emerging area, host range and vector species, are both segmented RNA arboviruses of inconclusive origin and serious impact on livestock. These converging aspects warrant the present work clarifying and discussing more specifically the pathogenesis of both diseases.

To do so, six experimental infections in cattle or sheep were implemented. The current thesis is therefore separated in three parts:

1) Contribution to the development of a reliable and standardized animal infectious model to study the pathogenesis of BTV and SBV in ruminant target species (studies 1 and 2);
2) Studies of the pathogenesis and teratogenic potential of BTV and SBV in pregnant animals (studies 3 and 4);
3) Cross-reactivity assessment, super-infection and serotype comparison with a particular emphasis on BTV serotypes historically present in Europe mainland (studies 5 and 6).

The study 1 highlighted the suitability of culture grown virus that was passaged a limited number of times for a standardized BTV infection model. In study 2 we concluded that both subcutaneous and intradermal inoculation routes were suitable to properly reproduce a SBV infection similar to field infection.

We reported in study 3 haemorrhages of the pulmonary artery – considered as almost pathognomonic of BTV infection - in calves born from infected dams prior to mid-gestation, despite the lack of RNAemia at birth. These results were consistent with the actual low frequency of BTV induced malformation in natural infections. Likewise in study 4, following the infection of ewes at 45 or 60

days of pregnancy no lambs showed any malformation suggestive of SBV infection and none of them had RNAemia or anti-SBV antibodies prior to colostrum uptake. However most of them had at least one extraembryonic or placental structure with positive SBV RNA detection.

The main objective of study 5 was to evaluate the effect of a BTV1 superinfection in naïve and BTV8-vaccinated calves. The inoculum appeared to be unexpectedly contaminated with BTV15. Then we further characterized the BTV1-BTV15 co-infection in addition to a BTV1 and BTV15 comparison in single serotype challenges. This study provided new insights in heterologous protection between BTV1, BTV8 and BTV15 and on their adaptation to cattle. The study 6 describes the clinical, serological and virological parameters after calf experimental infections with 5 European BTV serotypes (1, 2, 4, 9, and 16). Experiments showed (i) the limited virulence of the selected viral strains for bovines, (ii) a partial cross-reactivity, (iii) a potential weak cross-protection induced by BTV8 vaccination and/or infection. In addition, we could confirm that BTV serotypes are differently adapted to young bovines (BTV1, 16 and 9 more adapted than 2 and 4).

The current work provided renewed contributions to BTV and SBV experimental infections design, reproductive defects pathogenesis and updated serological cross-relationships between European BTV serotypes. These aspects are finally discussed in the light of the most recent experimental data and litterature. Despite the harsh biosafety constrains, the ethical concerns and the cost of such experiments, the value of the data provided and the lack of alternative models adequately reproducing BTV and SBV pathogenesis prove ruminant host-species to remain necessary.

General preamble

As human, animal, or zoonotic pathogens, RNA viruses represented a major source of emerging diseases during the last 30 years. The genetic adaptability and variability of these viruses is mostly driven by a high mutation rate and, in case of segmented genome, reassortment.

Mainland Europe underwent in the past 15 years the outbreak of two major pathogens affecting cattle and sheep: Bluetongue virus (BTV) and Schmallenberg virus (SBV). These outbreaks were singular in different ways: the diseases were either never reported in such Northern locations before (bluetongue virus) or just newly discovered (Schmallenberg virus); their emergence still has unexplained features; both viruses displayed the ability to cross the placental barrier and they confirmed that palearctic endemic *Culicoides* species could support and spread BTV and SBV epizootic.

Experimental infections of mammalian hosts proved to be a highly valuable tool to study the pathogenicity, the virulence, the pathogeny and transplacental infections since the dawn of the study of infectious diseases. The design of the *in vivo* models evolved and were usefully complemented with *in vitro* and *in silico* approaches to better comprehend the host-pathogen interactions.

Prior to study the pathogenesis of BTV and SBV in ruminants, including their teratogenic potential, an experimental model reproducing the disease had to be found. Palearctic *Culicoides* spp. are the vectors of both BTV and SBV in Northern and Western Europe. These midges are telmophagous insects: they lacerate the skin to feed on the effusion into this injury (hence considered as “pool” feeders), which includes blood, skin cells and lymph. To date there are no lab-adapted colonies of Palearctic *Culicoides*. Therefore, given the feeding behavior of *Culicoides* in natural infection and the lack of lab-adapted colonies, investigating the most adapted route of inoculation is of prime importance to ensure standardization and repeatability of challenge experiments. Amongst the other important pathogenesis factors to consider while designing experimental infections the origin of the inoculum and its passage history has to be carefully evaluated. Indeed the number of passages, the cell culture system used to grow the inoculum or by contrast the source animals for infectious blood or serum are central to achieve an experimental infection matching the virological, clinical and serological parameter of field infection with wild-type virus.

The timeframe, pathogenesis of *in utero* infection and teratogenic potential of both viruses were still poorly documented and mostly based on closely related viruses. Although both ruminant species the placenta of cattle and sheep differs. The uterine epithelium, historically believed to be missing in the placenta of small ruminant species, actually persists albeit in syncytial form in sheep and goat. By contrast an actual cellular epithelium (in cow and deer) lines the maternal side interdigitating with the fetal chorion. Obviously the gestation duration of the two species is an important factor of variability in the critical time frame at risk for the development of congenital defects. Likewise, the maturation of the immune system is tightly connected with the organogenesis and the timing of the related *in utero*

events in cattle and sheep could be looked deeper into trying to better comprehend the outcomes of BTV and SBV infection of pregnant ruminants.

In the context of a changing BTV epidemiological system in Europe, more insight and updated data on the cross-reactivity between different serotypes and potential reassortment would be of great value. Indeed very regularly figures based on experiments carried on in the 90's are displayed to illustrate the serological relationship between BTV serotypes, whereas molecular virology and sequencing clearly entered a new era. BTV can no longer be characterized solely on their serotypes to allow an adequate evaluation of their pathogenic potential.

The last part of the manuscript presents a general discussion about BTV and SBV converging and diverging aspects in matters of evolutionary dynamics, epidemiology, clinical picture and pathogenesis. Animal welfare concerns are also put in perspective.

The central challenge of the present thesis is indeed to integrate altogether results from pathogenesis studies targeting two different pathogens. Despite obvious and significant differences between BTV and SBV, from taxonomy to clinical picture or replication mechanisms, these differences and even more importantly their similarities worth to be discussed with the benefit of the hindsight of the last 10 years.

Introduction

1 History

1.1 Bluetongue disease, from Southern Africa to BTV8 emergence in Europe

From 1781 to 1784, the young François Levaillant (sometimes written as Le Vaillant – "The Valiant") travelled extensively in South-Africa inland. As an explorer and zoologist, he reported the damages caused by some of the most common cattle pathogens on the development of human settlement. Among other cattle diseases he described the "*Tong-Sikte*" afflicting the cattle (Gutsche, 1979). About one century later Duncan Hutcheon, then Colonial Veterinary Surgeon, described in his annual report in 1880 a very accurate clinical picture of the disease, called by that time "Malarial Catarrhal Fever". Indeed Hutcheon believed that the agent was an arthropod-transmitted intracorpuscular protozoon. Later on Spreull suggested the name "Bluetongue" to characterize the disease with respect to the cyanotic aspect of the tongue affected sheep could present. "Bluetongue" being itself the translation of the Dutch "*Blaauwtong*", also often named "*Bekziekte*" (mouth-sickness) (Spreull, 1905). Theiler and Robertson demonstrated that the aetiological agent of BT was filterable, thus indicating the viral origin of the disease. Then in 1906-1907 Theiler described the production of a first vaccine against BT. The method consisted of a single virus strain (BTV4) that was attenuated by serial passage through sheep and reported the absence of death following infectious challenge after 10 generations in the host (Theiler, 1908b). This blood vaccine was used by sheep farmers in South Africa for over 40 years despite evidence that the vaccine was not safe and that the resultant immunity was not adequate (Gorman, 1990). Protection failures from the vaccine prepared from the Theiler strain drove Neitz to investigate the antigenic differences of BTV strains in the field by cross-protection tests in sheep. These studies confirmed that each strain produce solid homogenous immunity, but only partial to no protection against infection with heterologous field strains (Neitz, 1948). Bluetongue is historically an African disease, and although it has not been reported from all African countries, it has probably been endemic in wild ruminants in sub-Saharan Africa since antiquity (Vervoerd and Eramus, 2004). In South Africa highly susceptible fine-wool European sheep breeds like Merino sheep act as sentinel animals and are indicative of clinical outbreaks. Scientists working in southern Africa contributed for a huge amount to clarify BT aetiology, epidemiology and vector-borne transmission, in the early years after the discovery of the virus as well as more recently through the last key findings.

The first official outbreak out of Africa was reported in 1943 in Cyprus (Gambles, 1949). Then the disease reached the shores of Palestine, Turkey, Syria and Israel during the winter of the same year (Shimshony, 2004). The European expansion of the virus started with an extensive outbreak of BTV serotype 10 in Portugal and Spain in 1956-1957 (Manso-Ribeiro et al., 1957). This first epizootic in Europe was characterized by an unusual severity, with sheep experiencing a mortality rate

of about 75%. The disease disappeared from the Iberic peninsula within 4 years, and the following European BTV episode only occurred in 1979-1980 in Greek islands (Mastroyanni et al., 1981).

BTV underwent from 1940s to 1980s an unprecedented expansion out of Africa into Europe but also in North America, Australia and the Indian subcontinent. It has been now identified on all continents but Antarctica (Maclachlan, 2011). This global spread associated with potential dramatic damages on livestock and massive economic losses mostly due to indirect costs such as trade restriction justified to include BTV in the list of notifiable diseases (formerly List A) of the OIE (*Office International des Epizooties*; also known as World Organisation for Animal Health) (Maclachlan, 2011).

The tipping point of the BTV epidemiology in Europe is the end of the 1990s. From 1998 to 2005 at least five serotypes (BTV1, 2, 4, 9 and 16) were involved in outbreaks in at least 12 countries, including Greece, Turkey, Cyprus and the Balkans (Purse et al., 2005). During the summer of 2000 the disease (identified as BTV2; BTV serotype 2) reached Italy and caused there serious damages to the local flocks (Calistri et al., 2004). BTV circulated continuously in Italy until November 2005. The nearby island Corsica got affected in the year 2000 as well (BTV2), with continuous detection until September 2004 (Breard et al., 2004). Outbreaks of BTV2 and BTV4 were later reported in Spain and Portugal in 2000 and 2004, respectively (Gomez-Tejedor, 2004). BTV2 was later in 2005 isolated in Spain from a herd of sentinel bovines and seroconversions to BTV4 were reported in Portugal the same year (Mellor et al., 2009).

On the 14 August of 2006, a farm in Kerkrade – South of Netherlands close to the Belgian and German borders - reported clinical signs in sheep suggesting an outbreak of BTV (World Organization for Animal Health, 2006). Four days later came the laboratory confirmation along with the official international press release from the Dutch agriculture ministry. The disease then spread rapidly: the 19 August BTV hit Belgium, and on 21 August Germany also announced its first BTV outbreak, in both countries in cattle herds. France announced the first BTV confirmed case on 31 August (Mellor et al., 2009). By the end of August 2006 scientists at the Pirbright Institute showed that the BTV causing disease in the Netherlands was serotype 8. This serotype has not previously been identified in Europe (International Society for Infectious Diseases, 2006). By the end of 2006, over 2000 outbreaks had been declared in five different countries (Netherlands, Germany, Belgium, France and Luxemburg) (International Society for Infectious Diseases, 2007b).

Eventually the disease disappeared in winter. However the success of the overwintering of BTV8 was confirmed when a sentinel bovine seroconverted in April 2007 (International Society for Infectious Diseases, 2007a). BTV8 then spread further to UK, Switzerland, Denmark and the Czech Republic (Zientara and Sanchez-Vizcaino, 2013). By the spring of 2009, the disease reached Hungary,

Austria, Sweden and eventually its northernmost location in Norway. In southern Europe BTV8 was present in Spain from its incursion in 2008 to its last report in 2010 (de Diego et al., 2014). Likewise, BTV8 hit Italy during the spring of 2008.

France gained its BTV free status in 2012 two years after a last reported case in 2010 (Courtejoie et al., 2017). Quite unexpectedly BTV8 re-emerged in 2015 after 5 years of absence; BTV8 was detected in a sick ram showing clinical signs of BTV infection in central France (Sailleau et al., 2017a). Sequencing demonstrated that the BTV8 currently circulating is similar to the strain that circulated back in 2008 (Breard et al., 2016). Moreover a recent retrospective serological study clearly suggests that the virus actually kept circulating undetected at low level (Courtejoie et al., 2017). The mechanism of re-emergence in 2015 is incompletely understood. It might involve a combination between the progressive decrease of the residual protection in local ruminant population and the maintained low level BTV circulation (possibly locally increased as a consequence of a higher vector activity). Indeed in non-naïve livestock clinical signs might remain undetected as long as the herd immunity does not fall under a specific threshold (Meroc et al., 2008).

1.2 The unexpected emergence of Schmallenberg virus in Western Europe

Schmallenberg virus (SBV) was discovered in November 2011 by the Friedrich Loeffler Institute (FLI, Riems Island, Germany) following the metagenomic analysis of a pool of blood samples from a farm in the city of Schmallenberg (North Rhine-Westphalia, Germany). These analyses were carried out following an abnormally high rate of decline in milk production associated with hyperthermia, severe diarrhoea and sometimes abortion in cattle from the area since August 2011. The percentages of nucleotide homology presented by the genetic sequences identified made it possible to classify this novel virus back then in the family *Bunyaviridae*, genus *Orthobunyavirus*, serogroup Simbu. Gerhauser et al. (2014) reported the lack of Schmallenberg virus in ruminant brain tissues archived from 1961 to 2010 in Germany underlining the recent introduction of the virus in Europe, most likely from tropical or subtropical regions (Gerhauser et al., 2014). However, other orthobunyaviruses were identified in Europe, either sporadically by the analysis of mosquito pools (*Batai* virus in Germany)(Jost et al., 2011), or due to an endemic presence (*Tahyna* virus) (Bennett et al., 2011).

Between November 2011 and mid-March 2012, the virus was detected in sheep, goats and cattle in Germany, the Netherlands, Belgium, United Kingdom and France, in a goat in Italy, lambs and calves in the Grand- Duché of Luxembourg and in a lamb in Spain, thus constituting the first occurrence of circulation of an *Orthobunyavirus* of the *Simbu* serogroup in Western Europe. SBV almost disappeared in 2013. However, since late summer 2014, new cases have occurred in adult cattle in Germany and in France.

In Belgium, the drop in cattle herds seroprevalence in 2012 (from 86 to 65 %) (Meroc et al., 2013) was however followed by the detection of SBV RNA in three aborted calves in 2016 leading to the assumption of a continued low-level circulation or to a re-introduction in late autumn of 2015 (Delooz et al., 2017; Sohier et al., 2017b). Moreover, the increase in ruminant seroprevalence against Schmallenberg virus during the summer of 2016 indicated the most extensive recirculation of SBV since its original emergence in 2011 (Sohier et al., 2017a).

2 Taxonomy, morphology and viral replication of Bluetongue virus

2.1 Taxonomy and structure of BTV

Bluetongue virus (BTV) causes the eponymous bluetongue disease (BT). BTV belongs to the family *Reoviridae*, subfamily *Sedoreovirinae* and represents the type specie of the *Orbivirus* genus (Mertens et al., 2005). The family *Reoviridae* currently contains fifteen genera of multi-segmented dsRNA viruses, including pathogens of a wide range of vertebrates (including humans), arthropods, plants and fungi (Mertens et al., 2004).

Unlike the other reoviruses, all orbiviruses are arthropod-borne viruses (arboviruses). This genus currently contains 22 species as well as 10 unclassified “orbiviruses” (Attoui et al., 2009). The three economically most important orbiviruses are bluetongue virus, African horse sickness virus and epizootic haemorrhagic disease virus all of which are transmitted by *Culicoides* species. The icosahedral virus particle, ≈ 80–90 nm in diameter, contains seven distinct proteins, from Viral Protein (VP)1 to VP7.

BTV is a non-enveloped virus with a genome of approximately 19 200 base pairs composed of ten linear segments of double-stranded RNA (dsRNA) (Schwartz-Cornil et al., 2008). The virus particle has been extensively studied at high resolution by cryo-electro-microscopy and X-ray crystallography techniques (Grimes et al., 1998; Nason et al., 2004). The ten linear segments of the dsRNA genome are packaged as exactly one copy of each segment (identified as Seg-1 to Seg-10 in order of decreasing molecular weight) within a three layered protein capsid (Sung and Roy, 2014). Characteristics of various proteins encoded by the different genome segments of BTV are shown in Table 1.

Table 1. Genome segments, proteins encoded by BTV segments and their functions (sizes for BTV serotype 10, modified from (Roy, 1992).

Segment (size in base pairs)	**Protein/s encoded (size in kilo Daltons)**	**Protein function**
S1 (3954)	VP1 (150 kDa)	RNA dependent RNA polymerase
S2 (2926)	VP2 (111 kDa)	Receptor binding and cell entry
S3 (2772)	VP3 (103 kDa)	Sub-core structural protein, localises viral polymerase complex
S4 (2011)	VP4 (76 kDa)	Capping enzyme
S5 (1769)	NS1 (64 kDa)	Viral protein translation enhancer
S6 (1638)	VP5 (59 kDa)	Membrane permeabilization protein
S7 (1156)	VP7 (38 kDa)	Core structural protein and receptor binding protein for *Culicoides* cells
S8 (1124)	NS2 (42 kDa)	Concentrator of core components, viral inclusion body formation
S9 (1046)	VP6 (36 kDa) NS4 (17 kDa)	Helicase Viral fitness to interferon response
S10 (822)	NS3 (26 kDa) NS3A (25 kDa)	Adaptor protein facilitating egress

The innermost "sub-core" layer is composed of 12 copies of triangular decamers of VP3(Grimes et al., 1998). VP3 can be considered as a "pseudo T = 2" icosaedral lattice, which encloses the ribonucleoprotein "transcriptase complexes", comprising an individual genome segment associated with three minor proteins involved in transcription and replication, namely the RNA-dependent RNA polymerase (VP1, Pol, 149 kDa), the RNA capping enzyme and transmethylase (VP4, CaP, 76 kDa) and the dsRNA viral helicase (VP6, Hel, 36 kDa) (Rao et al., 2017). The BTV sub-core layer can self-assemble when VP3 is synthesized separately from the other viral proteins (e.g. as expressed by a recombinant vector system) (Mertens et al., 2009).

The intermediate layer consists of the major immunodominant VP7 structural protein (38 kDa), organized in 260 trimers forming a T = 13 icosahedral lattice covering the sub-core (Roy, 1992). The VP3-VP7 interaction provides additional rigidity and strength to the sub-core layer. The core (700 Å in diameter), composed of VP7 and VP3 and the three minor proteins VP1, VP4 and VP6 is transcriptionally active and produces capped mRNA from which all BTV proteins are translated (Ramadevi et al., 1998).

The core is surrounded by an "outer capsid" composed of two structural proteins, 60 trimers of VP2 (111 kDa) and 120 trimers of VP5 (59 kDa). The trimers of the VP2 form "triskelion" motifs (three interlocked spirals) on the outer layer (Grimes et al., 1998). VP2 is responsible for receptor binding, hemagglutination and eliciting serotype-specific neutralizing antibodies (Schwartz-Cornil et al., 2008). VP2 is the major determinant of BTV serotype, with a minor role for VP5 (Mertens et al., 1989). It is also the most variable protein of BTV.

VP5 is more conserved than VP2 and also trimeric. It has a more globular structure and VP5 trimers are located above VP7 trimers on the core surface layer (Hewat et al., 1992a). VP5 has been shown to display membrane penetration and fusion activities that mediate release of viral particles from endosomal compartments into the cytoplasm (Hassan et al., 2001). The structure of the BTV particle is shown in Figure 1.

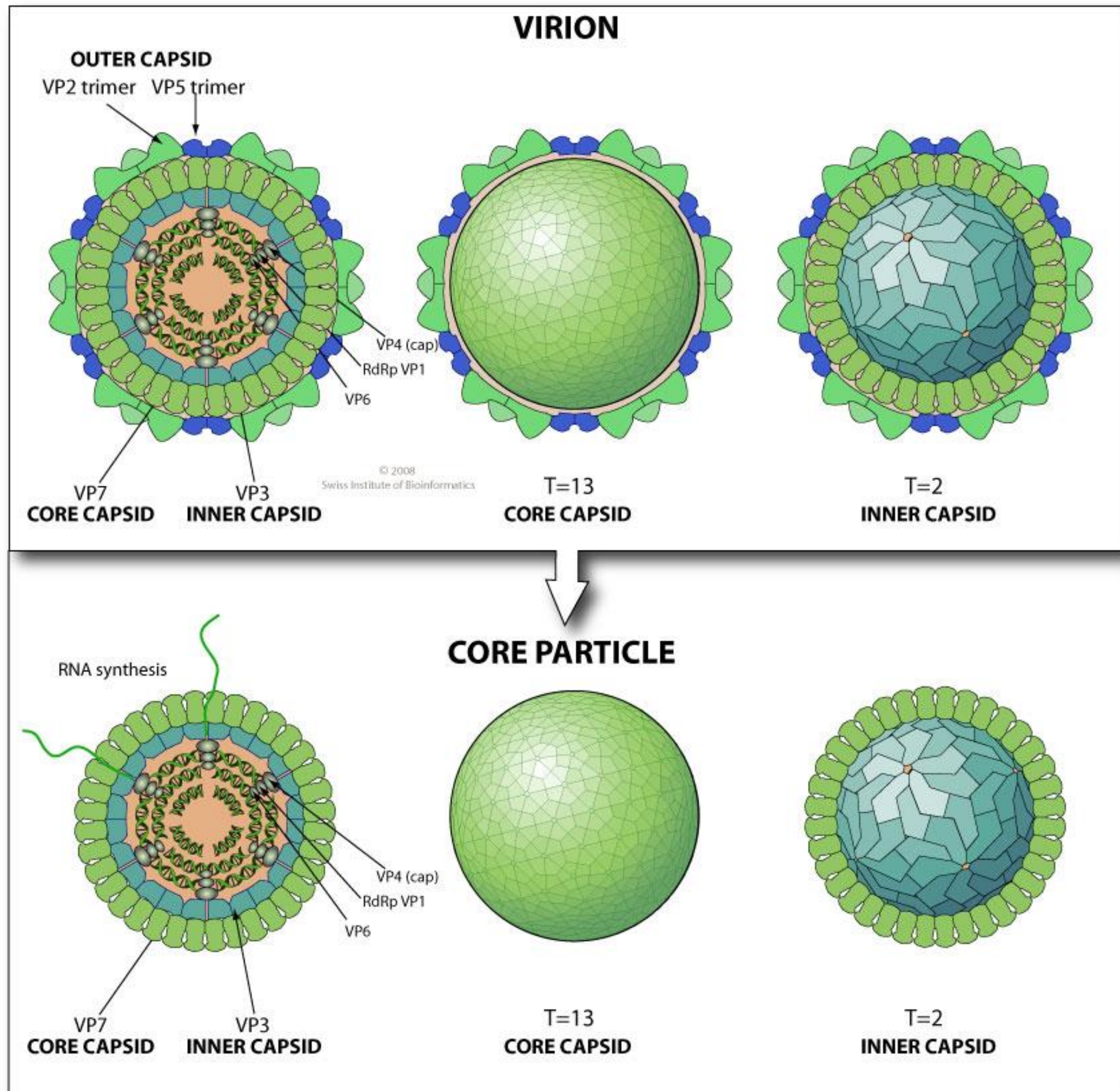

Figure 1. BTV structure. BTV is a non-enveloped, icosahedral, virus with a triple capsid structure, about 80 nm in diameter. The intermediate capsid has a T=13 icosahedral symmetry, the inner capsid a T=2* icosahedral symmetry (Gasteiger et al., 2003).

The virus has 6 or seven non-structural proteins: NS1, NS2, NS3, NS3a, NS4, NS5 and possibly NS5a. Four non-structural viral proteins (NS1–NS4) are only expressed in infected cells and have essential functions in the coordination of viral assembly, virus translocation within the cell and in modulating host cell responses to virus infection (Belhouchet et al., 2011).

NS1 is a 64-kDa protein expressed from BTV genome segment 5 (Mertens et al., 1984). It forms “tubules” of unknown function which are observed within BTV infected cells as well as in cells infected with other orbiviruses (Huismans and Els, 1979). No other viral factor is required to their assembly since they form in the absence of any other BTV protein (Hewat et al., 1992b). NS1 is

identified as a positive regulator of viral protein synthesis and viral morphogenesis (Owens et al., 2004; Boyce et al., 2012).

NS2 is the RNA binding protein and is also the major component of virus encoded inclusion bodies (VIBs), which are believed to be virus assembly sites (Kar et al., 2007).

NS3 (25.5kDa) and its shorter form NS3a (24kDa) are highly expressed in insect cells whereas they are almost undetectable in mammalian cells (they accumulate but at a very low level (Bansal et al., 1998)). They appear to be associated with both smooth intracellular membranes and plasma membranes. These proteins contribute to virus release thanks to viroporin-like properties by permeabilizing mammalian cell membrane. In addition NS3 also allows BTV to egress from infected cells through a budding mechanism. This mechanism might be prominent in insect cells as it induces a lesser cytopathic effect (Han and Harty, 2004; Schwartz-Cornil et al., 2008). Therefore NS3 amino acid sequence might be of importance for an efficient spreading within the insect vector (Riegler, 2002). NS3 also represses the production of INF-I by inhibiting the RLR-dependent signalling pathway in non-haematopoietic cells (Chauveau et al., 2013).

Although BTV replicates exclusively in the cytoplasm, NS4 is localized in the nucleoli of both mammalian and insect infected cells. NS4 is not required for viral replication (Ratinier et al., 2011). However NS4 is an interferon antagonist and confers a replication advantage to counteract the host's innate immune response. In particular it contributes to the virulence of BTV favoring replication in sheep (Ratinier et al., 2016).

BTV genome segment 10 potentially expresses a small protein overlapping the NS3 ORF in the +1 position. Likewise NS4 this putative NS5 protein seems to be localized in the nuclei of the host cells. Viral replication *in vitro* or pathogenicity *in vivo* in mouse models does not seem to be affected by NS5 (Stewart et al., 2015). This newly discovered protein requires further investigation but it might provide some evolutionary advantage since it is well conserved among BTV serotypes, inhibits gene expression and tolerate amino acid change possibly allowing adaptation to changes in the host cell (Stewart et al., 2015).

So far 32 distinct serotypes of BTV were described including four potential novel BTV serotypes: one detected in a Sheep Pox vaccine preparation in Israel (Bumbarov et al., 2016), an additional strain isolated from an alpaca in South Africa (Wright, 2014), another one in healthy goats in Sardinia (Savini et al., 2017) and the last one possibly circulating in goats in Italy in the Piedmont region and related to Toggenburg virus (Marcacci et al., 2018).

2.2 Viral replication and assembly of BTV

Being non-enveloped orbiviruses are quite resistant in the environment, particularly in the presence of protein (Erasmus, 1990). However when compared to rotavirus and reoviruses BTV is relatively fragile as it is readily inactivated by disinfectant containing acid, alkali, sodium hypochlorite and iodophors (Howell and Verwoerd, 1971; Roy and Noad, 2006).

BTV is transmitted between its ruminant hosts almost exclusively by the bites of hematophagous midges belonging to the genus *Culicoides* (Mellor et al., 2000). Virus is inoculated into the host bloodstream by *Culicoides* saliva during a blood meal. Mononuclear phagocytes and endothelial cells constitute the first target of the infectious virions before being disseminated via the host blood stream (Mohl and Roy, 2014). BTV replication can be divided in somehow classical steps that include adsorption, viral uptake, fusion/uncoating, transcription, protein translation, core assembly, and viral maturation/egress (Coetzee and Venter, 2015). Figure 2 depicts an overview of this process.

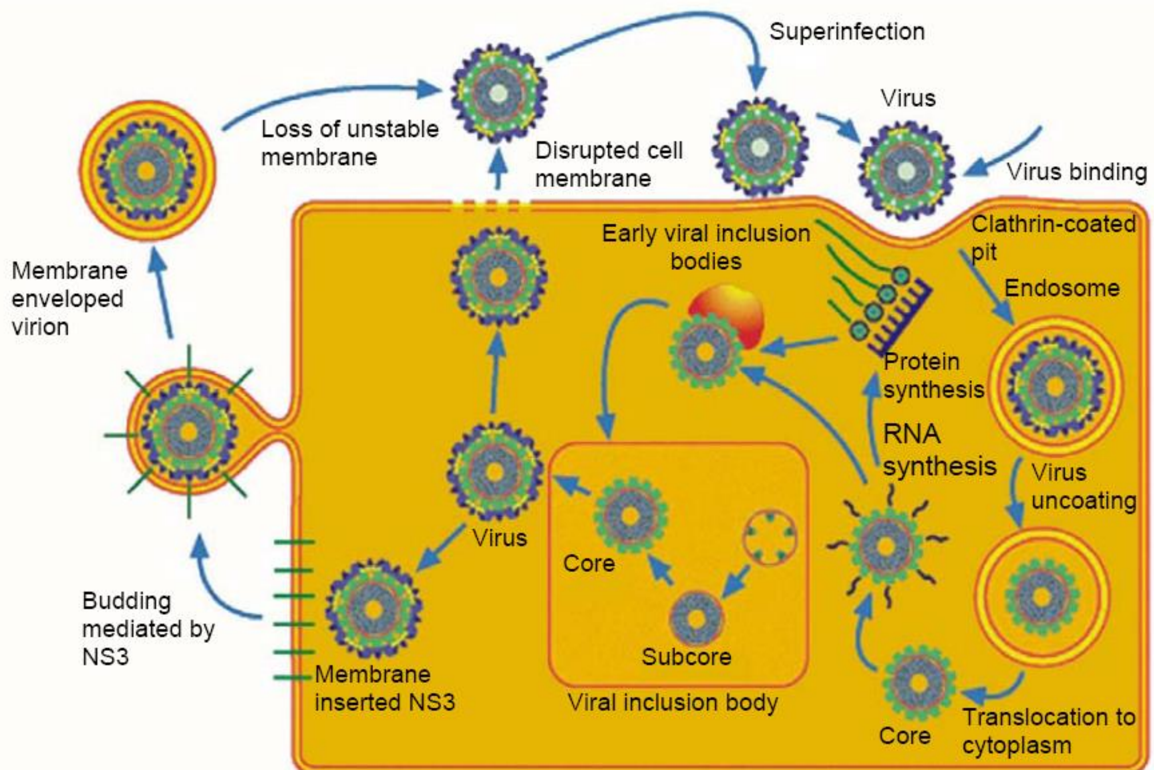

Figure 2. Bluetongue virus cycle. From (Mertens et al., 2004).

Virus entry in mammalian cells is initiated with the interaction of VP2 with cell surface receptors via sialic acid binding. The exact target receptor has to be determined but is most likely a glycoprotein (Hassan and Roy, 1999). Following attachment BTV virions are internalized through a

clathrin-mediated endocytosis pathway (Forzan et al., 2007). Acidification of the endosome is essential to allow entering of BTV particles since VP5 fusogenic activity is enabled by low-pH environment (Eaton et al., 1990). Within the early endosome the outer coat (composed of VP2 and VP5) is quickly lost.

Since VP2 and VP5 were demonstrated to be able to trigger apoptosis in mammalian cells the cell entry and uncoating has to be fast in order to outpace the activation of the NF-κB path (Mortola et al., 2004). Indeed adsorption and penetration were reported to occur within 10 min. following the infection (Lecatsas, 1968).

By contrast, VP7 trimers are responsible for insect cell binding activity (Xu et al., 1997; Tan et al., 2001). In addition BTV particles lacking the VP2-VP5 outer-core displayed a higher oral infectivity in insects (Mertens et al., 1996). Since VP2 also mediates the BTV binding to erythrocytes (Hassan and Roy, 1999), this stresses the potential importance of host serum or insect gut proteases in the initiation of the infection of the vectors.

Infected endosomes translocate the BTV core into the cytoplasm. This again occurs quite readily after infection as viral particles were detected in the cell cytoplasm within 1 hour post-infection (Huismans et al., 1987b; Forzan et al., 2007).

The outer-core removal activates the transcription functions of the core particle. In order to escape the cell defense mechanisms induced by the recognition of dsRNA by the host, the ten genome segments are synthesized within the core itself (Eaton et al., 1990). In total BTV genome is about 19200 base pairs (bp), ranging from 3954 for segment 1 to 822 bp for segment 10; however the exact length depends on the serotype (Fukusho et al., 1989). The viral RNA-dependent RNA polymerase VP1, the capping enzyme VP4 and and the helicase VP6 are located at the inner side of VP3 (Nason et al., 2004). Each of these proteins can work independently and together constitute the transcription complex.

Quite interestingly, BTV along with epizootic haemorrhagic disease virus (EHDV) and African horsesickness virus (AHSV) do transcribe mRNA optimally at 28°C (Van Dijk and Huismans, 1982). The temperature of VP1 activity range underlines the adaptation of the virus to both insects and mammalian cells (Schwartz-Cornil et al., 2008).

To allow the proper transcription of the BTV genome segments VP1 needs to get a clear access to the viral dsRNA. VP6 binds and unwinds the dsRNA ahead of VP1 and also separates parental and newly synthesized RNAs (Stauber et al., 1997). VP1 proteins transcribe positive sense ssRNA (mRNAs) from each of the genome segments (Boyce et al., 2004). Capping of the mRNAs is

achieved by the triple enzymatic activity of VP4: RNA triphosphatase, followed by guanylyltransferase and finally a methyltransferase (Patel and Roy, 2014).

High resolution crystallography and electronic microscopy allowed to identify conformational changes at the five-fold axes of the VP3 layer. The VP3 decameric structure therefore defines pores that can be used for the trafficking of viral nucleic acids and substrate from the core to the host cell cytoplasm (Gouet et al., 1999). Based on findings regarding rotaviruses assembly (Periz et al., 2013), it is suggested that each segments are associated with a single transcription complex. BTV has 10 segments and the VP3 decamer structure defines twelve vertices, leaving two potential pores unused. Therefore this model requires confirmation.

BTV transcripts are extruded from the core through these pores to the cell cytoplasm. There, they act as templates for translation and for negative ssRNA to further synthetize genomic dsRNA (Mertens et al., 1984; Diprose et al., 2001). As a consequence of the major role of the viral polymerase, VP1 and its corresponding segment are amongst the most conserved proteins/RNA segments considering the different BTV serotypes and even in the *Reoviridae* family (Mertens et al., 2004).

Viral mRNAs are translated using the host cellular machinery. In the infected mammalian cell BTV specific proteins can be detected 2 to 4 hours post-infection with an increasing production rate until 11 to 13 hours post infection (Verwoerd and Huismans, 1972; Eaton et al., 1990).

Core assembly mostly takes place in the viral inclusion bodies (VIB) near the nucleus. NS2 composes the VIB where encapsidation of the segments takes place in the VP3 shell (Kar et al., 2007).

Reassortment – the exchange of homologous genome segments with closely related viruses – is a powerful evolutionary mechanism for segmented viruses and BTV in particular. It was described from the field between wild and vaccine strains in any possible combinations (Batten et al., 2008b; He et al., 2010; Maan et al., 2010; Maan et al., 2012b) as well as *in vitro* (Samal et al., 1987; Gould and Hyatt, 1994).

The exact packing mechanism of the viral proteins and segments remains still unclear. Still, there is only one copy of each genome segment that is selected for packaging within the VP3 subcore. Outer core proteins VP2 and VP5 seem to be added to the progeny core as it leaves the VIB (Mertens et al., 2004).

The mature virions are exported by exocytosis mediated by the interaction between VP2 and vimentin (Bhattacharya et al., 2007). They reach host cell membrane where NS3 interacts with calpactin and acts as a bridge molecule facilitating cell membrane trafficking (Celma and Roy, 2011).

Egress of the mature viral particles is achieved through budding or rupture of the cytosolic membrane possibly leading to cell death (Patel and Roy, 2014). Production of viral particles is reported to be exponential between 8 and 24 hours post-infection (Schwartz-Cornil et al., 2008).

3 Taxonomy, morphology and viral replication of Schmallenberg virus

3.1 Taxonomy and structure of SBV

Until the recent changes in viruses nomenclature implemented by the International Committee on Taxonomy of Viruses (Adams et al., 2017) SBV was part of the *Bunyaviridae* family, genus *Orthobunyavirus*, grouped within the serogroup Simbu along with at least 27 other virus species. The members of the Simbu serogroup show cross-reactions to the complement fixation test but are distinguished by seroneutralization (Kinney and Calisher, 1981) and by genetic sequence analysis. Two members of this group are of particular medical importance, the Akabane (AKAV) and Oropouche (OROV) viruses, respectively in veterinary and human medicine.

Yet still part of the *Orthobunyavirus* genus, SBV, AKAV and Aino virus (AINOV) are now considered exemplar viruses of the species Sathuperi *orthobunyavirus,* Akabane *orthobunyavirus*, and Shuni *orthobunyavirus* respectively (De Regge, 2017). These belong to the new order *Bunyavirales,* family *Peribunyaviridae* (formerly *Bunyaviridae*)*,* which comprises the genus *Orthobunyavirus* and *Herbevirus* (host range limited to insects). Viruses belonging to the *Peribunyaviridae* family are enveloped viruses with a segmented RNA genome. The viral genome is single-stranded negative sense and SBV-virions are spherical, about 100 nm in diameter (Elliott, 2009).

Their genome consists of 3 segments: S (Small), M (medium) and L (Large), these names reflecting their respective length in terms of number of nucleotides (Walter and Barr, 2011).

The S segment of all Orthobunyaviruses encodes the nucleocapsid protein N and also a non-structural NSs protein, which plays a role in mediating the antiviral response of infected cells.

The M segment encodes a membrane protein precursor that will be cleaved by cellular proteases to form the two viral glycoproteins Gn and Gc, which play an essential role in the maturation of new viral particles and attachment to sensitive cells. These two glycoproteins were also referred to as G1 and G2, respectively (Saeed et al., 2001). This segment further encodes an NSm protein, derived from the same protein precursor as Gn and Gc, which also seems to play a role in viral morphogenesis.

A single protein is encoded by the L segment, a large complex protein that constitutes the viral RNA-dependent RNA polymerase (Figure 3).

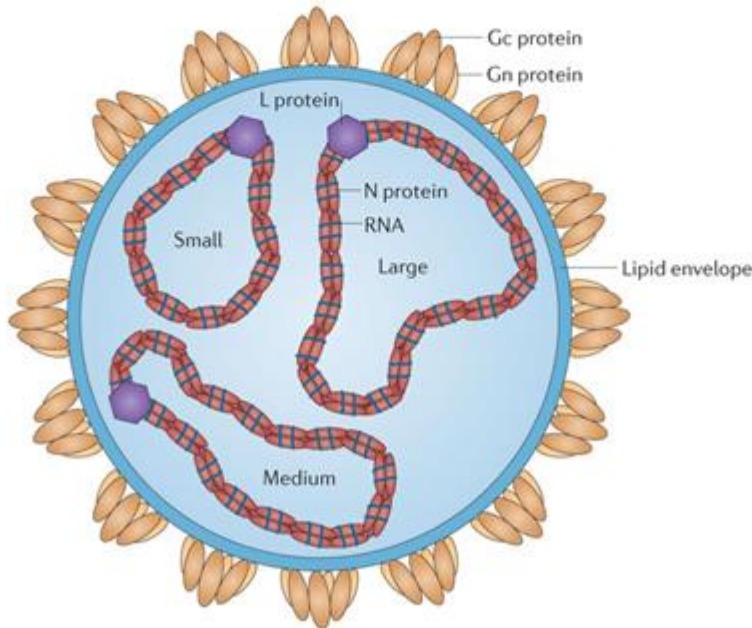

Figure 3. SBV structure. Enveloped, spherical. Diameter from 80 to 120nm. The three genomic RNA segments (small, medium and large) are encapsidated by the N protein to form three ribonucleoprotein (RNP) complexes that associate with the RdRp and are contained within the lipid envelope of the particle that is derived from the host cell Golgi complex, modified by insertion of the viral glycoproteins Gn and Gc (Elliott, 2014).

The first data from sequencing of the three genomic segments of SBV reported a nucleotide homology of 97% with the Shamonda virus (SHAV), 71% with AINOV, and 69% with AKAV respectively for the S, M and L segments (Hoffmann et al., 2012). This greater phylogenetic proximity to the SHAV has led FLI researchers to initially talk about Shamonda-like viruses to characterize SBV. Further and more recent sequencing of three genomic RNA segments of Sathuperi virus (SATV), SHAV and Douglas virus (DOUV) revealed that the M RNA segment of SATV and DOUV have a high degree of sequence identity with that of SBV, but the S and L RNA segments closely matched those of SHAV (Figure 4). Phylogenetic analysis suggested that SBV is a reassortant, with the M RNA segment from SATV and the S and L RNA segments from SHAV (Yanase et al., 2012). Genetic investigations covering additional Simbu serogroup viruses indicated that SBV should be classified within the species Sathuperi *virus* (Goller et al., 2012). SBV is likely to be the ancestor of SHAV, which is in contrast a reassortant virus comprising the S and L segments from SBV and the M segment from another virus, as proposed previously by Saeed *et al.* (Saeed et al., 2001).

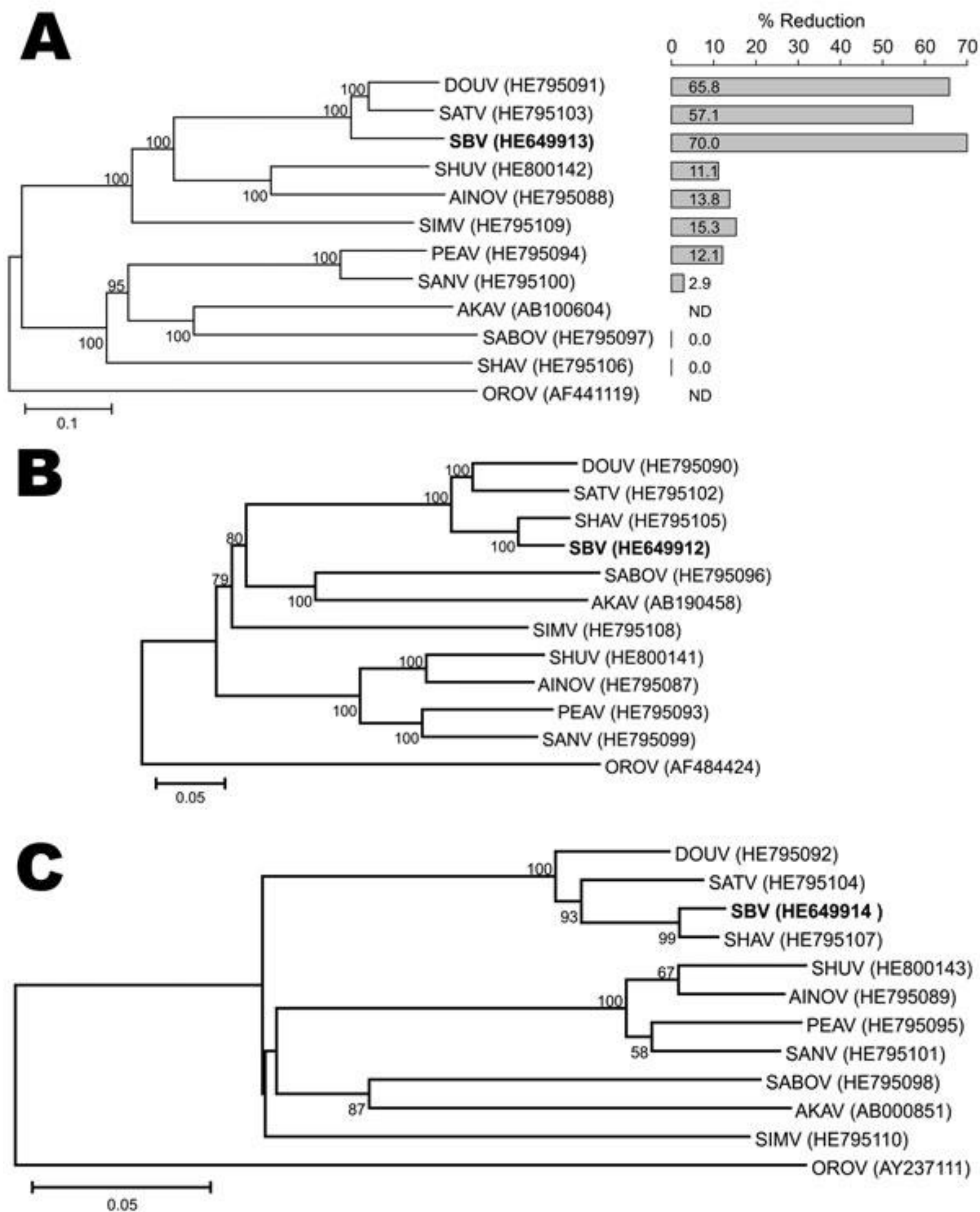

Figure 4. Phylogenetic relationships of Simbu serogroup viruses for the M (A), L (B), and S (C) coding regions. DOUV, Douglas virus; SATV, Sathuperi virus; SBV, Schmallenberg virus; SHUV, Shuni virus; AINOV, Aino virus; SIMV, Simbu virus; PEAV, Peaton virus; SANV, Sango virus; AKAV, Akabane virus; SABOV, Sabo virus; SHAV, Shamonda virus; OROV, Oropouche virus. ND, not determined. Cross neutralization of SBV antibodies against 9 Simbu serogroup virus strains were tested (upper right A panel). DOUV and SATV were well neutralized whereas SHAV titers (as well as AKAV titers) were not reduced at all. These results support the M gene phylogeny. Moreover, as SBV, DOUV and SATV cluster for all three segments but SHAV only for L and S

segments, it appears that SHAV, and not SBV, is a reassortant within the Simbu serogroup. From (Goller et al., 2012).

3.2 Viral replication and assembly of SBV

The life cycle of SBV is largely based on the knowledge gathered studying other *Peribunyaviridae*, since SBV was quite recently discovered. Virus attaches to the host cell through Gn-Gc glycoprotein dimer interactions with membrane surface receptors (Figure 5). These receptors are currently unknown, however dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) was reported as a potential candidate receptor (Lozach et al., 2011). Following attachment cell entry is likely mediated by clathrin-dependent endocytosis (Hollidge et al., 2012). Acidification of endocytic vesicles leads to the uncoating of the viral particles. The viral envelope then fuses with the endosomal membrane (Plassmeyer et al., 2007). Ribonucleoproteins (RNPs) segments are released in the cytoplasm and accumulate in the Golgi. Virus factories form and there viral RNA-dependent RNA polymerase (RdRp) starts the primary transcription of viral mRNAs. Transcription is initiated when RdRp cleaves a 10 to 18 nucleotides sequence from the 5' end of host mature mRNAs. These cellular sequences are subsequently used to prime the transcription of capped viral mRNAs in the cytoplasm (Patterson et al., 1984). Quite a particular feature is the need of on-going translation within the host cell to allow proper transcription of peribunyaviruses (Kolakofsky et al., 1987). The origin of this mechanism remains unclear (Elliott, 2014). Viral proteins are synthetized by host cell ribosomes (Doceul et al., 2013).

It seems that peribunyaviruses start to replicate once the quantity of N proteins in the cytoplasm reaches a threshold level. RdRp and N protein are the only required viral protein for transcription and replication (Dunn et al., 1995). As previously described for BTV, reassortment is a common phenomenon for segmented viruses. Given the tripartite genome of peribunyaviruses, coinfection with two different viruses could lead to 6 different theoretical reassortants. However as stated here above it was recently reported that to allow proper replication and transcription a functional promoter has to be recognized by RdRp and N proteins. Hence reassortants whose L and S segments came from the same virus tend to benefit from their higher replication rate (Tilston-Lunel et al., 2017). As a consequence reassortant progeny viruses display at a higher frequency L and S segments from a same origin.

Assembly and budding occur in tube-like virus factories comprising both cellular and viral (NSm) components that are built around the Golgi complex (Murphy et al., 1973; Doceul et al., 2013). Dimerized viral glycoproteins Gn and Gc accumulate in Golgi stacks and associate with RNPs. Maturation of viral particle is achieved by the migration through Golgi compartments. The progeny

virions are transported to the cell surface by exocytosis. Following virus release, further maturation occurs resulting in full infectivity (Walter and Barr, 2011; Elliott, 2014).

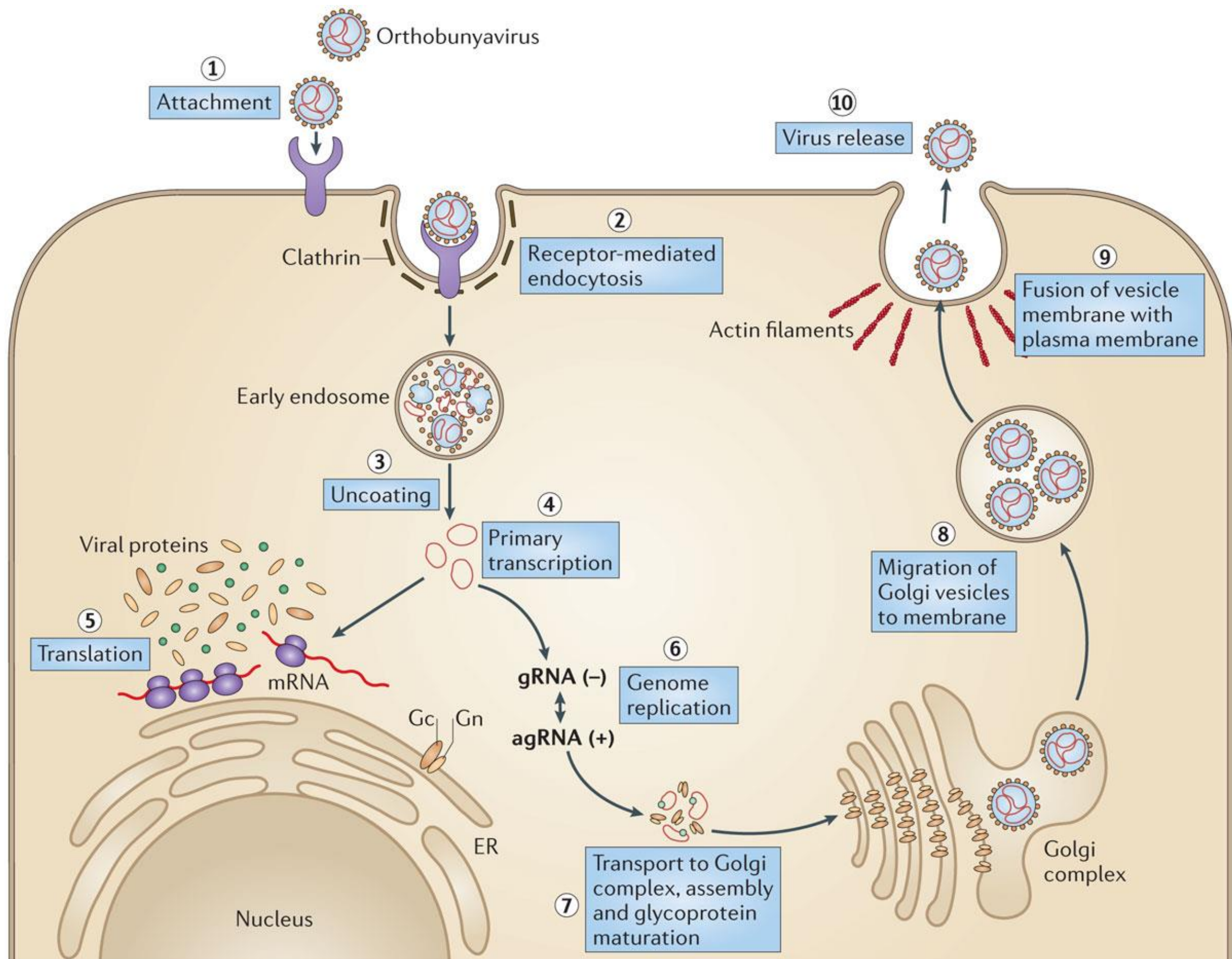

Figure 5. Overview of Orthobunyaviruses life cycle (Elliott, 2014).

4 Epidemiology

4.1 Introduction routes

Arboviruses can be introduced from one affected area to a naïve area following 4 main mechanisms: i) animal movement (domestic and wild, legal or illegal) or animal product transport (semen, embryos), ii) infected vectors carried by various living beings (animals or plants) or fomites; iii) active movement (flight of the vector in the case of BTV and SBV) and iv) through passive transportation (by wind for long-distance dissemination for BTV and SBV) (Saegerman et al., 2008).

Mintiens et al. reviewed in 2008 the possible routes of introduction of BTV8 at the Epicentre of the 2006 North-Western Epidemic regarding movements of ruminants, horses, semen or embryos (Mintiens et al., 2008). No obvious origin could be determined. In addition, introduction of infected vectors by wind, plants, aircraft or through illegal animal trade were considered unlikely to be the cause of the introduction of BTV8. Indeed, the best matches with the original BTV8 European strain were isolates from sub-saharian African countries, namely South Africa (reference strain), Kenya and especially Nigeria (Maan et al., 2008). From Nigeria to the Netherlands not much commercial exchanges exist and no cattle trade was registered between 2001 and 2011 (FAO, 2018).

Retrospective studies assessing the initial occurrence of BTV8 led to hypothesize an original introduction of BTV somewhere in 2006 near the National Park of *Hautes Fagnes et Eifel* at the border between Belgium and Germany (Saegerman et al., 2010). In addition, the likely use of unlicensed incompletely attenuated live vaccine resulted in the transitory appearance of BTV6 and BTV11 in the Netherlands, Germany and Belgium. Both serotypes appeared during the 2008 vector season (De Clercq et al., 2009; Maan et al., 2010; van Rijn et al., 2012). The exact source of these serotypes remains unknown and they both spontaneously disappeared without the implementation of any particular control measure.

SBV introduction in Western Europe is even more puzzling. The use of immunohistochemistry and *in-situ* hybridization in ruminant brain tissue archived between 1961 and 2010 in Germany did not allow to find any evidence of SBV proteins or RNA in the tested samples. Therefore it was hypothesized that SBV was only recently introduced from tropical or subtropical regions (Gerhauser et al., 2014). Indeed, several studies from South-Africa, Tanzania, Mozambique and China reported serological results suggesting SBV circulation in livestock possibly before the onset of the European epizootic (Leask et al., 2013; Blomstrom et al., 2014; Mathew et al., 2015; Zhai et al., 2018). However the SBV circulation outside Europe mainland has to be seen only as potential since serological cross-reactivity within the Simbu serogroup has been described based on ELISA results (mostly detecting anti-N protein antibodies) as well as on seroneutralization results (detecting anti-Gn/Gc antibodies) (Kinney and Calisher, 1981; Mathew et al., 2015).

4.2 Bluetongue Virus and Schmallenberg Virus are arboviruses

The European Palearctic Culicoides species constitute the main BTV and SBV transmission route. *Culicoides* are telmophagous insects: they feed from a pool of blood caused by tissue laceration, which includes blood, skin cells and lymph (Pages et al., 2014). *Culicoides* saliva contains vasodilator, anticoagulant and other components inhibiting leucocytes response (Perez de Leon et al., 1997; Perez de Leon et al., 1998; Bishop et al., 2006). Once bitten, the host recruits inflammatory cells that might be targets for viruses (Takamatsu et al., 2004).

In the field, culicoides become infected with an arbovirus only *via* a blood meal on an infected vertebrate host, and unlike other insect groups such as sandflies, mosquitoes and ticks (Walker and Davies, 1971), there is no scientific evidence to suggest vertical or horizontal transmission of viruses in infected culicoides (Mellor et al., 2000). Persistence of the virus in long-lived parous female midges is a more likely mechanism for overwintering of BTV in Europe (Mellor et al., 2000; Osborne et al., 2015). However, recently Larska et al. (2013) suggested a possible trans-ovarian transmission of SBV by Culicoides (Larska et al., 2013). The time period that spans from the ingestion of a virus during an infected blood meal to the transmission capacity is called an "extrinsic incubation period". During this time, the virus replicates in the midgut epithelial cells of the insect (Figure 6), and then diffuses to infect the secondary target organs. The virions thus produced will disperse in the insect via hemolymph.

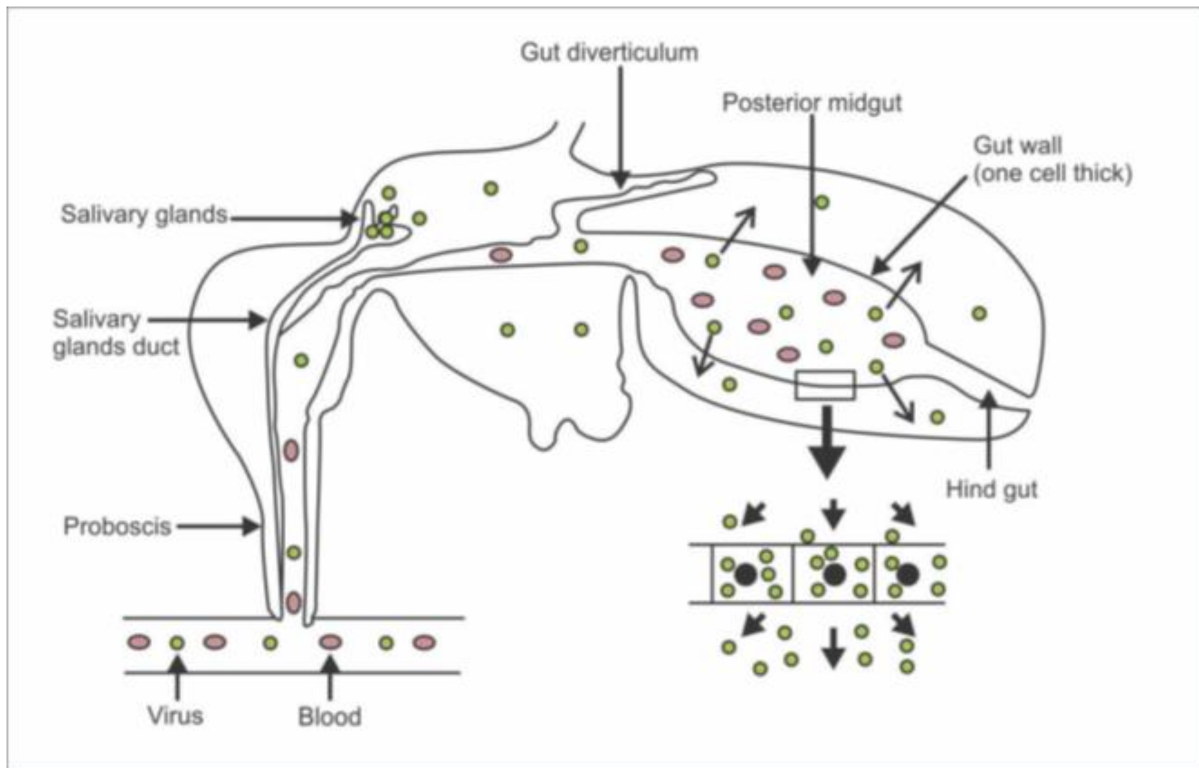

Figure 6. Schematic cycle of arbovirus infection in Culicoides species (Venter, 2014).

SBV had an apparent higher rate of spread in matter of geographic coverage by time unit when compared to BTV (Sedda and Rogers, 2013). However in this latter study case definition differed for each disease, with BTV cases defined as clinical disease in adult ruminants and SBV cases defined as fetal malformation cases. As a consequence SBV actual spread speed may have been overestimated since unapparent infection of adult ruminants were not recorded.

Vectorial capacity is the measure of the ability of a vector population to transmit a pathogen to a population of susceptible hosts (Garrett-Jones, 1964; Christofferson and Mores, 2011). The vector capacity is calculated using a combination of the biting rate, host preference, frequency of blood meals, vector competence, daily survival of the vector and extrinsic incubation period (Gerry et al., 2001).

Vector competence refers to the ability of a vector to support virus infection, replication and/or dissemination. It is estimated by the proportion of vector midges actually getting infectious following a meal of infectious blood. It is dependent upon genetics of the vector and environmental factors (Saegerman et al., 2008; Venter, 2014).

A competent vector may have a low vectorial capacity due to low biting rates or survivorship. By contrast, a low vector competence could be compensated by an aggressive behavior and high biting rate (Venter, 2014).

Indeed, data regarding the respective spread of orthobunyaviruses versus orbiviruses are not always consistent. A seroprevalence study in Central Sudan revealed different spread dynamics between Akabane and BTV and other orbiviruses, with sporadic occurrences over the years for the former and enzootic state for the latter (Mohamed et al., 1996). In Turkey as well Akabane virus has spread neither on the northeast coast nor on the Anatolian plateau, unlike BTV. Therefore these two viruses might be transmitted locally by different vectors, or if *Culicoides imicola* is their only vector, that it is more competent for BTV than for the Akabane virus (Taylor and Mellor, 1994). Kirkland by contrast reported a higher level of transmission efficiency for Akabane virus when compared to BTV. Vector competence, infection rate and attack rate are key elements to mitigate the transmission of viruses to the mammalian host and are species dependent (Kirkland, 2015).

Given the *Culicoides* vector species are similar for BTV and SBV infection rate during an epidemic has an important influence on disease transmission speed and extent. In areas where the classical *C. imicola* and palearctic *Culicoides* species are sharing the same ecosystem, higher BTV infection rates were associated with *C. scoticus*, *C. newsteadi* when compared to *C. imicola* (Foxi et al., 2016). In the lab BTV infection rate of *Culicoides* blood fed on membrane reach usually about 10-30 % (Tabachnick, 1996; Fu et al., 1999; Gerry et al., 2001) and up to 51.6% (Jennings and Mellor, 1987). In the field however captured *Culicoides* usually display an infection rate significantly lower and frequently below 1% regarding *Culicoides sonorensis* (Gerry et al., 2001). Minimum infection rate (MIR) of the *obsoletus* complex was reported to be about 2.6% (Vanbinst et al., 2009), which is much higher than the ones reported in two Italian studies about the BTV2 epidemic of 2002 and in the more recent study of Foxi et al. In these studies infection rates of 0.05% for *C. obsoletus/C. scoticus* (Savini et al., 2005), 0.22% for *C. obsoletus/C. scoticus*, *C. dewulfi* and *C. chiopterus* (De Liberato et al.,

2005) and 0.058 for *C. scoticus* (Foxi et al., 2016) were reported. In the US the estimated overall infection rate ranged from 0.08 % to 0.4% for *Culicoides sonorensis* (Gerry and Mullens, 2000; Gerry et al., 2001). It has however to be stressed that BTV infection of culicoides leads to an aversion towards light, therefore possibly underestimating the actual infection rate of the vector population as light traps are among the most commonly used traps (McDermott et al., 2015).

Considering that each SBV positive pool of *C. obsoletus* caught in the region of Antwerp in the months of September and October 2011 only contained one positive midge, infection rate would have reached 3.61% in this species in this region (De Regge et al., 2012). In Southern Belgium infection rate reached at least 2.86 % during the summer of 2012 (De Regge et al., 2014). To this respect SBV infection rate in European palearctic *Culicoides* species seems to be higher when compared to BTV infection rate. Regarding the *Orthobunyavirus* genus the potential difference in transmission efficiency between SBV and Akabane virus might rely on the sequence of the M segment. Indeed the importance of the M segment in determining vector competence and vector range was studied and reported in the California serogroup (Horne and Vanlandingham, 2014).

SBV RNA could be detected in Culicoides caught in Denmark in October 2011 (ProMED-mail, 2012b), in Belgium in a pool of *C. obsoletus* caught in early September 2011 and a pool of *C. dewulfi* caught at the beginning of October 2011(ProMED-mail, 2012a), as well as in Italy, on 6 Culicoides pools belonging to the obsoletus complex captured between September and November 2011. In Belgium, the RTqPCR were made only on culicoides heads. In this way, insects whose positivity is linked to a recent blood meal taken on viraemic animals are discarded. Thus, a positive result suggests the presence of virus in the salivary glands of the culicoides and reflects a possible active transmission of the virus with biological amplification by the vector (De Regge et al., 2012).

By contrast the role of mosquitoes or other arthropods in the transmission and epidemiology of SBV is currently highly unlikely (Doceul et al., 2013). In mosquitoes the salivary gland barriers seem able to prevent the further dissemination of SBV within the insect (Wernike et al., 2014b; Manley et al., 2015).

4.3 Vertical transmission

Vertical transmission from pregnant dams to their offspring is of major consequences for both SBV and BTV, especially BTV8 and lab-adapted strains (Wouda et al., 2009; Veldhuis et al., 2014a). Many pathogens are capable of crossing the placenta to cause fetal injury. Most maternal virus infections are not transmitted to the fetus; however, certain viruses are capable of crossing the placental barrier possibly causing developmental defects (teratogenesis). Teratogenesis is the production of a permanent abnormality in structure or function, restriction of growth, or death of the

embryo or fetus (Gilbert-Barness, 2010). The outcome of *in utero* infection depends on the susceptibility of the fetus to the infecting virus which, in turn, is a reflection of the gestational age of the fetus at exposure as well as the virulence characteristics of the infecting virus (MacLachlan et al., 2000).

In cattle the SBV transmission rate leading to congenital defects is assumed to be low, around 0.5 % (Veldhuis et al., 2014a). By contrast, subclinical transmission might be underestimated and could be as high as 12.5% (Wernike et al., 2014a) and up to 28 % (Garigliany et al., 2012a). During the BTV epizootic of 2007-2008, Darpel et al., estimated transplacental infection rate of 33 %, which is consistent with the latter result (Darpel et al., 2009).

The lesions potentially presented by the calves affected *in utero* by SBV could be distinguished according to two entities: a hydrocephaly / hydranencephaly syndrome and a torticollis / arthrogryposis syndrome. By analogy with Akabane virus the infection during the first 6 months seems to be critical: an infection of the fetus between 76 and 104 days usually gives rise to hydranencephaly / porencephaly type lesions, and from 103 to 174 it is predominantly arthrogryposis (Kirkland et al., 1988). The latest lesions have been observed for infection at 249 days of gestation, and it appears that fetuses less than 2 months old (after conception) could be protected from *in utero* infection (Kirkland et al., 1988).

By contrast with SBV, BTV *in utero* infection is usually associated to BTV lab adapted strains (i.e. passaged on cell culture like modified live vaccine strains) or more recently with the European BTV8 wild type virus. Indeed early studies reported a readily crossing of the placental barrier when ewes were infected with lab-adapted BTV or modified lab vaccine strains (Schultz and Delay, 1955; Cordy and Shultz, 1961). The highest susceptibility was then observed around 35-42 days of pregnancy (Kirkland and Hawkes, 2004) and infections after day 75 resulted in much lighter consequences (Osburn et al., 1971). Placental crossing, depending on the gestational stage, the BTV serotype and the inoculated dose, was reported to cause congenital defect in up to 40% of the offspring of infected ewes (Anderson and Jensen, 1969).

In cattle not so much studies were carried over however similar nervous lesions were reported when BTV lab-adapted or modified live-vaccine strains were involved (Richards et al., 1971; MacLachlan et al., 2000). By the end of the XXth century, at least five BTV serotypes (BTV4, BTV10, BTV11, BTV13, BTV17) were reported to be able to cross the placental barrier and possibly causing teratogenic effects (MacLachlan et al., 1985b; Richardson et al., 1985; Vercauteren et al., 2008). *In utero* infection caused by wild-type strains was considered uncommon (Waldvogel et al., 1992b) yet documented (McKercher et al., 1970).

4.4 Transmission through direct contact

Oral contamination of dogs was reported for the BTV closely related African Horse sickness virus (AHSV) following the ingestion of infectious horse meat (Van Rensberg et al., 1981). First occurrence of a potential BTV8 oral transmission to cattle was reported in 2008 supposedly following the ingestion of infectious placenta (Menzies et al., 2008). However prior to this first study Jauniaux et al. described the case of two lynx that died most likely of bluetongue disease. These animals were used to be fed with fetuses, stillborn and placentas of cattle at a time and in an area highly prevalent with BTV (Jauniaux et al., 2008). Oral infection was also documented for BTV8 in calves, but the study involved the consumption of spiked milk in quantities unlikely to match a possible field situation (Backx et al., 2009). In the USA non-productive perinatal BTV11 infection was reported. However the infection did not lead to seroconversion, viraemia or long lasting nucleic acid detection. The source of the contamination was confirmed to be colostrum (Mayo et al., 2010).

Animals can also get bluetongue disease as a nosocomial infection: bitches aborted and subsequently died of heart failure and respiratory distress following routine vaccination against distemper vaccine (Evermann et al., 1994). Unfortunately the vaccine was tainted with BTV11, already known for its ability to cross the placental barrier (Waldvogel et al., 1992a).

Some more unclear direct transmissions were also described in the supposed absence of vector midges and contamination through feed: Van der Sluijs et al. first reported the transmission of BTV8 to a control pregnant ewe housed with challenged animals (van der Sluijs et al., 2011). Two years later the same author also described the transmission in similar condition of BTV1 to a control pregnant ewe (van der Sluijs et al., 2013). Other BTV serotypes revealed their potential for direct contact transmission. Once every other likely routes of infection were ruled out (midges, feed or iatrogenic through the use of contaminated needles) Batten et al. concluded that BTV26 could possibly be transmitted by direct contact between goats (Batten et al., 2013b). An additional study confirmed in 2014 that BTV26 is readily transmitted through direct contact in caprine and is likely to be refractory to replication in the insect host (Batten et al., 2014). Such transmission was also described in red deer with BTV1 but then since during the experiment the animals were fighting quite seriously for dominance it was hypothesized that transmission to a control animal in such circumstances might be related to wound to wound contact or oral to wound contact (Lopez-Olvera et al., 2010). BTV2 adds up interestingly as a control ewe, kept in a different pen (70 cm away) from the challenge animals, displayed RNAemia and seroconverted. The authors brought forward a potential undetected contamination via the oral route (Rasmussen et al., 2013). The recently discovered BTV27 also showed abilities to be transmitted by direct contact between goats, whereas sheep and cattle seem to be insensitive to the virus (Breard et al., 2018).

The risk of iatrogen transmission of BTV was assessed and demonstrated through the use of shared needles for subcutaneous inoculation. However the real importance of that mean of transmission has to be clarified and is believed to be rare under field condition since only 25 % of the recipient animals (cattle and sheep) became infected (Darpel et al., 2016).

So far there was no direct horizontal animal to animal transmission reported for SBV. Spread modelling taking into consideration latent period, duration of viraemia, probability of host-vector transmission and viral replication rate are satisfying explanatory variables to explain the rapid spread of the virus without the need of alternatives to the vector transmission such as direct transmission (EFSA, 2014; Gubbins et al., 2014).

4.5 Genetic evolution

Like other families of RNA viruses, *Peribunyaviridae*-RNA-dependent RNA polymerases and orbiviruses VP1 RNA-dependent RNA polymerase are prone to produce errors in the replication of the viral genome. Broadly speaking, RNA virus replication is characterized by high mutation rates (10^{-5} to 10^{-3} misincorporations per nucleotide copied), short generation times, and high progeny yields (Domingo and Holland, 1997). In addition, segmented RNA viruses also generate genomic variation through recombination and reassortment (Bonneau et al., 2001). Therefore RNA viruses form populations of closely related viral variants that started from a single clone: the quasispecies (Domingo et al., 1996; Biebricher and Eigen, 2006).

As SBV and BTV are transmitted by arthropod vectors, the viruses undergo typically an alternate two-host ‘life cycle’ and are therefore suggested to be more stable and to evolve slower than vector-independent viruses (Fischer et al., 2013).

Both steps may put selective pressures on the viruses, but it remains unknown whether sequence divergence is related to the mammalian or arthropod portion of the virus life cycle. However it has been demonstrated that despite the lack of changes in the consensus sequence, the passage of BTV in *Culicoides* cells induces an increase of the number of low-frequency variants as well as an increase in virulence (Caporale et al., 2014). The biological stage of the virus within the insect vector seems to act a major role in the development of genomic variability and possibly as a consequence, explains at least partly the virulence of BTV. This is in line with results of Heidner et al. that demonstrated the genetic stability of BTV in cattle throughout prolonged infection and despite a strong humoral immune response (Heidner et al., 1988). Moreover, previous studies reported a correlation between the lifespan of the vector and the mutation rate, with short-living arthropods like mosquitoes were accumulating more mutations by time unit when compared to ticks (Gould et al., 2003). Since Culicoides are blood-feeding biting midges with a life-cycle closer from mosquitoes than from ticks

(Verhoef et al., 2014), one may hypothesize that culicoides-borne viruses display quite a large variability in relatively short amount of time. By contrast, Carpi et al. demonstrated that BTV genome segments have substitution rates usually lower (0.5-7 X 10^{-4} nucleotides substitutions per site, per year) when compared to single stranded positive sense RNA arboviruses (Carpi et al., 2010).

SBV as well seems overall showing a high level of genetic stability when passaged in cell culture (Hofmann et al., 2015b) and indeed the cycle between arthropod vector and mammalian host as well tend to repress the genetic drift of the virus (Garigliany et al., 2012b). Nonetheless the outcome of passages in cell culture can be quite unexpected and interesting: Varela et al. generated a SBV clone passaged 32 times that happened to be more virulent in mice than wild type virus (Varela et al., 2013). Most of the interesting mutations were found in the part of the M segment coding the Gc protein. The Gc protein being exposed on the outer surface of the virion it is indeed the main target for SBV neutralizing antibodies. The involvement of the N-terminal region of the Gc glycoprotein as a major element of genetic variability was confirmed by other studies and that particular region of the M segment was then referred to as a mutation “hot spot” at the origin of an hypervariable region in the Gc protein (Coupeau et al., 2013a; Fischer et al., 2013).

Reassortment of gene segments has been shown to occur extensively within both families *Peribunyavirdae* and *Reoviridae* (Weaver, 2006). Antigenic shift is a particular case of reassortment where the phenotypic properties of the virus are changed. In presence of pathogenic and nonpathogenic strains the outcomes of reassortment could be an increase in the pathogenicity in a previously avirulent strain and vice-versa (Shaw et al., 2013). SBV was believed to be a field reassortant between Sathuperi and Shamonda viruses (Garigliany et al., 2012b; Yanase et al., 2012) before it was established it was actually part of the species Sathuperi and a possible ancestor of the reassortant Shamonda virus (Goller et al., 2012). Recently it was shown that reassortment between SBV and Oropouche virus, another *Orthobunyavirus* yet belonging to another species, is not unlikely to occur (Tilston-Lunel et al., 2017). *Peribunyaviridae* are so prone to reassortment that it was hypothesized that all current *Peribunyaviridae* are actually reassortant viruses (Briese et al., 2013).

BTV ease on reassortment was described for a long time in the field and through experimental infections of mammals and insects vector (el Hussein et al., 1989; Maan et al., 2012b; Shaw et al., 2013). The timing of the infection is of consequence in the success of the reassortment since synchrone or shortly delayed infection with two serotypes could lead to a high reassortment rate whereas later super infection restrains the chances of reassortant progeny viruses to be generated (el Hussein et al., 1989). It seems that reassortment can involve any genomic segment but the origin of the transmitted ones suggests it is not random with over and under representation of particular segments from specific parental origin (Ramig et al., 1989). Moreover, the tendency to transmit certain segments

at a higher frequency seems not to be host-specific as similar segregation patterns were reported in VERO cells as well as in vector insects (Ramig et al., 1989).

Although genetic drift and antigenic shift are key elements to drive the evolution of segmented RNA viruses, recombination is another mechanism potentially leading to the emergence of new strains (Nagy and Simon, 1997). Recombination events were described in BTV not as usual events but still acting as evolutionary forces allowing orbiviruses to leap forward in fitness in a single step (He et al., 2010). Recombination in *Peribunyaviridae* was described too, but so far only for a few viruses and recombination in SBV has to be demonstrated (Klimas et al., 1981; He and Ding, 2012).

Fast genotypic and phenotypic changes can also be promoted through founder effect: a small number of viral variants can be fixed and thereby give raise to a novel genotype (Chao, 1990). The long term low level RNAemia observed in BTV infection can lead to the random ingestion of a single variant from the quasispecies virus population by a culicoide, subsequently amplified and spread in the field (Bonneau et al., 2001).

5 Economic losses and impact on livestock industry

BTV infection can lead to tremendous financial loss, partly due to direct losses from mortality and reduced production but mostly from the ban of ruminant trade (Zientara et al., 2010). Global economic losses, thus mainly secondary to trade restrictions through limitations of livestock and germplasm movements to bluetongue free countries were previously estimated at as much as three billion dollars annually and 125 million dollars for the US alone (Tabachnick, 1996).

The Belgian ovine flock indeed paid a very heavy toll to BTV. The 2007 epizootic of BTV8 in Northern Europe was far more aggressive than in 2006. Between July and October 2007, around 25 000 more sheep died in Belgium than during the same period in 2006, representing approximately one-sixth of the national flock (Wilson and Mellor, 2009). By the end of 2007, well over 30 000 farms in northern Europe had been infected, causing over 150 million euros of damage through direct costs and way more in lost trade. The 2006 epizootic of BTV8 in Northern Europe is believed to have caused greater economic damage than any previous single serotype BTV outbreak (Wilson and Mellor, 2009).

In the Netherlands the BTV8 epizootic in 2006 and 2007 has been estimated to a cost of 32.4 and 164-175 million euros, respectively (Velthuis et al., 2010). Using the same model the financial impact has been assessed around 40 million euros for 2008 (Velthuis et al., 2011). Milk losses were estimated to potentially reach more than 3% of the annual production of dairy cows following the infection of a naïve herd, with a negative impact on production up to 4 months after the first detection of a case in an infected herd (Nusinovici et al., 2013).

In Switzerland a stochastic economic model applied to retrospective (2008-2009) and prospective (2011-2012) analyses estimated losses from 2.6 million euros in 2011 to 12.2 million for 2008 (Hasler et al., 2012).

In Belgium, the average estimated losses at the level of the entire Walloon Region for the period 2006-2007 were respectively 32.3 million euros (low assumption based on the census of officially declared households), 90 million euros (medium assumption based on a representative survey of veterinarians) and 104.8 million euros (high assumption based on four representative surveys of cattle and sheep farmers) (Hanon et al., 2009).

Regarding SBV, recent studies evaluated an impact ranging from 23 to 43 euros per cow per year versus 19 to 37 euros per ewe per year (Waret-Szkuta et al., 2017). Other authors evaluated an annual cost between £ 6.40 to £ 20.85 £ per ewe in the UK versus £ 4.75 to 17.20 in France (Alarcon et al., 2014). Likewise, the SBV cost has been evaluated from £ 8.2 to £ 51.4 per cow per year in the UK versus £ 9.7 to £ 48.6 in France (Hasler et al., 2015). In Switzerland the average losses after SBV infection at the farm level was CHF 1606 (EUR 1338) but could locally be higher than CHF 10 000 (8333 euros) (Wuthrich et al., 2016).

The lesser economic impact and the decrease in clinical manifestation of SBV through time led to a low uptake of the vaccine throughout Europe. The European livestock is therefore potentially susceptible to a new outbreak of fetal malformations (Stavrou et al., 2017).

6 *Culicoides* biting midges

The biological vectors of BTV and SBV are biting midges belonging to the family *Ceratopogonidae*, genus *Culicoides* Latreille 1809 (Du Toit, 1944; De Regge et al., 2012). These small midges (1-4 mm), of which about 1,400 species are currently described worldwide with the exception of Antarctica, New-Zealand, Patagonia and Hawaii (Mellor et al., 2000). In Europe Mainland and exclusive of *C. imicola*, the classical old world BTV vector, only 5 species of these midges are reported to be of epidemiological relevance in the transmission of these viruses, namely *C. obsoletus*, *C. scoticus*, *C. pulicaris, C. dewulfi* and *C. chiopterus* (Vanbinst et al., 2009; Meiswinkel et al., 2014; Rasmussen et al., 2014). *C. obsoletus*, *C. scoticus* and *C. chiopterus* are part of the obsoletus complex based on morphologic and phylogenic similarities *(Lehmann et al., 2012)*. These Palearctic *Culicoides* species mostly feed on mammalian hosts (Calvo et al., 2012). Usually, the bigger the target the greater the feast: cows are preferred to horses and horses come before sheep (Elbers and Meiswinkel, 2015). Some *Culicoides* species – especially the Scottish biting midge *C. impunctatus* Goetghebuer 1920 - are responsible for painful biting reactions in human (Logan et al., 2010).

Culicoides spp. are reported to be distributed from tropics to the tundra and from see level to 4000 m. (Mellor et al., 2000). *Obsoletus* group species is the most prevalent group up to an altitude of 1200 whereas Pulicaris group dominates above 1500 m. (Kaufmann et al., 2012).

All the BTV and SBV vector species are multivoltine and most likely to complete 4 to 6 generations annually (Meiswinkel et al., 2014). This is clearly an increase in the estimated number of cycle that prevailed a few decades ago (Rieb, 1982). The local weather and climate in general and the temperature in particular are key elements to determine lifespan and vectorial capacity of culicoides (Mellor et al., 2000; Lysyk and Danyk, 2007; Carpenter et al., 2011).

6.1 Biology and ecology of *Culicoides*

Culicoides (Diptera: *Ceratopogonidae*) are holometabolous insects (Harrup et al., 2015). The life cycle of *Culicoides* therefore includes egg, four larval stages, and an emergence of the adult or imago from a nymph (Figure 7). Only females are hematophagous and need blood to complete a gonotrophic cycle (White et al., 2017). However it has been reported that as most hematophagous insect vectors, the culicoides females can also use sugar from nectar or honeydew to enhance their longevity (Kaufmann et al., 2015). Most culicoides species are at rest at the level of vegetation during the day (Zimmer et al., 2014). Survival, activity and dispersion of these biting midges are strongly influenced by meteorological variables such as the temperature, the humidity, local winds and immature stages are very sensitive to desiccation and require a certain level of moisture to survive (Blackwell et al., 1994). The active dispersion of culicoides is quite limited (Mellor et al., 2000), ranging from 800 m to 4 Km (Lillie et al., 1985). However using ascending winds Culicoides behave as “aerial plankton” and can be transported as far as 700 Km over sea and 300-400 Km over land (Hendrickx et al., 2008). Nevertheless the long range dispersal of culicoides midges seems to play a marginal role in the spreading of disease since at least for BTV it has been shown that more than 90 % of the outbreaks occurred within 31 Km from a previous onset with a modal value for infections of less than 1 Km (Sedda et al., 2012).

Female midges fly into swarms of males to mate; spermatophores of males are stored in one to three spermathecae of the female. After mating, the female looks for a host to blood feed which is essential for the maturation of the eggs. In some species it is the male that goes to hosts likely to attract females looking for a blood meal; mating occurs when she finishes feeding (Ziani Hadj-Henni, 2014).

Eggs are small and elongated, laid in batches, about 400 µm long by 50 µm wide. They are white when laid but gradually turn dark brown. They are usually not resistant to drying and usually hatch within two to seven days giving tiny white larvae (Mellor et al., 2000).

The larvae are worm-shaped and measure 0.3 to 1 cm long. They are apneutical and eucephalic (Courtney et al., 2000). Larvae require water, air and food and are not strictly aquatic or terrestrial. They cannot develop without moisture and can be found in a wide range of substrate including silage residues, dung, leftover cattle feed, compost and decaying wood (Zimmer et al., 2014). They feed on organic material and can be predaceous to microorganisms or small invertebrates, such as nematodes or insect larvae (Aussel and Linley, 1993, 1994; Zimmer et al., 2014)

The four larval stages can last from as little as four to five days to several weeks, depending on the species, temperatures, and geographic area. In temperate countries the diapause behavior of *Culicoides* can reach several months since fourth-instar larvae are commonly found over winter (White et al., 2017).

During the pupal stage the *Culicoides* does not feed. It is brief and typically lasts two to ten days and the Imago finally emerges.

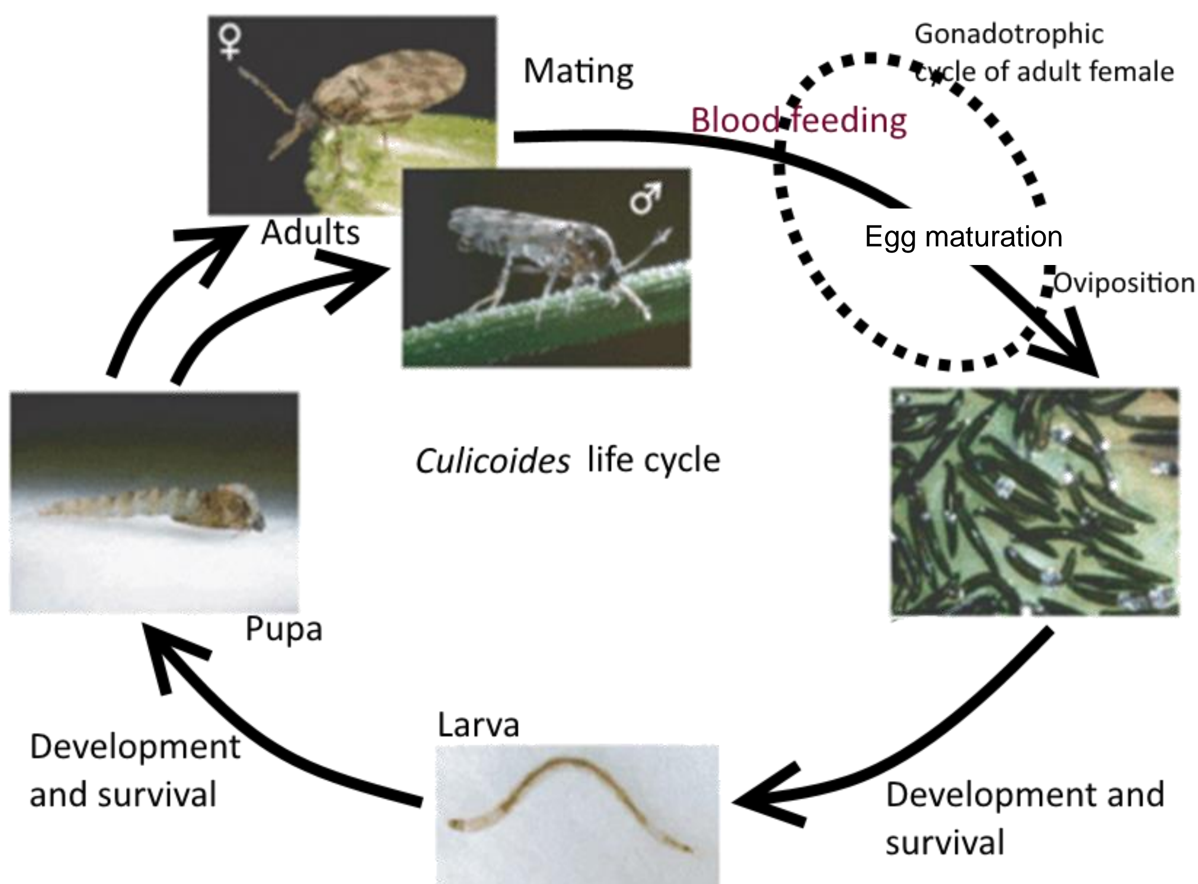

Figure 7. Culicoides life cycle. Adapted from (Purse et al., 2005)

6.2 Vector control

In Europe Mainland most of the work done on the control of *Culicoides* before the 2006 BTV outbreak was realized to limit the biting behavior of *Culicoides* species per se, as it causes nuisances to human and animals, especially horses (Carpenter et al., 2008). BTV and SBV emergences raised the need to limit the viral transmission rate as well. Most of the studies about the control of Palearctic *Culicoides* species were realized in the context of BTV emergence in Europe; yet most of their findings and conclusions should apply to SBV and any other arbovirus vectored by culicoides.

The current control strategies include: a) spraying of space and facilities with residual insecticides/repellents; b) Treatment of livestock with insecticides/repellents; c) Reducing biting risk; d) Management of breeding sites (Carpenter et al., 2008). The broad scale spraying of the environment with residual insecticides is clearly not realistic nowadays for pollution and residues concerns. Treatment of livestock was attempted in several studies. However quite often chemicals and insecticides were tested in laboratory facilities, which quite often does not meet the field conditions. Lambda-cyhalothrin was tested and killed efficiently culicoides. However the midges were brought into contact with treated wooden plates, which in no way is even close from *Culicoides* behavior (Schmahl et al., 2008). Likewise, deltamethrin pour-on treatment of ruminants was assessed by bringing into contact hair clipped from treated animals with culicoides (Mehlhorn et al., 2008). Permethrin and pirimiphos-methyl were tested on calves and authors well observed that a decrease in the number of midges leaving cattle is clearly different from a reduction in culicoides bite rate, since many midges can bite before being incapacitated some others biting again once revived (Mullens et al., 2000). Even though, treatment of sheep with deltamethrin led to a decrease in Culicoides feeding rate in field condition (Weiher et al., 2014), but since a single bite from an infected midge is sufficient to transmit BTV the actual impact on BTV transmission remains inconclusive (Baylis et al., 2008). When based on seroconversion of target ruminants in field conditions treatment with permethrin at a two weeks interval failed to reduce the exposure to BTV (Mullens et al., 2001).

Breeding site management and habitat removal has been demonstrated to have a negligible impact on BTV transmission. Indeed, breeding sites are ubiquitous and only require organic material and some degree of moist, which is usually abundant in the vicinity of ruminants (Mayo et al., 2014).

To prevent AHS in horses the stabling of equids especially at dusk was proven to be an appropriate strategy (Meiswinkel et al., 2000). Although being part of the recommendations formulated during the 2006 BTV8 outbreak, the efficacy of such a measure in the European context is disputable: i) cattle and sheep housing is usually more open than horse stalls, therefore it is easier for the midges to get to the animals; ii) the traditional AHSV vector, *C. imicola*, is clearly exophilic

whereas *C. obsoletus* and *C. pulicaris* just adapt to environmental factors and can behave either as exophilic or endophilic depending on circumstances (Kameke et al., 2017).

During the last decade biological means of control were also investigated. Among entomopathogen organisms, some fungus species are known to infect many insects worldwide and are used to control some major pests (Nicholas and McCorkell, 2014). *Metarhizium anisopliae* was studied on *Culicoides spp*. along with other species showing interesting properties to control larval and adult stages but so far no field trial were performed (Ansari et al., 2011; Narladkar et al., 2015).

Endosymbiotic bacteria were screened in European culicoides species. Indeed endosymbiotic bacteria are known to potentially increase (Graham et al., 2012) or decrease (Hedges et al., 2008) host susceptibility to viruses. Other endosymbionts have the ability to reduce the lifespan of their host therefore interrupting the viral transmission (McMeniman et al., 2009). So far the detection of endosymbiotic bacteria in European culicoides populations could not be linked with alteration in the vector competency of BTV or SBV vectors (Lewis et al., 2014).

Arboviruses are exposed to antiviral mechanisms in both arthropods and vertebrates. In the insect vector RNA interference (RNAi) is a sequence-specific RNA degradation mechanism that has been shown to play a major role in the antiviral response against arboviruses (Schnettler et al., 2013). Both BTV and SBV when used to infect culicoides-derived cell culture managed to generate virus derived small interfering RNAs (Schnettler et al., 2013). Moreover it was later shown than *Culicoides* have the required enzymatic machinery to properly perform RNA silencing through the production of RNAi (Mills et al., 2015). These findings open a way further to the establishment of midges refractory to viral replication (Harrup et al., 2016).

7 Pathogenesis and clinical signs of BTV and SBV

7.1 Bluetongue disease: a viral hemorrhagic fever affecting ruminants

It all starts when the culicoides take a meal of infected blood. Once the culicoides are infected, the virus persists in them throughout their lifespan (Tabachnick et al., 1996). After a blood meal, ingested virus passes through the midgut and spreads via the haemocoele, to the salivary glands. There it replicates and mature virions are released in the salivary ducts (Mellor et al., 2000). Only a fraction of *Culicoides* population gets permanently infected and an even smaller fraction is able to transmit the virus (Fu et al., 1999). Viral particles are then excreted in the saliva of the insect. As soon as four days post-infection viral load can be multiplied by 50 within the culicoides (Pages et al., 2014). Viral transmission occurs by an insect bite during a subsequent blood meal (Fu et al., 1999).
Once the virus reaches the blood flow of the ruminant, BTV multiplies first in the endothelium of blood vessels in the skin and simultaneously in regional lymphatic nodes draining the site of

inoculation (Darpel et al., 2012). It then spreads via blood and via lymph and infects secondary sites and organs, mostly the lung and spleen targeting vascular endothelium, and mononuclear phagocytes (Barratt-Boyes and MacLachlan, 1994). Virus replication occurs in mononuclear phagocytic, dendritic, and endothelial cells (ECs) (Drew et al., 2010b). Viraemia is highly cell associated to each cell type, especially to the erythrocytes (Bonneau et al., 2002). One particular feature is the persistence of BTV within invaginations of the erythrocytes cell membrane; this close association seems to contribute to protect BTV from the host immune system (Brewer and MacLachlan, 1994). Moreover, the extended lifespan of red blood cells might explain the prolonged but not persistent viraemia in the host as well as a more efficient transmission to the culicoides vector. Indeed, the median duration to obtain RTqPCR negative results on naturally BTV8 infected cattle was reported to be 195 days (Zanella et al., 2013). By contrast, infectious virus could only be isolated up to 39 days post infection (Di Gialleonardo et al., 2011). Viraemia and RNAemia length are clearly influenced by the considered serotype since previous studies described a viral isolation possible until 63 days post infection (Singer et al., 2001) and for BTV25 (Toggenburg Orbivirus) a RNA detection over a period of 19-25 months (Vogtlin et al., 2013).

BTV clinical picture, gross lesions and histopathologic lesions are all consequences of vascular injuries mostly of small caliber vessels (Mahrt and Osburn, 1986; MacLachlan et al., 2008). BTV causes direct virus-mediated endothelial injury with thrombosis and infarction as direct consequences of the virus replication in endothelial cells (EC). It is precisely those direct virus-mediated injury to the EC that is likely responsible for ischemia, necrosis and the characteristic cyanotic tongue sometimes reported in affected sheep (Maclachlan, 2011).

Cytokine and endothelial responses are the key elements to determine the outcome of BTV infection (Howerth, 2015). By contrast infection of macrophages and dendritic cells leads to the release of TNF and other vasoactive mediators, all contributing to the increase of vascular permeability, capillary leakage and edema. The redistribution of VE-cadherin, cytoskeleton and adherens junction alterations result in fluid leakage possibly leading to hypovolemic shock and subsequently in the most severe cases to death (Gowen and Holbrook, 2008), also often seen in AHS and other virus-induced haemorrhagic fever (Drew et al., 2010a). Proliferation of BTVspecific CD4+ and CD8+ T cells was demonstrated *in vitro* following the production of pro-inflammatory cytokines (IL-1 β, IL-6,IL-12) by infected conventional dendritic cells (Hemati et al., 2009). Macrophages produce additional vasoactive mediators such as TNF-α, IL-1 β and IL-8 (Drew et al., 2010b). BTV infection of lymph nodes draining the original area of the inoculations stimulates B cells proliferation and release of activated CD8+ T cells, most likely to control an early viral spread (Barratt-Boyes et al., 1995). The target cells of the secondary replication are mostly macrophages, dendritic cells, endothelial cells and lymphocytes (Maclachlan et al., 2014).

The various cytokine, prostanoids and proinflammatory mediators were suggested to trigger a so-called “cytokine storm” by analogy to the pathogenesis of fulminant hemorrhagic fevers (MacLachlan et al., 2008; Maclachlan et al., 2009). The prognosis of the disease is thus better assessed by the acute phase response than by the viral load (Sanchez-Cordon et al., 2013). Likewise, the increased severity of the affection on sheep versus cattle could be related to a higher ratio of plasma thromboxane to prostacyclin in sheep (DeMaula et al., 2002).

Group-specific antibodies are raised against VP7 since this protein is conserved amongst most BTV serotypes and strains (Maclachlan and Mayo, 2013), which can be used for pan-BTV detection by ELISA (Huismans and Erasmus, 1981). Neutralizing antibodies are targeted to regions of the VP2 outer capsid protein and generally only provide protection to homologous serotypes (serotype specific antibodies) (Huismans et al., 1987a; Gould and Eaton, 1990; Maclachlan et al., 2014). The capacity of an infected animal to raise neutralizing antibodies directed towards different serotypes does not seem to be infinite, especially in case of simultaneous inoculation of more than two serotypes. Indeed, possibly through interference mechanisms, usually not all inoculated serotypes do replicate in the host leading therefore to a partial coverage of the neutralizing antibodies generated against the inoculated serotypes (Jeggo et al., 1986). Some neutralizing epitopes are also conformationally dependent: they rely mostly on the interaction between VP2 and VP5, not only on VP2 (DeMaula et al., 2000). Moreover, several studies reported a better neutralization of BTV when both VP2 and VP5 were present (Lobato et al., 1997; Ma et al., 2012). Common neutralization epitopes were demonstrated by serological cross reaction to some extent (Dungu et al., 2004).

The level of antibodies doesn’t always correlate with the level of protection (Jeggo et al., 1984) and animals were reported to be protected even in the absence of circulating neutralizing antibodies (Stott et al., 1985; Sanchez-Cordon et al., 2015). CD8+ cytotoxic T lymphocytes (CTLs) are central in cell mediated immunity although their precise role is currently poorly defined (Andrew et al., 1995). BTV NS proteins are predominantly recognized by CTLs whereas VP2 and VP5 are not. However the extent of cross-reactivity between NS epitopes over different serotypes in not consistent (Jones et al., 1996) and despite the demonstration of CTLs cross-reactivity amongst different BTV serotypes (Calvo-Pinilla et al., 2012) the induction of CTLs targeting NS1 expressed in a recombinant adenovirus failed to confer protection to vaccinated animals (Maclachlan et al., 2014).

It has however to be clearly stated that in many cases BTV infection remains very mild or even subclinical. Sheep are clearly the most sensitive species, and fine wool breed like merinos or Sardinian sheep are particularly susceptible (Shimshony, 2004; Maclachlan et al., 2009; Verwoerd, 2012) although some more recent work suggested a lesser influence of the breed on BTV infection outcome than originally thought (Caporale et al., 2014). Other individual factors influence the clinical outcome of the disease, such as the immune status, the nutritional state and broadly speaking the

general health of the animal (Osburn, 1994a; Wackerlin et al., 2010). The usual asymptomatic display of BTV on cattle along with the prolonged RNA detection in this species led to consider bovine as the reservoir of the virus (MacLachlan, 1994).

Incubation period ranges from 2 to 15 days (Erasmus, 1990). Among the commonly reported clinical signs, some are not specific, such as transient and unsystematic hyperthermia, weakness, depression and anorexia (Maclachlan et al., 2009). Other clinical signs are more suggestive and include salivation, abundant serous or mucous-purulent nasal discharge with accumulation of crusts on the nostrils' wings, edema (especially of the head and distal end of limbs), congestion and ulceration of the oral mucosa, conjunctivitis and, rarely and mainly in sheep, cyanosis of the tongue, hence the name of the blue tongue disease (Cox, 1954; Elbers et al., 2008a; Dal Pozzo et al., 2009b). In lactating animals, teat ulceration leads to a steady decline in milk production that can last up to 4 months when infected with BTV8 and losses between 0.3 and 3.4 % (Santman-Berends et al., 2011a; Nusinovici et al., 2013). As a result of edema of limbs, stiffness and lameness are reported. In the most severe cases, death occurs within 8-10 days after the onset of clinical signs as a result of respiratory distress. The animal may also succumb later to a secondary bacterial pneumonia (Parsonson, 1990; MacLachlan, 2017).

Semen quality in rams has been reported to be decreased and correlated to viral RNA load following natural BTV8 infection (Leemans et al., 2012b). Affected semen reached back standard values 63-138 days after clinical bluetongue disease diagnosis (Kirschvink et al., 2009). Transient infertility in rams and bulls and viral shedding in semen has been indeed reported previously with other BTV serotypes as well (Osburn, 1994b). Viral nucleic acid found in the semen is well documented and seems to be clearly associated to the contamination of the sperm with red blood cells or mononuclear cells (Osburn, 1994b). Under experimental conditions the transmission of BTV to susceptible cows through contaminated semen could be demonstrated in a limited number of animals (Bowen et al., 1985). However this route of transmission is very unlikely to play an important role in BTV epidemiology. Moreover the hypothetic persistent infection or latent infection of calves born from dams infected during their pregnancy, once popular and mainly investigated from the late 70’s to the late 80’s, could not be supported by field and experimental data (Walton, 2004).

BTV8 exposure in naïve dairy herd has been associated to an increase of return-to-service rate from 8 to 21 % on a 90 days base return to service. Fertility was significantly decreased in cows inseminated from one month before to one month after the detection of the disease in the herd (Nusinovici et al., 2012).

The 2006 BTV outbreak in Central and Western Europe caused severe losses with mortality rates around 1-1.5 % in cattle and up to 40% in sheep and case fatality of about 3-5 % and up to 50 %,

respectively for cattle and sheep (Elbers et al., 2008b; Conraths et al., 2009; Santman-Berends et al., 2011b) .

BTV8 was characterized by an unusual ability to cross the placental barrier when compared to classical field wild type serotypes and strains (Desmecht et al., 2008; Wouda et al., 2008). The lesions described in new-born ruminants were similar to previously reported ones with infections with North American serotypes of BTV from 85 to 125 days of pregnancy (MacLachlan et al., 1985a). Namely, congenital cerebral cysts, thin-walled cerebral hemispheres, dilated cerebral ventricles were reported (porencephaly and hydanencephaly). As a consequence an increased number of “dummy calves” were also detected following the 2006 BTV8 outbreak (Wouda et al., 2008), sometimes associated with blindness and a “blue eye” syndrome (Holzhauer and Vos, 2009). When infection occurs later on pregnancy calves display encephalitis but usually no brain malformation (Waldvogel et al., 1992a).

7.2 Schmallenberg virus is an important teratogenic agent of ruminants

SBV infection starts as does BTV infection: by the bite of an infected *Culicoides* female midge (Rasmussen et al., 2014).

The influence of NSs protein on viral pathogenesis of *Peribunyaviridae* is established for several virus members of the family (Bridgen et al., 2001; Weber et al., 2001; Verbruggen et al., 2011). Since SBV is like BTV a potent INF inducer it has developed mechanisms to counteract the antiviral state of the infected cell. To that end NSs was demonstrated – at least partly thanks to its nucleolar localization - to promote the degradation of RPB1 (a subunit of RNA polymerase complex of the cell) and as a consequence hinders the global transcription level in the cell (Gouzil et al., 2017) . NSs represents so far the major identified virulence factor of SBV (Kraatz et al., 2015).

SBV infection of adult healthy cattle is usually quite similar to BTV infection since most animals only show mild clinical signs if any. The clinical picture is aspecific and includes transient fever, diarrhea, and anorexia (Conraths et al., 2013; Wernike et al., 2014c). However the milk production loss could range from barely noticeable (Veldhuis et al., 2014b) to a 50 % reduction lasting for 3-11 days (Doceul et al., 2013; Agerholm et al., 2015). Unlike BTV it seems that adult cows are more severely affected than sheep or goats (Doceul et al., 2013). SBV viral RNA can only be detected for a very limited amount of time in the blood, lasting for 2-6 days in cattle (Hoffmann et al., 2012). The main impact of SBV on ruminants is its teratogenic potential when the dams are infected during pregnancy and the virus crosses the placental barrier. The rate of effective transmission from the mother to the offspring showing pathological lesions seems to be low (about 0.5%) but the nature of the malformations could be dramatic and often fatal (Veldhuis et al., 2014a). By contrast, the risk of

SBV infection of the fetus after formation of the placenta was also estimated to be 28 % (Garigliany et al., 2012a).

The extent of the teratogenic effects depends on the timing of the infection and foetal development; fast dividing cells in the CNS are the target (Varela et al., 2013). Broadly speaking, the pathogenesis of SBV in sheep and cattle foetuses is similar to those of other ruminant orthobunyaviruses (Peperkamp et al., 2015). The cattle fetus builds-up its immunocompetency starting around 40 days of pregnancy up to 175 days whereas the lamb fetus starts at 20 days of pregnancy and ends around 115 days (Herder et al., 2013). During this time thymus and lymph nodes are colonized by immune cells; the fetus is nonetheless already capable of inflammatory response (Agerholm et al., 2015). The critical timeframe appears to match the period when CNS cells are differentiating the most, between 45 and 165 days of pregnancy in cattle (Wernike et al., 2014a). By analogy with AKAV infection of the fetus before 40 days of pregnancy might be unlikely since placentomes are considered to be key elements to allow the passage of the virus to the fetus and require to be vascularized and developed enough which is believed not to be the case this early in the gestation (Parsonson et al., 1988).

The lesions potentially presented by the calves affected *in utero* by SBV could be distinguished according to two entities depending on the stage of pregnancy when infection occurs: a hydrocephaly / hydranencephaly syndrome and a torticollis / arthrogryposis syndrome. The shorter gestation in small ruminants does not allow such a distinction and malformations are part of a usually joint hydranencephaly / arthrogryposis syndrome (Parsonson et al., 1981b). SBV appears to have an increased tropism for rapidly dividing immature cells such as those present in the nervous system and skeletal muscles of the fetus, directly inducing encephalomyelitis and necrotizing polymyositis. If the fetus survives, it will be by eventually presenting lesions of hydrocephaly / hydranencephaly, porencephaly, microcephaly, non-suppurating encephalomyelitis, arthrogryposis or torticollis (Tarlinton et al., 2012). Congenital lesions may also include dilation of the cerebral ventricles, paralysis of the tongue, brachygnatia inferior, blindness, deafness, general weakness, lameness, limb torsion, and muscle atrophy; kyphosis and spina bifida can be observed occasionally (Garigliany et al., 2012b; Garigliany et al., 2012c; Bayrou et al., 2014; Brulisauer et al., 2017). The most severe lesions occur after infection of mothers at earlier stages of gestation, reflecting the large population of vulnerable cells and the lack of a fully competent immune system. Microphthalmia can also be observed. Behavioral abnormalities similar to the “dummy calf/lamb syndrome” caused by BTV8 were also reported (van den Brom et al., 2012). The musculoskeletal malformations are considered to be secondary to the lesions of the CNS. Defects in the spinal chord would have consequences on a detrimental development of the ventral horns and subsequently arthrogyposis and vertebral column malformations (Parsonson et al., 1977; Peperkamp et al., 2015). As described earlier BTV8 and some

other BTV vaccine strains are known to induce hydranencephaly in domestic ruminants (Vercauteren et al., 2008). The lesions caused by the Border Disease Virus (BDV) and Bovine Viral Diarrhea Virus (BVDV), both pestiviruses, are characterized by hypomyelinogenesis and CNS dysplasia along with a reduction in the number of ventral motor neurons, like in Akabane virus infection (Clarke and Osburn, 1978). However in case of BTV, BDV or BVDV infection arthrogryposis cases are neither as frequent nor as severe as with SBV. In addition, primary involvement of muscle cells has been demonstrated in the fetus of sheep and cattle infected with Akabane virus (Kurogi et al., 1976; Kurogi et al., 1977; Parsonson et al., 1988). Thus, according to several authors, this increased frequency of musculoskeletal disorders in case of infection *in utero* with SBV could be explained in particular by the primary involvement of the fetal muscles (Kurogi et al., 1977; Edwards et al., 1989).

SBV RNA has been reported in bull semen several months after natural infection but no SBV infection by using SBV positive semen in AI has been demonstrated so far (Ponsart et al., 2014). SBV RNA positive semen has been used to subcutaneously inoculate cattle and interferon α/β receptor deficient (IFNAR -/-) mice with confirmed infection of several cattle; however the subcutaneous route is obviously different from the intrauterine route used in AI (Schulz et al., 2014).

After a natural infection, SBV specific antibodies remain in adult cows for at least two years. Colostrum-derived maternal antibodies are lost after 5-6 months (Elbers et al., 2014), which is close from the extent of the protection conferred by the colostrum-derived antibodies against AKAV (Tsutsui et al., 2009).

7.3 Host range: BTV and SBV affect many wild and domestic ruminants

All ruminants are susceptible to infection with BTV; clinical disease is most often manifested in sheep whereas cattle (and goats) are considered reservoir species. In wildlife a serious disease develops in white-tailed deer (*Odocoileus virginianus*) and pronghorn antelope (*Antilocapra Americana*) (Parsonson, 1990; Johnson et al., 2006; Drolet et al., 2016). The haemorrhagic syndrome caused by BTV was also reported in mule deer (*Odocoileus hemionus*), wapiti (*Cervus elaphus canadensis*), desert bighorn sheep, bison (*Bison bison*), elk (*Cervus elaphus*), mountain goat (*Oreamnos americanus*) along with African antelope species and other wild ruminants (Howerth et al., 2001). Camelids, Old World (Batten et al., 2011) as well as New World species (Schulz et al., 2012a; Schulz et al., 2012b). The African elephant, wildebeest and buffalo usually show a high seroprevalence (Coetzee et al., 2012). Outside North American wild ungulate species clinical disease was only reported in topi, or sassebi antelope (*Damaliscus lunatus*) (Wells, 1962). Experimental or natural infection of antelope, wapiti, musk, ox, bison, yak, white-tailed deer and African buffalo also produced clinical disease, whereas blesbock, mountain gazelle, roe deer, red deer and Eurasian elk did not show clinical sign after natural or experimental infection, yet BTV viral RNA or specific

antibodies could be detected (Niedbalski, 2015). The cases of iatrogen infections of lynxes and dogs was previously discussed, and BTV specific antibodies were also identified in African carnivores including lions, cheetahs, wild dogs, jackals, hyenas and large-spotted genets (Alexander et al., 1994). Similarly to dogs seropositive for AHSV (usually acquired by feeding with infected horse meat) (Braverman and Chizov-Ginzburg, 1996) these predators were most likely infected by ingestion of flesh and organs of BTV infected animals. In North America BTV specific antibodies have been found in Florida black bears (*Ursus americanus floridanus*) and Florida panthers (*Puma concolor coryi*) (Dunbar et al., 1998).

BTV has indeed never been demonstrated to infect humans (Hu et al., 2008). Nonetheless BTV might be of interest in human medicine. DsRNA viruses were demonstrated to display interesting oncolytic properties for at least 20 years (Strong et al., 1998). However and unfortunately, the very majority on those oncolytic viruses are also human cancer viruses (Wildner, 2003). BTV constitutes quite an exception: it was demonstrated to preferentially infect and destroy several types of human cancer cells, without prior genetic modifications and preserving normal human cells (Li, 2011). Therefore BTV based treatment represents an innovative perspective to treat human cancer, yet to be further investigated. BTV's potential in human medicine may also provide benefits to the treatment of AIDS. The interferon induction power of BTV leads to the production of many antiviral factors very useful in the resistance to AIDS, acting on different levels from preventing entry of HIV in host cells, restriction of HIV release and significant suppression of HIV in stimulated macrophages (Dai et al., 2015).
To date, SBV has never been related to a human disease, the viruses most closely related are non-zoonotic and serological studies realized on at risk human subject provided negative results (Ducomble et al., 2012; Reusken et al., 2012); for all these reasons the European Centre for Disease Prevention and Control declared the zoonotic risk of SBV as "very unlikely" (Tarlinton et al., 2012).
European livestock paid the greatest toll to SBV infection, mainly cattle, sheep and goats. In addition SBV has been isolated or confirmed by PCR in bison, roe deer and red deer, whereas SBV specific antibodies have been detected in roe deer, red deer, fallow deer, alpaca, mouflons, ibex, water buffalo and wild boar (Linden et al., 2012; Azkur et al., 2013; Garcia-Bocanegra et al., 2017; Rossi et al., 2017). Laloy et al. reviewed the seroprevalence in exotic ruminant species in zoos from the SBV epizootic area and found out 18 of these species could be affected by this virus, even when kept in urban areas. Animals born in Europe were also part of the sample therefore likely excluding a preliminary infection by a SBV-like virus (Laloy et al., 2016). Low and transient levels of SBV neutralizing antibodies could be detected in pigs in experimental infections (Poskin et al., 2014b). Recently a limited seroprevalence (5-7%) was demonstrated in horse population from Iran (Rasekh et al., 2018). By contrast, serological studies in United-Kingdom could not find evidences of SBV antibodies in local equids (EFSA, 2014). Evidences of SBV susceptibility were also found in zebras, Asian elephants and babirusa (Molenaar et al., 2015). Once again however these results usually based

on ELISA tests or VNT should be interpreted with the greatest caution. The ELISA multispecies kit mostly used in the above mentioned studies targets anti-nucleoprotein antibodies and cross-reactivity with other viruses from the Simbu serogroup was reported (see point 4.1 and figure 4). VNT results were also demonstrated to be at times inconsistent with ELISA results on the same samples, forward pointing to a SBV-like virus rather than SBV (Molenaar et al., 2015).

7.4 Viral Warfare and immune evasion

All pluricellular organisms display defence systems to prevent infections. In vertebrate the immune response can be divided in innate and adaptive responses. The innate immune response represents the first line of defence, with the objective to slow down, to interfere with viral replication in order to contain the infection and subsequently to allow the adaptive immune response to build up (Stanifer et al., 2017). Parts of the critical components of the innate immune response are the type I and type III interferon (IFN) families. The IFN proteins are cytokines related by structure, regulation and function (Levy et al., 2011). In most mammal species type I IFN proteins are encoded by more or less a dozen of genes for IFN-α, a single gene for IFN-β and several other genes for the other IFN proteins belonging to the type I family (Levy et al., 2011). They act as inducers of other antiviral effectors, display antiproliferative and proapoptotic properties, and contribute to the maturation of dendritic cells, NK cells, and cytotoxic T lymphocytes (Vitour et al., 2014). Both orbiviruses and orthobunyaviruses are known for decades to be very potent INF inducers, in particular BTV (Jameson et al., 1978; Raju and Kolakofsky, 1988). Indeed dsRNA viruses induce the production of type I interferon (IFN-α/β), among other inflammatory cytokines, both *in vivo* and *in vitro*, and is studied since at least 40 years (Kerr et al., 1974). The production of IFNs is also recognized to be a key determinant of peribunyaviruses pathogenesis (Elliott and Weber, 2009).

Following viral intrusion the infected cell recognizes pathogens associated molecular patterns (PAMP) and then start to produce IFN. dsRNA and ssRNA are types of well-known PAMP, along with non-methylated CpG DNA and envelop glycoproteins. Type I IFN cytokines are produced following the mediation of several distinct receptors named pattern recognition receptors (PRRs). The best characterized PRRs are i) the TLR3 (Toll-like receptor 3); ii) the RIG-I (retinoid acid-inducible gene I) or MDA5 (melanoma differentiation-associated gene 5)-MAVS (mitochondrial antiviral signalling) mitochondrial pathway (Broquet et al., 2011); iii) the PKR (dsRNA-activated protein kinase) pathway (Vitour et al., 2014), and (iv) the TRIF-dependent DexD/H-box helicases (Zhang et al., 2011).

Since the IFN signalling pathway is the cornerstone of the innate antiviral immune response, most viruses have elaborated evasion mechanisms. Viruses circumvent the IFN response through different mechanisms and among the most common are: (i) interfering with host cell gene expression and/or

protein synthesis; (ii) minimizing IFN induction by limiting the production of viral PAMPs and/or by specifically blocking IFN-induction cascades; (iii) inhibiting IFN signalling; (iv) blocking the action of IFN-induced effectors with antiviral activity (Randall and Goodbourn, 2008).

7.4.1 Interfering with host cell gene expression and/or protein synthesis

BTV was reported to induce different gene expression profiles whether the infected cells were conventional dendritic cells (cDC) or plasmacytoid dendritic cells (pDC). Among these two subset the cDC are specialized in antigen presentation whereas pDC are specialized in IFN-I production. During the course of the BTV infection the number of DC in blood decreases. The remaining pDC circulating in the blood flow were demonstrated to have overall pro-inflammatory genes up regulated whereas pDC and cDC found in lymph nodes expressed mostly an anti-inflammatory profile (Ruscanu et al., 2013). Cytoplasmic helicases RIG-I and MDA5 were demonstrated to mediate the expression of INF-β as well as the control of BTV infection in non-hematopoietic cells (Vitour et al., 2014). By contrast in hematopoietic cells like plasmocytoid dendritic cells (pDC) MyD88 adaptor, PKR and stress-activated protein kinase (SAPK)/Jun N-terminal protein kinase (JNK) were implicated (Ruscanu et al., 2012). Thus BTV reduces the number of pro-inflammatory pDC circulating in blood and modifies the gene expression of the remaining DC in the lymph nodes to an anti-inflammatory state, resulting overall in a lesser immune response.

Furthermore, BT is known to induce apoptosis through caspase-3 activation and trigger selective depletion in cell subsets, in particular CD8+ T lymphocytes (Umeshappa et al., 2010a). This also could be associated to a lower IFN-α expression. As a consequence, the destruction of IFN-producing infected cells could lead to immune-suppression and secondary bacterial infections, quite often described in BTV infected sheep (MacLachlan and Thompson, 1985; Umeshappa et al., 2010a).

Several studies involving other peribunyaviruses than SBV suggested that the NSs protein inhibits cellular mRNA transcription (Thomas et al., 2004). Barry et al. (2014) demonstrated that the SBV NSs protein targets the RPB1 subunit of the RNA Polymerase II for degradation, preventing transcription in infected cells and consequently *de novo* protein synthesis (see also point 7.2). The C-terminal region of NSs is critical for these inhibitory functions (Barry et al., 2014). The inhibition of cellular transcription leads to a reduction in IFN production as well that facilitates SBV replication and spread. The NSs of BUNV can also affect cellular apoptosis by reducing or delaying cell death (Kohl et al., 2003). LACV NSs by contrast tends to enhance apoptosis. The same authors reported a pro-apoptotic effect of SBV NSs, similar to LACV NSs.

7.4.2 Minimizing IFN production

Chauveau et al., (2013) reported the drastic inhibition of IFN-β activity triggered by NS3 (Chauveau et al., 2013). First, using a luciferase assay BTV was shown to greatly reduce the INF-β promoter activity. To identify the responsible viral protein(s) the different BTV8 ORFs were tested using tagged plasmids. NS3 induced a significant decrease in IFN-β promoter activity following stimulation of the RIG-1 pathway (Chauveau et al., 2013).

As part of the induction of an antiviral state in BTV infected and neighboring cells the activation and phosphorylation of interferon regulatory factor 3 (IRF3) was demonstrated. The nuclear translocation of IRF3 however was impaired in BTV infected cells. Interestingly BTV does not degrade IRF3 but sequesters it in cytoplasmic viral factories. Viral inclusion bodies are therefore not only believed to be the sites of viral replication and morphogenesis but could also take part of an immune evasion mechanism (Stanifer et al., 2017).

More recently it was demonstrated that BTV NS4 downregulates the host INF-I and mRNA levels, making NS4 a key determinant of viral virulence and possibly acting in synergy with NS3 (Ratinier et al., 2016). In addition, the inhibitory activity of NS4 was reported to be linked to its nucleolar localization, a rare feature for BTV proteins given the exclusive cytoplasmic replication. The recently discovered NS5 also shares a nucleolar localization and could act synergistically with NS4 (Stewart et al., 2015).

BTV passaged in insect vectors or in insect cell culture could induce less IFN than viruses derived from mammalian cells. Indeed, KC cells (derived from *C.sonorensis*) were reported to better sustain BTV replication (Anderson et al., 2014) and alphaviruses grown in mosquito cells induce less IFN than their mammalian grown counterparts (Shabman et al., 2007). Moreover, BTV8 transmitted *via* the bite of Culicoides induced a delayed IFN response when compared to needle infection and as a consequence led to a higher viraemia and an increased severity in the clinical picture (Pages et al., 2014).

SBV uses cap-snatching to keep the production of viral PAMPs to a minimum while initiating mRNA transcription. RdRp protein is believed to have cap-dependent endonuclease activity. In the cytoplasm, host mRNAs are cleaved from the 5’ end and the capped nucleotide residues are then used to prime transcription of the viral genome (Coupeau et al., 2013b). Once capped, viral mRNA is concealed from RIG-1 recognition (Pages et al., 2014).

7.4.3 Inhibition of IFN signalling

BTV is able to inhibit the expression of the IFN-stimulated genes following two mechanisms involving signaling pathways. The mechanism involved will depends on the timing of infection: in early infection BTV blocks the phosphorylation and nuclear translocation of STAT1. Later in the course of infection BTV will downregulate the Janus tyrosine kinase (JAK1) signal/transducer and TYK2 proteins expression (Doceul et al., 2014).

In OROV interference with IFN signaling was hypothesized as a specific mechanism involving NSs but to date there is no clear evidence of such an action for SBV NSs (Livonesi et al., 2007). RIG-I-like receptors (RLR) are a cytosolic receptor family shown to be involved in SBV viral RNA recognition (as for most of the negative single stranded RNA viruses)(Blomstrom et al., 2015). An alternative immune evasion strategy described for severe fever with thrombocytopenia syndrome virus (SFTSV) involves the action of NSs to relocalize RIG-I and some other signaling molecules into NSs induced endosome like cytoplasmic structures (Santiago et al., 2014). Whether or not such mechanisms do exist for SBV requires investigations.

7.4.4 Inhibition of INF-induced antiviral enzymes

In order to avoid host cell shut off and apoptosis, BTV produces capped mRNA internally within a core particle and releases ssRNA as the only viral genome in the cytoplasm of infected cells (Diprose et al., 2002). dsRNA is tightly associated with the BTV core as the release of viral dsRNA in the infected cell would be of dreadful consequences for the ongoing BTV infection.

8 Diagnosis

8.1 Clinical differential diagnosis

Since both BTV and SBV infect mostly the same species, are both arbovirus, both are teratogenic agents causing not dissimilar congenital lesions in the affected offspring and cause quite often mild and aspecific clinical signs in adult animals, they are clearly part of their reciprocal differential diagnosis.

SBV and BTV infection should be distinguished from other Orthobunyaviruses, such as the Akabane and Aino viruses, or Cache Valley virus (belonging to the Bunyamwera serogroup, circulating in North America). Other orbiviruses, such as Chuzan virus, belonging to the Palyam serogroup, isolated in Japan following a series of malformed calf births (Goto et al., 1988), are to be included in the differential diagnosis. Due to the potential for birth defects, BVDV, BDV and Wesselsbron disease virus are also to be considered (Rovid Spickler, 2010). *Neospora caninum* is an

important abortion agent in cattle throughout the world, and may also cause non-suppurating encephalomyelitis in congenital affection of calves. In this case, the condition is manifested by nervous disorders including proprioceptive deficits, arthrogryposis, and can lead to complete paralysis of the animal (De Meerschman et al., 2005). Nutritional causes (maternal deficiency in selenium and / or manganese at the beginning of gestation), toxic (ingestion of lupins between 40 and 70 days of gestation) or physical (exposure to ionizing radiations) can be envisaged (Oryan et al., 2011).

Regarding clinical Bluetongue disease in adult cattle or sheep, basically any condition showing oedema, haemorrhages and epithelial damage should be considered in the differential diagnoses. Moreover, it includes all the vesicular diseases, and some of them are notifiable diseases. In particular, foot-and-mouth disease, vesicular stomatitis, rinderpest and *peste des petits ruminants*, capripoxvirus, malignant catarrhal fever, BVD, IBR and bovine papular stomatitis should be considered in cattle. In sheep Orf should be considered additionally. For both species any other condition possibly leading to lameness, ocular or nasal discharge, respiratory distress and oedema should be taken into consideration (Williamson et al., 2008; Savini et al., 2011).

8.2 Laboratory diagnosis

The identification of BTV or SBV could be carried on following two different approaches:

1. Identification by direct diagnosis
2. Identification by indirect diagnosis

Direct diagnosis is based on the identification of viral antigen(s) by virological assays, classical virus isolation or detection of viral nucleic acids.

Indirect diagnosis is mostly based on the identification of antibodies by serological assays.

Specifics and details on some of the most common assays are provided for BTV in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2014).

Direct diagnosis detects actual presence of the virus itself (viral nucleic acids or antigen) and therefore is the most certain method of determining an infection. Viral isolation can be highly time consuming and expensive. RTqPCR, on the other hand can be achieved in a considerably shorter time and can be serotype specific in the case of BTV. Another direct serotype-specific direct diagnostic technique is the virus neutralization test (VNT). VNT requires the availability of a characterized immunized serum bank, and can become highly laborious depending on the epidemiological context (Hamblin, 2004). Moreover, high throughput is not possible to achieve.

Indirect diagnosis is based on the detection of the antibodies of interest from the serum of infected animals. Besides historical serological tests currently almost totally abandoned due to their relative lack of sensitivity or cross reactivity, ELISA remains the most recommended tests for both SBV and BTV due to their usually high sensitivity and specificity. The serotype identification is however not possible with such tests (Breard et al., 2017). To identify BTV to the serotype level seroneutralization test (SNT) is required. Like for VNT, several parameters complicate the standardization of SNT protocols and inter-lab repeatability (Worwa et al., 2013).

8.3 SBV direct diagnosis

SBV is diagnosed using RTqPCR to detect the viral nucleic acid (Doceul et al., 2013). The FLI has developed and disseminated across Europe two new RTqPCRs targeting either the S segment or the L segment currently used to detect SBV. However, RTqPCR is limited by the short-lived viremia presented in SBV-infected animals. Indeed, in case of congenital defects, malformations can be observed although the virus could be eliminated, thus making it impossible to detect antigens or nucleic acids of the virus. In the case of post-natal infection of cattle, the RNAemia is also brief, 2 to 5 days according to the first experimental data (Beer et al., 2013). Different kits targeting the segments S or L are commercially available.
Viral isolation has been successfully achieved from blood samples from clinically affected cows (Hoffmann et al., 2012).

Currently viral isolation is performed after a first passage on KC cells (larval cells of *Culicoides variipennis*) followed by inoculation of BHK-21 cells. The cytopathic effect is evident after 5 days of incubation (Hoffmann et al., 2012).

Interestingly, placentomes appear to be a tissue in which Orthobunyaviruses are more frequently isolated in case of *in utero* infection; Parsonson et al. hypothesized that the fetal-maternal interface may be a difficult access environment for neutralizing antibodies, and thus allow an increased replication (Parsonson et al., 1981b).
In case of clinical suspicion of SBV in adults, the etiology may be confirmed by RTqPCR. A negative viremia, because of the brevity of the latter, does not allow to permanently dismiss the suspicion of SBV.

8.4 SBV indirect diagnosis

The FLI has developed seroneutralization and indirect immunofluorescence tests. Researchers at CVI Wageningen have also developed a serum-neutralization test that was used in the first seroprevalence study on livestock in one of the affected countries. The development of serological

tests made possible to confirm the involvement of SBV in many cases of malformations in the absence of viral RNA detection. Using SBV lab isolated strains it has also been possible to detect neutralising antibodies in the serum of infected animals with virus neutralization tests (VNT) (Mansfield et al., 2013). However the classical VNT requires several days to be completed and cannot be automated, stressing the need of a faster mean to identify SBV. Therefore an indirect ELISA test was designed and developed by ID-Vet (Montpellier, France) later validated (Breard et al., 2013). Usually in the commercially available kits the plates are coated with SBV N protein (nucleoprotein). The major drawback is a described cross-reactivity towards SBV related orthobunyaviruses. Other commercial kits have been developed allowing to detect SBV antibodies on different matrixes (Humphries and Burr, 2012).

In the absence of positive RTqPCR results, the monitoring of antibodies specific to SBV by serology coupled at 3-week intervals (ELISA or seroneutralization test) may be necessary to complete the diagnosis.

8.5 Diagnosis of congenital SBV infection

In case of suspicion of congenital lesions or abortions caused by SBV, the first examinations to be carried out will be: i) the detection of antibodies specific for SBV in the serum of abortions or newborns before taking colostrum (ELISA or seroneutralization), (ii) the detection of SBV RNA by RTqPCR from a piece of placentome and, if possible, from the brain of aborted fetuses or neonates. Otherwise, EDTA blood and spleen can also be tested by RTqPCR, but the virus appears to be less frequently detected in these organs than in the CNS (personal data). In the aborted fetus, different combinations could be found: seropositive but vironegative, or mother seropositive and vironegative fetus (van Maanen et al., 2012).

If the approach is part of a diagnosis of abortion without particular suspicion of SBV, it may be useful to test the serum of the mother to detect SBV specific antibodies, their absence allowing to remove this virus from the potential etiologies of abortion in a context of a naïve livestock. In case of suspicion of SBV, the samples to be taken are presented in Table 2.

Table 2. Tissues and organs to sample in case of SBV suspicion. From (Parsonson et al., 1981a; Rovid Spickler, 2010; Martinelle et al., 2012).

	Adult/mother	Fœtus/stillborn	Storage	
			24-48 h	Long term
Serology	**Blood (dry tube)**	**Blood (dry tube) before/after colostrum intake**	2-20°C	-20°C
RTqPCR	**Blood (EDTA tube)**	**Blood (EDTA tube) before colostrum intake**	2-8°C	-80°C
		Brain		
		Peritoneal fluids		
		Placentome		
		Umbilical cord		
		Placental fluids		
		Spleen		
		Thymus		
Viral isolation and IHC	**Blood (EDTA tube)**	**Blood (EDTA tube) before colostrum intake**	2-8°C viral isolation) ; formaldehyd 10 % (IHC)	-80°C (viral isolation) ; formaldehyd 10 % (IHC)
	Encephal (cattle, if dead)	**Brain**		
		Placentome		
		Thymus		
		Spinal cord		
		Affected muscle		
		Spleen		
		Kidney		
		Heart		
		Lung		
		Lymph nodes		

8.6 BTV direct diagnosis

BTV can be isolated from blood, mainly the red blood cells fraction. The first step to successfully isolate BTV is to inoculate embryonated chicken eggs followed by passages on VERO or BHK-21 cells (Afshar, 1994). Indeed direct isolation on cell culture is tricky and can fail to isolate weak positive samples (Billinis et al., 2001).

First record of PCR being used for BTV diagnosis goes back to 1992 (Akita et al., 1992). In the late 90's and early 2000's several RT-PCR have been developed, targeting segments 2, 3, 6, 7 or 10 (Toussaint et al., 2007). Since 2006 molecular diagnosis techniques have considerably evolved. RTqPCR assays developed in Europe following the BTV8 outbreak aimed at detecting all BTV serotypes. Toussaint et al. designed a broadly used protocol and primer sets, targeting a fraction of S5 and later designated as "pan-BTV" (Toussaint et al., 2007; Zanella et al., 2013). Currently many commercial "ready-to-use" RTqPCR kits are available. Recently several of these kits were assessed and were found to be reliable and robust (Sailleau et al., 2017b). More recently serotype specific RT-PCR protocols detecting BTV1 to BTV26 (Maan et al., 2012a) and RTqPCR protocols amplifying the serotype specific segment 2 of serotypes 1 to 27 and 29 were developed (Maan et al., 2016).

8.7 BTV indirect diagnosis

Agar gel immunodiffusion (AGID) and competitive ELISA (cELISA) using VP7 as antigen were the first widely used diagnostic techniques. In-house ELISAs routines using commercial kits have been quickly developed following the 2006 BTV8 outbreak (Batten et al., 2008a; Batten et al., 2009) and AGID are practically no longer in use. Later sandwich (double-antigen) ELISAs have been introduced and provided evidences of their capacity to detect BTV antibodies earlier than cELISAs kits (Oura et al., 2009). However sandwich ELISA (sELISA) have been demonstrated at risk of false negative results if tested animals were infected longer ago: sELISA manage to detect BTV antibodies early in the course of the infection but are less sensitive than cELISA if the infection took place 4 weeks ago (Eschbaumer et al., 2011a).

Recently Bréard et al. developed a microsphere assay using fluorescent beads allowing a simultaneous detection of group and serotype specific antibodies (Breard et al., 2017).

9 Vaccines

Currently the best way to control BTV outbreaks remains vaccination (Maclachlan and Mayo, 2013). The ideal vaccine has to be safe, cheap, if not universal at least to prevent RNAemia and further transmission of not only one but several serotypes/strains and allows DIVA (Feenstra and van Rijn, 2017). To date, only modified live vaccines and inactivated vaccines are available for BTV whereas only inactivated vaccines are available for SBV. However commercial vaccines face two major issues: i) they are unable to allow the distinction between vaccinated and infected animals (Differentiating Infected from Vaccinated Animals – DIVA); ii) against BTV they fail to confer cross-protection to the ever growing number of different serotypes (Noad and Roy, 2009).

9.1 BTV and SBV modified live vaccines

Modified live vaccines (MLVs) have a long history since they were the first BTV vaccines to be developed (Theiler, 1908a). Most of these vaccines were developed in South Africa and since such vaccines are still currently in use, covering 15 different serotypes divided in three different vaccine formulation (Coetzee et al., 2012). Most of these vaccines are produced using BTV strains passaged between 19 and 70 times on embryonated chicken eggs and a few additional passages on BHK or VERO cells (Alpar et al., 2009). They are cheap to produce and induce a long lasting immunity with only one single shot (Monaco et al., 2004).

Nevertheless, BTV MLV vaccines present several potential disadvantages. As they do replicate very efficiently they could lead to severe clinical disease even possibly fatal (Veronesi et al., 2005). Safety is particularly at stake when under attenuated vaccines are used, like with vaccines that did not undergo a sufficient number of passages (Savini et al., 2008). Indeed this active replication can increase the viraemia to the point it could infect biting midges and as a consequence give rise to a vaccine-derived BTV outbreak; it may also revert to a virulent form and/or the segments in the vector may reassort with wild-type viral genes, resulting in a reassortant vaccine strain possibly infectious and pathogenic (Batten et al., 2008b). Teratogenic effects and abortions were described as well following the use of MLV (Savini et al., 2004). MLV seem to have a transient and limited impact on semen quality (Breard et al., 2007) and no effect on milk production (Giovannini et al., 2004). In addition, no DIVA strategy exists with MLVs.

Vaccination using a SBV mutant lacking NSs and a combined NSs/NSm deletion mutant has been assessed. The double deletion mutant caused no viraemia and vaccinated animals were fully protected when challenged with a wild-type SBV, which might open a new track for SBV MLV use (Kraatz et al., 2015). Nonetheless to date only SBV inactivated vaccines have been provided with a marketing authorization.

9.2 BTV and SBV inactivated vaccines

Regarding BTV, possibly the best strategic option for control of clinical outbreaks in the European endemic areas has been vaccination of susceptible livestock with inactivated vaccines to protect against disease and i) to exclude the possibility of reversion to virulence of the vaccine viruses and ii) avoid reassortment between vaccine and field strains of the virus (Saegerman et al., 2008). Inactivated vaccines were produced from whole virus preparation killed using an inactivating agent such as heat, gamma radiation, UV light or chemicals like binary ethylenimine (Savini et al., 2007). Inactivated vaccines are clearly safer than MLVs since they are theoretically unable to produce any viraemia, although there are reports of short-lasting BTV RNA detection in the blood of naïve then

vaccinated cattle (De Leeuw et al., 2015). Neutralizing antibodies raised following the use of an inactivated vaccine according to an appropriate protocol was demonstrated to last at least 4 years (Batten et al., 2013a). By contrast, naturally infected animal were tested 6 years post infection and still exhibited a strong humoral response; nonetheless this does not mean that vaccine-induced humoral immunity is in any way shorter (Eschbaumer et al., 2012). Quite surprisingly the change from one commercial inactivated vaccine to another between the primovaccination and the booster shot was reported to produce more neutralizing antibodies than using the same vaccine during the whole protocol along (Bartram et al., 2011).

The use of BTV inactivated vaccines has been associated to a 4 % increase of return-to-service in cattle when the animals were vaccinated between 3 days before and 16 days after AI (Nusinovici et al., 2011). By contrast, BTV8 vaccination of rams did not appear to cause any defects in semen quality (Leemans et al., 2012a).

SBV vaccines and immunological mechanisms are far less studied than BTV ones. SBV inactivated vaccines protect efficiently vaccinated animals (Wernike et al., 2013b). Moreover, as cattle need two vaccine shots, the complete protection of sheep can be achieved using a single vaccine dose (Hechinger et al., 2014). Inactivated vaccines against other closely related Orthobunyaviruses – namely AKAV, AINOV and Chuzan virus - were demonstrated not to be efficient against SBV (Hechinger et al., 2013).

Despite the numerous advantages of inactivated vaccines, they do have some limitations. They are more expensive to produce and require additional inactivation and purification steps. They are usually formulated with adjuvants and animals need several shots to be properly immunized (Mayo et al., 2017).

A summary of currently available and potential future vaccines is provided in table 3.

Table 3. A summary of BTV vaccine strategies. Adapted from (Mayo et al., 2017)

Vaccine strategy	Design Strategy	Advantages	Disadvantages
Inactivated vaccines	Virus killed by heat, UV radiation, or chemicals and mixed with adjuvant. The adjuvant non-specifically stimulates the immune system to respond to the viral antigens present on the killed viruses.	• Relatively safe, Cannot reassort with field strains	• Multiple doses required • Local reaction to adjuvant possible • Immunity may be transient

Modified live vaccines	Live virus attenuated by serial passage in alternate cell cultures or host(s). The viruses replicate (ideally) to a low level after vaccination, producing progeny viruses that stimulate virus-specific immunity.	• Cost effective • Single dose immunity possible • Immunity can last for years	• Reversion to virulence • Side effects (decreased lactation, abortion) • Reassortment with natural strains • Transmission by vector possible
Recombinant vector vaccines	Non-pathogenic viruses (other than BTV or SBV) are genetically modified to produce antigenic BTV or SBV proteins that stimulate specific antibodies. The modified viruses are administered in the vaccine, and they replicate to a low level within the host after vaccination.	• Strong neutralizing immunity possible • Potential for single dose immunity	• Difficult/expensive to design
Disabled infectious single cycle vaccines	Deletion of an essential gene results in BTV or SBV virions that can only replicate one time after vaccination. Purified single-cycle viruses are administered in the vaccine.	• Low risk of reversion to virulence	• Difficult/expensive to design • Multiple doses likely necessary
Virus-like particle vaccines	Genetically modified baculovirus vectors grown in insect cells express BTV or SBV structural proteins, which assemble into empty viral particles. The vaccine consists of purified viral particles and adjuvant.	• High stability • Low risk of side effects • No risk of disease	• Multiple doses required • Difficult/expensive to design and manufacture • Local reaction to adjuvant possible

9.3 Novel vaccines and perspectives

Next development in BTV and SBV vaccines would follow presumably three different paths: i) using genetically modified viruses; ii) using virus vectors to express immunogenic BTV or SBV proteins; and iii) using preparations of immunogenic proteins themselves (Mayo et al., 2017). The main goals of these next generation vaccines are to tackle some of the major drawbacks of inactivated and MLV. To limit the spread and the reversion to virulence and the ability to distinguish vaccinated from infected animals are part of these major objectives.

Disabled Infectious Single-Cycle (DISC) vaccines are infectious virus particle able to infect and replicate only once in the host cell due to the deletion of an essential gene (Feenstra and van Rijn, 2017). In the case of BTV the DISC vaccine lacks the expression of VP6 (Matsuo et al., 2011). Therefore DISC vaccines provide the efficacy of MLVs and the safety of inactivated ones. However, since all the viral proteins are present the perspective of DIVA development is unlikely.

Another type of newly designed vaccines generated by reverse genetics is the Disabled Infectious Single-Animal (DISA) vaccine. Basically, it pushes forward the knocking out of MLVs using genetic engineering to prevent unwanted spread and viraemia (Feenstra et al., 2014b). For BTV this is done by deleting NS3/NS3A, leading to complete avirulence, only local viral replication, and prevention of transmission to vector (Feenstra et al., 2014a; van Gennip et al., 2014). By contrast to DISC vaccines, since NS3/NS3A expression is lacking in DISA vaccines they are clearly in line with a potential DIVA approach, given the use of NS3 based ELISA tests (Feenstra et al., 2014a; Tacken et al., 2015).

DNA vaccines have been assessed in BTV and SBV. For both viruses protocols and reduction of viraemia did not fully reached the objectives. Furthermore the use of DNA vaccines is submitted to logistic limitations such as complex protocols, high vaccine load or increased number of boost shots (Li et al., 2015; Boshra et al., 2017).

Vectored vaccines use various combinations of immunogenic viral proteins (usually immunogenic capsid and envelop proteins) expressed through the replication of a different live attenuated virus. The non-pathogenic vector virus replicates at high level allowing the expression of a high number of immunogenic proteins in host cells (Boone et al., 2007). A lot of different recombinant vector viruses have been studied for BTV: capripox virus, canary poxvirus, fowlpox virus, equine herpesvirus 1, bovine herpesvirus 4, canine adenovirus, human adenovirus-5, vesicular stomatitis virus and vaccinia virus (Mayo et al., 2017).

Subunit vaccines and virus-like particles (VLPs) are non-replicating vaccines based on a similar principle: incomplete portions of the complete mature viral particle are sufficient to induce a protective immunity. BTV VP2 alone has been demonstrated to elicit a protective immune response in sheep (Huismans et al., 1987a). Subunit vaccines are produced by purification or on recombinant baculovirus or E.coli systems with the addition of adjuvants (Legisa et al., 2015; Feenstra and van Rijn, 2017). By contrast with subunit vaccines which are basically immunogenic proteins cocktails with adjuvants, VLPs are empty viral particles, with a conformation similar to fully functional mature virions yet lacking genetic material (Hewat et al., 1994). DIVA should be possible as replication complex and non-structural proteins are not present. Results so far have been very promising also *In vivo* on target species and the reasons no VLPs vaccines are currently marketed is unclear. It however seems that the production method is not cost effective enough when compared to currently marketed vaccines (Feenstra and van Rijn, 2017). Figure 8 illustrates the different generations and evolution of the characteristics of BTV vaccines.

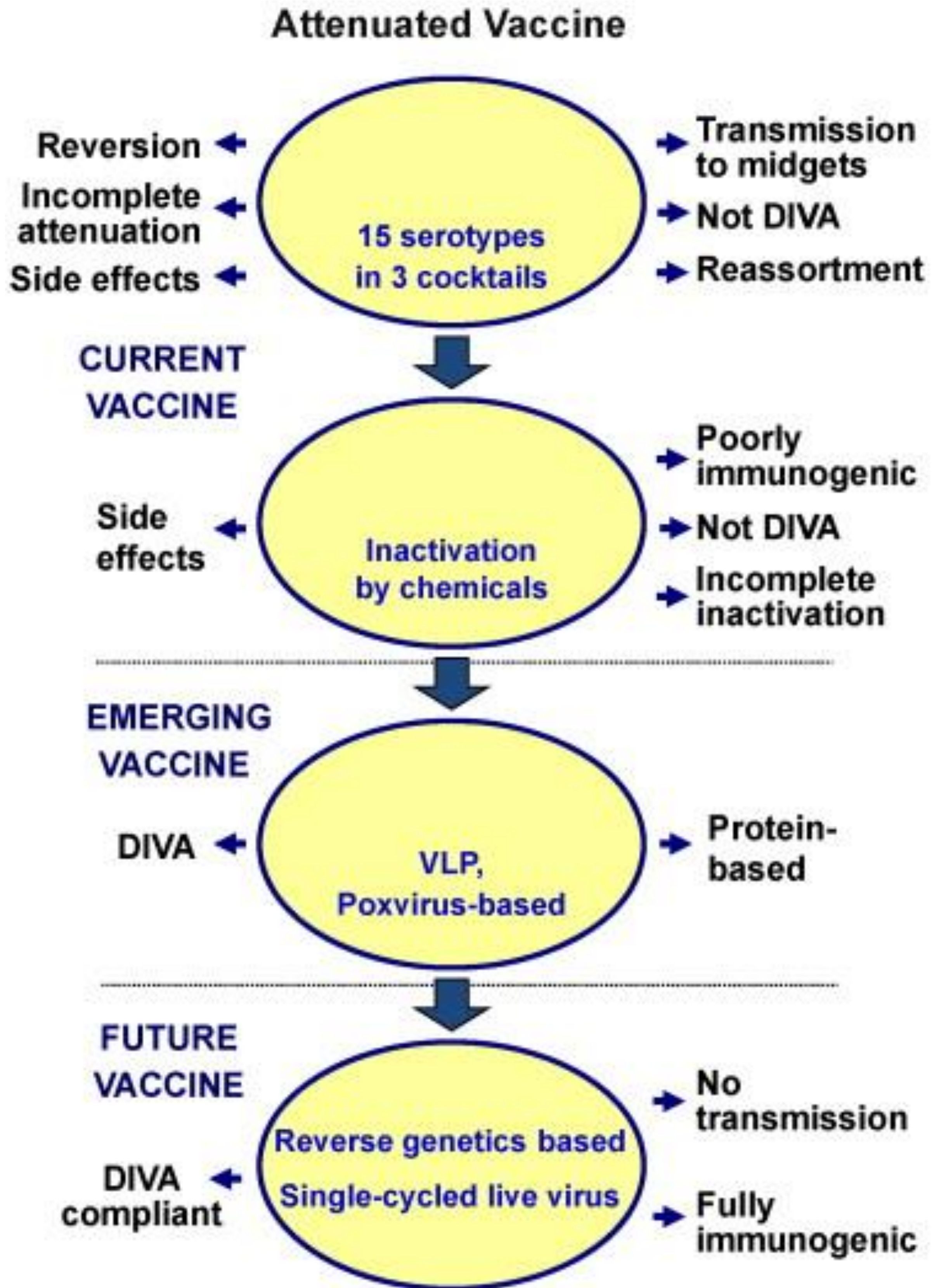

Figure 8. Characteristics and evolution from attenuated to future BTV vaccines. From (Noad and Roy, 2009).

Objectives

The unexpected emergence of BTV8 in Western and Northern Europe and the later spread of other serotypes, soon followed by the onset of SBV just a couple of years later stimulated policy makers both at European and Belgian levels to develop research programs aiming at a better understanding of the pathogenesis of these diseases and to clarify their potential impact on livestock industry.

General objective

Emerging and/or newly discovered viruses affecting domestic ruminants trigger the need for data acquired within the smallest time frame in order to manage efficiently the diseases.

The general objective of this thesis was to increase the knowledge about the pathogenesis of BTV and SBV in ruminant host-species and as a consequence to improve the decision making process in the context of vector-borne emerging diseases showing epizootic potential and possibly requiring high biosafety measures.

Specific objectives

As previously stated, the current thesis can be divided in three parts:

Part I: Gaining additional knowledge about the pathogenesis of BTV and SBV especially to develop reliable and standardized animal infectious models on ruminant host-species (studies 1 and 2).

Part II: Clarifying the pathogenesis of BTV and SBV in pregnant cattle and sheep, respectively (studies 3 and 4).

Part III: Studying cross-reactivity, super-infection and compare serotypes clinical outcome with a particular emphasis on BTV serotypes historically present in Europe mainland (studies 5 and 6).

More specifically with respect to the six studies presented in this manuscript the goals were:

1) Study 1: To better understand the kinetics of the appearance of the clinical signs of Bluetongue disease and to identify the most precocious ones (prerequisites for the establishment of an early warning system); moreover, to compare and discuss the clinical picture obtained with two different kind of inoculum (infectious blood and cell-cultured virus passaged a limited number of times) *versus* the disease as observed in the field.

2) Study 2: To assess the best way to reproduce *in vivo* SBV RNA detection and humoral immune response in sheep following experimental infection compared to field infection, with particular emphasis on the inoculation route.

3) Study 3: To study the mechanisms of protection and persistence of the BTV in pregnant dairy cattle and to clarify the circumstances of the placental crossing. To provide data about the teratogenic potential of BTV8.

4) Study 4: To obtain insight in the pathogenesis of SBV in pregnant sheep. This will allow having a better understanding in the vulnerable period in which SBV infection causes congenital malformations in lambs.

5) Study 5: To evaluate the effect of co- and superinfection with BTV1 on calves previously infected with BTV8. The fortuitous contamination of the inoculum with BTV15 added the supplemental objective of providing a preliminary discussion about the level of serotype-dependant adaptation to the mammal host.

6) Study 6: To compare the pathogenicity and virulence of BTV serotypes surrounding Western and Northern Europe on cattle, to assess the protection conferred by a preliminar vaccination against BTV8 and update serological cross-reactivity between BTV serotypes in the European epidemiological system.

Experimental section

Experimental section

Study 1 :

Two alternative inocula to reproduce bluetongue serotype 8 disease in calves

Vaccine 29 (2011):3600-3609

Martinelle L, Dal Pozzo F, Sarradin P, De Leeuw I, De Clercq K, Thys C, Ziant D, Thiry E, Saegerman C.

Preamble

This study has been realized in order to answer to an intriguing question involving bluetongue virus and the development of a reliable and reproducible experimental model, namely the role of the inoculum in the outcome of the disease. The unexpected emergence and subsequent rapid spread of bluetongue serotype 8 in Western and Northern Europe had been remarked for several reasons. Amongst these, the high virulence against bovine raised several questions around the pathogenesis and the protection mechanisms in this host.

Data relative to experimental BTV infection in the litterature are difficult to compare as different studies have used a variety of viruses of different serotypes, different passage history and inoculating different animal species/ breeds in different experimental settings.
In addition, the OIE still recommends to use infectious ruminant blood in BTV vaccine trials instead of cell culture adapted virus. The interesting question is whether BTV once isolated in cell culture loses virulence compared to BTV isolated from blood of an infected animal. This is a pertinent question because BTV can be attenuated after extensive passaging in cell culture. In addition, isolation of BTV in tissue culture from an infected animal is not straightforward and requires passaging of infected blood in embryonated eggs (or insect cells) before the virus can replicate in mammalian cells. In other words, BTV does not adapt easily in mammalian cells without prior passaging in embryonated eggs. Thus, it is clear that the viral "population" growing in tissue culture is somewhat selected from wild type BTV found in the blood of an infected animal and this could lead to a reduction of virulence.

In this paper we compare the effects of infectious blood and low cell-passaged virus on the outcome of the disease, and discuss their respective impact on the pathogenesis of BTV8 in bovine.

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Two alternative inocula to reproduce bluetongue virus serotype 8 disease in calves

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ABSTRACT

The aim of this study was to investigate the consequences in calves of two forms of inocula alternative to the use of wild type infectious blood. Two groups of five calves were infected with low cell-passaged virus and infectious blood issued from one animal passage of the same strain. A longitudinal study was implemented and characterised by clinical standardised observations, haematology, BTV RNA detection and viral isolation from blood, detection of serogroup and neutralising antibodies, cytokine expression and post-mortem examination 46 days post-infection (PI). Both tested inocula were able to reproduce clinical expression of the disease, in the bloodstream viral genome was detected until the end of the experiment while virus isolation was possible between days 7 and 31 PI. Humoral immune response developed earlier in calves infected with low cell-passaged virus, while in both groups a massive antibody production was confirmed by the immune balance between IL-4 and IFN-γ expression. Both tested inocula are presented as valid alternative to the use of wild type infectious blood in the study of the pathogenesis of BTV-8 or the efficacy of current and future vaccines.

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1. Introduction

Bluetongue (BT) is a non-contagious disease affecting ruminants and is caused by the bluetongue virus (BTV). BTV is transmitted by blood-feeding midges of the genus *Culicoides* (*Diptera Ceratopogonidae*) [1]. A broad spectrum of wild and domestic ruminants can be infected and severe clinical signs are mainly seen in certain breeds of sheep and some *Cervidae* species [2,3]. The severity of infection depends on various factors, such as species, breed, age, nutritional and immune status of animals, and environmental stresses, as well as the virulence of the BTV strain involved [4]. Although clear differences in virulence of BTV isolates are known, the virulence determinants are still poorly defined [4]. Clinical manifestations are closely linked to virus-induced vascular injuries and the role of species-specific endothelial cell-derived inflammatory and vasoactive mediators has been highlighted [5].

The European BTV-8 outbreak was characterised by peculiar features [6]. Among these features, a remarkable severity of the lesions in cattle was noticed [7]. Field evidence has demonstrated the ability of this BTV-8 strain to cross the placental barrier and subsequently to induce congenital infections in domestic ruminants, a feature previously attributed to cell-attenuated strains [3,8]. The striking pathogenesis of BTV-8 in Europe raises the question of the evolution undergone by this virus. In order to understand the virulence and the pathogenesis of the European BTV-8 strain in cattle as well as to assess the safety and the efficacy of current and future vaccines, a reliable animal model reproducing the features of the natural disease is required.

In the Bluetongue chapter of the *OIE Terrestrial Manual* 2009 (http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.03_BLUETONGUE.pdf), the use of wild type virus with no embryonated chicken eggs (ECE) or cell culture passages is recommended during the challenge of vaccinated animals in order to test the efficacy of the vaccine. Recently, when comparing infectious blood obtained from a naturally infected animal during the 2007 BTV-8 epidemic in Germany and culture-grown virus in experimentally infected sheep, the authors did not observe

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differences. They suggested the use of cell-passaged virus in BTV-8 challenge experiments [9].

In the course of a preliminary experiment with two calves infected with a low cell-passaged European BTV-8 inoculum, clinical expression of the disease, as well as viraemia and seroconversion were observed [10]. The current study was implemented to assess the accuracy and reproducibility of the described protocol using the same low cell-passaged inoculum. Furthermore, in parallel another group of calves were infected with viraemic blood issued from the preliminary experiment [10]. At the time of the experimental planning, no BTV-8 circulation was detected and consequently no viraemic blood from naturally infected animals was available. This condition represented a restriction to the progress of the study, while the use of a one animal passage inoculum could overcome this inconvenient. Furthermore, this approach has been successfully used to determine the duration of viraemia in experimentally infected calves [11].

In this study two groups of infected calves and one group of environmental control calves were monitored for 46 days and were subsequently euthanised for post-mortem examination. The clinical signs of the disease, the detection of viraemia and serogroup- and serotype-specific antibodies, a complete haematological examination and a preliminary investigation of cytokines expression have been realised.

2. Materials and methods

2.1. Animals

Twelve female Holstein calves aged between 5 and 6 months were used. They were bought 3 months before the beginning of the experiment in a French BTV-free area. The calves and their dams were tested to ensure their seronegative and non-viraemic status for BTV, conditions regularly verified during the next three months. Furthermore, during the three months of acclimatization an experienced veterinarian examined the calves to establish their healthy and asymptomatic condition (this was a thorough examination carried out according to the standardised checklist for the general clinical examination proposed by Jackson and Cockcroft [12]). Haematological analysis confirmed blood parameters within the physiological range. The calves were tested negative also for bovine viral diarrhea virus (BVDV) and for bovine herpesvirus 1 (BoHV-1).

2.2. Virus

BTV-8 strain BEL2006/01 isolated during the 2006 epidemic season in Belgium was used [13]. The virus was isolated from EDTA-blood of a symptomatic bovine on embryonated chicken eggs (ECEs) and was subsequently propagated on BHK-21 cell culture. The second passage of the virus on BHK-21 cells constituted the cell-passaged inoculum of this experimental protocol (BEL2006/01 BHK-21 P2). This inoculum was used to infect two calves (3179 and 3181), which became clinically ill, as reported in our previous work [10]. Viraemia was monitored and at the viraemic peak (8 days post-infection) blood was collected and stored at −80 °C. The infected blood of the experimentally infected and symptomatic calf 3181 was designated as BEL2006/01 calf 1.

The infectivity of BEL2006/01 BHK-21 P2 and BEL2006/01 calf 1 was assessed by inoculation of ECEs, as described by Bréard et al. [14]. Serial 10-fold dilutions of each viral inoculum were used to inoculate five ECEs/dilution and the viral titre was expressed as 50% embryo lethal dose (ELD_{50}/ml) [15].

2.3. Experimental design

Starting 3 months before the infection, calves were confined in an insect-secure biosafety level 3 zone at the Experimental Infectiology Platform (PFIE) of the National Institute for Agronomic Research (INRA) – Research Centre of Tours (Nouzilly, France). Animals were treated in accordance with the International Guiding Principles for Biomedical Research Involving Animals, as issued by the Council for the International Organizations of Medical Sciences. The experimental infection was performed from mid January to early March 2009, during the period of reduced vector activity (CIRAD, http://bluetongue.cirad.fr/FichiersComplementaires/2009-2_LettreInformationSurvCulicoides.pdf). Electrical insect killing light traps were installed at the entrance to the experimental zone. One week before the infection, the daily body temperatures of the calves were recorded and haematological analyses were performed for use as reference basal values.

Calves were separated into three physically isolated groups: group A (5 animals infected with BEL2006/01 BHK-21 P2), group B (5 animals infected with BEL2006/01 calf 1) and group C (2 animals, non-infected, as environmental controls). In both groups of infected calves, the administration route of the inoculum was strictly intravenous (IV) via the jugular vein. Calves of groups A and B were infected with respectively 1 ml inoculum and 15 ml of heterologous blood, corresponding to $10^{4.5} ELD_{50}$/animal for group A and 10^3 ELD_{50}/animal for group B. Animal management was carried out always starting with group C, followed by group A and group B. Between each group, operators changed their gloves and laboratory clothes, and dipped their boots in a quaternary ammonium/formaldehyde based footbath.

After 46 days PI, animals were euthanised following an IV injection of pentobarbital. Before slaughtering, body condition was evaluated and body condition scores were attributed to each calf (for details, see the necropsy and histopathological examination section).

2.4. Clinical examination

The animals were monitored daily by the same veterinarian during the entire course of the experiment. Body temperature and the development of clinical signs were examined daily and at the same time of day (in the morning) [12]. A standardised clinical report form for assessing BTV infection was used during the examination of the animals [16], which was performed as previously described [10]. Since the quantification of the severity of the clinical signs was a major objective of the study, a total clinical score was calculated for each animal daily, at the end of the experiment and for each group of clinical signs.

2.5. Haematology

Starting from the acclimatization week and for the first 3 weeks PI, a complete haemogram (Vet ABC, SCIL animal care company, France) was performed daily. Thereafter, haematology was performed once a week until the end of the experiment. In order to take into account individual variation in each parameter, the values during the acclimatization period were combined and expressed as a standardised baseline value for each animal (starting index equal to 100).

2.6. BTV detection in blood

EDTA-blood was collected daily until the end of experiment and immediately stored at −80 °C. After thawing, RNA extraction was achieved using the QIAamp Viral RNA Mini Kit (Qiagen, Germany).

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These samples were used to detect BTV RNA, and viral RNA denaturation, amplified segment, internal control and real-time RT-PCR (RT-qPCR) by itself were the same as described by Vandenbussche et al. [17]. Quantification cycle values (Cq) for each sample were used to compare the daily blood RNA viral load [18,19].

In order to detect and measure viable infectious virus in the bloodstream, EDTA-blood samples were used contemporarily for BTV isolation on ECEs [14]. BTV isolation trials were performed at the Belgian BTV reference laboratory by an experienced investigator, starting from the first day of BTV RNA detection. In order to detect the presence of viable virus during the viraemic peak, the following time points were systematically tested (7, 10, 15 and 31 days PI). In the presence of a positive isolation at 31 days PI, another attempt was made at 42 days PI. Five ECEs were used to isolate the virus at each time point.

2.7. Serology

Whole blood was collected daily until 15 days PI, then twice a week to the end of the experiment. Collected sera were subsequently stored at −20 °C. Group-specific anti-BTV antibodies were detected using a commercial competitive ELISA (ID Screen® Bluetongue Competition ELISA kit, ID Vet, France) according to the manufacturer's instructions.

Neutralising anti-BTV-8 VP2 antibodies were detected and measured in the sera of the infected calves at several time points (days 9, 11, 13, 18, 25, 32 and 45 PI). At each time point, serial 2-fold dilutions of the calf serum were tested in duplicate. The neutralising antibody titre was defined as the reciprocal of the serum dilution causing a 50% reduction in cytopathic effect. Serum samples with a titre <20, =20 and >20 were considered negative, doubtful and positive, respectively.

2.8. Cytokine expression

In order to investigate the immune balance between the type 1 helper T cell (Th1) and type 2 helper T cell (Th2) pathways in the three different groups of calves, we measured the evolution of INF-γ and IL-4 mRNA expression levels using two bovine reference genes, β-actin and glyceraldeyde-3-phosphate dehydrogenase (GAPDH), as an endogenous control. Relative quantification of gene expression was performed using the comparative Cq method [20].

Starting from fresh EDTA-blood collected during the first three weeks of infection, peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque PLUS (Amersham Biosciences, Belgium) following the manufacturer's instructions. After the last centrifugation step, PBMCs were suspended in 1 ml TRI Reagent solution (Applied Biosystems, USA) and stored at −80 °C. Total RNA isolation was performed according to the manufacturer's instructions and RNA quantity and quality were determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Belgium). Only samples with A_{260}/A_{280} ratios of between 1.8 and 2 were used for further analysis. To remove residual DNA, 1 μg of sample was digested using an RQ1 RNase-Free DNase (Promega, Netherlands). Reverse transcription of mRNA was carried out using the TaqMan Reverse Transcription Reagents (Applied Biosystems, USA), in a 10 μl volume and with a final concentration of 2.5 μM of oligo $d(T)_{16}$, 1.25 U/μl of MultiScribe Reverse Transcriptase, and 0.4 U/μl of RNase Inhibitor (Applied Biosystems, USA). Reverse transcription was performed with the following steps: 25 °C for 10 min, 48 °C for 30 min and 95 °C for 5 min. Real Time PCR was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems, USA) in the iCycler (BioRad, Belgium).

For each sample, bovine β-actin [13], bovine GAPDH and INF-γ [21] and bovine IL-4 [22] were amplified in separate tubes and in duplicate within the same PCR run using a first denaturation step at 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. Normalization to β-actin and to GAPDH was performed after verification of the absence of any influence of BTV infection on the expression levels of these two bovine reference genes. Furthermore, the efficiency of amplification of the target genes (INF-γ and IL-4) and the reference genes was equalised, by performing validation experiments on serial cDNA dilutions [20].

With the aim of standardizing individual variation, only samples with β-actin and GAPDH mRNA expression levels included in the confidence interval of 95% were considered. In order to normalize the expression level of INF-γ and IL-4 to the two endogenous reference genes, the ΔCq value was calculated (Cq value of the target gene subtracted from the Cq value of the reference gene). For each animal and at each time point, the balance between the Th1 and Th2 pathways was assessed by the ratio of the ΔCq of the IL-4 and the ΔCq of the INF-γ (Th2/Th1) (for the same reference gene).

2.9. Necropsy and histopathological examination

In order to estimate the effect of the disease on weight and growth, a body condition score was attributed to all the animals just before slaughtering.

Samples of spleen, lung, heart, bowel, tongue, thymus, femoral bone marrow, pulmonary artery, kidney, and of prescapular, submandibular, mediastinic and mesenteric lymph nodes were collected from infected and control calves and stored at −80 °C for virus detection. The same organs were stored in a 4% formaldehyde solution for histopathology analysis. Pictures of the organs were systematically taken. Necropsy scores (from 0 to 3) were attributed to organs showing gross lesions depending on the severity of the lesions.

In order to rule out the presence of concomitant pathogens, Gram staining and culture on blood and Gassner agar under aerobic condition were performed on pericardic effusion liquid when present.

BTV RNA detection was performed on all the collected organs starting from approximately 100 mg of tissue, which was processed using Trizol-LS reagent according to the manufacturer's instructions (Gibco Invitrogen, UK). BTV and bovine β-actin detection were performed by RT-qPCR as described above.

Histopathology examination was performed on haematoxylin–eosin stained sections of the organs preserved in formaldehyde and then paraffin embedded.

2.10. Data analysis

The trends in time of clinical scores by group of animals were analyzed using Spearman's rank correlation, referred to as r_s in the text [23]. A two-factor ANOVA with repeated measures on one factor (e.g., BTV antibody) was used to compare the kinetic of different parameters between groups of animals [24]. The validity conditions (homogeneity of variances and covariance matrixes) were preliminary tested [25]. For each specific time point, the comparison of quantitative parameters with unequal variance was compared with Welch's test and frequencies and proportions were compared with Fisher's exact test [23]. For all tests, P values < 0.05 were considered significant.

3. Results

3.1. Clinical scores and examination

While the 2 non-infected calves did not show any clinical signs of infection and remained healthy during the entire experiment, animals in both groups A and B showed clinical conditions compatible with BT disease. Compared to the acclimatization week, slight

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Table 1
Kinetic of the cumulative clinical score by category of clinical signs after BTV-8 infection of calves with low cell-passaged virus (group A) and infectious blood (group B).

	Day post infection																															Total score
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	42	43	44	45	
[a]*Body temperature*																																
A	3	1	2	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	11
B	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
[b]*Oral lesions*																																
A	0	0	2	3	3	3	4	4	3	1	1	2	2	8	6	6	6	6	6	4	2	1	1	1	0	0	0	0	0	0	0	75
B	0	1	4	5	6	7	6	7	7	6	5	4	4	3	3	6	8	6	4	4	4	0	0	0	0	0	0	0	2	2	0	104
[c]*Prescapular lymph node*																																
A	0	0	1	1	2	2	2	2	1	2	1	1	2	3	3	2	2	3	1	2	3	3	3	1	1	2	2	1	2	3	2	56
B	0	0	0	0	1	1	1	1	2	1	3	3	2	3	2	1	2	1	2	1	0	0	0	2	2	2	4	2	2	3	1	45
[c]*Submandibular lymph node*																																
A	0	0	1	0	1	1	2	2	1	2	1	1	1	2	2	1	2	2	1	1	1	1	1	1	2	1	2	2	0	1	0	36
B	0	0	0	0	2	2	3	2	3	2	3	3	3	1	2	0	0	0	0	0	0	0	0	0	2	3	4	2	5	2	0	44
[d]*Ocular signs*																																
A	0	0	1	2	2	1	1	0	0	0	1	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	11
B	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
[e]*Locomotor lesions*																																
A	0	0	1	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
B	0	0	0	0	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
[f]*Total clinical score*																																
A	3	1	8	7	9	9	9	8	6	5	4	4	8	14	12	9	10	11	8	7	6	5	6	3	3	3	4	3	2	4	3	194
B	1	1	5	7	9	10	11	10	12	9	12	11	10	7	7	7	10	7	6	5	4	0	0	2	4	5	8	4	9	7	1	201

[a] Body temperature score: 0 (body temperature ≤ 39.5 °C), 1 (body temperature ≥ 39.6 °C ≤ 39.9 °C), 2 (body temperature ≥40 °C < 41 °C), and 3 (body temperature ≥ 41 °C).
[b] Oral lesion score: 0 (absence of clinical signs), 1 (mucosal congestion), 2 (presence of erosion, ulcer or scabs not related to an ulcer's healing process). In the presence of a scab as a consequence of the healing process, a score of 1 was attributed.
[c] Lymph nodes: 1 (easily palpable, firm consistency, or swollen, warm or if painful) and 0 (other presentation).
[d] Ocular lesions score: 0 (absence of ocular clinical signs), 1 (ocular discharge), 2 (conjunctivitis was contemporarily observed), and 3 (with complicated ocular dermatitis).
[e] Locomotor system score: 0 (absence of clinical signs), 1 (congestion of the coronary band), 2 (congestion and oedema), and 3 (lameness).
[f] Total daily clinical score: equal to the sum of the previous categories of scores. Numbers are underlined when the sum of scores by group is equal to or higher than 10.

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pyrexia was observed in one animal in group A (calf 9103) at 2 and 4 days PI (40.3 °C) and in one animal from group B (calf 2125) at 3 days PI (39.7 °C). A mild increase in body temperature was measured at 7–10 days PI involving most of the infected calves (data not shown), although hyperthermia was never observed (Table 1). However, in the course of the study, no statistically significant difference was found in the recorded temperatures between the two groups of infected calves (two-factor ANOVA with repeated measures on one factor; $P > 0.05$).

The calves of both infected groups showed oral inflammation of various kinds, starting at 3 days PI. These lesions included reddening of the region around the lingual frenulum, red patches on the dental pad and the hard palate, ulcers, general erythema and oedema (more noticeable on the gums). Lesions evolved on the same pattern with an initial reddening of the frenulum of the tongue, followed by oedema of the gums and reddening of the inside of the cheeks. Calf 2125 (group B) showed foam-forming salivation, starting at day 9 PI and initially related to the feeding period, permanent the next day and lasting for 5 more days. The same calf had spontaneous bleeding in the oral cavity following handling of the tongue and gums for photographic purposes on day 5 PI. In calves from group A, lesions appeared to be less severe, mostly characterised by erythema and swelling of different levels in the mouth. Later lesions, beginning at 14 days PI, occurred mainly in the form of crusts on the dental pad, the nostril, the muzzle and the lower lip. No animal showed submandibular oedema. In addition, some animals showed slight conjunctivitis. No pastern swelling or any limping problems were observed.

The scores attributed to each animal in the course of the study are presented in Table 1. In the acute phase of the infection, total clinical scores of calves in group B were higher than in group A, whereas later on (after 14 days PI), a reversal was observed. Differences between the total clinical scores of groups A and B were not statistically significant during the entire period of observation ($r_s = 0.53$; $P = 0.001$), whereas the scores of both the infected groups showed significant differences when compared to control group C ($r_s < -0.23$; $P > 0.05$).

3.2. Haematology

The mean of the measured haematological parameters was calculated within each group and no variations were observed in the red blood cell count, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin concentration or enumeration of platelets (data not shown).

In both groups of infected calves, variations in the leucocyte population compared to the acclimatization week and to the non-infected calves were observed. While the number of lymphocytes was not affected, neutrophil and monocyte index counts underwent relevant fluctuations, contrasting with the stability of the same parameters in the non-infected calves. On the first days PI, a slight reduction, without statistical significant difference (two factor ANOVA with repeated measures on one factor, $P > 0.05$), in the neutrophil index count was measured in groups A and B compared to basal levels and to group C (Fig. 1A). Between 10 and 12 days PI, the neutrophil index count progressively increased in groups A and B, and exceeded the basal values. The index count of these two groups remained constantly and significantly higher than in group C for approximately 10 days (two-factor ANOVA with repeated measures on one factor; $P < 0.05$), before following a slow decrease, nevertheless not sufficient to get back to normal values within 44 days PI in infected groups. Interestingly, in both groups of infected calves, monocytes underwent a rise simultaneously to the neutrophils (between 10 and 12 days PI, Fig. 1B). The enhanced index count of monocytes remained steady for approximately 10 days (two-factor ANOVA with repeated measures on one factor; $P < 0.05$), before returning to reference value levels.

3.3. BTV viraemia

BTV RNA was detected in the EDTA-blood samples of both infected groups (A and B) but not in the control group (C). Table 2 shows the Cq values obtained in the course of the study. Bovine β-actin gene was constantly detected in the EDTA-blood samples of the three groups of calves (data not shown).

Viral RNA could be detected systematically throughout the experiment in four calves of both infected groups. BTV RNA was irregularly detected in one calf from each group (calf 2123 and 1629 from groups B and A respectively). In group A, viral RNA was detected significantly earlier (from the first 2–3 days PI onwards) than in group B (Fisher's exact test; $P < 0.0001$), but in both infected groups progressively lower Cq values were measured starting from 7 days PI, with the lowest Cqs (viraemic peak) situated between 10 and 12 days PI. Afterwards, Cq values increased and reached a plateau, with BTV RNA being detectable until the end of the experiment.

BTV isolation was obtained from the EDTA-blood samples collected between days 2 and 31 PI. Table 2 illustrates the results of the virus isolation on ECEs. Furthermore, BTV isolation was obtained from only three infected calves in group A. Indeed, although calf 7069 had a viraemia detectable by RT-qPCR, no virus isolation was achieved throughout the experiment. In group B, virus isolation was possible from the EDTA-blood collected from the four RT-qPCR positive calves. No viable virus was detected in the blood of calves 2123 (group B) or 1629 (group A), which were also characterised by a discontinuous and poor RNA detection. Between days 10 and 15 PI, the highest number of BTV positive ECEs were found in both groups, corresponding to the viraemic peak also described by RT-qPCR. At days 10, 15 and 31 when all animals were tested, a significant difference was observed only at day 10 PI. At this day point, a significantly higher percentage of BTV positive ECEs was observed in group B compared to group A (Fisher's exact test; $P < 0.03$). The presence of viable virus in the bloodstream of four calves (5892 and 9103 in group A; 2125 and 7498 in group B) was confirmed until day 31 PI.

3.4. Serology

No serogroup-specific antibodies were detected in the sera of calves in group C, whereas all the infected calves (in groups A and B) seroconverted in the course of the experiment. Seroconversion occurred at day 9 PI in calves in group A, with the exception of calf 1629, which had a late antibody response at day 21 PI. In group B, three calves seroconverted between days 10 and 11 PI, and two others between days 15 and 21 PI. A two-factor ANOVA with repeated measures on one factor was used to compare the results between these two groups and found significant earlier seroconversion (2 days in mean) of calves in group A ($P < 0.01$) as well as in group-time interaction ($P < 0.01$) (Fig. 2B).

Neutralising antibodies were measured in all infected calves (Fig. 2B). In group A, neutralising antibodies increased progressively starting from days 11 to 13 PI, while in group B their detection occurred between days 13 and 18 PI. A two-factor ANOVA with repeated measures on one factor was used to compare the titres of groups A and B but no significant difference was observed ($P > 0.05$) (Fig. 1B). By contrast at 13 dpi median titre of group A was already positive, whereas group B was only positive starting at the next tested time point, at 18 dpi.

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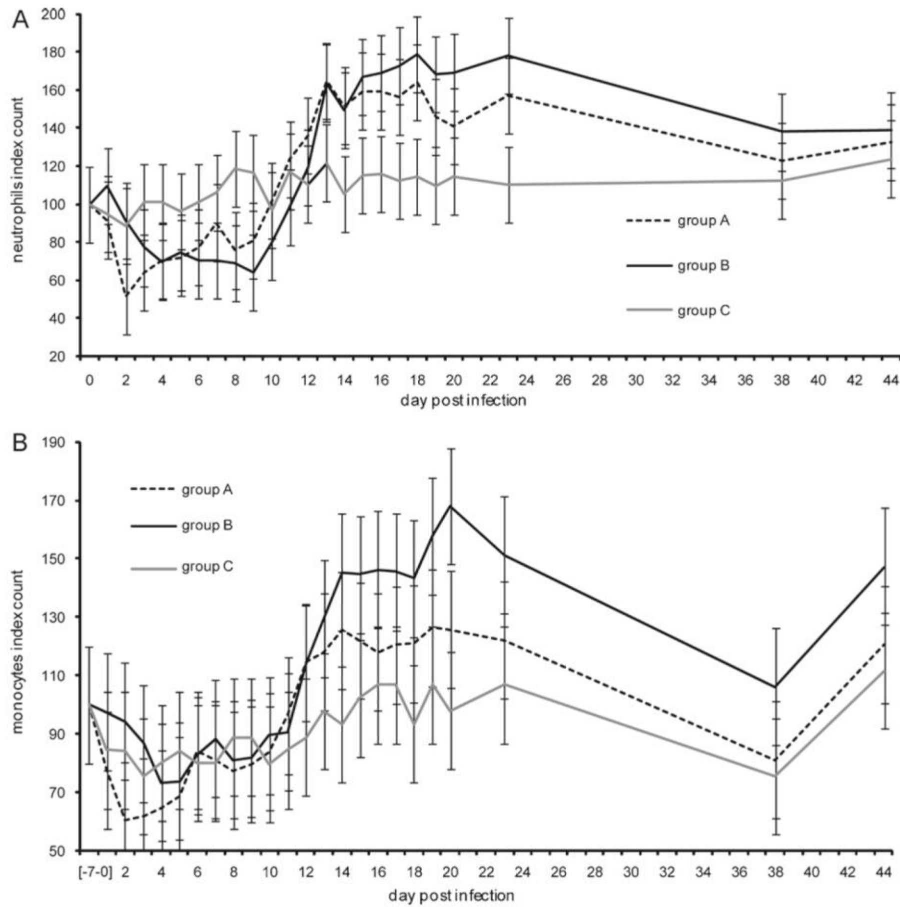

Fig. 1. Kinetic evolution of the mean ± one standard deviation neutrophils (A) and monocytes (B) indexes count. For each groups, a basal index was calculated as the mean value of the mean counts of days −7 PI to 0 PI ([−7–0] on the X-axis). The value of 100 was attributed to this index and mean daily counts were expressed as a percentage of this index, within each group. Dotted black lines: group A; black lines: group B; grey lines: group C.

Table 2
Longitudinal determination of the levels of viraemia by RT-qPCR and viral isolation on embryonated chicken eggs (ECEs), following intravenous infection of two groups of calves with BTV-8 European strain.

Day post infection	Group A (cell-passaged virus) Cq[a] and BTV positive ECEs[b]										Group B (infectious blood) Cq and BTV positive ECEs									
	1629		2129		5892		7069		9103		243		2123		2125		2126		7498	
	Cq	ECE	Cq	ECE	Cq	ECE	Cq	ECE	Cq	ECE	Cq	ECE	Cq	ECE	Cq	ECE	Cq	ECE	Cq	ECE
2	41.5	0	36.3	1	40.3	0	N/A		N/A		N/A		N/A		N/A		N/A		N/A	
3	40.4		34.9		35.7		N/A		37.1	0	N/A		N/A		N/A		41.7	0	N/A	
4	N/A		41.8		N/A		37.8		40		N/A		N/A		N/A		N/A		N/A	
5	40.9		37.3		35.7		41.4		38.7		N/A		N/A		N/A		39.4		N/A	
6	N/A		38.7		38.4		N/A		41.4		N/A		N/A		N/A		41.2		N/A	
7	N/A		34.8	1	34.9	1	37.9	0	36.2	1	36.2	1	38.7	0	37.4	0	36.4	1	38.7	1
8	41.5		31.8		31.4		35.7		31.4		33.2		38.7		36.3		34.6		33.3	
9	N/A		33.4		33.3		38.8		33.6		33.6		N/A		35.1		35.4		35.5	
10	N/A	0	30.6	2	29.6	1	35.6	0	29.7	4	29.9	4	40.8	0	32	4	31.5	5	31.4	3
11	N/A		30.4		31.4		36.6		31.8		31.2		42.2		31.7		31.9		31.8	
12	N/A		30.6		30		36		30.5		29.9		42.3		31.4		31.2		30.8	
13	N/A		33.2		33.1		39.2		34.6		33.8		N/A		33.5		34.7		33.9	
14	N/A		32.9		32.2		38		32.7		33.9		N/A		33.1		32.8		34.1	
15	N/A	0	34.6	5	36.9	3	41.8	0	36.4	5	34.5	3	N/A	0	35	2	35.9	4	35.6	3
18	N/A		39.5		37.9		41.6		40		40.6		N/A		40.5		38.7		40.2	
21	N/A		36.7		37.2		43.1		38.2		35.1		N/A		34.2		35		35.5	
24	N/A		40.4		37.2		40.3		38.5		38.4		N/A		39.8		40.2		41.1	
27	N/A		38.4		39.8		40.2		35.4		37.6		N/A		38.2		39.5		39.8	
31	N/A	0	35.3	0	33.6	1	39.7	0	37.6	1	33.8	0	42.3	0	35	1	35.6	0	35.6	1
37	N/A		42.8		40.7		N/A		39.4		41.3		N/A		43.5		N/A		41.7	
42	N/A		39.6		42.7	0	N/A		42.8	0	36.7		N/A		36.8	0	36.8		40.8	0

[a] Cq refers to the quantification cycle measured by RT-qPCR and performed with RNA purified from EDTA-blood samples.
[b] BTV positive ECEs refers to the number of embryonated chicken eggs where the virus could be successfully isolated. A total of five ECEs were inoculated each day of testing, with a corresponding EDTA-blood sample, starting with the first EDTA-blood sample shown to be positive by RT-qPCR.

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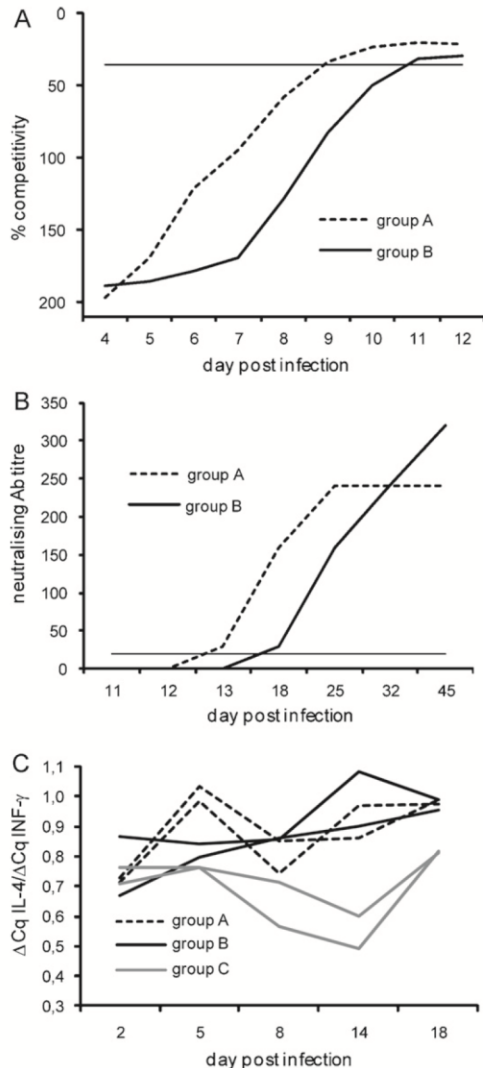

Fig. 2. Humoral and cell mediated immune response observed in the course of BTV-8 experimental infection of calves with low cell-passage virus (dotted line), infectious blood (black full line) and not-infected calves (grey full line). (A) The kinetic of the development of serogroup specific antibodies. The results are presented as the median of the percentage of competitivity obtained in the competitive ELISA. Horizontal black line represents the seroconversion cut-off corresponding to a competitivity under 35%. (B) The kinetic growth of neutralising antibodies in both infected groups of calves. The results are presented as the median values of the reciprocal of the serum dilution that causes a 50% reduction of cytopathic effect. Horizontal black line represents the seroneutralisation cut-off corresponding to a titre higher than 20. (C) The kinetic of the ratio between ΔCq IL-4 and ΔCq INF-γ after BTV-8 infection. IL-4 and INF-γ expression were normalized to GAPDH and β-actin (data not shown) and expressed as ΔCq values (see details in Section 2). Two calves per group fulfilled all the requirements necessary to analyze only representative and accurate samples (good RNA quality and β-actin and GAPDH mRNA expression levels included in the confidence interval of 95%).

3.5. Cytokine expression

In order to fulfil all the requirements necessary to analyze only accurate samples (good RNA quality and β-actin and GAPDH mRNA expression levels included in the confidence interval of 95%), the expression levels of INF-γ and IL-4 were monitored constantly for two calves in each group during the first three weeks PI (days 2, 5, 8, 14 and 18) (calves 2129 and 9103 for group A, calves 0243 and 2126 for group B and calves 3425 and 7012 for group C). All infected calves included in this analysis had clinical signs of disease and became viraemic. The results obtained using β-actin and GAPDH as reference genes were very similar and consequently only one series of results are presented (Fig. 2C). The immune balance between Th1 and Th2 pathways differed between the infected animals and the control animals (Fig. 2C). While the number of observations by group was limited ($n = 2$) – exploratory analysis – and the statistical power was weak, a lower Th2/Th1 ratio was found for animals of group C in comparison with both animals of groups A and B. At 14 days PI, the mean Th2/Th1 ratio was significantly lower for group C animals in comparison with group A (Welch test with unequal variance; $P = 0.004$) and group B (Welch test with unequal variance; $P = 0.02$) animals. No difference was observed between animals of groups A and B (Welch test with unequal variance; $P = 0.56$).

3.6. Necropsy, BTV RNA detection in organs and histopathological examination

After 46 days PI and before slaughtering, body condition was evaluated. Compared to the calves in group C, a reduced body condition score was highlighted in both infected groups (Table 3). At necropsy, none of the calves in group C showed macroscopic lesions, while calves in groups A and B had petechial haemorrhages in different lymphoid organs. Prescapular lymph nodes were systematically increased in volume and were characterised by disseminated petechial haemorrhages. Interestingly, among other regional lymph nodes, the mesenteric nodes were highly affected with an increased congestion and the presence of petechial haemorrhages. Analogous lesions were observed frequently in the thymus.

An abundant pericardic effusion was observed and collected in calves 5892 (group A), 2125 and 7498 (group B). Bacteriological analyses led to the isolation of *Micrococcus luteus* in animal 5892, considered as a contaminant without pathogenic potential. None of the samples from the two other calves gave rise to any bacterial growth. No lesions were observed at the pericardium, myocardium or endocardium of those calves. A dry pleurisy was documented in two calves from group A (1629 and 2129). The scores assigned to the organs in the course of necropsy are presented in Table 3. The comparison of the mean total scores assigned to groups A and B did not reveal statistically significant differences (Welch test, $P > 0.05$).

The tissue structures of the different organs sampled for histopathological examination were very well preserved. Of all the examined organ slides, only the mesenteric lymph nodes of calf 2125 (group B) and 7069 (group A) showed a few haemorrhagic foci (data not shown). The calf 7069 presented many small haemorrhagic areas on the thymus and a congestive spleen. The prescapular lymph node of animal 5892 (group A) showed only some haemorrhagic foci.

RT-qPCR performed on the total RNA extracted from the collected organs (spleen, lung, heart, bowel, tongue, thymus, femoral bone marrow, pulmonary artery, kidney, and prescapular, submandibular, mediastinic and mesenteric lymph nodes) as well as from inflammatory liquids (pericardic effusion) allowed the constant detection of the bovine β-actin gene, while BTV RNA could be detected only in some infected calves from the spleen, or from the prescapular and mesenteric lymph nodes (Table 3). No statistically significant difference was obtained when comparing the frequency of BTV RNA detection from the organs in the two groups (Fisher's exact test; $P > 0.37$). BTV RNA detection was systematically associated to high Cq values (>41), with the exception of the mesenteric lymph node of calf 2126, where a $Cq \leq 36$ was found (data not shown). Attempts at viral isolation on ECEs failed.

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Table 3
Necropsy scores, body condition scores and BTV RNA detection (when positive: score is underlined) in organs collected at the end of the experimental infection. Only organs showing necropsy lesions as a minimum are presented.

	Group A (cell-passaged virus)					Group B (infectious blood)				
	1629	2129	5892	7069	9103	243	2123	2125	2126	7498
Necropsy scores [a]										
Prescapular [b]	1	1	1	2	2	2	1	2	1	1
Sub mandibular [b]	0	0	0	1	1	0	0	0	0	2
Mediastinic [b]	0	0	0	1	0	0	2	0	0	2
Mesenteric [b]	2	2	0	2	1	2	1	3	2	1
Thymus	0	1	2	3	1	2	0	0	2	1
Spleen	0	0	0	0	0	0	0	0	0	0
Pericardic effusion [c]	0	0	1	0	0	0	0	1	0	1
Body condition [d]	1	1.5	0.5	1	1.5	0	1	1	0.5	0.5
Total score	5	6.5	4.5	10	6.5	6	5	7	5.5	8.5

[a] Necropsy score: on a 4 cm^2 defined area of each organ: 0 (absence of macroscopic lesions), 1 (less than 5 petechiae), 2 (from 6 to 10 petechiae), and 3 (more than 10 petechiae).
[b] Lymph node.
[c] Pericardic effusion: 0 (absence) and 1 (presence).
[d] Body condition: 0, good (tail head – fat cover over whole area and skin smooth but pelvis can be felt. Loin – end of horizontal process can only be felt with pressure; only slight depression in loin); 1, moderate (tail head – shallow cavity but pin bones prominent; some fat under skin. Skin supple. Loin – horizontal processes can be identified individually with rounded ends); 2, poor (tail head – deep cavity with no fatty tissue under skin. Skin fairly supple but coat condition often rough. Loin – spine prominent and horizontal processes sharp).

4. Discussion

In the current study we successfully reproduce BTV-8 clinical expression in calves using two different inocula: a low cell-passaged virus previously used in the course of a preliminary experiment (confirming already published observations) and infectious blood obtained after one animal passage of the same viral strain.

In the preliminary experiment the inoculum BEL2006/01 BHK-21 P2 was used to infect subcutaneously (SC) and IV two calves. The successful reproduction of specific BT clinical signs was attributed to the IV injection of a high titre low cell-passaged inoculum [10]. This conclusion was supported by the lack of BTV-8 clinical expression in calves infected SC and intradermally with another European BTV-8 strain [26]. Thereafter, in the current study, the IV route of infection was used to infect calves with BEL2006/01 BHK-21 P2 and BEL2006/01 calf 1. Both inocula reproduced BT disease with no significant differences observed in the pattern or in the severity of the lesions (total clinical scores). The initial different titre of the two inocula leads to the difficult comparison of their biological features, and it questions if the two inocula might have similar virulence.

In the course of our study, a variation from very slight to mild clinical signs was found within the calves of the same group. Analogous observations have previously been made in the field [7,27] and after experimental infection of bovines with the European BTV-8 strain [28]. Furthermore, without reference to any particular serotype or strain, BTV is known to induce a pleomorphic clinical presentation within the same herd, even when animals of the same age, breed or reproductive status are involved [4,29]. Early hyperthermia at 2–4 days PI followed by an increased body temperature at 7–10 days PI were described similarly in the course of our preliminary experiment [10]. Initial and transient hyperthermia has previously been described after several BTV experimental infections, without regard to the BTV serotype, the type of inoculum or the administration route [10,26,30]. In the present study, the second temperature rise temporally correlated to the secondary viraemia and to the active viral replication, most likely corresponds to the field observed pyrexia and to the development of the clinical signs of BT in bovines.

Daily clinical examination was coupled with haematological analysis in order to better define the general health condition of the three groups of animals. In the course of this longitudinal study, relevant variations occurred in the neutrophil and in the monocyte index count. To the authors' knowledge, this feature has not been investigated to date in the context of an experimental infection with the European BTV-8 strain. The mild neutropenia observed here within the first few days PI reflects the role of neutrophils in innate immunity, and their massive tissue infiltration during the early stages of viral infection [31]. Neutropenia has already been described as displaying a rapid onset after BTV experimental infection of calves [32]. In the present study, neutrophils and monocytes both showed a rise between 10 and 12 days PI, which corresponds to the viraemic peak measured by RT-qPCR and by viral isolation in both groups of infected calves. This increased number of neutrophils and monocytes could be attributed to their stimulation induced by cytokines released in the course of an immune response, such as TNF-α, TGF-β and IFN-γ [31]. In addition, in a study conducted by Ellis et al. [33], most of the cells expressing BTV were identified morphologically as monocytes concomitantly with the viraemic peak and with the development of neutralising antibodies. Moreover, it was expected that the variability in clinical disease would be, in part, a result of differential infection of these cells.

In the present research, a longitudinal study was performed in order to detail the duration of the viraemia in infected calves. An early BTV RNA detection was found in calves infected with the low cell-passaged virus. The statistical delayed BTV RNA detection in the calves infected with the blood inoculum could be explained by two hypotheses. The initial 1 log lower titre of the BEL2006/01 calf 1 inoculum could have influenced the initial progression of the infection. Nevertheless as we said above, the 2 log titre difference between the preliminary experiment and the calves of group A did not influence the dynamic of BTV RNA detection in the bloodstream. Another hypothesis explaining the delayed BTV RNA detection in calves infected with the blood inoculum could be the adsorption of BTV in indentations of the erythrocyte membranes [34,35]. Blood inoculum intravenously inoculated would act like a Trojan horse carrying BTV to the targeted lymphoid organs. Once there, the time required to destroy the remaining plasmic membrane to free BTV particles and initiate replication might explain the delay observed for BTV RNA detection. Furthermore, although a significantly higher isolation rate was found at 10 dpi in group B, no significant difference could be demonstrated between the two groups of calves, if the entire course of the experiment is considered. While BTV RNA could be detected until the end of the experiment, virus was isolated until 31 days PI, with no differences between the two groups of calves.

An analogous longitudinal approach was used to investigate the development of serogroup-specific antibodies and neutralising antibodies. The rise of the neutralising anti-VP2 antibodies occurred two days after the seroconversion in both infected groups. It has to be stressed that the 5 days difference in the crossing of the neutralising antibodies positivity threshold observed between groups A and B cannot be interpreted as a 5 days earlier development of neutralising antibodies in group A, as 13 and 18 dpi are successive bleeding point and there are no intermediate tested days. The serogroup-specific antibodies were developed significantly earlier in the group of calves infected with the low cell-passaged virus, reflecting the earlier BTV RNA detection found in this group. Antibodies anti-VP7 and anti-VP2 were found contemporarily to the presence of viable virus in the bloodstream of both groups of infected calves. In particular, co-circulation of neutralising antibodies with viable BTV was established for a maximum duration of 18 and 13 days for the calves infected with low cell-passaged virus and infectious blood, respectively. The localization of BTV viral particles in the indentations of the membrane of erythrocytes were found to have implications in the host immune response, allowing the virus to evade the presence of neutralising antibodies [35,36].

In the present study, an exploratory investigation of the balance of the Th1 and Th2 pathways in the course of the immune response to BTV-8 was performed. In the development of an efficient vaccine, it is crucial to know the role of the different lymphocyte populations in the course of the infection, in order to induce the most effective immune response. The expression levels of IFN-γ and IL-4 were used to evaluate the activation of the Th1 and Th2 pathways, respectively. In the course of the first week PI, the median Th2/Th1 ratios of all tested animals had a similar pattern, while at day 14 PI, the ratios of the infected animals were significantly higher than those of the control animals. Therefore, if although a balance in the expression of IFN-γ and IL-4 is observed during the first week of infection, a progressively increased ratio supports a constantly higher expression level of IL-4 compared to IFN-γ, or a reduced expression of IFN-γ with an unmodified expression of IL-4. Both hypotheses support the prevalence of a humoral immune response during the first two weeks PI and can be related to the massive development of antibodies observed in both infected groups. Nevertheless, the observed rise in neutrophils and monocytes testifies to their possible role in the organization of a phagocyte-dependent immune response.

Calves were euthanised 46 days PI and several lymphoid organs showed petechial haemorrhagic lesions. BTV RNA was also detected in lymphoid organs, such as the prescapular lymph nodes and spleen. To the authors' knowledge, this is the first report of a high rate of BTV RNA detection in the mesenteric lymph nodes, indicating a disseminated viral distribution. In this organ viral isolation using undiluted sample failed, and the additional attempts using dilutions gave negative results as well; however it cannot be excluded that the use of the undiluted sample led to non-specific inhibitor and/or toxic effect.

Three infected calves (2123, 1629 and 7069) were characterised by inconsistent BTV RNA detection and no virus isolation in the bloodstream, while seroconversion and development of neutralising antibodies were observed. Calves stayed healthy during the three months of acclimatization, while started to develop typical BT clinical signs after challenge. Calves 2123 and 7069 had necropsy lesions, and viral RNA could be detected in the mesenteric lymph node and in the spleen, whereas calf 1629 showed mild clinical manifestations of the disease and some necropsy lesions. A possible explanation could be found in a different individual receptivity to the infection, and the ability to instigate a more appropriate innate immune response with a rapid viral elimination. Likewise, Darpel et al. [26] observed important pathological lesions in bovines infected with a European BTV-8 strain although the animals failed to show any obvious clinical signs. Most of the lesions induced by BTV are a direct consequence of the virus's tropism for endothelial cells [37,38], leading to microvascular injury, oedema and haemorrhage. Infected endothelial cells produce several inflammatory mediators that may play a major role in the pathogenesis of the disease [5,39], causing clinical signs possibly without the effective presence of viral particles, as seen in human haemorrhagic fevers such as Ebola [4]. The inconsistent virological findings in calves 1629, 2123 and 7069, together with the presence of mild to moderate clinical signs, and necropsy lesions, could support the implication of vasoactive mediators in the pathogenesis of these lesions in the absence of viraemia.

In our study we present two experimental protocols alternative to the use of wild type infectious blood for the infection of calves with the European BTV-8. The results obtained confirm that BTV-8 after low numbers of cell passages or one animal passage maintains unmodified biological properties and a high pathogenicity for calves. The use of wild type infectious blood is possible only with samples coming from infected animals with well known sanitary conditions to avoid the concomitant inoculation of other pathogens. Furthermore, the standardization of this inoculum is difficult and comparison of the results obtained among different laboratories is hardly possible. The use of the cell-passaged virus as inoculum in experimental protocols requires the standardization of the number of passages in ECEs and in cell culture as well as the type of cells used in the adaptation. The tissue culture propagation has to be minimal to decrease the possibilities of viral antigenic changes. The use of the infectious blood as described in our study implies a preliminary experimental infection of animals with a known and adequate sanitary status.

The longitudinal observation of the three groups of calves allowed us to make original findings in the study of the pathogenesis of the European BTV-8 strain. The preliminary and exploratory results concerning the expression of cytokines in the course of bovine infection need to be further extended. In particular, the expression of more cytokines needs to be assessed in order to give more exhaustive information on the implication of the Th1 and Th2 pathways during the bovine immune response to BTV. Investigating the dynamics of monocytes and neutrophils led to unexpected findings, which may imply a greater role of these cells in the pathogenesis of BT. However, the importance of these cells in this process needs to be defined more accurately.

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Experimental section

Study 2 :

Three Different Routes of Inoculation for Experimental Infection with Schmallenberg Virus in Sheep

Transboundary and Emerging Diseases 64(2017):305-308

Martinelle L, Poskin A, Dal Pozzo F, Mostin L, Van Campe W, Cay AB, De Regge N, Saegerman C.

Preamble

Knowledge of the impact of different inoculation routes of SBV in experimental models is currently incomplete. Each route implies constrains and benefits, some of significant practical impact. It might be tempting to assume that different arboviruses sharing common vectors could be experimentally inoculated using similar routes, as vector feeding behaviour should be virus-independent. Based on this assumption the inoculation routes validated for BTV experimental infection are likely to be suitable for SBV experimental infection. However, since vectorial capacity, target cells and target tissues, and replication dynamics differs, the choice of the inoculation route should remain specifically evidence based. In order to complete the screening of the potential SBV inoculation routes we evaluated subcutaneous, intradermal and intranasal inoculations of sheep on clinical, virological and serological outcomes.

Intradermal and subcutaneous inoculations are classical routes broadly used in experimental infections. As developed in the introduction of the present thesis, BTV and SBV are arboviruses and the bite of infected culicoides clearly represents the epidemiologically relevant transmission route. However some direct BTV transmissions were also described in the supposed absence of vector midges. In 2011 Van der Sluijs et al. reported the first transmission of BTV8 to a control pregnant ewe housed with challenged animals, supposedly without iatrogenic inoculation. Therefore, in order to investigate a similar potential direct SBV transmission we also inoculated ewes by intranasal route.

Transboundary and Emerging Diseases

Transboundary and Emerging Diseases

SHORT COMMUNICATION

Three Different Routes of Inoculation for Experimental Infection with Schmallenberg Virus in Sheep

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Summary

Schmallenberg virus (SBV) is an emerging *Orthobunyavirus* affecting European domestic ruminants. In this study, three groups of ewes (n = 3) were inoculated with 1 ml of an SBV infectious serum, via the subcutaneous (SC), intradermal (ID) or intranasal (IN) route. The ewes were monitored for 10 days and no clinical signs were reported. IN inoculation failed to generate any detectable RNAemia. SC and ID inoculation induced typical SBV RNAemia and seroconversion upon day 6 post-inoculation in 3/3 and 2/3 sheep, respectively. In all the animals that showed RNAemia, the viral genome could be detected in spleen and mesenteric lymph nodes. Both the SC and ID routes seem suitable to properly reproduce field conditions, as comparable observations were reported regarding RNAemia, seroconversion and viral genome detection in organs.

Introduction

Schmallenberg virus (SBV; family *Bunyaviridae*, genus *Orthobunyavirus*) emerged in Europe in 2011 and is transmitted by *Culicoides* biting midges. It is associated with a mild non-specific syndrome in adult cattle, but most of the clinical impact is related to congenital malformations that can affect ruminant neonates following *in utero* infection (Herder et al., 2012). So far, in most of the experimental infections performed in host species, subcutaneous (SC) inoculation was chosen, but the intravenous (IV) (Hoffmann et al., 2012), oral (PO) and intramuscular (IM) routes were also evaluated (Wernike et al., 2013a,b). As *Culicoides* spp. are 'pool-feeder', that is blood feeding following laceration of the dermis of the host, superficial tissues are in first line of contact with the pathogen. Therefore, intradermal (ID) inoculation of SBV is worth investigating. In addition, intranasal (IN) inoculation has not been tested yet and could provide additional insights for potential SBV contact transmission. Thus, SC, ID and IN inoculations were compared based on their clinical, virological and serological outcomes.

Materials and Methods

Nine 'Mourerous' ewes of about 1 year of age were used. The Mourerous is a middle-size rustic breed from south of France. All were serologically and virologically negative for SBV. Three isolated groups of three ewes were randomly constituted in a BSL3 facility (CODA-CERVA Machelen, Belgium). Each animal was inoculated with 1 ml of the infectious serum via the IN way (ewes IN1 to IN3) in the right nostril, or subcutaneously (SC1 to SC3) in the left axilla or for the last group, intradermally on the left side of the neck (ID1 to ID3). ID inoculation was performed using a Dermojet® device (Akra Dermojet, Pau, France). The

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inoculum (graciously provided by Friedrich Loeffler Institute, Riems, Germany) contained about 2 × 10^3 50% tissue culture infective dose/mL ($TCID_{50}$/mL) and 7.3 × 10^6 RNA copies/mL of SBV S-segment. This study was approved by the Ethical Committee of the IPH-VAR (Scientific Institute of Public Health – Veterinary and Agrochemical Research Centre, number of project: 121017-01, date of approval: 11 February 2013).

A daily clinical examination was performed associated with blood, faecal and nasal sampling. Ewes were euthanized 10 days post-infection (dpi). The extraction of RNA from organs, faeces and serum, and the detection of SBV S-segment by a one-step reverse-transcription (RT) quantitative PCR (RT-qPCR) were performed as described previously (De Regge et al., 2013). The quantification cycle (Cq) values were converted into copy numbers (Poskin et al., 2014), and results were included only when negative extraction controls and negative and positive amplification controls were satisfactory.

Results and Discussion

No clinical signs were observed that could be related to an acute SBV infection in the inoculated sheep. No viral RNA was detected in the blood, nor in the organs of the animals from the IN group. Inoculation of ID3 did not result in a detectable RNAemia. Failures to reproduce or induce infection were previously described with the same inoculum in calves (Hechinger et al., 2013) and sheep (Wernike et al., 2013b) through SC route. Thus, the lack of infection is most likely not related to the ID route itself and could be related to a random failure of infection or individual factors of resistance. The observed RNAemia (Figs 1a and b), in both SC and ID groups, was comparable in duration (3–5 days) and in line with previous data (Hoffmann et al., 2012; Wernike et al., 2013b; Poskin et al., 2014) following IV and SC inoculations. The mean SBV genome copy numbers detected in the blood of sheep with RNAemia were not significantly different between SC and ID groups through time (two-way ANOVA with repeated measures; group effect: $P = 0.15$; group–time interaction: $P = 0.41$). In contrast, at viraemic peak, maximum copy number was significantly higher in the ID group versus SC group (Welch's test with unequal variance; $P = 0.026$).

The sporadic low-level detection of SBV genome in nasal swabs (Figs 1c and d) is highly unlikely to play a role in the epidemiology of the disease. As direct IN inoculation failed to cause RNAemia, an intermittent and low-level nasal

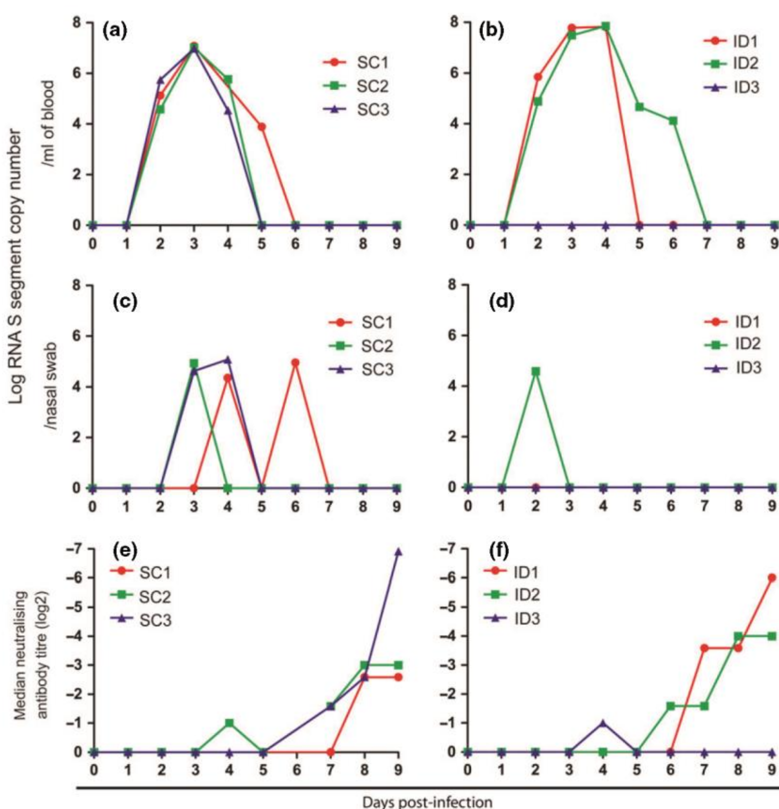

Fig. 1. Reverse-transcriptase quantitative PCR results in blood (a and b) and nasal swabs (c and d) for SC and ID groups. In panels (c) and (d), no sheep had positive detection in both nostril the same day; thus, right or left nostril origin is not specified. Panels (e) and (f) show the neutralizing antibody titres per mL in SC and ID groups, respectively. ID: Intradermal and SC: Subcutaneous. [Colour figure can be viewed at wileyonlinelibrary.com].

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shedding should be of very limited impact on the further spread of the virus. SBV genome was found in faeces of animals of all the three groups (Fig. 2), but only sporadically and inconstantly in animals of group IN. No SBV RNA was found in faeces of ewe ID3 that had no detectable viral RNAemia. Mean and maximum viral RNA copy numbers and duration of detection in faeces were not significantly different between SC and ID groups (Welch's test with unequal variance; $P = 0.13$, 0.12 and 0.48, respectively). In contrast with previous work (Wernike et al., 2013a), SBV genome remained detectable in faecal samples of SC and ID groups 1–2 days after the end of the positive detection in blood. The origin of the faecal positive detection, especially in IN group as no RNAemia could be detected, remains debatable. However, a gastrointestinal origin cannot be ruled out. Indeed, positive detection in IN group might be a consequence of a partial swallowing of the inoculum. Positive faeces in SC and ID groups might be related to infected cell loss or infected secretions in the gastrointestinal tract, further detected in faeces. Infected cells could reach gastrointestinal lumen through epithelium renewal or intestinal micro-bleeding caused by chronic parasitic infestation, or virus could be transferred from the blood to the bile and then to faeces. In addition, Wernike et al. (2013b) reported diarrhoea in a sheep following SBV infection, which could lead to a transient increase in intestinal permeability. Moreover, the 1–2 days delay between blood and negative faeces detection might be caused by the transit inertia, as mean ingesta retention time in sheep is about 48 h (Hadjigeorgiou et al., 2003). Other *Bunyaviridae* like hantaviruses are well known to be detected in faeces of infected animals, possibly longer than in the blood (Voutilainen et al., 2015).

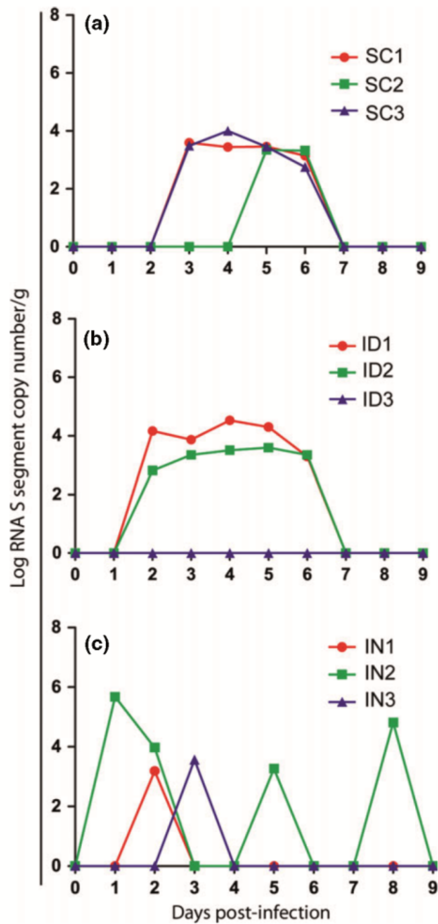

Fig. 2. Reverse-transcriptase quantitative PCR in faeces of subcutaneously (a), intradermally (b) and intranasally (c) inoculated animals. [Colour figure can be viewed at wileyonlinelibrary.com].

The presence of neutralizing anti-SBV antibodies was assessed by seroneutralization (SNT, De Regge et al., 2013). Only ewes that had RNAemia seroconverted within the duration of the experiment. Seroconversion occurred between 6 and 8 dpi in SC and ID groups (Figs 1e and f). As previously reported, the decrease of SBV RNA in the blood correlated with seroconversion (Poskin et al., 2014). However, as the animals were only tested for 10 days, a later seroconversion might have been possible in ewes without RNAemia.

At post-mortem examination, no particular gross lesions were reported. No SBV genome could be detected in the central nervous system or genital tract. Within the same ani-

Table 1. Log copy number of SBV S-segment by weight (g) of organ for each ewe

Organ	SC1	SC2	SC3	ID1	ID2	ID3	Mean by organ[a]
Lung	0.00	5.37	0.00	5.91	0.00	0.00	5.64 (0.38)
Spleen	6.46	6.70	6.72	7.69	7.44	0.00	7.00 (0.53)
Mesenteric lymph node	6.69	7.30	6.75	7.91	7.51	0.00	7.23 (0.52)
Submandibular lymph node	7.20	5.87	4.74	0.00	0.00	0.00	5.94 (1.23)
Superficial cervical lymph node	5.54	0.00	0.00	7.09	0.00	0.00	6.32 (1.10)
Mean by route[a]		6.3 (0.81)			7.26 (0.72)		

[a]Means were calculated only considering positive organs. Standard deviation is in curved brackets.

mal, SBV RNA detection in serum was systematically associated with detection in spleen and mesenteric lymph nodes.

Table 1 shows the log of copy number of SBV RNA in each positive tested organ for each animal. Considering animals that showed RNAemia, more organs were detected positive in SC group (11/15) when compared to ID group (6/10), however, this difference was not significant (Fisher's exact test for count data = 1.79; P = 0.67). Comparing organs that were detected positive in both ID and SC groups (lung, spleen, mesenteric and superficial cervical lymph nodes), SBV RNA copy numbers were significantly higher in organs of ID group (two-way ANOVA with repeated measures; P = 0.03).

Conclusion

IN inoculation failed to generate any detectable RNAemia. In both SC- and ID-inoculated animals, viral RNA could be detected in faeces 1–2 days longer than in the blood. The cause of this prolonged faecal viral detection is currently uncertain but not uncommon in *Bunyaviridae* and it might be a consequence of the digestion time in sheep. Both of the SC and ID routes seem to properly reproduce field conditions, as similar outcomes for parameters such as RNAemia, seroconversion and viral genome detection in organs were found. SBV RNA copy number was significantly higher in organs of the ID group, but this result has to be interpreted with circumspection as the sample size was small and one ewe had no RNAemia at all. However, to be properly performed, ID inoculation requires the use of a regular syringe, with more care while injecting and a high risk to go SC if the animal is not perfectly calm, or specific devices such as syringe tuberculin pistol or jet injectors (like the Dermojet® used in the current study) with the constraint of a limited volume to be injected per shot. Therefore, SC inoculation might be easier to deal with from a practical point of view.

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Experimental section

Study 3 :

Pulmonary artery haemorrhage in newborn calves following bluetongue virus serotype 8 experimental infections of pregnant heifers

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Martinelle L, Dal Pozzo F, Sarradin P, De Leeuw I, De Clercq K, Thys C, Thiry E, Saegerman C.

Preamble

The unexpected emergence and subsequent rapid spread of bluetongue virus serotype 8 (BTV8) in Western and Northern Europe led to dramatic repercussions on sheep and cattle livestock. Many features were specific of the BTV8 European outbreak: an increased severity in cattle, the discovery of competent local vectors, and the astonishing ability of the virus to cross the placenta barrier. Several experimental infections were performed to date, involving sheep or cattle, but only a part of them met the biosafety requirements to deal with BTV8 and in most of time they didn't exceed 4 to 5 weeks.

We decided to implement a longitudinal study of repeated experimental infections of pregnant heifers, including calving and follow-up of their offspring for 2 months. Half of the infected heifers were vaccinated against BTV8. Our work, running for nearly one whole year, investigated the effects of successive challenges on vaccinated and non-vaccinated pregnant heifers, with particular emphasis on transplacental infection and the efficacy of the vaccine to protect the foetus.

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Pulmonary artery haemorrhage in newborn calves following bluetongue virus serotype 8 experimental infections of pregnant heifers

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ABSTRACT

The emergence of bluetongue disease (BT) among livestock in Europe in 2006 raised many questions including the occurrence and epidemiological significance of foetal infections in cattle. To clarify these aspects, vaccinated and unvaccinated pregnant heifers were sequentially infected twice in an isolation facility (biosafety level 3) with a northern European outbreak strain of Bluetongue virus serotype 8 (BTV-8). The study was terminated 2 months after calving with necropsy of the dams and their offspring. The cattle were monitored throughout the study by clinical scoring and for the presence of circulating neutralising antibodies, and after calving for the presence of infectious virus and viral RNA in blood and milk. Four calves, one born from a vaccinated dam and three from non-vaccinated ones, that were infected at 120 days of gestation had obvious haemorrhage of the pulmonary artery at necropsy. Although haemorrhage of the pulmonary artery is highly characteristic of BT, viral RNA was not detected in any of these calves. Furthermore, although none of the calves born from heifers infected prior to mid-gestation had teratogenic BTV typical brain lesions, some had lesions at birth suggestive of *in utero* BTV infection. Despite the lack of viral RNA detection, the presence of haemorrhage of the pulmonary artery deserves to be reported as a new observation in the context of the multiple investigations having as main subject the BTV placental crossing in cattle.
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1. Introduction

Bluetongue virus (BTV) causes the eponymous bluetongue disease (BT), belongs to the family *Reoviridae* and represents the type species of the *Orbivirus* genus (Mertens et al., 2005). BTV is an arbovirus transmitted to susceptible mammals, mostly wild and domestic ruminants, by the bite of haematophagous female midges of the *Culicoides* genus. The unexpected introduction of the so far exotic BTV serotype 8 (BTV-8) in the core of Western Europe in 2006 and its rapid spread, constituted the major sanitary event in animal health of the last years. In addition to a surprising severity of the clinical expression of the disease in cattle, abortions and nervous abnormalities were observed in the offspring of affected ruminants (Vercauteren et al., 2008). Foetal infection in cattle and sheep is long ago described (Schultz and Delay, 1955), but is very rarely related to wild type virus natural infection (Housawi

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et al., 2004). In the very vast majority of cases, laboratory adapted strains, like modified live vaccine viruses, are involved (MacLachlan et al., 2000). Placental crossing represents an additional feature increasing the considerable economic losses related to BTV-8 (Velthuis et al., 2010).

With regards to BT epidemiology in Europe, vaccination had been rapidly considered a strategic option inevitable to stop the spread of the disease, to control BT clinical outbreaks in endemic areas and to allow safe movement of animals (Zientara et al., 2010). Therefore, several Member States and Switzerland launched massive vaccination campaigns, starting in 2008 with unregistered monovalent inactivated vaccines under temporary use authorisation. Efficacy and safety qualities of the vaccines were assessed through large scale use (Eschbaumer et al., 2009; Gethmann et al., 2009). In March 2009, the European Medicine Agency granted in the European Union the use of one of these vaccines against BTV-8 (BTVPUR Alsap 8, Mérial, Lyon, France). In general, populations of Palaearctic *Culicoides* species raises in spring and peak in temperate regions in late summer (Takken et al., 2008). As a consequence, cattle are likely submitted to several natural infectious challenges during the same vector-active period.

A recent work (van der Sluijs et al., 2012) investigated the protection conferred by a commercial inactivated vaccine (Bovilis BTV-8, Intervet) against foetal infection in pregnant ewes and heifers. In that study, the animals were euthanized 3 weeks after BTV-8 inoculation, allowing the evaluation of the consequences of the infection after a short period from the virus exposition.

In our study, pregnant heifers, separated in three groups, vaccinated, non-vaccinated and control, were infected through two successive BTV-8 challenges. Inoculations were realised 4 months apart, in order to correspond to the times of the year with the highest vectorial activity. BTV-8 placental crossing and the efficacy of the vaccine were investigated. To further evaluate the modalities of trans-placental transmission, calves were monitored during 2 months after their birth regarding clinical, virological and serological parameters. The presence of BTV-8 induced malformations was particularly explored by an extensive necropsy of the offspring at the end of the experiment.

2. Materials and methods

2.1. Ethics statement

All the animals were confined for the whole length of the study in an insect-secure biosafety level 3 zone (BSL3) at the Experimental Infectiology Platform (PFIE) of the National Institute for Agronomic Research (INRA) – Research Centre of Tours (Nouzilly, France). Experiments were approved by INRA's Committee on the Ethics of Animal Experiments and conform to the International Guiding Principles for Biomedical Research Involving Animals as issued by the Council for the International Organisations of Medical Sciences.

2.2. Animals and group constitution

Twelve Holstein heifers aged between 1 and 2 years were used. All the animals were tested seronegative and non viraemic for BTV and bovine viral diarrhoea virus (BVDV), and seronegative for bovine herpes virus 1 (BoHV-1), *Neospora caninum* and *Coxiella burnetii*. A thorough general clinical examination was carried out on all the animals by a veterinarian before including them to the study, to confirm their asymptomatic state, in accordance to physiological standards (Jackson and Cockcroft, 2002).

Heifers were randomly separated in three fence-isolated groups. These 3 groups were constituted as following: 5 animals in the non-vaccinated group (identified further as NV); 5 animals in the vaccinated group (V) and 2 animals in the control group (C). When an individual animal is mentioned, it is referred as its group name followed by its identification number. Once the heifers gave birth, their offspring was named as "O" followed by the vaccination status of the mother (NV, V or C) and a number depending on the chronologic order of calving within its group. When necessary the identification number of the dam was added in brackets.

The vaccinated heifers received for both injections of the vaccination protocol a 1 ml BTVPUR AlSap 8 (Merial, Lyon, France) dose, subcutaneously in front of the left shoulder. Heifers were vaccinated following the manufacturer's recommendations, with a first injection 78 days before the first infection (−78 dpi) and revaccinated 28 days later (−50 dpi). The first infectious challenge took place 50 days after second vaccine injection (referred later as 0 dpi) and the second challenge at 121 dpi (Fig. 1).

All animals were introduced in the BSL3 facility 1 week before the beginning of the experiment to allow their acclimatisation. Euthanasia was realised by intravenous injection of pentobarbital sodium (Dolétal, Vétoquinol, Lure, France), 10 g/animal, followed by bleeding once the corneal reflex disappeared. Heifers and their offspring were euthanatized at 223 dpi and necropsied (Fig. 1).

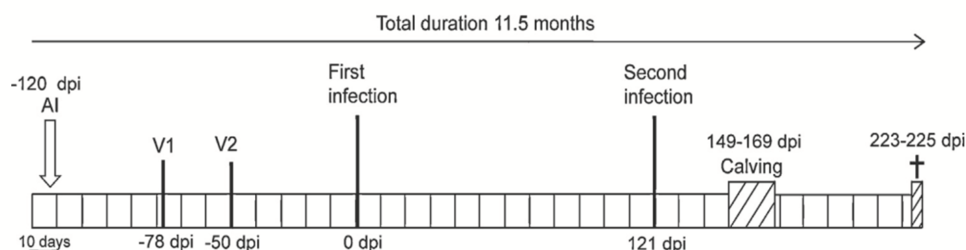

Fig. 1. Experimental timeline. Successive vaccinations (V1 and V2) and infections are represented. Artificial insemination (AI), calving period and euthanasia (†) are also represented.

2.3. Pregnancy and calving

All the heifers were synchronised using an intravaginal progesterone insert (CIDR EAZI BREED, Zoetis, Paris, France) following manufacturer's instructions. Heifers were artificially inseminated at −120 dpi, 48 h after CIDR removal, with semen from two clinically healthy Normand bulls, with a confirmed serological negative status for BTV, during the vector free period. Pregnancy was first diagnosed with a progesterone radio immune-assay (RIA) 20 days post artificial insemination (AI), following an INRA internal RIA protocol. Then, pregnancy-associated glycoproteins (PAG) were measured at 31 and 34 days post AI by homologous and heterologous RIA systems. From 60 to 150 days post AI, pregnancy was evaluated by B-mode ultrasonography (Tringa linear Vet, Pie Medical, Maastricht, the Netherlands, equipped with a 5 MHz transducer) once a month.

Calving was monitored using an intravaginal thermometer coupled to a GSM base (Velphone system, Médria, Chateaugiron, France). Immediately after birth and prior to colostrum intake, EDTA and sera samples were taken from calves. They were then separated from their dam in another close BSL3 stable. General clinical examination, weighing, cardiovascular, respiratory and nervous systems clinical examinations were performed (Jackson and Cockcroft, 2002). Dams were milked and colostrum density was evaluated with a colostrometer, sampled, and calves were given 2 L colostrum or more, to be adapted if a total of 100 g of gammaglobuline/calf could not be reached with only 2 L. Two more litres were given maximum 12 h later. To ensure that colostral immunity was properly transmitted, calves' serum's density between 4 and 7 days post-partum (dpp) was measured with an electronic refractometer. In addition, colostrum fed to each calf was screened for BTV RNA detection by real time quantitative polymerase chain reaction (RTqPCR). Calves were gathered depending on their mother's group, bucket fed with milk from their own dams for 15 days, and then fed with powdered milk from automatic milk feeders until the end of the experiment. From birth to euthanasia, daily EDTA and sera samples were taken from calves.

2.4. Virus

Infectious blood obtained in the course of a preliminary experimental infection was used to inoculate the animals (Dal Pozzo et al., 2009). Briefly, a calf being inoculated with a BTV-8 strain passaged twice in BHK-21 cells (BEL2006/01 BHK-21 P2) and showing clinical signs, was blood sampled at the viraemic peak (8 dpi). The EDTA blood was frozen at −80 °C and subsequently used as inoculum. All the infected animals were intravenously inoculated *via* the jugular vein with 15 ml of blood, corresponding to a titre of 10^3 ELD_{50}.

2.5. Clinical examination

Every day the severity of the infection was quantified by calculating clinical scores per system and per animal, leading to daily and overall clinical scores by groups and animal. For this purpose, a standardised clinical form adapted from Saegerman et al. was used (Saegerman et al., 2008).

2.6. BTV detection in blood

EDTA blood tubes were frozen at −80 °C. After thawing, RNA extraction, RNA denaturation and reverse transcription followed by RTqPCR were performed as previously described (Martinelle et al., 2011). Briefly, RTqPCR amplified a pan-BTV region of segment 5 and bovine β-actin as control (Toussaint et al., 2007). Viral load for each sample was expressed as quantitative cycle value (C_q). To allow statistical analysis, a C_q value of 44 was attributed to negative results. Calves were tested on 0 (before colostrum intake), 1, 2, 5, 10, and 15 dpp.

2.7. Antibody detection

VP7 antibodies (Abs) were detected using a commercial competitive ELISA kit (ID Screen® Bluetongue Competition ELISA kit, ID Vet, France) according to the manufacturer's instructions. From the day of the first vaccination to the end of the study, heifers were regularly tested. Calves were tested at day of birth before and 6–12 h after colostrum intake, and then regularly until the end of the experiment.

Neutralising Abs were titrated by seroneutralisation (SNT). Two-fold serial dilutions of the sera (1:10–1:1280) were tested in the presence of 100 $TCID_{50}$ of BTV8 isolate 2006/01, as previously described (Martinelle et al., 2011). Sera were titrated regularly during the experiment, with a closer monitoring around vaccinations and infections.

VP7 Abs were measured in individual milk by using a commercial indirect ELISA kit (ID Screen® Bluetongue Milk Indirect, ID Vet, France) following manufacturer's instructions.

2.8. BTV detection in organs and histopathology

Samples of prescapular and mesenteric lymph nodes, spleen, thymus and any organ with gross lesions to be likely related to BTV infection were collected from the heifers and their offspring, and stored at −80 °C. If showing lesions, additional samples were taken and stored in a 4% formaldehyde solution for histopathology analysis. About 100 mg of all the collected organs were tested to detect BTV nucleic acid; viral RNA extraction was performed using Trizol-LS reagent according to the manufacturer's instructions (Gibco Invitrogen, UK). BTV and bovine β-actin detection were performed by RTqPCR as described above.

Formaldehyde fixed organs were embedded in paraffin and stained with haematoxylin-eosin for histological examination. Moreover, Perls staining was performed on pulmonary artery slides, to reveal the presence of haemosiderophages. Micrographs were taken with a BX51 microscope and a DP50 camera (Olympus).

2.9. Statistical analysis

The trends of clinical pattern over time and by group of clinical signs were compared using Spearman's rank

correlation, referred to as r_s in the text (Dagnelie, 1998). A two-factor ANOVA with repeated measures on one factor (*e.g.*, antibody to BTV) was used to compare the kinetics of parameters considered between groups (Petrie and Watson, 2006). The validity conditions (*i.e.* homogeneity of variances and covariance matrix), had been previously verified (Howell, 1998). For each specific time point, the comparison of quantitative parameters with unequal variance was compared with Welch's test and frequencies and proportions were compared with Fisher's exact test (Dagnelie, 1998). For all tests, P values <0.05 were considered significant.

3. Results

3.1. Clinical and laboratory observations of the heifers

With the exception of V-8379 (see below), all the other heifers gave birth under natural circumstances and pregnancy duration were not significantly different by groups (Welch test, $P > 0.1$; Supplementary file 1). All the heifers had excellent quality colostrum, with a measured density systematically higher than 125 g/L. All colostrum samples were negative regarding BTV RNA.

Concerning the heifer V-8379, the first ultrasonography performed 16 days after first vaccination (corresponding to 60 days post AI) showed an embryonic death. Heifer V-8379 was re-inseminated 75 days apart from the other animals. The second AI of V-8379 resulted in a regular pregnancy, but because of a limited length of the experiment, calving of V-8379 was induced at 264 days of pregnancy, using a classical dexamethason and prostaglandin analogue combination. Birth occurred in this animal after 266 days of pregnancy.

From the beginning to the end of the experiment, control heifers and vaccinated animals did not show any clinical signs that could be related to BTV infection. After the first challenge, clinical signs showed by the non-vaccinated heifers were slight, consisting nearly exclusively in oral lesions. NV-1903 only had a slight reddening of the conjunctival mucosa at 13 dpi. The highest occurrence of clinical signs was obtained from 13 to 19 dpi. No heifer had hyperthermia. After the second challenge no clinical manifestations or temperature rise could be detected in any animal. BTV RNA was never detected in the EDTA-blood samples of control and vaccinated heifers. Table 1 shows the C_q values measured in non-vaccinated heifers in the course of the study. After the viraemic peak a progressive increase in C_q values was measured and no significant variation was observed after the second challenge (at 121 dpi). BTV RNA could be detected starting from 7dpi in two out of five non-vaccinated heifers (NV-1893 and NV-1903; Table 1). Generalised detection of the viral genome in all non-vaccinated heifers was possible from 9 to 153 dpi. NV-1893 and NV-1903 had detectable viraemia until 174 dpi. VP7 and neutralising Abs titres of the control heifers were undetectable at every tested time points.

All the vaccinated animals seroconverted at the latest 7 days after second vaccine injection. Then, most of the vaccinated animals kept very high amounts of circulating

Table 1
C_q values in blood of non-vaccinated heifers after BTV-8 infections.

dpi1	dpi2	Animals				
		1893	1903	1905	2200	8381
7	−114	30	42	N/A	N/A	N/A
8	−113	29.1	31.2	28.9	30.2	N/A
9	−112	30.3	31.8	27.2	29.9	31.1
10	−111	30.2	30.7	26.8	31.4	32.3
11	−110	32.8	33.9	29.3	33	32.4
12	−109	29.7	27.8	33.4	30.9	33.5
13	−108	34.6	30	29.8	32.3	31.9
14	−107	29.5	28.5	28.9	31.1	28.4
15	−106	30.2	31.1	28.8	31.9	29.8
16	−105	30.5	29.4	30.1	32.2	31.4
17	−104	31.6	29.6	30.4	32.7	32
18	−103	31.8	30.8	32.1	32.9	32.7
19	−102	33.8	32.8	34.6	33	34.2
24	−97	37.6	34.9	33.8	37.8	34.7
31	−90	36	35.1	34.7	36.9	34.7
48	−73	34	32.8	32.4	34.1	32.3
69	−52	37.9	37.1	36	38.9	35.3
90	−31	36.8	38.1	37	41.5	36.2
111	−10	42	38.6	37.6	38.8	37.8
122	1	41.3	39.2	37.6	39.3	38.2
125	4	40.5	39.4	37.4	41.6	38.1
146	25	38.2	38.4	37.8	39	37.4
153	32	37.3	39	37.2	39.3	37.3
155	34	36.1	38.8	35	N/A	36.1
160	39	38.2	N/A	40	N/A	36.7
174	53	38.6	N/A	N/A	N/A	39
175	54	N/A	N/A	N/A	N/A	N/A

dpi1: day after the first infection; dpi2: day after the second infection. Results are expressed as C_q values. N/A: negative result.

antibodies until the end of the experiment without significant variations after the two challenges. However V-5198 became seronegative before the 2nd infection, which induced a rise in the levels of VP7 Abs. Then once again V-5198 became seronegative, as detected at 202 dpi, and was still seronegative at the end of the experiment. Non-vaccinated heifers were confirmed seropositive at the latest at 18 dpi, with no significant variations until the end of the experiment ($P > 0.05$; Supplementary file 2).

First vaccination of the heifers did not induce detectable neutralising Abs, which rose at 10 and 14 days after the booster vaccination (Fig. 2). First infection clearly increased neutralising antibodies titres through the first month post-infection. Maximal median titres were found between 38 and 83 dpi. The effect of vaccination was quite obvious after first infection, as median titre of the vaccinated group rose clearly faster, reaching a level at 10 dpi only reached about 25 days later in the non-vaccinated group. In the latter group, median titres of neutralising antibodies titres rose very regularly after first infection, reaching a plateau and detected from 38 to 83 dpi. Likewise for vaccinated animals, the day of the second challenge median neutralising Abs titres had decreased. After the second challenge, evolution of median neutralising Abs of the animals was irrespective of their vaccination condition. Vaccinated and non-vaccinated heifers had titres showing a strong boost until 153 dpi, while afterwards these titres stayed high until the end of the experiment. Milk of the infected heifers was strongly positive after calving, regarding VP7 antibodies (Table 2). Colostrum and milk at 1 dpp could not be tested because of

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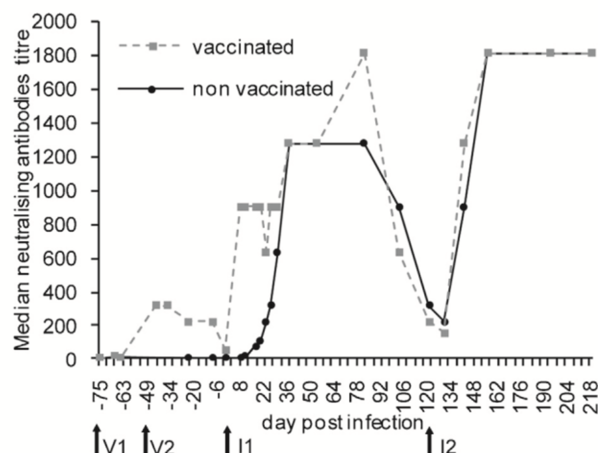

Fig. 2. Evolution of BTV-8 neutralising antibodies titres. Median neutralising antibodies titres are shown for heifers, after vaccinations and challenges. The two vaccine injections are represented as arrows followed by V1 and V2 respectively, and first and second challenge are represented as arrow followed by I1 or I2, respectively.

cream-like aspect that impaired proper pipetting and led to inconsistent results.

At the necropsy of the heifers, petechial haemorrhages on prescapular lymph nodes (NV-8381 and 1905 with a marked severity in the latter) and thymus (NV-8381) could be found. Histopathologic examination of the lymph nodes revealed multiple haemorrhagic foci, congestion, and sinus filled with inflammatory cells and hypertrophic cell rows compatible with a humoral response.

3.2. Clinical and laboratory observations of the offspring

All of the calves' sera attested a good immunity transfer, with density values ranging from 52 to 88 g/L, and mean densities by groups were not significantly different (Welch test, $P > 0.33$; data not shown).

Among the offspring, all the calves, with the exception of ONV-1 and 3, were born asymptomatic and at birth their mean body weights by groups were not significantly different (Welch test, $P > 0.52$, Table S1). ONV-1 and ONV-3 showed a reddening of the muzzle at birth, lasting for 3–4 days (Fig. 3). ONV-3's nose was homogenously hyperaemic, whereas ONV-1 only had congestion between the nostrils. These lesions were evident immediately after birth and were clearly different from wounds caused by reciprocal muzzle suckling of the calves and occurring right after milk consumption from the bucket.

No BTV RNA or positive viral isolation could be found in calves, whichever the considered time-point.

All the calves were seronegative at birth and then apart of control animals offspring, got highly seropositive after colostrum intake.

None of the calves had detectable neutralising Abs before colostrum intake (Table 2). Three calves from non-vaccinated (ONV-1(2200), ONV-3(1905), ONV-5(1893)) and one from vaccinated (OV-2(5211)) dams showed obvious haemorrhages of the pulmonary artery, involving from the inner half (OV-2) to the whole thickness of the vessel (ONV-3), and clearly visible from the pericardic face of the artery (Fig. 4).

Histopathological examination confirmed the presence of multiple haemorrhagic foci. Red blood cells were found among collagen and elastin fibres through the artery wall, especially around the intramural capillaries of the *vasa vasorum*. No viral RNA could be amplified in any of the tested organs, neither in heifers nor in offspring. Numerous haemosiderophages could be found in all the pulmonary arteries with haemorrhages following Perls staining (Fig. 4). Haemosiderophages were grouped in the periphery of dense haemorrhagic areas.

4. Discussion

Vaccination with the inactivated BTV-8 vaccine used in this study resulted in a total clinical and virological

Table 2
Antibodies in the milk and in the serum of the calves before/after colostrum uptake.

Calf	Milk 2 dpp	Serum before colostrum		Serum 1 dpp	
	%S/P (iELISA)	%N (cELISA)	Titre (SNT)	%N (cELISA)	Titre (SNT)
OV1	225	146	<8	6.3	452
OV2	316	136.8	<8	5.9	904
OV3	265	132.6	<8	6.4	450
OV4	173	137.3	<8	10.8	113
OV5	223	137.2	<8	11	452
ONV1	235	140	<8	6.5	904
ONV2	229	142.9	<8	6	319
ONV3	211	139.9	<8	3.8	452
ONV4	229	137	<8	5.5	904
ONV5	248	139.9	<8	5.2	638
OC1	1	145	<8	138.8	<8
OC2	21	133.9	<8	138.6	<8

dpp: day post-partum.
%S/P: Optical density, measured at 450 nm, of the sample, divided by the optical density of the positive kit control, X100%. %S/P ≥ 110% is positive.
%N: percentage of negativity (%N). %N ≤ 35 is positive; 35 < %N ≤ 45 is doubtful; %N > 45 is negative.
SNT: Seroneutralisation. Titre was defined as the reciprocal of the serum dilution causing a 50% reduction in cytopathic effect. titre <20 is negative; =20 is doubtful; and >20 is positive.
iELISA: indirect ELISA.
cELISA: competitive ELISA.

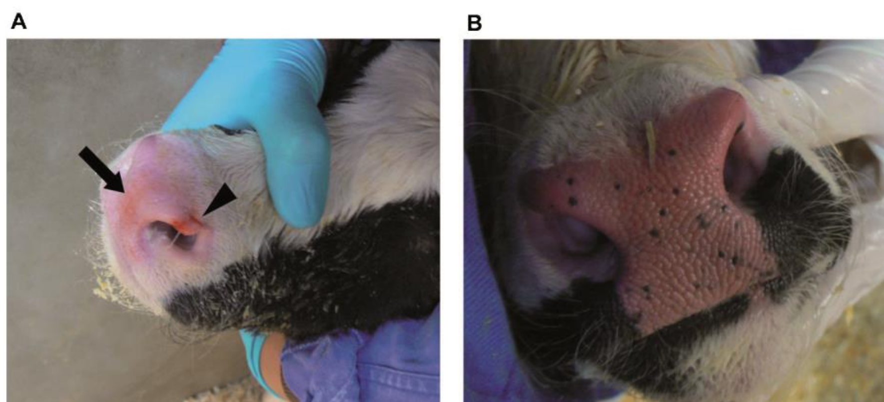

Fig. 3. Congenital reddening of the muzzle in two newborn calves. Two newborn calves from non-vaccinated dams were born with a slight reddening of the muzzle. Newborn calf ONV-1 (A) showed lesion for 3 dpp. Wounds caused by reciprocal suckling between calves (arrow head) have to be distinguished from actual congenital reddening, mostly concentrated between the nostrils (arrow). By contrast, ONV-3 had a uniform congestion of the muzzle that last for 4 dpp (B). The pictures were taken within 6 h post-partum.

protection of the heifers following two challenges with a homologous BTV serotype. In line with field data (Hund et al., 2012), vaccination only elicited the production of moderate neutralising Abs titres, which were fairly lower than those obtained after infection. However, it has been previously reported that clinical and virological protection may occur also even without detectable neutralising Abs prior to BTV inoculation (Eschbaumer et al., 2009; Savini et al., 2009). The very short positivity periods shown by V-5198 after each challenge should lead to consider this animal as a poor responder as defined by Outteridge (1993). The occurrence of embryonic death in heifer V-8379 was not a consequence of vaccination. An *a posteriori* dosage of PAG revealed that out of all the pregnant heifers, V-8379 had the lowest PAG level at 35 and 38 days of pregnancy, respectively 13 and 10 days prior to the first vaccination. PAG reached a concentration <0.8 ng/ml, (which constitute the threshold for positive pregnancy diagnosis (Sousa et al., 2008)) 4 days after vaccination (Supplementary file 3). Regarding the half-life of PAG at this time of pregnancy (4 days), embryonic death can be set the week before the first vaccine injection, excluding the embryonic death to be a vaccination side effect.

Concerning non-vaccinated heifers, most of the evaluated parameters were consistent with existing literature data, such as the detection of viral RNA in the blood from 7 to 174 dpi (Bonneau et al., 2002) and the occurrence of seronconversion between 9 and 12 dpi (Dal Pozzo et al., 2009; Darpel et al., 2007). Viraemia was not influenced by the 2nd infection because of the immunity induced by the 1st challenge. As previously described (Darpel et al., 2007; Martinelle et al., 2011), we noticed, only in some non-vaccinated heifers, a contrast between the mildness of clinical signs and an unexpected severity of histopathological lesions, despite a longer gap between infections and euthanasia. No similar lymphatic tissue activation could be seen in control animals or vaccinated heifers. Recent studies of experimental infections highlighted the risk of incidental inoculums contamination also using cell-infected supernatants (Breard et al., 2011; Dal Pozzo et al., 2013; Eschbaumer et al., 2011). The use of a blood inoculum from a BTV-8 infected calf, tested negative for BVDV and BoHV-1, held in a BSL-3 facility and submitted to a daily close clinical examination however limits the likelihood of an unnoticed contamination.

In this longitudinal study, the most intriguing finding was the haemorrhage of the pulmonary artery found as a pathological marker in 4 calves. No neurological signs, viraemia or BTV-8 Abs before colostrum intake were reported. Nevertheless, haemorrhage of the pulmonary artery was present in 3 out of 5 calves born from non-vaccinated heifers and in 1 out of 5 calves born from vaccinated heifers. In addition, 2 out of 3 of the non-vaccinated born calves with haemorrhage of the pulmonary artery showed at birth another BTV associated lesion (Backx et al., 2009), the reddening on the muzzle, which disappeared within 3–4 days. In this study infections were planned at times prone to generate nervous malformations but even when placenta crossing is certified, the lack of nervous lesions is not totally unforeseen, as recently demonstrated in goats (Coetzee et al., 2013).

Haemorrhages of the pulmonary artery in ruminants have been considered almost pathognomonic to BT (Erasmus, 1975; Worwa et al., 2010). Nevertheless, the lesion was associated with septicaemic pasteurellosis in sheep (Lujan et al., 2005) and *Streptococcus gallolyticus* subspecies *gallolyticus* infection in deer (Velarde et al., 2009). Each of these cases was related with death or severe affection of the animals, and no involvement of cattle. A similar lesion is also reported in deer infected with haemorrhagic adenovirus, but it has been shown that cervid adenovirus is unable to produce lesions in calves (Woods et al., 2008). In the present study, bovine neonatal pancytopenia can also be excluded, because of its case fatality rate (up to 90%), the lack of clinical signs and the age of the calves when euthanatized (Bastian et al., 2011). In addition, the heifers were not vaccinated with PregSure®, an inactivated vaccine against BVDV whose role in bovine neonatal pancytopenia has clearly been established during the past two years (Bastian et al., 2011). BTV related cases of haemorrhage of the pulmonary artery are generally limited to sheep, with only one known cattle

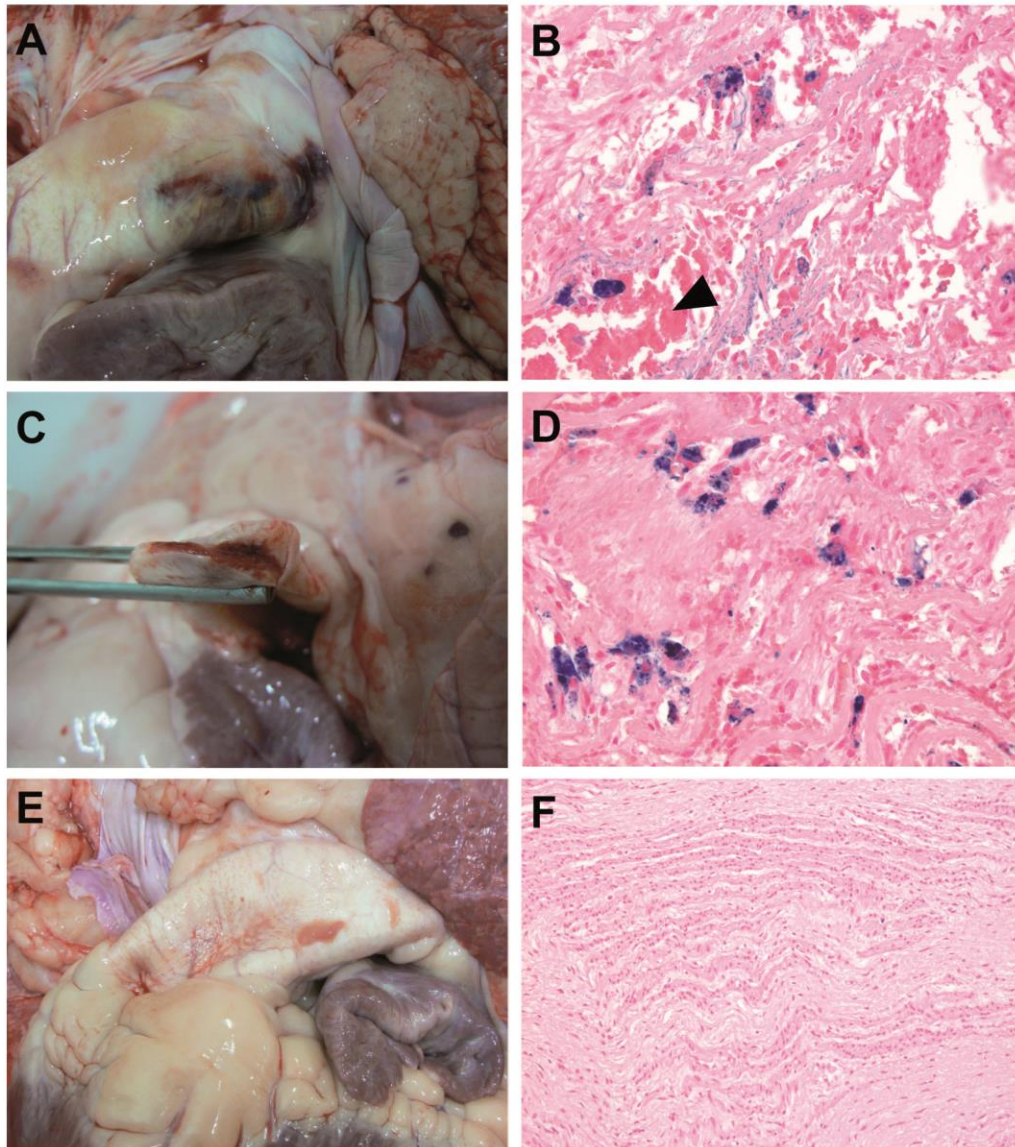

Fig. 4. Haemorrhages of the pulmonary artery in three calves. (A), (C), and (E) respectively macroscopic view of the pulmonary artery seen at necropsy in calves ONV-1, OV-2 and OC-1. Once the pericardium was removed, in cases (A) and (C) haemorrhage was easily visible. (B), (D), (F) microscopic sections of the pulmonary artery of ONV-1, OV-2, and OC-1, respectively (Perls staining 400×). (B) the artery shows diffuse haemorrhagic foci with erythrocytes disrupting conjunctival fibres (arrow head). (D) numerous haemosidereophages can be seen in the erythrocytes surrounding areas. (E) and (F) No macroscopic haemorrhage or ferritin deposit could be seen in the pulmonary artery of OC-1.

exception in literature (Baldwin et al., 1991). The intra mural perivascular localisation of the haemorrhages seen in 4 calves supports a bleeding origin from the *vasa vasorum*, as also reported in recent studies involving infection with BTV8 and BTV23 in sheep (Umeshappa et al., 2011; Worwa et al., 2010). Another outstanding feature is the acute or even hyperacute feature of the pulmonary artery lesions, observed several months after the last challenge. Extravasation of red blood cells should be followed by erythrophagocytosis within at least a few days (Geisbert et al., 2003). This is confirmed in this study by Perls staining, which also prove the long-lasting characteristic of these acute lesions and allows to rule out a recent origin of the bleeding. In human, macrophages containing haemosiderin can first be seen in tracheal aspirates at least 50 h after an acute pulmonary haemorrhage (Sherman et al., 1984). In a murine model, no haemosiderin-laden alveolar macrophages could be detected prior to day 3 after intranasal instillation of blood, and persisted for two months. In our study, the precise mechanism of the haemorrhagic process was not determined, but the haemorrhages were ancient enough to allow the deposit of haemosiderin in the macrophages, and leakage was probably still going on by the time of euthanasia as illustrated by the numerous red blood cells within the arteries wall.

Typical bluetongue pulmonary artery lesions, macroscopically detected (multifocal haemorrhages) or as histopathological alterations (lymphocytic arteritis), were reported in sheep infected with BTV-8 (Worwa et al., 2010) or BTV-25 (Toggenburg Orbivirus) (Chaignat et al., 2009), despite an only mild clinical presentation. In the latter study, two inoculated sheep had clinical signs consistent with BTV infection, including haemorrhage of the pulmonary artery, with viral RNA undetectable throughout the whole experiment. In the two above mentioned studies, animals were euthanatized using captive bolt pistol stunning plus bleeding, thus a different technique compared to pentobarbital injection plus bleeding used in the current work. The euthanasia method itself therefore cannot be the cause of the acute haemorrhage and the haemosiderin deposits in the macrophages excludes the lesions to be an agonic process consequence.

Out of the 4 calves with haemorrhages of the pulmonary artery, ONV-1 and ONV-3 had also a congenital reddening of the muzzle, a clinical observation previously reported by Backx et al. in a BTV-8 transplacentally infected calf (Backx et al., 2009).

None of the BTV induced lesions or clinical signs can be regarded as pathognomonic (Mertens et al., 2009). However, BTV clinical signs and lesions are characteristic and amongst them haemorrhages in the wall of the pulmonary artery and reddening of the muzzle are highly evocative. In this study, a total of 4 calves showed haemorrhages at the base of the pulmonary artery, and two of them also had congenital erythematous muzzle. On the other hand, no viral RNA could be detected in these animals, whichever blood or organs were tested. In addition, at birth before colostrum intake, no calves had detectable anti BTV-8 Abs. Other studies previously reported lesions in bovine (ovarian lesions) attributed to BoHV-1 (Miller and Van der Maaten, 1987), Akabane virus (Parsonson et al., 1981) and BTV-1 (de la Concha-Bermejillo et al., 1993) despite the failure to isolate these viruses or to detect viral antigens.

A random, incidental origin of the lesions seems unlikely, as 40% of the infected born calves showed haemorrhages of the pulmonary artery. Another explanation would be the lesions to be related to another pathogen than BTV. Indeed it is virtually impossible to strictly rule out this hypothesis, as it is impossible to test the inoculum and the calves for every single existing cattle disease. However, the clinical monitoring of the calf whose blood was used as inoculum did not suggest any infection but BT (Dal Pozzo et al., 2009). Plus, that blood was tested negative for BVDV and BoHV-1. Furthermore, there is very little literature (if any) that relates haemorrhages of the pulmonary artery to any pathogens that leave the calves clinically unaffected.

A last explanation would be the transitory BTV infection of the foetuses, without production of Abs. Bovine foetuses are able to respond to an antigenic stimulation since the fourth month of pregnancy (Rosbottom et al., 2011), which matches here with the time of the first infection. The use of a frozen then thawed blood inoculum through intravenous inoculation could allow a quick circulation of released viral particles in maternal plasma, allowing a direct invasion of the foetal tissues (Arvin, 1997). So calves born without VP7 Abs might have been infected prematurely to be properly immunocompetent, therefore unable to synthesise Abs. Besides, viral particles could have been eliminated before birth by cell mediated and innate immunity, playing an important role in protection against BTV (Umeshappa et al., 2010). This hypothesis assumes that the time period when heifers were infected constituted some kind of hinge in the course of the disease pathogenesis, with foetuses not mature enough to build up a humoral immune response detectable at birth, but already able to get rid of the virus through their cellular immune response. This is supported by a recent study from Coetzee et al., where malformed goat foetuses, also showing gross lesions including haemorrhage of the pulmonary artery, were Abs negative. These foetuses had only very low viral RNA load, and the authors suggested that BTV nucleic acid would have been undetectable at birth (Coetzee et al., 2013). However as none of the kids were kept until birth, the outcome of their serological status remains hypothetic. As it is currently considered that *in utero* infected foetuses eventually seroconvert, it is not possible to be more assertive in this way.

One of the main outstanding aspects of the BT pathogenesis is the now clearly established increased placenta crossing ability of European wild type BTV-8 when compared to other non lab-adapted strains (De Clercq et al., 2008). It is widely accepted that in cattle any immune response from the foetus owes itself alone, as maternal immunoglobulins and cytokines are usually not able to cross the placental barrier (Osburn et al., 1982). In this study we found BTV very typical lesions in calves born from BTV infected heifers. Nonetheless, no viraemia or Abs prior to colostrums uptake could be detected in these animals. Additional investigations would be required to clearly establish these haemorrhagic lesions to be a consequence of BTV-8 infection despite the lack of viral RNA detection.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2013.08.016.

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Supplemental material

Supplementary file 1

[1]: expressed in days after AI

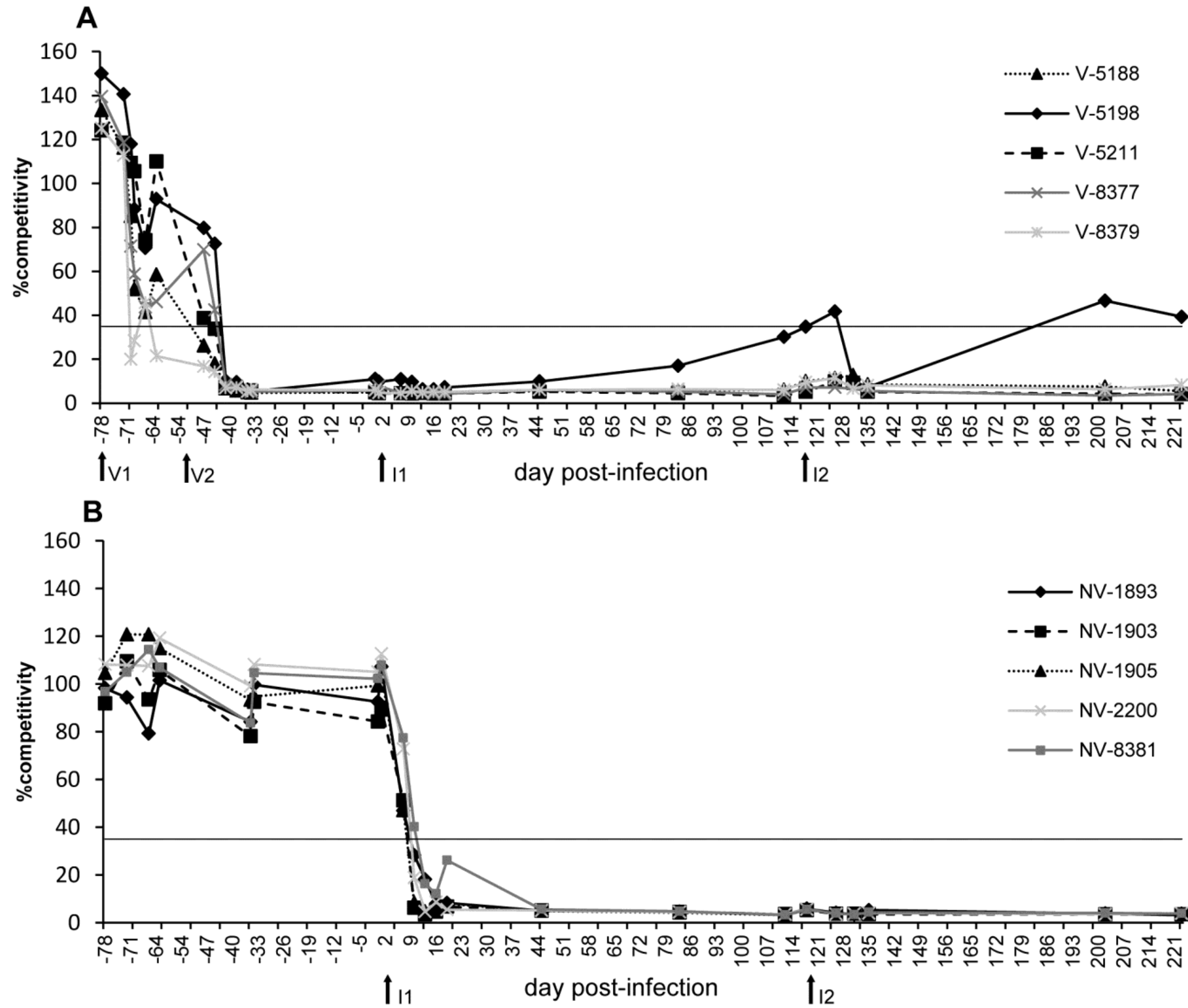
[2]: the number in brackets corresponds to the standard deviation

[3]: expressed in kg

[4]: because of the artificial induction of calving and subsequent shorter pregnancy in V-8379, OV-5 was not included in means and standard deviations calculations

Dam	Calf	Sex	Pregnancy duration[1]	Mean duration/group[1, 2]	Birth weight[3]	Mean weight/group[2, 3]
V-5188	OV-1	M	269	275.25 (5.9)	48	45.5 (5.6)
V-5211	OV-2	M	273		45	
V-8377	OV-3	M	276		51	
V-5198	OV-4	F	283		38	
V-8379	OV-5[4]	F	266		32	
NV-2200	ONV-1	F	283	284 (3.8)	46	44 (3.7)
NV-1903	ONV-2	M	285		50	
NV-1905	ONV-3	M	287		50	
NV-8381	ONV-4	M	279		41	
NV-1893	ONV-5	F	289		47	
CH-5213	OC-1	F	277	282.2 (1.4)	39	46.8 (7)
CH-5199	OC-2	F	279		49	

Supplementary file 2



Supplementary file 3

[1]: the number in brackets corresponds to the standard deviation

Calf	dpp	g/L	Mean by group [1]
OV-1	5	64	68.4 (4.72)
OV-2	6	74	
OV-3	7	66	
OV-4	7	65	
OV-5	4	73	
OC-1	5	72	62 (14.14)
OC-2	4	52	
ONV-1	7	88	73 (8.75)
ONV-2	5	73	
ONV-3	7	67	
ONV-4	6	67	
ONV-5	4	70	

Supplementary file 4

[1]: PAG antisera from bovine origin
[2]: PAG antisera from caprine origin

dpAI	dpi	dpV1	RIA-497[1]	RIA-706[2]
34	-88	-10	1.029	1.34
45	-77	1	1.008	1.161
46	-76	2	0.992	1.041
47	-75	3	0.902	0.827
48	-74	4	0.774	0.485
49	-73	5	0.582	0.654
50	-72	6	0.68	0.764
51	-71	7	0.601	0.514
52	-70	8	0.288	0.347
53	-69	9	0.332	0.547
54	-68	10	0.449	0.489

Experimental section

Study 4 :

Experimental Infection of Sheep at 45 and 60 Days of Gestation with Schmallenberg Virus Readily Led to Placental Colonization without Causing Congenital Malformations

PLoS One 10(9) 2015

Martinelle L, Poskin A, Dal Pozzo F, De Regge N, Cay B, Saegerman C.

Preamble

The main impact of SBV on livestock consists in reproductive disorders, with teratogenic effects, abortions and stillbirths. Several experimental infections were performed to date, involving target species or laboratory animal models, but by the time we implemented this study none of those were carried out on pregnant animals. The outcome of teratogenic viral infections of the fetus depends on the susceptibility of the fetus to the infecting virus, which, in turn, is dependent on the gestational age of the fetus. Congenital abnormalities following infection with the closely related Akabane virus were thoroughly described, stressing out the tight relationship between the stage of gestation and the effects of the infection on the fetus. For SBV the characteristics of placental crossing remain currently poorly understood but as well are critical to establish the limits of the infectivity window that lead to congenital malformation in ruminant offspring. To set these boundaries is especially important in sheep since SBV had a greater impact on sheep flock than in cattle herds in several European countries. As a matter of fact, dairy cattle give birth basically all year round while lambing is mostly seasonal. Sheep breeding seasons are widely overlapping with some of the highest vector activity periods in Europe. Thus considering the breeding season as a risk factor in correlation with the vector activity, the management of the breeding season could be a key element to avoid congenital malformations and to get ewes infected before or after the critical period of susceptibility for the fetus.

Therefore we decided to implement an experimental infection of pregnant ewes, inoculated with SBV during the first third of pregnancy, at 45 or 60 days of gestation. Ewes were separated in three groups: eight and nine ewes were subcutaneously inoculated with 1 ml of SBV infectious serum at 45 and 60 dg, respectively (G45 and G60). Six other ewes were inoculated with PBS as control group.

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RESEARCH ARTICLE

Experimental Infection of Sheep at 45 and 60 Days of Gestation with Schmallenberg Virus Readily Led to Placental Colonization without Causing Congenital Malformations

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Abstract

Background

Main impact of Schmallenberg virus (SBV) on livestock consists in reproductive disorders, with teratogenic effects, abortions and stillbirths. SBV pathogenesis and viral placental crossing remain currently poorly understood. Therefore, we implemented an experimental infection of ewes, inoculated with SBV at 45 or 60 days of gestation (dg).

Methodology

"Mourerous" breed ewes were randomly separated in three groups: eight and nine ewes were subcutaneously inoculated with 1 ml of SBV infectious serum at 45 and 60 dg, respectively (G45 and G60). Six other ewes were inoculated subcutaneously with sterile phosphate buffer saline as control group. All SBV inoculated ewes showed RNAemia consistent with previously published studies, they seroconverted and no clinical sign was reported. Lambs were born at term via caesarian-section, and right after birth they were blood sampled and clinically examined. Then both lambs and ewes were euthanazied and necropsied.

Principal Findings/Significance

No lambs showed any malformation suggestive of SBV infection and none of them had RNAemia or anti-SBV antibodies prior to colostrum uptake. Positive SBV RNA detection in organs was rare in both G45 and G60 lambs (2/11 and 1/10, respectively). Nevertheless most of the lambs in G45 (9/11) and G60 (9/10) had at least one extraembryonic structure SBV positive by RTqPCR. The number of positive extraembryonic structures was significantly higher in G60 lambs. Time of inoculation (45 or 60 dg) had no impact on the placental

Competing Interests: The authors have declared that no competing interests exist.

colonization success rate but affected the frequency of detecting the virus in the offspring extraembryonic structures by the time of lambing. SBV readily colonized the placenta when ewes were infected at 45 or 60 dg but infection of the fetuses was limited and did not lead to congenital malformations.

Introduction

In summer 2011, a new unspecific clinical syndrome was first described in adult cattle in Germany causing febrile disease, milk drop and diarrhoea [1], later attributed to a novel *Orthobunyavirus* (family *Bunyaviridae*) named Schmallenberg virus (SBV). Palearctic telmophagous midges of the genus *Culicoides* [2–4] were identified as the vector of SBV. However the most striking consequence of SBV spreading throughout Europe was the epizootic of malformations in ruminant offspring, leading to abortions, peripartum mortality and stillbirths. Indeed, SBV was linked to an arthrogryposis / hydranencephaly syndrome in ruminant newborns following *in utero* infection [5].

According to literature about Akabane virus (AKAV), a closely related *Orthobunyavirus*, the moment of the gestation when the mother gets infected influences the outcome of an *in utero* infection. In sheep, Parsonson et al. suggested that AKAV might only be able to reach the fetus if placenta is developed and vascularized enough [6], whereas Hashiguchi et al. considered congenital malformations highly unlikely to occur if infection took place after 50 days post insemination [7]. So far, susceptible gestation periods leading to congenital malformations need to be clarified in the different SBV host species.

In several European countries, SBV had a greater impact on sheep flock than in cattle herds [8, 9]. As a matter of fact, dairy cattle give birth basically all year round while lambing are mostly seasonal, with mating of the sheep concentrated in July-August or October-November, depending on the breed [10]. Indeed, sheep breeding seasons are widely overlapping with some of the highest vector activity periods in Europe [11]. Moreover, increased odds of malformations were reported in sheep flock with an early mating season in 2011 [12]. Thus considering the breeding season as a risk factor in correlation with the vector activity, the management of the breeding season could be a key element to avoid congenital malformations and to get ewes infected before or after the critical period of susceptibility for the fetus. Based on the pathogenesis of Akabane virus and epidemiological studies of the SBV outbreaks, it is assumed that teratogenic infection takes place in the first trimester. Stockhofe et al. performed SBV experimental infection of sheep at 38 and 45 days of pregnancy [13], however the infected ewes were slaughtered one week after inoculation. The aim of the current study was to investigate the occurrence of malformation at term in ewes infected later in the course of gestation. Thus we implemented an experimental study performed on groups of pregnant ewes subcutaneously infected with infectious SBV serum at day 45 and 60 of gestation. Therefore we could get a better insight on SBV pathogenesis in pregnant ewes and rate of transplacental transmission and colonization following two different gestation times of inoculation.

Materials and Methods

Ethical statements

The experiments, maintenance and care of ewes complied with the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and other

Scientific Purposes (CETS n° 123). The protocol used in this study was approved by the Ethical Committee of the IPH-VAR (Scientific Institute of Public Health—Veterinary and Agrochemical Research Center (VAR), number of project: 121017–01) on the 11th February 2013. All surgery was performed using xylazin (Paxman®, Virbac, France) and local anesthesia (procaine hydrochloride 4%, VMD, Belgium), and all efforts were made to minimize suffering.

Animals

A total of 23 "Mourerous" ewes of about 1 year-old and originating from a SBV free area in France were used in this experiment (original sheep flock from the *Alpes-Maritimes* department (ISO code FR-06), animals selected after a last serological screening carried out on the 08/11/2012). The Mourerous is a middle-size rustic breed from south of France. All the animals were serologically and virologically negative for SBV as determined by ELISA, SNT and RTqPCR (see below) before and after arrival at the experimental animal centre of CODA--CERVA where they were kept in Biosafety Level 3 facilities. The ewes also tested negative for bluetongue virus (BTV) and Maedi-Visna virus. The animals were only implemented in the experiments after a thorough clinical examination to ensure their asymptomatic state and good clinical condition in range with the physiological parameters [14].

Inoculum

The infectious serum used for inoculation of the sheep was obtained from Friedrich Loeffler Institute (Riems, Germany) and was already successfully tested in calves and sheep [1, 15, 16]. Briefly, the infectious serum originated from a heifer sampled two and three days after inoculation with infectious whole blood from a SBV-positive cow. The inoculum contained about 2 x 10^3 50% tissue culture infective dose/ml ($TCID_{50}$/ml) [15] and 7.3 x 10^6 RNA copies/ml of SBV S-Segment as determined by a RTqPCR detecting the SBV-S segment (see below).

Insemination and diagnosis of gestation

The animals were synchronised using Veramix sponges (60 mg Medroxyprogesterone Acetate, Zoetis®, Louvain-La-Neuve, Belgium). The sponges were removed 12 days after insertion and the ewes received 500 IU Pregnant Mare Serum Gonadotrophin through intramuscular route. Intracervical artificial insemination (AI) has been realized 52 hours after sponge removal, with a double dose of semen.

A total of four rams, from 1 to 6 years old, were used to get semen doses: 3 *Ile-de-France* and one crossbred Texel X Ile-de-France. These rams originated from a Maedi-Visna negative flock, and despite being SBV seropositive, their semen has been tested negative twice, one week apart, for SBV RNA (RTqPCR). A sample of each of the semen doses used for the actual AI has been also tested negative for SBV RNA *a posteriori*.

Gestation diagnostic has been performed by detection of pregnancy associated glycoprotein (PAG) by radio immune-assay (RIA) at 27 days post AI as previously described [17] and then by ultrasonography at 40/55 and 95/110 dg for the groups inoculated at 45 and 60 dg, respectively.

Experimental design

During the whole duration of the experiment insect light/glue traps were displayed and regularly removed and replaced, allowing identification of the caught insects to ensure the absence of *Culicoides*.

The ewes were inoculated subcutaneously (SC) in the left axilla with 1 ml of infectious serum. Eight ewes were inoculated at 45 dg (G45, ewe1 to ewe8) and nine ewes at 60 dg (G60, ewe9 to ewe17). Two control groups of three animals each were inoculated SC with sterile phosphate buffer saline respectively at 45 and 60 dg (control G45 and control G60, ewes18 to 20 and ewes21 to 23).

During the first two weeks of the experiment, clinical, virological and serological monitoring was realized on a daily basis, then once a week until the end of the experiment.

At the expected lambing period for each respective group, ewes were closely monitored and when first signs of labor could be identified, the ewe was anesthetized with xylazin (Paxman®, Virbac, France) intravenously (IV) and local anesthesia (procaine hydrochloride 4%, VMD, Belgium), and lambing was perform by Caesarian section. Once the Caesarian section was realized, the ewe was euthanatized with IV injection of 5 ml of T61® (MSD Animal Health BVBA, Belgium) and subsequently necropsied.

Lambs were identified following their mother (ewe1 gives birth to lamb1; plus a, b or c in case of multiple gestation). When the lambs were born alive they were revived to allow the evaluation of any neurological trouble. Ability of standing up and suckling reflex were evaluated and a morphologic examination, with special emphasis on skull and limbs, was also performed. Blood samples were taken and lambs were then euthanatized with 1 ml T61® IV and necropsied. Tissue samples were taken from extraembryonic structures (intercotyledonary membrane, placentome, umbilical cord, amniotic fluid, meconium), and organs including CNS (brain, cerebellum, brainstem, spinal cord), lymphoid organs (prescapular, mesenteric, submandibular, mediastinic lymphnodes, thymus and spleen), other inner organs and tissues (gonads, adrenal gland, liver and lung) and musculoskeletal structures (femoral cartilage, *Musculus Semitendinosus*) for viral RNA detection. Placentomes were collected, longitudinally cut and separated in two halves for subsequent SBV RNA detection. The Intercotyledonary membrane was sampled in the area surrounding the collected placentomes. Samples (2 cm long) of the umbilical cord were collected 4–5 cm distal to the newborns.

SBV detection by RTqPCR

The extraction of RNA from organs, feces, blood and serum and the detection of SBV-S segment by a one-step RTqPCR were performed as previously described [18].

The Cq values were converted into S-segment copy numbers using a RNA standard curve that was tested in each PCR run [19]. Briefly, RNA was extracted from SBV infectious serum with the RNeasyMini kit (Qiagen) following manufaturer's instructions and reverse transcribed with M-MLV Reverse Transcriptase (Life Technologies). A PCR amplification using primers targeting a 839bp fragment of the S gene of SBV (forward: 5′ -CTAGCACGTTGGATTGCTGA-3′ ; reverse: 5′ -TGTCCTTGAGGACCCTATGC-3′ ; Integrated DNA Technologies) was performed using the FastStart PCR Master kit (Roche). The fragment was cloned in the 2.1-TOPO cloning vector (Life Technologies) and transformed into competent *Escherichia Coli* TOP10 cells and multiplied. The plasmids were isolated and then linearized with BamHI, followed by *in vitro* transcription with the TranscriptAid T7 High Yield kit (Thermo Scientific) following manufacturer's instructions. Remaining DNA plasmids were eliminated by 2 successive Turbo DNA free treatments (Life Technologies) following manufacturer's instructions and the RNA was purified using the RNeasyMini kit (Qiagen). The copy number was calculated based on the predicted molecular weight of the RNA transcripts. Aliquots of the RNA transcripts were stored at −80°.

The RNA standard curve consisted of a ten-fold serial dilution of the RNA transcripts in TE buffer. The dilution series ranging from 3.9×10^7 to 3.9 copies/μL were run together with

samples of blood or organ and the standard curve was constructed by plotting the Cq values against the log of the input RNA copy number. A linear regression was fitted to the scattered points and was used to calculate the number of copies in the samples ran during the same RTqPCR.

Serology

The presence of neutralizing anti-SBV antibodies was assessed by seroneutralization (SNT), following the method described by De Regge et al. [20] and using an SBV isolate obtained from brain tissue of a lamb aborted in Belgium in 2011 which was passaged four times in Vero cells. Two positive and one negative control were added systematically to each assay. The titer was determined as the reciprocal of the highest serum dilution in which the entire monolayer was still intact. Sera were considered positive if the titer was ≥ 4 (specificity of 100% [20]).

Statistical analysis

Differences in RNAemia of the different groups through time were assessed by two-way ANOVA with repeated measures. Difference between mean maximum copy numbers by group was evaluated with Welch test for unequal variance. Differences in positive detection of viral RNA in lambs, organs and reproductive performances (viability at birth, prolificacy) were evaluated by Chi^2 test or Fisher's exact test for count data depending on the sample size.

P values ≤ 0.05 were considered significant. Statistical analyses were performed using the R software/environment (R-3.0.1, R Foundation for Statistical Computing, http://www.r-project.org/).

Results

Clinical impact and lambing

No clinical impact following inoculation was measured in ewes. The temperatures stayed within the physiological range upon euthanasia. All the animals conserved a good appetite and remained in good general condition.

Table 1 reports sex ratio, prolificacy rate and the percentage of living lambs at birth. No significant different was reported in the viability of the offspring between infected groups and between infected and control groups (Chi^2 test = 0.7, $P = 0.4$ and Chi^2 test = 0.3, $P = 0.6$, respectively; df = 1). Prolificity rate was also not significantly different between infected groups and between infected and control groups (Chi^2 test = 0.1, $P = 0.74$ and Chi^2 test = 0.008, $P = 0.93$, respectively; df = 1). None of the lambs had malformation evocative of SBV infection (arthrogryposis-hydranencephaly syndrome, stiff neck, scoliosis, brachygnathism). Only one lamb was born three days before the expected date and was in good health. All other lambs were born at term, no malformation was observed and they were able to stand up and showed a good suction reflex.

SBV RNA detection

SBV detection in the blood and organs of the ewes. No viral RNA was detected in the blood of the ewes from the control groups. SBV genome was first detectable at 2 dpi in both G45 and G60 groups and could be detected for maximum 5 days (Fig 1). The mean SBV genome copy numbers detected in the blood of sheep with RNAemia were not significantly different between G45 and G60 groups through time (two-way ANOVA for repeated measures; group effect: $P = 0.4$; group-time interaction: $P = 0.44$). The mean of the maximal SBV RNA

Table 1. Reproductive performances.

Group	Number of lambs	Sex ratio	Weight at birth (Kg)[a]	Prolificacy rate (%)	% of healthy living lambs
G45	11	0.8	4[a](0.7); 3.1[b](1.1); 2[c](0.3)	137.5	63.6
G60	10	1.5	4[a](0.5); 3.3[b](0.5)	111.1	80.0
Control G45	4	1.0	5[a](1)	133.3	50.0
Control G60	3	2.0	4.5[a](0.5); 3[b](1.8)	100.0	67.0
Total	28	1.2	3.7(1.2)	121.7	67.9

Sex ratio is the ratio of male lambs to female lambs. Prolificacy is defined as the number of progenies born per parturition.

[a]: Single lambs

[b]: Twins

[c]: Triplets

Standard deviation follows in curved brackets.

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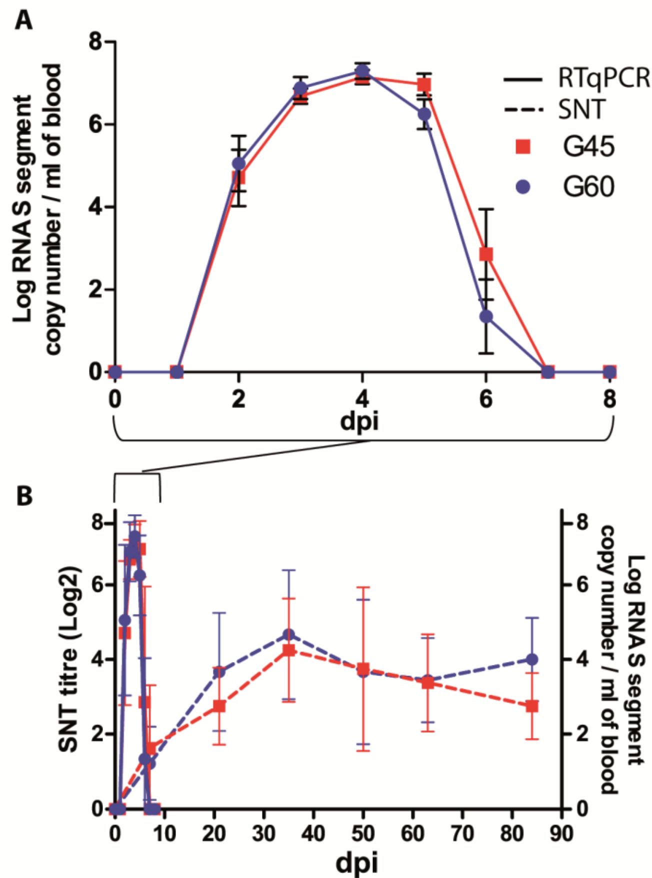

Fig 1. SBV viral RNA detection and neutralizing antibodies detection in the serum of the pregnant ewes. Detection of SBV genome in serum for G45 and G60 ewes (A). Results of SNT for G45 and G60 ewes and SBV genome detection downscaled to 90 days (B). Full line represents SBV genome detection and dashed line represents SNT.

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copy number was not significantly different between G45 and G60 (Welch test for unequal variance; $P = 0.83$).

SBV genome could be detected in the ovaries of ewe9, in the ovaries and the spleen of ewe11 and only in the spleen ewe16, all belonging to G60. No SBV nucleic acid could be detected in any of the G45 ewes or control groups' organ samples.

SBV detection in the blood and organs of the lambs. No SBV RNA could be detected in the blood of any of the lambs, from any of either the infected or control groups.

SBV RNA was shown in the brainstem and spinal cord of lamb10 and in the femoral joint cartilage, prescapular lymph node and muscle *M. semitendinosus* of lamb11 (both G60); in G45 viral genome could only be demonstrated in the lung of lamb4b (Fig 2).

SBV detection in extraembryonic structures. Intercotyledonary membrane was the most frequently SBV positive extraembryonic structure in both G45 and G60, (50% of tested samples; 5/10) followed by amniotic fluid (38,9%; 7/18), umbilical cord (38.5%; 5/18) and placentomes (33.3%; 6/18) (Fig 2). Percentage of positive detection was higher in G60 (47.2%; 17/36) versus G45 (19.4%; 6/31) for each of the latter structures, and overall difference was significant (Chi^2 test = 5.74, df = 1, $P = 0.017$). For individual extraembryonic structure, detection was only significantly more frequent in G60 (55.6%; 5/9) versus G45 (10%; 1/10) for placentome (Chi^2 test = 4.55, df = 1; $P = 0.033$). Meconium of G45 lambs was found more often positive than G60 ones (7/11 versus 3/10), but that difference was not significant (Chi^2 test = 3.38, df = 1, $P = 0.12$).

The rate of placenta colonization, as defined as the number of lambs with at least one positive extraembryonic structure divided by the total number of lamb, was however not significantly different between G60 (90%; 9/10) and G45 (82%; 9/11), respectively (Fisher's exact test for count data = 1.94; $P = 1$).

Serology

All the inoculated ewes seroconverted within three weeks, with first positive detection at 7 dpi for several ewes of both G45 and G60. Then all the inoculated ewes remained seropositive until the end of the experiment (Fig 1B).

No newborn lamb had anti-SBV neutralizing Abs before colostrum uptake.

Discussion

Viral placental crossing is defined as the passage of the virus from the mother through the placenta to fetal tissues. Thus, placental colonization does not necessarily imply passage of the pathogen to the fetus itself [21]. In this study SBV placental colonization could be demonstrated after experimental infection in most of pregnant ewes at 45 and 60 days of gestation. Extraembryonic structures were found frequently positive, as previously described after natural infection [18, 20].

However no teratogenic effect could be reproduced. Congenital malformations caused by SBV remain a rare phenomenon in the field; teratogenesis associated with SBV infection has been observed only in 1 malformed calf out of 72 ones born from infected dams in natural conditions [22]. In addition, Veldhuis et al. (2014) estimated in cattle the malformation rate at about 0.5% of infected fetuses [23]. Therefore with respect to the number of newborn lambs considered in the current study, the lack of malformations is not especially surprising, despite lambs being thought to display more often malformations caused by SBV than calves [9, 24–25].

In both G45 and G60, the percentage of successful placental colonization was high (9/11–82% and 9/10–90%, respectively) and consistent with previous experimental data reporting a

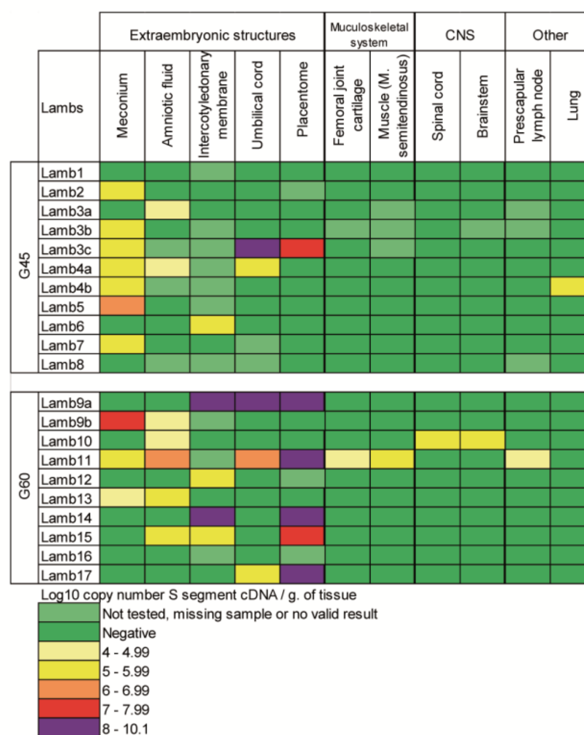

Fig 2. Mondrian matrix of SBV viral RNA detection in organs at necropsy. Rectangles color-coded according to SBV detection level. CNS: Central nervous system.

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100% placental colonization with or without placental crossing in ewes infected with SBV at 38 or 42 dg [13]. However, in our study significantly more positive samples, from both extraembryonic structures and lamb organs, were found in newborn lambs originating from ewes that were infected at 60 dg compared to those infected at 45 dg. It is likely that infection in most of G45 fetuses was about to be resolved with SBV eliminated from many organs whereas G60 fetuses could not clear the virus as much, the infection being more recent in the latter. Indeed the G60 group had a shorter period to eliminate the virus (85–87 days for G60 instead of 100–102 for G45). Another explanation could derive from the hypothesis emitted by Parsonson et al. regarding AKAV, a close related *Orthobunyaviruses*. These authors hypothesized the efficiency of the placental crossing being related to the development and vascularization of the placentomes, possibly explaining the highest detection rate in G60 when compared to G45 [6]. The higher development of placentomes at 60 dg might provide more target cells to infect and therefore lead to prolonged positive detection in the infected tissues. According to Parsonson et al., fetal trophoblastic cells constitute privileged target cells for AKAV, and SBV was reported to readily replicate in ovine trophoblast cells both *in vitro* [26] and *in vivo* [8], possibly being important target cells for SBV as well. Lawn et al. [27] reported that in sheep placenta examined at 42 days of gestation, placentomes are rather undifferentiated with only a few displaying rudimentary villi and crypts. Therefore placentomes at 45 days of gestation might be insufficiently developed to support intensive viral replication, yet allowing placental colonization. By contrast, placentomes at 60 days of gestation could better sustain SBV replication, as reported for AKAV [6].

A strong correlation between malformations and precolostral anti-AKAV antibodies has been reported [28], and growing evidences suggest that this pattern is shared by SBV, with 79 to 91% of naturally infected malformed lambs reported to be seropositive before colostrum uptake [29, 30]. Therefore in the current study, with no lamb showing SBV-associated abnormalities it is not surprising that all the precolostral sera were negative.

To produce Abs, the fetus has to be immunocompetent and the virus has to reach the systemic blood circulation of the fetus. In sheep *in utero* acquisition of humoral immune competence can be roughly estimated to span from 66 to about 100 dg [31]. For AKAV production of neutralizing antibodies is considered to start at 65–75 dg in sheep [32]. However Parsonson et al. reported ewes infected between 30 and 36 dg giving birth to malformed lambs with precolostral neutralizing antibodies against AKAV [33]. In the latter study a significant individual variation in humoral response of fetuses to AKAV was reported and no stage of gestation could be associated to seroconversion in 100% of the infected animals.

Therefore, despite a frequent successful placental colonization of SBV, infection of the fetus itself might be far less common, since SBV RNA could only be detected in some organs of lamb4b (G45), lamb10 (G60) and lamb11 (G60). Together the rare productive infection of the fetus and the individual variation could explain the absence of precolostral Abs against SBV in this study.

Although SBV RNA was detected at least once in almost all the extraembryonic structures of the lambs born from infected ewes, SBV positive detection in central nervous system (CNS) was very scarce with only one brainstem and one spinal cord sample found positive from lamb10 (G60 lamb). In ruminant newborns showing congenital defects suggestive of SBV infection, CNS is very often positive by RTqPCR [18, 20]. Here, despite SBV detected in several organs but CNS (with the exception of lamb10 with SBV RNA in spinal cord and brainstem), no malformations were reported, supporting a CNS invasion and SBV genome detected at birth possibly being associated to congenital malformations.

From the infected pregnant ewe to the CNS of the fetus the virus has to cross two major histological obstacles, namely placenta and blood-brain barriers (BBB).

It has been suggested that the development of the BBB was the reason that adult animals only show asymptomatic or very mild SBV infection [26]. In sheep BBB to sucrose (small and metabolically inert molecule) starts to develop around 50–60 dg, undergo a large decrease of permeability at about 70 dg and keep developing until 123 dg [34]. Nevertheless no malformed lambs were found in G45 and G60 although G45 ewes were infected before the assumed establishment of the BBB. Orthobunyaviruses are considerably larger (about 100 nm in diameter [35]) when compared to sucrose (0.44–0.53 nm); hence viral particles crossing might be hindered already at an earlier stage of gestation. A delayed transmission from the mother to the fetus is unlikely since Stockhofe et al. reported a 100% placental crossing 7 days after the infection of pregnant ewes with SBV [13]. The critical period of gestation leading to malformation in lambs might be narrower, possibly closer to the lower estimate for AKAV, from 28 to 36 dg [8], or to the one reported by Sedda et al. for SBV, between 37 and 42 dg [36]. Herder et al. [37] described SBV antigens in ruminants CNS mostly detected in temporal and parietal lobes, mesencephalon and hippocampus. Besides, cerebellar hypoplasia was frequently reported. Several critical neurologic events related to these structures occur during the first third of gestation in sheep: post-proliferative zone appears in the cerebellum between 21 and 31 dg; hippocampus CA1/CA3 neurogenesis spans from 22 dg to 59 dg, and neurogenesis of the cortical layer VI peaks around 40 dg [38]. Moreover in the spinal cord motor neurons are differentiated and develop mostly between 31–35 dg, corresponding to the time the embryos become motile [39]. Thus most of these decisive steps were over or about to be finished by 45 dg. As a consequence

SBV infecting fetuses after 45 dg might be able to cross the BBB but might fail to invade CNS because of the scarcity of developing target cells.

It was assumed that SBV induced malformations are secondary to CNS lesions since SBV shows a clear tropism for nervous tissues [29, 33]. Infection by pestiviruses like border disease virus and bovine viral diarrhea virus are characterized by CNS dysplasia and hypomyelinogenesis in a similar way than following AKAV infection [40]. However since arthrogryposis induced by pestiviruses is neither as frequent nor as severe than in cases caused by AKAV infection, authors hypothesized that the increased frequency of musculoskeletal troubles following *in utero* AKAV infection might be related not only to CNS damages but also to primary infection of fetal muscles [41, 42]. Indeed, primary infection of muscle cells was confirmed in ruminant fetuses infected by AKAV [6, 43]. The presence of SBV in muscle tissues is less frequently evaluated, and if so, quite often by IHC or *in situ* hybridization, with frequent negative results [30, 26, 44]. In the current study one muscle sample from a G60 lamb was found positive by RTqPCR, without signs of gross pathology. The lack of SBV positive detection in the serum of any of the lambs excludes a blood origin by the time of the test. In line with this result, Balseiro et al. reported a higher detection level of SBV RNA in muscles than in the brain or spinal cord of malformed calves [45]. These data suggest that primary infection of fetal muscles might act as an auxiliary mechanism to CNS infection in the pathogenesis of the hydranencephaly/arthrogryposis syndrome caused by SBV, as well as speculated for AKAV.

Yet not statistically significant, an intriguing finding was the SBV RNA higher detection rate in meconium from G45 lambs (7/11) than from G60 ones (3/10). Meconium was reported to be a matrix either rarely or frequently found positive by RTqPCR, depending on the considered study [18, 22]. It is noteworthy to underline that at the same time, amniotic fluid was found more often positive in G60 lambs (5/10) than in G45 lambs (2/11) but not statistically significant as well. As meconium is an end-product of the amniotic fluid being swallowed by the fetus during gestation time, it is likely that SBV detection in the meconium could reflect the former transient presence of the virus in the amniotic fluid. Fecal shedding was reported following SBV experimental infection in heifers [46] and ewes [47]. Hence positive SBV RNA detection in meconium might be the *in utero* version of the previously reported positive detection in the feces of adult animals.

Conclusion

The current study demonstrated a very high placental colonization rate when infection occurred at 45 or 60 days of gestation in an experimental context. As an extension of field data where congenital malformations were highly associated with SBV RNA detection in CNS, the absence of gross pathology evocative of SBV infection was reported along with RTqPCR negative results in CNS. In most of the cases, SBV did not reach CNS or could not produce an infection lasting long enough to be detected at birth. In addition, the lack of reported malformations supports the need of an established SBV infection in the CNS of the fetus to become teratogenic. Quite surprisingly, as the placental barrier along with the increasing immunocompetence were assumed to decrease the likelihood of a positive RNA detection at birth, the closer the infection from the term, the higher was the frequency of organs still positive. SBV could infect the placenta of most of the infected ewes. By contrast, viral genome could only be detected in organs of three new-born lambs, of which only one showed positive detection in CNS. No anti-SBV Abs could be detected before colostrum uptake. These results suggest SBV can readily colonize the placenta, but subsequent infection of the fetus is either rare or short lasting. Consequently CNS infection is even less common and so are malformations, at least following infection of the ewes at 45 or 60 days of gestation. *In situ* hybridization and

immunohistochemistry might provide more detailed characterization of the cells involved in placental and fetal colonization. Additional experimental infections of pregnant ewes at earlier times in gestation would allow a further clarification of the limits of the specific stages of gestation leading to congenital defects.

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Author Contributions

Conceived and designed the experiments: LM AP FDP NDR BC CS. Performed the experiments: LM AP. Analyzed the data: LM AP CS. Contributed reagents/materials/analysis tools: LM AP NDR BC CS. Wrote the paper: LM AP FDP NDR BC CS.

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Experimental section

Study 5 :

Experimental bluetongue virus superinfection in calves previously immunized with bluetongue virus serotype 8

Veterinary Research 47(2016) (1) :73

Martinelle L, Dal Pozzo F, Sarradin P, Van Campe W, De Leeuw I, De Clercq K, Thys C, Thiry E, Saegerman C.

Preamble

The 2008 BTV8 epizootic in Northern Europe is believed to have caused greater economic damage than any previous single serotype BT outbreak. At the same time, BTV1 was circulating in Spain, Portugal and South-West of France. Moreover, herds simultaneously BTV1 and BTV8 positive were detected at the overlapping fringes of the BTV1 and BTV8 affected areas. As a consequence, cattle could be infected by both serotypes at the same time or sequentially. The current study investigates the impact of a BTV1 superinfection on cattle previously immunized against BTV8 following experimental challenges on BTV8 vaccinated and non-vaccinated cattle, with particular emphasis in cross-reactivity between serotypes. The inoculum happened to be contaminated with BTV15, so far exotic to Europe mainland. As a consequence we further characterized the BTV1-BTV15 co-infection. In addition we compared BTV1 and BTV15 in single serotype challenges.

This study provides new insights in heterologous protection between BTV1, BTV8 and BTV15. The impact of the different challenges on clinical signs and viraemia is also discussed.

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VETERINARY RESEARCH

RESEARCH ARTICLE Open Access

Experimental bluetongue virus superinfection in calves previously immunized with bluetongue virus serotype 8

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Abstract

The effect of a superinfection with bluetongue virus serotype 1 (BTV1) was evaluated on two groups of four calves. One group received a commercial inactivated BTV serotype 8 (BTV8) vaccine. This group and the non-vaccinated group of calves were challenged twice (4 months apart) with the European BTV8 strain isolated during the 2006–2007 epidemics. Calves were then infected with a BTV1 inoculum which was found to be unexpectedly contaminated by BTV serotype 15 (BTV15). BTV1 and BTV15 single infections were performed on two other groups of three BTV naïve calves. A severe clinical picture was obtained after superinfection with BTV1/BTV15 in both vaccinated and non-vaccinated animals and after challenge with BTV8 in non-vaccinated animals. BTV1 and BTV15 single infection caused only very slight clinical signs. After superinfection and at the viraemic peak, there were an average of above 1000 times more BTV15 genomic copies than BTV1 ones. BTV1 RNA could be detected only in the spleen of one calf whereas BTV15 RNA was found in 15 organs of seven different animals. BTV8 immunization whether it was acquired through vaccination and challenges or challenges alone did not change BTV1 or BTV15 RNA detection in superinfected animals. However in these animals a partial cross neutralization between BTV8 and BTV1 might be involved in the lower BTV1 replication versus BTV15. Infection with different serotypes can occur also in the field. Interference between virus strains, genetic reassortment and cross-protection were considered as mechanisms to explain the clinical outcomes and the other virological and immunological findings in the course of BTV1/BTV15 superinfection.

Introduction

Bluetongue (BT) is a non-contagious disease affecting ruminants and is caused by the bluetongue virus (BTV), the type species of the genus *Orbivirus*. BT is a World Organization for Animal Health reportable disease and is of considerable socioeconomic concern and of major importance in the international trade of animals and animal products [1]. Economic losses associated with BTV infection are caused directly through reductions in animal productivity and death, implementation of control measures, and more importantly, indirectly through trade losses due to animal movement restrictions [2].

Within each different *Orbivirus* species, several virus serotypes are identified, based on the specificity of reactions with the neutralizing antibodies generated by their mammalian host [3]. These reactions are dependent to a large extent to VP2 and also VP5, which are the most variable proteins in BTV; VP2 especially contains the most epitopes that drive neutralizing antibodies production and therefore is the main determinant of the serotype [4]. To date, 27 serotypes have been identified, including BTV25 identified in Switzerland in 2007 [5], BTV26 from Kuwait in 2010 [4] and BTV27 detected in goats in Corsica (France) in 2014 [6]. In addition, two putative new serotypes, respectively BTV28 and BTV29 were recently detected [7]. Indeed serological cross-reactions between different serotypes are described [8] and evidences of

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possible heterologous cross-protection do exist [9, 10], but their influence on the epidemiology of the disease is not sufficiently understood.

From 2006 to 2015, seven BTV serotypes were detected in Western and Central Europe, namely BTV1, 6, 8, 11, 14, 25, and 27. Most of the economic losses have to be attributed to BTV8, and to a lesser extent, BTV1, with respectively over 27 000 and 6000 holdings affected only in 2008. BTV8 alone was responsible for the death of more than 20 000 sheep in Belgium, which represents 5–10% of the national flock [11]. The 2008 BTV8 epizootic in Northern Europe is believed to have caused greater economic damage than any previous single serotype BT outbreak [12]. By contrast re-emergence of BTV8 in France in 2015 was only of limited impact [13].

On the other hand, BTV1 that circulated contemporarily in Southwest Europe, was described as a virus leading to subclinical or mild disease in cattle [14]. As a consequence of this epidemiologic context, domestic ruminants in the field could be sequentially infected by these two serotypes, as it was reported in France and Spain [15].

In this paper the results of a 9 month-long experiment are shown. Calves were originally divided in two groups, with one group being vaccinated against BTV8, and were subsequently both challenged with a homologous BTV8 European strain. In order to mimic the occurrence of repetitive infections according to studies reporting several peaks of vector activity during the course of the year [16], the same calves were infected a second time with the same BTV8 strain and later with BTV serotype 1 (BTV1) (superinfection). The aim of this study was to analyse the outcome of these successive challenges, taking into account the influence of vaccine immunity as well as natural post-infection immunity. The BTV1 inoculum appeared to be contaminated with BTV15 [17]. In order to evaluate any in vivo cross-protection, the consequences of a BTV1 and BTV15 single infection in BTV naïve calves were also considered.

Materials and methods

BTV8 successive infections

Ten Holstein female calves, about 6–7 months old, were used. All the animals were tested seronegative and non viraemic for BTV and bovine viral diarrhoea virus (BVDV), and seronegative for bovine herpesvirus 1 (BoHV1). A thorough general clinical examination was carried out on all the animals by a veterinarian before including them in the study, to confirm their asymptomatic state, in accordance to physiological standards [18].

The calves were housed in an insect-secured BSL3 zone at the Experimental Infectiology Platform (PFIE) of the INRA centre of Tours (Nouzilly, France). The local ethical committee approved the experimental protocol (dossier n.2011-10-1). Three groups were created: a group of four non-vaccinated calves (group NV, calves 1–4), a group with four vaccinated calves (group V, calves 5–8), and an environmental control group with two calves (group C, calves 15 and 16). Vaccination against BTV8 was performed using the inactivated commercial vaccine BTVPUR AlSAP 8 (Merial, Lyon, France) following manufacturer's instructions.

Calves of group V and NV were infected twice, 4 months apart, using the same BTV8 inoculum described in a previous experimental infection [19]. Briefly, a calf inoculated with a BTV-8 strain passaged twice in baby hamster kidney fibroblasts (BHK-21) cells (BEL2006/01 BHK-21 P2), was blood sampled at the viraemic peak, showing clinical signs. The first infection took place 50 days after the second vaccine shot, and the second challenge 120 days later (Figure 1A). Each time, half of the dose was inoculated via the jugular vein and half subcutaneously. For each one of these challenges, all eight infected animals were administered a total of 15 mL of blood, corresponding to a titre of 10^3 embryo lethal dose 50 (ELD_{50}).

The animals were daily examined and sampled for EDTA-blood and whole blood on dry tubes during the 3 first weeks after infection, then twice a week until superinfection. For clarification purpose, days post infection regarding BTV8 successive infection are mentioned as dpi_{BTV8}, with day of first challenge as dpi_{BTV8} 0.

BTV superinfection

About 5 months (160 days) after the first BTV8 infection, and 40 days after the second one, calves of groups NV and V were challenged with BTV1 infectious blood, kindly provided by the Friedrich-Loeffler Institute. Each animal received $10^{6.15}$ tissue culture infective dose 50% ($TCID_{50}$) of virus, half intravenously and half subcutaneously. An incidental contamination of the inoculum with BTV15 was discovered during the course of the study [17].

The animals were daily examined and sampled for EDTA-blood and whole blood on dry tubes until the end of the experiment.

Calves were euthanized 21 days after the superinfection and necropsied (Figures 1A and B). Days post infection for superinfection is mentioned as $dpi_{BTV1/15}$.

BTV1 and BTV15 single infections

Eight Holstein male calves, about 6–7 months old, were housed in the BSL3 facility, at CODA-CERVA's (Veterinary and Agrochemical Research Centre, Uccle, Belgium) experimental centre (Machelen, Belgium). These animals fulfilled the same inclusion criteria of the BTV8

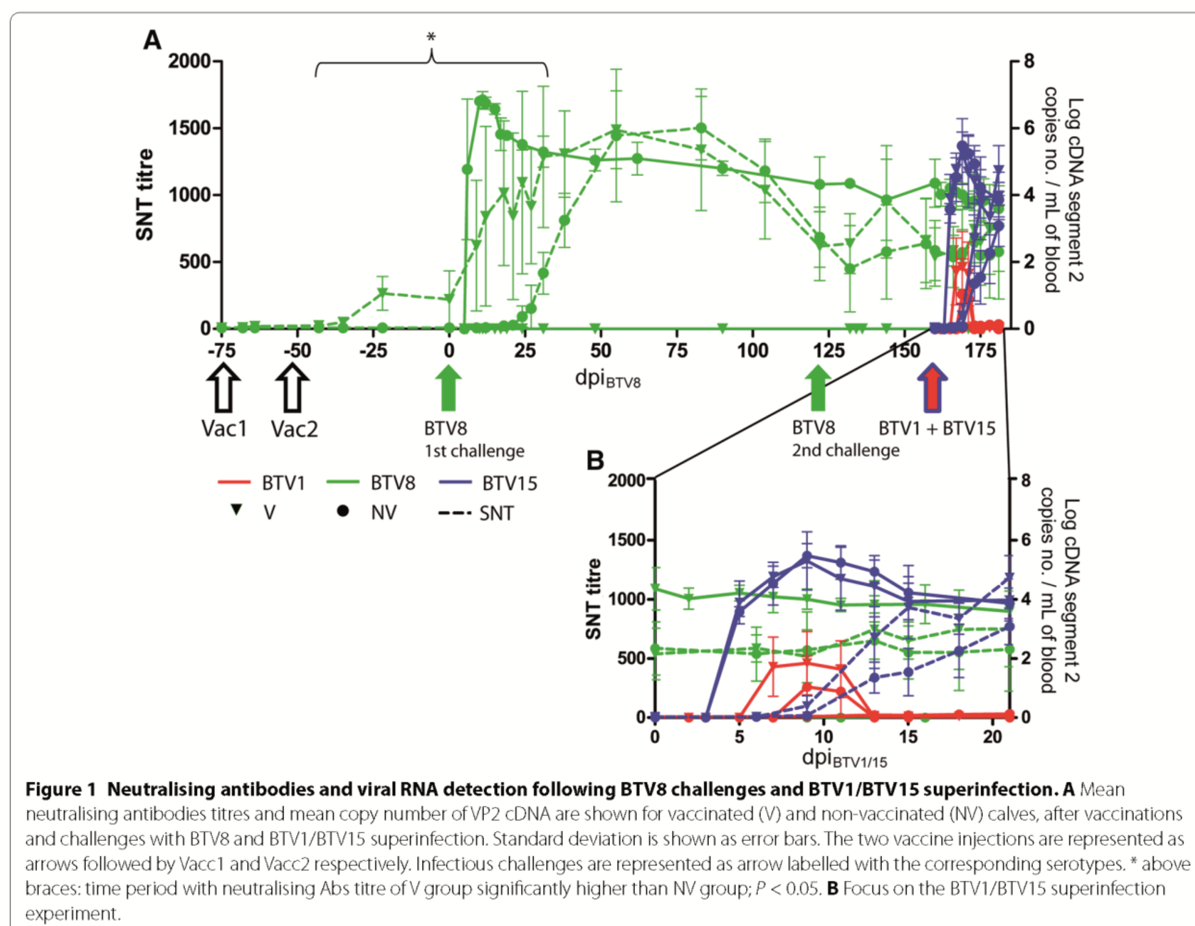

Figure 1 Neutralising antibodies and viral RNA detection following BTV8 challenges and BTV1/BTV15 superinfection. A Mean neutralising antibodies titres and mean copy number of VP2 cDNA are shown for vaccinated (V) and non-vaccinated (NV) calves, after vaccinations and challenges with BTV8 and BTV1/BTV15 superinfection. Standard deviation is shown as error bars. The two vaccine injections are represented as arrows followed by Vacc1 and Vacc2 respectively. Infectious challenges are represented as arrow labelled with the corresponding serotypes. * above braces: time period with neutralising Abs titre of V group significantly higher than NV group; $P < 0.05$. **B** Focus on the BTV1/BTV15 superinfection experiment.

successive infections and were naïve for BTV, BVDV and BoHV-1. The experimental protocol was reviewed by the competent authority (Ethical Committee of the Institute of Public Health-Veterinary and Agronomical Research Centre) and subsequently approved (ref. 110228-01 RT 10/10 BLUETONGUE).

After an acclimatization period of 2 weeks, calves were divided in three groups [BTV1: calves 9–11, and BTV15: calves 12–14, single infections; mock-infection: four calves (calves 17–20)]. The infection was performed with a volume of 1 mL of virus diluted in Dulbecco's modified eagle medium (DMEM, Lonza BioResearch, Belgium), half intravenously and half subcutaneously.

The BTV1 strain has been provided by CODA-CERVA, from sub-saharian origin, derived from the European Community Reference Laboratory for bluetongue at the Pirbright Laboratory, UK collections, and subsequently passaged two times in BHK-21 cells. The infection was performed with 10^6 $TCID_{50}$ per animal.

BTV15 was provided by CODA-CERVA, derived from the European Community Reference Laboratory for bluetongue at the Pirbright Laboratory, UK and was then passaged twice on BHK21 cells at CODA-CERVA. Calves were infected with 10^4 $TCID_{50}$.

Mock-infected calves were inoculated with sterile DMEM following the same routes and volume.

The animals were daily examined and sampled for EDTA-blood and whole blood on dry tubes until the end of the experiment. Calves were euthanized 35 dpi and necropsied. Days post infection for single infections are mentioned as dpi_{single}.

Clinical and post-mortem examination

After each challenge, the individual body temperature and the clinical signs were monitored for 3 weeks. The severity of the infection was quantified by calculating clinical scores on a daily basis, leading to overall cumulative clinical scores by groups and animal. For this

purpose, a standardised clinical form adapted from Saegerman et al. was used [20]. As BTV1 and BTV15 single infections only involved three animals whereas other groups had four calves each, total clinical score was pondered to allow direct comparison of clinical scores from different experiments.

Samples of spleen, thymus, prescapular and mesenteric lymph nodes were collected from infected and control calves and stored at −80 °C for virus detection.

Serology

Neutralizing antibodies (Abs) were titrated by seroneutralization (SNT). Two-fold serial dilutions of the sera (1:10–1:1280) were tested in the presence of 100 $TCID_{50}$ of virus, as previously described [21]. The neutralizing antibody titre was defined as the reciprocal of the serum dilution causing a 50% reduction in cytopathic effect. Serum samples with a titre <20, =20 and >20 were considered negative, doubtful and positive, respectively.

In order to identify in vitro cross neutralization between BTV1, BTV8 and BTV15, the serum of the calf infected with BTV8 and showing the highest anti-BTV8 antibody titre was tested in the presence of BTV1 or BTV15. Similarly, the serum of the calves infected with BTV1 and BTV15 following single serotype infections and showing the highest neutralizing antibody titres against the correspondent virus were tested against heterologous serotypes. In vitro cross neutralization was measured using the percentage of neutralization obtained using heterologous serotypes with immune serum and compared to homologous neutralization as reference (100%).

In the course of the two BTV8 challenges, and after BTV1 and BTV15 single infections, seroconversion against VP7 antibodies was also evaluated using a commercial competitive ELISA kit (ID Screen® Bluetongue Competition ELISA kit, ID Vet, France). Results were expressed as % of negativity (PN) compared to the negative kit control and transferred to a positive, doubtful or negative result according to the cut-off settings provided by the manufacturer (PN ≤ 35 is positive; 35 < PN ≤ 45 is doubtful; PN > 45 is negative).

BTV RNA detection

Viral RNA extraction from the blood was achieved using the QIAamp Viral RNA Mini Kit (Qiagen, Germany). Viral RNA denaturation and reverse transcription followed by qPCR were performed as previously described [22]. BTV RNA was detected by serotype specific RTqPCR, using a fragment of BTV segment 2 as the target. Serial dilutions of in vitro constructed plasmids (pGEM®-T Easy Vector, Promega, The Netherlands) carrying the target part of the segment 2, specific for each serotype, allowed the absolute quantification of the viral cDNA equivalent in samples. Quantification was expressed in cDNA copy number/mL of blood. RTqPCR cycling conditions, primers and probes were similar to the ones described by Vandenbussche et al. [22] for BTV1 and BTV8 (RTqPCR_BTV1_S2 and RTqPCR_BTV8_S2, respectively), and Eschbaumer et al. [18] for BTV15 (RTqPCR_BTV15_S2). In all the RTqPCR of this study, bovine beta-actin was contemporaneously amplified as internal control (RTqPCR_ACT) [23].

BTV RNA detection was performed on all the collected organs starting from approximately 100 mg of tissue, which was processed using TRI reagent according to the manufacturer's instructions (Life Technologies Europe BV, Gent, Belgium). BTV and bovine beta-actin detection were performed by RTqPCR as described above.

Viral growth assay

In vitro replication of BTV1, BTV8 and BTV15 were compared on VERO and Bovine Pulmonary Endothelial cells (BPAEC). BTV8 was the same as in the BTV8 successive infections, and BTV1 and BTV15 were the same as in single infections. These viruses were used for a growth assay following a protocol previously described [24]. Briefly, VERO and BPAEC confluent cells in 24 wells plates were inoculated with a multiplicity of infection (M.O.I.) of 0.05, and after 0, 8, 24, 48 and 120 h post infection (hpi), supernatant was removed and stored at −80 °C. Each virus underwent three independent assays on each cell type. Supernatants were then titrated by end-point dilution and titres expressed as Log10 of $TCID_{50}$/mL.

Statistical analysis

Mean cumulative clinical scores were analysed using linear mixed model, with calf as random effect. Viraemia and serological results were compared using two-way ANOVA with repeated measures. RNA detection in organs at necropsy, frequencies and proportions were compared with Fisher's Exact Test for count data [25]. For all tests, P values <0.05 were considered significant. In case of multiple comparisons, a Bonferroni correction was applied to reduce the risk of type I error (conservative approach) and a Holm correction was applied when more than four comparisons had to be tested. Statistical analyses were performed using the R software/environment (R-3.1.2, R Foundation for Statistical Computing, [26]) and SAS software, Version 9.3 TS level 1M2 of the SAS System for Unix, and SAS University Edition (SAS Institute, Cary, NC, USA).

Results

BTV8 successive infections

Clinical examination

From the beginning to the end of the experiment, control calves and vaccinated animals (V) did not show any clinical signs that could be related to BTV infection. After the first challenge, clinical signs showed by NV calves were slight, mostly consisting in ocular lesions and to a lesser extent by oral lesions. Clinical signs consistent with BTV8 infection could be reported from 7 to 21 dpi_{BTV8}. After the second challenge no clinical manifestations or temperature rise could be detected in any animal.

Serology

VP7 and anti-BTV8 neutralizing Abs in control calves could not be detected at any tested time points.

First vaccination of the calves did not induce detectable neutralizing Abs, which could only be detected 7 days after the booster vaccination. In NV group, neutralizing Abs were first detected at 18 dpi_{BTV8} (Figure 1A). The titre of anti-BTV8 neutralizing Abs of the V group was significantly higher between −43 dpi_{BTV8} (thus 35 days after the first vaccine shot) and 27 dpi_{BTV8} ($P < 0.005$), and then Abs titres of both NV and V groups followed a similar trend until the end of the experiment (Figure 1A).

Following the second BTV8 challenge, neutralizing Abs titres underwent a boost in both NV and V groups until 33 dpi_{BTV8} (Figure 1A).

The use of an ELISA allowed the detection of anti-VP7 Abs in all the vaccinated animals as soon as 3 days after second vaccine injection (Figure 2). Then PN of vaccinated animals did not evolve significantly until the end of the measures at 180 dpi_{BTV8} (Additional file 1). Non-vaccinated calves were confirmed seropositive between 10 and 19 dpi_{BTV8}, with no significant variations until the end of the measures at 180 dpi_{BTV8} (Additional file 1, $P > 0.05$).

BTV RNA detection

BTV8 RNA was never detected in the EDTA-blood samples of control and vaccinated calves during the course of the two BTV8 infections. In the NV group of calves, BTV RNA could be detected starting from 5 dpi_{BTV8}. After the viraemic peak (11–15 dpi_{BTV8}) a progressive decrease in BTV8 RNA was measured (Figures 1A and B), until the end of the experiment.

BTV superinfection

Clinical and post-mortem examination

In the NV as well as in the V group, lesions following BTV1/BTV15 superinfection were mainly conjunctivitis

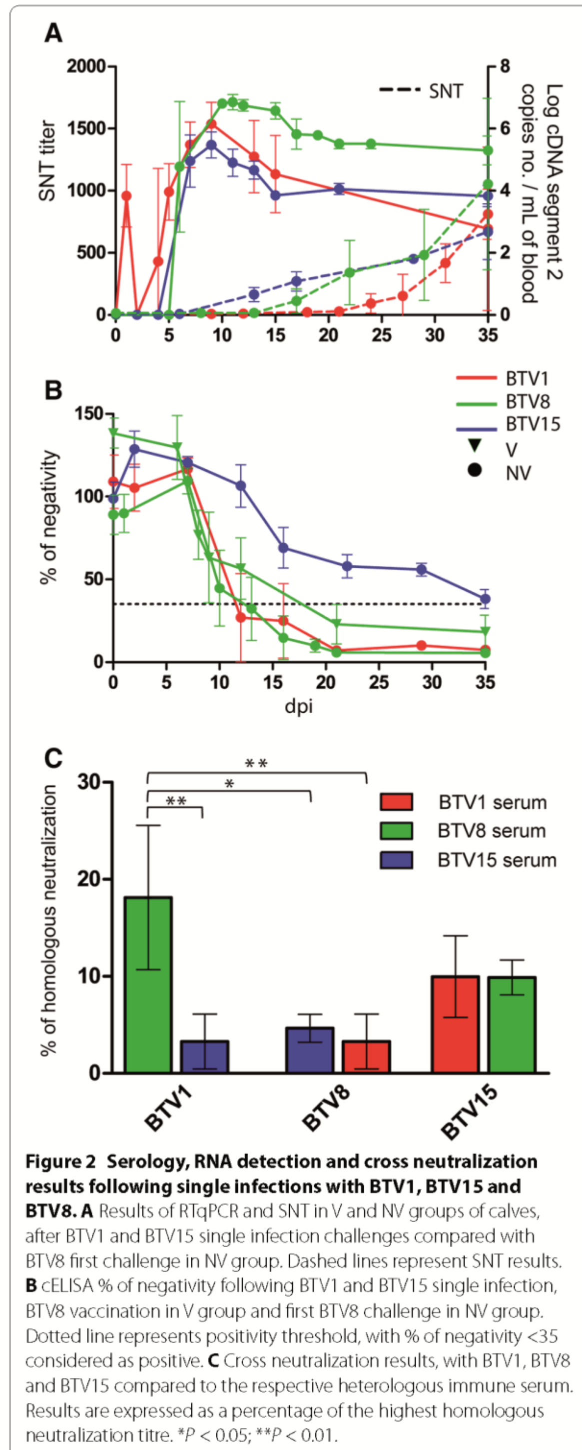

Figure 2 Serology, RNA detection and cross neutralization results following single infections with BTV1, BTV15 and BTV8. A Results of RTqPCR and SNT in V and NV groups of calves, after BTV1 and BTV15 single infection challenges compared with BTV8 first challenge in NV group. Dashed lines represent SNT results. **B** cELISA % of negativity following BTV1 and BTV15 single infection, BTV8 vaccination in V group and first BTV8 challenge in NV group. Dotted line represents positivity threshold, with % of negativity <35 considered as positive. **C** Cross neutralization results, with BTV1, BTV8 and BTV15 compared to the respective heterologous immune serum. Results are expressed as a percentage of the highest homologous neutralization titre. *$P < 0.05$; **$P < 0.01$.

with serous to purulent discharge. Erosions of the muzzle, erosions and ulcerations of the gums and the dental pad and later crusts on the muffle or on the cutaneous-mucous junction were commonly notified. Reddening and swelling of the coronal margin and interdigital space were also mentioned. Conjunctivitis and congestion of the lower limb were mild to severe and respectively less severe and absent in the previous BTV8 infection. At the end of the experiment cumulative clinical score of the NV BTV1/BTV15 superinfected group was higher than in NV BTV8 and V BTV1/BTV15 groups, however the difference was not significant ($P > 0.4$, Additional file 2).

The necropsy revealed petechial haemorrhages of limited extent in prescapular and submandibular lymph nodes, and thymus, at least in one of these organs in all the superinfected calves. BTV8 RNA could be detected in prescapular lymph node of one calf and in the spleen of another one, both from NV group; BTV1 RNA could only be detected in the spleen of one vaccinated calf and BTV15 RNA could be detected with a significant higher frequency ($P < 10^{-5}$) in 15 organs belonging to 7 different calves (5/15 in NV group and 10/15 in V group; Figure 3).

No BTV RNA could be detected in any of the tested organs from control animals.

Serology

After the superinfection, high levels of residual neutralizing antibodies against BTV8 were found throughout the experiment in all the infected animals, with a roughly steady level (Figures 1A and B). There were no significant differences between NV and V groups through time in anti BTV8 neutralizing antibodies (two-way ANOVA with repeated measures, group effect: $P = 0.78$, group time interaction: $P = 0.84$). By contrast, BTV1 only gave rise to very low titres of neutralizing antibodies.

BTV15 neutralizing antibody titre increased regularly after infection, with a positive titre detectable in most of the animals at 9 $dpi_{BTV1/15}$ (Figure 1B). BTV15 neutralizing Abs followed an increasing trend until the time of euthanasia; in previously vaccinated animals a higher and significant earlier raise of neutralizing antibody was found compared to non-vaccinated ones (two-way ANOVA with repeated measures, group effect: $P < 0.02$, group time interaction: $P < 2 \times 10^{-4}$) (Figure 1B).

BTV RNA detection

At the time of the superinfection, a residual BTV8 RNAemia was detected in the NV group of calves. BTV8 RNAemia decreased through the time of the experiment, but was still detectable in all the NV animals by the time of euthanasia (180 days after the first BTV8 challenge—Figure 1).

The superinfection inoculum contained respectively $10^{6.8}$ and $10^{7.6}$ copies of segment 2 cDNA per mL of blood, for BTV1 and BTV15.

After superinfection in the NV and the V groups, BTV1 could only be detected inconstantly, from one to 3 days amongst all the tested day-points and at lower

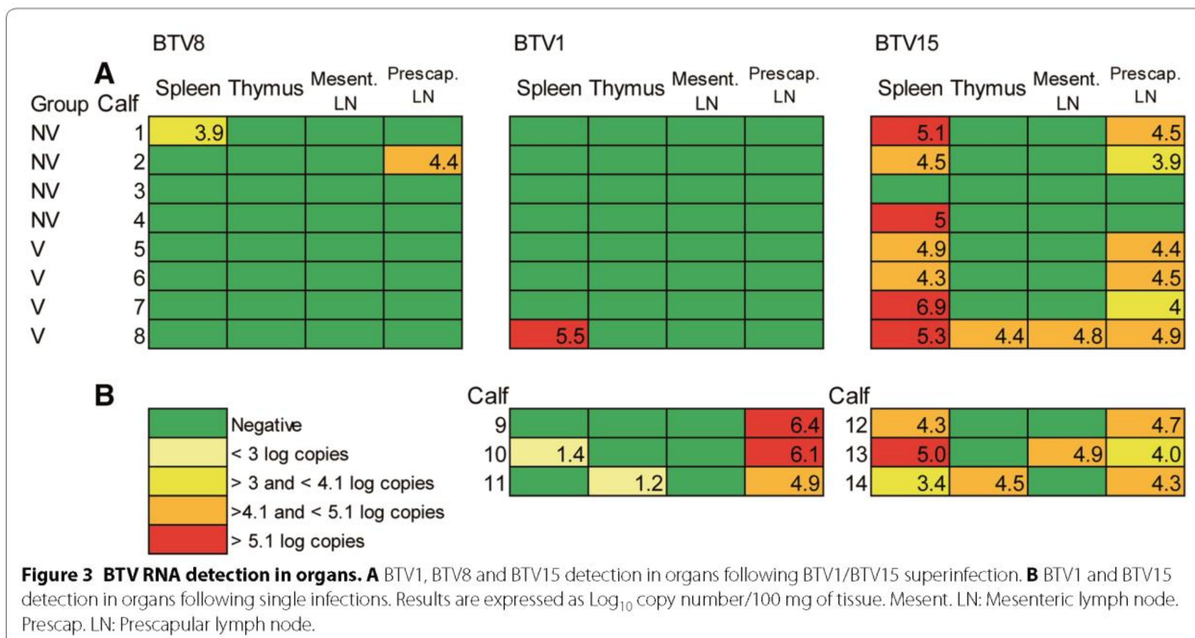

Figure 3 BTV RNA detection in organs. A BTV1, BTV8 and BTV15 detection in organs following BTV1/BTV15 superinfection. **B** BTV1 and BTV15 detection in organs following single infections. Results are expressed as Log_{10} copy number/100 mg of tissue. Mesent. LN: Mesenteric lymph node. Prescap. LN: Prescapular lymph node.

copy number than BTV15 (Figure 1B). On the contrary, BTV15 could be easily detected in both groups and among all the infected calves. RNAemia through time was significantly different between BTV15 and BTV1 whichever the considered vaccination status of the animals ($P < 10^{-8}$). Mean copy number at viraemic peak was $10^{5.4}$ (±0.7 Log) cDNA copy number/mL of blood for BTV15 and $10^{2.4}$ (±1.8 Log) for BTV1. BTV15 could be detected until the end of the experiment (Figure 1B). Between V and NV groups, detection of BTV1 and BTV15 was not significantly different ($P = 0.18$ and $P = 0.86$ for BTV1 and BTV15 respectively).

No viral RNA could be detected in control animals (data not shown).

BTV1 and BTV15 single infections

Clinical and post-mortem examination

During BTV1 single infection, one calf underwent sporadic hyperthermia and all the three calves of the group had mild oral and ocular lesions. No systemic impact was reported in any of these animals.

During BTV15 single infection, infected animals showed very mild clinical conditions compatible with BT, including congestion and crusts on the nostrils and oral mucosa. One calf showed hyperthermia at 7 dpi (39.6 °C) and at 14 (40 °C) and 15 dpi (40.7 °C), with no other lesion throughout the experiment (Additional file 2). No hyperthermia was recorded in any of the other cattle at any stage of the experiment.

Overall, the sum of clinical scores in BTV1 and BTV15 single infected groups were not significantly different when compared to control animals or to the V group after BTV8 challenge ($P > 0.14$), but were significantly lower when compared to first BTV8 challenge in NV calves and the superinfection with BTV1/15 inoculum in both V and NV groups ($P < 0.002$).

Necropsy for BTV1 single infection revealed moderate petechial haemorrhages in mesenteric and mediastinic lymph nodes in one calf, prescapular and mediastinic lymph nodes in another one and no lesions in the last one.

In the calves with BTV15 single infection, no BTV specific lesions could be found at necropsy. Petechial haemorrhages were reported on the thymus and the prescapular lymph node of two calves, respectively, in both cases on a limited amount.

All the three BTV1 infected calves had a positive BTV1 detection in prescapular lymph nodes. In addition, viral RNA could be detected in the thymus and spleen of two other calves, respectively (Figure 3).

RTqPCR revealed BTV15 positive detection in the spleen and the prescapular lymph nodes of all of the three BTV15 infected calves. In addition, thymus and mesenteric lymph node were shown to be positive in two calves, respectively (Figure 3). The frequency of positive detection in organs was not different between BTV1 and BTV15 (Fisher's Exact Test for count data, $P > 0.4$).

No specific lesions or BTV RNA detection could be found in control animals. A few non-specific abscesses could be found in the lung of one of these calves.

Serology

During BTV1 single infection anti-BTV1 neutralizing antibodies could be detected for the first time at the 16 dpi_{single} tested time point and then increased regularly until the end of the experiment (Figure 2A). Similarly, also anti-BTV15 neutralizing antibodies were measured starting from 16 dpi_{single} in the course of BTV15 single infection and the titres increased regularly until the time of euthanasia (Figure 2A).

There was no significant difference between SNT titres of BTV1 and BTV15 single infection and BTV8 after first infection in NV group during the first 35 days ($P > 0.24$).

BTV1 infected calves seroconverted regarding anti-VP7 antibodies between 7 and 16 dpi_{single}, and were still all seropositive at the end of the experiment.

Anti-VP7 Abs in BTV15 infected animals clearly increased between 10 and 15 dpi_{single} in all infected calves (Figure 2B). However, only 1/3 calves seroconverted at the end of the experiment at 35 dpi_{single}, whereas the two other calves remained slightly out the positivity limit (mean PN = 38 ± 5.7, Figure 2B). At 35 dpi_{single} PN of BTV15 infected group was significantly higher when compared to BTV1 single infection group and BTV8 groups at 35 dpi_{BTV8} ($P < 0.007$).

In vitro cross neutralization assay only showed limited cross reactivity between BTV8 immune serum against BTV1 virus (18% ± 7.4% of the BTV8 immune serum homologous neutralization, Figure 2C). However, this cross reactivity was significantly higher than the one measured between BTV1 versus BTV15 immune serum, BTV8 versus BTV1 and BTV15 immune serums ($P < 0.02$). BTV1 and BTV8 immune serum elicited a limited cross reactivity toward BTV15 (10% ± 4.5% and 10% ± 1.8%, respectively).

BTV RNA detection

In the course of BTV1 single infection BTV RNA could be detected as soon as 1 dpi in all the calves of the group, but then could only be detected again at 2 and 3 dpi for respectively one and two calves.

During BTV15 single infection viral RNA could be detected starting from 7 dpi in all three calves. BTV15 RNA could be detected until the end of the experiment (Figure 2A). The levels of RNA peaked in the blood of

both BTV1 and BTV15 groups between 9 and 11 dpi_{single}. BTV1 cDNA copy number detected in single infected calves was significantly higher than in BTV1 superinfected ones, no matter their vaccination status ($P < 10^{-4}$). By contrast, there were no significant differences in cDNA copy numbers between BTV15 superinfected (V and NV groups), BTV1 and BTV15 single infected calves ($P > 0.2$). Moreover, BTV1 and BTV15 cDNA copy numbers were not significantly different from BTV8 cDNA copy number during first infection in NV group ($P > 0.13$).

Viral growth assay

From 0 to 24–48 hpi, BTV1 showed a faster replication, however not significant, whichever the considered cell line (Figure 4). In VERO cells BTV15 grew less efficiently than BTV1 from 0 to 48 hpi ($P < 10^{-5}$), but finally reached by 120 hpi similar titres to BTV1 and BTV8 in VERO cells ($P > 0.14$) and to BTV1 in BPAEC ($P > 0.9$). Homologous viral growth was not significantly different between cell types ($P = 0.15$), and there was no significant differences between serotypes in BPAEC ($P > 0.3$).

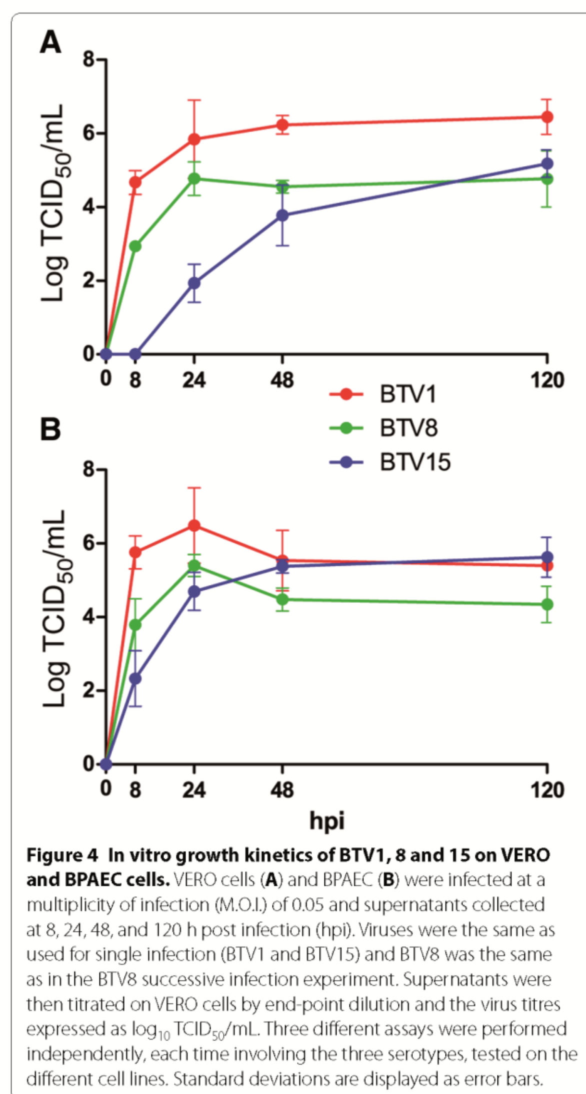

Figure 4 In vitro growth kinetics of BTV1, 8 and 15 on VERO and BPAEC cells. VERO cells (**A**) and BPAEC (**B**) were infected at a multiplicity of infection (M.O.I.) of 0.05 and supernatants collected at 8, 24, 48, and 120 h post infection (hpi). Viruses were the same as used for single infection (BTV1 and BTV15) and BTV8 was the same as in the BTV8 successive infection experiment. Supernatants were then titrated on VERO cells by end-point dilution and the virus titres expressed as log_{10} $TCID_{50}$/mL. Three different assays were performed independently, each time involving the three serotypes, tested on the different cell lines. Standard deviations are displayed as error bars.

Discussion

The influence of the existent active immunity towards the European BTV8 strain on the outcomes of a superinfection with BTV1 was evaluated. The BTV1 inoculum appeared to be contaminated with BTV15 thus the animals were actually infected with a mixed BTV1–BTV15 inoculum. BTV8 active immunity was evaluated either by vaccination followed by infectious challenges or by infectious challenges alone. Two successive infections with the same BTV8 strain were realized 4 months apart. In line with field data [9], vaccination only elicited the production of neutralizing Abs detectable after the second vaccine boost. Vaccinated animals underwent a significantly earlier detection of neutralizing antibodies after the first BTV8 challenge when compared to non-vaccinated calves. In the NV BTV8 group, the detection of BTV8 RNA lasted until 180 dpi_{BTV8} (end of the experiment), which is consistent with currently existing literature data [27, 28]. Non-vaccinated calves infected with BTV8 showed a slight to mild clinical picture. A moderate impact of the disease caused by BTV8 on cattle is not unusual, in experimental infections [22, 29] as well as in the field [30–32].

After the unexpected contamination of the BTV1 inoculum with BTV15, the influence of both viruses on the outcome of the infection was investigated. In the inoculum used for the superinfection, the copy number of BTV1 segment 2 cDNA per mL of inoculum was about tenfold lower than BTV15. After superinfection BTV1 could be found irregularly and only at a few tested time-points in the blood of the calves while BTV15 was detected with high levels of RNAemia until the end of the experiment in both V and NV groups. The overwhelming replication of BTV15 versus BTV1 is in line with results reported by Eschbaumer et al. [18]. Domination of one serotype on another during mixed infection has been previously reported [33] and the same authors observed that about 5% of progeny viruses were actually reassortants. Any genome segment can be involved in reassortment which is readily generated, as demonstrated by Shaw et al. [34]. As in the current study viral RNA was based on segment 2 quantification, it is not possible to rule out that some of the segment 2 detected by RTqPCR being actually part of reassortant viruses. This was also one of the hypotheses brought to light by Dal Pozzo et al. [25] to explain the predominance of BTV8 on BTV1 and

BTV15 in the course of a triple co-infection. Another possible explanation is the viral interference, occurring between two or more viruses infecting simultaneously the same host.

BTV8 was recently used to study underlying IFN-I control mechanisms by the virus [35]. To investigate whether the different tested serotypes would also show different replication patterns in vitro or not, growth curves of BTV1, BTV8 and BTV15 were established in two common cell lines, BPAEC and VERO cells. VERO cells are deficient for IFN-I production [36]. BTV15 did not replicate as much as BTV1 in VERO cells during the first 48 h. Nevertheless, in IFN-I competent cells such as BPAEC primary line, both BTV serotypes replicated following a similar pattern through time. These results are not inconsistent with the hypothesis of BTV15 to be better adapted to IFN-induced state when compared to BTV1, possibly explaining the relatively more efficient replication of BTV15 in vivo or in vitro in IFN-competent cell lines. Another hypothesis to explain the difference between BTV1 and BTV15 RNAemia after superinfection might be related to the influence of BTV8 immunity on these two serotypes. The level of heterologous reactivity as assessed by SNT between BTV8 immune serum and BTV serotypes 1 and 15 was low, yet higher for BTV1 ($18 \pm 7.4\%$ versus $10 \pm 1.8\%$ for BTV1 and BTV15, respectively; Figure 2C). This result is in line with Hund et al., which reported partial cross neutralization between BTV8 positive serum and BTV1, despite the genetic distance between these serotypes [9].

In addition, no significant difference was reported between RNAemia of BTV15 superinfected calves (from V and NV groups, both immunized against BTV8) and RNAemia of BTV15 single infected calves. Thus this low in vitro humoral cross reactivity between BTV8 immune serum and BTV15 seemed to have no significant influence on BTV15 RNAemia in vivo in contrast to the BTV1 RNAemia.

BTV1/BTV15 superinfection led to clinical disease in both V and NV animals. On the contrary, during BTV1 and BTV15 single infection the calves had very low clinical scores. The reason of this difference remains uncertain; however individual variability could be part of the explanation.

After superinfection, BTV1 neutralizing Abs only reached very low levels, as a consequence of the very low BTV1 RNA detection. By contrast, BTV15 neutralizing Abs extended to high titres, either in BTV1/BTV15 superinfected animals or in BTV15 single serotype infected calves.

Despite high neutralizing Abs detection following BTV15 single serotype infection, ELISA detecting VP7 Abs showed a mean PN at 35 dpi just above the positivity threshold. This is consistent with previous reports showing that significant immunological differences exist between BTV15 and other BTV serotypes and that monoclonal antibodies raised against BTV1 VP7 failed to react with BTV15 VP7 [37]. When assessing diagnostic tools aiming at non-serotype specific detection, it would be therefore advisable to include distantly related strains in the test panel to cover most of the genetic variability displayed by BTV proteins.

Vandenbussche et al. suggested to use the ID Vet cELISA kit with a cut off of 66 PN instead of 35 for BTV8, as recommended by the manufacturer, to achieve optimal accuracy for both screening and diagnostic [38]. Taking into account this suggestion, all of the three calves would have been considered as seropositive by day 21 post infection with BTV15.

Unlike the European BTV8, known for its increased virulence in bovine, BTV1 and BTV15 have been associated with subclinical or very mild disease in this species. Numerous factors are known to influence the severity of BT in individual ruminants; nutritional status, immune status and age, breed, environmental stresses such as high temperature and ultraviolet radiation [39]. In this study, the accidental co-infection with BTV1 and BTV15 and the obtained severe clinical outcome underlined the potential higher pathogenicity of a co-infection.

The main objective of this study was to observe the outcomes of a superinfection in calves previously immunized with the European BTV8. BTV1 or BTV15 RNA detection in superinfected animals was not different whether BTV8 immunization was acquired through vaccination and challenges or challenges alone. Furthermore, a low cross neutralization was measured between BTV8 and BTV1, and between BTV8 and BTV15. Taken all together in the context of the European BT epidemiological situation, the results could suggest that an infection or a vaccination with the European BTV8 strain would not efficiently protect the bovines from a superinfection with the BTV1 or BTV15 strains used in the study.

Additional files

Additional file 1. BTV group specific anti-VP7 antibodies, after vaccination against BTV8 and BTV8 challenges, for non-vaccinated, control and vaccinated calves. BTV group specific anti-VP7 antibodies as the % of negativity. Dashed line represents the cut off value. A % of negativity under the cut off (35%) is considered positive. NV: non vaccinated; V: vaccinated. The two vaccine injections are represented as arrows labelled Vac1 and Vac2 respectively, and first and second BTV8 challenges are represented as green arrows. Standard deviations are represented as error bars.

Additional file 2. Cumulative clinical scores. Cumulative clinical scores after the first BTV8 challenge in NV and V animals, BTV1/BTV15 superinfection and BTV1 and BTV15 single infections. Sd: Standard deviation.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
CS, ET, FDP, LM, KDC, and PS conceived and designed the experiments. LM, FDP, PS and WVC performed the experiments in vivo. LM, FDP, CT and IDL performed the experiments in vitro. LM, FDP and CS analysed the data. LM, FDP and CS wrote the paper. All authors read and approved the final manuscript.

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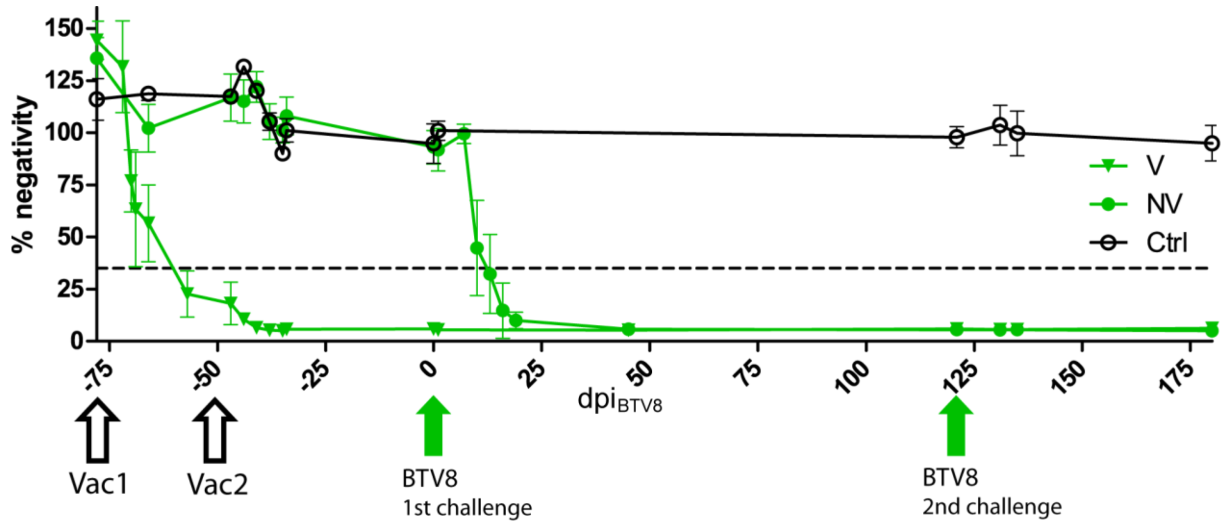
Supplemental material



Additional file 1. BTV group specific anti-VP7 antibodies, after vaccination against BTV8 and BTV8 challenges, for non-vaccinated, control and vaccinated calves. BTV group specific anti-VP7 antibodies as the % of negativity. Dashed line represents the cut off value. A % of negativity under the cut off (35%) is considered positive. NV: non vaccinated; V: vaccinated. The two vaccine injections are represented as arrows labelled Vac1 and Vac2 respectively, and first and second BTV8 challenges are represented as green arrows. Standard deviations are represented as error bars.

animal	group	D0	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15	D16	D17	D18	D19	D20	D21	group mean	group sd
3272	NV_BTV8	0	0	0	0	2	2	2	4	6	8	10	12	16	18	19	21	23	25	27	29	29	29	30,5	17,2
3643	NV_BTV8	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	2	3	6	7	8	9	9		
8059	NV_BTV8	0	0	0	0	1	1	1	2	3	5	7	9	11	13	15	17	19	21	23	25	29	33		
9026	NV_BTV8	0	0	0	0	1	2	4	6	8	10	12	15	19	23	27	31	35	39	42	45	48	51		
3272	NV_BTV1/BTV15	0	1	2	3	5	9	12	15	18	21	24	27	30	33	35	37	40	42	42	43	43	44	43,3	19,7
3643	NV_BTV1/BTV15	0	0	0	2	3	4	9	16	20	24	28	32	36	40	43	48	54	58	62	64	66	68		
8059	NV_BTV1/BTV15	1	1	1	1	1	1	1	1	1	1	2	4	7	10	14	20	22	27	31	35	38	41		
9026	NV_BTV1/BTV15	0	0	0	0	0	0	0	0	1	3	6	9	11	13	14	15	16	16	17	18	19	20		
2137	V_BTV1/BTV15	0	0	0	0	2	2	3	6	9	13	16	19	22	24	26	28	29	30	31	31	31	31	33	11,7
3217	V_BTV1/BTV15	0	0	0	2	3	5	8	11	13	15	16	17	19	21	23	25	25	27	28	30	31	31		
9077	V_BTV1/BTV15	0	0	1	4	5	10	14	16	18	20	22	25	29	33	35	37	39	41	43	46	48	49		
9108	V_BTV1/BTV15	0	0	1	1	1	4	7	9	10	11	13	14	14	14	15	16	18	20	21	21	21	21		
169	BTV1	2	2	2	2	2	2	2	4	4	4	4	4	4	6	7	7	7	7	8	9	10	10	8,3	3,8
6712	BTV1	0	0	0	0	0	0	1	2	4	4	4	4	4	4	4	4	4	4	4	4	4	4		
1004	BTV1	0	0	2	4	4	4	5	7	8	8	8	8	8	8	8	9	10	11	11	11	11	11		
3598	BTV15	2	2	2	2	2	2	3	3	3	3	3	3	3	3	5	7	7	7	7	7	7	7	6,7	5,5
4401	BTV15	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1		
6336	BTV15	0	2	4	4	4	4	4	4	4	4	4	4	4	4	5	6	7	8	9	10	11	12		
2137	V_BTV8	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	8,3	3,8
3217	V_BTV8	0	0	0	0	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2		
9077	V_BTV8	0	0	0	0	0	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2		
9108	V_BTV8	0	0	0	0	0	1	2	2	2	2	2	2	2	2	2	2	2	3	3	3	3	3		
1317	ctrl	0	0	0	0	0	0	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2,2	1,1
3884	ctrl	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1		
1894	ctrl	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	2	2	2		
2004	ctrl	0	0	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2		
7494	ctrl	0	0	0	1	1	1	1	1	2	2	2	2	2	2	2	3	4	4	4	4	4	4		

Additional file 2. Cumulative clinical scores. Cumulative clinical scores after the first BTV8 challenge in NV and V animals, BTV1/BTV15 superinfection and BTV1 and BTV15 single infections. Sd: Standard deviation.

Experimental section

Study 6 :

Assessment of cross-protection induced by a bluetongue virus (BTV) serotype 8 vaccine towards other BTV serotypes in experimental conditions

Veterinary Research, (2018) 49:63

Martinelle L, Dal Pozzo F, Thys C, De Leeuw I, Van Campe W, De Clercq K, Thiry E, Saegerman C.

Preamble

From 1998 to 2007, in addition to BTV8, Europe had to face the emergence of BTV1, 2, 4, 9, and 16, spreading in countries where the virus has never been detected before. These unprecedented outbreaks trigger the need to evaluate and compare the clinical, virological and serological features of the European BTV serotypes in the local epidemiological context. In addition, current established serological cross-reactivity between different BTV serotypes is mostly based on experimental infections and field data prior to the XXth century BTV circulation in Europe mainland. These serological relationships could benefit from an update in the light of the European epidemiological context.

The current study describes the clinical, serological and virological parameters after calf experimental infections with these 5 European serotypes of BTV (1, 2, 4, 9, and 16). Moreover, cross-protection resulting from prior vaccination with commercial inactivated BTV8 vaccine was assessed.

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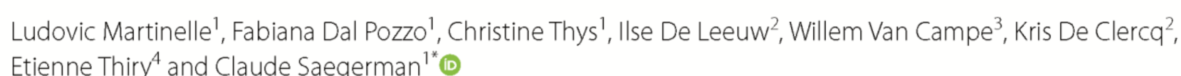
VETERINARY RESEARCH

RESEARCH ARTICLE

Open Access

Assessment of cross-protection induced by a bluetongue virus (BTV) serotype 8 vaccine towards other BTV serotypes in experimental conditions

Ludovic Martinelle[1], Fabiana Dal Pozzo[1], Christine Thys[1], Ilse De Leeuw[2], Willem Van Campe[3], Kris De Clercq[2], Etienne Thiry[4] and Claude Saegerman[1*]



Abstract

Bluetongue disease is caused by bluetongue virus (BTV) and BTV serotype 8 (BTV8) caused great economic damage in Europe during the last decade. From 1998 to 2007, in addition to BTV8, Europe had to face the emergence of BTV1, 2, 4, 9, and 16, spreading in countries where the virus has never been detected before. These unprecedented outbreaks trigger the need to evaluate and compare the clinical, virological and serological features of the European BTV serotypes in the local epidemiological context. In this study groups of calves were infected with one of the following European BTV serotypes, namely BTV1, 2, 4, 9 and 16. For each tested serotype, two groups of three male Holstein calves were used: one group vaccinated against BTV8, the other non-vaccinated. Clinical signs were quantified, viral RNA was detected in blood and organs and serological relationship was assessed. Calves were euthanized 35 days post-infection and necropsied. Most of the infected animals showed mild clinical signs. A partial serological cross reactivity has been reported between BTV8 and BTV4, and between BTV1 and BTV8. BTV2 and BTV4 viral RNA only reached low levels in blood, when compared to other serotypes, whereas in vitro growth assays could not highlight significant differences. Altogether the results of this study support the hypothesis of higher adaptation of some BTV strains to specific hosts, in this case calves. Furthermore, cross-protection resulting from a prior vaccination with BTV8 was highlighted based on cross-neutralization. However, the development of neutralizing antibodies is probably not totally explaining the mild protection induced by the heterologous vaccination.

Introduction

Bluetongue virus (BTV) represents the type species of the *Orbivirus* genus, family *Reoviridae* and causes bluetongue disease (BT) in susceptible species [1, 2]. BTV is usually transmitted to domestic and wild ruminants by the bite of haematophagous female midges of the *Culicoides* genus yet direct transmission was demonstrated at least for serotype 26 [3]. From 1998 to 2006, Europe had to face an unprecedented emergence of BTV serotypes 1, 2, 4, 9 and 16 (BTV1, 2, 4, 9, 16) throughout the Mediterranean Basin, including several countries where the virus was never detected before. August 2006 is a tipping point in BTV epidemiology, with a first detection of BTV8 in Europe Mainland [4] and a subsequent wide spread throughout Europe during the following 2 years. BTV8 emergence was easily spread through Culicoides species that were not known as the historic BTV transmission species, i.e. *Culicoides obsoletus* complex species [5]. This epidemic—affecting abundantly cattle whereas previous outbreaks largely occurred in small ruminants—is considered to have caused greater economic damage than any previous

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single serotype outbreak [6]. Most of the countries involved in the beginning of the BTV8 epidemic and that paid the heaviest toll were declared bluetongue-free in 2012 (Belgium, the Netherlands, Germany, France [7, 8]).

Bluetongue virus virulence and transmission potential is not serotype driven thus outcome of the infection cannot be predicted based on the serotype alone [9]. Within a serotype, the geographical origin can be used to define topotypes with different pathogenicity. As an example, some Australian strains were reported to be less virulent than their Western counterparts [10]. The presence of competent palearctic vectors and several serotypes recently described in Europe mainland, with non-immunized livestock, trigger the need to evaluate and compare the clinical, viral and immunological features of the European BTV serotypes in cattle. In addition, since the European BTV8 showed an unusual virulence in cattle, the emergence of another serotype could take place in an area with local cattle possibly already immunized against BTV8.

Serological relationships between the different BTV serotypes were mostly established more than 25 years ago based on plaque reduction tests and cross-protection experiments in sheep [11]. It is assumed that there is partial or no cross-protection between the different BTV serotypes, therefore the need of serotype specific vaccination strategies. At the moment, a total of 27 serotypes have been recorded [12], possibly 29 [13]. As a consequence, developing and implementing multi-serotype prophylactic approaches to tackle BTV is one of the major challenges in the control of the disease. Cross-reactivity between BTV1 and BTV23 [14], BTV1 and BTV8 [15] or more recently between BTV16 and multivalent serum of sheep vaccinated against BTV9, 2 and 4 [16] was reported. These serotypes are however traditionally considered as poorly related.

The current study was implemented to pursue two main objectives. First, to assess and compare the virulence of some of the BTV serotypes threatening Europe mainland—namely BTV1, BTV2, BTV4, BTV9 and BTV16—in controlled conditions in calves. Second, to evaluate the extent of cross-protection granted by BTV8 vaccination in calves infected with these serotypes. In addition, in vitro humoral cross-reactivity was determined.

To these ends, each of the tested serotypes (BTV1, BTV2, BTV4, BTV9, and BTV16) was used to infect two groups of calves. One group was vaccinated against BTV8 using a commercial inactivated vaccine, and the other group was not. The clinical, pathological and virological consequences of the infection with these different serotypes, whether the animals were vaccinated or not, were compared, and serological relationships were assessed.

Materials and methods

Animals

Animals were treated in accordance with the International Guiding Principles for Biomedical Research Involving Animals, as issued by the Council for the International Organizations of Medical Sciences and EU Directive 2010/63/EU for animal experiments.

A total of 35 Holstein male calves, about 5.5–6 months old, were used. All the selected animals were tested seronegative (ELISA and seroneutralization) and non viraemic (RTqPCR) for BTV and Bovine herpesvirus 1 (BoHV1). In addition these calves were also born from BTV naïve dams (seronegative and RTqPCR negative). They were introduced in an insect secured BSL3 facility 1 week before the beginning of the experiment to allow their acclimatization.

Virus

BTV1, BTV2, BTV4, BTV9 and BTV16 were all derived from the reference strains of the Onderstepoort Veterinary Institute. These strains underwent further passages at The Pirbright Institute (TPI); passage history is available at the RNAs and Proteins of dsRNA Viruses [17]. The BTVs were subsequently passaged at Sciensano, Ukkel, Belgium (formerly CODA–CERVA) between two and four times on BHK-21 cells. BTV8 originated from a field sample (BEL2006/01) afterwards passaged 6 times in BHK-21. Each serotype has been tested by RTqPCR specific of the serotypes used in the study to rule out potential contamination of the inocula.

Experimental design

Five animals were kept as environmental control, and were inoculated with Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Gent, Belgium). Groups were identified by their vaccination status against BTV8 (V_ or NV_, respectively vaccinated and non-vaccinated) followed by the subsequently inoculated BTV serotype (BTV1, BTV2, BTV4, BTV9 or BTV16). For each tested serotype, two groups of three calves each were used. The animals from the first group were vaccinated against BTV8 (BTVPUR AlSap 8, Merial, Lyon, France) following manufacturer instructions, with the second vaccine injection 33 days before challenge. In the other group the animals were not vaccinated.

To be infected, the animals received between 2.5 and 4 mL of inoculum, properly diluted to a normalized titre of 10^6 $TCID_{50}$/animal. Inoculations were realised through the subcutaneous route, on the left side of the neck. Daily examination of the calves included temperature and

clinical signs monitoring for 35 days post-infection (dpi). The severity of the infection was quantified by calculating clinical scores per system and per animal, leading to overall clinical scores by groups and animal, following a standardised clinical form adapted from Saegerman et al. [18]. Briefly, clinical signs were summed up according to their nature (general signs versus localised clinical signs on muzzle, mouth, limbs and eyes) and intensity (crust, ulcerations or necrosis, oedema or inflammation). The calves were euthanatized at 35 dpi by captive bolt stunning followed by bleeding. Extensive necropsy has been performed, and spleen, thymus, prescapular and mesenteric lymph nodes, testicle and lung were sampled from infected and control calves, to detect BTV RNA by RTqPCR.

BTV RNA detection

Viral RNA extraction from the blood was achieved using the QIAamp Viral RNA Mini Kit (Qiagen, Antwerp, Belgium). In the organs, about 100 mg of tissues per organ were processed; viral RNA extraction was performed using Trizol reagent according to the manufacturer's instructions (Gibco Invitrogen, UK). Viral RNA denaturation and reverse transcription were adapted from previously published protocols [19] with slight modifications, as denaturation was realised in presence of random hexamers. Serotype specific RTqPCR assays were carried out for BTV1, BTV2, BTV4, BTV8, BTV9 and BTV16 using LSI VetMAX European BTV Typing Real-Time PCR Kits (ThermoFisher Scientific, Gent, Belgium), following manufacturer's instructions.

RTqPCR reactions were run on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories N.V., Temse, Belgium) using the following cycling conditions: heat inactivation at 95 °C for 10 min, 50 cycles consisting of denaturation at 95 °C for 15 s and annealing/elongation at 58 °C for 30 s.

To allow absolute quantification of the viral RNA content in blood and organ samples, standard curves of serotype specific plasmids (pGEM®-T Easy Vector, Promega, The Netherlands), carrying the target part of the segment 2, were constructed. Quantification was expressed in cDNA copy number/mL of blood.

Anti-BTV antibodies detection

For each tested serotype neutralizing antibodies (Abs) were titrated by seroneutralization (SNT). Two-fold serial dilutions of the sera (1:10–1:1280) were tested in the presence of 100 $TCID_{50}$ of BTV, as previously described [20]. The neutralizing antibody titre was defined as the reciprocal of the serum dilution causing a 50% reduction in cytopathic effect. Serum of all the animals has been tested at several time points with the homologous virus.

Anti-VP7 antibodies circulation was also evaluated using a commercial competitive ELISA kit (ID Screen® Bluetongue Competition ELISA kit, ID Vet, France). Results were expressed as % negativity (PN) compared to the negative kit control and transferred to a positive, doubtful or negative result according to the cut-off settings provided by the manufacturer ($PN \leq 35$ is positive; $35 < PN \leq 45$ is doubtful; $PN > 45$ is negative). As these cut-off values were rather designed for screening purposes [21], the cut-off suggested by Vandenbussche et al. [19] (negative when $PN > 66$) has been also considered as a tool for individual diagnostic, with respect of the limited number of animals.

Haematology

Starting during the acclimatization period and until the end of the experiment, a complete haemogram (Vet ABC, SCIL animal care company, France), including total leukocytes, monocytes, lymphocytes, neutrophils, eosinophils and basophils was performed on EDTA blood samples on a regular basis.

Cross-neutralization assay

In each non-vaccinated group, the individual serum sample with the highest homologous neutralizing titre has been selected and subsequently tested by SNT against all the other inoculated BTV serotypes. In order to avoid potential bias due to low humoral response against any of the serotype, heterologous neutralization results for each tested serotypes were expressed as a percentage of the titre reached when the immunised serum was tested with the homologous serotype. BTV8 immune serum was obtained from an experimentally infected heifer in a previous study [22].

In vitro kinetic growth of BTV serotypes

In vitro growth properties of the 6 BTV serotypes (i.e. BTV1, BTV2, BTV4, BTV8, BTV9 and BTV16) used in this study were compared using their replication kinetics in VERO cell culture, following a protocol adapted from Dal Pozzo et al. [23]. Briefly, all the inocula were used in a one-step growth assay, with confluent VERO cells, at a multiplicity of infection (m.o.i.) of 0.05. After 0, 8, 24, 48, 72, 96 and 120 h incubation, the supernatant was removed and stored −80 °C. For each time point, the virus titre was determined at least in triplicate by plaque assay [24] and expressed as Log $TCID_{50}$/mL.

Infectivity

For each serotype, the original inoculum plus two serial 1:10 dilutions were tested by RTqPCR. Knowing the infectious titre of each inoculum, for each serotype a mean ratio of segment 2 (S2) cDNA/$TCID_{50}$ was then

calculated. The infectious titre of the blood samples was then extrapolated using RTqPCR results multiplied by the mean segment 2 cDNA/$TCID_{50}$ ratio and expressed as $TCID_{50}$/mL, for each tested time points. $TCID_{50}$/mL titres were then converted in PFU/mL to assess the level of infectivity of each serotype based on the estimate of the minimal PFU/mL required to infect vector *Culicoides* according to Dungu et al. [25].

Statistical analysis

For viraemia levels and infectivity assessment between vaccinated and non-vaccinated groups, the comparison of quantitative parameters was performed using pair-wise t tests or Welch test, as appropriate. One-way ANOVA with post hoc Tukey test were used to analyse haematological values within the same group with respect to baseline values at 0 dpi. Differences between control and infected groups at the same time-point were analyzed using a two-way mixed model ANOVA with Bonferroni post-test. Two-way mixed model ANOVA were calculated using the statistical analysis program GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego California USA). Other statistical analyses were realized using the R software/environment (R-3.2.1, R Foundation for Statistical Computing). For all tests, P values < 0.05 were considered significant.

Results

Clinical examination

Two calves from the control group showed 1–2 days-lasting hyperthermia between 6 and 12 dpi, without other systemic problem or clinical signs evocative of bluetongue disease. In infected animals, clinical signs were mild, and would probably go unnoticed in the field, as appetite was conserved and general condition unchanged. Nevertheless, typical bluetongue clinical signs were observed, including facial oedema, swelling and reddening of the odontoid papillae, crusts and erosions at the muco-cutaneous junction, nasal discharge and purulent conjunctivitis. V_BTV9 group and NV_BTV1 group clinical scores were significantly higher than control group (P<0.05). V_BTV9 and NV_BTV16 groups had a significantly higher clinical score than their counterparts infected with the same serotype but different vaccination status. There were no significant differences between clinical scores of other infected groups when compared to each other, to the control group or when comparing groups infected with the same serotype but with different vaccination status (Figure 1). A great individual variability was observed in the clinical outcomes within each group, as in 6 out of 10 infected groups, one single animal totalized 50% or more of the total clinical score of the group.

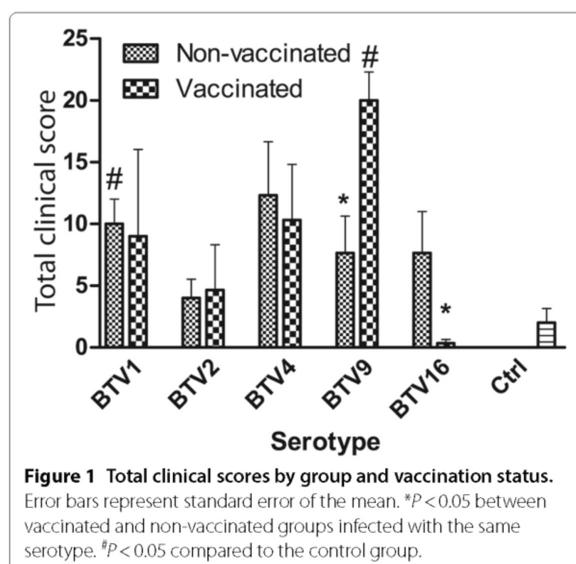

Figure 1 Total clinical scores by group and vaccination status. Error bars represent standard error of the mean. *P < 0.05 between vaccinated and non-vaccinated groups infected with the same serotype. #P < 0.05 compared to the control group.

BTV RNA detection

No viral RNA was detected in any of the control animals at any time point. Inoculated animals showed different viraemia patterns depending on the inoculated serotype and their vaccination status. The earliest BTV RNA detection in the blood occurred at 1 dpi in NV_ and V_BTV1 groups. The latest onset of viraemia occurred in one calf of V_BTV2 group at 11 dpi. In calves infected with serotypes 2 and 4 BTV RNA could only be detected inconsistently and RNAemia reached moderate levels when compared to calves infected with BTV1, 9 and 16 (Figure 2). After the challenge of vaccinated animals, the Log copy number of viral RNA was significantly lower in BTV2 and BTV4 groups when compared to BTV9 and BTV16 groups (Two way ANOVA with repeated measures, P < 0.004). In addition, BTV1 RNA detection was also significantly lower than BTV9 (P < 0.013). After the challenge of non-vaccinated animals viral RNA detection was significantly lower in BTV2 and BTV4 groups when compared with BTV9 and BTV1 groups (P < 0.003), and BTV4 RNA detection was significantly lower than BTV16 (P < 0.005). Regarding homologous serotypes, only BTV1 showed a lower RNA detection in V group versus NV group (P < 0.016). Vaccinated animals had an RNAemia ranging from 79.4 to 95.5% of the max RNAemia level of the non-vaccinated animals at the viraemic peak. At the end of the experiment viral RNA was still detectable in 40% of the vaccinated animals versus 73% in the non-vaccinated calves. However this difference was not significant (χ^2, P = 0.065).

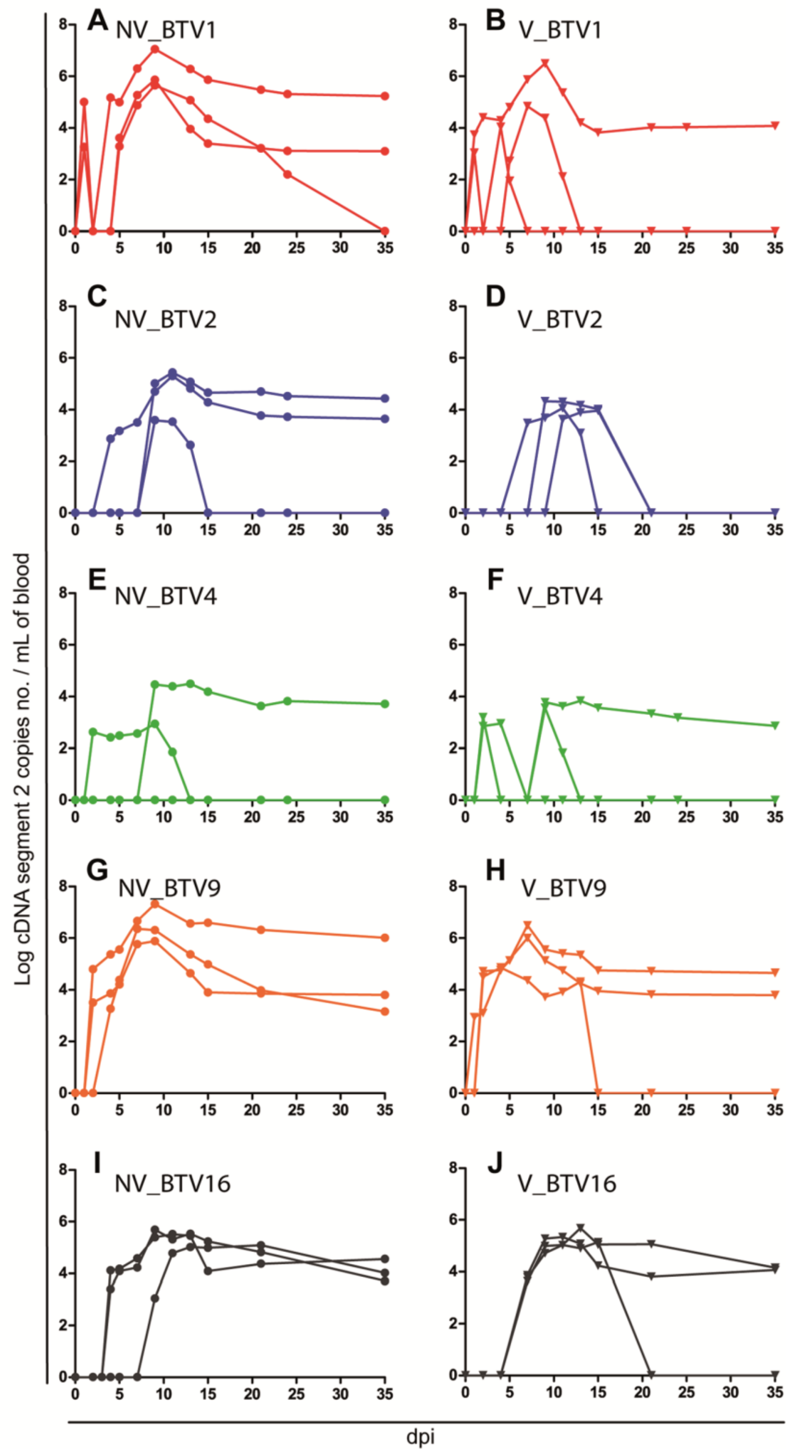

Figure 2 Individual daily viral genome load in calves' blood. Results are expressed as the Log copies of BTV segment 2 cDNA per mL of blood. **A** and **B** BTV1; **C** and **D** BTV2; **E** and **F** BTV4; **G** and **H** BTV9; **I** and **J** BTV16. **C**, **E**, **G** and **I** are non-vaccinated groups whereas **B**, **D**, **F**, **H** and **J** are vaccinated groups; dpi: day post-infection.

Anti-BTV antibodies detection

All the inoculated animals produced homologous neutralizing antibodies that started generally to be detected two to 3 weeks post-infection (Figure 3). In previously BTV8 vaccinated calves, the rise of neutralizing antibodies against the virus serotype used in the challenge was earlier detected and reached higher levels compared to non-vaccinated calves however only significant for BTV2 infected animals ($P < 0.035$). In vaccinated animals, anti-BTV8 neutralizing antibodies were contemporaneously circulating (Figures 3B–J) and anti-BTV8 titres were significantly higher than in NV animals for all serotypes. Whereas no significant increase in anti-BTV8 neutralizing antibodies could be detected in non-vaccinated animals, vaccinated ones from groups inoculated with BTV2 and 16 underwent a slight boost in anti-BTV8 neutralizing antibodies titres, despite the heterologous nature of inoculated serotype ($P < 0.006$).

Neutralizing antibodies titres had no correlation with viral RNA detection level ($r = 0.12$, $P = 0.11$) and maximal SNT titres had no correlation with maximal BTV RNA copy number ($r = 0.09$, $P = 0.63$).

The cELISA kit managed to detect Anti-VP7 antibodies for each tested serotype. The time of seroconversion was in line with previously published data [20, 26, 27], between 10 and 21 dpi for all serotypes (Figure 4). By the time of inoculation, 2 V_BTV2 and 2 V_BTV4 calves had a PN above the positivity threshold (thus considered negative) determined by the manufacturer ($PN \leq 35$), however considered positive if taking into account of the threshold defined by Vandenbussche et al. [19] ($PN \leq 66$). All these 4 animals had PN that reached values under 35 within 7 dpi.

Haematology

Monocyte counts increased shortly after infection in all groups but control animals independently of their vaccination status. The increase started between 4 and 11 dpi, peaked at 15 or 18 dpi and then came back to baseline levels by 35 dpi (Figure 5). All NV groups showed peaks significantly higher than baseline value (0 dpi) or control value at the same time point (Figure 5A, $P < 0.05$, two way ANOVA with repeated measures) whereas in V groups only peaks of V_BTV1, BTV2, and BTV9 were significantly higher (Figure 5B, $P < 0.05$, two way ANOVA with repeated measures).

Lymphocyte count followed a different trend, decreasing in the first dpi, from about 4–11 dpi, then recovering and even significantly exceeding baseline values (Figure 5D, V_BTV4: 18 dpi, $P < 0.05$, two way ANOVA with repeated measures) or control values at the same time point (Figures 5C and D, NV_BTV2: 25 dpi and V_BTV16: 21, 25 and 35 dpi, $P < 0.05$, two way ANOVA with repeated measures).

Necropsy and BTV RNA detection in organs

At the necropsy, lesions were sporadically reported, including haemorrhage and petechial haemorrhage on several lymph nodes, endocardic suffusion, abscesses and petechial haemorrhage in the lung, and a slight haemorrhage in the wall of the pulmonary artery of one calf in the NV_BTV9 group.

No viral RNA could be detected in any organs of the animals of the BTV2 and BTV4 groups, whether they were vaccinated or not (Table 1). The proportion of positive organs was significantly higher in BTV9 infected groups (pairwise Fisher test, $P < 0.002$). However there was no significant difference between BTV9 V and NV groups regarding organ detection ($P = 0.075$). Considering all serotypes all together, viral RNA was most commonly detected in prescapular lymph node (Table 1). In vaccinated animals infected with BTV9, detection of BTV RNA in organs was associated with an Odds Ratio of 0.16 ($P < 0.03$, [0.029–0.691]).

Cross neutralization assay

Different degrees of in vitro cross neutralization could be found using all immunized sera (Table 2). However, immunized sera of BTV2 and BTV9 showed the least degree of in vitro cross neutralization against the other tested viral serotypes. On the contrary, BTV8 immune serum had a higher neutralization effect in vitro on the growth of BTV4, reaching a titre equal to 25% against BTV4 when compared to the titre reached against BTV8 itself. BTV1 immune serum reached a similar level of partial seroneutralization against BTV8. A lesser cross neutralization has been reported with the BTV16 serum towards BTV1 virus (Table 2).

In vitro kinetic growth of BTV serotypes

The mean virus titres measured for each serotype at the different time points were compared to all the other serotypes using a two-factor ANOVA with repeated measures on one factor, showing no significant difference between the different kinetic growth curves ($P = 0.41$, Figure 6).

BTV1, 2, 6 and 16 had earlier replication as cytopathic effect (CPE) has been reported since 8 h post-infection (hpi), whereas BTV4, 8 and 9 showed CPE starting at 24 hpi.

Infectivity

At viraemic peak S2 copies number ranged from $10^{6.52\ (\pm 0.73)}$ for NV_BTV9 to $10^{3.39\ (\pm 1)}$ for NV_BTV4

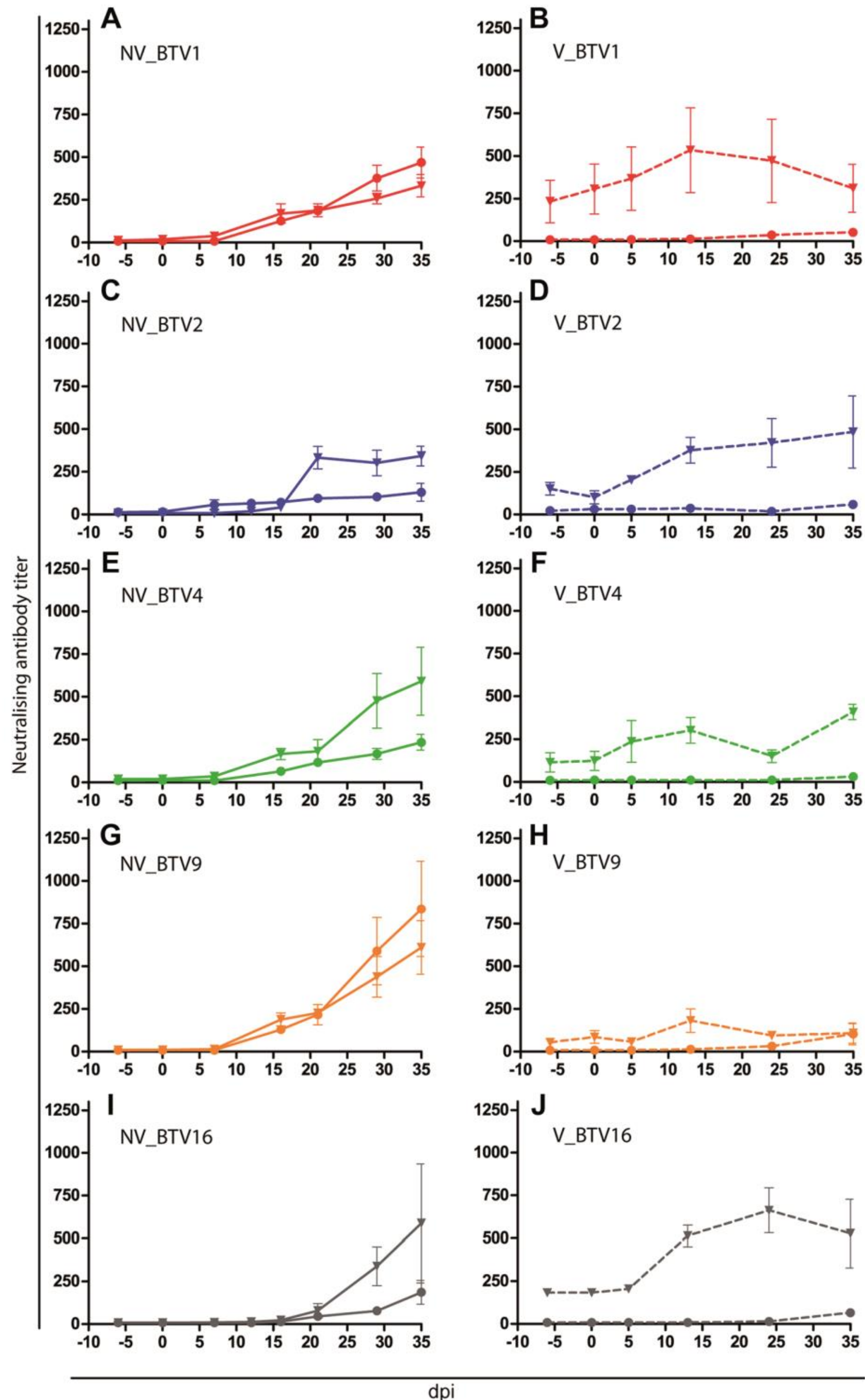

Figure 3 Evolution of neutralising antibodies titres against BTV8 and homologous inoculated serotypes. **A**, **C**, **E**, **G** and **I** (full lines): mean neutralising antibodies titres per group against respectively BTV1, 2, 4, 9 and 16. In each panel vaccinated (triangle) and non-vaccinated (filled circle) groups are represented. **B**, **D**, **F**, **H**, **J** (dashed lines): mean neutralising antibodies titres against BTV8 in vaccinated (triangle) and non-vaccinated (filled circle) animals in respectively BTV1, 2, 4, 9 and 16 inoculated groups. Error bars represent standard error of the mean. dpi: day post-infection.

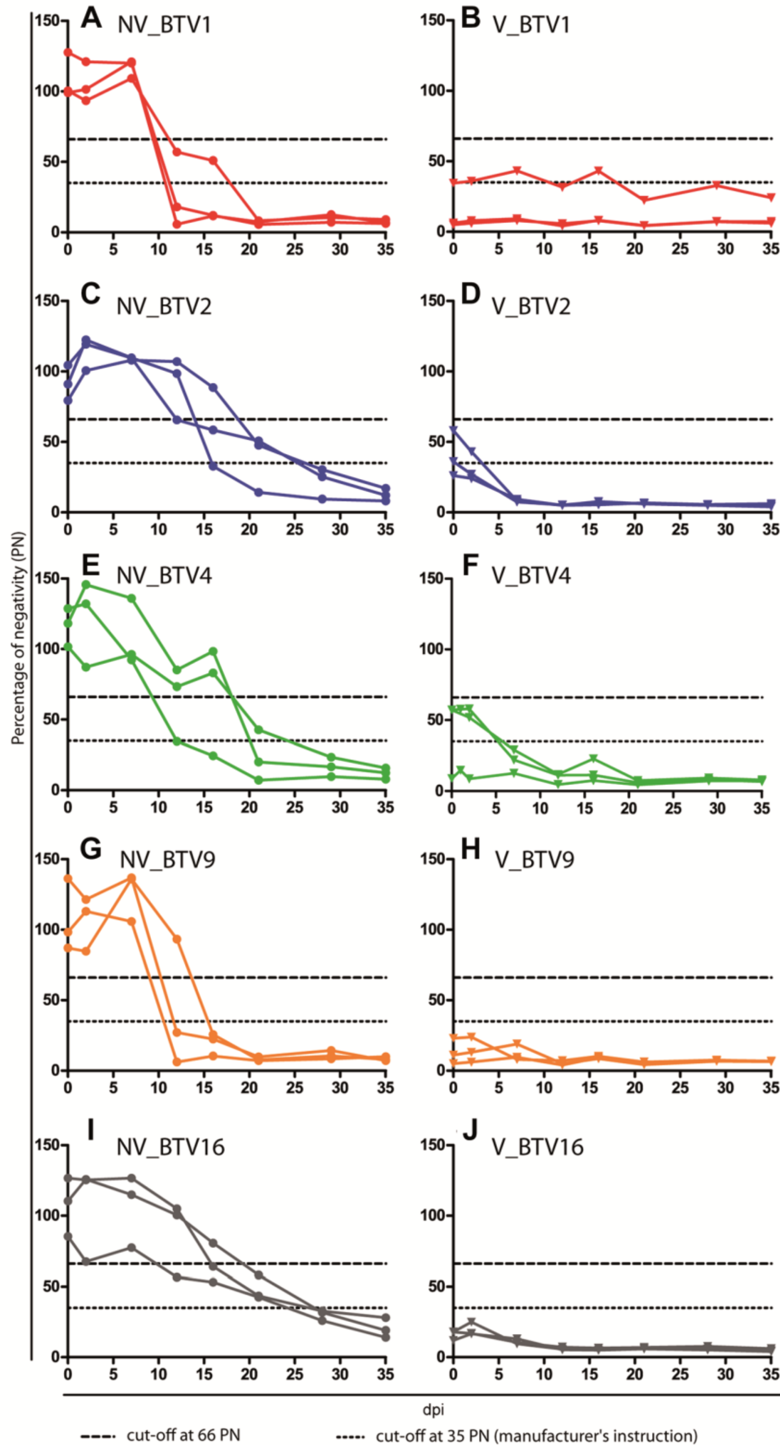

Figure 4 Evolution of serogroup specific antibodies. The results are presented for each animal as the percentage of negativity (PN) obtained in the competitive ELISA. Data for non-vaccinated (filled circle) and vaccinated (triangle) animals are shown for BTV1 (**A**, **B**), BTV2 (**C**, **D**), BTV4 (**E**, **F**), BTV9 (**G**, **H**) and BTV16 (**I**, **J**). Dotted line represents the cut-off value recommended by the manufacturer and dashed line the one suggested by Vandenbussche et al. [18]. dpi: day post-infection.

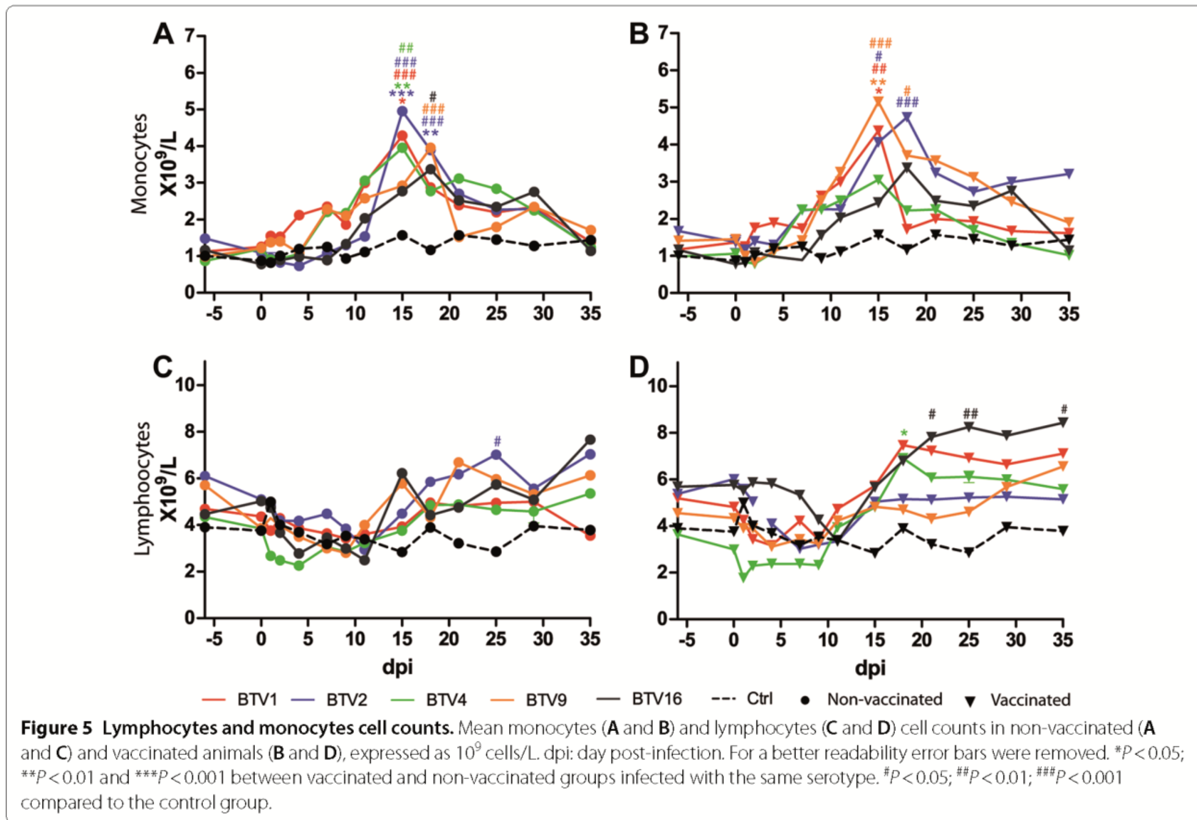

Figure 5 Lymphocytes and monocytes cell counts. Mean monocytes (**A** and **B**) and lymphocytes (**C** and **D**) cell counts in non-vaccinated (**A** and **C**) and vaccinated animals (**B** and **D**), expressed as 10^9 cells/L. dpi: day post-infection. For a better readability error bars were removed. *$P<0.05$; **$P<0.01$ and ***$P<0.001$ between vaccinated and non-vaccinated groups infected with the same serotype. #$P<0.05$; ##$P<0.01$; ###$P<0.001$ compared to the control group.

Table 1 BTV RNA detection in organs at necropsy

	Calf ID	Spleen	Mesent. LN	Prescap. LN	Thymus	Testicle	Lung
NV_BTV1	169	NEG	NEG	7.41	NEG	NEG	NEG
	1004	NEG	NEG	6.27	NEG	3.33	NEG
	6712	NEG	NEG	4.92	NEG	NEG	NEG
V_BTV1	2038	NEG	NEG	0.40	NEG	NEG	NEG
	2044	1.42	NEG	18.96	NEG	NEG	NEG
	5093	NEG	NEG	NEG	0.15	NEG	NEG
NV_BTV9	1058	NEG	4.73	57.43	28.61	2.23	0.57
	2071	12.22	4.36	18.78	NEG	1.89	1.30
	3045	2.00	1.14	5.72	0.52	4.59	NEG
V_BTV9	2740	NEG	7.40	NEG	24.48	1.71	NEG
	3934	0.96	0.88	2.09	NEG	NEG	NEG
	4935	11.84	NEG	9.46	NEG	NEG	NEG
NV_BTV16	5583	NEG	NEG	2.25	NEG	NEG	NEG
	8606	NEG	NEG	NEG	NEG	NEG	NEG
	9535	NEG	0.23	NEG	17.34	NEG	NEG
V_BTV16	2461	NEG	NEG	NEG	NEG	NEG	NEG
	3150	NEG	NEG	NEG	97.22	NEG	NEG
	5077	NEG	NEG	2.70	NEG	NEG	NEG

Results are expressed a BTV RNA copy number per 100 mg of tissue.

No positive detection could be found in any tested sample in BTV2 and BTV4 vaccinated or non-vaccinated groups.

NV_: non vaccinated group; V_: vaccinated group; NEG: negative result; Mesent. LN: mesenteric lymph node; Prescap. LN: prescapular lymph node.

Table 2 Percentages of relative homologous and heterologous seroneutralization titers

Immunised serum	BTV serotype					
	BTV1	BTV2	BTV4	BTV8	BTV9	BTV16
Control	0.52	1.11	0.55	0.44	0	3.54
BTV1	100	1.11	8.85	25	0	3.54
BTV2	1.3	100	3.15	0.44	0	3.54
BTV4	0.65	0.88	100	0.44	0	12.5
BTV8	10.36	12.5	25	100	6.25	3.54
BTV9	2.61	3.1	0.77	0.44	100	3.54
BTV16	18.34	12.39	0.83	0.44	0	100

(Figure 7A), which correspond to a titre of $10^{3.74\ (\pm 0.42)}$ $TCID_{50}$/mL for NV_BTV9 group and $10^{0.58\ (\pm 0.11)}$ $TCID_{50}$/mL for NV_BTV4 (Figure 7B). Only BTV9 and BTV16 groups had consistently max viraemia higher than 10^{3155} $TCID_{50}$/mL (threshold value under which oral infection of *Culicoides* is supposed to be impossible according to Dungu et al. [25]) in all animals. Amongst BTV1 infected calves only NV_BTV1 calf 0169 had a max viraemia above the threshold. No animals in the BTV2 or BTV4 groups had a viraemia above 10^{3155} $TCID_{50}$/mL whichever was the considered time point or vaccination status.

Discussion

In this study BTV inoculation resulted from asymptomatic affection to mild clinical signs. Although controversial [20, 28, 29] the passage history of the virus used in the current study could influence the clinical expression of the disease. Individual susceptibility could be another explanation [1].

Unlike in many other viral diseases [30–32], it was not possible to demonstrate a statistically significant relation between level and duration of viraemia, and clinical presentation. However this is not surprising in BTV infection, as RNAemia lasts longer in cattle than in sheep despite the more severe outcome in the latter species [33]. Furthermore, BTV clinical picture in cattle is usually subtle and does not allow to make any correlation with the viral RNA detection in the blood.

On the other hand, BTV replication level has been correlated to the adaptation of the virus to the host [34]. In this study and based on the levels of viral RNA detection serotypes were ranked as follows from the most to the less adapted in calves: BTV9, 1, 16, 2 and 4 in non-vaccinated groups, and BTV9, 16, 1, 2 and 4 in vaccinated ones. The limited circulation of BTV2 and BTV4 RNA in blood might explain the absence of positive detection in organs at necropsy. The hereinabove suggested host adaptation ranking based on BTV serotypes might involve non-serotype specific virulence factors, in line with findings from Caporale et al. [35].

For standardization purposes the same doses were inoculated, regardless of the serotype. It has to be stressed that different BTV strains or serotypes, as a consequence of their large variability, their origin, could indeed be non-equivalently adapted to their hosts. Dal Pozzo et al. described a dominance of BTV8 in cattle when compared to BTV1 and BTV15 [23]; North American serotype 10 replicates more efficiently in sheep than serotype 17 [36], and comparing BTV1 and BTV15, BTV1 seems to be better adapted to sheep whereas BTV15 is better adapted to cattle [34]. In addition, the modified live vaccine (MLV) required doses to grant protection in sheep have been determined to be different depending on the considered serotype [37]. Indeed the low replication level of BTV4 and BTV2 observed in this study might rely on an inoculation

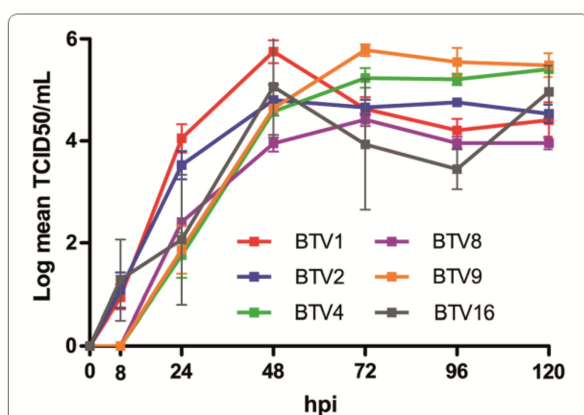

Figure 6 In vitro kinetic growth of BTV1, 2, 4, 9, and 16. Growth curves of BTV1, 2, 4, 8, 9 and 16 in VERO cells. Cells were infected at a MOI of 0.05 and supernatant collected at 8, 24, 48, 72, 96 and 120 h post-infection (hpi). Then supernatants were titrated on VERO cells by end-point dilution assay and expressed as the Log ($TCID_{50}$/mL). Each growth curve has been established independently at least in triplicate for each serotype.

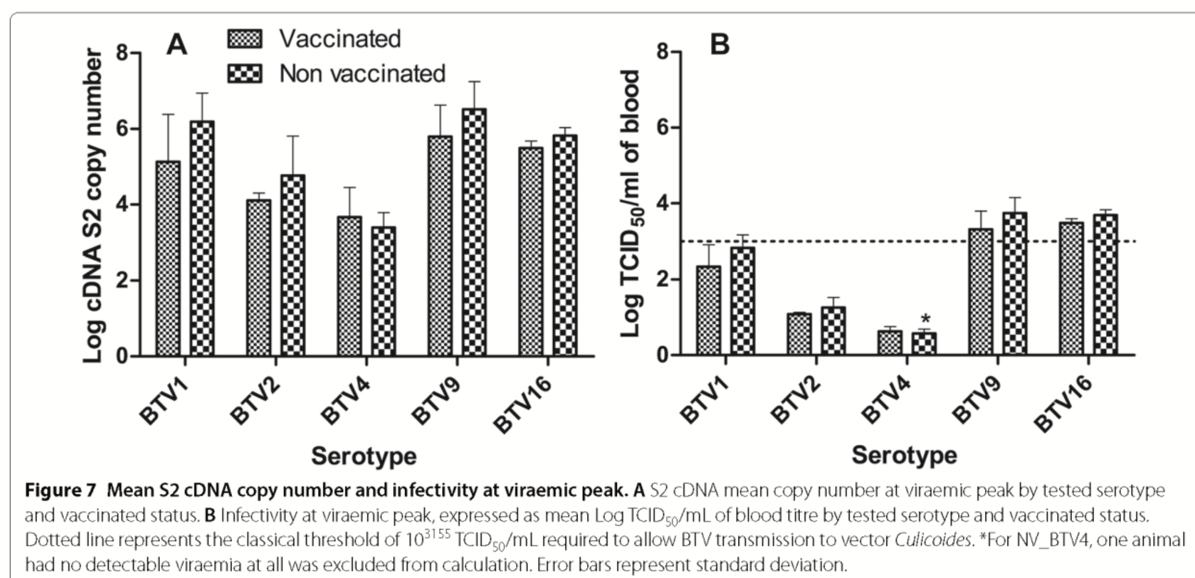

Figure 7 Mean S2 cDNA copy number and infectivity at viraemic peak. **A** S2 cDNA mean copy number at viraemic peak by tested serotype and vaccinated status. **B** Infectivity at viraemic peak, expressed as mean Log $TCID_{50}$/mL of blood titre by tested serotype and vaccinated status. Dotted line represents the classical threshold of 10^{3155} $TCID_{50}$/mL required to allow BTV transmission to vector *Culicoides*. *For NV_BTV4, one animal had no detectable viraemia at all was excluded from calculation. Error bars represent standard deviation.

dose insufficient for these serotypes and support the hypothesis of a poor adaptation to cattle.

Amongst the serotypes used in this study, Erasmus described serolological relationship, based on cross-protection tests in sheep or heterotypic antibody responses, between serotypes 2 and 1, 1 and 9, and 9 and 4; serotypes 8 and 16 being quite serologically isolated [11]. In the current study cross neutralization tests revealed moderated antigenic relationships mainly between serotypes 4 and 8, 1 and 8 and to a lesser extent 2 and 9. Partial cross neutralization has been previously reported between serotypes 1 and 8 [15]. The discrepancies among the above mentioned studies have to be interpreted carefully, as the origin of the used isolates varies greatly, from African strains to European field isolates. In addition, the intra-serotype VP2 nucleotide sequence variation can be up to more than 30% [38]. The quasispecies nature of BTV, its evolution through genetic drift and founder effect could explain the divergences between VP2 amino acids sequences and in vitro cross neutralization assays.

In this study the serological cross-reactivity with BTV8 was limited to BTV4 and BTV1. Consequently, the differences observed in the course of the current experiment between vaccinated and non-vaccinated groups are most likely unrelated to humoral immunity. Numerous studies have described the importance of cell-mediated immunity in the course of BTV infection [14, 39]. Cell-mediated immunity is suggested to rely to an important extent on non-structural proteins [40]. Despite non-structural proteins not being part of the viral particles used to produce inactivated vaccines, vaccinated animals were reported to develop antibodies against NS proteins [41]. Therefore, in this study, the shorter RNAemia and lower BTV detection frequency in organs of the vaccinated animals might reflect a partial NS proteins based protection. In addition, VP7 peptides have also been shown to be recognized by CTL in natural host and are considered to share similar sequences for several BTV serotypes [42]. This might be one of the underlying mechanisms that could explain the partial cross-protection of BTV1 immunization against BTV23 challenge described by Umeshappa et al. [14]. This is not contradictory to the findings of the present study, as BTV8 is as remotely related to the other European tested serotypes than BTV1 is related to BTV23, with respect to Erasmus description [11].

In our study no differences were observed among the haematology parameters measured after challenge among vaccinated and non-vaccinated groups. Monocytosis starting from 4 dpi and can be directly linked to BTV infection which induces transcriptional activation of bovine monocyte-derived macrophages [43]. The increase of monocyte count in infected cattle might be part of the mechanisms explaining the moderate clinical picture in bovine, by contrast to the severity of the disease in sheep [44]. Indeed, differences in BTV pathogenesis in sheep and cattle were reported to be related to different production levels of vasoactive mediators [44]. Simultaneous in vivo experimental infection of cattle and sheep could be carried out along with a detailed characterization of the cytokines produced in each species to clarify this hypothesis.

The minimum level of required viraemia to infect a vector has been established for several arthropod borne pathogens of major concern, like West Nile Virus [45], Dengue virus [46] or Chikungunya virus [47]. For BTV, MLVs are expected to lead to a viraemia lower than 10^3 PFU/mL, which is about 10^{3155} $TCID_{50}$/mL, supposed to prevent oral infection of ***Culicoides*** [48], even if it has been clearly reported that many MLVs could give raise to higher titres in experimental conditions and possibly lead to MLV circulation in the field in Europe [49, 50]. In the current experiment, only BTV9 and BTV16 infection could reliably reproduce a viraemia above 10^{3155} $TCID_{50}$/mL at some points. It is however important to stress that the putative 10^{3155} $TCID_{50}$/mL threshold is largely discussed and remains debatable. End-point titration relies on the cell-lines used and on the time of incubation before staining, both critical parameters that have no gold standard. Therefore, although useful within a study itself, direct comparison of end-point titration values from different sources would provide not much more than coarse approximation of infectivity potential. In addition, it has been reported on several occasions that vector could clearly acquire BTV from animals with a lower viraemia, sometimes even undetectable through classical isolation techniques [51, 52]. Moreover, viraemia levels only make epidemiological sense in the light of the considered serotype or strain [45] and vectors biology, i.e. biting rates and oral susceptibility, as these parameters are species dependent and likewise population dependent [53]. These results also highlight the interest of using natural host species; indeed the tested serotypes had in VERO cell culture replication properties that did not significantly differ whereas in cattle in the current study BTV1, 9 and 16 appear to be better adapted to cattle than BTV2 and BTV4. In addition, Coetzee et al. recently reported the absence of correlation between replication levels in VERO cells and virulence in ruminant host [54]. Therefore, as useful as could in vitro tests or mice models be to clarify pathogenicity mechanisms or to allow preliminary vaccine evaluations, they are unable to fully replace natural host experimental infections, which has to be chosen with special emphasis on the specie that represent the most relevant economical issue.

Broadly speaking, the results only indicated minor significant differences between the vaccinated/non-vaccinated groups although some quite obvious differences between the different serotype groups. One of the factors surely attributing to the non-significant differences is the fact the number of animals per group was low thus limiting the power of the statistical analysis. Further study focussing on some particular aspects using a higher number of individuals could clarify some of those points.

Amongst the BTV serotypes evaluated in this study, BTV1, BTV9 and BTV16 appeared to be better adapted to cattle host than BTV2 and BTV4. None of the tested serotypes could cause serious clinical disease. Viral RNA copy number was higher at viraemic peak in non-vaccinated animals. Viral detection at the viraemic peak and in the organs at necropsy suggests a partial and minor protection of BTV8 vaccination against infection with European heterologous serotypes in an experimental context. The very limited serological cross reactivity between the different tested serotypes most likely suggests cellular based mechanisms. It has been recently reported that West Nile virus lineages that induce different mortality rates in the field could cause similar mortality in experimental conditions, the discrepancy putatively thought to be linked to a different host competence among these strains [55]. Vector borne viruses are in permanent interaction with environment, hosts and vectors; therefore the epidemiological meaning of the potential effect of a mass anti-BTV8 vaccination to (partially) protect cattle livestock from heterologous serotypes remains uncertain.

Abbreviations

BTV: bluetongue virus; BT: bluetongue disease; ELISA: enzyme linked immunosorbent assay; RTqPCR: reverse transcription quantitative polymerase chain reaction; BoHV1: bovine herpesvirus type 1; BSL3: biosafety level 3; TPI: The Pirbright Institute; CODA–CERVA: Centrum voor Onderzoek in Diergeneeskunde en Agrochemie–Centre d'étude et de recherches vétérinaires et agrochimiques; BHK: baby hamster kidney; DMEM: Dulbecco's modified eagle medium; $TCID_{50}$: tissue culture infectious dose 50; dpi: day post-infection; RNA: ribonucleic acid; VP7: viral protein 7; PN: percentage of negativity; SNT: seroneutralization; cDNA: complementary deoxyribonucleic acid; S2: segment 2; PFU: plaque forming unit; ANOVA: analysis of variance; NV and V: non-vaccinated and vaccinated; CPE: cytopathic effect; MLV: modified live vaccine; CTL: cytotoxic lymphocyte; VERO: verda reno.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LM, FDP, CS and KdC designed the experimental protocol. CS and KdC allowed the coordination of the project. KdC and IDL provided the inocula. LM and WVC carried out experimental infections, cattle sampling and clinical follows-up. LM and CS performed the necropsy. CT, LM and IDL carried out the laboratory analyses. CS and LM realized the statistical treatment of the data. LM, FDP, CS, KdC and ET contributed to the manuscript drafting. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Experimental protocol has been approved by the ethical committee of the IPH-VAR (Institute of Public Health—Veterinary and Agrochemical Research, Groeselenberg 99, 1180 Uccle, nr. of the project 110228-01 RT 10/10 BLUETONGUE).

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Discussion - Perspectives

Starting in 2006, BTV serotype 8 was responsible in Western and Northern Europe for a major epizootic. One must go back to the last major outbreaks of foot-and-mouth disease in the 1980s to encounter such a plague. The magnitude and diffusion of the disease were surprisingly high, leaving the different affected countries with no other options than to reduce the movement of herds, trying to use insecticides to prevent contamination of healthy animals or to house them within the barns at dusk. All these measures only had a limited impact on the disease transmission. The fight against BTV only took a new turn with the marketing of BTV8 inactivated vaccines in 2008 and the subsequent implementation of national vaccination campaigns in most of the affected countries.

During the late summer of 2011 a first cluster of reduced milk yield, fever and sometimes diarrhea was reported in the Netherlands. In December of the same year first congenital malformations occurred and Schmallenberg virus was identified and named in March 2012, becoming one of the very few orthobunyaviruses distributed in Europe with Batai virus (Jost et al., 2011; Hofmann et al., 2015a).

By the time of their emergence, both viruses were lacking pathogenesis and epidemiology data in the European context (naïve ruminants, Palearctic vectors, European climate) and most assumptions were extrapolated based on scientific knowledge on other related viruses and/or other regions of the World.

To study and determine the pathogenesis, the dynamic of these viruses, to clarify their ability to cross the placental barrier, standardized, repeatable models potentially displaying field-like outcome are required.

A proper experimental infection needs a proper inoculum

An adequate inoculum to use in infectious challenges to study viral pathogenesis should be:

1) Safe, meaning it should have been screened for contaminations, adventitial agents or other pathogens (Speder, 2014);
2) Easily available, practical and standardised;
3) Contain a virus the closest from wild-type virus found in the field displaying similar replication and virulence properties;

Infectious blood versus cell passaged inoculum

An infectious inoculum can be used for several purposes, from the investigation of *in vivo* characteristics of recently discovered viruses (Breard et al., 2018) to vaccine efficacy requirements or to investigate certain specific aspects of the pathogenesis (MacLachlan et al., 1994). In the context of an emerging pathogen with an epizootic potential it is quite obvious that time is of the essence and since standardization is not critical in the very first steps of *in vivo* characterization using an infectious animal product such as blood or serum could be both faster and more secure to reproduce expected

clinical signs and viraemia. Nevertheless it appears that in most of the most recent experimental infections involving BTV or SBV, culture grown inocula were used in a majority of challenges (table 4). The main reasons to use cell-passaged virus can be summarized as follow:

1) The original isolate or any strain of particular interest can be distributed throughout the world, leading to great improvement of standardization;
2) Viral amplification by cell-passages allows a high increase in viral titre, subsequently allowing to inoculate lower volumes;
3) Screening for contamination or other pathogens is easier in cell culture and eliminate some veterinary public health concerns about using ruminant blood to infect other ruminants;
4) Virulence in cell culture can be easily standardized.

Virus	Type of inoculum	Host Species	Cell type	Nb of passages	Inoculation route	Volume (ml)	Titre (TCID50/ml)	Ref
BTV	Cell-passaged; blood (goat)	Cattle, sheep, goats	BSR; ECE+BSR	3; 1+3 or 1+2	SC; IV (blood)	2, 3 or 4; 1 (blood)	10^3-10^4.67	Bréard et al., 2017
BTV	Cell-passaged	Cattle, sheep, goats	KC+BHK-21	1+1	SC	2-4	10^6	Schultz et al., 2018
BTV	Reverse genetic	Sheep, goats	/	/	SC+IV	1	10^5	van Rijn et al., 2016
BTV	Cell-passaged	Cattle	BHK-21	2	SC+IV	1-4	10^4-10^6.15	Martinelle et al., 2016
BTV	Cell-passaged	Cattle, sheep	KC	2	SC, ID	1	10^7	Darpel et al., 2016
SBV	Cell-passaged	ECE	KC+BHK-21+HmLu-1	1+5+2	Yolk sac	0.2	5x10^2-5x10^6.4	Collins et al., 2018
SBV	Serum (cattle)	Goats	/	/	SC	1	/	Laloy et al., 2017
SBV	Cell-passaged	IFNAR mice	KC+BHK-21	1+1	SC	0.1	10^3	Tauscher et al., 2017
SBV	Cell-passaged	IFNAR mice	KC+BHK-22	1+2	SC	0.1	10^8	Boshra et al., 2017
SBV	Cell-passaged	Sheep	KC+BHK-21	1+1	SC	1	2x10^3	Poskin et al., 2015

Table 4. Inocula characteristics used in the 5 most recent experimental infection studies on BTV and SBV (as searched on PubMed with keywords “experimental infection bluetongue” and “experimental infection Schmallenberg).

In most of the studies presented in the current manuscript, the clinical signs reported in the BTV infected animals were of a lesser extent than those reported from the field (Martinelle et al., 2011; Martinelle et al., 2013; Martinelle et al., 2015; Martinelle et al., 2016; Martinelle et al., 2017). Since we used mostly cell-passaged inocula and as modified live vaccines gain their attenuation through serial cell passages the first and most obvious hypothesis to explain the mild severity of bluetongue disease in our experiment was the use of culture grown virus. Moreover, passage history of the inocula we used involved mostly ECE, BHK-21 and VERO cells. It was reported that BTV grown on KC cells (derived from *Culicoides sonorensis*) could induce a greater severity of the clinical signs

(Moulin et al., 2012). One hypothesis is that KC cells may better mimic natural vector-borne infection compared to virus passaged in other cell lines (Anderson et al., 2014).

As a matter of fact, it was also reported that the inoculation of infectious material from field isolates rarely produce a clinical picture as severe as in natural infection (Drolet et al., 2015). An additional hypothesis would be that the *Culicoides* saliva might act as a catalyzer to enhance the ability of BTV to produce severe clinical signs. Indeed *Culicoides* saliva was demonstrated to contain a trypsin-like protease able to cleave VP2, leading to the formation of infectious subviral particles with enhanced infectivity (Darpel et al., 2011). Moreover, in the field and no matter the care and experience of the farmers there are always some animals that would be more sensitive to viral diseases within the herd due to individual poor immune defences, genetic variability or metabolic status. By contrast in an experimental context the health and sanitary status of every single animal is very strictly controlled and monitored, inducing a bias toward the selection of very healthy and vigorous animals most likely able to face and recover from BTV infection.

We demonstrated nonetheless the suitability of BTV passaged a few times on cell culture to both reproduce clinical signs and RNA detection (Martinelle et al., 2011). Other authors concluded to the benefits of culture grown virus to be used in experimental challenges in ruminants (Eschbaumer et al., 2010) as well. Despite converging result the policy of the OIE remains unchanged regarding recommended vaccine efficacy requirements, i.e. challenging vaccinated and unvaccinated sheep with a virus “passaged only in ruminant animals and with no or limited ECE or cell culture passages” (OIE, 2014).

Results regarding SBV do not show that much consistency. Indeed, in cattle Wernike et al. reported a reduced viral replication of culture-grown SBV when compared to natural host-passaged inoculum (Wernike et al., 2012). By contrast, one year later the same team concluded to the suitability of both infectious serum and low passage cell culture material for SBV experimental challenges in sheep (Wernike et al., 2013a). Besides the passage history the origin of the isolated virus seems to be of importance as CNS originating virus failed to reproduce RNAemia in inoculated animals (Wernike et al., 2013a). Successive serial passages in cell-culture indeed are well known to usually result in decreased virulence. However regarding SBV Varela et al. reported an increased pathogenicity in a SBV strain passaged 32 times in INF-incompetent sheep CPT-Tert cells, associated with a faster spread of the virus in the brain of suckling mice (Varela et al., 2013). SBV was demonstrated to grow efficiently in several cell lines including sheep CPT-Tert, bovine BFAE, human 293T, dog MDCK, hamster BHK-21, BSR, KC and VERO cells (Varela et al., 2013; Kraatz et al., 2015). Whereas serial passages in CPT-Tert led to the accumulation of a variety of mutations mostly in the M and S segments, the porcine cell line SK-6 proved to be highly susceptible and to allow the genetic stability of SBV throughout successive passages (Hofmann et al., 2015b). Therefore, depending on the cell line

used to grow SBV, serial passages can lead to attenuation, increased virulence, or efficient propagation with a low frequency of nucleotide exchanges.

A matter of doses and routes

When it comes to arboviruses the choice of the route of inoculation can be driven by two main considerations:

1) The need for a route that will mimicry the most the behavior of the vector in the wild. Usually haematophagous arthropods are either telmophagous or solenophagous; depending on the vector species the route might be intradermal (ID), subcutaneous (SC) or intravenous (IV). In experimental infections given the size of the arthropods and the size of their mouthparts the inoculated viral load and volume are usually way higher than the ones inoculated through naturally occurring feeding (Venter et al., 2007). Another drawback is the lack of vector saliva components, which can modify the structure and infectivity of *Reoviridae* and *Bunyavirida*e viral particles (Darpel et al., 2011; Horne and Vanlandingham, 2014). We used SC route in study 4 and 6.
2) The need for a route that will ensure the virus to reach blood stream. Quite obviously this is the intravenous route. We used the intravenous route in studies 1 and 3. Since vector saliva components can enhance the infectivity of arboviruses there is a risk that the inoculation of the virus alone or at a distal site from the vector feeding site would result in a failed infection (Le Coupanec et al., 2013). Therefore the option to by-pass the skin to readily reach the bloodstream may be relevant.

Several authors including us (study 5) used mixed routes to overcome the respective disadvantages of each approach (Table 4; (Dal Pozzo et al., 2009a; Dal Pozzo et al., 2013)). In study 2 we compared intranasal, intradermal and subcutaneous routes for experimental infections with SBV. Intradermal is an interesting yet underused route: indeed most haematophagous arthropods do not make it through the skin and their mouthparts only allow them to feed intradermally. Most of the cellular and fluid exchanges between the skin and the blood do occur in the dermis (Nicolas and Guy, 2008). In addition, there are some evidences suggesting that intradermal inoculation can be more appropriate to reproduces many aspects of natural infection, including clinical disease, viral and immune responses (Umeshappa et al., 2011). However to perform an actual intradermal inoculation the volume to be injected has to be limited, the dermis being mostly composed of a network of collagen fibres. Therefore it is required to multiply the inoculation sites to reach the desire total inoculum volume and infectious titre. To realize the inoculation itself the most practical tools are the Dermojet® (Akra Dermojet) or a special syringes for intradermal injections (used to perform bovine tuberculosis skin tests as an example). These devices allow usually volumes between 0.1 to 0.4 ml, thus the need of

multiple injections to reach the common 1-4 ml inoculation volume used in ruminant infectious challenges experiments (Table 4). Moreover, with both systems the inoculum has to be transferred from its original vial to a small tank part of the body of the dermojet or to a special cartridge to be used with the intradermal syringue. This extra step increases the number of handlings, which should be limited especially in the case of BSL3 pathogens. In the present thesis the intranasal route was mostly investigated to test whether or not a potential direct contamination between sheep could be achieved. Indeed regarding BTV several authors reported unexpected and inconclusive direct horizontal transmission with different serotypes (BTV8, BTV1 and BTV26 at least) (van der Sluijs et al., 2011; van der Sluijs et al., 2013; Batten et al., 2013b; Batten et al., 2014).

Broadly speaking, several authors reported a direct link between the inoculated viral doses and the onset of clinical signs and viraemia, i.e. the higher the dose the sooner the clinical signs and viral RNA detection (Alexandersen et al., 2003; Quan et al., 2004; Howey et al., 2009). In another study we evaluated four 10-fold dilutions of a SBV infectious serum inoculum on ewes (See Annex 1, (Poskin et al., 2014a)). The undiluted original inoculum had a titre of 2 X 10^3 $TCID_{50}$/mL. It appears there is a critical dose to be inoculated to successfully reproduce field-like virological and immunological parameters, and once this threshold is over there no dose-dependent effect anymore. Indeed, in the productively infected animals no statistical differences between the different inoculation doses were found in the duration or quantity of viral RNA circulating in blood, nor in the amount of viral RNA present in virus positive lymphoid organs. Likewise Di Gialleonardo *et al*. compared three groups of cattle inoculated with 100 fold dilutions of BTV8; no significant differences in viraemia kinetics could be found (Di Gialleonardo et al., 2011).

Inoculation by the bite of Culicoides was reported to be more efficient than intradermal inoculation, especially by delaying the early immune response of the host despite a generally lower inoculated viral dose when compared to needle inoculation (Pages et al., 2014). Several mechanisms were hypothesized to explain this apparently enhanced infectivity in Culicoides transmitted BTV:

1) The *Culicoides* saliva contains proteases able to cleave VP2, leading to the formation of infectious subviral particles (ISVP) displaying higher infectivity in KC cells and culicoides (Darpel et al., 2011);
2) The ratio of infectious BTV particles versus defective virions produced within culicoides might be higher when compared with cell culture grown BTV (Pages et al., 2014);
3) Pharmacological agents contained in Culicoides saliva might affect the host's immune response by anti-proliferative effects on leucocytes (Bishop et al., 2006) or a reduced INF alpha/beta expression, as demonstrated with vesicular stomatitis virus and mosquito saliva (Limesand et al., 2003).

Nonetheless, the use of *Culicoides* to perform experimental challenges remains highly limited by practical constraints: to date besides *C.nubeculosus, C. riethi* and *C.sonorensis* no other

Culicoides species were successfully establish as lab-adapted colonies (Boorman, 1974; Veronesi et al., 2013), the alternative being insects caught in the wild. In addition, prior to the infectious challenge on the ruminant host the infection of culicoides is particularly tricky given the size of the insect and the exact amount of virus delivered to each ruminant cannot be known.

Altogether the subcutaneous route seems to represent the best compromise for BTV and SBV. The dose itself has to be sufficient but there is no gain in using massive viral load.

Beware of contaminations!

Bluetongue disease history is scarred with incidents of contamination of biological samples. In 1992, modified live vaccines against canine distemper, adenovirus type 2, Parainfluenza, and parvovirus, reconstituted with a killed canine coronavirus vaccine, led to abortions in several bitches. A virus could be isolated and was eventually identified as bluetongue serotype 11 (Evermann et al., 1994; Wilbur et al., 1994). More recently, a case of BTV11 contamination was reported by ANSES (*Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail*, Maison-Alfort, France), in the context an experimental infection of goats with BTV8, and appeared to be very closely related to the BTV11 isolated in Belgium (Breard et al., 2011). The BTV15 contamination that we discussed in study 5 (Martinelle et al., 2016) has been previously involved in two other experimental infections. Eschbaumer *et al*. used BTV1 culture supernatant, issued from ANSES, that was then passaged once on VERO cells before being injected to calves and sheep (Eschbaumer et al., 2011b). That inoculum has been subsequently used by Dal Pozzo *et al.* (Dal Pozzo et al., 2013), with the exact same outcome, namely discovery of the BTV15 contamination. Thus, ANSES received a BTV11 tainted BTV8 challenge virus from FLI, and involuntarily sent a BTV15 contaminated BTV1 strain to FLI. BTV inoculums were not only contaminated with BTV heterologous serotypes: Rasmussen et al. reported the use of a BTV2 inoculum contaminated with Border Disease Virus in sheep (Rasmussen et al., 2013).

So far literature does not report experimental infections with a SBV inoculum that was contaminated by another virus belonging to the same or a different family. Broadly speaking contamination routes are most likely related to i) laboratory contamination during sample preparation or ii) natural multiple infection of the original donor animal (Vandenbussche et al., 2015). Given the potential dramatic consequences of such contamination incidents, inocula should indeed be tested for major pathogens affecting the host species used in challenge experiments but also for a set of BTV serotypes considered to be the most at risk. Despite the transient circulation of BTV6 (van Rijn et al., 2012), BTV11 (Vandenbussche et al., 2015) and BTV14 (Orlowska et al., 2016) of vaccine origin in Europe the BTV11 contamination here above mentioned happened to be similar to BTV11 reference strain. Hence the contamination of the inoculum is far from being necessarily related to an ongoing viral circulation even though it might remain silent because of the lack of clinical consequences. Thus

to rule out any potential BTV contamination all known BTV serotypes should be tested for. Such a recommendation would inevitably increase the constrains and costs of quality control of inocula prior to their use in experimental infections. Extensive screening could however be considered on a case-by-case basis.

Placental crossing and teratogenesis

Both *Reoviridae* and *Peribunyaviridae* are families of viruses able to cross the placental barrier, infect the foetus and potentially cause teratogenic effects in the central nervous system or muskuloskeltetal defects (arthrogryposis) (Doceul et al., 2013; Maclachlan and Osburn, 2017). It is generally considered that the age of the foetus (in gestational age) is a key criterion to determine the extent of the congenital defects as differentiating nervous tissues are important targets for both BTV and SBV : usually the younger the foetus, the more severe the lesions (Doceul et al., 2013; Maclachlan and Osburn, 2017).

However, to colonize the foetus viruses need a way in; therefore it is considered that SBV *in ute*ro infection can only occur once the first placentomes are established, around day 30 of pregnancy in cattle and slightly earlier in sheep(Parsonson et al., 1988; Charles, 1994; Garigliany et al., 2012a).

Small ruminants and cow have slightly different definitive placental structure. In 1909 Grosser classified the mammalian placentas according to the number of tissue layers between foetal and maternal blood after implantation. The ruminants were considered to have a syndesmochorial placenta, *i.e.* where the uterine epithelium is removed and the chorion is in contact with the maternal connective tissue (Amoroso, 1961). Other authors reclassified it as epitheliochorial since the uterine epithelium subsisted [498]. From these historical and oversimplified categories the ruminant placenta was later considered to be characterized by the migration of the foetal chorionic binucleate cells and their fusion with the uterine epithelial cells. The ruminant placenta is a mix between the syndesmochorial and epitheliochorial placentation and the uterine epithelium subsists as a fetomaternal syncytium (Wooding, 1992). In small ruminants more than 95 % of the placenta forms a fetomaternal syncytium. In cows multinucleate cells appear only transiently once the uterine epithelium has regrown after implantation. This type of placenta is called synepitheliochorial (Wooding and Burton, 2008).

At implantation several changes occur: the papillae in the uterine glands immobilize the conceptus and it starts to elongate (cow: 15 days post coitum (dpc); sheep: 13-16 dpc). Subsequently the cells of the trophectoderm and the uterine epithelium get interdigitated, binucleate cells start to be seen. Then binucleate cells start to differentiate and to migrate (cow: 20-22 dpc; sheep: 16-18 dpc). Foetal villi develop in the caruncular areas starting at 24-26 dpc in small ruminants and 28-30 dpc in cow, thus defining the end of the implantation and the start of the placental development (King et al., 1979). Table 5 summarizes some of the essential events in the course of the prenatal development in cattle and sheep.

Event	Timing in cow (dpc)	Timing in sheep (dpc)
Blastocyst hatching from zona pellucida	9	9
Elongation of the blastocyst, establishment of the primitive streak, emergence of the notochord	17-18	13-14
Appearance of neural folds, closure of the neural groove	17-19	15-16
Implantation begins	16-19	15-18
Neurula	20-21	17
Neural tube complete; optic and otic vesicles present	21-23	19-20
Placentation begins	22-23	17-22
Three brain vesicles visible	24-25	17
Placentoma are detectable	32-36	21
Lymphoid development of the thymus	42	36
Spleen development	55	43-44
Peripheral lymph nodes	60	45
IgM containing cells	59	65
Myelin sheath acquisition (starting)	60	54-63
IgG containing cells	145	87

Table 5. Key events in sheep and cow embryos/foetuses with particular emphasis on nervous and immune systems. Compiled from (Bryden et al., 1972; Evans and Sack, 1973; Jordan, 1976; Maddox et al., 1987; Spencer et al., 2004; Assis Neto et al., 2010; Khaksary-Mahabady et al., 2018)

In the current work we decided to infect with BTV8 vaccinated and non-vaccinated pregnant heifers at 120 days of pregnancy (study 3, (Martinelle et al., 2013)). We also challenged pregnant ewes with SBV at 45 and 60 days of pregnancy (study 4, (Martinelle et al., 2015)). Thus for both viruses the experimental infection took place within the critical timeframe, between 30 and 150 days for cattle and between 30 and 70 days of pregnancy for sheep (Figure 9, (Charles, 1994)). Moreover, in experimental conditions the highest BTV transplacental infection was found at mid-term gestation, around 70 days of pregnancy in sheep (van der Sluijs et al., 2011; van der Sluijs et al., 2013). The prenatal period can be divided into four main periods: i) fertilization; ii) blastogenesis; iii) embryogenesis and iv) fetogenesis (Szabo, 1989). The embryo sprouts and develops tissues and organ structures from the three original germ layers (ecto-, meso-, and endoderm). By the end of the embryogenesis the conceptus became a “miniature” version of the adult animal, displaying all its specific features. Once the organs are differentiated the embryo becomes a fetus (Coppock and Dziwenka, 2017). The fetal phase is characterized by a fast growth of the conceptus. In cattle and sheep the fetogenesis starts around 45 and 38 dpc, respectively (Evans and Sack, 1973).Thus the critical timeframe for BTV and SBV infection overlaps the end of the embryo stage and the beginning of the fetal stage. Moreover, although in ruminants ɣ-globulins are unable to go through the placental barrier from the mother to the foetus it is admitted that cow and sheep fetuses become sequentially and

increasingly immunocompetent to a larger variety of antigens throughout the pregnancy (Silverstein et al., 1963; Schultz et al., 1973). The critical timeframe for BTV and SBV infection spans over the course of several important events in immune system development (Table 5). Although the sequence of antigens to be successively and progressively recognized by the fetal ruminant through pregnancy seems to be quite conserved between individuals, these antigens can be recognized starting with a difference of a few days between individuals (Fahey and Morris, 1978). This individual variability could explain the findings by De Clercq et al.,(2008), who reported all possible combinations of serological status/RTqPCR results in dam/calf pairs in a context of high BTV8 suspicion along with results which were interpreted as apparent immunotolerance (De Clercq et al., 2008). Likewise, malformed calves and lambs were found SBV viropositive or vironegative with or without SBV antibodies, suggesting the possibility of an *in utero* clearance of the virus. Moreover, most of the malformed calves that were negative in both SBV antibodies and RTqPCR were born from seropositive mothers (De Regge et al., 2013).

The range of teratogenic lesions, congenital defects and other reproductive disorders caused by BTV and SBV was quite extensively described in the introduction of the present thesis. Table 6 summarizes the most common malformations and nervous lesions induced by some of the most common viruses inducing such lesions in ruminants.

As a matter of fact, none of these respective lesions were reported either in the BTV experimental infection of heifers (study 3) or in the SBV infection of ewes (study 4).

In study 3 we reported reddening of the muzzle and haemorrhages in the wall of the pulmonary artery in calves born from non-vaccinated mothers. These findings were associated with the absence of any anti-BTV antibodies prior to the colostrum intake.

Lesion	Definition	BVDV	SBV	BTV	AKAV/AV
Hydranencephaly	Extensive loss of cerebral tissue with replacement by clear fluid	+	+	+	+
Porencephaly	Cystic fluid filled cavities in the brain tissue	+	+	+	+
Hydrocephalus	Dilation of the lateral ventricles by cerebrospinal fluid	+	+	+	-
Microencephaly	Reduced size of the cerebrum	+	+	+	+
Cerebellar hypoplasia	Reduced size of the cerebellum	+	+	+	
Kyphosis	Dorsal vertebral column curvature	-	+	-	-
Lordosis	Ventral vertebral column curvature	-	+	-	-
Scoliosis	Lateral vertebral column curvature	-	+	-	-
Torticollis	Twisted cervical vertebral column curvature	-	+	-	-
Arthrogryposis	Joint contraction of the limbs	-	+	+/-	+

Table 6. Summary of some of the most common central nervous and musculoskeletal lesions following in utero infection with bovine virus diarrhea virus (BVDV), SBV, BTV, Akabane virus (AKAV), or Aino virus (AV). Adapted from (Agerholm et al., 2015).

In study 4, out of the 22 born-alive lambs none had any anti-SBV neutralizing antibodies prior to colostrum intake.

In both these experiments timing of inoculation was optimal to achieve transplacental infection of the foetus with regard to data available from the literature yet no malformations could be seen. No antibodies against the virus used to infect the mothers could be detected as well. These striking results might even question the success of the infection, notwithstanding the positive RNA detection in the mothers. In study 3, the report of similar lesions and serology results in another experiment on goats (Coetzee et al., 2013), and in study 4 the detection of SBV nucleic acids in organs of several lambs and many extraembryonic structures provide support to an actual transplacental infection. In addition, in another study (see Annex 2 (Poskin et al., 2017)) we managed to isolate SBV from foetal envelopes in the animals from study 4 at birth, thus 90 and 105 days post infection. The very low ratio of precolostral seroconversion in immunocompetent fetuses was also confirmed following the infection of pregnant cattle with SBV (consortium, 2014).

Transplacental transmission of BTV8 based on field data was reported to range from 16 % (van Wuijckhuise et al., 2008; Santman-Berends et al., 2010b) to 35 % (Desmecht et al., 2008; Darpel et al., 2009). In experimental infections passage of BTV8 from the mother to the foetus could be demonstrated in 43 % of infected ewes whereas BTV1 could be detected in up to 67 % of the foetus

(van der Sluijs et al., 2013). Other authors cited a BTV placental crossing in ewes of about 40 %. The observed BTV8 vertical transmission rate in goats infected at 61 days of pregnancy was also 33 % (Belbis et al., 2013).

SBV vertical transmission seems to be lower when compared to BTV, especially in cattle (Wernike et al., 2015). As indicated in the introduction, the rate of malformations caused by SBV was reported to be about 0.5 % in cattle (Veldhuis et al., 2014a) although the rate of intrauterine infection – based on serological results of the calves prior to colostrum intake – was reported to be about 28 % (Garigliany et al., 2012a). Other authors documented field data about congenital malformations affecting 3 % of the calves but 8-10 % of the lambs in farms at the beginning of the SBV epizootic (Dominguez et al., 2012; Dominguez et al., 2014). In Belgium based on a survey targeting farmers we also found an estimated 10 % of malformed sheep in SBV positive flocks (see Annexes 3 (Saegerman et al., 2014)).

A)

B)

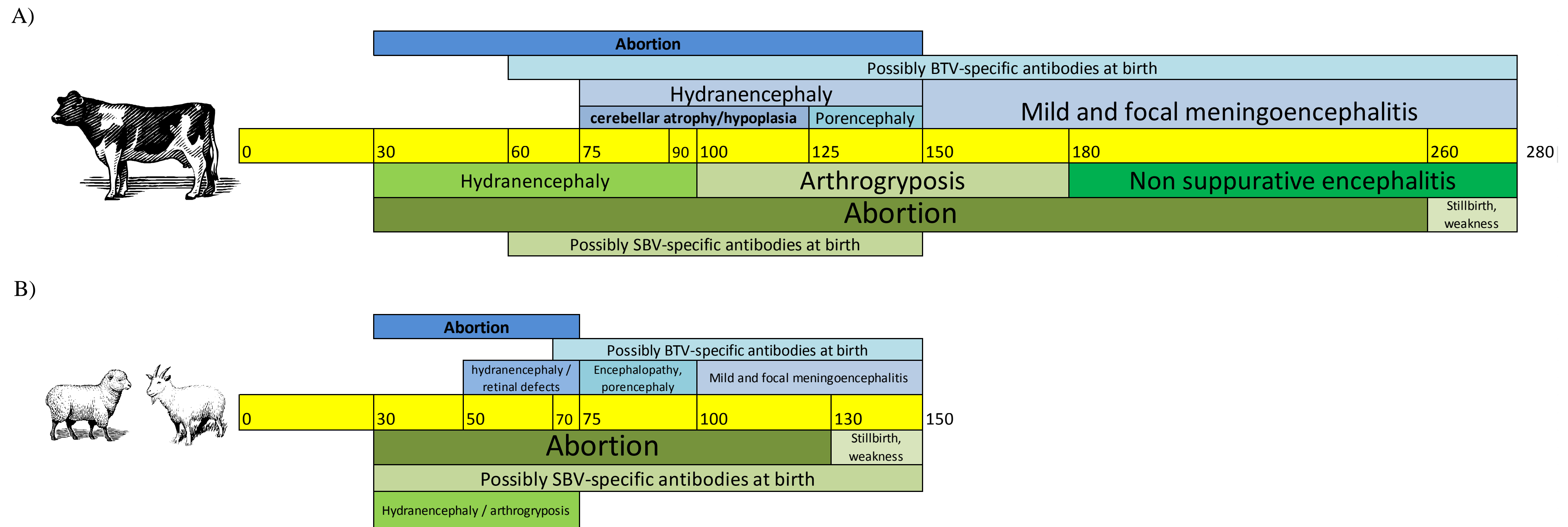

Figure 9. Suggested timeframe for BTV and SBV in utero infection causing defects in cattle and small ruminants offspring (Charles, 1994; Martinelle et al., 2012; Martinelle et al., 2013; Afonso et al., 2014; consortium, 2014; Martinelle et al., 2015; Maclachlan and Osburn, 2017). See also Annex 4.

In utero potential BTV (green shades) and SBV induced defects (blue shades) following infection of the pregnant dams along the whole gestation time for cattle (A) and small ruminants (B).

Embryonic losses represent a key factor affecting ruminant production systems. In cattle as well as in sheep most of the spontaneous embryo mortalities occur in the early embryonic life, namely before 16-18 dpc (Dixon et al., 2007; Diskin and Morris, 2008). In cattle early embryonic losses were reported to range from 20 to 44 % whereas in sheep in ranges from 12 to 30 %, with a clear increase of embryo deaths with the ovulation rate (Geary, 2005; Dixon et al., 2007). The impact of both BTV and SBV on reproductive parameters other than teratogenesis is well documented. BTV8 was reported to increase the 56-days-return to service rate and the number of AI required to achieve a pregnancy (Santman-Berends et al., 2010a; Nusinovici et al., 2012). During the SBV epizooty the number of AI to get cattle pregnant was slightly yet significantly increased regardless of whether or not they were part of a herd reporting malformations indicative of an actual infection (Lechner et al., 2017). Although SBV and especially BTV had a tremendous economic impact on livestock industry, it is remarkable to highlight the relative poor efficiency of the placental crossing and more specifically the low rate of congenital deformities induced by those viruses. It also seems clear that congenital malformations underestimate the actual rates of BTV and SBV transplacental infections (Afonso et al., 2014).

BTV variability and vaccine induced cross-protection

The interrelationships between at least the 24th first discovered serotypes were established more than 25 years ago by plaque reduction tests, cross protection tests and heterotypic antibody responses (Erasmus, 1990). Homologous protection relies mostly on neutralizing antibodies and T cells targeting VP2 and VP5; serological response is however not exclusively protective against homologous serotypes (Jeggo et al., 1983; Schwartz-Cornil et al., 2008). Cytotoxic T cells were also demonstrated to support heterologous protection by targeting NS1 and inner core proteins.

Several recent experiments actually provided interesting results not exactly in line with the diagram of serological relationships suggested by Erasmus in 1990. Indeed, heterologous protection involving serotypes not necessarily seen as close from each other was reported following the use of inactivated vaccine (Umeshappa et al., 2010b) or virus-like particle vaccines (Perez de Diego et al., 2011). An attempt of cross protection using BTV4 – considered as an ancestor serotype – Modified Live Vaccine resulted of the protection of the vaccinated sheep against BTV9 and 11, and to a limited extend against BTV10 but not BTV1 (Zulu and Venter, 2014).

Besides, the serological relationships were built, when based on results from experiments using an animal model, solely on sheep. In study 5 and 6 we demonstrated that the tested serotypes displayed various levels of adaptation to cattle. A host-dependent virulence was also reported previously (Eschbaumer et al., 2011b). Moreover, since BTV immunogenicity not only is based on serotypes but also on topotypes it is fully justified to renew and update serotype relationships within new epidemiological systems (Dahiya et al., 2004).

The serial passages of BTV in mammalian cells were reported to lead to a decrease in the genetic diversity of the viral population which was linked to a decrease of the virulence of that population. Therefore, apart from screening for contaminants and other pathogens, standardizing titres and volumes, checking for passages history, it might be interesting to screen for viral variability prior to launch infectious challenges with different BTV serotypes (Caporale et al., 2014).

Surveillance, prevention and control

The emergence of exotic or newly discovered vector-borne viruses follows common sequential steps: arrival, establishment and spread (Randolph and Rogers, 2010). Traditional surveillance systems involves disease reporting (by veterinarians, laboratories, farmers) followed by epidemiological and laboratory investigation. The objective is to allow an early detection and subsequent early decision making process to manage the emerging syndrome.

In the case of Bluetongue disease, the minimum requirements for monitoring programs to be implemented by Member States are defined in the EC No 1266/2007. They consist in i) passive clinical surveillance; ii) serological surveillance; and iii) entomological surveillance (Kyprianou, 2007).

By contrast with Bluetongue disease, the disease caused by SBV is not classified as notifiable to the OIE. In Belgium both diseases are part of the Abortion Protocol, a mandatory reporting system implemented in the context of Brucellosis surveillance: any fetuses displaying evocative lesions are tested for BTV and SBV.

Vaccination of susceptible livestock has been shown to be an efficient way to prevent clinical disease and transmission of BTV and SBV (for details please see the Chapter I.9) and complies with OIE recommendations. Highly sensitive molecular methods are now available to detect both viruses in many matrixes (please see Chapter I.8).

When it comes to emerging and/or rare disease, the actors involved at the different levels of the surveillance network might lack the awareness to properly report unusual syndromes or clinical picture. The limits between normality and abnormality could be missed by veterinary practitioners since they cannot rely efficiently on a previous experience, especially when clinical disease is overall rare. In another study we suggested the use of a method called “classification and regression tree” (CART), which is based on the analysis of field clinical observations (Saegerman et al., 2012) (Annex 5). Such approaches could improve the detection of emerging arboviruses in livestock by ranking clinical signs based on their importance with respect of the dynamics of the emergence.

In another paper we discussed the use of sentinel animals, in particular in the case of BTV surveillance (Saegerman et al., 2009) (Annex 6). Sentinel animals are animals chosen within their usual environment or kept in a specific one, and closely followed up through time in order to detect as soon as possible, in a quantitative or qualitative manner, the exposition to a given pathogen. It is part of a specific prospective surveillance. BTV and SBV being both vectorised by the same arthropods showing a seasonal activity pattern, sentinel animals could be used especially to detect the re-emergence of these pathogens. With respect to economic concerns and the short RNAemia in SBV infected ruminants, ELISA tests appear to be the most cost-effective option.

The critical time frame for *in utero* infection with BTV and SBV causing typical congenital malformations ranges from 30-150 and 30-75 days of pregnancy for cattle and small ruminants, respectively (Figure 9).

In Belgium, two major lamb production systems coexist:

- The production of "pasture lamb" represents about ¾ of the Walloon production. Lambs are often kept inside the barn during the first month. Around the age of one month, when in addition to suckling they start to eat solid feed, they go out in meadows with their mother. They are weaned around the age of 3 months and slaughtered at the age of 6 months, when they reach a weigh of 40 to 45 kg. Mating season usually starts in September to end in November.
- The production of "barn lambs", about ¼ of Walloon production. The lambs are raised exclusively in barns or sheepfold and are slaughtered at the age of 3 months at a weight of 35 to 38 kg. Mating season takes place in April-May through summer.

Birth rate of cattle is also seasonal, especially regarding meat cattle (Figure 10). Vector species are mostly active from April to November (De Regge et al., 2015).

Given these data, critical time of the year for *in utero* infection would be October-November and May to October for pasture and barn lambs, respectively. For meat cattle the high risk period would span from June to October.

Unless a very strict oestrus synchronization and insemination management, expecting to protect pregnant animals from the most deleterious teratogen lesions of BTV and SBV based on the use of insect repellent and housing practices is delusional. Vaccination remains the most efficient measure to avoid vertical transmission of BTV and SBV.

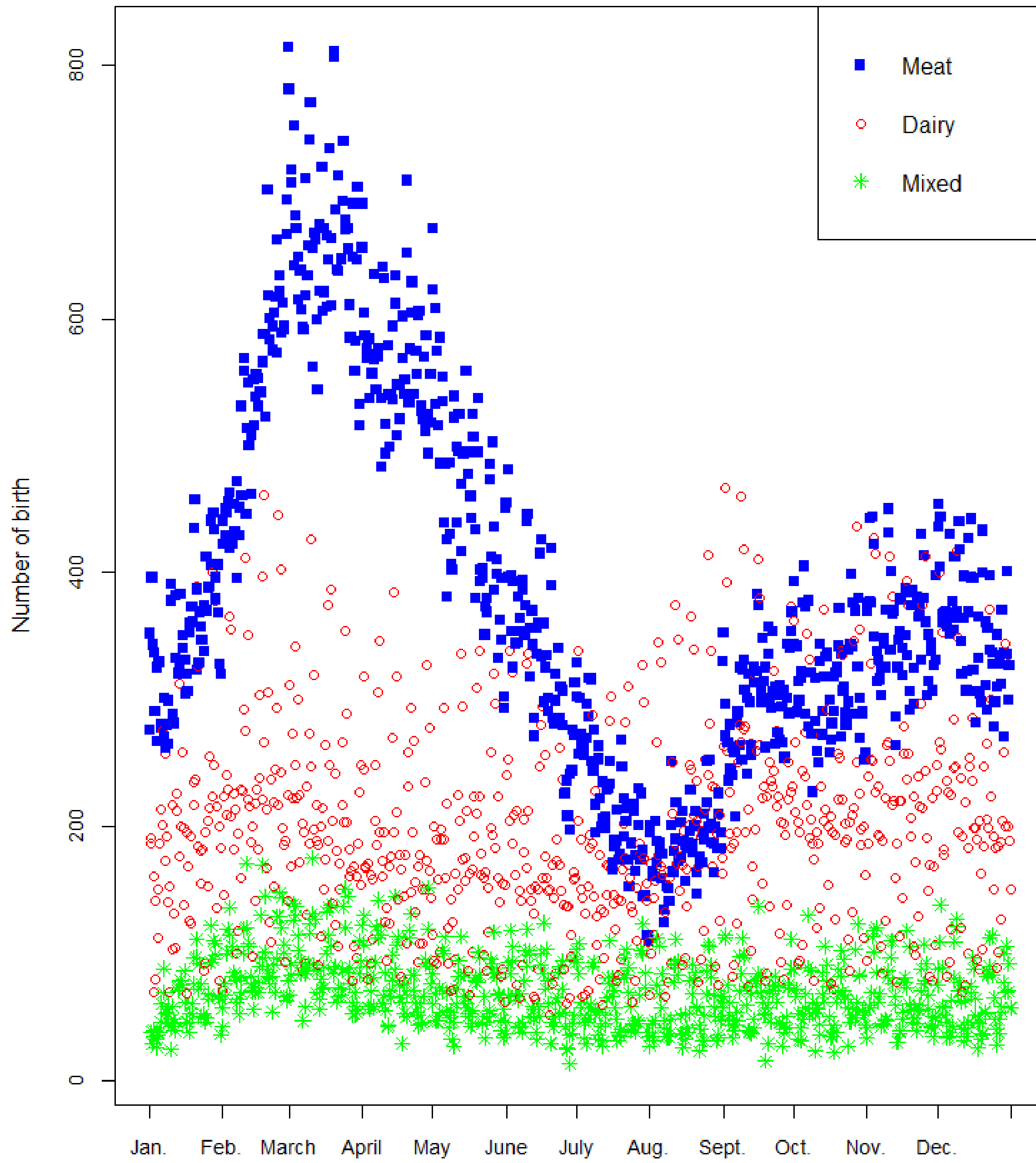

Figure 10. Daily calving distribution in the Walloon Region in 2015 (Martinelle et al., 2018)

Conclusion and recommendations

In conclusion, targeting ruminant host species in experimental infections especially with BSL3 pathogens is very expensive, time consuming, subject to stringent animal welfare constraints and critical sample size analysis to meet optimal statistical requirements …

The number of ruminants used in experimental infection is chosen based on welfare and statistical concerns but also quite unfortunately on economic and practical grounds (Coetzee et al., 2014). In the present thesis we worked with BTV in BSL3 facilities and with SBV in BSL2+/BSL3 facilities depending the phase of the experiment. Indeed, the Belgian Service of Biosafety and Biotechnology classes BTV as a class 3 pathogen whereas there is no recommendation for SBV. However we based our experimental infection with SBV by analogy with AKAV, also classified as a class 3 pathogen (Belgian Biosafety Server, 2018). Domestic ruminants being herd animals, they need to be housed in groups or at least not individually. Euthanasia methods have to be the most humane as possible and clear end points have to be defined. Given the scarcity of clinical signs caused by BTV and SBV in the field and the individual variations in the response to the infection the number of animals to be included has to be chosen very carefully to comply with the Reduction objective (3 Rs concept) but has to be sufficient to limit the risk of not being able to provide useful data in the context on the ongoing scientific investigation. This is particularly striking regarding experimental infection of pregnant ruminants with low malformation rates following transplacental transmission.

…Yet ruminant model remains unavoidable to grasp the impact and assess the pathogenesis of emerging vector-borne viruses.

The most objective parameter to assess a vaccine efficacy against a virus and especially a RNA vector-borne virus is the evaluation of the viral RNA detection by RTqPCR in the host target (Eschbaumer et al., 2010). BTV and SBV virulence were demonstrated to vary depending on the ruminant host whether it is cattle, sheep or goat. In addition, pregnancy length differs between cattle and small ruminants; the placentation and the development of the foetal envelopes present slight differences (Spencer et al., 2016). As a consequence, to study any of the specific aspect related to a ruminant species there are no other animal model or any alternative able to mimic the natural situation in a proper way (Coetzee et al., 2014).

The results presented in this thesis provide new insights about viruses that raveled through Europe causing severe losses in the livestock industry. In addition these aspects open new perspectives to expand the knowledge on emerging vector-borne virus targeting ruminants. More specifically:

1) According to our experiments, subcutaneous route with an inoculum passaged a limited number of times on cell culture seems to represent the best compromise between a high

probability to reproduce an infection similar to what happens in the field and logistics surrounding the preparation/storage/management of the inoculum. To prevent the loss of viral variability isolation and limit the risks of attenuation isolation of BTV could be done on KC cells (Moulin et al., 2012) whereas SBV could benefit regarding the same aspects from an isolation on the highly susceptible SK-6 cell line (Hofmann et al., 2015b). Screening for concomitant pathogens should be considered on a case by case basis, if required. The dose should be chosen based on literature data yet no advantage is provided by inoculating a massive viral load.

- Since some data from other authors suggest a better reproduction of the diseases with intradermal inoculation, it could be further investigated, especially if more user-friendly devices could hit the market.
- A major breakthrough would be the successful adaptation of a colony of Palearctic BTV and SBV vector *Culicoides* species (*C.obsoletus/scoticus, pulicaris*) to laboratory conditions and subsequent use in infectious challenges.
- Vector-borne transmission of BTV implies the puncture of the skin at some point. There are growing evidences that under certain circumstances additional routes of transmission can be observed: a goat was reported to be infected by BTV2 without direct contact (Rasmussen et al., 2013). The recently discovered BTV26 also displayed the ability to infect goats through direct contact (Batten et al., 2014). In other experimental infection control ewes were found positive with BTV1 and BTV8 (van der Sluijs et al., 2011; van der Sluijs et al., 2013). The study of the virus factors affecting this modified/underreported transmission feature should allow a better understanding of the epidemiology of the disease.

2) The teratogen potential of BTV and SBV expresses only very rarely and the current work contributed to clarify the timeframe and consequences of the infection of pregnant ruminants with these viruses. Moreover, depending on the timing of the infection a transplacental infection with BTV or SBV is far from necessarily implying congenital malformations; actually seroconversion is inconsistent even though the foetus might be immunocompetent by the time of infection. SBV can readily infect and remain in foetal envelopes without causing any defects in the foetus but a transient viraemia with SBV nucleic acid possibly being detected in some offspring’s organs at birth. BTV can cause slight *in utero* lesions with no BTV RNA or anti-BTV antibodies being detected at birth. As expected, the use of BTV8 inactivated vaccine successfully protected both the dams and foetuses from BTV8 infection.

   - Despite growing evidences of an improved transplacental transmission around mid-gestation (van der Sluijs et al., 2011) the lower time-limit to successfully reproduce a SBV or BTV intrauterine infection by inoculating the mother (30 days of pregnancy; time required for the placentomes to develop and become functional) remains mostly based on

previous work on Akabane virus or non-European BTV serotypes. In study 4 the earlier time we evaluated was 45 days of pregnancy. The Central Veterinary Institute in Wageningen infected pregnant ewes at 38 and 45 days of pregnancy; SBV passed the placental barrier easily and percentage of SBV viral RNA in umbilical cord was even higher in the group infected at 38 days (consortium, 2014). The earliest BTV8 transplacental transmission was described in ewes infected at 40-45 days of pregnancy (van der Sluijs et al., 2011). To the best of our knowledge there are no data about the consequences of experimental infections prior to those times.

- The BTV1 epizootic in Europe had a far lesser extension and consequences than the BTV8 one. It was demonstrated that BTV1 was able to induce transplacental transmission to a higher incidence compared to BTV8, causing more severe pathology and nervous malformations (van der Sluijs et al., 2013). Likewise, BTV2 was found to cross the placental barrier quite efficiently (Rasmussen et al., 2013). The transplacental passage being overall a rare phenomenon and the onset of malformations even rarer in the field, it is not unlikely that other wild type European serotypes – transmitted less readily in adults – might also be able to cross the placental barrier.

3) In study 6 we showed (i) the weak virulence of the selected viral strains for bovines, (ii) a partial serological cross-reactivity between BTV8 and BTV4, and BTV1 and BTV8, (iii) a potential weak cross-protection induced by BTV8 vaccination. According to *in vivo* virological parameters, we could confirm that BTV serotypes are differently adapted to young bovines (BTV1, 16 and 9 more adapted than 2 and 4). We also demonstrated a striking dominance of BTV15 over BTV1 in cattle in study 5, whereas BTV1 was shown to prevail over BTV15 in sheep (Eschbaumer et al., 2011b).
   - Further studies of the mechanisms of reassortment and the respective contribution of insects and mammalian hosts are required to clarify the epidemiological dynamics of BTV evolution in Europe;
   - Additional cross-challenges and cross-vaccination in ruminants with selected European serotypes in accordance with the epidemiological situation should help to refine the relationships between these serotypes and might undercover unexpected cross reactivity.
   - The optimization of reverse genetic protocols would greatly improve the understanding of reassortment, virulence factors and host adaptation. The applications of this technique are vast and are of the greatest help to further tackle the threat posed by BTV.

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Annexes

Annex 1

Dose-dependent effect of experimental Schmallenberg virus infection in sheep

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Short Communication

Dose-dependent effect of experimental Schmallenberg virus infection in sheep

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ABSTRACT

Schmallenberg virus (SBV) is an orthobunyavirus affecting European domestic ruminants. In this study, the dose-dependent effect of experimental infection of sheep with SBV was evaluated. Four groups of three ewes were each inoculated subcutaneously with 1 mL of successive 10-fold dilutions of an SBV infectious serum. The ewes were monitored for 10 days, but no clinical signs were observed. The number of productively infected animals within each group, as evidenced by viraemia, seroconversion and viral RNA in the organs, depended on the inoculated dose, indicating that a critical dose has to be administered to obtain a homogeneous response in infected animals under experimental conditions. In the productively infected animals, no statistical differences between the different inoculation doses were found in the duration or quantity of viral RNA circulating in blood, nor in the amount of viral RNA present in virus positive lymphoid organs.

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Schmallenberg virus (SBV) is newly emerged orthobunyavirus transmitted by *Culicoides* spp. (De Regge et al., 2012) that causes abortions, stillbirths and malformations in domestic ruminants (Herder et al., 2012; Hoffmann et al., 2012). In a recent study in cattle, subcutaneous inoculation with a 1/100 dilution of an SBV infectious bovine serum induced a longer duration of viral RNA circulating in blood compared to inoculation with undiluted infectious serum (Wernike et al., 2012). The present study was conducted to determine if a similar dose-dependent effect occurs in sheep.

Twelve 1-year-old Mourerous ewes, negative for SBV by ELISA, serum neutralisation test (SNT) and quantitative reverse transcriptase PCR (qRT-PCR), were included in the study. Three randomly selected ewes in each of four groups were inoculated subcutaneously in the left axilla with 1 mL undiluted, or 1/10, 1/100 or 1/1000 diluted, SBV infectious bovine serum in phosphate buffered saline (PBS). The infectious serum was obtained from Friedrich Loeffler Institute and had been tested in cattle and sheep (Hoffmann et al., 2012; Wernike et al., 2012, 2013). The inoculum contained 2 $\times 10^3$ 50% tissue culture infectious doses/mL ($TCID_{50}$/mL), as determined by end-point titration on baby hamster kidney (BHK) cells (Wernike et al., 2012) and was sent to CODA-CERVA on dry ice under appropriate transport conditions. The study was approved by the joint Ethical Committee of the Belgian Scientific Institute of Public Health and CODA-CERVA (project number 121017-01; date of approval 11 February 2013).

During the 10 day period following infection, clinical examinations of ewes were performed daily and blood was collected from the jugular vein. Two ewes, inoculated with the undiluted or the 1/1000 diluted inoculum had rectal temperatures of 40 °C 1 day post-inoculation (dpi), but the average and median temperatures in the groups stayed in the normal range (38.3–39.9 °C). No other clinical signs were detected throughout the experiment.

The presence of SBV RNA in serum and whole blood was determined by detection of the SBV S segment using a one-step qRT-PCR (De Regge et al., 2013). In case of doubtful results, RNA extracts were retested in a two-step PCR with the same primers, as described previously (De Regge and Cay, 2013). Cycle threshold (Ct) values were converted into S segment copy numbers using an RNA standard curve (see Appendix A: Supplementary material).

All ewes inoculated with the undiluted or 1/10 diluted SBV infectious serum, along with one ewe inoculated with the 1/100 infectious serum, were positive by qRT-PCR for viral RNA in blood (Fig. 1). No SBV RNA could be detected in other ewes by qRT-PCR during the experiment. The number of ewes in each group that were positive for viral RNA in blood decreased significantly as a function of the inoculated dose (Fisher's exact test; $n = 12$; $P = 0.045$), providing evidence that a critical dose needs to be administered to induce a homogenous productive infection in sheep. When the

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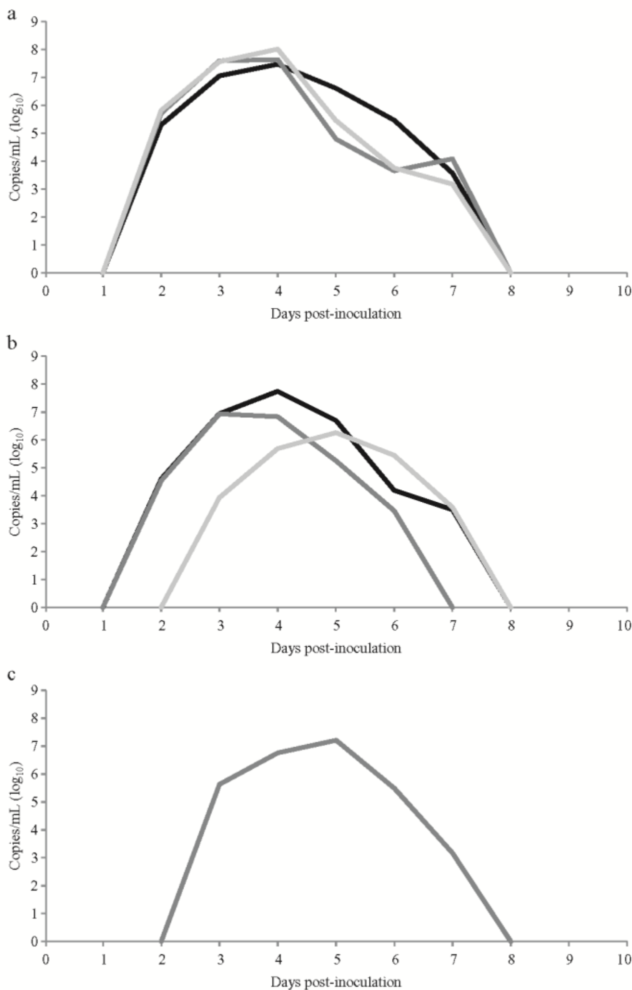

Fig. 1. Detection of Schmallenberg virus (SBV) S segment RNA by qRT-PCR (copy number/mL) in the blood of sheep (three ewes in each group: ewe 1 ▬, ewe 2 ▬, ewe 3 ▬) inoculated subcutaneously at day 0 with undiluted (a), or 1/10 (b) or 1/100 (c) dilutions, of SBV infectious bovine serum. None of the animals inoculated with a 1/1000 dilution became positive for SBV RNA by quantitative reverse transcriptase PCR. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Spearman–Karber method was applied to the data (Hierholzer and Killington, 1996), the undiluted serum contained at least $10^{1.83}$ sheep infectious doses per mL.

It would be interesting to see if inoculation of other sheep breeds with SBV would have similar results, since differences in breed susceptibility have been described for another bunyavirus, Rift Valley fever virus (Busquets et al., 2010). The influence of the inoculum should be considered when planning future experiments in sheep and there is a need to be careful with extrapolation of $TCID_{50}$ values used in this experiment. Previous studies have shown that the origin of the virus and the way it has been passaged might strongly influence the outcome of an experimental infection, even if high inoculation doses are used (Wernike et al., 2013).

In all sheep that became positive by qRT-PCR for viral RNA in blood, SBV RNA could be detected from 2 to 7 dpi. The duration of detection of viral RNA in blood by qRT-PCR and the SBV copy number at the peak of detection were not significantly different between groups inoculated with undiluted or 1/10 diluted infectious bovine serum (two-sample t tests with unequal variances; $n = 6$; $P = 0.14$ and 0.26, respectively). The copy number at the peak of detection of viral RNA by qRT-PCR in blood in sheep inoculated with 1/100 diluted infectious serum reached a similar level. Comparable results were obtained when the presence of SBV RNA was determined in whole blood samples (data not shown).

All ewes were euthanased at 10 dpi. No gross lesions were observed at postmortem examination. Portions of cerebrum, cerebellum, brain stem, lung, spleen, left superficial cervical and mesenteric lymph nodes, tonsils and ovary were collected. Virus was detected in the spleen, and the superficial cervical and mesenteric lymph nodes, in all seven ewes, and in the lungs of two ewes, that were positive by qRT-PCR for viral RNA in blood (Table 1). There was no significant difference in the SBV RNA copy number in the superficial cervical and mesenteric lymph nodes, or spleens, between sheep inoculated with the undiluted and 1/10 diluted infectious serum (two-sample t test with unequal variances; $n = 6$; $P = 0.30$, 0.99 and 0.38, respectively). The copy numbers in the three different lymphoid organs of the sheep that were positive by qRT-PCR for viral RNA in blood following inoculation with 1/100 diluted infectious bovine serum reached similar levels.

These observations raise the question of the importance of the lymphatic system in the pathogenesis of SBV in sheep. Interestingly, similar observations were obtained after SBV infection of other sheep breeds (Wernike et al., 2013). However, as little is known about the pathogenicity of orthobunyaviruses of veterinary importance (Doceul et al., 2013), it remains difficult to interpret these data. Further studies quantifying SBV in these lymphatic tissues over time are needed to clarify this issue.

Table 1
Detection of Schmallenberg virus (SBV) S segment RNA in inoculated sheep at postmortem examination.

	Undiluted			1/10 dilution			1/100 dilution		
	Ewe 1	Ewe 2	Ewe 3	Ewe 1	Ewe 2	Ewe 3	Ewe 1	Ewe 2	Ewe 3
Cerebrum	Neg	Neg	Neg	NA	Neg	Neg	Neg	Neg	Neg
Cerebellum	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Brain stem	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Mesenteric lymph nodes	5.60×10^7	2.90×10^7	1.90×10^7	1.00×10^7	9.30×10^6	8.70×10^7	Neg	4.30×10^6	Neg
Superficial cervical lymph nodes	2.90×10^5	4.30×10^5	1.90×10^6	6.83×10^6	2.41×10^6	8.10×10^5	Neg	1.30×10^7	Neg
Spleen	7.40×10^6	5.10×10^7	1.10×10^7	1.00×10^7	4.80×10^6	8.50×10^6	Neg	8.10×10^5	Neg
Tonsil	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Ovary	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Lung	Neg	Neg	Neg	Neg	Neg	6.80×10^4	Neg	7.60×10^5	Neg

Neg, negative; NA, not available.
SBV S segment RNA (copies/g) detected at 10 days post-inoculation by qRT-PCR in different organs of ewes inoculated subcutaneously with different doses (undiluted or 1/10, 10/100 or 1/1000 dilution) of an SBV infectious serum. All samples from ewes inoculated with a 1/1000 dilution were negative.

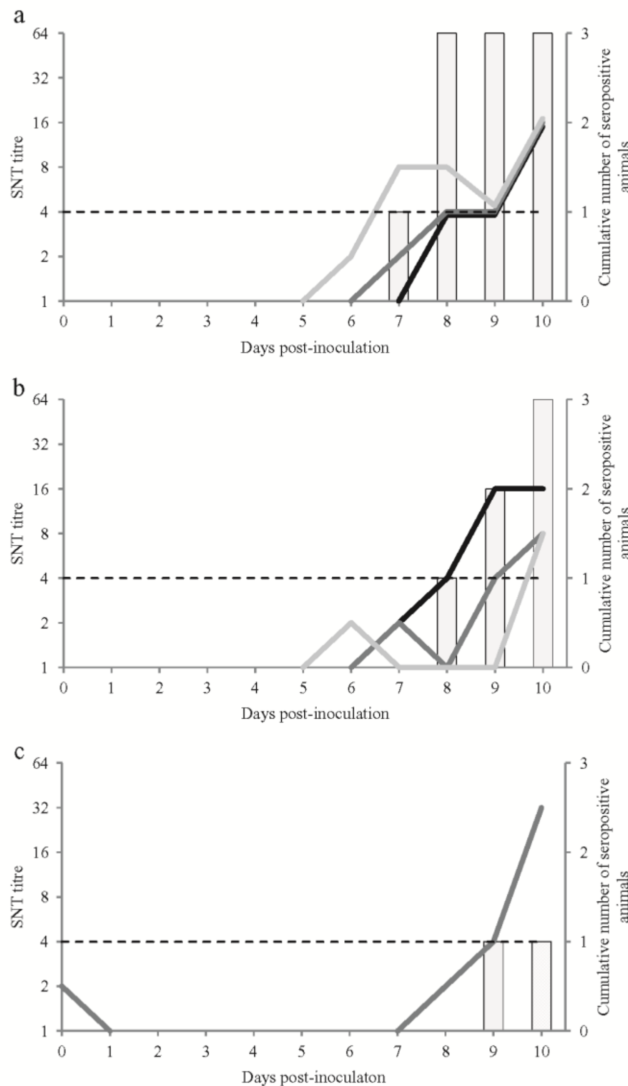

Fig. 2. Seroconversion in Schmallenberg virus (SBV) inoculated animals. Titres of neutralising anti-SBV antibodies measured in serum from four groups, each of three ewes (ewe 1 ▬, ewe 2 ▬, ewe 3 ▬), inoculated subcutaneously at day 0 with undiluted (a), or 1/10 (b) or 1/100 (c) dilutions, of an SBV infectious serum. None of the animals inoculated with a 1/1000 dilution seroconverted. The dashed line indicates the cut-off value of the serum neutralisation test (SNT). Sera were considered to be positive if the SNT titre was ≥4 (specificity 100%, De Regge et al., 2013). The columns (□) represent the cumulative number of ewes which had seroconverted at different days post-infection. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The presence of neutralising anti-SBV antibodies was assessed by SNT (De Regge et al., 2013). All ewes that were positive by qRT-PCR for viral RNA in blood seroconverted between 7 and 9 dpi (Fig. 2), while the other ewes were negative. The number of SBV antibody positive animals by group decreased significantly as a function of the inoculated dose (Fisher's exact test; $n = 12$; $P = 0.045$). Serum samples collected on the day of euthanasia were also tested by the ID Screen Schmallenberg virus Indirect Multi-Species ELISA (IDVet); all samples were negative. This discrepancy is probably because the SNT can detect immunoglobulin (Ig) M antibodies with neutralising capacity, while the ELISA only detects IgG due because it uses an anti-multi-species IgG-horseradish peroxidase conjugate.

The productively infected animal in the 1/100 dilution group was inoculated with a calculated dose of, at most, 20 $TCID_{50}$. It seems reasonable to assume that infectious doses of this magnitude can be delivered by SBV-infected *Culicoides* spp. during feeding. For BTV, another disease transmitted by *Culicoides* spp., a single midge can transmit 0.32–7.79 $TCID_{50}$ (Fu et al., 1999). Recent reports of Ct values of around 30 for the SBV S segment (obtained using the same qRT-PCR) in the saliva of SBV-infected *Culicoides sonorensis* (Veronesi et al., 2013) indicate that this could also be realistic for SBV.

In conclusion, this experiment provides evidence that a critical dose needs to be administered to induce a homogeneous productive infection in sheep. However, when a sufficient dose is administered, no dose dependent effect was observed, either in the duration and quantity of viral RNA detected by qRT-PCR in blood, or in the amount of viral RNA present in the lymphoid organs.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Appendix: Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tvjl.2014.05.031.

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Annex 2

Genetically stable infectious Schmallenberg virus persists in foetal envelopes of pregnant ewes

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Genetically stable infectious Schmallenberg virus persists in foetal envelopes of pregnant ewes

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Abstract

Schmallenberg virus (SBV) is a recently emerged vector-borne virus, inducing congenital defects in bovines, ovines and caprines. Here we have shown that infectious SBV is capable of persisting until the moment of birth in the foetal envelopes of ewes infected with SBV-infectious serum at day 45 (1/5 positive) and 60 (4/6 positive) of gestation. This persistence of at least 100 days is a new aspect of the SBV pathogenesis that could help to explain how SBV overwinters the cold season in temperate climate zones. Furthermore, sequencing of the M segment shows that the persisting virus in the foetal envelopes is genetically stable since only a few mutations compared to the inoculum were found. This supports the hypothesis that persisting virus could start the infection of new hosts. Finally, neutralization tests showed that infectious SBV present in the foetal envelopes at birth can be neutralized by the humoral immunity present in the infected ewes.

Schmallenberg virus (SBV) emerged in Europe in the summer of 2011 [1]. SBV belongs to the family *Bunyaviridae*, genus *Orthobunyavirus*, and its genome consists of three segments of single-stranded negative-sense RNA, which are named according to their size: small (S), medium (M) and large (L) [2].

It was first identified in adult cattle showing hyperthermia, drop of milk production and diarrhoea [1]. The virus is also responsible for congenital disease in calves, lambs and kid goats [3–5]. Malformation, abortion and stillbirth were the most frequently observed clinical symptoms, namely in lambs [6]. SBV is transmitted by small haematophagous insects called *Culicoides* [7].

SBV RNA has been detected in organs from experimentally infected adult sheep and cattle for a long time after inoculation [8–10], and SBV RNA was also identified in organs of calves and lambs that were aborted and stillborn under field conditions [11]. In a previously performed *in vivo* infection experiment (Ethical Committee approval number: 121017–01) at our laboratory, 11 ewes were inoculated with an SBV-infectious bovine serum (gift from Martin Beer of the Friedrich-Loeffler Institute containing 2×10^3 $TCID_{50}$ ml^{-1} SBV) at day 45 (five ewes) and 60 (six ewes) of gestation and kept till the moment of lambing [12]. The ewes gave birth to 17 lambs. At the moment of birth, a total of 31 organs and fluids originating from the ewes (four samples), lambs (six samples) and foetal envelopes (21 samples) were found to be positive for SBV RNA in quantitative reverse transcription PCR (qRT-PCR) [12]. Here, we tested all of these 31 SBV qRT-PCR positive samples (Table 1) in virus isolation to evaluate whether the detected SBV RNA originates from infectious virus or only represents remaining non-infectious viral RNA.

After homogenization of organ samples with the Ultra-Turrax and filtration through 0.8 µm Millipore filters, SBV isolation was attempted on Vero cells grown in six-well plates. The inoculum was incubated with the cells for 2 h, washed away, replaced by 3 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 1000 IU penicillin ml^{-1}, 50 µg ml^{-1} gentamicin, 250 µg ml^{-1} Fungizone amphotericin B and 10 % foetal calf serum and incubated for 4 days. Two or three passages were performed. During each passage, the cell monolayer was analysed for the presence of lysis plaques under the light microscope and SBV isolation was confirmed by qRT-PCR, which was described before [12]. Virus isolation from amniotic fluids and from serum collected at the peak of viraemia was carried out as described above with the exception that those samples were directly added to the Vero cells without homogenization or filtration.

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Keywords: Schmallenberg virus; sheep; persistent infection; virus isolation; sequencing; overwintering.
Abbreviations: CI, Confidence interval; p.i., post inoculation; qRT-PCR, quantitative reverse transcription PCR; SBV, Schmallenberg virus; VNT, virus neutralization test.
The GenBank/EMBL/DDBJ accession numbers for the M segments of Schmallenberg virus sequenced in this article are KX034187 to KX034203.
One supplementary table is available with the online Supplementary Material.

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Table 1. Study samples and result of virus isolation

In a previously described experimental infection study [12], 11 ewes were inoculated with SBV at day 45 or 60 of gestation. At the moment of birth, organs from all ewes and all 17 born lambs were collected, including different parts of the foetal envelopes. This table provides an overview of those samples from that experiment that are analysed in more detail in this study. It is the serum collected from the ewes at the peak of RNAaemia and all 31 tissues and fluids that tested positive for SBV in qRT-PCR at the moment of birth. Samples originate from ewes (E), lambs (L) or foetal envelopes (FE). Samples that were sequenced were given a code that is used throughout the manuscript. The result of the virus isolation (+, positive; −, negative) is indicated. Also the obtained isolates were given a code (starting with an 'I'), which is used throughout the manuscript.

Day of gestation at SBV inoculation	Ewe no.	Sample	Sample code	SBV isolation	Sample code of isolate
45	1	FE-Amniotic fluid		−	
	2	FE-Umbilical cord		−	
	2	FE-Placentome	2-Pl	−	
	2	FE-Amniotic fluid		−	
	2	L-Lung		−	
	2	FE-Umbilical cord	2-UC	+	I2-UC
	2	E-Serum (5 days p.i.)	2-Se_5	+	I2-Se_5
60	3	E-Ovary		−	
	3	FE-Intercotyledonary membranes		−	
	3	FE-Umbilical cord		−	
	3	FE-Placentome	3-Pl	+	I3-Pl
	3	E-Serum (4 days p.i.)	3-Se_4	+	I3-Se_4
	4	FE-Amniotic fluid		−	
	4	L-Spinal cord		−	
	4	L-Brainstem		−	
	5	E-Ovary		−	
	5	E-Spleen		−	
	5	L-Cartilage		−	
	5	L-Superficial cervical lymph node		−	
	5	L-Muscle		−	
	5	FE-Umbilical cord		−	
	5	FE-Placentome	5-Pl	−	
	5	FE-Amniotic fluid		−	
	5	E-Serum (5 days p.i.)	5-Se_5	+	I5-Se_5
	6	FE-Intercotyledonary membranes		−	
	7	FE-Amniotic fluid		−	
	8	FE-Placentome	8-Pl	−	
	8	FE-Intercotyledonary membranes	8-IM	+	I8-IM
	8	E-Serum (4 days p.i.)	8-Se_4	+	I8-Se_4
	9	FE-Intercotyledonary membranes		−	
	9	FE-Amniotic fluid		−	
	9	FE-Placentome	9-Pl	+	I9-Pl
	9	E-Serum (5 days p.i.)	9-Se_5	+	I9-Se_5
	10	E-Spleen		−	
	10	E-Serum (4 days p.i.)	10-Se_4	NT	
	11	FE-Umbilical cord		−	
	11	FE-Placentome	11-Pl	+	I11-Pl
	11	E-Serum (5 days p.i.)	11-Se_5	+	I11-Se_5

days p.i., days post inoculation; NT, not tested.

None of the four and six SBV RNA positive organs of ewes and lambs, respectively, was found to be positive in virus isolation (Table 1). This suggests that SBV RNA detected in maternal organs collected at 90 and 105 days post-inocualtion (p.i.) probably represents remaining non-infectious viral RNA. The incapability to isolate SBV from foetal tissues is in contrast with earlier successful isolations of SBV from brain material of newborn malformed lambs [13, 14] and can probably be explained by the small number of only low positive SBV RNA foetal tissues found in the current experiment.

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In contrast, five isolates were obtained from 21 samples belonging to the foetal envelopes (Table 1), showing the capacity of SBV to persist for at least 100 days in pregnant ewes. One isolate (I2-UC) was obtained from an umbilical cord (sample 2-UC) of a ewe inoculated at day 45 of gestation (sample collected at 105 days p.i.). In the group of ewes inoculated at day 60 of pregnancy (samples collected at 90 days p.i.), three SBV isolates (I3-Pl, I9-Pl, I11-Pl) were obtained from the placentome (samples 3-Pl, 9-Pl, 11Pl) of three ewes and one (I8-IM) from the intercotyledonary membranes (sample 8-IM) of another ewe (Table 1). Therefore, SBV isolation seems more successful from foetal envelopes of ewes inoculated with SBV at day 60 of pregnancy (4/6; 67 %) than on day 45 of pregnancy (1/5; 20 %), although this was not statistically significant (Fisher's exact test; P=0.24). Since this persistence in foetal envelopes occurred in the presence of a strong neutralizing humoral immune response [12], it further supports the role of the placenta as an immunosuppressed zone where different immune suppressive mechanisms prevent the foetus from being rejected by the maternal immune system. Several mechanisms have been described to contribute hereto: the placental barrier function, the absorption or blocking of noxious antibodies or the synthesis of non-specific systemic and local suppressor factor-like immunosuppressive proteins [15].

No infectious SBV could be recovered from the amniotic fluids. This seems to be in line with the observation that the mean SBV RNA copy number is a minimum of 1000-fold lower in amniotic fluids (3.06×10^5) than in umbilical cords (4.69×10^8), placentomes (4.83×10^9) and intercotyledonary membranes (1.44×10^9) (Kruskal–Wallis test; P=0.014). Although the incapability to isolate SBV from the amniotic fluid suggests that this externally shed fluid does not represent an important risk for virus transmission, we advocate that more research is performed regarding this subject before this is accepted as a general conclusion. Other studies have reported much higher viral RNA loads in amniotic fluids of field-aborted lambs than those found in our study [16].

SBV isolates (I2-Se5, I3-Se_4, I8-Se_4, I9-Se_5, I11-Se_5) were also obtained from the sera collected at the peak of RNAaemia (4 or 5 days p.i.) from the five ewes from which SBV was successfully isolated from the foetal envelopes (Table 1) and used in experiments described further on in this manuscript.

Previous studies have shown that the S and L segment of SBV strains found at different locations and moments in time are highly genetically stable [2, 14, 17–21]. More genetic variability has however been found in the M segment of the virus. This segment encodes a polyprotein precursor that is later cleaved into the non-structural (NSm) protein and the two envelope glycoproteins named Gn and Gc [22]. Several authors even identified a hypervariable region in the coding sequence of the putative Gc protein based on sequencing studies performed on organ and brain samples collected from aborted lambs and calves [2, 14, 17]. It was hypothesized that this hypervariable region might be important for the evasion of the host immunity [2].

Therefore, we next analysed whether the persisting virus in the foetal envelopes had mutated between the moment of inoculation and the moment of birth. We sequenced the M segment of (1) the inoculum, (2) viral RNA present in serum at the peak of RNaemia, (3) viral RNA present in placentomes at birth and (4) the virus isolates obtained from placentomes. Sequencing was performed using methods described in Fischer *et al.* [2] and using primers described in Coupeau *et al.* [17]. Sequences were submitted to GenBank and received accession numbers KX034187 to KX034203. The positions of the nucleotide (nt) and the predicted amino acid (aa) sequences were aligned with the complete M segment sequence published by Hoffmann and colleagues [1] (GenBank accession number HE649913.1).

Overall, SBV RNA present in serum collected at the peak of RNAaemia and in placentomes at the moment of birth showed a high sequence stability of the M segment. A mean homology of 99.92 % [95 % confidence interval (CI) =99.89 –99.95 %] with the inoculum was observed (Fig. 1 and Table S1, available in the online Supplementary Material). One to five nucleotide substitutions compared to the inoculum were found in SBV RNA present in the serum samples collected at the peak of RNAaemia from seven ewes. In three out of seven samples (3-Se_4, 10-Se_4, 11-Se_5) the corresponding aa sequence was completely homologous to the inoculum, while in three (2-Se_5, 5-Se_5, 9-Se_5) and one (8-Se_4) other serum samples, respectively, these nucleotide substitutions resulted in one and four aa substitutions. After persistence in the placentomes for between 90 and 105 days, between two and nine nucleotide substitutions compared to the inoculum were found in the M segment of SBV RNA present in the six placentome samples. In four out of six placentome samples (3-Pl, 8-Pl, 9-Pl, 11-Pl), these nucleotide substitutions did not result in any predicted aa sequence change compared to the inoculum.

Taken together, these sequencing results show that the persisting virus in foetal envelopes is highly stable since only a few mutations compared to the initial inoculum were found. Therefore, the previously reported high number of mutations found in the hypervariable region of the M segment of SBV isolates obtained from the brain tissue of malformed lambs [2, 17] are probably not acquired during the virus passage in the ewe or foetal envelopes. Our results suggest that it is more probable that those mutations might either be requisite to allow crossing of transplacental barriers and/or accumulate during the successive replication cycles in different foetal tissues before entering the brain of developing lambs. They might also be acquired during replication within the brain tissue of the lamb to evade the local immune pressure in that compartment. The genetic stability of the persisting virus is also in line with the results of Wernike *et al.* [20] who described a high genetic homology in all segments, including the M segment, between SBV strains present in field-collected blood samples after its first

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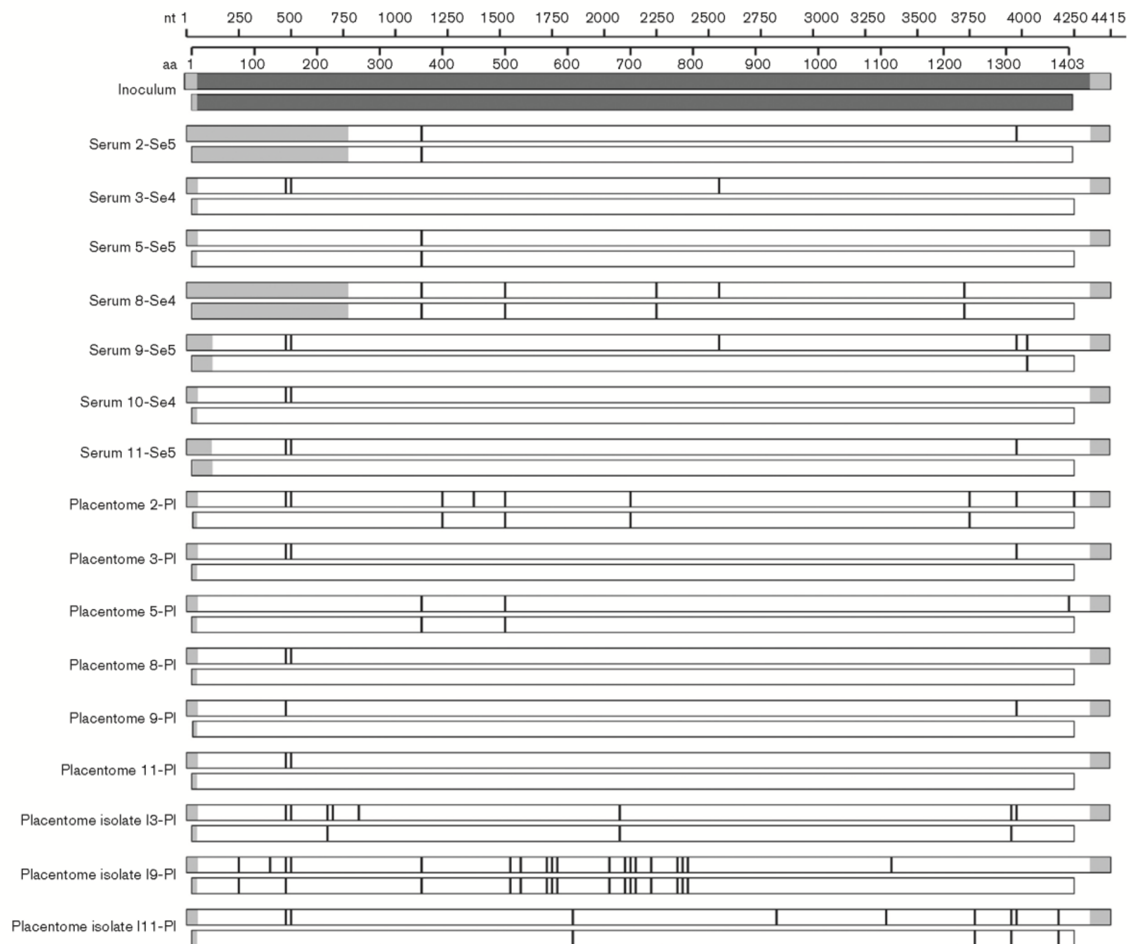

Fig. 1. RNA and aa alignment of the SBV M segment. The RNA (upper bar associated with each sample) and corresponding aa (lower bar associated with each sample) sequences of the M segment of SBV present in the initial inoculum, in the serum at the peak of viremia, in placentomes at the moment of birth and in SBV isolates from placentomes were aligned and mutations compared to the sequence of the initial inoculum are indicated by vertical black lines. Nucleotide (nt) and aa positions are predicted based on the complete M segment sequence HE649913.1 [1]. No sequences were obtained for areas indicated by a light grey colour.

emergence in 2011 and its renewed circulation in 2014 [20]. This is probably caused by the fact that SBV needs to replicate in both insect and mammalian hosts, meaning that only mutations that do not impact its fitness in both hosts might be maintained. This phenomenon was described before for West Nile and dengue viruses, which exhibit a lower than predicted mutation rate [23–26].

Interestingly, all three isolates that were obtained from placentomes (I3-Pl, I9-Pl, I11-PL) originated from samples (3-Pl, 9-Pl, 11-Pl) containing SBV that had an aa sequence of the M segment that was 100 % homologous to the inoculum. Passaging the virus four times in Vero cells during the isolation process had, however, a variable influence on the sequence of the M segment. Two isolates acquired eight (I3-Pl) and nine (I11-Pl) nucleotide substitutions, respectively, leading to three and four aa substitutions. The third isolate (I9-Pl) was more severely affected and acquired 19 nucleotide substitutions, leading to 16 aa changes in the predicted protein sequence. In total, 14 of the 16 aa changes were located in the previously defined hypervariable region of the M segment. This finding agrees with a previous report showing that repeated passaging of SBV in BHK-21 cells leads to an accumulation of mutations in the hypervariable region of the M segment [17]. Hofmann *et al.* [19] reported that this might be overcome by performing SBV isolations in porcine SK-6 cells.

In a final experiment, it was evaluated whether the induced humoral immunity upon SBV infection was capable of neutralizing the infectious SBV that was recovered from the placenta at the moment of birth (being at 90 or 105 days after infection) in order to assess whether this persisting virus would be capable of escaping the neutralizing immune

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response upon renewed release in the circulation. In this respect, the capacity of serum collected at 35 days p.i. (samples 3-Se_{35}, 8-Se_{35}, 9-Se_{35}, 11-Se_{35}) and 90 days p.i. (samples 3-Se_{90}, 8-Se_{90}, 9-Se_{90}, 11-Se_{90}) to neutralize SBV isolated from the placentome (samples I3-Pl, I9-Pl and I11-Pl), intercotyledonary membranes (I8-IM) and serum at the peak of RNAaemia (I3-Se_4, I8-Se_4, I9-Se_5, I11-Se_5) from the corresponding animal was determined using a virus neutralization test (VNT). The VNT titres for the different combinations of serum (35 or 90 days p.i.) and SBV isolates (serum or organ) were determined in triplicate, as previously described [11].

The VNT titres measured in serum collected at 35 and 90 days p.i. against the SBV isolates obtained from serum at the peak of RNAaemia and from the foetal envelopes of four ewes were always positive ($\geq$4), suggesting that the infectious virus present in the foetal envelopes at birth would be neutralized when released in the bloodstream. The mean $\log_2$ titre for the VNT conducted with the serum isolates was 3.5 at 35 days p.i. and 4.7 at 90 days p.i. The mean $\log_2$ titre for the VNT conducted with the placentome isolates was 3.5 at 35 days p.i. and 4.8 at 90 days p.i. (Fig. 2). The use of a linear mixed model to estimate the mean effect of time on the SBV-specific antibody response showed that the VNT titres measured in serum collected at 90 days p.i. were significantly higher than those found in serum collected at 35 days p.i., and this against both serum (P=0.007)

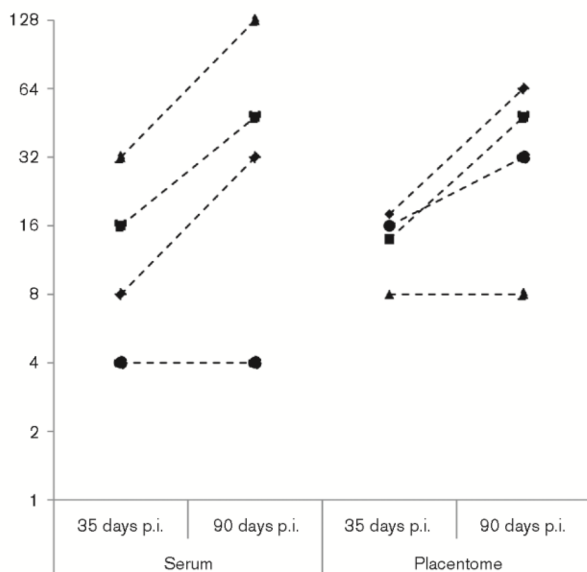

Fig. 2. Neutralizing capacity of serum collected at different time points after SBV inoculation of pregnant sheep. The neutralizing antibody titre of serum collected from four ewes at 35 and 90 days p.i. was determined against SBV isolates originating from those particular ewes. For each ewe, one isolate originated from the serum collected at the peak of viraemia and the other from the placentome collected at birth.

and placentome (P=0.004) isolates. This probably reflects the well-known affinity maturation of the humoral immune response [27].

The most challenging question related to these results is whether the persisting virus in the placentomes could play a role in the transmission and overwintering of SBV. This will have to be evaluated in future studies, but several hypotheses can be formulated on how this persistent virus might perpetuate infection. A first hypothesis could be that infected placentas serve directly or indirectly as a food source for overwintering *Culicoides* larvae after being discarded on dung heaps, a preferred *Culicoides* larval habitat [28–30]. Another hypothesis could be that unprotected seronegative ewes could become infected after contact with infected placental material. Previous studies have, however, shown that oral inoculation of sheep and cattle with SBV-containing fluids was unsuccessful in transmitting infection under experimental conditions [9, 31], making this route of transmission rather unlikely. Disease transmission after uptake of infected placental or foetal material has already been reported for bluetongue virus, another *Culiciodes*-borne virus [32, 33], but more studies will be necessary to evaluate this for SBV. Other options for overwintering that cannot be excluded at this point are the potential role for immature or adult vectors, other than *Culicoides*, to transmit SBV after feeding on infected foetal envelopes, or the existence of viraemic newborn lambs that could serve as a food source for adult *Culicoides*.

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Conflicts of interest

The authors declare that there are no conflicts of interest. Y. V. d. S. is currently employed with the European Food Safety Authority (EFSA) in the BIOCONTAM Unit that provides scientific and administrative support to EFSA's scientific activities in the area of Microbial Risk Assessment. The positions and opinions present in this article are those of the authors alone and are not intended to represent the views or scientific works of EFSA.

Ethical statement

This study uses material collected during an experimental animal infection study that was previously published (Martinelle et al. [12]). That study was approved by the Joined Ethical Committee of CODA-CERVA and the Institute of Public Health Belgium (Ethical Committee approval number: 121017–01).

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Annex 3

Preliminary survey on the impact of Schmallenberg virus on sheep flock in South of Belgium

Transboundary and Emerging Diseases 61 (2014) 469-472

Saegerman C, **Martinelle L**, Dal Pozzo F, Kirschvink N

Transboundary and Emerging Diseases

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SHORT COMMUNICATION

Preliminary Survey on the Impact of Schmallenberg Virus on Sheep Flocks in South of Belgium

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Keywords:
Schmallenberg virus; sheep; epidemiology; survey; livestock impacts; economic impacts

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Summary

Between late February and May 2012, a preliminary anonym survey was conducted among sheep farmers in south of Belgium in order to contribute to future estimations of the economic losses caused by Schmallenberg virus (SBV). Based on clinical signs consistent with SBV infection, this survey involved 13 meat sheep flocks considered as positive flocks with subsequent SBV detection by RT-qPCR [SBV-positive flocks (PF); total of 961 animals], and 13 meat sheep flocks considered as negative flocks (NF; total of 331 animals). These preliminary results indicated several significant characteristics that were more present in PF than in NF. These include an increased rate of abortions (6.7% in PF versus 3.2% in NF), of lambs born at term but presenting malformations (10.1% in PF versus 2.0% in NF) and of dystocia (10.1% in PF versus 3.4% in NF). Lamb mortality during the first week of life was reported more frequently in PF (8 of 13 PF, 61.5%) than in NF (1 of 13 NF, 7.7%). In PF, the observed prolificacy rate was 2-fold lower (93%) than expected (186%). The implementation of a survey at larger scale, including a high number of breeders, is necessary to allow a more detailed analysis of the SBV impact in the sheep sector.

Introduction

A new virus of the family *Bunyaviridae*, genus *Orthobunyavirus* has recently emerged in Europe. It has been provisionally named Schmallenberg virus (SBV), following the location of its first identification in Germany (Hoffmann et al., 2012). Schmallenberg virus was initially diagnosed by RTqPCR, while serological tests have been developed more recently. The SBV is not a reportable disease to the World Organization for Animal Health [*Office International des Epizooties* – (OIE)]. Under-reporting and under-detection are prejudicial to an accurate estimation of the impact of the disease caused by SBV on livestock industry (Martinelle et al., 2012). Therefore, gathering farmers' estimations in matters of apparent reproductive and clinical consequences of SBV infection could help to more accurately delineate the effects of the disease on sheep flocks.

Materials and Methods

In 2010, a total of 1223 sheep flocks (with professional incomes) were registered in Walloon Region (South of Belgium), including 48 000 animals (DGARNE, 2012).

Five hundred Walloon breeders are identified as members of the inter-professional federation of goats and sheep in the south of Belgium [*Fédération Interprofessionnelle Caprine et Ovine Wallonne* (FICOW)]. Among these, 367 members hold meat sheep flocks.

A solicitation to participate to an anonymous survey was sent by the Journal '*Filière ovine et caprine*' to all members of the FICOW (Vandiest, 2012). The purpose of this survey was to gather first field clinical observations, including any disorders encountered during the lambing period.

This survey took place in south of Belgium between late February and May 2012. The time period of the reported

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lesions corresponds to the period from May 2011 until February 2012. A SBV-positive flock (PF) was defined as a flock for which at least one suspected animal with clinical signs consistent with SBV infection was submitted to the laboratory with subsequent positive RT-qPCR result (USDA, 2012). A SBV-negative flock (NF) was defined as a flock for which no clinical signs consistent with SBV infection were observed. Attempts of SBV detection were performed using the brain stem and cerebellum of the foetuses (Cay et al., 2012). Recommendations issued in the note accompanying the survey explicitly specified that all flocks could participate, regardless of their SBV status (PF or NF).

The comparison of prolificacy rates and the comparison of the number of breeding females in PF and NF were realized using a paired non-parametric Wilcoxon signed-rank test and a non-parametric Mann–Whitney *U*-test, respectively (the hypothesis of normality of the distributions could not be verified). The frequency of clinical signs has been assessed with Pearson's chi-squared test or a Fisher's exact test depending of conditions of use (Petrie and Watson, 2006).

Results and Discussion

Responding farmers were divided in two groups, depending on the detection of SBV (confirmed by RT-qPCR) in their herds: 13 SBV-positive meat sheep flocks (PF; total of 961 animals) and 13 SBV-negative meat sheep flocks (NF; total of 331 animals). In total, it represents a sample of 5% (i.e. 26/500) of all members of the FICOW or 7% (i.e. 26/367) of meat sheep breeders that are members of the FICOW. This rate represents the lower limit of what is expected for this type of investigation (Dufour, 1994). The farmers who participated in the survey are from all provinces of the Walloon Region (south of Belgium; Fig. 1). In addition, farmers who responded reported variable levels of losses in their flocks (with first report at January, 2012). In this condition, the presence of bias (i.e. over representation of severely affected flocks) had probably a limited impact.

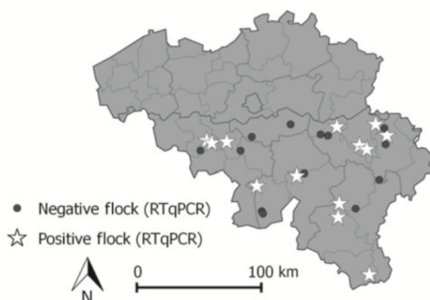

Fig. 1. Localization of the flocks originating from the south of Belgium included in the survey in relation to their Schmallenberg virus (SBV) status.

The numbers and characteristics of sheep considered in this survey are listed in Table 1. Different sheep breeds used for meat production were represented with a predominance of Texel.

The observed and the expected prolificacy rates for each flock were estimated considering the breed and aggregated by group (PF and NF; Babo, 2000; Laignel and Benoit, 2005). No difference occurred in NF between the observed and the expected values (Wilcoxon signed-rank test; $P = 0.12$) but for PF, the aggregated observed prolificacy rate was significantly lower (93%) than the aggregated expected prolificacy rate (186%; Wilcoxon signed-rank test; $P = 0.01$). This represents a 2-fold reduction in the expected prolificacy. No significant difference was observed between PF and NF in term of the starting date and duration of the first lambing period. However, it appears that the number of breeding females was significantly higher in PF (average of 41, median of 23, minimum of 5 and maximum of 154) compared with NF (average of 11, median of 8, minimum of 2 and maximum of 26; Mann–Whitney *U*-test; $P = 0.01$; Fig. 2). This finding should be in accordance with the hypothesis of a wide exposition of flocks to the SBV and a higher probability to detect SBV in flock with an increased number of breeding females.

Clinical signs encountered in the two groups were compared. It appeared that the observation frequency of stiff joints was significantly higher in PF (11/13) compared with NF (2/13) (Fisher's exact test, $P = 0.045$). The abortion rate (χ^2 test, $P = 0.04$) and the number of stillborn or lambs dying right after being born (χ^2 test, $P < 0.001$) were significantly higher in PF compared with NF (Table 2). Reported Schmallenberg virus-associated lesions were similar to those attributed to SBV in previous reports (Herder et al., 2012; van Maanen et al., 2012).

The flock dystocia rate was significantly higher (mean 18.5%, median 13%, minimum 0% and maximum 66.7%) in PF compared with NF (mean 6.4%, median 0%, minimum 0% and maximum 83% in only one flock of small size; Fisher's exact test, $P < 0.001$). In addition, lamb

Table 1. Characteristics of sheep being monitored by the participating sheep farmers

	Number of animals present in sheep flocks with		
Sheep category	PF	NF	Total
Meat sheep < 1 year	422	153	575
Meat sheep over 1 year	496	167	663
Breeding rams	43	11	54
Total	961	331	1292

PF, SBV-positive flocks; NF, SBV-negative flocks. Status of the flocks was assigned based on RT-qPCR results.

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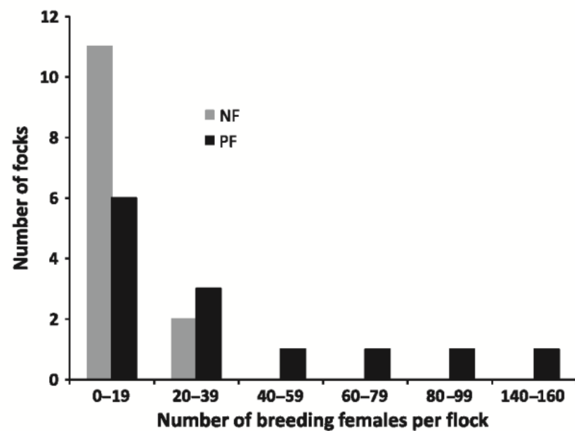

Fig. 2. Number of breeding females in function of Schmallenberg virus (SBV) flock status PF, SBV-positive flocks; NF, SBV-negative flocks. Status of the flocks was assessed based on RTqPCR results.

Table 2. Comparison of reproductive and clinical parameters

Variable	PF (N = 13)	NF (N = 13)
Number of pregnant primiparous ewes	22	26
Number pregnant multiparous ewes	505	119
Number of abortions	35 (6.7%)	8 (3.2%)
Number of clinically healthy	366 (70.0%)	216 (85.4%)
Number of stillborn or died at birth lambs	69 (13.2%)	24 (9.5%)
Number of lambs born at term but malformed	53 (10.1%)	5 (2.0%)

PF, SBV-positive flocks; NF, SBV-negative flocks. Status of the flocks was assigned based on RT-qPCR results.

mortality during the first week of life was reported more frequently by farmers in PF (8 of 13 PF, 61.5%) than in NF (1 of 13 NF, 7.7%; Fisher's exact test, P = 0.01).

A symptomatic treatment (antibiotics and/or anti-inflammatory) was administered occasionally after dystocia in 10 of 13 PF. The average duration of treatment was 3.5 days (minimum 2 and maximum 6 days). The mean percentage of treated animals per flock was 18.5% (minimum 0% and maximum 67%). The cost of treatment per animal averaged € 50.4 (median 50, minimum 8 and maximum € 124.5).

The number of lambs born at term but deformed was significantly different between PF and NF (χ^2 = 16.4; P < 0.001) and reached 10% in PF compared with 2% in NF. This percentage is not significantly different from that observed in France and obtained with a greater number of PF, that is, 11.7% (χ^2 = 1.33, P = 0.25; Dominguez et al., 2012).

Schmallenberg virus affection does not figure among reportable diseases list (Royal Order, 20.11.2009); therefore, it is hard to achieve a representative view of the real situation because of the risk of underreporting (Martinelle et al., 2012). Moreover, the detection by RTqPCR is also limited by the short length of the viraemia, ranging from 2 to 5 days in experimentally infected adult cattle (Hoffmann et al., 2012). In addition, organ distribution of SBV-RNA in malformed newborns, especially in lambs, is an important component to take into account to allow an increase of the sensitivity of the diagnostic strategy as demonstrated by Bilk and collaborators (2012). Furthermore, in a recent study, Hahn et al. (2013) found that only 12% of RT-qPCR positive calves were also positive by *in situ* hybridization, most likely because of a lower sensitivity of the latter technique, unsuitable to detect SBV mRNA in tissues with low SBV mRNA copy number and/or reduced viral load. In addition, in another study, van Maanen et al. (2012) reported that only 42% ELISA positive foetuses were also positive by RT-qPCR. This percentage was even lower for animals without malformations and provides support to the superiority of ELISA as a reliable and sensitive diagnostic test. Therefore, it is likely that some negative flocks may have been misestimated. Consequently, the zootechnical impact of SBV infection might be slightly different than these preliminary results suggest.

In contrast to bluetongue disease, the emerging disease caused by SBV was characterized by a very large and fast geographic spreading (Beer et al., 2012). In sheep, in acute phase of the disease, no particular alteration was observed in adults. This fact participates to the silent spreading of the disease. This is supported by a recent EFSA report, which highlights the underreporting of SBV cases (European Food Safety Authority, 2012). Retrospective epidemiologic studies would bring to light useful data to clarify more accurately spatio-temporal circumstances of SBV emergence in Belgium.

This preliminary survey allowed a better characterization of SBV-related economic losses in meat sheep flocks and will allow refining questionnaires for a larger scale use.

Conclusion

Measuring the extent of the episode of SBV on livestock and zootechnical performances requires further research efforts. As these results are preliminary and exploratory, an implementation of the survey on a larger scale, including a larger number of farmers, is needed to allow a more detailed analysis of the impact of SBV in the sheep sector.

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Annex 4

Le virus Schmallenberg ou l'émergence du premier *Orthobunyavirus* du sérogroupe *Simbu* en Europe

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Martinelle L, Dal Pozzo F, Kirschvink N, De la Grandiere M A, Thiry E, Saegerman C

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Le virus *Schmallenberg* ou l'émergence du premier *Orthobunyavirus* du sérogroupe *Simbu* en Europe

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RÉSUMÉ : Le virus *Schmallenberg* (SBV) a été identifié en Allemagne en novembre 2011. Il s'agit d'un virus de la famille des *Bunyaviridae*, genre *Orthobunyavirus*, appartenant au sérogroupe *Simbu*. L'analyse métagénomique d'échantillons prélevés sur des bovins adultes a permis de rapprocher le SBV des virus *Akabane, Aino* et *Shamonda*. La maladie se manifeste chez le bovin adulte par une chute de la production laitière, de la fièvre, une diarrhée pouvant être sévère et parfois des avortements. Une atteinte congénitale de type arthrogrypose/hydranencéphalie est décrite chez des agneaux, des chevreaux et des veaux. La maladie causée par le SBV est considérée comme non contagieuse, à transmission vectorielle, vraisemblablement par des moucherons du genre *Culicoides*. L'atteinte clinique est décrite aux Pays-Bas et en Allemagne depuis l'été 2011 chez les bovins adultes, et c'est depuis décembre que des cas d'atteinte congénitale avec détection du SBV ont été rapportés d'abord en Allemagne, aux Pays-Bas, et en Belgique, puis au Royaume-Uni et en France et enfin, plus récemment, en Italie, au Grand-Duché de Luxembourg et en Espagne. Le SBV a été jusqu'à présent essentiellement diagnostiqué par réaction en chaîne par polymérase en temps réel. Des tests sérologiques ont été développés récemment. Le risque zoonotique est considéré comme très faible. L'émergence du SBV constitue un évènement majeur en santé animale et un nouveau défi pour les vétérinaires et chercheurs européens.

INTRODUCTION

Le virus *Schmallenberg* (SBV) a été découvert en novembre 2011 par le *Friedrich Loeffler Institute* (FLI, Ile de Riems, Allemagne) suite à l'analyse métagénomique d'un pool d'échantillons sanguins en provenance d'une ferme de la ville de Schmallenberg (Rhénanie du Nord-Westphalie, Allemagne). Ces analyses ont été menées à la suite de la constatation, par les éleveurs et vétérinaires de la région, d'une fréquence anormalement élevée de baisse de production laitière associée à de l'hyperthermie, de la diarrhée pouvant être sévère et parfois des avortements chez les bovins, et ce, depuis le mois d'août 2011. Les pourcentages d'homologie nucléotidique présentés par les séquences génétiques identifiées ont permis de classer ce nouveau virus dans la famille des *Bunyaviridae*, genre *Orthobunyavirus*, sérogroupe *Simbu*. Les virus appartenant à ce groupe sont non contagieux, transmis par des arthropodes hématophages, notamment des moustiques et des moucherons du genre *Culicoides*. Entre le mois de novembre 2011 et la mi-mars 2012, le virus a été mis en évidence chez des ovins, des caprins et des bovins en Allemagne, aux Pays-Bas, en Belgique, au Royaume-Uni et en France, chez une chèvre en Italie,chez des agneaux et des veaux au Grand-Duché de Luxembourg et chez un agneau en Espagne, constituant ainsi la première occurrence de circulation autochtone d'un *Orthobunyavirus* du sérogroupe *Simbu* en Europe occidentale. Cependant, d'autres *Orthobunyavirus* ont été identifiés en Europe, soit sporadiquement par l'analyse de pools de moustiques (cas du virus *Batai* en Allemagne) (Jöst *et al.*, 2011), soit dû à une présence endémique (cas du virus *Tahyna*) (Bennett *et al.*, 2011). La maladie associée à l'infection par le SBV se manifeste chez le bovin adulte par une baisse de la production laitière, de la fièvre, une diarrhée pouvant être sévère et parfois des avortements. Une atteinte congé-

nitale est également décrite chez des agneaux, des veaux et des chevreaux, caractérisée par des malformations de type arthrogrypose/hydranencéphalie.

Dans cette revue, la phylogénie, la pathogénie et les mesures applicables au contrôle des *Orthobunyavirus* du groupe *Simbu* sont présentées en y incluant les données disponibles à l'heure actuelle sur le SBV, arrêtées au 24 avril 2012. Les données épidémiologiques relatives à la circulation du virus en Europe sont également résumées. Le risque zoonotique présenté par le SBV est enfin discuté.

TAXONOMIE ET PHYLOGÉNIE

Les virus de la famille des *Bunyaviridae* sont des virus enveloppés, à ARN monocaténaire de polarité négative, de forme sphérique et mesurant environ 100 nm de diamètre (Elliott, 2009). A l'heure actuelle, cette famille regroupe 95 espèces réparties en 5 genres, sur base de leurs propriétés sérologiques et biochimiques : *Orthobunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus* et *Tospovirus* (*International Committee on Taxonomy of Viruses*, 2009). Les 4 premiers genres comprennent des virus qui infectent des hôtes vertébrés alors que le dernier infecte des plantes (Elliott, 1997). Leur génome est constitué de trois segments : S (*Small*), M (*medium*) et L (*Large*), ces appellations reflétant la longueur respective de ces segments en terme de nombre de nucléotides (Walter et Barr, 2011). Le segment S de tous les *Bunyaviridae* encode la protéine de nucléocapside N. Pour les *Orthobunyavirus* (ainsi que pour les *Phlebovirus* et *Tospovirus*), ce segment encode également une protéine NSs, non structurale, qui joue un rôle dans la médiation de la réponse antivirale des cellules infectées. Le segment M encode un précurseur protéique membranaire qui sera clivé par des protéases cellulaires pour former les deux glycoprotéines virales Gn et Gc, qui jouent un rôle essentiel dans la maturation des nouvelles particules virales et l'attachement aux cellules sensibles. Ces deux glycoprotéines étaient aussi dénommées respectivement G1 et G2 (Saeed *et al.*, 2001a).

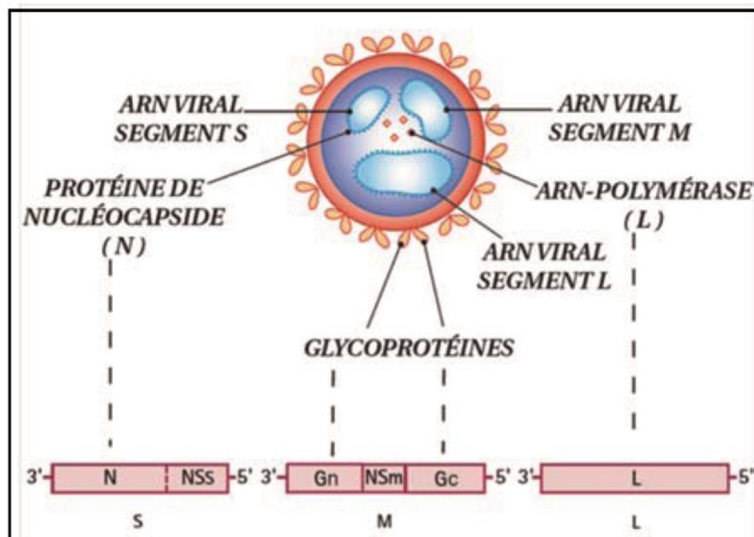

Figure I : Les virus de la famille des *Bunyaviridae* sont enveloppés et généralement sphériques, et les hétérodimères Gn-Gc sont extériorisés selon une matrice propre au genre du virus. Leur génome est constitué de trois segments d'ARN (S, M et L) adoptant une conformation circulaire en association avec les protéines virales de nucléocapside N. Le segment S code la protéine de nucléocapside N et, pour la plupart des membres des genres *Orthobunyavirus, Tospovirus* et *Phlebovirus*, également la protéine NSs, qui intervient dans la modulation de la réponse antivirale des cellules infectées. Le segment M encode un précurseur polyprotéique membranaire, qui sera clivé en glycoprotéines virales Gn et Gc ainsi que, chez les *Orthobunyavirus*, *Tospovirus* et *Phlebovirus*, en une protéine NSm, impliquée dans la morphogénèse virale. Pour tous les *Bunyaviridae*, le segment L code une unique protéine complexe, constituant l'ARN polymérase virale dépendant de l'ARN (d'après Thiry, 2007). .

Ce segment code encore une protéine NSm, issue du même précurseur protéique que Gn et Gc, qui semble également jouer un rôle dans la morphogénèse virale. Une seule protéine est encodée par le segment L, une grande protéine complexe qui constitue l'ARN polymérase virale dépendant de l'ARN (figure 1). À l'origine, sur base de données sérologiques, les *Orthobunyavirus* ont été séparés en 18 sérogroupes, parmi lesquels se trouve le sérogroupe *Simbu* (d'après le nom du virus-type du groupe, le virus *Simbu*). Ce groupe comprend 27 virus (sans tenir compte du SBV), qui ont été isolés jusqu'à présent sur tous les continents à l'exception de l'Europe. Les membres du sérogroupe *Simbu* présentent des réactions croisées au test de fixation du complément mais se distinguent par séroneutralisation (Kinney et Calisher, 1981) et par l'analyse des séquences génétiques. Deux membres de ce groupe sont d'une importance médicale particulière, les virus *Akabane* et *Oropouche*, respectivement en médecine vétérinaire et humaine (tableau I).

Les données issues du séquençage des trois segments génomique du SBV ont permis d'établir une homologie nucléotidique de 97 % avec le virus *Shamonda*, 71 % avec le virus *Aino*, et 69 % avec le virus *Akabane* respectivement pour les segments S, M et L (Hoffmann *et al.*, 2012). Cette plus grande proximité phylogénique avec le virus *Shamonda* a conduit les chercheurs du FLI de parler de virus *Shamonda-like* pour caractériser le SBV. À l'heure actuelle, seules les séquences nucléotidiques du segment S des virus *Aino*, *Akabane*, *Tinaroo* et *Oropouche* ont été complètement publiées, aussi toute caractérisation plus précise du SBV ne pourra se faire pour l'instant que sur base de ce segment.

Le virus Akabane

Le virus *Akabane* est un des *Orthobunyavirus* les plus étudiés, en raison de son impact économique parfois considérable, à cause non seulement des pertes liées à la naissance de jeunes non viables mais également suite à la diminution de production laitière faisant suite à l'épisode clinique, cette perte pouvant s'élever chez la vache laitière à plus de 25 % sur une lactation (Horikita *et al.*, 2005). Il aura fallu plus de 15 ans pour parvenir à associer ce virus, initialement isolé

en 1959 dans le village japonais éponyme à partir de moustiques *(Aedes vexans, Culex tritaeniorhynchus)*, avec le syndrome d'arthrogrypose-hydranencéphalie qui frappait régulièrement le Japon (Kurogi *et al.*, 1975). Des anticorps spécifiques ont été découverts chez le bovin, le cheval, l'âne, le mouton, la chèvre, le dromadaire, le buffle (*Bubalus bubalis*) et le porc. La maladie a été décrite chez les bovins, ovins et caprins (Huang *et al.*, 2003).

La distribution géographique du virus *Akabane* s'étend sur une bande allant des latitudes 35° Nord à 35° Sud. La côte égéenne turque (Yonguc *et al.*, 1982) jusqu'au Japon et l'Australie, en passant par la Malaisie et le sous-continent Indien sont concernés. Plusieurs études sérologiques prouvent également la circulation du virus en Afrique (Kenya et Afrique du Sud), bien qu'aucune malformation congénitale n'ait été rapportée (Metselaar et Robin, 1976 ; Theodoridis *et al.*, 1979). Les malformations congénitales caractéristiques de type arthrogrypose, hydranencéphalie et microcéphalie ont été décrites en Australie, au Japon, à Taïwan, en Israël, en Corée et en Turquie (Kobayashi *et al.*, 2007).

Au sens large, les zones d'endémicité du virus *Akabane* et du virus de la fièvre catarrhale ovine (BTV, *Bluetongue virus*) sont globalement comparables, même si les espèces de culicoïdes vectrices peuvent être localement différentes (Taylor et Mellor, 1994). Au sein de cette zone, les femelles des espèces de ruminants sensibles sont généralement atteintes avant leur première gestation, et ainsi les nouveau-nés sont habituellement indemnes de malformations (Taylor et Mellor, 1994). Les atteintes cliniques sont surtout rapportées aux marges de cette zone, ou à l'occasion de l'importation de bétail naïf en zone endémique (Jagoe *et al.*, 1993). Des souches d'origine géographique distinctes, indiscernables sérologiquement, peuvent néanmoins présenter un pouvoir pathogène différent (McPhee *et al.*, 1984 ; Parsonson *et al.*, 1988).

<u>*Le virus Aino*</u>

Ce virus a été découvert en 1964 au Japon (Takahashi *et al.*, 1968) et a été impliqué dans des cas de malformations congénitales, d'avortements et de mortinatalités chez le bovin (Yoshida *et al.*, 2000). Les lésions ont été reproduites au cours d'infections expérimentales (Tsuda *et al.*, 2004). Ce virus est largement répandu dans l'est et le sud-est de l'Asie ainsi qu'en Australie (Yanase *et al.*, 2010). L'impact économique moindre du virus *Aino* explique une littérature nettement moins abondante que pour le virus *Akabane*. De plus, ce virus est réputé franchir la barrière placentaire moins fréquemment que le virus *Akabane* (Tsuda *et al.*, 2004). Lors d'une infection par un *Orthobunyavirus*, les anticorps neutralisants sont dirigés contre certains épitopes de la glycoprotéine Gc. Cette dernière, encodée par le segment M, est ainsi spécifique de chaque espèce virale. C'est par ailleurs la protéine la plus variable chez les *Orthobunyavirus* (Briese *et al.*, 2006). Par conséquent, le pourcentage d'homologie nucléotidique modéré relevé

Tableau I : les 28 virus du sérogroupe *Simbu*, avec classification selon la lignée et l'embranchement, abréviation, année de premier isolement, et distribution géographique des isolements viraux positifs sur arthropodes vecteurs et hôtes vertébrés après infection naturelle (d'après Kinney et Calisher, 1981 ; Seymour et *al.*, 1983 ; Aguilar et *al.*, 2011 ; Saeed et *al.*, 2001a; 2001b ; Hoffmann et *al.*, 2012).

Les lignées et embranchements ont été établis sur base de la séquence nucléotidique du cadre de lecture ouverte de la protéine N (voir Saeed et al., 2001a). En cas d'absence de données disponibles, la case a été laissée vide. Pour le virus Schmallenberg (SBV), la nomenclature est temporaire. Abrév. : Abréviation.

Lignée	Embranchement	Virus	Abrév.	Année d'isolement	Répartition géographique	Arthropode vecteur	Hôte vertébré
I	Ia	Aino	AINO	1964	Japon, Australie	Culicoïdes, moustiques	Bovin
		Kaikalur	KAI	1971	Inde	Moustiques	
		Peaton	PEA	1976	Australie	Culicoïdes	Bovin
		Sango	SAN	1965	Nigéria, Kenya	Culicoïdes, moustiques	Bovin
		Shuni	SHU	1966	Nigéria, Afrique du Sud	Culicoïdes, moustiques	Homme, bovin
	Ib	Akabane	AKA	1959	Australie, Japon, Taïwan, Israël, Corée et Turquie, Kenya, Afrique du Sud	Culicoïdes, moustiques	Bovin
		Sabo	SABO	1966	Nigéria	Culicoïdes	Chèvre, bovin
		Tinaroo	TIN	1978	Australie	Culicoïdes	
	Ic	Douglas	DOU	1978	Australie	Culicoïdes	Bovin
		Sathuperi	SAT	1957	Inde, Nigéria	Culicoïdes, moustiques	Bovin
		Shamonda	SHA	1965	Nigéria	Culicoïdes	Bovin
	Id	Simbu	SIM	1955	Afrique du Sud, Cameroun, République Centrafricaine,	Moustiques	
II		Jatobal	JAT	1985	Brésil		Coati
		Oropouche	ORO	1955	Amérique du Sud (Trinidad, Brésil, Pérou, Panama)	Culicoïdes, moustiques	Homme
III		Ingwavuma	ING	1959	Afrique du Sud, Inde, Nigéria, République Centrafricaine, Thaïlande, Taïwan, Guyane	Moustiques	Oiseaux, porc
		Mermet	MER	1964	États-Unis	Moustiques	Oiseaux
IV		Facey's Paddock	FP	1974	Australie	Moustiques	
V		Buttonwillow	BUT	1962	États-Unis	Culicoïdes	Lapin
		Inini	INI	1973	Guyane		Oiseaux
		Iquitos	IQT	1995	Pérou	Culicoïdes, moustiques	Homme
		Manzanilla	MAN	1954	Trinidad		Singe
		Nola	NOLA	1970	République Centrafricaine	Moustiques	
		Para	PARA				
		Schmallenberg	SBV	2011	Allemagne		Bovin
		Thimiri	THI	1963	Inde, Égypte, Australie	Culicoïdes	Oiseaux
		Utinga	UTI	1965	Brésil, Panama	Culicoïdes, moustiques	Paresseux
		Utive	UTIV	1975	Panama	Culicoïdes	Paresseux
		Yaba	YABA	1963	Nigéria	Moustiques	

Tableau II : foyers déclarés par espèce et pays atteints, arrêtés au 20 avril 2012, d'après Dominguez, 2012

Pays	Date	Foyers (total)	Foyers ovins		Foyers bovins		Foyers caprins	
			nombre	% du total	nombre	% du total	nombre	% du total
Allemagne	20.04.12	1265	845	66,8	375	29,6	45	3,6
France	20.04.12	1303	1096	84,1	188	14,4	19	1,5
Belgique	16.04.12	412	167	40,5	243	59,0	2	0,5
Espagne	12.03.12	1	1	100,0	0	0,0	0	0,0
Pays-Bas	20.04.12	296	107	36,1	183	61,8	6	2,0
Royaume-Uni	20.04.12	249	217	87,1	32	12,9	0	0,0
Luxembourg	2.04.12	12	6	50,0	6	50,0	0	0,0
Italie	16.02.12	1	0	0,0	0	0,0	1	100,0

entre les segments M des virus *Aino* et *Schmallenberg* n'a rien d'exceptionnel.

Le virus Shamonda

Ce virus a été isolé pour la première fois au Nigeria en 1965, à partir de sang de bovin (Causey *et al*., 1972). Il a également été isolé au Nigeria quelques années plus tard chez *Culicoides imicola* (Lee, 1979). L'isolement viral le plus récent a été réalisé au Japon en 2002 à partir d'un mélange d'espèces de culicoïdes capturés au moyen de pièges lumineux (Yanase *et al*., 2005b). Il n'existe pas à l'heure actuelle de preuve directe d'un lien entre le virus *Shamonda* et des manifestations cliniques chez l'homme ou l'animal, mais celui-ci a été suggéré sur bases sérologiques chez des veaux au Japon atteints d'arthrogrypose/hydranencéphalie (Yanase *et al*., 2005b). Le segment S du virus *Shamonda* présente le plus haut niveau d'homologie avec celui du SBV. Ce segment code notamment la protéine N, qui est la plus conservée au sein du sérogroupe *Simbu*. Sur base de la séquence de cette protéine, les virus du sérogroupe *Simbu* partagent une identité nucléotidique variant de 65 à 96 %. Cinq lignées phylogénétiques ont ainsi pu être définies (I à V). Les virus *Akabane*, *Aino* et *Shamonda* ont tous les trois été classés dans la lignée I (respectivement aux embranchements Ib, Ia, Ic) (Saeed *et al*., 2001a) (tableau II).

VOIE DE TRANSMISSION HORIZONTALE ET VECTEURS

Avec plus de 350 isolats disposant d'une nomenclature, la famille des *Bunyaviridae* est probablement la plus vaste des familles d'arbovirus (Hart *et al*., 2009). En effet, la plupart des *Orthobunyavirus* sont transmis par des moustiques et des *Culicoides*. Les *Nairovirus* sont quant à eux transmis essentiellement par des tiques, les *Phlebovirus* par des phlébotomes, des moustiques ou des tiques. Les *Tospovirus* se transmettent aux plantes par l'intermédiaire des thysanoptères (petits insectes phytophages). Les *Hantavirus* ne se transmettent pas par l'intermédiaire d'arthropodes, mais se maintiennent dans la nature grâce à plusieurs espèces de rongeurs, réservoirs de ces virus, et infectent leurs hôtes par voie aérogène, suite à l'inhalation par ces derniers d'aérosols de sécrétions de rongeurs contaminés (Ulrich *et al*., 2008).

Comme pour d'autres virus à ARN segmenté, tels les *Orthomyxoviridae* ou les *Reoviridae*, il existe un risque réel de réassortiment entre *bunyavirus* présentant une homologie de séquence nucléotidique suffisante. Ainsi, le virus *Ngari*, responsable de cas de fièvres hémorragique chez l'homme, est un virus réassortant entre les virus *Bunyamwera* et *Batai*, responsables quant à eux d'une atteinte fébrile non hémorragique (Gerrard *et al*., 2004 ; Briese *et al*., 2006). Le virus *Iquitos*, découvert en 1995 à Iquitos au Pérou, s'est avéré être un virus réassortant entre les segments S et L du virus *Oropouche* et le segment M d'un *Orthobunyavirus* inconnu à l'heure actuelle. Ce segment M est reconnu pour son rôle dans la dissémination systémique du virus de La Crosse (*Orthobunyavirus*, sérogroupe California) dans l'organisme du moustique *Aedes triseriatus*, vecteur naturel de ce virus (Beaty *et al*., 1982 ; 1985). Ainsi, la compétence vectorielle *d'A. triseriatus* est notamment déterminée par la séquence en acides aminés des protéines codées par le segment M (glycoprotéines virales et protéine NSm) et, de cette façon, le changement d'un nombre restreint d'acides aminés peut faire évoluer le spectre de vecteurs que le virus peut infecter (Elliott, 2009). Par ailleurs, un taux élevé de réassortiment a été observé dans le vecteur après coinfection simultanée ou à intervalle très proche (maximum 2 jours) avec différents mutants du virus de La Crosse (Beaty *et al*., 1985).

À l'instar des autres familles de virus à ARN monocaténaire, les ARN polymérases dépendant de l'ARN des *Bunyaviridae* sont enclines à produire des erreurs lors de la réplication du génome viral. Par conséquent, il est licite de postuler que la création de virus mutants favorise l'adaptation à de nouveaux vecteurs.

Vecteurs et transmission horizontale du virus Akabane

Le virus *Akabane* peut être transmis par des moustiques (Oya *et al*., 1961 ; Metselaar et Robin, 1976), mais surtout par des culicoïdes : *Culicoides brevitarsis* en Australie (Doherty *et al*., 1972) et *Culicoides oxystoma* au Japon (Kurogi *et al*., 1987). La réplication du virus a été attestée chez *Culicoides variipennis* et *Culicoides nubeculosus* en conditions expérimentales (Jennings et Mellor, 1989). Le virus *Akabane* a également été isolé chez *Culicoides imicola* au Sultanat d'Oman (al-Busaidy et Mellor, 1991). Cette espèce est également présente en Turquie, où elle constitue le vecteur principal de la fièvre catarrhale ovine (FCO) (Erturk *et al*., 2004). Cependant en Turquie, le virus *Akabane* ne s'est étendu ni sur la côte Nord-Est ni sur le plateau anatolien, contrairement au BTV. Il est ainsi probable que ces deux virus soient transmis par des vecteurs différents, ou si *Culicoides imicola* est leur unique vecteur, que celui-ci soit plus compétent pour le BTV que pour le virus *Akabane* (Taylor et Mellor, 1994).

Vecteurs et transmission horizontale du virus Aino

Bien qu'initialement isolé chez des moustiques, le virus Aino possède comme principaux vecteurs des *Culicoides*, notamment *Culicoides oxystoma* au Japon (Yanase *et al*., 2005a).

Vecteurs et transmission horizontale du virus Shamonda

Culicoides imicola est un vecteur reconnu du virus *Shamonda*, mais d'autres espèces de culicoïdes sont impliquées. En effet, le virus est bien

présent au Japon, en dépit de l'absence de *Culicoides imicola* sur l'archipel.

Jusqu'à présent, seule la voie vectorielle a été décrite pour la transmission horizontale de ces virus. La brièveté de la virémie dans les espèces étudiées ne permet pas de définir d'espèce réservoir. La transmission verticale, suite au passage transplacentaire du virus, est certes prépondérante en termes de pertes économiques et d'impact zootechnique, mais son rôle dans le maintien du virus dans la nature reste à préciser. En effet, le fœtus infecté *in utero* subit l'avortement ou a généralement éliminé le virus une fois à terme, à moins d'une infection en fin de gestation. Dans le cas du SBV, si les espèces de culicoïdes paléarctiques endémiques d'Europe occidentale sont confirmées dans leur rôle de vecteur, l'importance épidémiologique des ruminants en virémie congénitale est sans doute encore moindre. En effet, les petits ruminants, plus enclins à présenter de la virémie à la naissance en raison de leur gestation plus courte que celle du bovin, naissent en général en période d'inactivité vectorielle, rendant fortement improbable une transmission ultérieure. L'inactivité vectorielle dont il est question ici n'est pas absolue mais correspond à la définition fournie au Journal Officiel de l'Union Européenne du 27/10/2007, à savoir absence de capture de *C. imicola* et capture de moins de 5 femelles pares de culicoïde sur l'ensemble du territoire de l'État membre considéré (*Commission of the European Communities*, 2007). Les bovins qui naissent quant à eux plus tard dans la saison, même si l'activité vectorielle a repris, devraient en général avoir éliminé le virus à la naissance. Pour le virus *Akabane*, l'apparition d'anticorps neutralisants est décrite dès 76 jours de gestation chez le fœtus bovin, ce qui supporte l'hypothèse d'une forte improbabilité de détection virale dans les tissus du nouveau-né à la naissance (Hartley *et al*., 1977).

Tableau III : prévalences dans les troupeaux testés par RTqPCR, au 24 avril 2012, en Belgique et aux Pays-Bas, selon l'espèce considérée

Troupeaux	Belgique			Pays-Bas		
	Troupeaux testés	Troupeaux positifs	Prévalence de troupeaux testés (%)	Troupeaux testés	Troupeaux positifs	Prévalence de troupeaux testés (%)
Bovins	831	275	33,1	1187	203	17,1
Ovins	250	167	66,8	344	107	31,1
Caprins	11	2	18,2	36	6	16,7
Total	1092	444	40,7	1567	316	20,2

Vecteurs et transmission horizontale du SBV

L'hypothèse d'un rôle central des espèces de culicoïdes paléarctiques dans la transmission du SBV est étayée par plusieurs études rétrospectives récentes. En effet, l'ARN du SBV a pu être détecté dans des culicoïdes capturés au Danemark en octobre 2011 (*International Society for Infectious Diseases*, 2012f), en Belgique dans un pool de *C. obsoletus* capturés début septembre 2011 et un pool de *C. dewulfi* capturés début octobre 2011 (*International Society for Infectious Diseases*, 2012g), ainsi qu'en Italie, sur six pools de culicoïdes appartenant au complexe *obsoletus* capturés entre septembre et novembre 2011 (*Instituto G. Caporale*, 2012). En Belgique, les RTqPCR ont été réalisées uniquement sur les têtes des culicoïdes. De cette façon, les insectes dont la positivité est liée à un repas sanguin récent pris sur des animaux virémiques sont écartés. Ainsi, un résultat positif suggère la présence du virus dans les glandes salivaires du culicoïde et reflète une possible transmission active du virus avec amplification biologique par le vecteur.

Par ailleurs, le rôle des moustiques ou d'autres arthropodes dans la transmission et l'épidémiologie du SBV ne peut être formellement écarté à l'heure actuelle.

EPIDÉMIOLOGIE DESCRIPTIVE

Les sections suivantes détaillent la chronologie de l'épizootie, étape par étape, jusqu'à la notification des cas en Italie, au Grand-Duché de Luxembourg et en Espagne. La situation épidémiologique des pays affectés au 24 avril 2012 est synthétisée dans le tableau II (foyers déclarés), le tableau III (prévalence des troupeaux suspects testés par RTqPCR en Belgique et aux Pays-Bas) et la figure 2 (distribution géographique du SBV à travers l'Europe en fonction de l'espèce).

Premiers cas cliniques aux Pays-Bas

Depuis août 2011, le Service de Santé Animale néerlandais à Deventer enregistrait un nombre anormalement élevé de cas de diarrhée aqueuse, fièvre (jusqu'à 41°C), et baisse de la production laitière (*International Society for Infectious Diseases*, 2011d). Plus de

Figure II : Régions NUTS (*Nomenclature of Territorial Units for Statistics*) européennes avec au moins un troupeau ovin (A), caprin (B) et bovin (C) confirmé atteint par le SBV au 19 mars 2012 (d'après l'*European Food Safety Authority*, 2012).

80 élevages laitiers, situés à l'extrême est du pays, en zone frontalière avec l'Allemagne, avaient rapporté ce type d'atteintes touchant un pourcentage variable de leurs vaches (*International Society for Infectious Diseases*, 2011a). Les premiers soupçons des éleveurs de la région se sont dirigés vers l'Allemagne. En effet, début mai 2011, des déchets de cuves à méthanisation utilisés comme engrais verts et épandus sur les champs ont été incriminés dans des cas supposés de botulisme chronique. Une présentation clinique différente permit néanmoins d'écarter rapidement cette hypothèse. Les tests réalisés par le Service de Santé animale et l'Université de Wageningen sur des échantillons fécaux, incluaient des techniques de culture, la microscopie électronique, la RTqPCR, ainsi qu'un screening à l'aide de la biopuce Epizone Biochip 5.1 (reconnaissant plus de 2000 espèces virales), mais aucune de ces méthodes n'a permis d'identifier l'agent responsable des signes cliniques décrits (*International Society for Infectious Diseases*, 2011d).

Premiers cas en Allemagne et identification du virus

En Allemagne également, depuis août 2011, des éleveurs et des médecins vétérinaires de Rhénanie du Nord-Westphalie (ouest du pays, en zone frontalière avec les Pays-Bas) avaient rapporté une fréquence inhabituellement élevée d'atteintes cliniques chez des bovins caractérisées par de la diarrhée sévère, de la fièvre (> 40°C) associées à une baisse de la production laitière et, dans quelques cas, des avortements. En Allemagne, c'est un nouvel épisode de FCO qui a été dans un premier temps envisagé (*International Society for Infectious Diseases*, 2011b).

Les analyses menées au FLI ont permis d'écarter les virus de la FCO, de la maladie hémorragique épizootique, de la fièvre aphteuse, de la diarrhée virale bovine, de la rhinotrachéite infectieuse bovine ainsi que ceux responsables de la fièvre de la vallée du Rift et de la fièvre éphémère bovine, comme agents étiologiques de cette maladie. Le 18 novembre 2011, c'est finalement grâce à une analyse métagénomique d'un pool de 3 échantillons sanguins prélevés dans une ferme de la ville de Schmallenberg (d'où le nom – provisoire – de virus *Schmallenberg*) que des séquences génomiques virales présentant des homologies avec le genre *Orthobunyavirus* de la famille des *Bunyaviridae*, ont pu être mises en évidence. Une RTqPCR nouvellement développée, ciblant le segment L et testée sur une centaine d'échantillons issus de 14 fermes où la maladie avait été rapportée a permis la détection de neuf cas positifs dans quatre fermes différentes (*International Society for Infectious Diseases*, 2011b).

Confirmation de la présence du virus aux Pays-Bas, premières atteintes congénitales

Pendant ce temps aux Pays-Bas, les données requises pour la réalisation de la RTqPCR de la FLI avait été transmises. L'Institut vétérinaire central (CVI, Lelystad) avait testé 50 échantillons issus de 8 fermes où des problèmes de diarrhée avaient été rapportés. Simultanément, 115 échantillons de contrôle étaient testés. Le 8 décembre 2011, sur 50 échantillons testés, 15 se sont révélés positifs, alors que tous les contrôles étaient restés négatifs.

Depuis décembre 2011, une augmentation du nombre de naissances d'agneaux malformés avait été rapportée aux Pays-Bas, à travers tout le pays (*International Society for Infectious Diseases*, 2011e). Ces animaux, mort-nés ou non viables, présentaient des anomalies de type torticolis, hydrocéphalie/hydranencéphalie et/ou arthrogrypose. Malgré le faible nombre d'échantillons testés alors, l'exclusion préalable des pathogènes classiques pouvant donner lieu à de telles lésions et la confirmation de la présence d'ARN du SBV chez deux agneaux malformés de la même ferme ont permis de renforcer l'existence d'un lien de causalité entre le virus et ces anomalies.

Premières atteintes congénitales en Belgique

En Belgique, c'est aussi à partir de décembre 2011 que les premiers cas d'anomalies congénitales chez des agneaux ont été signalés. La présence du SBV a été confirmée le 22 décembre 2011 par RTqPCR dans le thymus de 3 agneaux originaires d'une ferme de la province d'Anvers, près de la frontière hollandaise, qui présentaient de l'hydranencéphalie ou de l'hypoplasie cérébrale. Dans cette ferme de 180 brebis, parmi les 60 qui mirent bas pendant cette période, 20 donnèrent des agneaux présentant des anomalies congénitales (*International Society for Infectious Diseases*, 2011c). Les lésions observées étaient comparables à celles décrites aux Pays-Bas : torticolis, arthrogrypose, scoliose, hydranencéphalie ou hypoplasie cérébrale. Le 19 janvier 2012, le SBV était détecté pour la première fois en Wallonie dans le cerveau d'un avorton mâle blanc-bleu belge à 6 mois de gestation. L'animal, dépourvu de malformations évidentes, présentait néanmoins de l'œdème sous cutané et de l'hydranencéphalie à l'autopsie (*International Society for Infectious Diseases*, 2012d). Il était issu d'un élevage d'environ 300 têtes, essentiellement des blanc-bleu belges, ainsi que des vaches laitières, situé dans le sud du pays, près de la frontière française. La brucellose, la diarrhée virale bovine – maladie des muqueuses, l'avortement mycotique et les principales maladies bactériennes abortives ont été exclues de l'étiologie de cet avortement (*International Society for Infectious Diseases*, 2012c).

Bilan en Belgique (fin du mois de janvier)

Au 25 janvier 2011, 272 fermes ayant déclaré des épisodes d'avortement, de mortinatalité ou de malformations congénitales ont été testées en Belgique. Parmi celles-ci, 189 élevages bovins (un résultat positif), 81 élevages ovins (55 élevages positifs) et deux chèvreries, toutes deux négatives. Au sein d'un troupeau de moutons positif, le pourcentage de brebis donnant naissance à des agneaux infectés varie d'environ 32 %, à, dans une autre bergerie, 75 % (International Society for Infectious Diseases, 2012a).

Bilan aux Pays-Bas (fin du mois de janvier)

Au 26 janvier aux Pays-Bas, un total de 311 fermes a été testé, dont 159 troupeaux bovins, 136 ovins et 16 caprins. Deux troupeaux bovins, 73 troupeaux ovins et 3 caprins se sont révélés positifs (Nederlandse Voedsel - en Waren Autoriteit, 2012).

Bilan en Allemagne (fin du mois de janvier)

En Allemagne, d'août 2011 au 20 janvier 2011, le virus a été détecté dans 32 élevages au total (Friedrich-Loeffler-Institute, 2012a). Douze échantillons bovins ont été confirmés positifs (parmi lesquels un jumeau mort *in utero* 10 jours avant le terme), répartis dans 6 fermes. Des cerveaux

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d'agneaux malformés se sont révélés positifs dans 25 fermes (14 en Rhénanie du Nord-Westphalie, 10 en Basse-Saxe et une en Hesse). Enfin, une chèvrerie a également été testée positive. De plus, le premier cas détecté en Rhénanie-Palatinat (24 janvier 2012) concerne une femelle bison et son avorton *(International Society for Infectious Diseases,* 2012e*)*.

Premiers cas au Royaume-Uni

Le Royaume-Uni est le 4e pays à avoir détecté le SBV sur son territoire (*International Society for Infectious Diseases,* 2012d). En effet, la présence du virus a été confirmée le 23 janvier 2012 dans quatre fermes (deux fermes dans le comté de Norfolk, une dans les comtés de Suffolk et d'East Sussex), à partir d'échantillons d'ovins présentant des malformations congénitales comparables à celles observées dans les pays où le virus avait été précédemment détecté.

Premiers cas en France

La France a suivi de peu, puisque les deux premiers cas d'infection au SBV chez des agneaux ont été confirmés le 25 janvier 2012. Ces cas sont issus des départements de la Meurthe-et-Moselle et de la Moselle, tous deux dans l'est de la France (Direction générale de l'Alimentation, 2012a).

Premiers cas en Italie, au Grand-Duché de Luxembourg et en Espagne.

Un premier cas de SBV a été confirmé en Italie le 16 février 2012 dans une petite exploitation de chèvres (*World Animal Health Information System*, 2012a) et le 17 février 2012 dans une exploitation ovine du Grand-Duché de Luxembourg (World Animal Health Information System, 2012b). Le premier, et pour l'instant unique cas de SBV en Espagne, a été déclaré à l'OIE le 13 mars 2012. Il s'agissait d'un agneau avorté le 06 mars 2012 présentant des lésions compatibles avec une atteinte par le SBV, dont la présence a été confirmée par RTqPCR *(World Animal Health Information System,* 2012c*)*.

ÉVOLUTION DE L'ÉPIZOOTIE

Les zones où le virus a été détecté pour la première fois, quelque soit le pays considéré, sont remarquablement superposables avec celles où la FCO a également fait son apparition en 2006-2007, ce qui tend à accréditer l'hypothèse d'une transmission vectorielle par les culicoïdes.

Si les culicoïdes sont effectivement les vecteurs du SBV, compte tenu de la période d'inactivité vectorielle des *Culicoides* endémiques et du pic d'atteinte clinique observé chez les bovins adultes, il est vraisemblable que la majorité des infections des mères ait eu lieu en août-septembre 2011. Par conséquent, en raison de la durée de gestation de la brebis (environ 147 jours), il était permis de postuler que la proportion d'agneaux atteints allait diminuer après le 1er février (International Society for *Infectious Diseases*, 2012c). De plus, les brebis infectées entre les 30e et 50e jours de gestation semblent être les plus susceptibles de donner naissance à des agneaux malformés.

Par contre, le service de santé de l'état de Rhénanie du Nord-Westphalie s'attendait pour les premiers mois de 2012 à un pourcentage de veaux nouveau-nés infectés de 15 à 20 % (*International Society for Infectious Diseases*, 2012b). En outre, selon cette hypothèse, le pic de naissance de veaux atteints de lésions congénitales aurait du se produire autour du mois de février 2012, ce qui est cohérent avec les données issues du terrain (anonyme, 2012). Aux Pays-Bas, les premiers résultats de la première étude de séroprévalence ont été rendus publics récemment (*International Society for Infectious Diseases*, 2012h). Les échantillons sanguins testés, prélevé sur 1123 vaches laitières entre le 1er novembre 2011 et le 1er février 2012 dans le cadre de la surveillance de la FCO, ont révélé qu'environ 70 % des bovins laitiers hollandais étaient séropositifs envers le SBV. La prévalence intra troupeau peut être très élevée. Dans deux exploitations ovines et deux exploitations bovines testées de manière exploratoire, entre 70 et 100 % des animaux possédaient des anticorps spécifiques dirigés contre le SBV. Le futur du SBV en Europe ne pourra être déterminé que lorsque la capacité du virus à passer l'hiver aura été établie, et que la séroprévalence du bétail à l'échelle européenne aura été précisée.

SIGNES CLINIQUES ET LÉSIONS

Signes cliniques causés par le SBV chez le bovin adulte

Les premières descriptions de signes cliniques d'atteinte par le SBV chez le bovin adulte rapportaient une hyperthermie (> 40°C) transitoire, une chute de production laitière significative (jusqu'à 50 %), une diarrhée sévère et parfois des avortements (*International Society for Infectious Diseases*, 2011b).

Première infection expérimentale de bovins avec le SBV

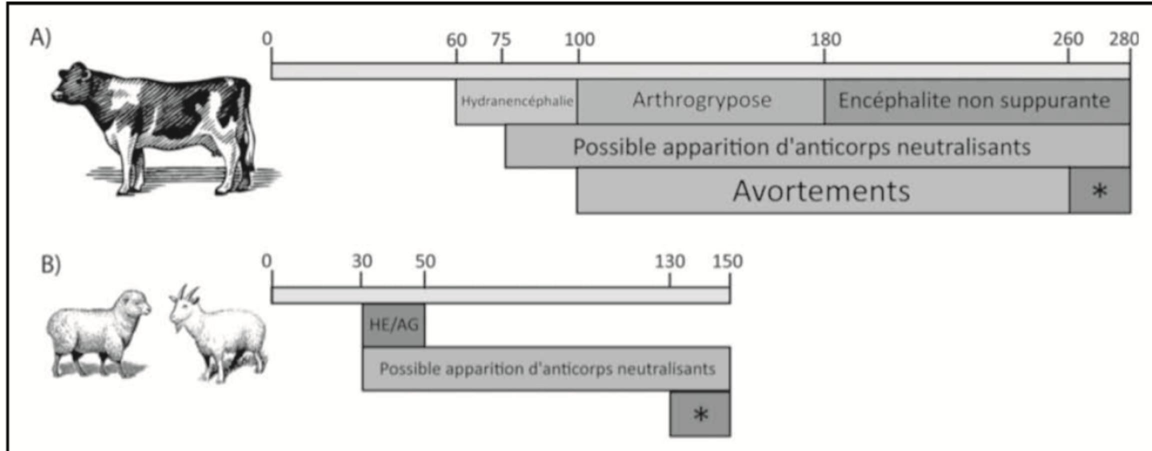
Très récemment, l'équipe allemande du Friedrich Loeffler Institute (FLI) qui a identifié le SBV pour la première fois, a publié des données relatives à la première infection expérimentale de bovins avec le SBV (Hoffmann *et al.*, 2012). Trois veaux d'environ 9 mois ont été infectés par voie intraveineuse et/ou sous-cutanée. La virémie détectée par RTqPCR s'est étendue de 2 à 5 jours après l'infection, avec une virémie maximale au jour 4. Un animal a développé une hyperthermie (40,5°C) et un autre une diarrhée muqueuse persistant plusieurs jours. Le sérum testé à 21 jours s'est révélé positif par séroneutralisation.

Signes cliniques causés par le virus Akabane chez le bovin

De par la similarité des lésions causées et la proximité génétique avec le virus *Akabane*, un mécanisme pathogénique comparable peut être envisagé pour le SBV. Les lésions potentiellement présentées par les veaux atteints *in utero* par le virus *Akabane* ont pu être distinguées selon deux entités : un syndrome hydrocéphalie/hydranencéphalie et un syndrome torticolis/arthrogrypose. L'infection au cours des six premiers mois semble être critique : une atteinte du fœtus entre 76 et 104 jours donne généralement lieu à des lésions de type hydranencéphalie/porencéphalie, et de 103 à 174 c'est l'arthrogrypose qui prédomine (Kirkland *et al.*, 1988). Les lésions les plus tardives ont pu être observées pour une infection à 249 jours de gestation, et il semble que les fœtus âgés de moins de 2 mois sont protégés (Kirkland *et al.*, 1988). De la microphtalmie peut également être observée (Brenner *et al.*, 2004). Lors d'épizootie, de 4 à plus de 40 % des veaux nouveau-nés peuvent être atteints (Inaba *et al.*, 1975 ; Kalmar *et al.*, 1975 ; Parsonson *et al.*, 1981c). Une étude australienne rapporte une fréquence d'anomalies congénitales chez des veaux naïfs de 30 à 54 % (Jagoe *et al.*, 1993). Dans un contexte

Figure III : Conséquences hypothétiques d'une infection *in utero* par le virus *Schmallenberg* (SBV), pour les bovins et les petits ruminants.

Les différentes fenêtres d'infection *in utero* par le SBV sont présentées, selon l'espèce concernée : bovins (A) ou petits ruminants (B). Les durées de gestation sont indiquées en jours. HE/AG : hydranencéphalie/arthrogrypose. * : prématurité, mort-nés, jeunes faibles, mortinatalité.



expérimental, des lésions comparables ont pu être obtenues chez des veaux nouveau-nés dont les mères avaient été infectées entre 62 et 96 jours de gestation (Kurogi *et al*., 1977b). Dans cette dernière étude, des avortements vers 100-120 jours de gestation ont été décrits, alors que des infections plus tardives seront également à l'origine de mises-bas prématurées ou de veaux mort-nés, présentant éventuellement des lésions d'encéphalomyélite non suppurante (Charles, 1994).

De manière plus synthétique, pour une période d'activité vectorielle donnée avec transmission du virus effective, les veaux à naître les premiers, et donc infectés en fin de gestation, présenteront éventuellement des lésions d'encéphalomyélite non suppurante ; les veaux nés vers le milieu de la période de vêlage, infectés vers la mi-gestation, présenteront des altérations musculaires avec arthrogrypose et réduction du nombre de neurones dans la corne ventrale de la moelle épinière. Ils pourront présenter également un épaississement des parois vasculaires du système nerveux central (SNC) et des cavités kystiques dans l'encéphale. L'hydranencéphalie occupe une place centrale dans le tableau clinique des veaux nés en fin de période de vêlage (et donc infectés tôt pendant la gestation) (Konno *et al*., 1982).

En se basant sur les données disponibles pour le virus *Akabane*, il est possible d'estimer les conséquences d'une infection par le SBV, selon l'espèce, et en fonction du stade de gestation où a lieu l'infection (figure 3).

Le virus *Akabane* semble présenter un tropisme accru pour les cellules immatures en division rapide telles celles présentes dans le système nerveux et les muscles squelettiques du fœtus, induisant directement de l'encéphalomyélite et de la polymyosite nécrosante. Si le fœtus survit, ce sera en présentant à terme des lésions d'hydrocéphalie/hydranencéphalie, de porencéphalie, de microcéphalie, d'encéphalomyélite non suppurante, d'arthrogrypose ou de torticolis. Les lésions congénitales peuvent encore inclure de la dilatation des ventricules cérébraux, de la paralysie de la langue, de la cécité, de la surdité, une faiblesse générale, de la boiterie, des torsions de membres et de l'atrophie musculaire (Kurogi *et al*., 1977b *; Kitani et al., 2000*). Une cyphose et des *spina bifida* peuvent s'observer à l'occasion (Rovid Spickler, 2010).

Les lésions les plus sévères s'observent suite à l'infection des mères aux stades plus précoces de la gestation, reflétant l'importante population de cellules vulnérables et l'absence de système immunitaire pleinement compétent. Parsonson et collaborateurs (1988) suggèrent que, chez le mouton, le virus passe de la mère au fœtus par les placentomes, qui pour cela doivent être suffisamment développés et vascularisés. Selon le même auteur, les cellules trophoblastiques fœtales constituent une cible privilégiée pour la réplication du virus *Akabane* à ce stade de l'infection.

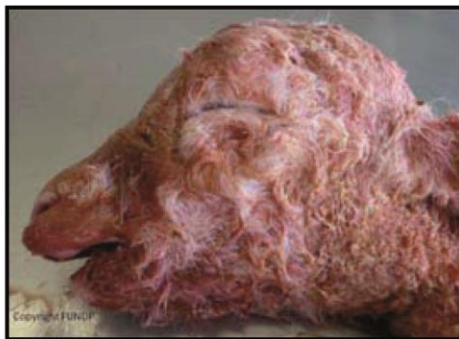
De manière assez paradoxale, s'ils ne sont pas morts dès la mise-bas, les veaux présentant une atteinte nerveuse même sévère survivent régulièrement plus longtemps que ceux atteints d'arthrogrypose/torticolis. En effet chez ces derniers la mise-bas est souvent problématique et ils se révèlent rapidement incapables de téter.

L'infection post-natale de veaux ou de bovins adultes par le virus *Akabane* est généralement asymptomatique, même si certaines souches (souche *Iriki* et apparentées) peuvent être la cause d'atteintes nerveuses, avec hyperesthésie, tremblements, ataxie, nystagmus et opisthotonos, en l'absence d'hyperthermie et avec conservation de l'appétit. Des épidémies de ce type, d'ampleur modérée, ont été décrites au Japon, en Corée et à Taïwan (Liao *et al*., 1996). Des lésions d'encéphalomyélite ont pu être mises en évidence chez ces animaux (Kono *et al*., 2008 ; Lee *et al*., 2002). Les études d'infection expérimentales indiquent, comme dans le cas de l'infection à SBV, une virémie transitoire de courte durée, présente entre les jours 1 et 6 et détectable pendant quatre jours (Kurogi *et al*., 1977b).

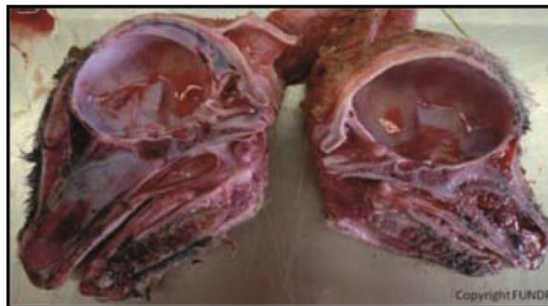
Signes cliniques causés par le virus Akabane chez le mouton

En conditions expérimentales, jusqu'à 36 % des agneaux nés de mères infec-

Figure IV: Agneau né vivant à l'âge d'un jour, incapable de se mettre debout. L'animal présente un réflexe de tétée bien développé et une vision normale. La motricité des muscles de l'encolure et du dos ainsi que la sensibilité du tronc sont normales. Le membre antérieur droit [1] est normalement formé et présente une motricité et une sensibilité normales. Le membre antérieur gauche [2] présente une arthrogrypose au niveau du carpe et un déficit moteur (paralysie flasque) à partir de l'épaule alors que la sensibilité du membre est normale. Les deux membres postérieurs [3 et 4] présentent un déficit moteur (paralysie flasque touchant tout le membre), un déficit sensoriel et de l'arthrogrypose touchant toutes les articulations



(A)



(B)

Figure V: Tête d'un agneau présentant du brachygnathisme et un hydrocéphale (A). Coupe sagittale de la tête du même animal (B). La cavité crânienne présente un volume accru. On note l'absence quasi-totale de l'encéphale et un tronc cérébral de taille très réduite.

tées entre le 30e et le 36e jour de gestation peuvent naître avec des anticorps neutralisants dans leur sérum avant prise de colostrum (Parsonson *et al.*, 1977). Le lien entre les lésions et le virus n'est pas systématiquement évident, puisque des agneaux présentant des malformations typiques peuvent naître dépourvus d'anticorps neutralisants. De même, des agneaux cliniquement sains et sans lésions anatomopathologiques peuvent quant à eux naître avec des anticorps neutralisants, particulièrement si les mères ont été infectées à 50 jours de gestation ou plus tard. Les malformations peuvent être très fréquemment observées chez les agneaux infectés entre 30 et 36 jours de gestation (pouvant toucher jusqu'à 80 % des agneaux infectés d'après Parsonson *et al.*, 1981a), et la présence d'anomalies congénitales suite à une infection après 50 jours de gestation est considérée comme improbable (Hashiguchi *et al.*, 1979).

Les lésions congénitales de l'agneau sont comparables à celles qu'il est possible d'observer chez le veau, mais les lésions nerveuses et musculo-squelettiques semblent plus fréquemment coexister : arthrogrypose et agénésie du cerveau ou hydranencéphalie, porencéphalie, brachygnatisme (figure 4), scoliose, avec également dans certains cas de l'hypoplasie des poumons et de la moelle épinière. Au niveau histopathologique des lésions de dégénérescence et d'atrophie musculaire ont été rapportées, et dans le système nerveux central, atrophie cérébrale, épanchements kystiques et malacie, œdème généralisé, gliose sousépendymaire, manchons périvasculaires et plaques minéralisées sont décrites. Des lésions semblables ont été retrouvées dans le cervelet, le tronc cérébral ainsi que dans la moelle épinière (Parsonson *et al.*, 1981b).

L'arthrogrypose, chez l'homme comme chez les espèces domestiques, est généralement neurogénique, secondaire à des lésions *in utero* des motoneurones ventraux du SNC (Mayhew, 1984 ; Edwards *et al.*, 1989). Ces lésions consistent généralement en une réduction de la myélinisation et du nombre des neurones de la corne ventrale de la moelle épinière (Parsonson *et al.*, 1977). D'autres virus tératogènes peuvent être à l'origine d'importantes lésions du SNC, comme le BTV, le virus de la maladie des frontières (*Border disease virus*, BDV) et le virus de la diarrhée virale

bovine-maladie des muqueuses (*bovine viral diarrhoea virus*, BVDV), pourtant l'arthrogrypose n'est que rarement rapportée en cas d'atteinte par ces virus (Clarke et Osburn, 1978 ; Edwards *et al*., 1989 ; Maclachlan *et al*., 2009). Le sérotype 8 européen et certaines souches vaccinales atténuées du BTV sont reconnues pour pouvoir induire de l'hydranencéphalie chez les ruminants domestiques (Vercauteren *et al*., 2008). Les lésions causées par les *pestivirus* BDV et BVDV sont caractérisées par de l'hypomyélinogénèse et de la dysplasie du SNC, avec comme dans les cas d'atteinte par le virus *Akabane*, réduction du nombre des motoneurones ventraux (Clarke et Osburn, 1978). Cependant, lors d'atteinte par le BTV, le BDV ou le BVDV, les cas d'arthrogrypose ne sont ni aussi fréquents, ni aussi sévères qu'en cas d'atteinte par le virus *Akabane*. Par ailleurs, l'atteinte primaire des cellules musculaires a été prouvée chez le fœtus de mouton et de bovin infecté par le virus *Akabane* (Kurogi *et al*., 1976 ; 1977b ; Parsonson *et al*., 1988). Ainsi selon plusieurs auteurs, cette fréquence accrue de troubles musculosquelettiques en cas d'infection *in utero* par le virus *Akabane* pourrait s'expliquer notamment par l'atteinte primaire des muscles fœtaux (Kurogi *et al*., 1977b ; Edwards *et al*., 1989).

Chez le mouton adulte l'infection au virus *Akabane* apparaît subclinique en conditions naturelles ou expérimentales. Chez la brebis gestante les anticorps neutralisants sont détectables entre 5 et 10 jours après l'infection et la virémie est détectable entre les jours 1 et 5 après l'infection (Parsonson *et al*., 1981a).

Signes cliniques causés par le virus Akabane chez la chèvre

L'inoculation expérimentale de dix chèvres avec le virus *Akabane* a été réalisée entre 30 et 55 jours de gestation. Aucun signe clinique n'a pu être mis en évidence chez les adultes. Les mères ont présenté une virémie durant 2 à 4 jours après l'infection. Les anomalies fœtales se sont révélées rares, avec seulement quelques chevreaux faibles, une momification, et des malformations sur un avorton (mère infectée à 40 jours de gestation) récupéré *in utero* à 120 jours de gestation (Kurogi *et al*., 1977a).

Pouvoir pathogène du virus Aino

Plus récemment, une infection expérimentale de bovins gestants avec le virus *Aino* a été réalisée (Tsuda *et al*., 2004). Le passage transplacentaire n'a pu être démontré, mais l'inoculation intra-utérine a conduit à la naissance de veaux prématurés, faibles ou avec des lésions congénitales comparables à celles décrites chez les agneaux atteints par le SBV. Ce virus reste associé à un syndrome d'arthrogrypose/hydranencéphalie proche de celui induit par le virus *Akabane*. En effet, des anticorps neutralisants contre le virus *Aino* ont été mis en évidence au Japon et en Australie chez des bovins présentant ce type de lésions, et des antigènes du virus *Aino* ont également été détectés dans des cellules gliales d'un avorton bovin au Japon. Le virus a été ensuite isolé à partir du même avorton (Coverdale *et al*., 1978 ; Noda *et al*., 1998; Uchinuno *et al*., 1998 ; Yoshida *et al*., 2000).

Pouvoir pathogène du virus Shamonda

À la connaissance des auteurs, il n'existe à l'heure actuelle aucune preuve formelle du pouvoir pathogène du virus *Shamonda*, quelle que soit l'espèce considérée.

DIAGNOSTIC

Diagnostic clinique

Le contexte épidémiologique et clinique peut faire suspecter une atteinte par le SBV. Chez le bovin, des épisodes anormalement fréquents de diarrhée, baisse d'appétit et de production laitière, hyperthermie associés éventuellement à des avortements, et suivi en période de vêlage par la naissance de veaux atteints d'arthrogrypose et/ou d'hydranencéphalie (ou de troubles nerveux associés), sont évocateurs. En admettant le très probable rôle des culicoïdes dans la transmission du virus, l'atteinte clinique des adultes devrait pouvoir être observée pendant la période d'activité vectorielle, soit entre avril et novembre en Europe occidentale. L'atteinte préalable des mères peut passer inaperçue sans préjuger des conséquences sur la progéniture. En France, la plateforme de surveillance épidémiologique en santé animale définit comme cas suspect, dans le bandeau nord-est (Alsace, Lorraine, Nord Pas de Calais, Picardie, Champagne Ardennes), « tout bovin, ovin ou caprin, *(i)* avorton ou nouveau-né, malformé (arthrogrypose, raccourcissement des tendons du jarret, déformation de la mâchoire, hydranencéphalie torticolis, etc.) ou *(ii)* nouveau-né présentant des troubles neurologiques (paralysie flasque, mouvements exagérés, hyperexcitabilité, difficulté à téter, ataxie,etc.) ». Pour le reste du pays il s'agit de tout second cas au cours du même trimestre dans une même exploitation présentant les mêmes signes cliniques qui sera considéré comme suspect (Direction générale de l'Alimentation, 2012b).

Chez les petits ruminants, l'atteinte des adultes n'a pas été décrite jusqu'à présent. La naissance d'agneaux et de chevreaux présentant de l'arthrogrypose, du brachygnathisme, de l'hydranencéphalie, mort-nés ou très faibles, justifie la poursuite d'analyses au niveau sérologique ou virologique (figures 4 et 5).

Bien que les lésions observées chez les avortons et nouveau-nés ne puissent être considérées comme pathognomoniques, elles demeurent tout à fait évocatrices et ont probablement une valeur prédictive positive supérieure à celle des tests visant à détecter le virus ou l'ARN viral. Des analyses épidémiologiques complémentaires seront requises pour préciser cet aspect de la maladie.

Diagnostic différentiel

L'atteinte par le SBV doit être distinguée d'une atteinte par d'autres *Orthobunyavirus*, tels les virus *Akabane* et *Aino*, ou le virus de Cache Valley (appartenant au sérogroupe *Bunyamwera*, circulant en Amérique du Nord). Des *Orbivirus*, comme le BTV ou le virus *Chuzan*, appartenant au sérogroupe *Palyam*, isolé au Japon à la suite d'une série de naissances de veaux malformés (Goto *et al*., 1988), sont à inclure dans le diagnostic différentiel. En raison des malformations congénitales qu'ils sont susceptibles de provoquer, les BVDV, BDV et le virus de la maladie de Wesselsbron sont aussi à considérer (Rovid Spickler, 2010).

Neospora caninum est un agent d'avortement d'importance chez les bovins à travers le monde, et peut égalementêtre à l'origine d'encéphalomyélite non suppurante chez les veaux en cas d'atteinte congénitale. Dans ce

Tableau IV : échantillons à prélever en cas de suspicion d'atteinte par le virus *Schmallenberg* (d'après Parsonson et al., 1981a ; Rovid Spickler, 2010 ; International Society for Infectious Diseases, 2012a).

En gras, les échantillons à prélever en priorité. IHC : immunohistochimie (non disponible pour le moment). La réaction en chaîne par polymérase en temps réel (RTqPCR) et l'isolement viral seront idéalement réalisés endéans les 24-48h.

	Adulte/mère	Fœtus/avorton/nouveau-né	Conservation	
			24-48 h	Long terme
Sérologie	**Sang sur tube sec**	**Sang sur tube sec avant/après prise de colostrum**	2-20°C	-20°C
RTqPCR	**Sang sur tube EDTA**	**Sang sur tube EDTA avant prise de colostrum**	2-8°C	-80°C
		Encéphale		
		Liquide péritonéal		
		Placentome		
		Rate		
		Liquide placentaire		
		Thymus		
		Liquide péricardique		
		Liquide pleural		
Isolement viral et IHC	**Sang sur tube EDTA**	Sang sur tube EDTA avant prise de colostrum	2-8°C (isolement viral) ; formol 10 % (IHC)	-80°C (isolement viral) ; formol 10 % (IHC)
	Encéphale (bovin, si mortalité)	**Encéphale**		
		Placentome		
		Thymus		
		Moelle épinière		
		Muscle atteint		
		Rate		
		Rein		
		Cœur		
		Poumon		
		Nœuds lymphatiques		

cas, l'affection se manifeste par des troubles nerveux incluant des déficits proprioceptifs, de l'arthrogrypose, et pouvant conduire jusqu'à la paralysie complète de l'animal (De Meerschman *et al*., 2005).

Des causes nutritionnelles (carences des mères en sélénium et/ou manganèse en début de gestation), toxiques (ingestion de lupins entre 40 et 70 jours de gestation) ou physiques (exposition à des radiations ionisantes) peuvent être envisagées (Oryan *et al*., 2011).

<u>*Diagnostic de laboratoire*</u>

Le FLI a développé et diffusé à travers l'Europe deux nouvelles RTqPCR, ciblant soit le segment S, soit le segment L, utilisées actuellement pour détecter le SBV. La RTqPCR est cependant limitée par la brièveté de la virémie présentée par les animaux atteints par le SBV. En effet, lors d'atteinte congénitale, les malformations peuvent être constatées bien que le virus ait pu être éliminé, rendant ainsi impossible la détection des antigènes ou des acides nucléiques du virus. En cas d'atteinte post-natale chez les bovins la virémie là aussi est brève, 2 à 5 jours d'après les premières données expérimentales (cf. supra « Première infection expérimentale de bovins avec le SBV »). Différents kits, ciblant les segments S ou L, sont disponibles dans le commerce (ADIAVET™ Schmallenberg Virus, Adiagène® ; TaqVet™ Schmallenberg Virus – S Gene - kit (SBVS), LSI® ; AnDiaTec® BoVir® Schmallenberg virus *real time* RT-PCR Kit, Andiatec®).

L'isolement viral a, pour l'instant, été réussi à partir de sang de bovin adulte cliniquement atteint (Hoffmann *et al*., 2012). Il est probable que, comme c'est le cas pour le virus *Akabane*, cet isolement soit difficile en cas d'atteinte congénitale, à moins qu'il soit réalisé sur un avorton expulsé simultanément à (ou peu de temps après) l'atteinte de sa mère ou suite à une infection *in utero* proche du terme. Actuellement, l'isolement viral est réalisé après un premier passage en aveugle sur cellules KC (cellules larvaires de *Culicoides variipennis*) suivi par l'inoculation de cellules BHK-21. L'effet cytopathogène est manifeste après 5 jours d'incubation (Hoffmann *et al.*, 2012). Par analogie avec les virus *Aino* et *Akabane*, l'isolement sur souriceaux (âgés de 1 à 2 jours, après inoculation intracérébrale) et sur cellules pulmonaires de hamster (HmLu-1) pourraient être des méthodes suffisamment sensibles pour le SBV (Kurogi *et al*., 1977c ; Yoshida *et al*., 2000). Le tropisme d'autres *Orthobunyavirus* pour les cellules neuronales et astrogliales a été démontré, par immunohistochimie ou immunofluorescence après infection naturelle (Noda *et al.*, 1998 ; 2001) ou en cultures primaires (Kitani et al., 2000).

De manière intéressante, les placentomes semblent constituer un tissu au sein duquel les *Orthobunyavirus* sont plus fréquemment isolés en cas d'infection *in utero*. Parsonson et collaborateurs (1981a) ont émis l'hypothèse que l'interface fœto-maternelle pourrait constituer un environnement difficile d'accès pour les anticorps neutralisants, et ainsi permettre une réplication accrue.

Le système nerveux central est également constitué d'organes à privilégier en cas de recherche de SBV. Les premiers résultats tendent à prouver par exemple que la RTqPCR est plus sensible lorsque réalisée sur le cerveau que sur le thymus (*International Society for Infectious Diseases*, 2012a).

Le développement d'outils sérologiques devrait permettre de confirmer l'implication du SBV dans de nombreux cas de malformations en l'absence de détection d'ARN viral. En effet, l'activité d'anticorps neutralisants contre le virus *Akabane* a pu être prouvée *in utero* chez le fœtus bovin dès 76 jours de gestation (Parsonson *et al*., 1981a), par consé-

quent, la détection de ces anticorps dans le sérum du veau nouveau-né, prélevé avant la prise de colostrum, constituerait une preuve du passage transplacentaire du virus. Cela dit, ici encore par analogie avec la pathogénie du virus *Akabane*, l'absence d'anticorps chez le nouveau-né ne devrait pas pour autant exclure le SBV du diagnostic étiologique (Parsonson *et al*., 1977). L'absence d'anticorps chez la mère par contre, compte tenu de la naïveté du cheptel européen envers le SBV, l'exclut évidemment. Lors d'une infection par le virus *Akabane* chez le veau, l'analyse de persistance des anticorps d'origine maternelle indique la baisse sensible de ces derniers vers 4-5 mois (chez les veaux de race laitière et allaitante, respectivement, d'après Tsutsui et collaborateurs (2009), et pourrait être comparable en cas d'infection par le SBV. Pour le virus *Akabane*, il existe en Europe un kit ELISA de compétition qui détecte les IgG1, commercialisé par la société ID Vet (Montpellier, France).

Démarche diagnostique

En cas de suspicion d'atteinte clinique causée par le SBV chez les adultes, l'étiologie pourra être confirmée par RTqPCR. Une ARNnémie négative, en raison de la brièveté de cette dernière, ne permet pas d'écarter définitivement le SBV. Le suivi des anticorps spécifiques du SBV par sérologie couplée à trois semaines d'intervalle (test ELISA ou séroneutralisation) peut s'avérer nécessaire pour compléter le diagnostic.

En cas de suspicion d'atteintes congénitales ou d'avortements causés par le SBV, les premiers examens à réaliser seront : i) la détection d'anticorps spécifiques du SBV dans le sérum des avortons ou des nouveau-nés avant prise de colostrum (ELISA ou séroneutralisation), ii) la détection de l'ARN du SBV par RTqPCR à partir d'un morceau de placentome et si possible de l'encéphale des avortons ou nouveau-nés. À défaut, le sang prélevé sur EDTA et la rate peuvent être également testés par RTqPCR, mais le virus semble moins fréquemment détecté dans ces organes que dans le SNC (données personnelles).

Si la démarche s'inscrit dans un diagnostic d'avortement sans suspicion particulière de SBV, il peut être utile te tester le sérum de la mère pour détecter les anticorps spécifiques du SBV, leur absence permettant d'écarter ce virus de l'étiologie de l'avortement.

Pour le SBV, le FLI a déjà mis au point des tests de séroneutralisation et d'immunofluorescence indirecte. Un test ELISA est également en cours de développement (Ministère fédéral de l'Alimentation, 2012), et un kit ELISA indirect du commerce (ID Vet, Montpellier, France) a été récemment validé par l'ANSES et est actuellement disponible (*International Society for Infectious Diseases*, 2012i). Les chercheurs du CVI de Wageningen ont eux aussi mis au point un test de séroneutralisation utilisé très récemment dans la première étude de séroprévalence sur le bétail d'un des pays affecté (*International Society for Infectious Diseases*, 2012h). En cas de suspicion de SBV, les échantillons à prélever sont présentés dans le tableau IV.

CONTRÔLE

La transmission horizontale vectorielle est la seule décrite pour le SBV à l'heure actuelle, et compte tenu de la période d'inactivité vectorielle, il est vraisemblable que le SBV n'a pas circulé en Europe au cours de l'hiver 2011-2012. Aussi les mesures de contrôle sont vaines pour une maladie dont les conséquences se font ressentir en l'absence de circulation virale effective. En termes de désinfection, le SBV étant enveloppé, la plupart des désinfectants usuels sont suffisants pour l'inactiver, comme l'eau de Javel, la chlorhexidine, les détergents et les produits de nettoyage à base d'alcool et de phénol. Pour la saison à venir et en cas de persistance du virus au cours de la période hivernale, la seule mesure susceptible de diminuer les cas est un usage accru des répulsifs anti-insectes sur les animaux sensibles (Friedrich-Loeffler Institut, 2012b).

Il n'existe pas de vaccin contre le SBV à l'heure actuelle. Seuls existent des vaccins contre le virus *Akabane* (sans aucune preuve de protection croisée contre le SBV, celle-ci est improbable en raison de la faible identité nucléotidique entre les deux virus). Des vaccins atténués sont disponibles en Corée du Sud (Himmvac Bovine *Akabane* Live Vaccine, BoviShot® *Akabane*) et au Japon. Récemment, un vaccin trivalent inactivé contre les maladies causées par les virus *Akabane*, *Aino* et *Chuzan* a été développé et est commercialisé au Japon. Les souches vaccinales utilisées ont été inactivées au formol ou à l'éthylèneimine binaire et du Montanide IMS 1314 (Seppic, France) est utilisé comme adjuvant (Kim *et al.*, 2011). Les vaccins atténués contiennent en général la souche TS-C2, issue de la souche OBE-1 (isolée à partir d'un fœtus bovin naturellement infecté en 1974) passée à basse température (30°C) sur cellules HmLu-1 (Kurogi *et al.*, 1979).

En date du début du mois d'avril 2012, le SBV n'est pas considéré comme un agent de maladie à déclaration obligatoire, et ce, à travers toute l'Europe. En l'absence de réglementation, aucunes mesures ni barrières commerciales ne sont prévues pour le moment. Cela dit, dès le 1er février, la Fédération de Russie a commencé à imposer des restrictions temporaires sur l'importation d'animaux et de matériel génétique en provenance de l'Allemagne, des Pays-Bas, de la Belgique et de la France. Les autorités russes ont maintenu en outre les restrictions imposées auparavant sur l'importation d'animaux en provenance du Royaume-Uni et ont introduit à partir du 1er février les (mêmes) restrictions sur le matériel génétique en provenance de ce pays. Le Mexique de son côté a suspendu l'importation de matériel génétique de tous les ruminants en provenance des mêmes pays.

RISQUE ZOONOTIQUE

À la fin du mois de décembre 2011, l'Institut national de Santé publique et d'Environnement néerlandais (RIVM) a publié un avis officiel concernant le risque présenté par le SBV pour l'homme (Braks *et al*., 2011). Ce risque a été évalué comme très faible, bien que ne pouvant pas être définitivement exclu. En effet, à l'heure actuelle, aucun cas humain n'a été à déplorer, tant chez les vétérinaires que chez les fermiers des régions concernées. Un rapport de l'Institut fédéral allemand pour l'évaluation des risques (BfR) estime que, bien qu'il ne soit pas encore possible de délivrer des conclusions définitives, on ne doit pas s'attendre à ce que le SBV puisse être transmis à l'homme, soit par contact direct, soit par l'alimentation, qu'il s'agisse de viande ou de produits laitiers (Bundesinstitut für Risikobewertung, 2012). De plus, la plupart des virus du sérogroupe *Simbu* sont des pathogènes exclusifs des animaux. Cependant, les virus *Oropouche* et *Iquitos* (en fait, un réas-

sortant du virus *Oropouche* (Aguilar *et al.*, 2011)) sont reconnus pour avoir un potentiel zoonotique. Dans le cas du virus *Oropouche*, la maladie consiste essentiellement en un syndrome ressemblant à la dengue, associé éventuellement à de la photophobie et à un rash cutané (Grimstad, 1988). La guérison survient généralement en 2 à 3 semaines, spontanément, sans séquelles ni mortalités rapportées jusqu'à présent (LeDuc et Pinheiro, 1989). Ce virus est à l'origine de plusieurs foyers en Amérique du Sud (Tesh, 1994). L'atteinte par le virus *Iquitos* peut inclure une composante digestive, avec vomissement, diarrhée et nausée (Aguilar *et al.*, 2011).

Or, au cours de l'hiver 2011-2012, les cas cliniques de SBV chez les bovins adultes ne sont plus rapportés. L'apparition plus récente d'atteintes congénitales chez des veaux et agneaux correspond à une infection *in utero* contractée au cours des mois précédents. Par conséquent, compte tenu du mode de transmission retenu à l'heure actuelle (piqûre d'insectes et le plus vraisemblablement de culicoïdes) et la saison, l'émergence de cas humains est hautement improbable pour le moment.

CONCLUSIONS

Il est particulièrement remarquable que le virus *Schmallenberg* et celui de la FCO, aient, apparemment, émergé à la même période de l'année, dans une zone géographique superposable. En Europe occidentale, le BTV et le SBV n'ont pas été retrouvés aux limites de l'aire de répartition de virus homologue ou apparentés, mais sont tous deux apparus au cœur d'une zone où les troupeaux étaient naïfs, sans continuité géographique avec des zones d'endémicité (ou d'endémicité de virus apparenté pour le SBV). La question de leur introduction reste tout à la fois importante et irrésolue. Rien n'exclut cependant pour l'instant une circulation du SBV non détectée, préalable à l'été 2011.

Les virus du sérogroupe *Simbu* peuvent infecter une grande variété d'espèces. Leur importance vétérinaire est liée aux pertes économiques conséquentes qu'on peut leur imputer suite à l'infection du bétail domestique, mais il est également reconnu que plusieurs de ces virus peuvent toucher la faune sauvage. Sugiyama et collaborateurs (2009) ont démontré qu'un peu plus de 10 % des sangliers dans la région de Kyushu au Japon étaient positifs aux virus *Akabane* et *Aino*. Par ailleurs aux États-Unis, chez plusieurs espèces d'oiseaux migrateurs (*Progne subis, l'hirondelle noire, ou Agelaius phoeniceus*, le carouge à épaulette, entre autres), il a été possible d'isoler le virus *Mermet*, un autre *Orthobunyavirus* du sérogroupe *Simbu* (Calisher *et al.*, 1969). Une évaluation sérologique des suidés sauvages et de certaines espèces d'oiseaux d'Europe envers le SBV serait intéressante et pourrait éventuellement fournir d'utiles informations sur la circulation du virus préalablement au mois d'août 2011. À ce titre, l'application d'une démarche similaire à celle développée dans le cadre de la FCO, pour circonscrire le moment et le lieu d'émergence les plus probables du SBV pourrait se justifier (Saegerman *et al.*, 2010).

L'expérience acquise lors de l'émergence de la FCO aidant, il apparaît clairement que pour la gestion de ces maladies non contagieuses, transmises notamment par des moucherons du genre *Culicoides*, la vaccination, associée à un zonage circonstancié efficace, demeurent des options de gestion à ne pas négliger. Bien que la transmission à l'homme soit hautement improbable, vétérinaires et éleveurs doivent rester attentifs en cas de problèmes de santé inhabituels.

SUMMARY

Schmallenberg virus (SBV) has been identified in Germany in November 2011. It belongs to the family *Bunyaviridae*, genus *Orthobunyavirus*, of the serogroup *Simbu*. Metagenomic analysis of samples taken from adult cattle allowed to establish its close relationship with *Akabane*, *Aino* and *Shamonda viruses*. The main clinical signs in adult cattle are fever and a significant drop of milk yield for several days, in some cases also diarrhoea and abortions. A congenital arthrogyposis/hydranencephaly syndrome is also described in lambs, kids and calves. The infection is considered as non contagious, most likely propagated among ruminants by biting midges of the genus Culicoides. Clinical cases were reported in adult cattle in Germany and the Netherlands since summer 2011, and congenital affections with SBV detection since December, first from Germany, the Netherlands and Belgium, then United-Kingdom and France, and more recently in Italy, Luxembourg and Spain. So far SBV was most frequently diagnosed using real-time quantitative polymerase chain reaction. Serological tests have been developed recently. Zoonotic risk cannot be excluded but is considered unlikely. SBV emergence is a major event in animal health and is a new challenge for European veterinarians and researchers.

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Annex 5

Evidence-based early clinical detection of emerging diseases in food animals and zoonoses: two cases

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Evidence-Based Early Clinical Detection of Emerging Diseases in Food Animals and Zoonoses: Two Cases

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KEYWORDS

- Epidemiology
- Evidence-based veterinary medicine (EBVM)
- Classification and regression tree analysis
- Early clinical detection
- Bovine spongiform encephalopathy
- Bluetongue virus serotype 8 (BTV-8)

Evidence-based veterinary medicine (EBVM) is the application of evidence-based medicine (EBM) to the veterinary field.[1] By definition, it is the conscientious, explicit, and judicious use of the best scientific evidence to inform clinical decisions with a view to improve the clinical outcome at the individual level.[2,3] However, in the veterinary profession, a great deal of time is spent in making diagnostic, therapeutic, and preventive decisions in a complex and uncertain environment where optimal evidence is often lacking.[4]

Medical care is the art of making decisions without adequate information.[5] Medical decision making has been studied extensively and follows a mainstream trend, labeled "rational optimizing."[6] It is usually based on cognitive rational models, such as decision analysis, decision tables, decision trees, and Bayes' theorem.[7–11] When *decision* refers to *diagnosis,* the consideration of the possible causes of a disease, its prevalence, and an initial evaluation of clinical signs will lead to a differential diagnosis about which clinical judgment, informed by evidence clinical data, is exercised.[3]

The authors have nothing to disclose.

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Diagnosis may involve the choice and interpretation of an appropriate confirmatory diagnostic test.

To detect and identify emerging or rare diseases, a good clinical approach is essential as few biological and epidemiologic data and/or laboratory tests are available. The approach aims at establishing the limits between normality and abnormality as veterinarians cannot relate the clinical signs to those of a known disease or to their experience. These limits should be built on the ability to detect biological variations in physiologic and environmental conditions. The various actors involved in epidemiosurveillance networks (eg, breeders, veterinarians, and slaughterhouse staff) should be prepared for this clinical approach to fulfill their responsibility in health monitoring.[12] Part of this training should develop knowledge of disease biology and epidemiology, and skills in a rigorous, standardized, and evidence-based clinical approach including that of differential diagnosis.[13–16]

Because, with emerging diseases, the implementation of classic EBVM is difficult as a result of few published cases are available and/or accessible via web searches, other options are necessary.

The current report aims to describe a method to improve the early clinical detection of emerging diseases in food animals and zoonoses. This approach is based on the analysis of field clinical observations collected on the first cases suspected of disease using a method called “classification and regression tree” (CART) (Zanella G, Martinelle L, Guyot H, et al. Clinical pattern characterisation of cattle naturally infected by BTV-8. Unpublished data, 2011.).[17,18] Those clinical facts become the only evidence available. Two practical examples are developed to illustrate the feasibility of the method in cattle. Future prospect is also proposed like the implementation of a structured, well-informed and interactive veterinary web clinical data mining platform.

CASE DESCRIPTION

Two examples are developed to illustrate the use of CART analysis for stimulating the early warning of emerging animal diseases. This is a key parameter of health control strategy.[19] CART analysis is a nonlinear and nonparametric model fitted by binary recursive partitioning of data (including clinical signs). Using CART 6.0 software (Salford Systems, San Diego, California), the analysis successively splits the dataset into increasingly homogeneous subsets until it is stratified and meets specified criteria (clinical signs) (**Fig. 1**). Further details about CART are presented in previously original articles or reviews (Zanella G, Martinelle L, Guyot H, et al. Clinical pattern characterisation of cattle naturally infected by BTV-8. Unpublished data, 2011.).[17,18,20]

Case 1: Early Detection of Bovine Spongiform Encephalopathy

Background

Bovine spongiform encephalopathy (BSE) emerged in 1986.[21] It is a neurodegenerative disease characterized by a very long incubation period compared to the life of the host species.[22] BSE started a dramatic chain of events in the United Kingdom and subsequently in other countries.[23] The peak of interest was the discovery of its potential zoonotic character after the first description of a new variant of Creutzfeldt-Jakob disease (*v*CJD) in 1996.[24–26] The presence of clinical signs seems to be linked to the localization and degree of vacuolization of neurons. The main warning signs are psychic disorders (apprehension, temperament change, abnormal ear position, and abnormal behavior), sensory disorders (exaggerated responses to stimuli, excessive licking), as well as postural and locomotion abnormalities (ataxia and tremors). Their

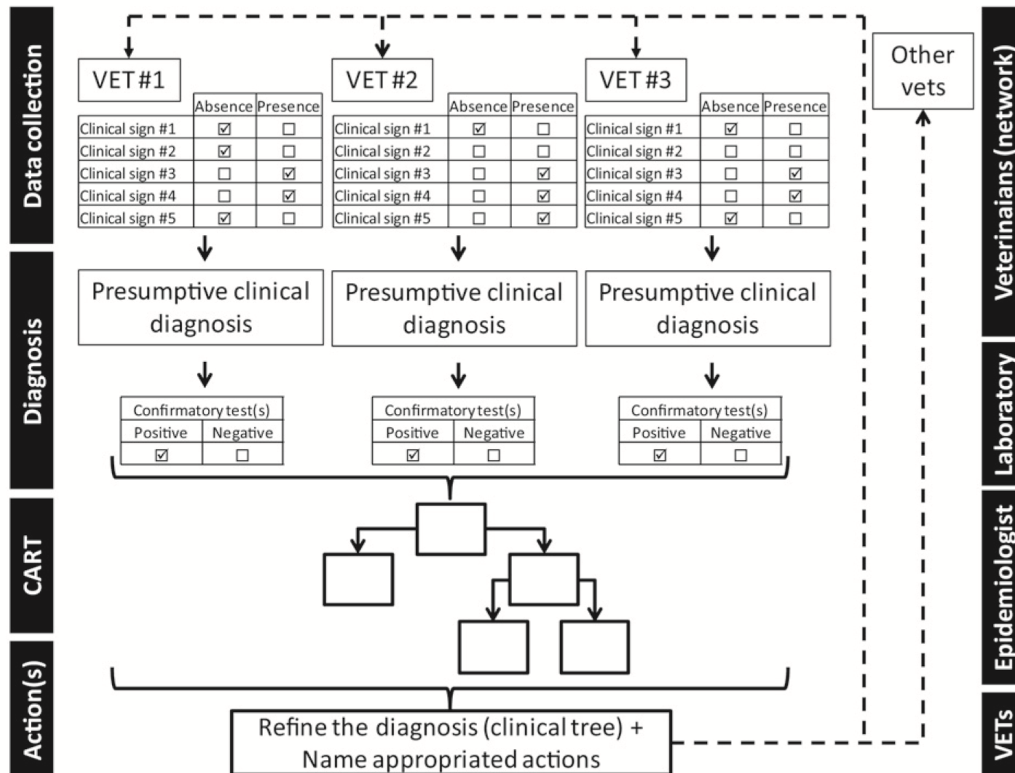

Fig. 1 Flowchart of the CART approach with implication of veterinarians (*left*, process; *right*, actors involved).

identification requires a clinical approach: a thorough veterinary clinical examination of the animal when on a halter and when moving in an uncustomary environment.[16]

Now the evolution of BSE incidence in many European countries is in decline.[27] Because of the favorable BSE epidemiologic situation of most Member States in the European Union, a lowering of control measures, by reducing testing procedure, was recently suggested. However, in such a context, the reporting of clinically suspected cattle by the veterinarians is the most common method for detecting sporadic cases of BSE.[18] The improvement of clinical diagnosis and decision-making remains crucial.

Veterinary data collection

A comparison of clinical patterns captured by veterinarians, consisting of 25 clinical signs, was carried out among BSE cases confirmed in Belgium before October 2002 (N = 30) and 272 suspected cases that were subsequently determined to be histologically, immunohistochemically, and scrapie-associated-fiber negative.[10]

Epidemiological methods and findings

Seasonality in reporting suspected cases was observed, with more cases being reported during wintertime when animals were kept indoors. The median duration of illness was 30 days. Using odds ratio, the 10 most relevant signs of BSE were kicking in the milking parlor, hypersensitivity to touch and/or sound, head shyness, panic-stricken response, reluctance to enter in the milking parlor, abnormal ear movement or carriage, increased alertness behavior, reduced milk yield, teeth grinding, and

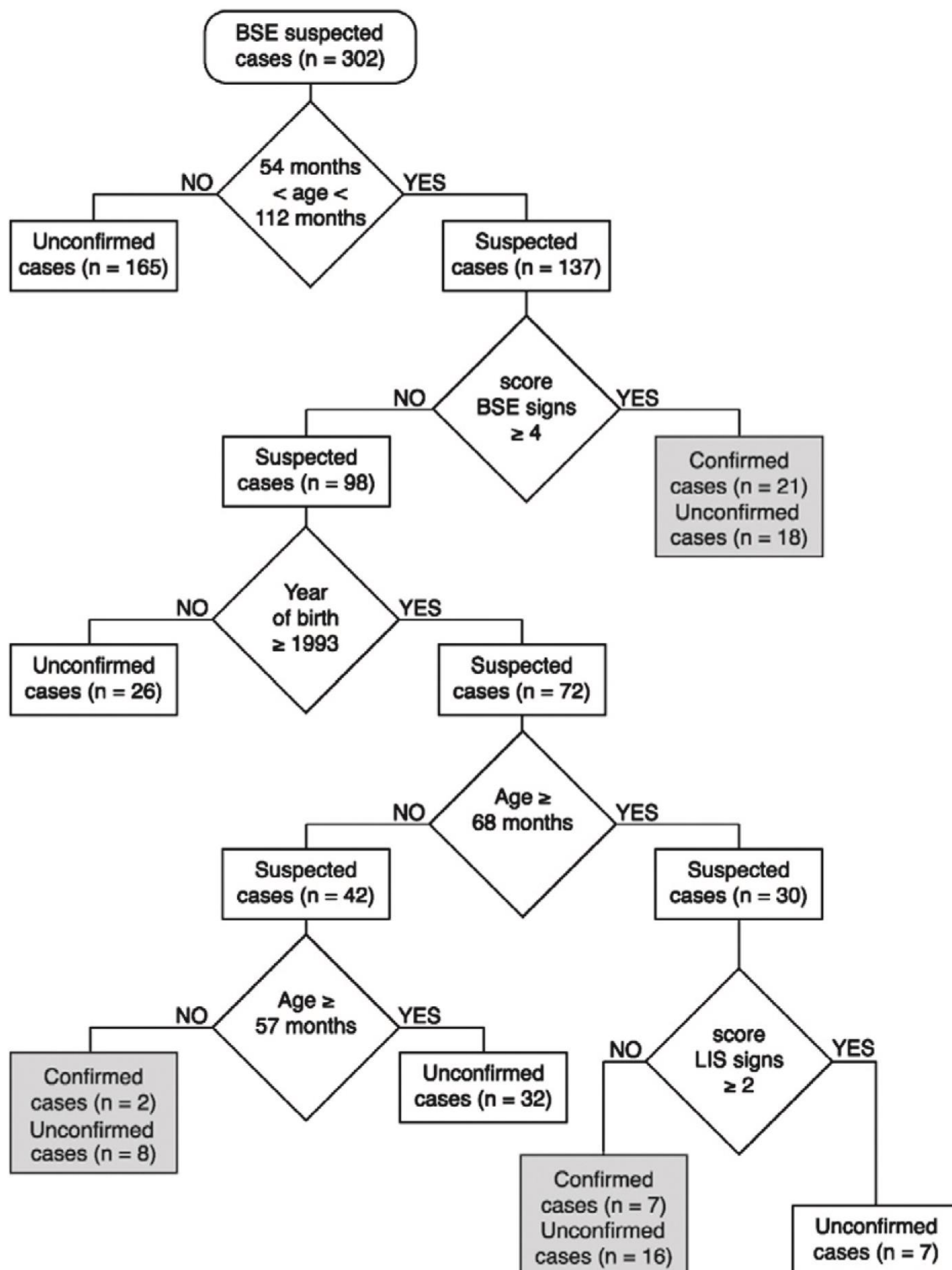

Fig. 2 CART modeling for clinically suspected BSE cases in Belgium.[10] LIS, listeriosis; Score, number of clinical signs that are present.

temperament change. Ataxia did not appear to be a specific sign of BSE. A classification and regression tree was constructed by epidemiologists using the following 4 features: age of the animal, year of birth, number of relevant BSE signs noted, and number of clinical signs typical of listeriosis reported. The model presented a 100% sensitivity and an 85% specificity (**Fig. 2**).

Veterinary significance
The originality of the approach resides in the fact that, first, it involved both veterinarians and epidemiologists. Second, it offered an explorative and interactive tool based of clinical observations (evidence) captured by veterinarians and, then, the results and conclusions are independent of BSE prevalence, through the use of odds ratios. This feature is especially appealing for rare events. A similar decision tree, allowing the distinction between “highly suspected BSE cases” and all other suspected BSE cases, could be applied in other countries, with or without the use of rapid tests. The continued addition of standardized clinical data by veterinarians would permit further improvement of the current model tree, even if the clinical BSE pattern was modified in time. Based on the CART analysis results, veterinarians could more appropriately identify affected cows and retrieve them from the food chain in a public health perspective.

Case Study 2: Early Detection of Bluetongue

Background
Bluetongue (BT) is a noncontagious disease affecting ruminants and is caused by the bluetongue virus (BTV). BTV is transmitted by blood-feeding midges of the genus *Culicoides* (Diptera: *Ceratopogonidae*).[28] A broad spectrum of wild and domestic ruminants can be infected and severe clinical signs are mainly seen in certain breeds of sheep and some *Cervidae* spp.[29,30] The severity of infection depends on various factors, such as species, breed, age, nutritional and immune status of animals, and environmental stresses, as well as the virulence of the BTV strain involved.[31] Although clear differences in virulence of BTV isolates are known, the determinants of virulence are still poorly defined.[31] Clinical manifestations are closely linked to virus-induced vascular injuries and the role of species-specific endothelial cell–derived inflammatory and vasoactive mediators has been highlighted.[32] The European BTV-8 outbreak was characterized by peculiar features.[33] Among these features, a remarkable severity of the lesions in cattle was noticed.[34]

Veterinary data collection
Forty-one cattle from 7 Belgian farms and 2 French farms confirmed as infected with bluetongue virus serotype 8 (BTV-8) were monitored from the onset of clinical signs to describe the disease pattern (Zanella G, Martinelle L, Guyot H, et al. Clinical pattern characterisation of cattle naturally infected by BTV-8. Unpublished data, 2011.). On each visit, a standardized clinical form was filled for each animal by a veterinarian (**Box 1**).[35]

Epidemiological methods and findings
A clinical score was calculated for every week until the end of clinical signs. A CART analysis was conducted by epidemiologists to determine the most important clinical signs every week for the first 7 weeks. The highest scores were recorded within 2 weeks of clinical onset. The first recorded clinical signs were quite obviously visible (conjunctivitis, lesions of nasal mucosa, and nasal discharge). Skin lesions, a drop in milk production, and weight loss appeared later in the course of the disease. A biphasic pattern regarding nasal lesions was noticed: the first peak concerned mainly congestive and ulcerative lesions, whereas the second peak mainly concerned crusty lesions.

Veterinary significance
These results should ensure a more accurate detection of BT in cattle by veterinarians to increase the early detection of emerging diseases (**Table 1**).

Box 1
Bluetongue standardized clinical form for the use in different species[35]

General information: Identification number of the herd; identification number of animal; animal species; breed; sex; date of birth; date of last calving; stage of pregnancy; date of clinical examination; name of clinician

General clinical signs: Hyperthermia; decreased milk production; wasting, emaciation, weight loss; tiredness; edema of head, ears, submandibular region, or the periorbital region; hypertrophied lymph nodes

Clinical signs of skin and annexes: Lesions of the muzzle, lips (congestion > ulcers > necrosis); conjunctivitis, tears, periocular dermatitis; photosensibilization-like lesions; presence of petechiae, contusions, ecchymoses; erythema, inflammation of the skin, crusts; cyanosis of the skin or limbs; skin lesion of the udder, teats or vulva; scrotal skin lesions; wool loss (sheep)

Clinical locomotor signs (musculoarthoskeletal): Incapacity to lift up or prostration; reluctance to move or limited movement; lameness, stiffness of front limbs; lameness, stiffness of hind limbs; edema of coronary bands; swelling of pastern, fetlock, cannon, carpal or hock joint; pododermatitis; contracture of front limbs; contracture of hind limbs; arched back; amyotrophy; torticollis or neck bended

Digestive clinical signs: Loss of appetite; anorexia; difficulties in grasping the food; regurgitation; congestion, erythema of the oral mucosa; ulcerative lesions of the oral mucosa, excoriations; salivation, drooling, foam out of the mouth; edema and/or protrusion of the tongue; cyanosis of the tongue; hemorrhagic stool; diarrhea

Respiratory clinical signs: Ulcerative lesions of the nasal mucosa; purulent nasal discharge; mucous, serous, aqueous nasal discharge; halitosis or bad breath; dyspnea, oral breathing, stridor

Neurologic clinical signs: Apathy, lethargy; generalized weakness, paresis, or paralysis

Reproductive clinical signs: Anestrus; abortion or premature calving; stillbirth; abnormalities of newborns

Duration of evolution: Date of the first clinical signs; comments on the evolution of the disease within the herd

Post-mortem (PM): Has a PM examination been performed? If “yes,” please attach a copy of the PM record(s) (with the animals identification mentioned)

Concomitant pathologie(s)

Data from Saegerman C, Mauroy A, Guyot H. Appendix. Bluetongue in ruminants: a standardised clinical report form for the use in different species. In: World Organization for Animal Health and University of Liege, editors. Bluetongue in northern Europe. Paris: 2008. p. 82–7.

DISCUSSION AND SUMMARY

The clinical expression of a disease in an animal depends on several parameters: the nature of the causal agent (dose, virulence),[36] the location of induced lesions,[37] the host (resistance, general condition, immune status), and the environment; some clinical signs may be exacerbated when the environment of the animal is altered.[38,39] The quality of observation plays an essential role and is proportional to the breeders’ and veterinarians’ level of information, awareness, and training. The intensity of observation is also important, and seems to depend directly on herd size. According to the US National Animal Health Monitoring System (NAHMS), the rate of neurologic problems in breeding females in beef herds, expressed in affected cattle per 1000, doubles when herd size is <100 heads, and is nil when herd size is >300 heads.[40] In addition to these parameters, there is a degree of variability that depends on the

Table 1
Variable importance in CART analysis during the first 7 weeks of cattle naturally infected by BTV-8

	Variable Importance						
Clinical Sign	**Week 1**	**Week 2**	**Week 3**	**Week 4**	**Week 5**	**Week 6**	**Week 7**
Conjunctivitis, lacrimation, periocular dermatitis	100	38		33		100	
Ulcerative lesions of nasal mucosa, crusts	32	100	100	91			76
Mucous, serous, aqueous nasal discharge	26	1		100		28	
Congestion, erythema, redness of buccal mucosa and/or muzzle	21			19		61	
Loss of appetite	18		71	18	3	28	27
Purulent nasal discharge	14			6		13	10
Ulcerative lesions of buccal mucosa, excoriation	11		24	44	0		0
Swelling of coronary bands	7					62	
Skin lesions of udder, teat, or vulva	1			9	32	18	
Swelling of the head, tongue, submaxillary area, jaws			18	22		16	
Lameness or generalized stiffness				2		5	3
Incapacity to stand up, prostration			2	1			3
Anorexia				6			
Tiredness, limited walking				2		47	
Salivation, ptyalism, mouth foam				6		7	
Weight loss			3	62	100	5	41
Arching of back			3				
Muscular atrophy			9	36			
Anoestrus				53		9	5
Milk loss				34	69	78	100
Dyspnea, buccal breathing, loud breathing				5		19	

individual animal and the observer (clinical picture, prepatent phase, and course of the disease). To improve knowledge regarding diseases, especially (re-)emerging animal diseases, it is important (1) to improve awareness, training, and information available for breeders and veterinarians, (2) to use a uniform method for clinical examination by veterinarians, (3) to make more systematic use of confirmatory diagnostic tests, (4) to create sentinel networks of highly motivated breeders and veterinarians, (5) to transcribe the results of observations in a codified and standardized form, regarding both nature and course, (6) to compile and validate existing information by epidemiologists, (7) to enrich a relational database, and (8) to discuss actual experience in a focus group.

In case of early clinical detection of emerging animal diseases, an EBVM approach is difficult to perform. However, an alternative approach based on new structured and harmonized clinical observations (evidence) should be used (standardized clinical form compiled by veterinarians). With 2 practical examples, we demonstrated the usefulness of joint effort involving veterinarians and epidemiologists in CART analysis to improve the early clinical detection of (re-) emerging animal diseases. The strategy is based on analysis of clinical observations (evidences) captured by veterinarians in the field. Selection criteria are based on signs captured by a structured and harmonized clinical form. A presumptive clinical diagnosis performed by veterinarians implies confirmatory diagnostic test(s). Results are analyzed taking into account all clinical signs registered. The CART analysis carried out by epidemiologists allows producing a robust clinical tree that improves the early clinical detection of diseases by any veterinarian who has not faced the considered emerging disease before.

The CART approach is characterized by (1) its exploratory and interactive aspects, (2) its independence from sample size and disease prevalence, which is usually imperfectly known, and (3) its spatiotemporal universality (adaptation is possible when the clinical profile of disease evolves in function of time or region; adaptation is also possible for other diseases). The use of tools to improve the detection of (re-)emergent diseases will lead to more effective veterinary epidemiosurveillance networks. The efficacy of these networks requires regular evaluations together with the elaboration and a continuous follow-up of performance indicators. The recent episodes of both human and animal (re-)emergent diseases have also highlighted the important role of global health information systems. These systems require abilities, resources, collaborative, and coordinated actions of medical and veterinary regulatory authorities.

To improve early clinical detection of (re-)emerging diseases, a future prospect should consist in developing a veterinary structured and informed clinical platform. Whilst some interesting diagnostic support systems for veterinary medicine exist, like the "Consultant" support system from the Cornell College of Veterinary Medicine,[41] no interaction and partition of clinical data are currently available.

Facing the emergence of diseases, the translation of the support system to an interactive platform should be interesting. Involving sentinel veterinarians in this platform is crucial. Veterinarians should be stimulated in a pilot research project to ensure the collection of field clinical data through the filling of structured and harmonized clinical forms. The connection between validated clinical data and results of confirmatory diagnostic tests using CART analysis by epidemiologists permits building useful clinical decision trees to improve the evidence-based early clinical detection of diseases of food-producing animals in the field.

More interactions between veterinarians and epidemiologists should be stimulated in a clinical perspective.

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Annex 6

Animaux sentinelles et Fièvre Catarrhale Ovine en Europe

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ANIMAUX SENTINELLES ET FIÈVRE CATARRHALE OVINE EN EUROPE *

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RESUME

Le recours à des animaux ou unités sentinelles est une forme de surveillance spécifique reposant sur un protocole prospectif. Il s'agit d'une stratégie couramment utilisée pour la surveillance de la fièvre catarrhale ovine à travers le monde. Cet article présente les premiers résultats d'un réseau de bovins sentinelles mis en place en Belgique en 2007 et d'un système d'ovins sentinelles mis en place à la plate-forme d'infectiologie expérimentale de l'INRA de Tours-Nouzilly (France) en 2008. Ces systèmes ont permis de répondre à l'objectif de détecter une réapparition de la fièvre catarrhale ovine. Toutefois, une amélioration de la standardisation, de l'évaluation et de la gouvernance de tels réseaux est souhaitable afin de satisfaire l'ensemble des critères de qualité d'un système de surveillance tels que ceux édictés par les Centres de prévention et de contrôle des maladies aux Etats-Unis.

Mots-clés : Fièvre catarrhale ovine, animal sentinelle, bovins, ovins, épidémiologie, Belgique, Europe.

SUMMARY

The recourse to sentinel animals or sentinel units is a type of specific surveillance based on a prospective protocol. This is the strategy usually applied to the surveillance of bluetongue disease around the world. This article presents the initial results of a sentinel network in cattle implemented in Belgium in 2007 and of a system of sheep sentinel system set up in 2008 at the INRA Experimental Infectiology Platform I – Research Center in Tours-Nouzilly (France). These systems made possible the detection of bluetongue reoccurrence. However, improvements in standardisation, assessment and management are required to meet all the quality criteria of a surveillance system similar to that enacted by the centres of disease prevention and control in the United States.

Keywords : Bluetongue, Sentinel animal, Cattle, Sheep, Epidemiology, Belgium, Europe.

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I - INTRODUCTION

Il n'est pas inutile de rappeler la définition *sensu stricto* proposée d'un animal sentinelle sanitaire. Il s'agit d'un « *Animal choisi dans son milieu ou placé volontairement dans un milieu et suivi au cours du temps afin de détecter précocement, de manière qualitative ou quantitative, une exposition à un agent pathogène donné* » [Toma, 2009].

Le recours à des animaux ou unités sentinelles est une forme de surveillance spécifique reposant sur un protocole prospectif. Il s'agit d'une stratégie couramment utilisée pour la surveillance de nombreuses maladies infectieuses et parasitaires comme, par exemple, certaines maladies zoonotiques [VerCauteren *et al.*, 2009 ; Heyman et Saegerman, 2009] ou vectorielles y compris la fièvre catarrhale ovine (FCO) [Gorch *et al.*, 2002 ; Racloz *et al.*, 2007 ; Hoffmann *et al.*, 2008].

Dans le cas spécifique du virus de la FCO, les unités sont constituées de groupes d'animaux non antérieurement exposés au virus, se trouvant sur des sites fixes et régulièrement soumis à des prélèvements pour détecter toute nouvelle infection par ce virus [Organisation mondiale de la santé animale, 2009] (Annexe). Etant donné les préférences trophiques des *Culicoïdes* inféodés à nos régions pour les bovins [Bartsch *et al.*, 2009], ceux-ci constituent des animaux sentinelles de choix [Organisation mondiale de la santé animale, 2009].

Depuis l'émergence du virus de la FCO de sérotype 8 (BTV-8) en Europe du nord et centrale, en août 2006 [pour une revue, voir par exemple Saegerman *et al.*, 2008a, 2008b], un système d'exploitations sentinelles a été mis en place dans bon nombre d'Etats membres de l'Union européenne dont la Belgique et la France. Un tel système visait à détecter l'apparition d'une nouvelle activité vectorielle dès le printemps 2007.

La présente étude avait pour objectif initial de comparer les résultats de deux réseaux sentinelles en Europe ; ceux mis en place en Belgique et en France. Les deux pays étaient intéressants à comparer du fait d'une dynamique spatio-temporelle d'infection différente [Saegerman *et al.*, 2008b]. Les données du réseau sentinelle belge étaient centralisées et standardisées. Les données concernant le réseau sentinelle français n'ont pas pu être obtenues. La raison invoquée a été un défaut de centralisation et de standardisation.

Toutefois, un jeu de données, de portée géographique limitée, a pu être obtenu auprès de la plate-forme d'infectiologie expérimentale de l'INRA de Tours-Nouzilly. Ces données ont été analysées en collaboration avec l'INRA et le CIRAD.

II - MATERIELS ET METHODES

1. SYSTEME SENTINELLE MIS EN PLACE EN BELGIQUE1

1.1. BUT DE LA SURVEILLANCE

Le but du système de surveillance mis en place était la détection rapide de la réapparition du BTV-8 en Belgique en 2007.

1.2. POPULATION ECHANTILLONNEE

La population correspondait aux bovins issus d'exploitations laitières (contention journalière) pratiquant la vente de produits laitiers à la ferme et à réponse négative au test ELISA de compétition réalisé lors de la campagne hivernale de dépistage de la FCO en janvier ou février 2007 [Méroc *et al.*, 2008]. Ceci représentait un total de 18 542 prélèvements sanguins pour lesquels un résultat ELISA était disponible. Des contrôles ultérieurs avaient également été programmés en deuxième intention (voir point 1.4.). Ces prélèvements réalisés en seconde intention ont induit un biais de recrutement puisque les éleveurs ayant procédé à ceux-ci

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ont modifié leur comportement en ce qui concerne le rythme des prélèvements initialement prévus. Pour cette raison et le fait que ces contrôles ultérieurs auraient biaisé l'incidence de la FCO, seuls ceux correspondant aux prélèvements réalisés en première intention ont été pris en compte dans l'analyse des résultats (N = 16 375).

1.3. Estimation de la taille de l'echantillon

La taille de l'échantillon était fondée sur les critères de surveillance préalablement édictés [Commission européenne, 2007]. Elle doit permettre de détecter une incidence mensuelle de séroconversion de 2%, avec un niveau de confiance de 95%, dans chaque unité géographique de référence. En Belgique, l'unité de référence a été définie par un quadrillage comportant des cellules de 45 Km de côté (2 025 km^2) et revient à prélever 150 échantillons par unité. Comme une province représente environ deux de ces unités géographiques, cela portait le nombre de prélèvements à 300 par province. La taille ainsi obtenue a été ensuite multipliée par un coefficient de 1,5 afin de tenir compte des exploitations qui étaient susceptibles d'abandonner le suivi en raison de la durée du programme sentinelle (plusieurs mois). Pour des raisons pratiques, l'échantillonnage final était de 15 bovins par troupeau sentinelle et 30 troupeaux par province [7] (270 troupeaux pour la Belgique), soit au total 4 050 animaux soumis à des prélèvements par mois.

Lorsque des fermes initialement prévues ne participaient pas au suivi, celles-ci ont été remplacées jusqu'au 30/05/07, soit par le choix de l'exploitation la plus proche en distance de celle à remplacer, soit par le choix permettant la meilleure répartition géographique possible.

1.4. Tests de diagnostic utilises

Le test utilisé en première instance était un test ELISA compétitif de détection d'anticorps dirigés contre la protéine VP7 du virus de la FCO (protéine très conservée parmi l'ensemble des sérotypes). Si un résultat positif était constaté, les 15 animaux du troupeau étaient contrôlés à nouveau par le même test. La virémie (ARNnémie) des animaux séropositifs lors de ce second contrôle a été évaluée à l'aide d'une réaction de polymérisation en chaîne après transcription inverse (RTqPCR). Les protocoles de ces deux tests ont été décrits antérieurement [De Clercq *et al.*, 2008].

Les tests ont été réalisés dans la semaine de réception des échantillons au CERVA-CODA après avoir été transmis, au sud du pays, par l'association régionale de santé et d'identification animales (ARSIA) et, au nord par la Dierengezondheidszorg Vlaanderen (association similaire à la précédente).

1.5. Organisation prevue du programme

Le monitoring devait être répété chaque mois. Les prises d'échantillon devaient être réalisées entre le 20ème et le dernier jour du mois et ce, à partir du 20 mars 2007. Chaque vétérinaire d'exploitation concerné recevait de l'unité provinciale de contrôle (site provincial de l'Agence fédérale belge pour la sécurité de la chaîne alimentaire) une liste avec les exploitations et les animaux à échantillonner.

1.6. Base de donnees

Les données du suivi sentinelle étaient collectées dans des fichiers Excel® séparés. Ils ont été convertis en une base de données relationnelle Access® afin de faciliter la validation des données et les extractions nécessaires à l'analyse de celles-ci.

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7 Il y a dix provinces en Belgique. Les provinces de Brabant wallon et Brabant flamand ont été regroupées pour les besoins du suivi sentinelle étant donné leur relative faible superficie.

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2. SYSTEME SENTINELLE MIS EN PLACE A LA PLATE-FORME D'INFECTIOLOGIE EXPERIMENTALE DE L'INRA DE TOURS-NOUZILLY (département 37)

Un lot de huit ovins de la plate-forme d'infectiologie expérimentale de l'INRA de Tours-Nouzilly - non vaccinés pour raison expérimentale - a été laissé en pâture du 21 mai 2008, après traitement à l'Ectotrine® dont le principe actif est la cyperméthrine et la rémanence de sept à huit semaines, jusqu'à fin octobre 2008. Pour le suivi de ces animaux - qui n'étaient pas, à l'origine, destinés à constituer un lot sentinelle - seul un test ELISA compétitif a été mis œuvre. Les informations générées par ce suivi ont été comparées à l'existence de foyers environnants de FCO.

3. ANALYSE DES DONNEES

L'approche utilisée était soit comparative (contrôle de qualité des procédures), descriptive (répartition spatio-temporelle) ou analytique (évaluation de la pertinence des résultats sérologiques positifs).

III – RESULTATS

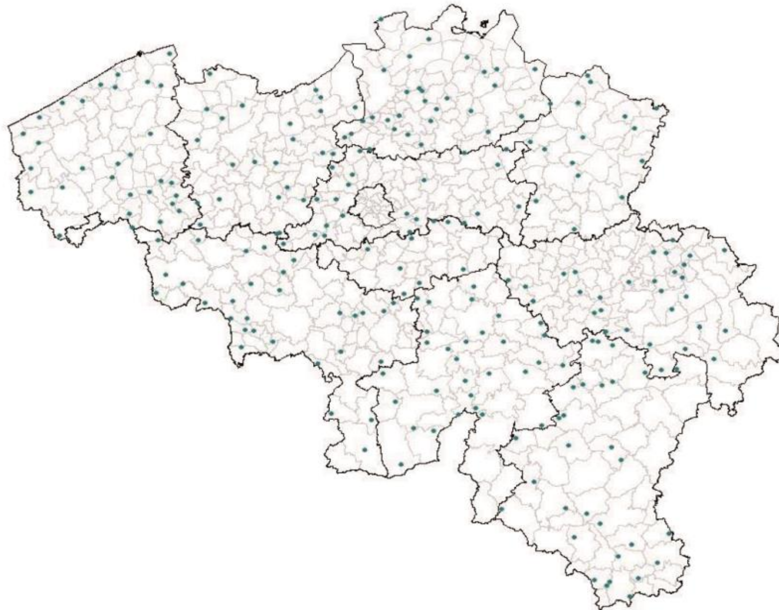
1. SYSTEME SENTINELLE MIS EN PLACE EN BELGIQUE

1.1. Repartition des exploitations sentinelles

La répartition géographique des 270 exploitations sentinelles était uniforme sur toute la Belgique (figure 1).

Figure 1

Localisation des 270 exploitations sentinelles sélectionnées en Belgique en mars 2007 en vue de détecter une nouvelle activité du virus de la fièvre catarrhale ovine
[Source : AFSCA]



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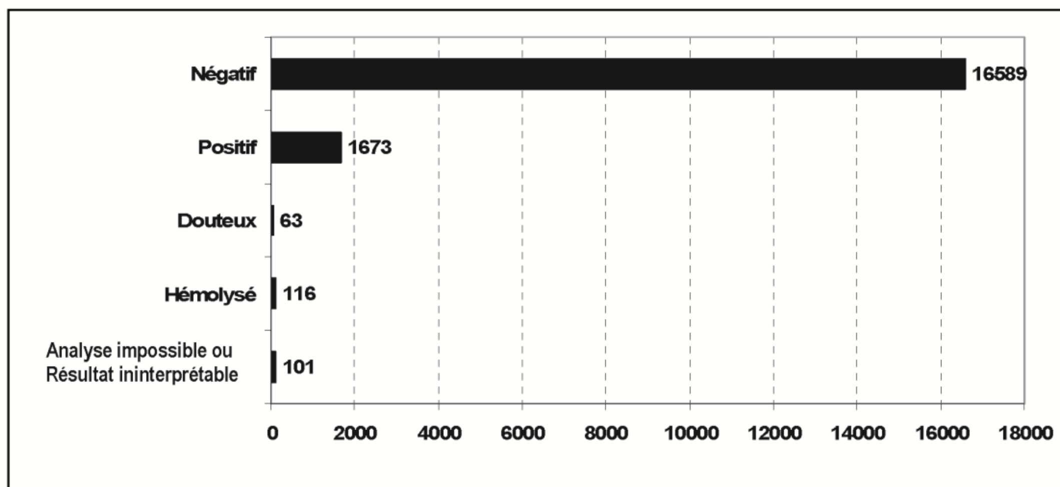
1.2. CONTROLE DE QUALITE DES PROCEDURES INITIALEMENT PREVUES

Quelques indicateurs de performance qualitatifs et quantitatifs ont été retenus.

L'évaluation qualitative concernait l'état des sérums (figure 2). Sur un total de 18 542 prélèvements, un pourcentage de résultats douteux (0,34%) et de sérums hémolysés, d'analyses impossibles ou de résultats ininterprétables (1,17%) a été constaté.

Figure 2

Répartition des sérums selon le résultat d'analyse et leur qualité (*N* = 18 542)



L'évaluation quantitative concernait le degré de complétude du programme sentinelle. Trois paramètres ont été analysés :

- les dates de réalisation des prélèvements par rapport à celles prévues initialement (figure 3) ;
- le nombre de répétitions des prélèvements sanguins par bovin (tableau 1) ;
- et le nombre total de prélèvements sanguins mensuels par rapport au nombre prévu initialement (figure 4).

De l'analyse, il ressort que :

- des pics de fréquence sont clairement identifiés aux dates de prélèvement prévues initialement. Dans 75% des cas, les dates ont été respectées. La marge de progrès est de 25% (figure 3) ;
- le nombre de répétitions des prélèvements sanguins par bovin (prélèvement mensuel) n'a pas été respecté (tableau 1) ;
- et le nombre total de prélèvements sanguins mensuels n'a atteint pleinement l'objectif retenu qu'au mois d'avril. Le degré de complétude n'a été que d'un peu moins de 75% durant les mois de mai à juillet 2007 (figure 4).

1.3. EVOLUTION TEMPORELLE DU RATIO ENTRE BOVINS SEROPOSITIFS ET BOVINS SERONEGATIFS

Le ratio entre les nombres de bovins séropositifs et séronégatifs est représenté à la figure 5. D'un niveau de base entre mars et juin 2007, le ratio a augmenté progressivement à partir de juillet pour atteindre un sommet en septembre 2007.

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Figure 3

Répartition des prélèvements sanguins en fonction de la date de prélèvement et des résultats relatifs à la fièvre catarrhale ovine (Belgique, année 2007, *N* = 16 375)

Légende : Axe des X, date de prélèvement ; Axe des Y, nombre de prélèvements ;
■, résultat sérologique négatif ; ■, résultat sérologique positif.

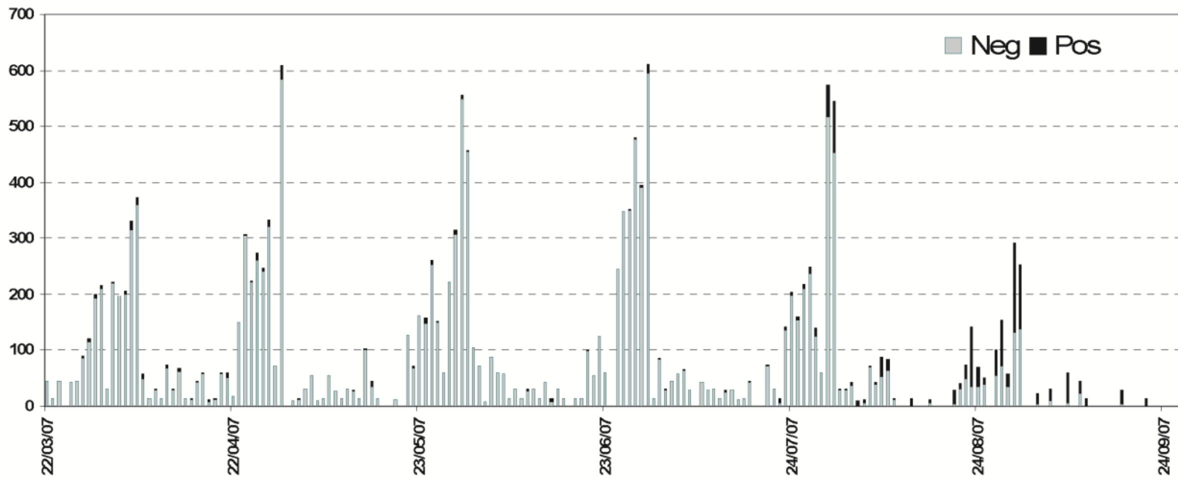
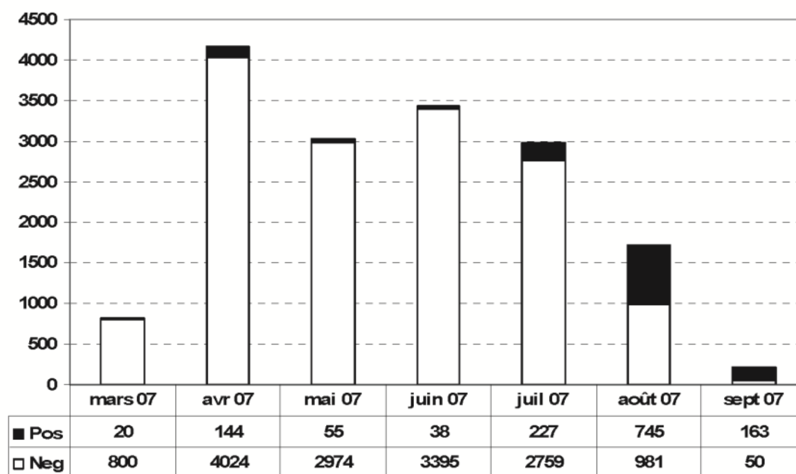


Figure 4

Répartition des prélèvements sanguins en fonction du temps (mois) et des résultats relatifs à la fièvre catarrhale ovine (Belgique, année 2007, *N* = 16 375)

Légende : Axe des X, mois ; Axe des Y, nombre de prélèvements.



	mars 07	avr 07	mai 07	juin 07	juil 07	août 07	sept 07
■ Pos	20	144	55	38	227	745	163
□ Neg	800	4024	2974	3395	2759	981	50

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Figure 5

Evolution mensuelle du ratio entre bovins séropositifs et séronégatifs au test ELISA pour la détection d'anticorps dirigés contre le virus de la fièvre catarrhale ovine (Belgique, année 2007, *N* = 16 375)

Légende : Axe des X, mois de l'année; Axe des Y, ratio entre bovins séropositifs et séronégatifs.

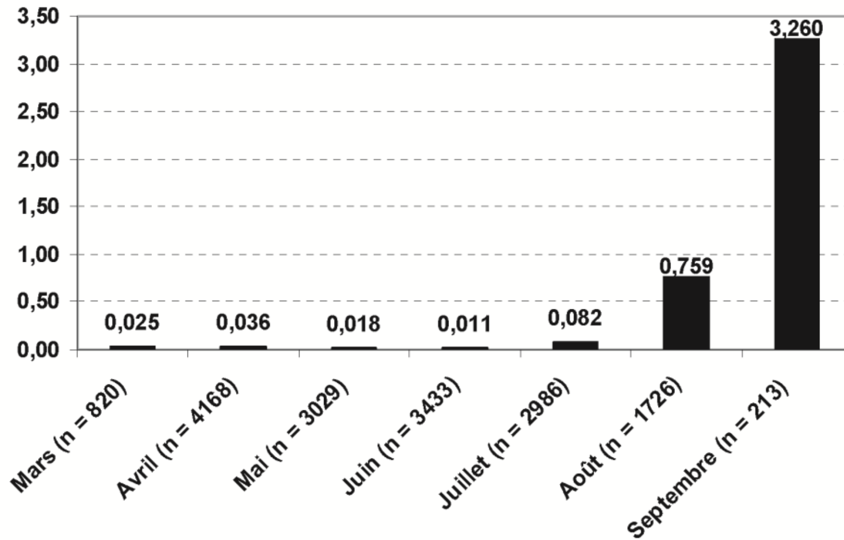

Tableau 1

Nombre de bovins en fonction du nombre de prélèvements réalisés par bovin (*N* = 16 789)

Nombre de prélèvement(s) par bovin [A]	Nombre de bovins concernés [B]	Total [A] x [B]
1	2 689	2 689
2	870	1 740
3	681	2 043
4	926	3 704
5	905	4 525
6	348	2 088
Total général		**16 789**

1.4. Investigation des resultats seropositifs

1.4.1. Prise en compte de la spécificité du test ELISA compétitif

Le niveau de base du ratio entre les nombres de bovins séropositifs et séronégatifs indique des séroconversions régulières mais à bas bruit. Ces séroconversions pourraient être attribuables soit à un défaut de spécificité du test ELISA, soit à une activité vectorielle à bas bruit, soit encore à des erreurs d'échantillonnage. Sur base d'une analyse bayésienne d'échantillons, collectés sur le terrain en Belgique durant l'épizootie de BTV-

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8, provenant d'animaux de statut sanitaire inconnu et contrôlés avec un test ELISA de compétition et une RTqPCR, la spécificité du test ELISA de compétition a été estimé à 98,2% avec un intervalle de confiance 95% variant de 96,3 à 99,6% [De Clercq *et al.*, 2008]. Tenant compte de la borne inférieure de cet intervalle de confiance, les taux enregistrés à partir de juillet traduisent très certainement un processus dynamique d'infection à BTV-8. Par contre, les taux de séroconversion des mois de mars à juin 2007 pourraient être compatibles avec un défaut de spécificité du test observé.

1.4.2. Prise en compte du pourcentage d'inhibition obtenu au test ELISA de compétition (résultats bruts)

Les résultats du test ELISA de compétition sont exprimés en pourcentages d'inhibition et ceux-ci ont également été analysés lorsqu'ils étaient disponibles (*N* = 4 187). L'évolution des paramètres statistiques de ce pourcentage au cours du temps a été suivie sur une base mensuelle (figure 6). Il ressort que ce pourcentage diminue également à partir du mois de juillet pour atteindre un plancher en septembre 2007, témoignant d'une réactivation du BTV-8.

1.4.3. Prise en compte des résultats du test RTqPCR

Un moyen d'évaluer si les séroconversions observées entre mars et juin 2007 provenaient d'un défaut de spécificité du test était de croiser les informations avec des résultats RTqPCR provenant des mêmes animaux. En particulier, l'évolution temporelle du ratio entre bovins « positifs » et « négatifs » au test RTqPCR de détection de l'ARN du virus de la fièvre catarrhale ovine peut être suivie (figure 7). Dès le mois d'avril 2007, une augmentation progressive de ce ratio est observée pour atteindre un sommet en juillet 2007, ce qui témoigne d'un début d'activité virale.

Figure 6

Evolution temporelle du pourcentage d'inhibition du test ELISA de compétition dans le cadre de la détection de la fièvre catarrhale ovine en Belgique entre avril et septembre 2007 (paramètres statistiques exprimés sous forme de boîtes à moustaches, plus communément appelées « boxplots »)

Légende : Axe des X, mois de l'année 2007 exprimés de 4 à 9 (avril à septembre) ; Axe des Y, pourcentage d'inhibition du test ELISA de compétition (plus le pourcentage est faible, plus la quantité d'anticorps détectés est élevée).

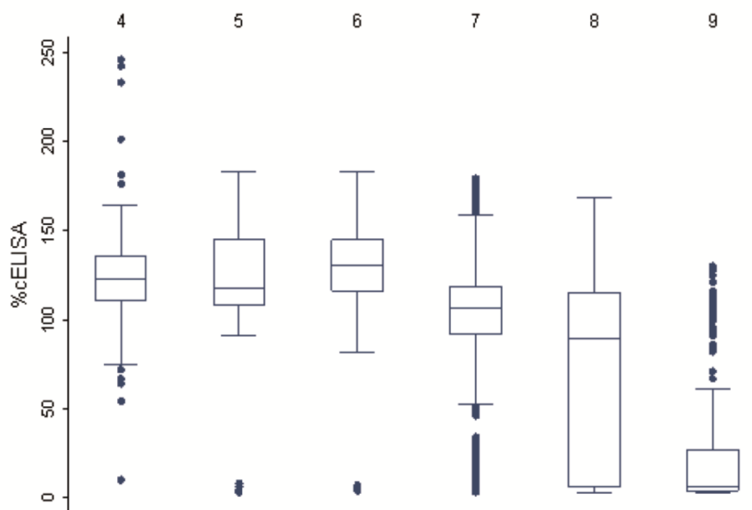

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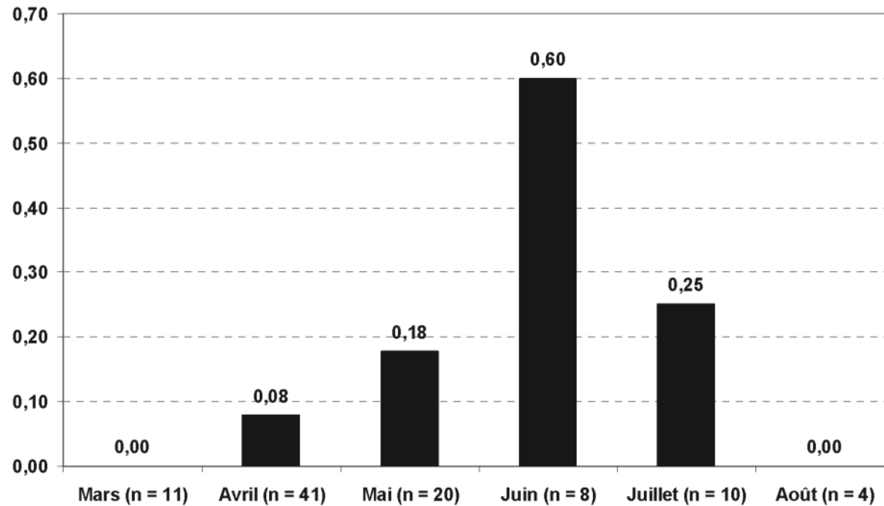
Figure 7

Evolution mensuelle du ratio entre bovins à réponse positive ou négative au test RTqPCR de détection de l'ARN du virus de la fièvre catarrhale ovine (*N* = 94)

Légende : Axe des X, mois de l'année ; Axe des Y, ratio entre bovins positifs et négatifs au test RTqPCR.



2. SYSTEME SENTINELLE DE LA PLATE-FORME D'INFECTIOLOGIE EXPERIMENTALE DE L'INRA DE TOURS-NOUZILLY (DEPARTEMENT 37)

Le suivi sentinelle de quelques ovins non vaccinés qui sont restés en pâture a permis d'observer une séroconversion entre le 10 juillet (semaine 28) et le 4 août 2008 (semaine 32) (tableau 2). Les foyers de FCO dans un rayon de 25 km autour du centroïde de la commune de Nouzilly ont été recherchés entre les semaines 28 à 32 (tableau 3). La séroconversion observée était bien un reflet de l'apparition de nombreux cas cliniques incidents de FCO dans un rayon de 25 km autour du centroïde de Nouzilly.

Tableau 2

Suivi sérologique de huit ovins sentinelles à la plate-forme d'infectiologie expérimentale INRA de Tours-Nouzilly

Ovin	14-mai-08			13-juin-08		10-juil-08		4-août-08		1-sept-08		22-sept-08		16-oct-08	
	Date	ELISA	PCR	Date	ELISA	Date	ELISA	Date	ELISA	Date	ELISA	Date	ELISA	Date	ELISA
1				13-juin-08	-	10-juil.-08	-	4-août-08	-	1-sept.-08	-	22-sept.-08	-	16-oct.-08	-
2										1-sept.-08	-	22-sept.-08	-	16-oct.-08	-
3								4-août-08	-	1-sept.-08	-	22-sept.-08	-	16-oct.-08	-
4										1-sept.-08	-	22-sept.-08	-	16-oct.-08	-
5	14-mai-08	-	-	13-juin-08	-	10-juil.-08	-	4-août-08	-	1-sept.-08	-	22-sept.-08	-	16-oct.-08	-
6	**14-mai-08**	-	-	**13-juin-08**	-	**10-juil.-08**	-	**4-août-08**	+	**1-sept.-08**	+	**22-sept.-08**	+	**16-oct.-08**	+
7										1-sept.-08	-	22-sept.-08	-	16-oct.-08	-
8				13-juin-08	-	10-juil.-08	-	4-août-08	-	1-sept.-08	-	22-sept.-08	-	16-oct.-08	-

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IV - DISCUSSION

L'analyse des résultats des systèmes animaux sentinelles mis en place indique qu'ils permettent d'alerter les parties prenantes d'une réapparition de la FCO (figures 5 à 8).

Bien que d'ampleur limitée, le suivi d'ovins mis en place à la plate-forme d'infectiologie expérimentale de l'INRA (qui n'étaient pas, à l'origine, destinés à constituer un lot sentinelle), a permis d'être le reflet de l'apparition de nombreux cas cliniques incidents de FCO dans un rayon de 25 km autour du centroïde de la commune de Nouzilly. Comme les animaux suivis n'ont pas fait l'objet de prélèvements systématiques (tableau 2), il est difficile d'évaluer le potentiel de précocité de la détection par rapport aux exploitations environnantes où des cas cliniques de FCO avaient été constatés (tableau 3).

Concernant le système belge d'exploitations sentinelles mis en place, celui-ci a bien permis de détecter la réapparition de la FCO en 2007 (figure 8). Cependant, tenant compte du niveau de qualité énoncé au point 3.1.2., la sensibilité du réseau sentinelle n'était pas des plus élevée puisque les premiers foyers cliniques ont été détectés au même moment que l'augmentation d'incidence des séroconversions observées dans le réseau sentinelle, en juillet 2007.

Tableau 3

Foyers de fièvre catarrhale ovine déclarés dans un rayon de 25 km autour du centroïde de la commune de Nouzilly durant les semaines 28 à 32

Commune (département)	Espèce	Date de suspicion	Type de suspicion
CHEMILLE-SUR-DEME (37)	Ovin	13/07/2008	Clinique
SAINT-ARNOULT (41)	Bovin	17/07/2008	Clinique
MONNAIE (37)	Bovin	18/07/2008	Clinique
LES HERMITES (37)	Bovin	21/07/2008	Clinique
VILLEDIEU-LE-CHATEAU (41)	Bovin	22/07/2008	Clinique
LES HERMITES (37)	Bovin	22/07/2008	Clinique
LES HAYES (41)	Bovin	26/07/2008	Clinique
MONTREUIL-EN-TOURAINE (37)	Bovin	28/07/2008	Clinique
SAINT-LAURENT-EN-GATINES (37)	Ovin	29/07/2008	Clinique
NEUVY-LE-ROI (37)	Bovin	29/07/2008	Clinique
COUTURE-SUR-LOIR (41)	Ovin	29/07/2008	Clinique
AUTHON (41)	Ovin	30/07/2008	Clinique
SAINT-MARTIN-DES-BOIS (41)	Ovin	1/08/2008	Clinique
VILLEDIEU-LE-CHATEAU (41)	Bovin	1/08/2008	Clinique
LES HERMITES (37)	Bovin	2/08/2008	Clinique
SAINT-MARTIN-DES-BOIS (41)	Bovin	2/08/2008	Clinique
COUTURE-SUR-LOIR (41)	Ovin	4/08/2008	Clinique
TREHET (41)	Caprin	4/08/2008	Clinique

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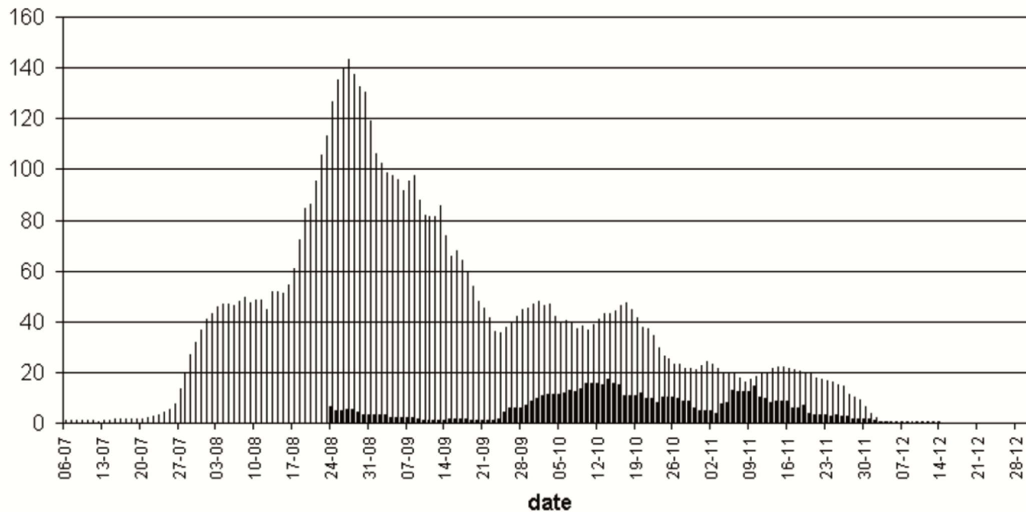
Figure 8

Evolution temporelle des notifications officielles des cas de fièvre catarrhale ovine en Belgique durant l'année 2006 (en grisé foncé) et 2007 (en grisé clair) [Source : AFSCA]

Légende : Axe des X, date ; Axe des Y, nombre de troupeaux infectés par la fièvre catarrhale ovine.



Plusieurs éléments sont à mettre en exergue à propos du réseau sentinelle :

- la bonne *représentativité* des troupeaux sentinelles (adéquation avec le Règlement (CE) N° 1266/2007) ;
- la *rapidité* du système de détection en tenant compte des caractéristiques des tests de diagnostic utilisés (*sensibilité* et *spécificité*). Toutefois, cette rapidité est tributaire d'une analyse proactive des résultats du système sentinelle mis en place. Un point crucial est de tenir compte des résultats quantitatifs (par exemple, le ratio entre les nombres de résultats positifs et négatifs au test ELISA et au test RTqPCR ou l'évolution temporelle des pourcentages d'inhibition du test ELISA compétitif, …). De plus, lorsque le taux de résultats positifs est inférieur à 100% moins la valeur de la borne inférieure de l'intervalle de confiance de la spécificité du test (c'est-à-dire : 100% - 96,3% = 3,7%), la mise en œuvre de tests RTqPCR permet d'attribuer ou non les sérologies observées à des animaux ayant rencontrés du virus de la FCO. Une réflexion doit avoir lieu sur le choix du test ELISA et du test RTqPCR à utiliser. Ces tests doivent, en première instance, pouvoir détecter un large panel de sérotypes possibles. En cas de résultats positifs à une RTqPCR capable d'identifier l'ARN de tous les sérotypes, il y a lieu d'investiguer ultérieurement, avec des PCR spécifiques, le ou les sérotypes circulants. Ceci est de nature à permettre une certaine *flexibilité* du système sentinelle à d'autres sérotypes que ceux initialement prévus ;
- la séparation stricte entre les prélèvements programmés mensuellement et les contrôles ultérieurs est à conseiller en vue d'assurer une *simplicité* maximale de la procédure ainsi que la *stabilité* du système de surveillance. Idéalement les contrôles, en particulier les premiers, devraient absolument être réalisés par un épidémiologiste chevronné qui, par une visite sur le terrain, pourra réaliser une enquête épidémiologique assortie de prélèvements en vue de confirmer ou d'infirmer le ou les résultats séropositifs et en vue d'investiguer l'activité vectorielle locale ;
- un retour d'information à destination de tous les acteurs impliqués dans le réseau

sentinelle devrait être mis en œuvre systématiquement et périodiquement en vue d'une meilleure *acceptabilité* du système par les acteurs de la surveillance. Ce retour d'information est susceptible d'améliorer le suivi mensuel d'un maximum de troupeaux inclus dans le réseau.

Outre ce qui précède, des éléments additionnels d'amélioration peuvent être proposés :

- une mise en place du système plus tôt dans l'année. En effet, plusieurs éléments plaident en cette faveur tels que, par exemple, la preuve d'une activité vectorielle dès le mois de février [Hoffmann *et al.*, 2009] et la récente découverte de sites d'émergence de *Culicoïdes* à l'intérieur des étables même en hiver [Zimmer *et al.*, 2010] ;
- Une analyse des résultats en temps réel. Pour cela, un animateur du réseau sentinelle devrait être clairement identifié ;
- La mise en place d'indicateurs de performances concernant l'évaluation de l'activité du réseau sentinelle, à l'instar de ce qui existe déjà pour les réseaux d'épidémiosurveillance [par exemple, Ouagal *et al.*, 2004], est suggérée ;
- Enfin, une analyse coûts/bénéfices du système mis en place devrait être initiée, en particulier en vue de comparer ce dernier au réseau d'épidémiosuveillance passive (clinique).

V - CONCLUSION

Un suivi d'exploitations sentinelles concernant l'émergence de nouveaux sérotypes ou la réémergence de sérotypes connus du virus de la FCO reste d'actualité en Europe. Une réflexion doit avoir lieu afin d'adapter les réseaux sentinelles existant à la situation épidémiologique et ce, dans le cadre d'une stratégie vaccinale. L'efficacité et la pérennité de tels réseaux ne peuvent être appréciées qu'à travers des évaluations régulières et/ou l'élaboration et le suivi en continu d'indicateurs de performance [Centers for disease control and prevention, 2001]. Ces systèmes sentinelles nécessitent des compétences, des ressources et des efforts coordonnés et synergiques entre toutes les parties prenantes [Saegerman *et al.*, 2003]. Enfin, il convient de rappeler qu'un système d'animaux sentinelles n'est pas simple à gérer et que celui-ci vient en support du réseau d'épidémiosurveillance clinique et ne peut en aucun cas le supplanter.

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ANNEXE

Règles internationales concernant l'usage d'animaux sentinelles dans le cadre de la fièvre catarrhale ovine [Organisation mondiale de la santé animale, 2009]

Le recours à des animaux ou unités sentinelles est une forme de surveillance spécifique reposant sur un protocole prospectif. Il s'agit de la stratégie préférée pour la surveillance du virus de la fièvre catarrhale du mouton. Les unités sont constituées de groupes d'animaux non antérieurement exposés au virus, se trouvant sur des sites fixes et régulièrement soumis à des prélèvements pour détecter toute nouvelle infection par ce virus.

Le principal objectif d'un programme reposant sur l'utilisation d'animaux sentinelles est de détecter les infections dues au virus de la fièvre catarrhale du mouton sur un site donné. Ainsi, les unités sentinelles peuvent être localisées par exemple sur les limites habituelles des zones infectées pour détecter les changements de distribution virale. Ces programmes permettent par ailleurs d'observer les facteurs temporels et la dynamique qui caractérisent les infections.

Les programmes faisant appel à des animaux sentinelles doivent retenir des animaux dont l'origine et l'historique des expositions sont connus, doivent maîtriser les paramètres de soins tels que l'usage des insecticides et la stabulation (en fonction de l'épidémiologie du virus de la fièvre catarrhale du mouton dans le secteur considéré) et doivent rester souples dans leur conception en termes de fréquence des prélèvements et de choix des tests.

Les sites des groupes sentinelles doivent être choisis avec soin. L'objectif est de maximiser les chances de détecter une activité du virus de la fièvre catarrhale du mouton sur le lieu géographique où le site sentinelle joue le rôle de point de prélèvement. L'effet de facteurs secondaires susceptibles d'influencer les résultats dans chaque site (facteurs climatiques par exemple) peut également être analysé. Pour éviter les biais, les groupes sentinelles doivent être constitués d'animaux d'âge et de sensibilité similaires. Les bovins constituent les sentinelles les mieux adaptées, mais d'autres espèces de ruminants domestiques peuvent également être utilisées. La localisation géographique doit être la seule caractéristique qui doit différencier les groupes sentinelles entre eux.

Les prélèvements sériques recueillis dans le cadre des programmes faisant appel à des animaux sentinelles doivent être conservés méthodiquement dans une banque de sérums afin de permettre la conduite d'études rétrospectives en cas d'isolement de nouveaux sérotypes. La fréquence des prélèvements dépend des raisons expliquant le choix du site. Dans les secteurs enzootiques, l'isolement des virus permet de surveiller les sérotypes et les génotypes des virus circulant au cours des différentes périodes. Les limites entre les secteurs infectés et non infectés peuvent être définies par la mise en évidence sérologique de l'infection. Les prélèvements sont habituellement réalisés une fois par mois. Le fait de placer des animaux sentinelles dans des zones déclarées indemnes permet de confirmer que les infections provoquées par le virus de la fièvre catarrhale du mouton ne passent pas inaperçues. Dans ce cas, des prélèvements effectués avant et après la période potentielle de transmission sont suffisants. L'isolement et l'identification du virus apportent une conclusion définitive sur les virus de la fièvre catarrhale du mouton circulant dans un pays ou une zone donné(e). S'il est nécessaire d'isoler le virus, les animaux sentinelles doivent faire l'objet de prélèvements suffisamment fréquents pour avoir la certitude que des échantillons sériques sont obtenus durant la période de virémie.

« La vraie langue d'une science est celle qui en facilite l'étude, en mettant ses principes à la portée de tout le monde. Le pédant qui ne cherche qu'à en imposer et non à enseigner, surcharge ses leçons de termes inutiles, et sème à chaque pas des difficultés qui finissent par dégoûter celui que son inclination portait naturellement à l'étude, et qui y aurait pris goût si on lui avait aplani les routes de la science, au lieu de les hérisser d'épines : manière que n'ont adopté, au reste, nos petits charlatans scientifiques que pour éblouir les sots qui, entendant des mots qu'ils ne comprennent souvent pas, regardent ceux qui les débitent comme des êtres doués d'un savoir supérieur. »

François Levaillant (1753-1824), discoverer of the Bluetongue disease.

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