University of Liege - Faculty of Veterinary Medicine
Department of Infectious and Parasitic Diseases,
Fundamental and Applied Research on Animal and Health (FARAH)
Research Unit of Epidemiology and Risk Analysis Applied to Veterinary Sciences (UREAR-ULg)

Epidemiology of Schmallenberg virus in Belgium and study of its pathogenesis in sheep

Epidémiologie du virus Schmallenberg en Belgique et étude de sa pathogenèse chez le mouton

Antoine Poskin

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I would like to dedicate this manuscript to my lovely Emeline…

ABSTRACT

During summer 2011, cattle presented severe hyperthermia combined with dropped milk yield and diarrhoea from unknown origin. In October 2011, blood was collected from cattle presenting these clinical signs in Schmallenberg, a small city in West Germany. A new *Orthobunyavirus*, responsible for these unspecific clinical signs was identified and named Schmallenberg virus (SBV). Upon November 2011, an epizootic outbreak of abortion, stillbirths and malformed new-born was observed in bovine, ovine and caprine herds in Europe due to transplacental transmission of SBV to the foetus. The SBV vectors are small hematophagous midges of the gender *Culicoides.*

This work contributed to estimate the impact of the SBV epidemic in Belgium (Study 1). On the basis of farmer's observations, between 0.5% and 4% of calves were aborted, stillborn or malformed due to SBV in 2011-2012. Abortions and stillbirths were not clear consequences of the SBV outbreak in cattle. In sheep, between 11% and 19% of lambs were aborted, stillborn or malformed due to SBV in 2011-2012. Deformed animal was the most important finding of SBV outbreak at herd level and an essential condition for the farmer to send suspected samples to the National Reference Laboratory (NRL) for SBV analysis. The results gathered from the study indicate that SBV surveillance and monitoring should be implemented by SBV RNA detection with rRT-PCR in organs collected from stillborn and deformed calves and lambs born in big herds.

The high impact of SBV highlighted in the Study 1 was putatively explained by unknown host supporting the SBV activity. In this respect, the role of pigs had never been evaluated. This was essential considering the suggested role of the domestic pigs in the life-cycle of the SBV-closely related Akabane virus (AKAV) (Huang *et al*., 2003). The absence of RNAemia after experimental infection of piglets with SBV realized in the Study 2 of the

thesis suggests the absence of obvious role of domestic pigs in SBV life-cycle. The absence of RNAemia is indeed a strong indication that further spread of SBV from the pigs to the *Culicoides* during a blood meal of the vector is not likely to occur, therefore making impossible an SBV transmission. The limited and temporary seroconversion observed after SBV inoculation in only half of the inoculated piglets and the absence of seroconversion reported in a limited number of field collected samples support this consideration.

To prevent SBV progression, it was crucial to further study the pathogenesis of SBV. The Study 1 proved that the most important clinical impact of SBV was the consequence of the malformed new-born; hereto it was particularly crucial to improve the knowledge on the development of the SBV-related teratogenic effects. In this respect, experimental infection of pregnant sheep with SBV constituted an appropriate research approach. An experimental model was therefore essential to standardize. This thesis contributed to the standardization of *in vivo* experiments (in collaboration with another working group) by determining the minimum infectious dose of an SBV infectious inoculum. This reference infectious serum must contain approximately 20 $TCID_{50}$ to induce a homogeneous effective infection in sheep. This dose is rather low and could be inoculated by a single *Culicoides* under natural conditions. Beyond this minimum infectious dose, no dose dependent effect was observed in productively inoculated ewes, either in the duration of the RNAemia, the quantity of SBV RNA detected by rRT–PCR in the blood, or in the number of SBV RNA copies present in the organs collected at necropsy.

The experimental model developed (partly) in the Study 3 was used to inoculate pregnant ewes at day 45 and 60 of gestation, and increase the knowledge on SBV transplacental transmission. The inoculation induced the persistence of SBV RNA in placental organs until birth. Schmallenberg virus RNA was recovered from the organs collected at birth from the lambs of both groups. However, the chance to obtain SBV RNA positive placental

organs was significantly higher when the infectious inoculum was inoculated at day 60 of gestation. Positive organs in lambs included CNS and muscle, but no malformation was observed in new-born lambs. This absence of malformations suggests that SBV inoculation must occur earlier than the day 45 of gestation to produce teratogenic effects in sheep. Also, the persistence of SBV RNA in the foetal envelope is indicative of a putative mean for SBV overwintering.

The Study 4 highlighted a 6 month persistent seroconversion in the absence of SBV surinfection. In the meantime, SBV circulation drastically dropped on the field and the absence of SBV circulation could induce the sheep to become seronegative under natural conditions. In the Study 5, the experimental model developed in the Study 3 was used to demonstrate that one single SBV inoculation can induce a protective immunity in sheep that persists during a minimum period of 15 months. This experiment highlights that 2 successive periods of SBV circulation, spared of one year, is not likely to induce malformations on the field the second year.

Based on the experience gathered with the closely related AKAV, recurrent outbreaks of congenital events can be expected for a long period. Vaccination of seronegative animals could be used to prevent the deleterious effects of SBV in case of SBV re-emergence. During this epidemic, different surveillance approaches including syndromic surveillance, sentinel herd surveillance, cross-sectional seroprevalence studies and pathogen surveillance in vectors have proven their utility and complementarity and should be considered to continue in the future in order to monitor the SBV dynamic.

RESUME

Durant l'été 2011, des vaches ont présenté des hyperthermies sévères combinées avec des chutes de production laitière et de la diarrhée d'origine inconnue. En octobre 2011, du sang a été prélevé sur des vaches présentant ces signes cliniques à Schmallenberg, une petite ville dans l'Ouest de l'Allemagne. Un nouvel *Orthobunyavirus*, responsable de ces signes cliniques aspécifiques a pu être identifié, et fut nommé virus Schmallenberg (SBV). A partir de novembre 2011, une épizootie d'avortements, de mortinatalités, et de nouveau-nés malformés a été observée chez les bovins, les ovins et les caprins en Europe des suites de la transmission transplacentaire du SBV vers le fœtus. Les vecteurs du SBV sont des petits insectes hématophages appelés *Culicoides*.

Ce travail a contribué à l'estimation de l'impact du SBV en Belgique (Etude 1). Sur base des observations faites par les éleveurs, entre 0.5% et 4% des veaux étaient avortés, mort-nés ou malformés à cause du SBV en 2011-2012. Les avortements et les mortinatalités n'étaient pas des conséquences claires de l'émergence du SBV chez les bovins. Chez les moutons, entre 11% et 19% des agneaux étaient avortés, mort-nés ou malformés à cause du SBV en 2011-2012. Les animaux malformés constituaient la principale conséquence clinique de l'émergence du SBV au niveau du troupeau et une condition essentielle pour que le fermier suspecte la présence du SBV et que des échantillons puissent être envoyés au laboratoire national de référence (LNR) pour des analyses SBV. Les résultats de l'étude montrent que la surveillance et le monitoring du SBV devraient être mise-en-œuvre par la détection d'ARN de SBV dans des organes prélevés chez des veaux et des agneaux mort-nés ou malformés dans les troupeaux de grande taille.

L'impact élevé du SBV (Etude 1) était potentiellement expliqué par la présence d'espèces hôtes non-révélées expliquant l'expansion du SBV. En tenant compte de ces

considérations, il était essentiel d'étudier le rôle potentiel de l'espèce porcine dans le cycle de vie du SBV, puisque le porc a un rôle suggéré dans le cycle de vie du virus Akabane (AKAV) (Huang *et al*., 2003), un virus proche du SBV (Etude 2). L'absence d'ARNémie après une infection expérimentale de porcelets avec du SBV suggère cependant que le porc ne joue pas de rôle évident dans le cycle de vie du SBV. L'absence d'ARNémie rend en effet impossible une transmission vers un *Culicoides* lors d'un repas sanguin, et donc empêche la transmission. La séroconversion faible et temporaire observée chez la moitié des porcelets inoculés, ainsi que l'absence de séroconversion identifiée dans un nombre limité d'échantillons sanguins de porcs domestiques prélevés sur le terrain supportent par ailleurs cette conclusion.

Pour entraver la progression du SBV, il est crucial d'accroitre la connaissance sur la pathogenèse du SBV, en particulier sur ses effets tératogènes, qui constituent le principal impact clinique du SBV (Etude 1). Pour cela, une infection expérimentale de brebis gestantes avec le SBV était l'approche scientifique la plus adaptée. Il s'avérait dès lors essentiel de développer un modèle expérimental d'infection par le SBV. Durant cette thèse, un modèle expérimental *in vivo* a été développé et standardisé (en collaboration avec un autre groupe de recherche) par le biais d'une étude de la dose minimale infectieuse d'un sérum infectieux de SBV (Etude 3). Ce sérum infectieux de référence doit contenir approximativement 20 $TCID_{50}$ pour induire une infection productive chez le mouton. Cette dose est plutôt faible et pourrait être inoculée par un seul *Culicoides* en conditions naturelles. Au-delà de cette dose, aucun effet dose-dépendant sur la durée de l'ARNémie, sur la quantité d'ARN de SBV détectée dans le sang, ou dans le nombre de copies d'ARN de SBV dans les organes prélevés à l'autopsie n'a été observé.

Le modèle expérimental développé (en partie) dans l'Etude 3 a été utilisé pour accroitre la connaissance sur la transmission transplacentaire du SBV. L'inoculation de brebis gestantes avec un sérum infectieux de SBV aux jours 45 et 60 de la gestation a été mise-en-

œuvre (Etude 4). Dans cette expérience, la persistance d'ARN de SBV a été constatée dans les organes placentaires à la naissance des agneaux. La probabilité d'obtention d'organes contenant de l'ARN de SBV était significativement supérieure lorsque le sérum infectieux était inoculé au jour 60 de la gestation. Les organes positifs chez les agneaux étaient le système nerveux central et les muscles. Aucune malformation n'a été observée chez les agneaux nouveau-nés. L'absence de malformations après une inoculation au jour 45 et 60 de la gestation suggère que l'infection doit avoir lieu avant le jour 45 de la gestation pour induire des effets tératogènes. Par ailleurs, la persistance d'ARN de SBV dans les enveloppes fœtales suggère un potentiel nouveau moyen d'hivernation du virus.

L'Etude 4 a mis en exergue une séroconversion persistant au moins 6 moins, en l'absence de surinfection. En parallèle, la circulation de SBV a chuté de manière drastique sur le terrain, cette absence de circulation virale laissait craindre que des moutons puissent redevenir séronégatifs. Dans l'Etude 5, le modèle expérimental développé (Etude 3) a été utilisé pour démontrer qu'une seule infection avec du SBV induit une immunité protectrice et persistante pendant au moins 15 mois. Une seule inoculation est donc suffisante pour prévenir la transmission du SBV et l'apparition de malformations chez les durant cette période.

Sur base de l'expérience tirée de l'AKAV, des épisodes récurrents d'évènements congénitaux dus au SBV peuvent être craints. La vaccination des animaux séronégatifs devrait être réalisée en prévention des effets délétères du SBV en cas de réémergence virale. Pendant cette épidémie, différentes approches de surveillance, incluant une surveillance syndromiques, une surveillance *via* des troupeaux sentinelles, des études de séroprévalence cross-sectionnelles et la surveillance des pathogènes dans les vecteurs ont démontré tout leur intérêt et complémentarité, et devraient être poursuivis à l'avenir.

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I had a lot of special colleagues at ENZOREM, but Sara, you are the one I spent most of my time with. I cannot remember a bad memory or a day when you were not smiling. As a matter of fact, they were not and you have a contagious happiness. It was a pleasure to share an office with you in all our good moments. I wish you all the best for the following months. You are the next on the list of PhD.

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I would like to thank all the friends from Belgium and France for the great memories during these three years. A happy private life allowed me to accomplish my work with pleasure and passion.

I would like to finish this acknowledgment section thanking to next special person to come in my life, Baptiste, who I can wait to finally meet after nine months of incubation, you have great parents and you are already special to me.

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LIST OF ABBREVIATIONS

aa	Amino acid
AI	Artificial insemination
AIC	Aikake Information Criterion
AINO	Aino virus
AKAV	Akabane virus
ARSIA	Association Régionale de Santé et d'Identification Animales
BBB	Blood–brain barrier
BHK	Baby hamster kidney
BSL 3	Biosafety level 3
BTV	Bluetongue virus
BVDV	Bovine viral diarrhea virus
CNS	Central nervous system
CODA–CERVA	Veterinary and Agrochemical Research Centre
Cq	Quantification cycle
cRNA	Complementary sense RNA
Ct	Cycle threshold
DGZ	Dierengezondheidszorg Vlaanderen
DOUV	Douglas virus
DMEM	Supplemented Dulbecco's Modified Eagles Medium
dpi	Days post–infection
EMC	Porcine encephalomyocarditis
ER	Endoplasmic reticulum
FLI	Friedrich Loeffler Institute
G45	Group inoculated at day 45 of gestation
G60	Group inoculated at day 60 of gestation
HRP	Horseradish peroxidase
IFN	Interferon
Ig	Immunoglobulin
IPH–VAR	Scientific Institute of Public Health – Veterinary and Agrochemical Research Center
L segment	Large segment
LNR	Laboratoire national de référence
M segment	Medium segment

Mab	Monoclonal antibodies
MALDI-TOF MS	Matrix-assisted laser desorption/ionization-time of flight spectrometry
MEM	Minimum Essential Medium
MIR	Minimum infectious rate
MID	Minimal infectious dose
mRNA	messenger RNA
N protein	Nucleocapsid protein
NRL	National Reference Laboratory
NSm	Non–structural protein M
NSs	Non–structural protein S
nt	Nucleotide
OD	Optical density
ORO	Oropouche virus
ORF	Open reading frame
OVI	Onderstepoort Veterinary Institute
PAG	Pregnancy associated glycoprotein
PBS	Phosphate buffered saline
PCV2	Porcine circovirus 2
PEAV	Peaton virus
PI	Persistently infected
PMSG	Pregnant mare serum gonadotrophin
PRRSV	Porcine reproductive and respiratory syndrome virus
RdRp	RNA–dependant RNA polymerase
RIA	Radio immune–assay
RNP	Ribonucloprotein complex
RPB1	Largest subunit of the RNA polymerase II
rRT–PCR	Real–time reverse transcription polymerase chain reaction
RTVOL	Réseau Technique Vétérinaire Objectif Lait
S segment	Small segment
S1	Season 1
S2	Season 2
S3	Season 3
SATV	Sathuperi virus
SABOV	Sabo virus
SANV	Sango virus

SBV	Schmallenberg virus
SC	Subcutaneously
SHAV	Shamonda virus
SHUV	Shuni virus
SIMV	Simbu virus
SIV	Swine influenza virus
$TCID_{50}$/ml	50% tissue culture infective dose/ml
TMB	Tetramethylbenzidine
ULg	Université de Liège
VNT	Virus neutralization test
vRNA	viral RNA

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PART ONE : REVIEW OF LITERATURE

CHAPTER 1 : GENERAL INTRODUCTION

1.1 Identification of a new emerging virus in Europe

During summer 2011, several dairy cattle herds in Germany experienced high fever, decreased milk yield and severe diarrhea from unknown origin. In October 2011, blood samples were collected from cattle showing these clinical signs in Schmallenberg, a small city located in Western Germany. Metagenomic analysis identified a novel *Orthobunyavirus* which was subsequently isolated from blood of affected animals and that was probably responsible for this unspecific syndrome. This new virus was provisionally named Schmallenberg virus (SBV) according to the place of its first identification (Hoffmann *et al*., 2012). Retrospective studies did not identify SBV presence in Germany before 2010 (Gerhauser *et al*., 2014). Starting from November 2011, a wide outbreak of aborted, stillborn and malformed newborns due to transplacental infection with SBV was observed in cattle, sheep and goat (Garigliany *et al*., 2012b).

1.2 Virology of Schmallenberg virus

Taxonomy and phylogenetic relationships of Schmallenberg virus

Sequencing analysis classified SBV in the family *Bunyaviridae* (Hoffman *et al*., 2012). The *Bunyaviridae* family contains 350 viruses, classified into five genera: *Orthobunyavirus, Hantavirus, Nairovirus, Phlebovirus* and *Tospovirus* (Doceul *et al*., 2013). *Bunyaviruses* of veterinary importance are Rift Valley fever virus (Phlebovirus), Akabane virus (AKAV, *Orthobunyavirus*) and Nairobi sheep disease virus (*Nairovirus*); Bunyaviruses affecting humans include hantaviruses (haemorrhagic fever with renal syndrome and cardio-

pulmonary syndrome) and Crimean-Congo haemorrhagic fever virus (*Nairovirus*) (Doceul *et al*., 2013). Schmallenberg virus is an *Orthobunyavirus*, and a member of the Simbu serogroup to which Aino virus (AINO), AKAV, Douglas virus (DOUV), Oropouche virus (ORO), Sathuperi virus (SATV) and Shamonda virus (SHAV) belong (Figure 1) (Hoffmann *et al*., 2012).

Contradictory results have been published on the origin of SBV. First sequencing analysis conducted by Hoffmann *et al*. (2012) identified a high degree of homology between SBV and SHAV S–segment, AINO M–segment and AKAV L–segment. Another study suggested that SBV is a reassortant between SATV M–segment and SHAV S and L–segments (Yanase *et al*., 2012), while a third study suggested that SBV belongs to the species SATV and is likely to be the ancestor of SHAV (Goller *et al*., 2012). As a matter of fact, the exact origin of SBV remains unclear, but the *Bunyaviruses* have a propensity for reassortment due to their segmented RNA genome (Lievaart-Peterson *et al*., 2015).

Akabane virus is probably the most studied virus within the Simbu serogroup. Since its first isolation in 1958, evidence of virus presence was reported in four continents: Asia, Oceania, Europe (Cyprus) and East–Africa (Markusfeld and Mayer, 1971; Nobel *et al*., 1971; Parsonson *et al*., 1977; Sellers and Herniman, 1981; Al–Busaidy *et al*., 1987; Taylor and Mellor, 1994; Kono *et al*., 2008). The last known emergence of the more restricted SATV and SHAV took place in Japan back in 1999 and 2002, respectively (Yanase *et al*., 2004 and 2005). SBV was therefore the first known *Orthobunyavirus* of veterinary importance to emerge in continental Europe (Saeed *et al*., 2001).

The AKAV is a *Culicoides*-borne virus inducing clinical signs similar to those observed with SBV infected hosts including the congential defects (arthrogryposis-hydranencephaly) and the drop of milk production, and infects the same species as SBV:

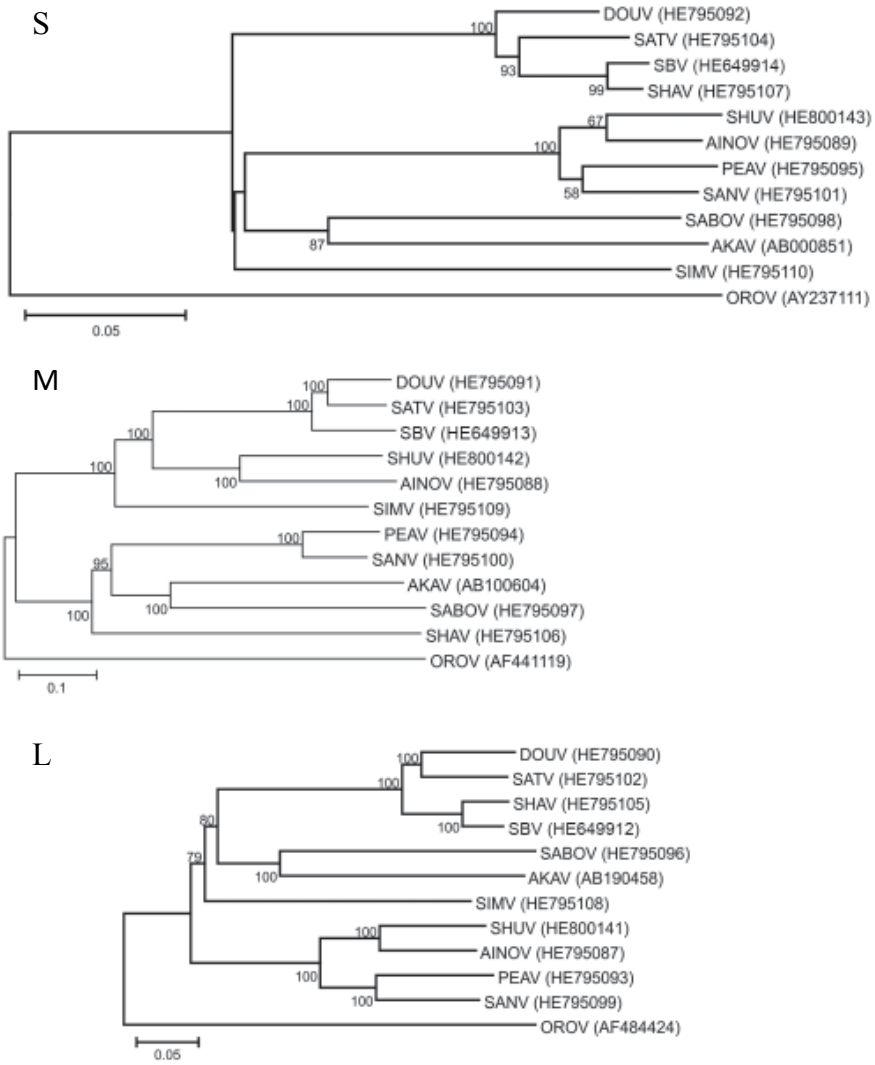

Figure 1: Phylogenetic relationships of Schmallenberg virus

The maximum–likelihood tree for phylogenetic relationship between the Schmallenberg virus (SBV) S segment (S); the SBV M segment (M); and the SBV L segment (L) and viruses belonging to the Simbu serogroup: Douglas virus (DOUV), Sathuperi virus (SATV), Shamonda virus (SHAV), Shuni virus (SHUV), Aino virus (AINOV), Peaton virus (PEAV), Sango virus (SANV), Sabo virus (SABOV), Akabane virus (AKAV), Simbu virus (SIMV), Oropouche virus (OROV). The numbers indicate percentage of 1000 bootstrap replicates (with value higher than 50). The phylogenetic inferences were deduced from coding sequences of the detailed segments. The tree was obtained from Goller *et al*. (2012).

cattle, sheep and goat (Martinelle *et al*., 2012). Upon experimental infection at day 30 and 36 of gestation, until 36% of seropositive lambs and 80% of malformed lambs can be observed, seropositive lambs can born with or without precolostrum antibodies, regardless of the malformations (Parsonson *et al*., 1977 and 1981). Malformations are highly unlikely to happen after infection at day 50 of gestation (Martinelle *et al*., 2012). Interrestingly, the pigs have a suggested role in the life-cycle of AKAV, hereto viraemia and virus replication in organs were observed upon oronasal infection with an infectious inoculum (Huang *et al*., 2003).

Virus structure and pathogenicity

Orthobunyaviruses are enveloped RNA viruses with a negative sense, single–stranded genome. The virion has a diameter of approximately 100 nm (Doceul *et al*., 2013). The genome is divided in three segments named as a function of their size small (S), medium (M) and large (L) respectively (Figure 2) (Elliott and Blakqori, 2011). The S segment is a genetically stable segment (Hulst *et al*., 2013; Coupeau *et al*., 2013; Fischer *et al*., 2013) which length is approximately 1kb (Elliott and Blakqori, 2011). The S segment is commonly targeted for SBV diagnosis with real–time reverse transcription polymerase chain reaction (rRT–PCR) (Bilk *et al*., 2012; De Regge *et al*., 2013). The S segment encodes two important viral proteins in one overlapping open reading frame (ORF): the nucleocapsid (N) protein and a non–structural (NSs) protein (Doceul *et al*., 2013). The N protein is approximately 25 kDa, it is the most abundant viral antigen protein of *Bunyaviruses* and it is commonly targeted in indirect SBV ELISA (Elliott and Blakqori, 2011; Bréard *et al*., 2013). The N protein is associated with the genome in the form of a ribonucleoprotein complex (RNP) forming three individuals S, M and L helical nucleocapsids (Schmaljohn and Nichol, 2007; Bréard *et al*.,

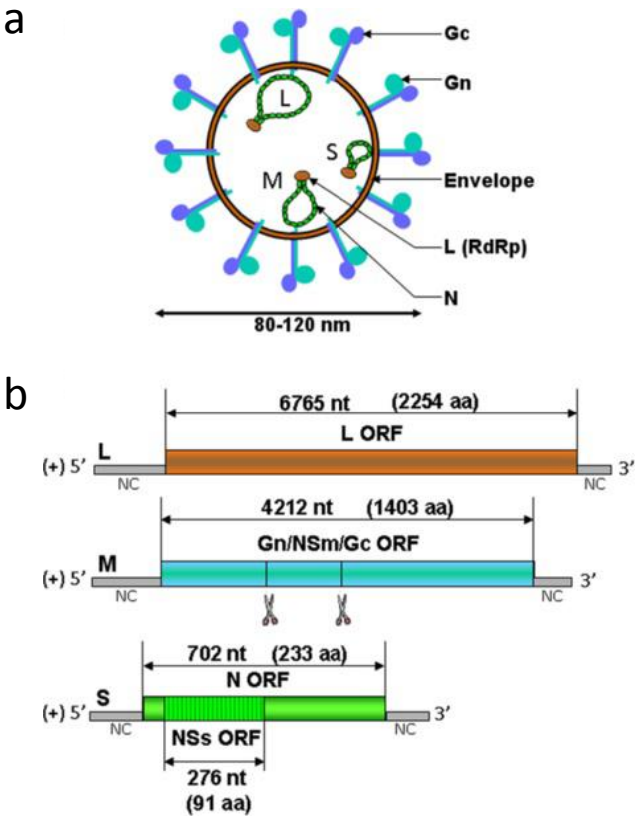

Figure 2: Schematic representation of the virion and the genome of Schmallenberg virus

The figures show (a) the schematic representation of Schmallenberg virus (SBV) gathered from the structure of *Bunyaviruses* that is given with the two envelope glycoproteins (Gn and Gc), and the three genome segments small, medium and large (S, L and M) associated with the N protein (N) in the form of ribonucleoprotein complexes (RdRp). The L protein constitutes a RNA dependant RNA polymerase in *Bunyaviruses*; (b) the approximate length of the open reading frame (ORF) is given for each segment with the numbers of nucleotide (nt) and amino acids (aa), and the encoded proteins. The putative cleavage site for the polyprotein precursor encoded by the M segment is indicated with scissors. The figure was obtained from Doceul *et al* (2013).

2013; Ariza *et al*, 2013). The RNP protects the genome from ribonucleases and reduces the host innate immune response (Dong *et al*., 2013b). The nucleoprotein interacts with viral genomic and antigenomic (replicative intermediate) RNA species to form ribonucleoprotein complexes (RNP) that are the functional templates for RNA replication and transcription (Elliott et al. 2011). The exact structure of the SBV N protein remains unclear, but two distinct models were proposed. The first one suggests that the SBV N protein forms a tetramer structure to which RNA is bound into a positively charged binding cleft (Dong *et al*., 2013a, Ariza *et al*., 2013). The second model suggests that the N protein forms a hexamer ring structure inside which RNA is wrapped, thereafter the RNA is exposed to the outside of the ring structure for replication and transcription by partial rotation of the N protein (Dong *et al*., 2013b). The N protein is also known to interact with the L protein and the two glycoproteins Gn and Gc, although its exact role remains to be elucidated (Schmaljohn and Nichol, 2007). The SBV NSs protein has a key role in SBV pathogenicity by degrading the largest subunit of the RNA polymerase II (RPB1) that reduces the host cell RNA production (Barry *et al*., 2014), and particularly the expression of virus induced interferon (IFN) only 16 hours after the cell-infection (Blomström *et al*., 2015). The SBV NSs protein has also a pro–apoptotic effect that enhanced the virus pathogenicity (Barry *et al*., 2014).

The M segment contains approximately 4.5kb (Elliott and Blakqori, 2011). The M segment encodes a polyprotein precursor that is cotranslationally cleaved into the non–structural (NSm) protein and two envelop glycoproteins named Gn and Gc (Elliott and Blakqori, 2011; Doceul *et al*., 2013). The SBV NSm protein potentiates the effect of the NSs protein in IFN production inhibition (Kraatz *et al*., 2015). The two envelope glycoproteins Gn and Gc constitute the basis of the virus envelope and form the characteristic spikes covering SBV (Doceul *et al*., 2013). Studies on La Crosse virus, another *Orthobunyavirus* affecting human, showed that the susceptibility to cell culture is related to the Gc protein (Schmaljohn

and Nichol, 2007). In addition, Gn is also important to determine entry in the central nervous system (SNC) (Flint *et al*., 2009). Consequently, the two glycoproteins play a key role in host–cell attachment and fusion process (Fischer *et al*., 2013).

The L segment is the longest of the three SBV segment with about 6.9kb (Elliott and Blakqori, 2011). The L segment constitutes, like the S segment, a genetically stable part of the genome and encodes the RNA–dependent RNA polymerase (RdRp) also called L–protein (Elliott and Blakqori, 2011; Coupeau *et al*., 2013; Hulst *et al*., 2013).

Bunyaviruses do not have matrix protein which role was proposed to be surrogated by the cytoplasmic tail of the Gn glycoproteins (Strandin *et al*., 2013)

Intracellular life–cycle

This section describes the most likely SBV intracellular life–cycle on the basis of information gathered from SBV itself and other closely related (*Ortho*)*bunyaviruses*. Consequently, information might be sometimes hypothetical and the life-cycle will be adapted with new specific knowledge in the future.

The virus enters the cell by endocytosis, mediated by the Gc and the Gn proteins (Plassmeyer *et al*., 2007; Doceul *et al*., 2013). Acidification of the endocytic vesicle induces conformational changes in the glycoproteins Gn and Gc resulting in the fusion of SBV with the endosome membranes. This fusion releases the virus genome and the RdRp in the cytoplasm (Schmaljohn and Nichol, 2007). Subsequently, 10 to 18 nucleotides are cleaved from the 5’ end of host messenger RNA (mRNA) by the RdRp and used as primers in a cap–snatching process to initiate RNA synthesis (Elliott and Blakqori, 2011, Doceul *et al*., 2013). The RdRp uses cytoplasmic free nucleotides to produce complementary copies of the whole

viral genome called antigenome used as template for viral RNA production (Schmaljohn and Nichol, 2007; Doceul *et al*., 2013). The RNA replication occurs probably in the perinuclear region because only the L and the N proteins are needed for RNA synthesis and these proteins are associated with perinuclear membranes (Dunn *et al*., 1995; Kukkonen *et al*., 2004; Schmaljohn and Nichol, 2007). Viral RNA is translated in proteins by host cell ribosomes and the M segment is cotranslationally cleaved into the Gn and the Gc glycoproteins (Schmaljohn and Nichol, 2007; Doceul *et al*., 2013). A heterodimer complex is formed by the glycoprotein Gn and Gc in the endoplasmic reticulum (ER) that is transposed to the Golgi apparatus to complete glycosylation. The transport to the Golgi occurs via a Golgi retention signal located on the Gn protein for most *Bunyaviruses* (Walter and Barr, 2011; Doceul *et al*., 2013). Newly–formed genome RNP are formed and accumulated in the Golgi apparatus where the RNP interact with the two glycoproteins Gn and Gc (Overby *et al*., 2007; Shi *et al*., 2007; Snippe *et al*., 2007; Hepojoki *et al*., 2010; Doceul *et al*., 2013). The accumulation of glycoproteins in the Golgi apparatus provoques a vacuolization and virus assembly occurs in viral factories around the Golgi complex. These factories comprise repetitive units of Golgi stacks, mitochondria, components of the rough endoplasmic reticulum (ER) and virus–derived tubular structures (Salanueava *et al*., 2003; Elliott and Blakqori, 2011). The virion must contain at least one of each ribonucleocapsids (S, M and L) to maintain its infectivity (Schmaljohn and Nichol, 2007). It is currently unknown how the *Orthobunyaviruses* package the full genome into a single infectious virion (Elliott and Blakqori, 2011). The mature virion is released from the cells by exocytosis (Schmaljohn and Nichol, 2007). The life–cycle of *Bunyaviruses* is summarized in Figure 3.

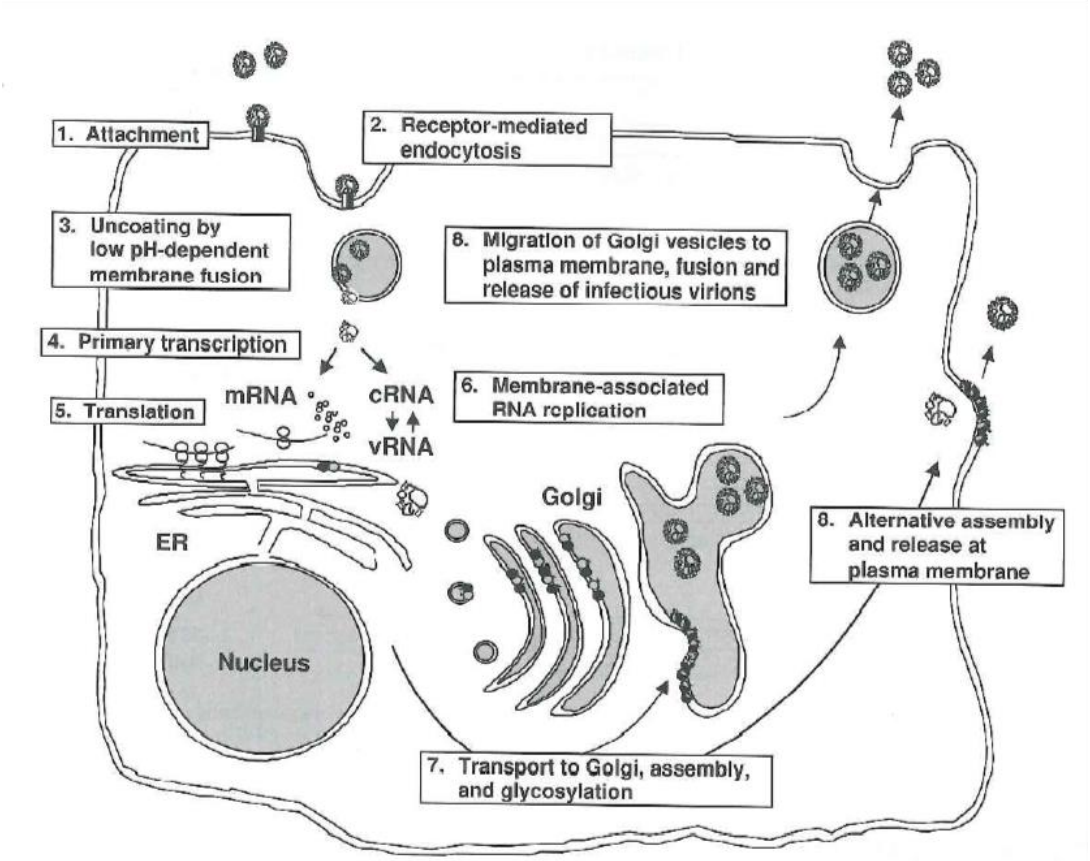

Figure 3: Intracellular life–cycle of *Bunyaviridae*

Schema obtained from Schmaljohn and Nichol (2007). ER: endoplasmic reticulum; cRNA complementary sense RNA; mRNA: messenger RNA; vRNA: viral RNA.

1.3 Clinical signs induced by Schmallenberg virus

The SBV induces an acute unspecific syndrome including high fever, drop of milk production and diarrhoea in adult cattle (Hoffmann *et al*., 2012; Muskens *et al*., 2012). Clinical signs were never reported under natural conditions in adult sheep and only few signs were observed after SBV experimental infection (Beer *et al*., 2013; Wernike *et al*., 2013e).

The teratogen effects due to SBV are mostly reported to affect the musculoskeletal system. Arthrogryposis, hydranencephaly, brachygnathia, scoliosis, kyphosis or lordosis were frequently reported during the epidemic (Herder *et al*., 2012). Also morphologically normal SBV infected animals were born presenting CNS alterations (Hahn *et al*., 2013). At necropsy, joints blocked by muscle contraction, muscle atrophy, muscle discoloration, petechiation of muscle, cerebellar hypoplasia, hypoplasia of the spinal cord, porencephaly, hydranencephaly or hydrocephalus were observed (Garigliany *et al.*, 2012a and b; Herder *et al.*, 2012; Hahn *et al.*, 2013; Varela *et al.*, 2013; Bayrou *et al*., 2014). Cerebellar hypoplasia and porencephaly are the most frequently observed consequence of SBV infection (Varela *et al*., 2013).

Histopathological data indicate that the lesions in the CNS were characterized by a lymphohystiocytic infiltration of the white and the grey matters, mainly observed in the perivascular regions. SBV also affects neurons as evidenced by the observation of neuroparenchymal degeneration and necrosis (Herder *et al.*, 2012; Hahn *et al.*, 2013; Peperkamp *et al*., 2014). The lesions of inflammation due to SBV in the CNS are more severe in lambs than calves (Peperkamp *et al*., 2014). Micromyelia induced by the loss of spinal ventral motor neurons was also reported (Peperkamp *et al*., 2014). Lesions due to SBV in peripheral organs were only demonstrated in muscle and consisted in myofibrillar hypoplasia

(Seehusen *et al*., 2014). Pictures of lesions reported in peer-reviewed literature are showed in Figure 4.

1.4 Laboratory diagnosis

The first rRT-PCR to be developed amplified a fragment of the SBV L segment and the β-actin or GAPDH as internal control of extraction and amplification (Hofman et al., 2012). Subsequently, a protocol targeting the SBV S segment was developed, the latter showed a higher sensitivity and was therefore preferred (van der Poel, 2012). A successful identification of SBV from blood is however difficult to complete with rRT-PCR considering the limited span of viraemia. The rRT-PCR should therefore be reserved to diagnosis in symptomatic animal, namely an animal presenting high fever (Beer *et al*., 2013). The rRT-PCR has also proven itself to detect SBV RNA presence in organs, hereto positive foetal organs (cerebrum, amniotic fluid, umbilical cord, spinal cord and brainstem) are supporting evidence for virus infection *in utero* (Bilk *et al*., 2012; Bréard *et al*., 2013; De Regge *et al*., 2013).

Schmallenberg virus isolation has lower implication for SBV diagnosis due to a high rate of unsuccesfull isolation attemps, although it has be achieved from brain, serum and blood with Vero cells (African green monkey kidney epithelial), BHK-21 cells (baby hamster kidney fibroblast) or KC cells (*Culicoides variipennis* larvae) (Bréard *et al*., 2013).

Due to the limited viraemia, evidence of virus passage is more easily provided by serological tests. Virus neutralization tests (VNT) were developed shortly after virus emergence (Loeffen *et al*., 2012). Also, different ELISA test were rapidly available. The most

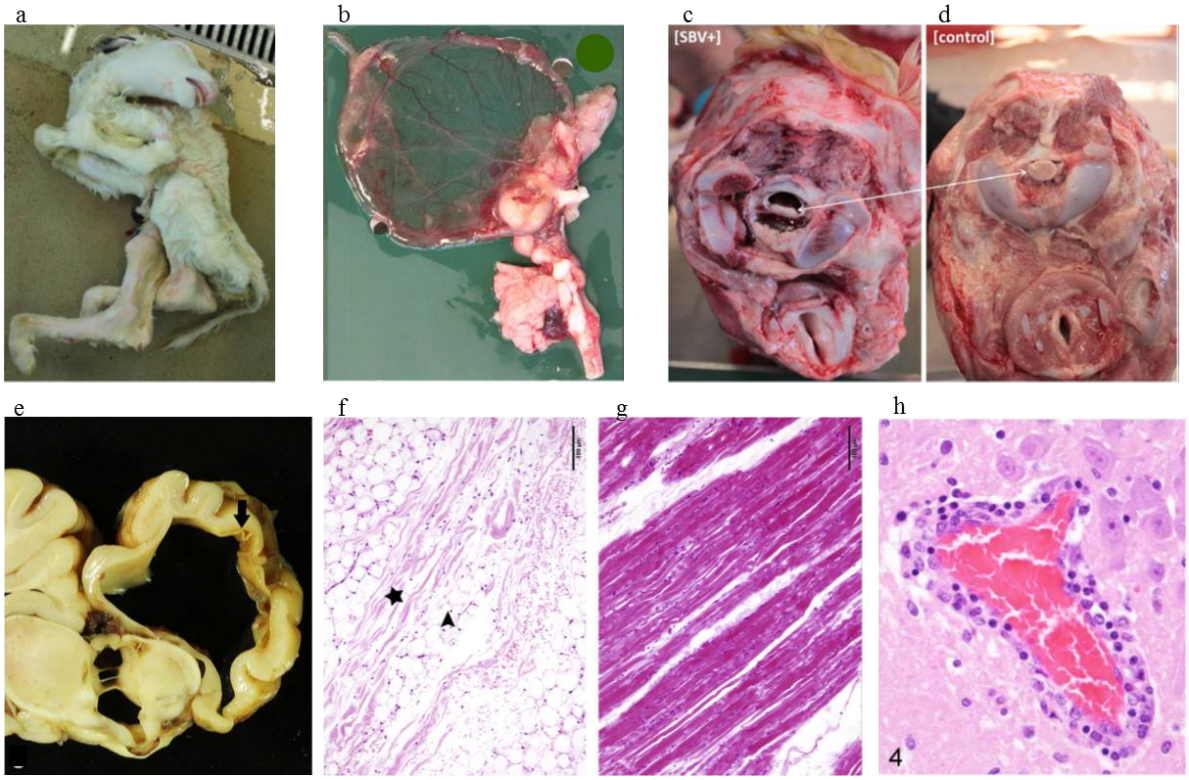

Figure 4: Macroscopic ([a] to [e]) and microscopic ([f] to [h]) findings in foetuses infected with Schmallenberg virus

[a] Musculoskeletal deformations in Schmallenberg virus (SBV) infected calves (Garigliany *et al*., 2012a). [b] Hydranencephaly in calves, here characterized by cerebrum and cerebellum hypoplasia (Garigliany *et al*., 2012a). [c] Spinal cord hypoplasia in SBV infected calf compared to [d] a control calf (Garigliany *et al*., 2012a). [e] Unilateral loss of cerebrum parenchyma in SBV infected calves (Varela *et al*., 2013). [f] Muscular hypoplasia in SBV infected animals evidenced by the observation of normal muscular fibres (star) replacement by adipose tissue (arrow) compare to [g] a healthy muscular tissue (Seehusen *et al*., 2014). [h] Perivascular infiltration of lymphocytes and macrophages in the central nervous system (Herder *et al*. 2012).

commonly used is an indirect ELISA based on a recombinant SBV nucleoprotein antigen (ID-VET, Montpellier, France) (Bréard *et al*., 2013). Virus neutralization test proved itself to be more sensitive during an European ring test, although rather laborious compared to ELISA that is therefore preferred for large seroprevalence screening (Méroc *et al*., 2013; van der Poel *et al*., 2014). Also, ELISA that can identify SBV specific antibodies in milk are available (Humphries *et al*., 2012; Balmer *et al*., 2013)

Immunofluorescence technique were successfully developed although demonstrating limited interest in lambs at birth probably because of the long span between assumed infection and birth (Hahn *et al*., 2013).

1.5 Culicoides vectors of Schmallenberg virus

The *Culicoides* are hematophagous insects that belong to the order *Diptera*, family *Ceratopogonidae* (Mellor, 2000). More than 1400 *Culicoides* species are known worldwide and transmit more than 50 viruses including human transmitted diseases (Mellor *et al*., 2000). In Europe, about 120 species are identified (Mellor *et al*., 2000). The species are identified by wing characters (Mathieu *et al*., 2012). Cutting-edge technique based on amplification with PCR of DNA markers and matrix-assisted laser desorption/ionization-time of flight spectrometry (MALDI-TOF MS) should facilitate the speciation in the future (Cetre-Sossah, 2004; Kaufmann, 2012). The *C. obsoletus* and *C scoticus*, frequently associated as the *Obsoletus* complex, are the most abundant species in European farms, excepted in the Mediterranean basin. The *C. dewulfi* and *C. chiopterus* are abundant along the English Channel and the North Sea in France, England and the Netherlands. Finally *C. impunctatus* and *C. newsteadi* are a very abundant species in northern Europe (Scotland) and the

Mediterranean basin, respectively (EFSA, 2014). *Culicoides imicola* was mostly restricted to Northern Africa until the end of the nineties. Under the influence of the climate change, this species is now present on most of the European side of the Mediterranean basin, causing growing concern on introduction of new viruses in Europe, namely the African horse sickness virus (Bethan *et al*., 2005). One should however interpret these results carefully; the capture technique influences the abundance of the species. Most catches of *Culicoides* are currently achieved with 'Onderstepoort Veterinary Institute' (OVI) trap. These traps are known to overestimate the biting rate of *C. obsoletus* and underestimate the biting rate of *C. dewulfi* (Carpenter *et al*., 2008). Also, captures are known to be influenced by the number of hosts present in the surroundings (Garcia-Saenz *et al*., 2011).

The life-cycle of *Culicoides* includes 4 successive stages: eggs, larva, pupa and adult (Figure 5). The *Culicoides* can complete their life-cycle inside animal enclosure (Koenraadt *et al*., 2014). Contradictory results have been published on adult abundance inside or outside stable of *Culicoides*. Zimmer *et al*. (2010) found inside stables to be the most abundant site of *Culicoides* capture, while Baylis *et al*. (2010) caught more *Culicoides* outside than inside. Bog lands (Obsoletus complex), wet forest, (Pulicaris complex) and feces constitute known Culicoides larvae habitats (Koenraadt *et al*., 2014). Zimmer *et al*. (2010) have shown that dried dung adhering to the stable walls and used animal litter is a breeding site for Belgian species of *Culicoides*.

The preferred host of the *C. obsoletus* complex, *C. dewulfi* and *C. scoticus* are horses, sheep, goats, cattle and poultry (Viennet *et al*., 2011). Different *Culicoides* species are also known to feed on human (Mellor *et al*., 2000).

The diffusion of the virus from the blood meal to the salivary glands of the vector, were the virus can be transmitted to a new host, is a complex process (Black *et al*., 2002). Only a portion of a *Culicoides* population will effectively replicate SBV and transmit the

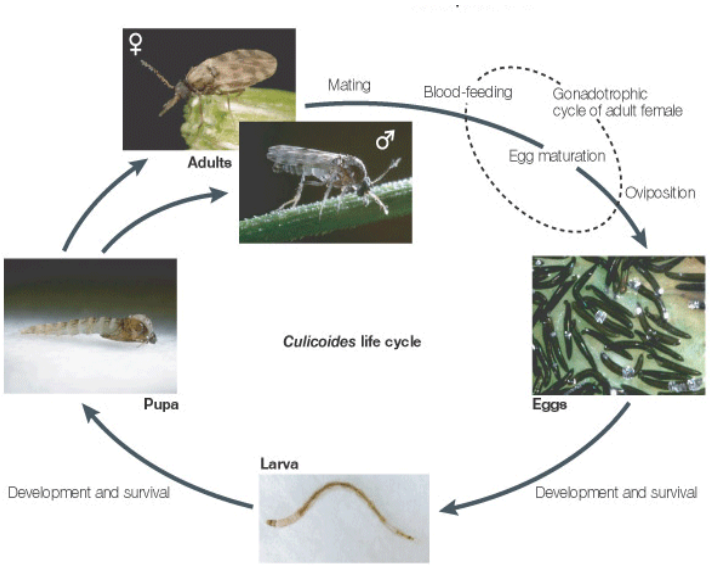

Figure 5: *Culicoides* life-cycle.

Schematic representation of the four successive stages of the *Culicoides* life-cycle: eggs, larvae, pupa and adults. Image obtained from Purse *et al*. (2005).

virus (Veronesi *et al*., 2013). Ten to 14 days are needed for bluetongue virus (BTV) transmission through *C. variipennis* (Mellor, 2000). A first 1-2 days decrease in virus titer is observed induced by virus inactivation in the gut and excretion in feces. The virus must subsequently multiply in the mesenteronal cells, pass the 'mid-gut' barrier, and finally escape in the haemocoel (Mellor *et al*., 2000). No further barrier is known in the *Culicoides*, and the lack of salivary barrier is established for BTV, therefore infection of the salivary glands and the infection of reproductive organs can occur upon 'mid-git' barrier passage (Koenraadt *et al*., 2014). No transovarial transmission has been demonstrated for *Culicoides*-transmitted virus so far (Mellor *et al*., 2000).

Proving the role of *Culicoides* as vector is not straight forward. Virus RNA identification is not sufficient to prove the vector capacity. The presence of viral RNA in midges, the presence of amount of viral RNA in midges comparable to the amount of viral RNA in hosts, the detection of viral RNA in the heads of the midges, and viral RNA presence on whole *Culicoides* are good indicators of vectorial role of *Culicoides*. In this context, much indirect evidence supports the role of the *Culicoides* in the wide SBV expansion. *C. obsoletus*, *C. scoticus*, *C. chiopterus* and *C. dewulfi* midges have been proposed to be putative vectors while the role of *C. pulicaris*, *C. nubeculosus*, *C. punctatus* and *C. imicola* remains to be clarified (De Regge *et al*., 2012; Rasmussen *et al*., 2012; Elbers *et al*., 2013; Goffredo *et al*., 2013; Larska *et al*., 2013; Balenghien *et al*., 2014; De Regge *et al*., 2014; De Regge *et al*., 2015; Elbers *et al*., 2015).

After the first identification in Germany in August 2011, SBV spread rapidly and widely over a large part of Europe (Figure 6). The exact place of SBV emergence remains unknown but the wide SBV expansion was most probably mediated by the transport of the

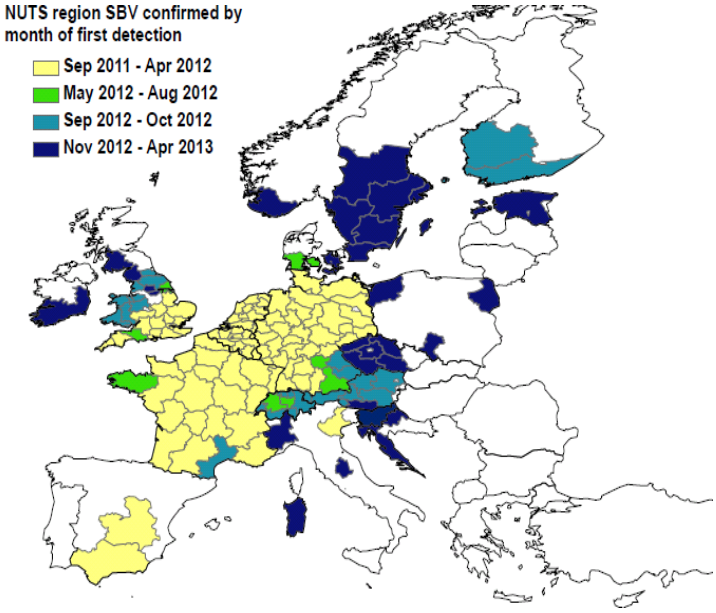

Figure 6: Schmallenberg virus expansion in Europe at the beginning of the epidemic

The map indicates the first confirmation of Schmallenberg virus (SBV) detection in different regions of Europe by month between September 2011 and April 2013. The map was obtained from Doceul *et al*. (2013).

Culicoides by the wind, as already proved for BTV (Hendrickx *et al*., 2008; Saegerman *et al*., 2008). In this respect, 70% of the farms were affected by midges transported by the wind (downwind movement) (Sedda and Roger, 2013). Rossi *et al*. (2015) observed a lower seroprevalence beyond an altitude of 800m. The protection conferred by altitude is however only partial because seroprevalence above 50% were reported at 1500m from the same study.

1.6 Embryology

The expansion of the blastocyst starts around day 13-15 of gestation, and the attachment to uterus wall occurs around day 15-18 of gestation (Reynolds and Redmer, 1992). The formation of the placentome begins at day 20-25 of gestation and the placentomes are well established around day 30 of gestation (Reynolds and Redmer, 1992). The placenta continues its development and remains poorly differentiated at day 42 of gestation (Lawn *et al*., 1969).

Angiogenesis starts upon day 14 of gestation, and angiogenic factors expression increased upon day 16 of gestation (Grazul-Bilska *et al*., 2010). Subsequently, vascular development increases upon day 14-20 of gestation and is maintained until day 30 (Grazul-Bilska *et al*., 2010). The blood flow in uterus is multiplied by 6 between day 11 and 30 of gestation (Reynolds and Redmer, 1992).

Brain develops in three phases, firstly a low increase between day 40 and 90 of gestation, followed by a rapid increase between day 90 and the birth. The brain development continues after the birth (McIntosh *et al*., 1979). The blood-brain barrier develops between day 70 and 123 of gestation (Evans *et al*., 1974). Muscles develop in three phases. The

primary muscle fibers of the skeletal muscle of lambs develop upon day 32 of gestation. The secondary fibers develop on the primary fibers upon day 38 of gestation. Finally, tertiary fibers develop on the secondary fibers upon day 62-76 of gestation (Byrne *et al*., 2014).

The development of the fetal immune system starts 19 days post fecundation and lasts until 115 days post fecundation; in the meantime, the humoral immune response is acquired between day 66 and 100 of gestation (Silverstein *et al*., 1963; Herder *et al*., 2013; Tizard, 2013; Martinelle *et al*., 2015).

The fœtal development has an impact on clinical outcome for teratogenic viruses. On the basis of information gathered from AKAV, foetus lambs infected with SBV between day 30 and 50 of gestation are susceptible to born with hydranencephaly arthrogryposis syndrome, while foetal infection between day 130 and 150 could induce premature, stillbirth-births and weak newborns (Martinelle *et al*., 2012).

1.7 Route for Schmallenberg virus infection: from the *Culicoides* saliva to the foetus

This paragraph describes the most likely scenario for SBV life–cycle from the *Culicoides* to the sheep host on the basis of information gathered from SBV and other closely related (*Ortho*)*bunyaviruses* (Figure 7). Consequently, information might be hypothetical and will have to be adapted with up-date knowledge in the future.

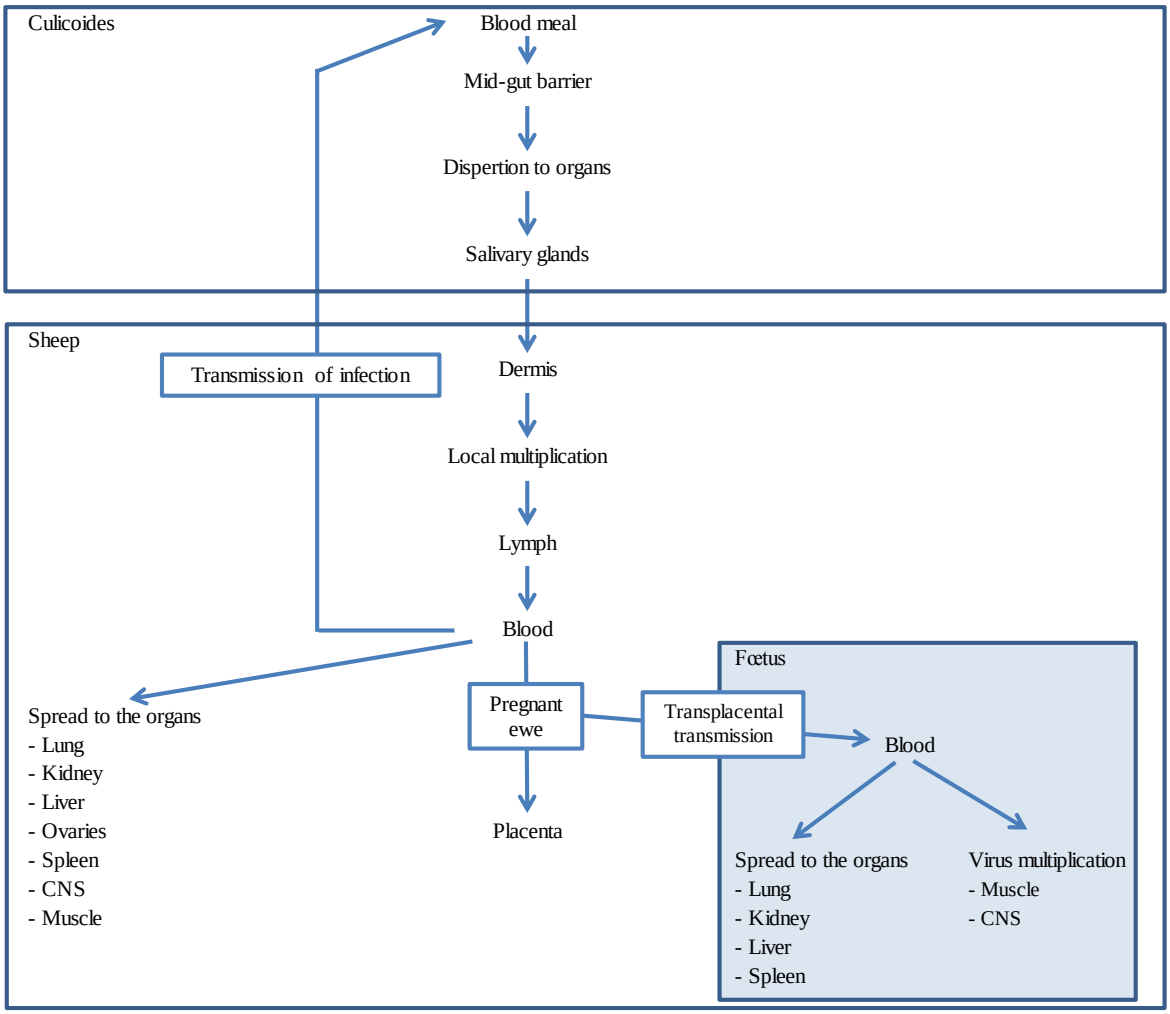

Figure 7: Proposition of Schmallenberg virus life-cycle

Complete extra–cellular life-cycle of Schmallenberg virus in *Culicoides* and sheep host. Information can be gathered from other (*Ortho*)*bunyaviruses* and will have to be confirmed in the future. CNS: central nervous system.

Culicoides takes a blood meal on an infected host during the viremia and SBV reaches the gut of the midge where it must replicate in the gut cells to reach the hemocoel (mid–gut barrier) (Mellor *et al*., 2000). The capacity of SBV to pass the mid–gut barrier could rely on genetic factors of the M segment (Schmaljohn and Nichol, 2007). High temperatures induce in *Culicoides* the leaky gut phenomenon (Mellor *et al* 2000). Schmallenberg virus could consequently by–pass the mid–gut barrier under favourable environmental circumstances. Once the mid–gut barrier crossed, the virus expands all over the *Culicoides*, including the salivary glands, since no other barrier was demonstrated in *Culicoides* so far (Mellor *et al*., 2000). The virus can finally be transmitted from the salivary glands to a new host during a blood meal (Veronesi *et al*., 2013).

Schmallenberg virus enters the host via its dermis considering that the *Culicoides* blood feed through laceration of the dermis (St-George, 2001). Local replication of SBV occurs in the muscle and the virus is drained towards the local lymph node, and to the blood at 2 dpi (Flint *et al*., 2009; Wernike *et al*., 2013). The virus disseminates into the host during the 4 to 5 days viremia (Wernike *et al*., 2013). Schmallenberg virus could replicate in skeletal muscle that is the major site of replication for *Orthobunyavirus* (Schmaljohn and Nichol, 2007). The exact role of the other SBV positive organs detected so far in adult animals (lung, lymph node, spleen, liver, ovary, kidney, CNS) remains to be clearly elucidated (Wernike *et al*., 2013e; Martinelle *et al*., 2015).

If the sheep is pregnant, SBV infects the placenta and transplacental transmission to the foetus occurs before 7 dpi (Stockhofe *et al*., 2013). The placenta and the foetus can be infected between 30 and 38 days of gestation at least (Stockhofe *et al*., 2013). In the foetus, SBV reaches the CNS and the muscle. In CNS, SBV induces pre–acute haemorrhage (48h after entering the cells) followed by malacia (72h) and finally vacuolation (96-120h) of the white matter leading to porencephaly and hydranencephaly (Varela *et al*., 2013). The

development of the musculoskeletal malformations could be the consequence of primary myositis or due to the central nervous loss inducing denervation and alteration of muscle development (Herder *et al*., 2012). Peripheral lesions due to SBV are only observed in muscle (Seehusen *et al*., 2014).

The development of the foetal immune system starts 19 days post fecundation and lasts until 115 days post fecundation, with the humoral immune response acquired between day 66 and 100 of gestation and AKAV specific antibody production that starts between day 65 and 75 of gestation (Silverstein *et al*., 1963; Herder *et al*., 2013; Tizard, 2013). It remains unclear why malformations were frequently reported in the presence of a seroconversion (De Regge *et al*., 2013). These difficulties to understand the underlying phenomenon of this process confirms that foetal SBV infection is a complex process that is probably multifactorial and depends on the age of the foetus at the time of infection, the maturation of the immune system, the virus strain or environment and host genetic factors (Varela *et al*., 2013).

1.8 Schmallenberg virus host range

The host range of SBV is mostly restricted to ruminant species. RNAemia with seroconversion was measured and experimentally reproduced in cattle, sheep and goats (EFSA, 2014). Schmallenberg virus was also detected by rRT–PCR in bison, deer, moose, alpacas and buffalos (EFSA, 2012). Schmallenberg virus specific antibodies were detected in alpacas, Anatolian water buffalo, elk, bison, red deer, fallow deer, roe deer, muntjac chamois and wild–boars (EFSA, 2012; Linden *et al*., 2012; Desmecht *et al*., 2013; EFSA, 2014).

It was demonstrated that the virus does not replicate in poultry (EFSA, 2014). Viral RNA was recovered from one puppy presenting torticollis at necropsy and originating from a French litter of five puppies showing neurological signs and early age death (Sailleau *et al.*, 2013). Two other seroprevalence studies in dogs were performed in Sweden and Belgium, respectively. The first one identified one seropositive dog among 86 bitches tested with ELISA and VNT (Wensman *et al.*, 2013). The second one failed to detect SBV infection with ELISA in 132 dog sera collected from April 2012 to January 2013 in a region with high seroprevalence for SBV (Garigliany *et al.*, 2013).

1.9 Reproduction performances of sheep

Most common sheep breeds have prolificacy rate between 1.2 and 2 (number of lambs born/number of lambing) and the percentage of neonatal mortality in sheep should not exceed 8% to 15%. Rustic breeds usually have a lower prolificacy rate, although their reproduction period is easily delayed. Lamb mortality rate is increased with heavy lambs and an elevated number of lambs in one gestation. First-lamb ewes, poor clinical conditions and alimentation of the ewes can also have a negative influence on mortality rate. Conversely, minimum loss is observed when ewes are aged between 4 and 5 years old and in the case of cross-breeding (reduction of the consanguinity) (Dudouet, 2003). Most of the embryonic losses occur during early pregnancy and reaches 30% of the fertilized ova (Reynolds and Redmer, 2001). The early gestation is therefore a critical period of pregnancy (Reynolds *et al*., 2010).

1.10 Introduction to Schmallenberg virus epidemiology in Belgium

The following paragraphs summarize the main findings of the SBV epidemic in Belgium during 2011–2015 (Poskin *et al*., 2016). This time span was divided in four time periods (seasons 1 to 4) ranging each from May till April of the next year. This coincides with the beginning of the *Culicoides* vector season and the end of the peak period of SBV associated congenital malformations induced by infections during that vector season (Figure 8).

1.10.1 May 2011 – April 2012: emergence of Schmallenberg virus in Belgium

Clinical suspicions of SBV

The exact time of SBV introduction in Belgium remains unclear. The earliest report of clinical signs that might be related to SBV infection were collected by three veterinarians of a veterinary surveillance network for dairy production called "Réseau Technique Vétérinaire Objectif Lait" (RTVOL). These veterinarians reported cases of milk drop syndrome of unknown origin that started mid–May 2011. Intriguingly, the clinical signs reported by the veterinarians were simultaneously observed at three different places in the Walloon region of Belgium: Somzée, Julémont and Fléron. These clinical signs consisted of high–hyperthermia, generally above 40.5°C and up to 41.3°C, and a drop of milk production, without any other remarkable symptom. Milk yield dropped with 50 to 90% and recovered almost entirely, within eight days after the first clinical signs. The farms were negative for piroplasmosis,

Syndromic surveillance
Vector surveillance
Cross-sectionnal seroprevalence studies
Sentinel herd surveillance

	Before the first quarter of 2011	Retrospective study on cattle sera collected in South Belgium indicate that cattle were seronegative before the first quarter of 2011 (Garigliany et al., 2012c)
Season 1	Mid-May 2011	Putative emergence of SBV in Belgium as observed on the basis of veterinarian's observations (Théron , unpublished data)
	August 8, 2011	First Belgian positive pool of Culicoides collected in Liège with rRT-PCR (De Regge et al., 2015)
	August -Septembre 2011	Increase in notifications of milk drop syndroms at regional animal health centers (De Regge et al., 2012)
	Septembre - Octobre 2011	First evidences of SBV presence in adult cattle (Promedmail, 20120117.1012402)
	Decembre 23, 2011	First deformed lambs due to SBV in Belgium (Promedmail, 20111223.3665)
	January-April 2012	Peak of SBV positive samples by rRT-PCR analysed at CODA-CERVA for season 1
	March 2012	Results obtained for seroprevalence in a Belgian cross-sectional seroprevalence study in cattle sera from 422 herds : within-herd: 86,3%, between-herd: 99,8% (Méroc et al., 2013a)
	April 2012	Results obtained for seroprevalence in a Belgian cross-sectional seroprevalence study in sheep sera from 83 flocks: within-herd 84,3%, between-herd: 98% (Méroc et al., 2014)
Season 2	End-July 2012 - Mid-Octobre 2012	SBV recirculation detected with rRT-PCR in lambs (Claine et al., 2013a)
	August 2012	Captures of SBV positive *Culicoides* in the second season of the epidemic at different locations of Belgium (Garigliany *et al*., 2012c)
	End-February 2013	Follow-up study of seroprevalence in cattle on sera collected in 188 herds representative for the Belgian cattle population: within-herd 65.66%, between-herd 100% (Méroc et al., 2013b)
	January-April 2013	Peak of SBV positive samples by rRT-PCR analysed at CODA-CERVA for season 2
Season 3	End-April 2014	Absence of SBV positive cases by rRT-PCR reported at CODA-CERVA during the third season of epidemic
Season 4	January 2015	Substantial increase in the number of positive VNT at CODA-CERVA

Figure 8: Key observations summarizing the Schmallenberg virus epidemic in Belgium

The review of the epidemiology of Schmallenberg (SBV) in Belgium was structured in four seasons of one year each (from May to April onwards). The beginning of this season corresponds to the beginning of the vector period and the end with the drop of the peak of congenital malformations induced by SBV infections during that vector season. The observations are classified according to the surveillance method that contributed to the understanding of the epidemic: syndromic surveillance, vector surveillance, cross–sectional seroprevalence studies and sentinel herd surveillance. Abbreviations used in the figure are rRT–PCR: real–time reverse transcriptase polymerase chain reaction; SBV: Schmallenberg virus; VNT: virus neutralization test.

BTV and Q fever. In the absence of a precise diagnosis, symptomatic treatments with non–steroidal anti–inflammatory drugs and antibiotics were implemented by the veterinarians from RTVOL, without satisfactory results. Subsequent diagnostic investigations were unsuccessfully oriented to erlichiosis and heat–stress. Consequently, the syndrome observed by the veterinarians was retrospectively considered to be the first hypothetical cases of SBV in Belgium, even though this could not be strictly confirmed because blood samples were no longer available (Théron, unpublished data). Passive surveillance networks based on field veterinarian observations were the first to suspect the emerging disease during spring 2011. The emergence of SBV in Belgium at that time is further supported by putative SBV cases reported by a veterinarian in July 2011, as revealed in a retrospective study (Martinelle *et al*., 2014). Also, the number of notifications of milk drop syndrome and diarrhoea from unknown origin increased in August and September 2011 at the Belgian regional Animal Health Care centres: Association Régionale de Santé et d'Identification Animales (ARSIA) and Dierengezondheidszorg Vlaanderen (DGZ) (De Regge *et al*., 2012). One should however be careful to attribute these clinical signs solely to SBV without a confirmed diagnosis and also other causes inducing high temperature and drop of milk production should be considered.

rRT–PCR confirmation of SBV circulation in livestock in 2011–2012

The earliest detection of SBV in Belgium via rRT–PCR was achieved on samples from adult cattle showing fever and milk–drop syndrome in September and October 2011 and in three sheep belonging to a sentinel herd surveillance of the University of Namur on September 6th, 2011 (ProMed–mail, 20120117.1012402; Claine *et al*., 2013b). The first PCR detection of SBV in a lamb born with signs of malformations due to SBV was reported later on December 23th, 2011 (ProMed–mail, 20111223.3665).

During the first season of the epidemic, after optimization of the diagnostic technique (De Regge and Cay, 2013), Veterinary and Agrochemical Research Centre (CODA–CERVA) received, from November 2011 onwards, 1282 suspected samples for SBV analysis in cattle of which 353 were positive. In sheep, these were 499 suspected samples of which 267 were positive. The median date for the reception of the rRT–PCR positive samples was mid–April 2012 in cattle and end–January 2012 in sheep (Figure 9). The observation of numerous SBV cases at the beginning of 2012 is also supported by different studies reporting SBV detection from January 2012 onwards. Schmallenberg virus RNA was identified in 10 lambs at the University of Namur in January 2012 (Kirschvink *et al*., 2012). At the University of Liège, 15 calves that were deformed or died without obvious reason were received during January to March 2012, and all scored positive in rRT–PCR for SBV (Bayrou *et al*., 2014).

The data from the diagnostic surveillance suggests that Belgian ruminant livestock was mainly affected during the end of the summer and autumn 2011. Combined with the peak of SBV positive malformed lambs and calves observed at the beginning of the year 2012, this leads to the hypothesis that infection during the first third of gestation induces the congenital malformations. This is supported by the observations done at the sentinel herd of Namur, which allows combining the period of SBV circulation in adult sheep (September–October 2011) with the dates of conception and the observations made at birth. Lambs conceived in spring 2011 and born in October 2011, and therefore infected during late stage of gestation, were all normal and presented SBV specific antibodies probably originating from colostrum. The lambs born in January 2012 originated from ewes mated between August 19th, 2011 and September 5th, 2011 and the ewes were therefore probably infected around the first third of gestation. Both still–born and deformed lambs were observed in this group. Another group of ewes was mated from October 13th, 2011 to October 26th, 2011 and were thus probably

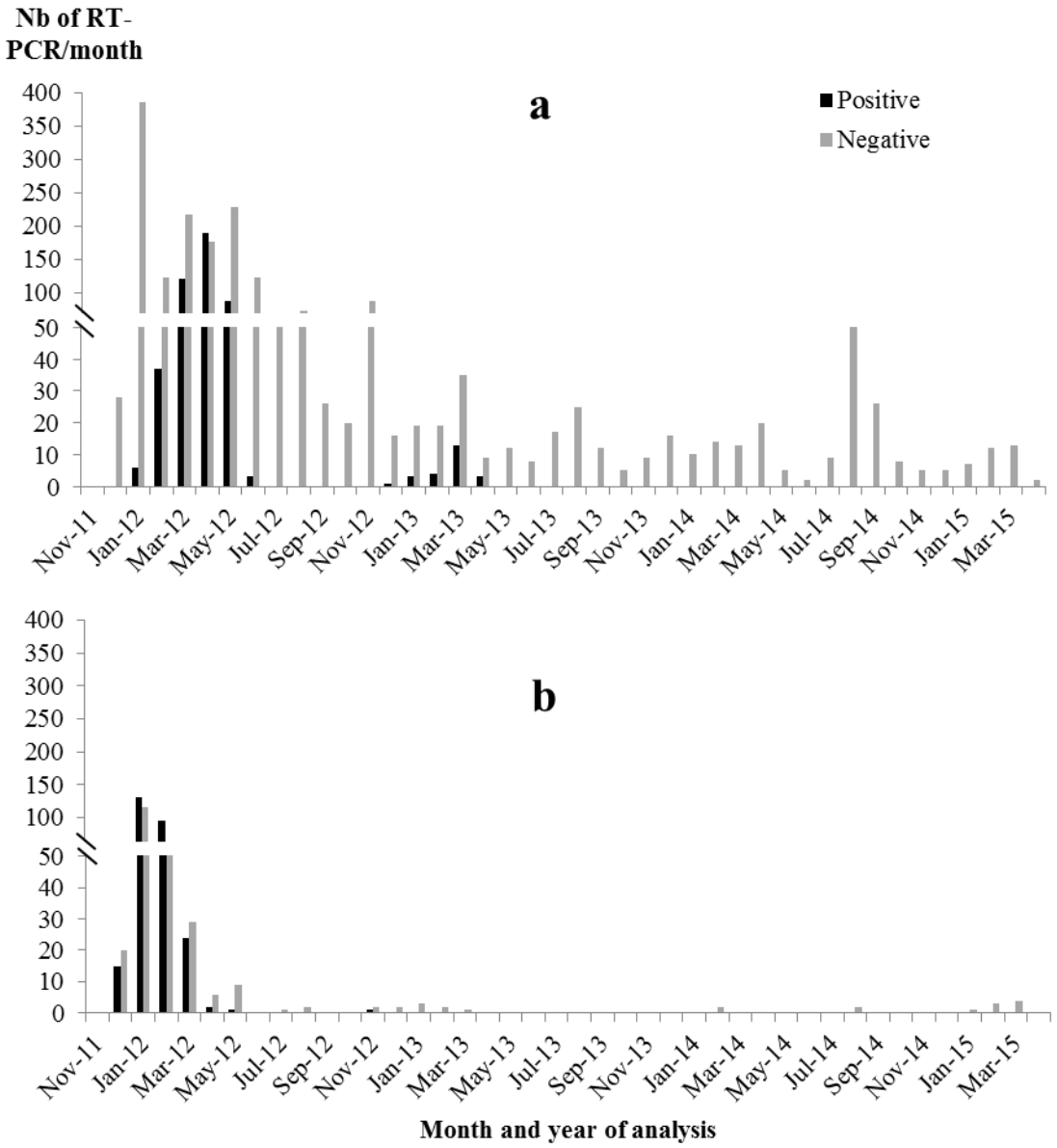

Figure 9: Schmallenberg virus samples received at CODA–CERVA for RT-PCR analysis in cattle (A) and sheep (B) between November 2011 and March 2015

Number of Schmallenberg virus (SBV) positive and negative samples monthly submitted to CODA–CERVA for real–time reverse transcription polymerase chain reaction (RT–PCR) diagnosis between November 2011 and April 2015 in a) cattle (n=2419) and b) sheep (n=535). The dark bars indicate the numbers of positive rRT-PCR results for each month and the grey bars indicate the number of negative rRT-PCR results for each month.

infected at the very beginning of gestation. A high percentage of aborted foetuses was reported (Claine *et al*., 2013b).

Seroprevalence in Belgium in 2011–2012

A limited number of adult cattle sera collected in spring 2010 (n=71) and during the first quarter of 2011 (n=40) were negative for SBV antibodies, supporting the absence of SBV circulation before that time in Belgium (Garigliany *et al*., 2012c). At the University of Namur, retrospective analyses were made on their sentinel flock. Seroconversion was first identified in September 2011 and seroprevalence increased strongly thereafter resulting in a seroprevalence of 99% (n=422) in January 2012 (Claine *et al*., 2013b).

Three large–scale seroprevalence studies based on annual cross–sectional surveys were conducted in Belgian farms using ELISA (ID Screen, ID Vet, Montpellier, France). The first study was carried out on 422 cattle farms (n=11635 bovines) between January 2nd, 2012 and March 7th, 2012. By that time the between–herd seroprevalence had already reached 99.8% and the within–herd seroprevalence was 86.3% (Méroc *et al*., 2013). The second study was conducted on 83 sheep flocks (n=1082) and eight goat farms (n=142) that were sampled between November 4th, 2011 and April 4th, 2012. A between–herd seroprevalence of 98% and a within–herd seroprevalence of 85.1% were found in sheep. In goat, the within–herd seroprevalence was 40.7% (Méroc *et al*., 2014). In a third study, cattle sera were collected in 209 farms (n=519) from the Walloon region between February 13th, 2012 and April 22nd, 2012. A seroprevalence of 90.8% was found (Garigliany *et al*., 2012c).

Interestingly, different seroprevalence studies indicated a lower SBV circulation among both cattle and sheep during the first season of the epidemic in the Ardennes. This is

an upland region in the south of Belgium, largely covered with dense forest neighbouring pastures. It usually has a prolonged winter and temperature is lower compared to the rest of the country (Méroc *et al*., 2013 and 2014), suggesting that factors like altitude, temperature, rainfall, host–availability and landscape related parameters has an impact on SBV transmission as has been suggested before for BTV transmission (Pioz *et al*., 2012; Faes *et al*., 2013).

Taken together, seroprevalence studies support a massive virus expansion in Belgium between the end of the summer 2011 and the beginning of 2012. This demonstrates the remarkable expansion capacity of SBV. The high seroprevalence observed after the 2011–2012 season suggested that a comparable circulation and spread would be difficult the following years.

Clinical impact of SBV in 2011–2012

Several studies tried to estimate the clinical and financial consequences of the SBV emergence. This was however not straight forward because case–control studies were difficult to design. One study tried to determine the clinical and economic impact on the basis of observations made by field veterinarians. Therefore a questionnaire was submitted via a local veterinary journal. Only 27 responses were collected. The median morbidity rate in cows, ewes and goat for SBV were respectively 7.5%, 5.5% and 3.5%, while in offspring this was 2%, 10% and 5%, respectively. At individual level, adult animal suspected of SBV infection had frequently been treated with anti–inflammatory drugs and antibiotics. A second study throughout the Walloon region estimated the impact on sheep based on information provided by a limited number of farmers (n=26). A questionnaire was transmitted to the farmers via a dedicated journal. Twenty–six questionnaires (originating from 13 negatives and 13 positives

farms) were collected between late February and May 2012. The proportion of abortion, still–born and malformed lambs were estimated to be 6.7%, 13.2% and 10.1%, respectively, in the positive flocks and 3.2%, 9.5% and 2%, respectively, in the negative flocks (Saegerman *et al*., 2014).

1.10.2 May 2012 – April 2013: second Schmallenberg virus season

Recirculation of SBV in Belgium in 2012–2013

Considering the seroprevalence measured after the first vector season, it was expected that recirculation of SBV would preferentially be observed in the Ardennes area where seroprevalence was lower. The first published evidence for recirculation was found in the sentinel sheep herd of the University of Namur. Fifty lambs were monitored between April and October 2012 and blood was collected twice a month. Three animals were found SBV positive by rRT–PCR on July 27th, 2012. Most rRT–PCR positive results were observed between August 8th and October 3rd, 2012 and all lambs monitored during the study had been infected before October 17th, 2012 (Claine *et al*., 2013a).

At CODA–CERVA, the first SBV positive sample for the second season of the epidemic was received on November 13th, 2012. The sample originated from a sheep farm located in the very South of the country, in the city of Chassepierre, nearby France. While this herd had been spared from SBV during the first season of the epidemic, it went through a severe SBV episode of congenital malformations that lasted three weeks. The farmer reported 120 gestations meaning that 223 lambs were expected that year taking 1.86 expected lambs

per gestation (Saegerman *et al*., 2014). In total, 7% of still–births lambs (15/223) plus 7% of deformed lambs (15/223) were reported (Poskin *et al*., unpublished data).

After this first case in Chassepierre, 831 cattle samples and only 24 sheep samples were received for SBV rRT–PCR analysis at CODA–CERVA and, respectively, 114 and two samples scored positive. Most of these positive SBV samples were received between January and March 2013 (Figure 9). In Liege, only a limited number of 12 calves highly suspected of SBV infection were presented between January and February 2013, and three were found SBV positive in rRT–PCR (Bayrou *et al*., 2013).

The decrease in the number of SBV cases reported to the authorities in 2012–2013 indicates a more limited virus circulation compared to the first season. It must however be emphasized that SBV has never been a notifiable disease in Belgium. It is hypothesized that farmers and veterinarian did not send all SBV suspected cases for diagnostic identification. Although reporting of aborted calves and lambs is mandatory in Belgium and SBV is routinely investigated as a potential cause for the abortion, it is estimated that less than one out of 30 aborted animals is reported (Delooz *et al*., 2011). Consequently, the real number of SBV cases observed during the second season of epidemic was probably underestimated.

Seroprevalence in Belgium in 2012–2013

Since only a limited number of samples was tested by rRT–PCR, seroprevalence studies were a better tool to evaluate virus circulation in 2012–2013. First VNT were conducted at CODA–CERVA from June 2012 onwards. The vast majority of the samples received at that time were positive (Figure 10) and most probably represent antibody

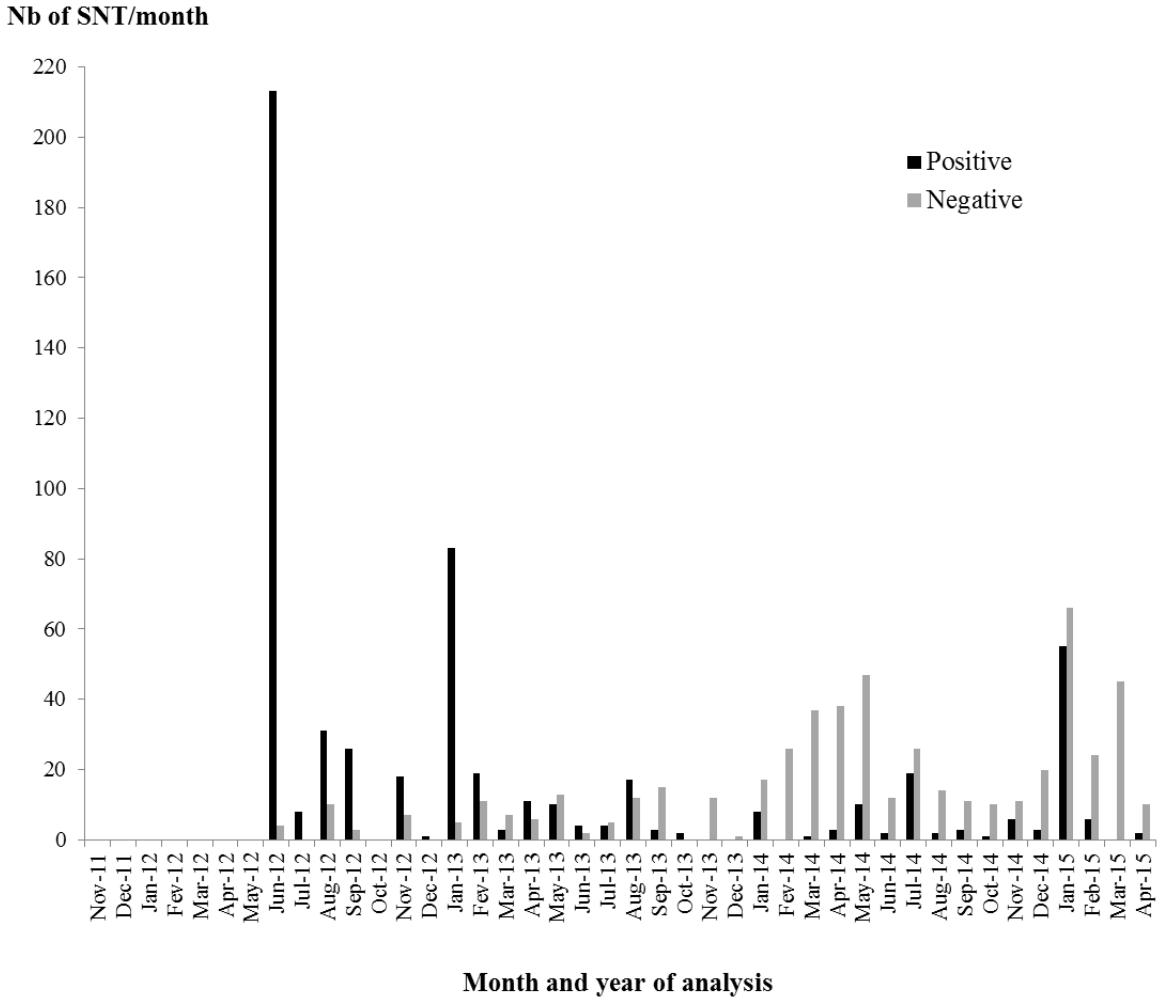

Figure 10: Number of sample received for Schmallenberg virus neutralization tests at CODA– CERVA between November 2011 and March 2015

Number of bovine samples analysed each month between November 2011 and April 2015 for the presence of Schmallenberg virus (SBV) specific antibodies with virus neutralization test (VNT) (n=1101). The dark bars indicate the numbers of positive VNT (VNT titre ≥4, De Regge *et al*., 2013) results for each month and the grey bars indicate the number of negative rRT-PCR results for each month.

persistence due to infection during the first season of the epidemic (Elbers *et al*., 2014a and b).

Evidence of renewed virus circulation was found in the sheep flock belonging to the University of Namur. Seroconversion of naive ewes occurred starting from July 2012 (Claine *et al*., 2013a) and the VNT values measured in previously immunized ewes increased between February 2012 and February 2013 suggesting a "booster effect" due to reinfection during the second season of SBV circulation (Claine *et al*., 2013c).

More conclusive and representative results were obtained via a follow–up cross–sectional serological survey in 188 Belgian herds sampled between January and February 2013 aiming to identify SBV circulation in young animals. The between–herd seroprevalence was estimated to be 100% in the entire cattle population, while the mean within–herd seroprevalence was 65.7% indicating a substantial decrease compared to 2011–2012 (Méroc *et al*., 2015). The seroprevalence rate of 20.6% in 6–12 months old calves in 2012–2013 indicate a recirculation during the second season of the epidemic, but was however clearly lower than the 64.9% found in 2011–2012 in the same age category (Méroc *et al*., 2013 and 2015). The lower within–herd seroprevalence observed in the entire cattle population was consequently due to the low percentage of infection in naive young calves. In conclusion, the seroprevalence studies support the occurrence of a new virus circulation in 2012–2013. This circulation was however more limited as indicated by the lower percentage of naïve calves infected. It further confirms that animals infected under natural conditions remain protected over a long period of time.

1.10.3 May 2013 – April 2014: third season of epidemic

In 2013–2014, due to the diminution of the SBV impact, less effort was done to follow the SBV situation in Belgium. Only a low number of SBV suspected samples (161 cattle samples and two sheep samples) were received at CODA–CERVA for SBV rRT–PCR analysis, and all samples were found negative (Figure 9). Similarly, few sera were received for VNT, and only a low number (52 out of 230 samples analysed in cattle and one out of three in sheep) was positive (Figure 10). These elements support the hypothesis that the number of cases was drastically reduced compared to the two previous seasons due to a lower virus circulation in 2013–2014. Again, the impact might be underestimated since there was no clear incentive for the farmers to send in suspected samples.

1.10.4 May 2014 – April 2015: fourth season of epidemic

As in 2013–2014, no specific SBV surveillance was put in place in 2014–2015. Interestingly, Germany and the Netherlands reported renewed SBV circulation during summer and autumn 2014 (ProMed–mail, 20141121.2978286, Wernike *et al*., 2015). Despite this resurgence, one Dutch study demonstrated that SBV was absent from the Netherlands at that time and that the seroprevalence had drastically dropped (Veldhuis *et al*., 2015).

Given the presumed low virus circulation in Belgium in 2013–2014 and the associated increase of the number of susceptible seronegative livestock, it was expected that SBV circulation would then also have occurred in Belgium. However, no SBV PCR positive

samples (n=145 in cattle and n=10 in sheep) have been found for the 4th season of epidemic at CODA–CERVA. In addition a substantial increase of the number of VNT positive samples was observed in January 2015 (55 positive samples out of 66 analyses) (Figure 10). Although this increase is suggestive of a virus circulation in Belgium at the end of summer and in autumn 2014, it cannot be excluded that these positive samples are the consequence of infections that occurred between 2011 and 2013 knowing that SBV antibodies (revealed by VNT) produced after natural infection are known to persist at least 24 months (Elbers *et al*., 2014b).

1.10.5 Schmallenberg virus monitoring in Culicoides

SBV in Belgian Culicoides in 2011–2012

Besides monitoring in host species, SBV has also been monitored in its *Culicoides* vector. The *Culicoides* were collected throughout 2011 with OVI traps located in the regions of Antwerp, Liege, Gembloux and Libramont. A total of 7305 *Culicoides* were pooled and tested for SBV presence in *Culicoides*' heads via rRT–PCR (De Regge *et al*., 2015). The absence of SBV in *Culicoides*, collected before August 2011, supports the indications described above and confirms that only limited virus circulation occurred during early summer 2011. SBV positive pools containing midges collected between August 10th, 2011 and October 28th, 2011 were found confirming SBV circulation at that time. In Liège, half of the pools containing midges collected in October 2011 were found positive, leading to a minimum infectious rate (MIR) in *C. obsoletus* complex of 3.1%. Similarly, a MIR of 3.6% in *C. obsoletus s.s.* midges was found in Antwerp in September 2011 (De Regge *et al*., 2015). In

contrast, only one positive pool containing midges collected on October 11th, 2011 was found in Gembloux and no positive pools were found in Libramont (De Regge *et al*., 2015). The absence of positive *Culicoides* at Libramont, which is located in the south of the country, correlates with the lower virus circulation in the Ardennes during the season 2011–2012 as observed in cross–sectional seroprevalence studies (Méroc *et al*., 2013 and 2014).

SBV in Belgian Culicoides in 2012–2013

A study whereby SBV was monitored in *Culicoides* collected in 2012–2013 with 12 OVI traps located in Antwerp, Liege, Gembloux and Libramont allowed to investigate whether the lower virus circulation observed in seroprevalence studies was correlated with a lower circulation of SBV in the *Culicoides* population.

Culicoides were collected between May 2012 and November 2012 and analysed for SBV RNA presence with rRT–PCR. Positive pools were found in all studied regions, but remarkably only in the month of August. The MIR of 0.4%, 0.3% and 0.2% in Antwerp, Gembloux and Liege, respectively, in the subgenus *Avaritia* were clearly lower than those observed the previous year (De Regge *et al*., 2012; De Regge *et al*., 2014). In contrast, the MIR was 2.86% and 3.26% in the subgenera *Avaritia* and *Culicoides*, respectively, in the South of the country in Libramont (De Regge *et al*., 2014). These results correlate with the increase of seroprevalence observed in the Ardennes after the first episode of 2011–2012 (Méroc *et al*., 2015) and with the outbreak observed in Chassepierre in 2012–2013.

These results show that despite its cost, virus surveillance in vectors is an interesting and powerful tool to study the SBV epidemic and was complementary to seroprevalence and

virus detection studies in host. The fact that SBV infected Culicoides were only found in August indicate however that this surveillance has to be performed continuously.

1.10.6 Schmallenberg virus in Belgian wildlife

The seroprevalence was evaluated in Belgian deer shot during the 2010 and 2011 hunting seasons in the different provinces of the Walloon Region. All collected sera were seronegative for SBV in 2010 but the seroprevalence was 47% in red deer (54/116) and 48% in roe deer (52/109) in 2011 (Linden *et al*., 2012). Desmecht and colleagues (2013) studied the seroprevalence in wild boars from Walloon region. Wild boars were seronegative in fall 2010 but reached seroprevalence of 27% in 2011. This decreased to 11% in 2012. Also 31 serum samples originating from wild boars shot in Flanders region during fall and winter 2012–2013 were tested with VNT by Poskin and colleagues (unpublished data) and revealed a low seroprevalence of 4%. These studies show that deer and wild boars could be infected with SBV and therefore act as a potential reservoir that helps the virus in its massive expansion. The exact role of these species in the epidemic remains however to be clearly determined.

PART TWO : OBJECTIVES

CHAPTER 2:

OBJECTIVES OF THE THESIS

When Schmallenberg virus emerged in 2011, it became rapidly obvious that SBV would have a significant impact in domestic livestock. Little knowledge was however available at that time on this recently emerged virus. In this respect, a European consortium (EU consortium, 2014) composed of English, French, German, Dutch and Belgian teams was set up in 2012. Their goal was to study the epidemiology, the pathogenesis and the diagnosis of SBV. Several aspects needed to clarified including the impact of SBV in Europe, the mean(s) of SBV transmission, the mean(s) of SBV overwintering and several aspects of SBV pathogenesis.

The work presented in this manuscript was acheived in the context of this European consortium (EU consortium, 2014).

General objective

The general objective of this thesis was to improve the global knowledge on the SBV epidemiology in Belgium and to study its pathogenesis in sheep.

Specific objectives

Five specific objectives were pursued and each was addressed in a distinct study:

- Specific objective 1: assess the impact of SBV in Belgium (Study 1);
- Specific objective 2: investigate the potential role of pigs in SBV life-cycle (Study 2);
- Specific objective 3: determine and standardized an SBV experimental model (Study 3);

- Specific objective 4: investigate the outcome of SBV infection in pregnant sheep (Study 4);

- Specific objective 5: evaluate the persistence and the kinetic of SBV specific antibodies in sheep (Study 5).

The Study 1 was conceived to improve knowledge on SBV impact in Belgium, and more generally to contribute to the estimation of the SBV impact in Europe. The Study 1 is a case-control study that was submitted to Belgian cattle and sheep farmers. The first part of the questionnaire aimed to identify the numbers of aborted, stillborn and malformed calves and lambs born due to SBV during the epizootic outbreak of congenital events and to study the impact of SBV in adult ruminants. It was also important to define more precisely what an SBV case is at herd level, an important feature for the attribution of clinical signs solely to SBV upon experimental infections. Also, questions were designed to identify the date and the reason leading a farmer to send samples to National Reference Laboratory (NRL) for SBV analysis. A last part of the questionnaire inquired managing practices and environment related parameters to identify potential risk factors for SBV infection at Belgian herd level.

The first epidemiological studies conducted at CODA-CERVA and the case-control study showed the high impact of SBV in Belgium. This high impact could be the (partial) consequence of a wide host range, potentially infected subclinically, and able to multiply the virus and contribute to its expansion. In this respect, the Study 2 evaluated the role of pigs in the SBV life-cycle. The role of pigs was important to study considering their suggested role in the life-cycle of the SBV closely related AKAV (Huang *et al*., 2003). Indeed, the role of pigs required clarification.

The Study 1 demonstrated that the impact of SBV was mostly imputed to the congenital defects observed in sheep. It was therefore essential to evaluate further the

pathogenesis of SBV according to the development of malformation *in utero*. In this respect, experimental infections of pregnant sheep with SBV were planned on a large number of ewes. Two preliminary experiments were firstly designed to standardize an experimental model that could reproduce faithfully a natural infection in the pregnant sheep. In the Study 3, the minimum infectious dose of an SBV inoculum was evaluated, while the impact of the route of inoculation was studied (Martinelle *et al*., 2015).

The Study 4 consisted in the inoculation of SBV into pregnant ewes at day 45 and 60 of gestation. The goal was to study the period of the gestation during which an SBV transplacental transmission must occur to induce congenital defects. The latter was required to improve the knowledge on SBV pathogenesis and fight efficiently further SBV spread.The inoculation was made according to the experimental infection model developed in the preliminary experiments. Also, the date were chosen in collaboration with a Dutch team to investigate an inoculation period covering the day 38 to 60 of gestation (Stockhofe *et al*., unpublished results).

The study 4 highlighted a probable short open-window for an SBV infection to induce the development of malformations. Interestingly, first evidence of a persistent and protective seroprotection upon one single infection was also observed. In this respect, it was important to unravel if this persistent seroconversion could prevent viraemia between 2 periods of SBV circulation, therefore preventing the SBV-induced teratogenic effects. In this respect, the production and the persistence of SBV specific antibodies after an experimental infection and the impact of surinfections on antibody titre was evaluated in the Study 5.

PART THREE : EXPERIMENTAL SECTION

CHAPTER 3: IMPACT OF SCHMALLENBERG VIRUS IN BELGIUM

3.1 Preamble

Schmallenberg virus emerged during summer 2011 (Hoffmann *et al*., 2012). Two cross-sectional seroprevalence studies conducted at CODA-CERVA highlighted the high seroprevalence encountered in Belgium at the end of the first vector season (Méroc *et al*., 2013 and 2014). The clinical impact of SBV in Belgium remained however unknown at national level. In this respect, a case–control survey was carried out in Belgian cattle and sheep herds during autumn 2012. The study aimed to determine the percentage of lambs and calves stillborn, malformed and aborted due to SBV in Belgium, and the clinical impact of SBV in adult ruminants. This was essential to study the manifestation of SBV infection in true field conditions, sounds knowledge being essential to allow appropriate evaluation of SBV clinical consequences in the experimental context. Also, the study aimed to increase knowledge on SBV diagnosis on the field, and subsequently guide farmers for SBV diagnosis and improve SBV surveillance on the field. Finally, it was expected to identify risk factor for SBV infection at herd level and allow to identification of farms or regions more susceptible to be infected by SBV in case of re-emergence.

3.2 Study 1: Schmallenberg virus in Belgium: estimation of impact in cattle and sheep herds.

This section constitutes the original article published in *Transboundary and emerging diseases*.

ARTICLE 1:

Poskin A., Méroc E., Behaeghel I., Riocreux F., Couche M., Van Loo H., Bertels G., Delooz L., Quinet C., Dispas M., Van der Stede Y. Schmallenberg virus in Belgium: estimation of impact in cattle and sheep herds. Transbound Emerg Dis, 2015, 60, 4–8.

3.2.1 Abstract

Schmallenberg virus emerged during summer 2011. SBV induced an unspecific syndrome in cattle and congenital signs (abortions, stillbirths and malformations) in domestic ruminants. To study the impact of SBV in Belgium, a phone survey was conducted upon September 2012. Hereto two groups of cattle farmers (A and B) and two groups of sheep farmers (C and D) were randomly selected. Farms from groups A (n = 53) and C (n = 42) received SBV–positive result at rRT–PCR in the Belgian NRL. Farms from groups B (n = 29) and D (n = 44) never sent suspected samples to NRL for SBV analysis but were however presumed seropositive for SBV after the survey.

Questionnaires related to reproduction parameters and clinical signs observed in new-born and adult animals were designed and addressed to farmers. As calculated on a basis of farmers' observations, 4% of calves in group A and 0.5% in group B were reported aborted, stillborn or deformed due to SBV in 2011–2012. The impact as observed by sheep farmers was substantially higher with 19% of lambs in group C and 11% in group D that were reported aborted, stillborn or deformed due to SBV in 2011 – 2012. Interestingly, abortions or

stillbirths were not clear consequences of SBV outbreak in cattle farms, and the birth of a deformed animal was an essential condition to suspect SBV presence in cattle and sheep farms.

This study contributes to a better knowledge of the impact of the SBV epidemic. The results suggest that SBV impacted Belgian herds mostly by the birth of deformed calves, stillborn lambs and deformed lambs. This work also demonstrates that the birth of a deformed calf or lamb was a trigger for the farmer to suspect the presence of SBV and send samples to NRL for further analyses.

3.2.2 Introduction

Among viruses which affected cattle and sheep populations in recent years, SBV is probably the most intriguing one after its first emergence in 2011 (Hoffmann *et al*., 2012). The first clinical signs related to SBV were notified during summer 2011 in cattle; an acute unspecific syndrome including high fever, drop of milk production and diarrhoea was observed (Hoffmann *et al*., 2012; Muskens *et al*., 2012). In the following months, a significant increase of congenital malformations, including arthrogryposis, torticollis, kyphosis, brachygnathia, hydrocephalia or lesions of the central nervous system, was observed in calves, lambs and kid goats (Garigliany *et al*., 2012a; Herder *et al*., 2012; van den Brom *et al*., 2012). In contrast to cattle, no clinical signs were reported in adult sheep under natural conditions so far (Beer *et al*., 2013).

Schmallenberg virus spread rapidly throughout Europe including Belgium (EFSA, 2012). Two serological studies from Méroc *et al*. (2013 and 2014) demonstrated that Belgian

sheep and cattle herds were extensively infected with SBV during the epidemic. In 2012–2013, data reported the resurgence of SBV in previously or newly infected countries: Belgium, Germany, Greece, Norway and Scotland (Anonymous, 2013; Claine *et al*., 2013a; Wernike *et al*., 2013d; Chaintoutis *et al*., 2014; Wisløff *et al*., 2014).

It is now commonly accepted that the Culicoides played a key role in the expansion of the epidemic (De Regge *et al*., 2012; Rasmussen *et al*., 2012; Elbers *et al*., 2013; Larska *et al*., 2013). It highlights once again the importance of Culicoides–borne diseases in Belgium after the BTV epidemic (Méroc *et al*., 2008 and 2009).

Increasing knowledge of the real impact of SBV in domestic livestock and risk factors associated with SBV infections is essential given the threats of re–emergence of SBV or emergence of other Culicoides–borne diseases (Beer *et al*., 2013). In Belgium, only two limited preliminary surveys studied the impact of SBV in cattle and sheep populations (Martinelle *et al*., 2014; Saegerman *et al*., 2014). In the Netherlands, a case–control survey identified grazing as possible risk factor for SBV (Veldhuis *et al*., 2014a). Finally, Veldhuis *et al*. (2014b) studied the impact of SBV on milk production and reproductive performance in the Netherlands and Germany.

This study describes the results of a phone survey within the Belgian cattle and sheep farms, conducted upon September 2012, aiming to evaluate the impact of SBV after the first introduction.

3.2.3 Material and methods

Study Population

A phone survey with a case–control design was carried out to identify possible risk factors for SBV infection. In this study, the original definition of 'case' and 'control' was maintained. Hereto two groups of Belgian cattle farmers, one case group (group A) and one control group (group B), were selected based on two sampling frames. The first sampling frame consisted of 408 cattle herds in which all herds had sent at least one sample to the NRL in Belgium with a confirmation of SBV infection diagnosed by rRT–PCR (De Regge *et al*., 2013). The second sampling frame consisted of 382 cattle herds that had never sent 'samples/material' to the NRL for SBV diagnosis at the time of the study. Respectively 71 (group A, SBV–confirmed by PCR) and 60 (group B, no SBV analysis performed) cattle herds stratified proportionally to the number of herds per province were randomly selected.

In sheep, two similar sampling frames were constructed (166 flocks and 83 flocks, respectively) and 70 case flocks (group C) and 74 control flocks (group D) were selected for the survey. All herds used in the sheep survey were different from those used in the cattle survey.

Although the survey had a case–control design, based on the upper specified definition, it seemed that the control herds in cattle (group B) and sheep (group D) were infected with SBV. Indeed, these herds had shown high levels of antibodies specific for SBV infection observed in two cross–sectional studies after this survey (Méroc *et al*., 2013 and 2014).

Questionnaire

To have an idea of the history of each selected farm, data related to three seasons were obtained: season 1 (S1, 01/09/09 – 31/08/10) represented the situation before the emergence of SBV. Season 2 (S2, 01/09/10 – 31/08/11) covered the period in which the first cases could retrospectively be associated with SBV infection in adult animal. Finally, season 3 (S3, 01/09/11 – 31/08/12) covered the period in which increased number of malformations was observed in calves and lambs (outbreak of SBV in Belgium) (EFSA, 2012).

The questionnaire was structured in two main sections. The first section (Q1.1 – Q1.8) was dedicated to the impact of SBV in relation to reproduction parameters, including congenital deformations reported in calves and lambs. The second section (Q2.1 – 2.3) was focused on the clinical impact of SBV in adult animals. A short description of the questionnaire can be found in Table I.

To estimate the impact of SBV, cattle and sheep farmers were asked to give estimates in relation to abortions (Q1.3), stillbirths (Q1.4) and deformed animals (Q1.5) they observed during the three last seasons: before the outbreak of congenital signs (S1 and S2) and in the SBV outbreak of 2011–2012 (S3).

To evaluate the temporal and spatial spread of SBV infected cattle and sheep farms in Belgium, the farmers were asked to provide the date at which they assumed for the first time that SBV infection was present in their herds (Q1.7.1) and to indicate the reason for this assumption (Q1.7.2). In addition, the farmers were asked to estimate the time period (first and last date) during which they observed the highest number of ‘SBV–suspected’ animals that were born in the herd (Q1.7.5).

			Cattle		Sheep	
			A	B	C	D
Section 1: global impact of SBV in the herd.						
1.1	Give the period of calving/lambing during each of the 3 last seasons (S1, S2, S3)		53	29	42	44
1.2	How many gestations did you have during each of the 3 last seasons (S1, S2, S3)		53	29	42	44
1.3	How many aborted calves/lams did you have during each of the 3 last seasons (S1, S2, S3)		53	29	42	44
1.4	How many stillborn calves/lams did you have during each of the 3 last seasons (S1, S2, S3)		53	29	42	44
1.5	How many deformed calves/lams did you have during each of the 3 last seasons (S1, S2, S3)		53	29	42	44
1.6	What malformations did you observed according the following systems:					
		members	50	21	42	44
		column	50	21	42	43
		head	50	21	40	43
		nervous system	41	20	42	44
1.7	Did you have a first suspicion of SBV?		53	29	42	44
	1.7.1	What was the date of the first clinical suspicion of SBV?	48	3	38	13
	1.7.2	What was the reason of the first clinical suspicion of SBV?	50	4	42	14
	1.7.3	Did "SBV suspected lambs/calves" die shortly after birth?	49	3	40	12
	1.7.4	After how long did they die?	25	1	29	11
	1.7.5	Indicate the period during which the highest number of "SBV suspected animals" was born?	41	3	41	12
1.8	Did you observe the birth of premature animals?		49	23	42	44
Section 2: impact on adult animals.						
2.1	Did you observe in adult animals of the farm high fever, degradation of the general state, loss of appetite, drop in milk production*, severe diarrhea or nervous symptoms		53	29	42	44
2.2	Did you observe the death of cows/ewes after giving birth to SBV calf/lamb?		43	0	37	13
2.3	Did you observe hydroallantois during calving/lambing?		37	3	37	13

*only asked to cattle farmers

Table I: Summary of the questionnaire submitted to cattle and sheep farmers aiming to identify Schmallenberg virus impact in Belgian ruminant livestock

The table summarizes the questions and gives the number of answers obtained for each question from the cattle farmers (column A, n=53) and sheep farmers (column C, n=42) who obtained a positive results for Schmallenberg virus (SBV) analysis with real–time reverse transcription polymerase chain reaction (rRT-PCR) at a National Reference Laboratory (NRL). The column B and D indicate the number of answers obtained from the cattle farmers (column B, n=29) and sheep farmers (column D, n=44) who never send suspected samples for SBV analysis with rRT-PCR at the NRL. All missing data from Q1.6 to Q2.3 were due to farmers who did not have opinion on the question or questions not applicable to the farm. The questionnaire was validated by a group of veterinarians and farmers to verify the terminology used and the feasibility to obtain the demanded administrative information of the farmer. The survey was conducted by phone in the mother tongue of the respondent (Dutch or French). The interviewers were not aware of the SBV status of the herds during the interview. The interviewers were veterinarians and/or veterinary assistants from ARSIA, CODA–CERVA and DGZ.

To evaluate the impact of SBV on adult animals, the second section contained questions on clinical signs compatible with SBV infection in adult cattle/sheep (Q2.1 – Q2.3).

A survey was considered complete and subsequently included in the analyses only if the interviewer obtained complete answers for Q1.1 – Q1.5. Incomplete questions from Q1.6 – Q2.3 were accepted and consisted of questions for which the farmer did not know the answers or for questions that were not applicable for the farmer. The non–responding farmers consisted of farmers who refused the interview or provided incomplete surveys.

Descriptive analysis

The mean number of gestations for each season (S1, S2 and S3) was calculated per group (A, B, C and D) taking the total of number of gestations (Q1.2) divided by the number of herds in the corresponding group.

The fraction of aborted/stillborn/deformed calves/lambs at animal level in each group was calculated taking the total number of aborted/stillborn/deformed calves/lambs (Q1.3 – 1.5) divided by the total number of expected new-born calves/lambs in the corresponding group. In cattle, one new-born calf was expected per gestation, while in sheep, 1.86 lambs were expected per gestation (Saegerman *et al*., 2014).

The percentage of aborted/stillborn/deformed calves/lambs for each season (S1, S2 and S3) was calculated per herd taking the number of aborted/stillborn/deformed calves or lambs observed by the farmer in the herd (Q1.3 – Q1.5) divided by the expected number of new-born calves or lambs in the herd within that period. In cattle, one new-born calf was expected per gestation, while in sheep, 1.86 lambs were expected per gestation, as stated by Saegerman *et al*. (2014).

To evaluate the percentage of aborted, stillborn and deformed calves and lambs due to SBV in S3 (estimated impact of SBV), the difference between the percentage of aborted/stillborn/deformed calves/lambs obtained before the outbreak of congenital signs (average of S1 and S2) and the percentage obtained during S3 was calculated for each herd.

A purely spatial normal model was used to scan for clusters of cattle/sheep farms using the dates of first suspicion of SBV infection (Q1.7.1). The model tested the null hypothesis that first suspicion date was homogeneously distributed among the farms. The method used a likelihood ratio test to identify clusters. To test the significance of this likelihood, 1000 Monte Carlo simulations were performed to obtain its distribution, and clusters with P–value <0.05 were to be considered as statistically significant. The test was performed on SaTScan 8.2.1. (Kulldorff, 1997).

Statistical analysis

The total number of animals present in cattle and sheep herds and the total number of adult animals present in the cattle herds in August 2012 were extracted from the Belgian system for animals identification, registration and follow–up (SANITEL). Data were not available for two cattle herds (one in group A and one in group B) and three sheep herds (one in group C and two in group D) and were consequently deleted for the result part. The mean herd size, the mean number of adult animals and the mean number of gestations during S3 (Q1.2) were compared between cattle groups A and B and between sheep groups C and D with Welch's t–test to evaluate the influence of herd size on the study population. P–value <0.05 were considered to be statistically significant.

3.2.4 Results

Response rate

The final survey consisted of 53 cattle surveys in group A, 29 cattle surveys in group B, 42 sheep surveys in group C and 44 sheep surveys in group D resulting in a responding rate of 75% (53/71), 48% (29/60), 60% (42/70) and 59% (44/74), respectively. The total number of animals included in the survey within each group is given in Table II. The geographical distribution of cattle and sheep farms is shown in Figure 11.

Descriptive analysis: impact on reproductive parameters

***Cattle/calves*:**

In cattle, the average calving period during S1, S2 and S3 was similar for groups A and B and covered early October to mid–July, independently of the predefined seasons (Q1.1, data not shown).

During the SBV outbreak in S3, the differences between group A and group B were significant in relation to the mean herd size, the mean number of adult animals per herd and the mean number of gestations ($P < 0.05$) (Q1.2, Tables II and III). The fraction of aborted/stillborn/deformed calves at animal level in group A and B for S1, S2 and S3 are given in Figure 12 (Q1.3 – Q1.5).

			Number of animals per herd				Grand total	Number of adult animals per herd (>24 months of age)				Grand total of adult
	group	n=	mean	95% CI [ ]	Min	Max	of animals	mean	95% CI [ ]	Min	Max	animals
Cattle	A	52	177	[143 — 211]	8	657	9200	96	[77 — 115]	2	393	4980
	B	28	102	[79 — 125]	5	203	2844	55	[42 — 69]	2	116	1551
Sheep	C	41	89	[34 — 145]	2	858	3669					
	D	42	44	[12 — 76]	2	613	1850					

Table II: Herd size according to the different groups used in the study aiming to identify Schmallenberg virus impact in Belgian ruminant livestock

Mean herd size and mean number of adult animals present in the herds are given for each group A, B, C and D: cattle (group A) and sheep (group C) farmers who obtained a Schmallenberg virus (SBV) positive real–time reverse transcription polymerase chain reaction (rRT-PCR) results in a National Reference Laboratory (NRL); cattle (group B) and sheep (group D) farmers who never send suspected samples to NRL for SBV analysis. The mean values are given with their associated 95% confidence interval (95% CI), minimum and maximum values. The total number of (adult) animals within each group is given as well. All data were extracted from the Belgian system for bovine and ovine identification, registration and follow–up (SANITEL) in August 2012. Results could not be obtained for two cattle herds and three sheep herds. The number of adult sheep is unknown.

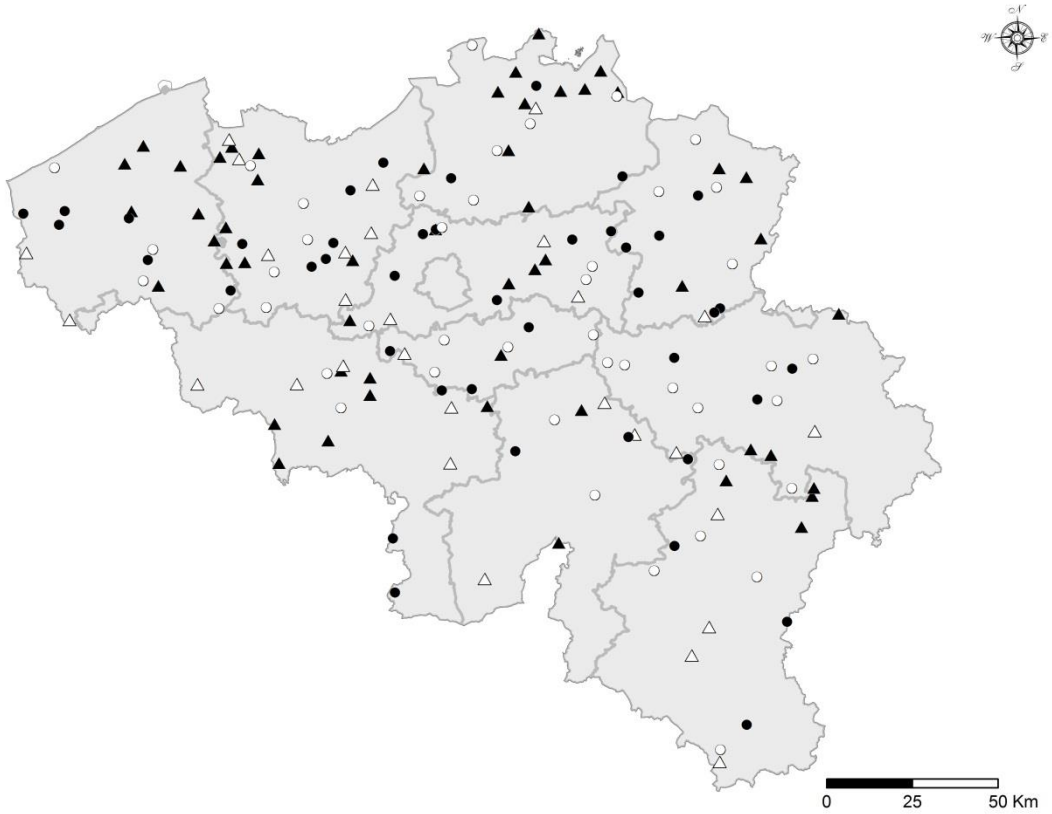

Figure 11: Farm used in the four groups used in the study aiming to identify Schmallenberg virus impact in Belgian ruminant livestock

Geographical localization of the farms in the study: group A (n = 53, ▲), group B (n = 29, D), group C (n = 42, ●) and group D (n = 44, ○). The groups were defined as follow: cattle (group A) and sheep (group C) farmers who obtained a Schmallenberg virus (SBV) positive real–time reverse transcription polymerase chain reaction (rRT-PCR) results in a National Reference Laboratory (NRL); cattle (group B) and sheep (group D) farmers who never sent suspected samples to NRL for SBV analysis.

		Cattle				Sheep			
		A		B		C		D	
	Season	Mean	95% CI [ ]	Mean	95% CI [ ]	Mean	95% CI [ ]	Mean	95% CI [ ]
Number of gestations	S1	77	[61 — 93]	51	[38 — 65]	62	[25 — 99]	31	[8 — 54]
	S2	79	[63 — 96]	52	[38 — 65]	62	[26 — 99]	33	[6 — 60]
	S3	77	[61 — 93]	50	[36 — 64]	68	[26 — 109]	30	[9 — 51]
Expected number of newborn lambs	S1					115	[46 — 183]	57	[15 — 100]
	S2					115	[48 — 184]	61	[10 — 111]
	S3					126	[49 — 203]	56	[18 — 95]

Table III: Number of gestations and new-born lambs per herd in the four groups used in the survey aiming to identify Schmallenberg virus impact in Belgian ruminant livestock study

Mean number of gestations per herd calculated in each group as the total number of gestations divided by the total number of herds in the group (Q1.2). The mean number of expected new-born lambs is given and was calculated per herd by multiplying the number of gestations reported in the herd by the expected prolificacy rate of 1.86 lambs per gestation (Saegerman *et al*., 2014). The expected number of new-born calves was calculated taking a one calf per gestation basis. It is consequently equal to the number of gestations. The data are given with their associated 95% confidence interval for the three predefined seasons: seasons 1 and 2 (S1 and S2, before the outbreak of congenital signs), and season 3 (S3 during the outbreak of congenital signs) and for the four groups: A (n = 53), B (n = 29), C (n = 42) and D (n = 44). The four groups were defined as: cattle (group A) and sheep (group C) farmers who obtained a Schmallenberg virus (SBV) positive real–time reverse transcription polymerase chain reaction (rRT-PCR) result in a National Reference Laboratory (NRL); cattle (group B) and sheep (group D) farmers who never send suspected samples to NRL for SBV analysis.

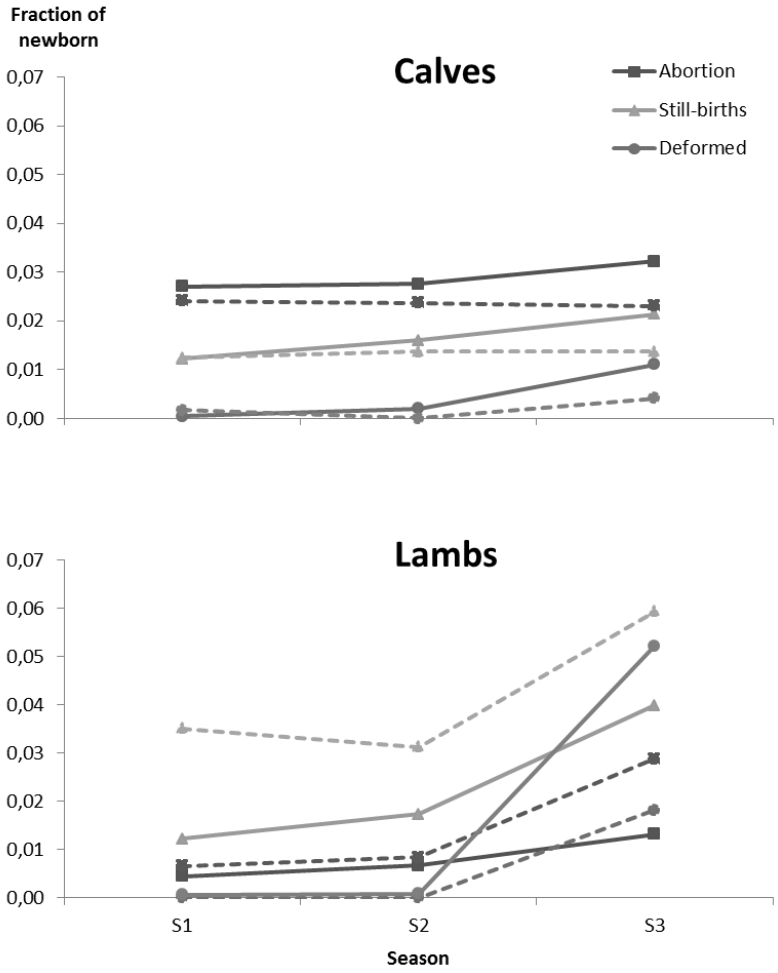

Figure 12: Fraction of congenital diseases in Belgium cattle and sheep herds in the four groups used in the study aiming to identify Schmallenberg virus impact in Belgian ruminant livestock

Fraction of aborted/stillborn/deformed calves/lambs at animal level in groups A, B, C and D calculated taking the total number of aborted/stillborn/deformed calves/lambs in the group (Q1.2 – Q1.5) divided by the total number of expected new-born calves/lambs in the corresponding group (Table III). The results are given for the cattle herds and the sheep herds during the three predefined seasons: seasons 1 and 2 [S1, S2: before the outbreak of congenital signs due to Schmallenberg virus (SBV)] and season 3 (S3: outbreak of congenital signs due to SBV). Results are given for the four groups: A (n = 53, solid lines), B (n = 29, dashed lines), C (n = 42, solid lines) and D (n = 44, dashed lines). The four groups were defined as: cattle (group A) and sheep (group C) farmers who obtained a SBV positive real-time reverse transcription polymerase chain reaction (rRT-PCR) result in a National Reference Laboratory (NRL); cattle (group B) and sheep (group D) farmers who never send suspected samples to NRL for SBV analysis.

The mean estimated impact of SBV on congenital signs in S3 indicates that in total, 4% of the calves in group A and 0.5% in group B were aborted, stillborn or deformed due to SBV in S3. The mean estimated impact of SBV in S3 was substantially different between groups A and B for stillborn and deformed calves; in contrast with the results in group A, the mean estimated impact of SBV for stillborn calves in group B was even lower in S3 compared with S1 and S2 (as indicated by the negative percentage) (Q1.3 – 1.5, Table IV).

In group A, deformations of the locomotor system, vertebral column, head and nervous signs were observed by respectively 88% (44/50), 66% (33/50), 46% (23/50) and 15% (6/41) of the farmers. In group B, this was respectively 10% (2/21), 10% (2/21), 10% (2/21) and 15% (3/20) of the farmers (Q1.6). The most observed clinical signs were scoliosis, twisted limbs and hydrocephalus.

The first retrospective suspicion of SBV as indicated by the cattle farmers was due to a calf born with deformations or stillborn and was notified during week 18 of 2011 in group A (Q1.7.1, Figure 13). This first suspicion was in a herd localized in the north–west of Belgium. No significant spatial cluster related to the date of first suspicion could be identified in the cattle group. In group A, 68% of the farmers (34/50) related that the first suspicion of SBV infection was due to calves born with deformations and/or stillborn, 24% of the farmers (12/50) related that the first suspicion was due to an abortion and 8% of the farmers (4/50) related that the first suspicion was due to an adult cattle, which presented clinical signs commonly attributed to SBV in adult animals. In group B, 14% of the farmers (4/29) retrospectively observed a first suspicion of SBV and this was always due to calves born with deformations and/or stillborn (Q1.7.2). Fifty–one per cent of the farmers (25/49) in group A and 33% of the farmers who reported a first suspicion of SBV (1/3) in group B stated that ‘SBV–suspected’ calves died shortly after birth (Q1.7.3). Moreover, the farmers from group

	Cattle				Sheep			
	A		B		C		D	
	Mean	95% CI [ ]	Mean	95% CI [ ]	Mean	95% CI [ ]	Mean	95% CI [ ]
Abortions	0.13%	[-1.16% —1.41%]	0.68%	[-1.47% — 2.83%]	2.77%	[0.26% — 5.29%]	2.6%	[0.95% — 4.24%]
Stillbirths	0.52%	[-0.79% — 1.83%]	-0.47%	[-1.55% — 0.61%]	8.31%	[4.52% — 12.11%]	5.37%	[3.02% — 7.73%]
Deformations	3.31%	[-0.47% — 7.1%]	0.25%	[-0.35% — 0.85%]	8.28%	[4.58% — 11.98%]	2.76%	[1.1% — 4.42%]

Table IV: Impact of Schmallenberg virus in the four groups used in the study aiming to identify Schmallenberg virus impact in Belgian ruminant livestock

The mean estimated impact of Schmallenberg virus (SBV) calculated as the difference between the percentage of respectively aborted/stillborn/deformed calves/lambs during S3 (during the outbreak of congenital diseases due to SBV) compared to the average of these percentages observed during S1 and S2 (before the outbreak of congenital diseases due to SBV) (Q1.2–1.5). Positive differences reflect the situation that percentages were higher during S3 compared to S1 and S2, negative differences indicate that percentages in S3 were lower than during S1 and S2. Results are given for the four groups: A (n = 53), B (n = 29), C (n = 42) and D (n = 44). The four groups were defined as: cattle (group A) and sheep (group C) farmers who obtained a SBV positive real–time reverse transcription polymerase chain reaction (rRT-PCR) result in a National Reference Laboratory (NRL); cattle (group B) and sheep (group D) farmers who never send suspected samples to NRL for SBV analysis.

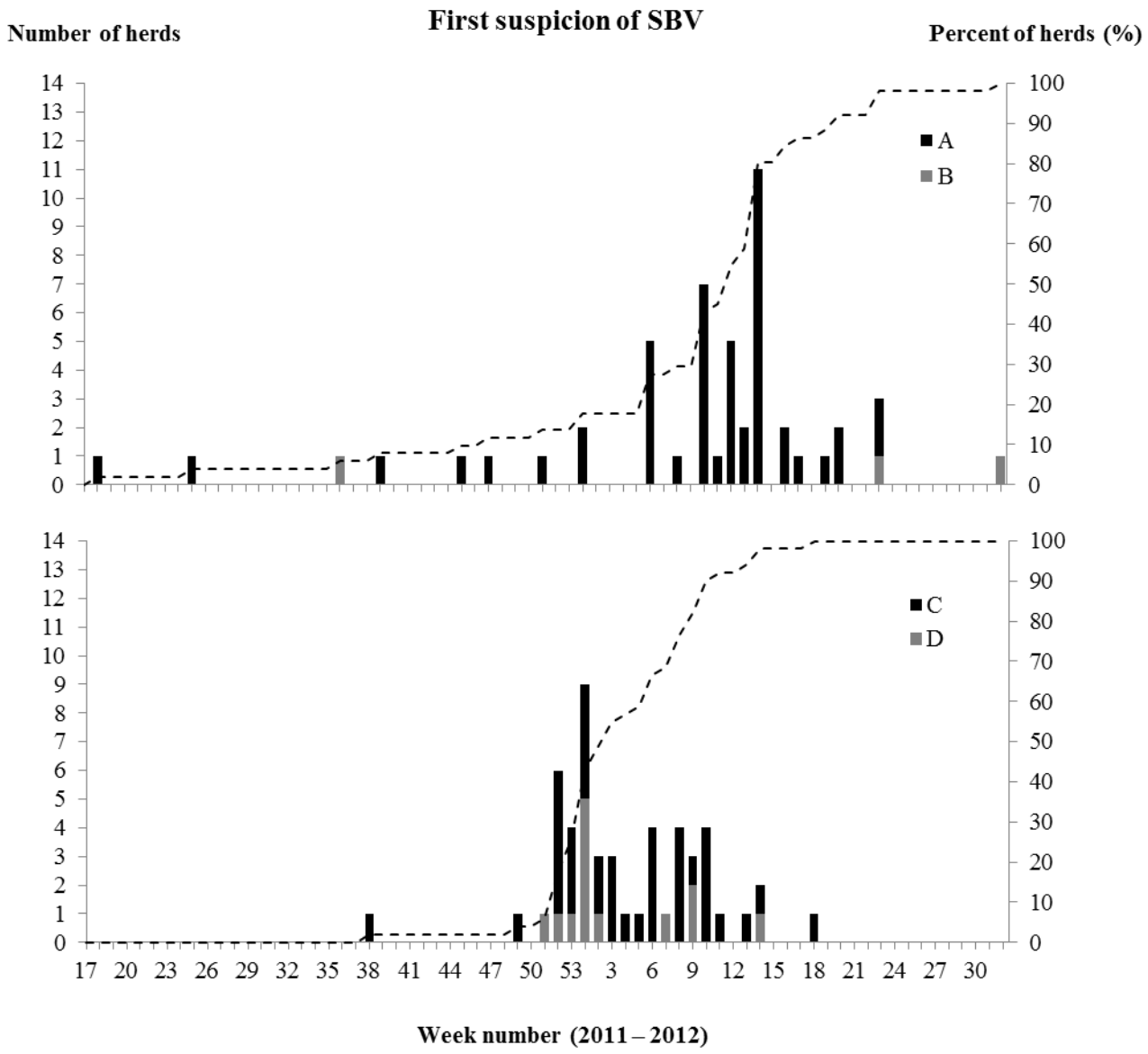

Figure 13: Number of herds newly infected with Schmallenberg virus in the four groups used in the study aiming to identify Schmallenberg virus impact in Belgian ruminant livestock

Number of herds perceived to be infected with Schmallenberg virus (SBV) by the farmers during 2011 and 2012 in the cattle herds and the sheep herds (Q1.7.1). Results are given for the four groups: A (n = 48), B (n = 3), C (n = 38) and D (n = 13). The four groups were defined as: cattle (group A) and sheep (group C) farmers who obtained a SBV positive real–time reverse transcription polymerase chain reaction (rRT-PCR) result in a National Reference Laboratory (NRL); cattle (group B) and sheep (group D) farmers who never send suspected samples to NRL for SBV analysis. The dashed line indicates the cumulative percentage of herds newly suspected of an SBV infection (——).

A stated that the death of these 'SBV–suspected' calves happened after an average period of 4 days (95% CI [1 – 8]) (Q1.7.4). Most of the SBV suspicious cases were reported on average from the beginning of March 2012 to the beginning of April 2012 in group A (Q1.7.5, Figure 14).

Twenty–two per cent of the farmers (11/49) from group A and 9% of the farmers (2/23) from group B reported that premature animals were born at farm during S3 (Q1.8).

***Sheep/lambs*:**

In sheep, the average lambing period was similar for groups C and D. The period covered mid–January to early April independently of the predefined seasons (S1, S2, S3) (Q1.1, data not shown).

The mean herd size and the mean number of gestation during S3 were substantially higher, however not significantly, in group C as compared to group D (Q1.2, Tables II and III). The fraction of aborted/stillborn/deformed lambs at animal level in group C and D for S1, S2 and S3 are given in Figure 12 (Q1.3 – 1.5).

The mean estimated impact of SBV on congenital signs in S3 indicates that, in total, 19% of the lambs in group C and 11% in group D were aborted, stillborn or deformed due to SBV in S3. The mean estimated impact of SBV in S3 was substantially different between groups C and D for stillborn and deformed lambs (Q1.3 – 1.5, Table IV).

In group C, deformations of the locomotor system, vertebral column, head and nervous signs were observed by 95% (40/42), 79% (33/42), 63% (25/40) and 29% (12/42) of the farmers, respectively. In group D, this was 25% (11/44), 19% (8/43), 9% (4/43) and 16%

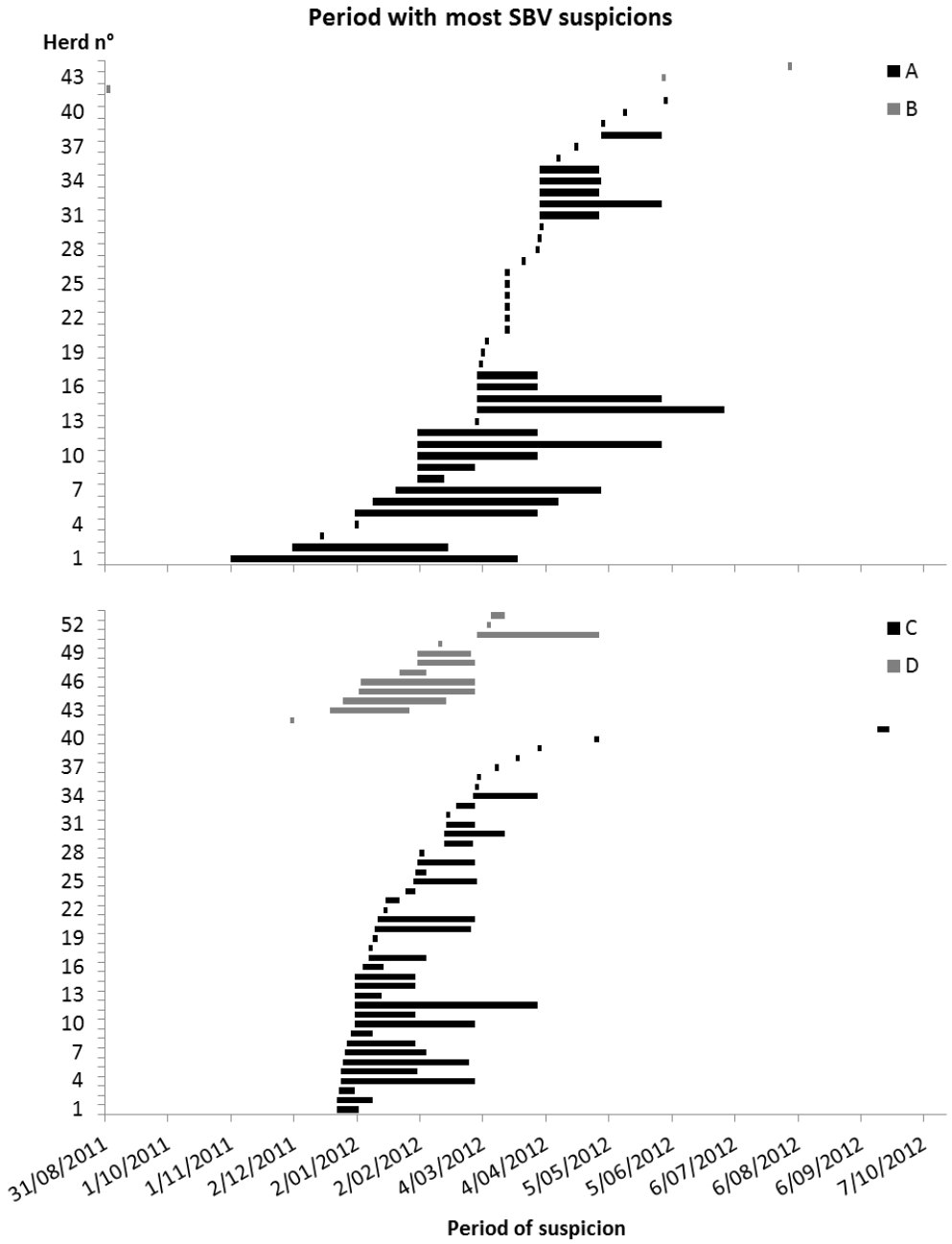

Figure 14: Peak of Schmallenberg virus suspicions in the four groups used in the study aiming to identify Schmallenberg virus impact in Belgian ruminant livestock

Period in which the highest number of calves and lambs were born and suspected of Schmallenberg virus (SBV) infection during 2011 and 2012 (Q1.7.5). Results are given for the four groups: A (herd numbers 1–41), B (herd numbers 42–44), C (herd numbers 1–41) and D (herd numbers 42–53). The four groups were defined as: cattle (group A) and sheep (group C) farmers who obtained a SBV positive real–time reverse transcription polymerase chain reaction (rRT-PCR) result in a National Reference Laboratory (NRL); cattle (group B) and sheep (group D) farmers who never send suspected samples to NRL for SBV analysis.

(7/44) of the farmers, respectively (Q1.6). Specifically, torticollis, twisted limbs and hydrocephalus were mostly related within the different systems.

The first retrospective suspicion of SBV as indicated by the sheep farmers was during week 38 of 2011 (Q1.7.1, Figure 13). It concerned a flock belonging to group C and localized in the middle–west of Belgium, about 15 km away from the Dutch border. No significant spatial cluster related to the date of first suspicion could be identified in the sheep group. In group C, among the reasons conducting to a first suspicion of SBV in the flocks, the birth of a deformed/stillborn lamb was invoked by 86% of the farmers (36/42). Twelve per cent of the sheep farmers (5/42) related a first suspicion of SBV due to an aborted lamb. Finally, only 2% of the sheep farmer (1/42) stated a first suspicion of SBV based on an adult sheep, which presented clinical signs commonly attributed to SBV in adult animals.

In group D, 32% of the farmers (14/44) retrospectively observed a first suspicion of SBV. For 71% of these farmers (10/14), the reason for this suspicion was a deformed/stillborn lamb, while the remaining 29% of farmers (4/14) suspected SBV on the basis of an aborted lamb. No sheep farmers from group D reported a first retrospective suspicion of SBV based on clinical signs commonly attributed to SBV in adult animals (Q1.7.2).

Sixty–five per cent of the farmers (26/40) in group C and 83% of the farmers who reported a first suspicion of SBV (10/12) in group D stated that ‘SBV–suspected’ lambs died shortly after birth (Q1.7.3). Moreover, the farmers estimated that these ‘SBV–suspected’ lambs died after an average period of 2.5 days in group C (95% CI [1.2 – 3.9]) and 1.6 days in group D (95% CI [0.5 – 2.8]) (Q1.7.4). Most of the SBV suspicious cases were reported in average from the end of January 2012 to the end of February 2012 in groups C and D (Q1.7.5, Figure 14).

Premature animals were reported by 7% of the farmers (3/42) in group C and 5% of the farmers (2/44) in group D (Q1.8).

Descriptive analysis: impact on adult animals

***Cattle*:**

During the interview, 28% of the cattle farmers (15/53) from group A and only 3% of the farmers (1/29) from group B declared having observed clinical signs commonly attributed to SBV in adult animals (Q2.1).

Two per cent of the cattle farmers (1/43) belonging to group A observed that at least one cow died shortly after giving birth to an 'SBV–confirmed' calf (Q2.2). In addition, 32% of the farmers (12/37) from group A observed hydroallantois in cattle which gave birth to 'SBV–confirmed' calves, while this was not observed in group B (Q2.3).

***Sheep*:**

Twelve per cent of the sheep farmers (5/42) in group C and only 2% of the sheep farmers (1/44) in group D declared to have seen clinical signs that can commonly be associated with an SBV infection in adult animals (Q2.1).

Twenty–four per cent of the sheep farmers (9/37) in group C and 38% of the sheep farmers (5/13) in group D declared to have at least one ewe that died after giving birth to an 'SBV–confirmed' lamb (Q2.2). Moreover, 24% of the sheep farmers (9/37) in group C

observed hydroallantois in ewe during lambing of 'SBV–confirmed' lambs. The latter was not observed in group D (Q2.3). Eleven per cent of the sheep farmers (4/37) in group C declared simultaneously that at least one ewe died after giving birth to an 'SBV–confirmed' lamb and that they observed hydroallantois in ewe during lambing of 'SBV–confirmed' lambs.

3.2.5 Discussion

This study describes the general impact of SBV perceived by Belgian cattle and sheep farmers after the emergence of SBV in 2011. The study was originally planned to be a case–control study. However, it seemed impossible to build a relevant control group. The between–herd seroprevalence in the Belgian cattle herds (99.76%; Méroc *et al*., 2013) and sheep herds (98.03%; Méroc *et al*., 2014) was indeed very high at the time the study was designed. This made it difficult to select available seronegative flocks for controls. Furthermore, in this study, 14% of the cattle farmers (4/29) and 32% of the sheep farmers (14/44) suspected SBV but did not provide any sample to the NRL for SBV analysis. Their presence in group B and D is thus questionable. These farmers could however not be included in group A or C because the SBV suspicions were not confirmed with rRT–PCR, and the latter was used as a classification criterion for cases and controls in this study.

The difficulties encountered for assigning herds into the case and control groups, which were also faced in other studies (EFSA, 2012, Veldhuis *et al*., 2014a), stress the importance of having a good definition of a case (herd) for emerging diseases.

Nevertheless, for some parameters, substantial and significant differences could be observed between the different groups of cattle and sheep defined in the current study (groups

A, B, C and D). In particular, herds belonging to groups A and C had a higher herd size and a higher number of gestations. This means that NRL obtained more rRT–PCR positive results from bigger herds (even though this should be confirmed in sheep with a bigger sample of herds). Stillbirths and deformed new-born are overall rare phenomenon. These congenital events have consequently more chances of being noticed in bigger herds. The latter is clearly shown by the higher estimated impact of SBV for stillborn and deformed animals observed in groups A and C (Table IV). The higher percentage of farmers who related deformations in the different organic systems and body parts in groups A and C (Q1.6) corroborates also this observation. It makes thus perfect sense that stillborn and deformed new-born, which were more observed in bigger herds, led to a suspicion of SBV and consequently pushed the farmers and the veterinarians to send samples to NRL (classification criteria for group A and C). These results stress that monitoring and surveillance of SBV can be focused in big herds because they are more likely to undergo stillbirths or deformed new-born due to SBV.

On the basis of our results, it can be observed that 41% of the cattle farmers (12/29) from group B and 11% of the sheep farmers (5/44) from group D did not send suspected material to NRL despite an abortion, which is mandatory in Belgium. This observation questions the efficacy of surveillance programme in Belgium as already demonstrated by Delooz *et al*. (2011).

Interestingly, abortions and stillbirths did not seem to be a clear consequence of SBV epidemic for the cattle farmers, in contrast with the situation in sheep, although high variability was observed between the herds. This difference results most probably from different susceptibility of the species (cattle or sheep) to SBV. This specificity was already reported in a preliminary impact study conducted in Belgium, which showed that sheep offspring was clearly more impacted by SBV than cattle offspring (Martinelle *et al*., 2014).

For both species, the birth of a deformed animal showed to be the typical sign for the farmers to suspect the presence of SBV in their farms. It seems logical because deformed animals were less observed during S1 and S2, the seasons before the outbreak of congenital signs due to SBV. Consequently, even if the percentages of aborted or stillborn calves or lambs increased during S3, the birth of a deformed animal constituted a remarkable phenomenon for the farmer. Interestingly, 86% of the farmers (25/29) from group B and 68% of the farmers (30/44) from group D did not mention the observation of an SBV suspicion in their herd (Q1.7). This is intriguing considering that all the herds from the groups B and D were seropositive for SBV (Méroc *et al*., 2013 and 2014). Nonetheless, among all farmers who did not suspect SBV (n = 25 in group B and n = 30 in group D), it is noticeable that not a single deformed calf or lamb was observed. The latter proves that the birth of a deformed animal was the essential condition for the farmers to suspect SBV at herd level.

Even if it is here assumed that the observation of deformed animals is a trigger to suspect SBV, to ensure a good diagnosis, this suspicion of SBV must be distinguish from the multiple causes, other than SBV, that can induce congenital deformations. Among these other causes, viruses [BTV, bovine viral diarrhoea virus (BVDV)] parasites (*Neospora caninum*), nutritional deficiency (manganese, selenium), toxic causes (lupin) or physic causes (radiation) must be investigated (Martinelle *et al*., 2012). Genetic defects must also be included in the differential diagnosis, particularly when it comes to the local Belgian blue breed (Weaver, 2004).

Most SBV suspicions were made by the farmers at the end of the year 2011 and the beginning of the year 2012. This is consistent with other studies relating a high number of SBV cases at that time in Belgium (De Regge *et al*., 2013; Bayrou *et al*., 2014). One cattle owner from group B reported however the birth of a calf still–born or deformed due to SBV as soon as week 18 of 2011 (early May 2011) suggesting an early SBV infection. This seems

very unlikely because evidences support a putative emergence of SBV during spring 2011, and clinical signs in offspring were not expected that soon (Garigliany *et al*., 2012c). It indicates that this stillborn/deformed calf was probably the consequence of another pathology or pathogen agent such as BVDV (Martinelle *et al*., 2012).

To the best of our knowledge, premature animals were never reported to be a consequence of SBV infection. This observation is consistent with the results of this study. Premature birth was indeed not a trigger for the farmer to suspect SBV at herd level. Premature animals should consequently not be used to monitor SBV presence in the future.

It is noticeable that during this survey, substantially more cattle farmers compared to sheep farmers observed clinical signs commonly attributed to SBV in adult animals. The higher percentage calculated in bovine species seems logical. Indeed, it is well described that after SBV infection, adult cattle suffer from an unspecific syndrome including the signs asked for in the questionnaire (Beer *et al*., 2013). It is however intriguing that some farmers reported clinical signs in adult sheep because clinical signs in adult sheep were never related under natural condition (Beer *et al*., 2013), and few clinical signs were observed after experimental infection (Wernike *et al*., 2013e). Hereto the proportion of farmers who observed clinical signs in adult animals was probably overestimated, particularly in sheep. SBV causes indeed a non–specific syndrome and the clinical signs described consequently to an SBV infection are commonly observed in other diseases. Subsequently, it is difficult to certify that the clinical signs observed in the herds were the actual consequence of an SBV infection and not the consequence of another pathogen agent. Furthermore, the clinical signs in adult animals happen weeks before the birth of deformed new-born, which was shown to be the true signal of SBV for the farmers. This time–gap between the observation of the clinical signs and the birth of deformed lambs, together with the non–specificity of the clinical signs and the absence or low awareness for the disease at that time (August–September of 2011) make the

link between SBV and clinical signs in adults difficult to certify for the breeders. In conclusion, the real impact of SBV on adult cattle seems to be low and was probably extremely limited in adult sheep. The clinical signs in adult animals were not a good indicator of SBV infection for the farmer.

More sheep farmers stated the death of ewes at lambing compared to cattle at calving. This can be associated with the fact that, proportionally, more deformed animals were born in sheep during the SBV epidemic. Deformed animals are susceptible to induce complications and dystocia, which could lead to a fatal issue (Mee, 2008). Also, less sheep farmers stated hydroallantois. This is probably due to a less closely monitoring during lambing as it is the case during calving in cattle. The deleterious effects of SBV in adult animal were in conclusion not only the direct consequence of SBV infection but also due to these dystocia, mainly in sheep.

Saegerman *et al*. (2014) carried out a similar impact study with sheep farmers. In that study, a case and a control group were defined. Comparison between both studies is difficult because in the present study, no credible control group was on hand. This could also be the case in the study of Saegerman *et al*. since the negative group was probably impacted by SBV for two reasons. Firstly, the percentages of aborted/stillborn/deformed lambs were high (3.2%, 9.5% and 2%, respectively) in the negative group of the study of Saegerman *et al*. compared to those observed during S1 and S2 in the present study that represented the Belgian situation before the outbreak of congenital signs due to SBV (Table III). Secondly, 50% of the surveys (13 sheep flocks) were classified in the negative group. Such a percentage of negative herds is however doubtful knowing that the seroprevalence in Belgium reached 98.03% in November 2011 (Méroc *et al*., 2014) during the period (April 2011 – February 2012) inquired by Saegerman *et al*. (2014). A comparison seems however relevant if only the positive groups from Saegerman *et al*. (2014) is used and compared with group C of the present study. Indeed,

using a similar approach (herds with a positive PCR result in sheep), the percentages of aborted/still-born/deformed lambs are higher with 2-3% compared to the present study. Different reasons can explain this discrepancy: the sample size (n = 168 and n = 26) as well as the geographical representativeness (Belgium versus only one region of Belgium) can induce a bias for comparing both studies.

This study described the general impact of SBV infection perceived by Belgian cattle and sheep farmers after the first emergence of SBV in Belgium. This descriptive analysis suggests that monitoring and surveillance of SBV can be implemented by monitoring the material from stillborn and deformed calves/lambs on large herds via rRT–PCR. It was also observed that SBV epidemic had minor impact on the percentages of aborted calves and lambs and stillborn calves, while the percentages of stillborn lambs and deformed calves and lambs were highly increased the year of the outbreak. The birth of a deformed calf/lamb was the most obvious trigger for the farmers to notify 'SBV–suspected material' to the laboratory. Finally, SBV had a limited impact in adult cattle and SBV probably did not affect adult sheep, with the exception of complications due to the birth of deformed lambs.

CHAPTER 4 : THE ROLE OF PIGS IN SCHMALLENBERG VIRUS LIFE-CYCLE

4.1 Preamble

Unravel the host range of a virus allows to understand its life-cycle and to identify potential unknown hosts. Schmallenberg virus proved itself a wide and rapid expansion in Belgium during cross-sectional studies and the Study 1 (Méroc *et al*., 2013 and 2014). Such an expansion was putatively explained by multiple hosts that could act as reservoir for SBV. The putative role of pigs in SBV epidemiology was therefore to be investigated considering the abundance of the species in Belgium. The importance to study the role of domestic pigs in the SBV life-cycle was supported by the suggested role of pigs in the epidemiology of the SBV closely related AKAV (Huang *et al*., 2003). Finally, local *Culicoides* species are known to blood feed on pigs, which supported also the importance of evaluating the putative role of pigs in SBV life-cycle (Ninio *et al*., 2011). To evaluate the role of pigs in the life-cycle of SBV, 4 piglets were infected with an SBV infectious inoculum and kept into contact with 4 non-infected piglets. The clinical impact of SBV in the piglets, the presence and the length of RNAemia, the seroconversion and the eventual horizontal transmission were evaluated.

4.2 Study 2: Experimental Schmallenberg virus infection of pigs

This section constitutes the original article published in *Veterinary Microbiology*.

ARTICLE 2:

Poskin A., Van Campe W., Mostin L., Cay B., De Regge N. Experimental Schmallenberg virus infection of pigs. Vet Microbiol, 2014, 170, 398–402.

4.2.1 Abstract

Schmallenberg virus is a newly emerged virus responsible for an acute non–specific syndrome in adult cattle including high fever, decrease in milk production and severe diarrhoea. It also causes reproductive problems in cattle, sheep and goat including abortions, stillbirths and malformations. The role of pigs in the epidemiology of SBV has not yet been evaluated while this could be interesting seen their suggested role in the epidemiology of the closely related AKAV. To address this issue, four 12 week old seronegative piglets were subcutaneously infected with 1 ml of SBV infectious serum provided by the Friedrich Loeffler Institute (FLI) and kept into contact with four non–infected piglets to examine direct virus transmission. Throughout the experiment blood, swabs and feces samples were collected and upon euthanasia at 28 dpi different organs (cerebrum, cerebellum, brain stem, lung, liver, iliac lymph nodes, kidney and spleen) were sampled. No clinical impact was observed and all collected samples tested negative for SBV in rRT–PCR. Despite the absence of viremia and virus transmission, low and short lasting amounts of neutralizing antibodies were found in two out of four infected piglets. The limited impact of SBV infection in pigs was further supported by the absence of neutralizing anti–SBV antibodies in field collected sera from indoor housed domestic pigs (n = 106). In conclusion, SBV infection of pigs can induce

seroconversion but is ineffective in terms of virus replication and transmission indicating that pigs have no obvious role in the SBV epidemiology.

4.2.2 Introduction

In December 2011, researchers from the FLI (Greifswald, Germany) isolated and identified a newly emerged virus responsible for an acute non–specific syndrome including high fever, decrease in milk production and severe diarrhoea in adult cattle during summer and autumn 2011 (Hoffmann *et al*., 2012). The virus was named SBV after the city where it was first identified (North Rhine–Westphalia, Germany; Hoffmann *et al*., 2012; Muskens *et al*., 2012). From December 2011 onwards, SBV was responsible for abortions, stillbirths and malformations such as the hydranencephaly–arthrogryposis syndrome in cattle, sheep and goat (Garigliany *et al*., 2012b; Herder *et al*., 2012; van den Brom *et al*., 2012). The identification of SBV in biting midges (Culicoides spp.) collected during summer and autumn of 2011 suggests an important role of these vectors in the wide and fast spread of SBV (De Regge *et al*., 2012).

Beside the animals mentioned above, SBV has been detected by rRT–PCR in bison, deer, moose, alpacas and buffalos and antibodies to SBV have been detected in fallow deer, roe deer, red deer, dogs and wild–boars (Linden *et al*., 2012; EFSA, 2012; Desmecht *et al*., 2013; Sailleau *et al*., 2013). To date, the role of pigs in the epidemiology of SBV has not yet been evaluated. This could however be interesting seen their suggested role in the epidemiology of AKAV, another virus of the Simbu serogroup that is closely related to SBV (Huang *et al*., 2003; Hoffmann *et al*., 2012). Seen the high density of pigs in the countries

affected by SBV and their economic importance, an experimental infection of pigs with SBV was performed to study their possible role in the SBV epidemiology.

4.2.3 Material and methods

Ethical statements

All experiments described in this study were approved by the Ethical Committee of the IPH–VAR (Scientific Institute of Public Health – Veterinary and Agrochemical Research Centre, project number 121017–01).

Experimental infection: animals, housing, inoculum and samples

Nine 12 weeks old Landrace breed pigs and one 10 months old ewe of the Mourerous breed were housed in the experimental animal BSL3 facilities of CODA–CERVA. All animals tested seronegative in a SBV VNT at the beginning of the experiment and the absence of SBV in their blood was confirmed by rRT–PCR. Eight piglets were housed together in one box, while one was kept apart as a negative control. Of the eight piglets, four were randomly selected and subcutaneously inoculated with 1 ml of SBV infectious serum and formed the positive group. The inoculum contained about 2 x 10^3 50% tissue culture infective dose/ml ($TCID_{50}$/ml) (kindly donated by the FLI, Germany; Wernike *et al.*, 2012b). The other four piglets in the box, as well as the negative control animal, were subcutaneously mock infected with 1 ml of phosphate buffered saline (PBS) and formed the transmission group. The ewe was housed separately and was subcutaneously inoculated with 1 ml of infectious serum (FLI)

and used as positive control of infection. A clinical examination of all animals was performed daily (temperature, appetite, respiratory and neurological signs, diarrhoea) and at days 0, 1, 2, 3, 4, 5, 7, 9, 11, 14, 17, 20, 24 and 28 for the pigs and 0, 1, 2, 3, 4, 9 and 17 for the ewe 2 blood tubes were sampled (one with EDTA and one without anticoagulant). One nasal swabs which was immediately immerged in 2 ml Minimum Essential Medium (MEM) (Dulbecco's, Sigma Aldrich, St. Louis, MO) and feces were also collected of the piglets. Serum was prepared from the blood tube without anticoagulant by 15 min centrifugation at 3000 rpm. All samples were stored at -80°C, except for EDTA blood that was stored at 4°C. The piglets were euthanized at 28 days post–infection (dpi) and following samples were collected during autopsy: cerebrum, cerebellum, brain stem, lung, liver, iliac lymph nodes, kidney and spleen.

Field collected samples from domestic pigs

One hundred and twenty–three sera collected from indoor housed pigs before the emergence of SBV (before 2011), that had continuously been stored at –20°C, were used to determine the VNT cut off value for porcine sera. Furthermore 106 sera collected from indoor-housed domestic pigs between 16 January 2012 and 12 April 2013 were used to evaluate the presence of neutralizing antibodies after the outbreak.

Serology

The presence of SBV specific antibodies was assessed by a VNT on porcine serum samples collected at -5, 0, 7, 14, 20 and 28 dpi during the experimental infection study and on field collected sera from domestic pigs. This was done following the protocol previously described by De Regge *et al*. (2013). Serum samples from the experimental infection study

were also tested by ELISA with the ID Screen Schmallenberg virus Indirect Multi–Species ELISA using an anti–multi–species IgG–horseradish peroxidase (HRP) conjugate (IDVet) following manufacturer's instructions. For each sample the S/P percentage was calculated. Test results were considered negative if S/P < 50%, doubtful if 50% < S/P<60% and positive if S/P > 60%.

SBV detection by rRT–PCR

The extraction of total RNA from blood, organs and feces was done as previously described (De Regge *et al*., 2013). Prior to RNA extraction, the immerged swabs were shaken at high speed for 60 min at 4°C, followed by total RNA extraction from 140 ml of MEM using the Qiamp Viral RNA mini kit (Qiagen, Hilden, Germany). Subsequent down–stream detection of the SBV–S segment RNA was done by rRT–PCR as previously described (De Regge *et al*., 2013). In each rRT–PCR run negative extraction controls and negative and positive amplification controls were included and tests were only validated when all controls were satisfactory.

4.2.4 Results

Clinical observations

None of the four infected piglets showed any clinical signs and the rectal temperatures stayed within the normal range of 38.7-39.2°C (Andersson and Jonasson, 1993) during the first two weeks after the inoculation with SBV. For one SBV infected piglet (number 4), the

rectal temperature started to rise at day 16 post–infection and peaked at day 19 post–infection at 41.6°C. Respiratory problems were observed from day 18 post–infection associated with translucent white nasal secretions and superficial breathing. This was followed by loss of appetite and apathy, finally leading to its death in the night between day 22 and 23 post infection. No gross pathological lesions were found at autopsy and the piglet tested negative for the following diseases in PCR assays: porcine circovirus 2 (PCV2), porcine encephalomyocarditis (EMC), porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV) and mycoplasma hyopneumoniae (data not shown). The negative control pig and the four transmission control pigs showed no clinical signs during all the experiment.

Virus replication, excretion and transmission

No macroscopic lesions were found in any of the piglets at autopsy. In addition, all blood samples, swabs, feces and organs of all infected and control pigs tested negative for SBV in rRT–PCR (data not shown). The infectivity of the inoculum was however confirmed by the clear viremia observed in the infected ewe between day 2 and 9 (data not shown).

Serology

The analysis of 123 pig sera dating from 2009 to the end of 2010 revealed sporadic titres up to 4 (Figure 15). Based on this result, the threshold value of the VNT for a positive SBV serology in piglets was set at a VNT titre of 8, corresponding to a specificity of 100%. In the infection experiment, two out of four SBV infected piglets had seroconverted by day 14 post-infection, but only low neutralizing antibody titres of 8 were found (Figure 16). By the

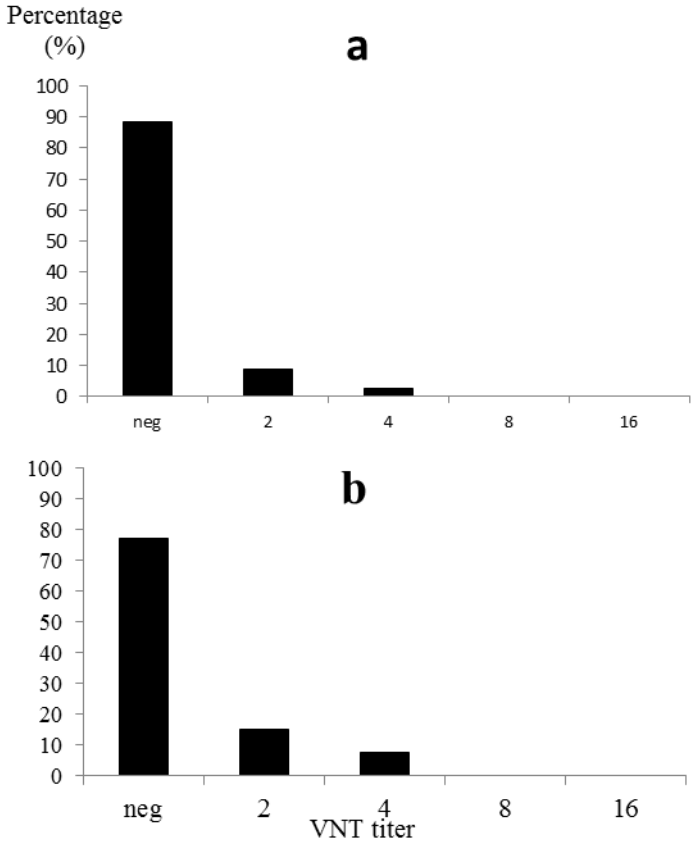

Figure 15: Schmallenberg virus specific antibody titres in domestic pigs housed indoor

Schmallenberg virus (SBV) specific antibody titres as measured by a virus neutralization test (VNT) are shown for sera collected from (a) domestic pigs housed indoor before the SBV emergence in 2011 (n = 123) and (b) domestic pigs housed indoor between January 2012 and April 2013 (n = 106).

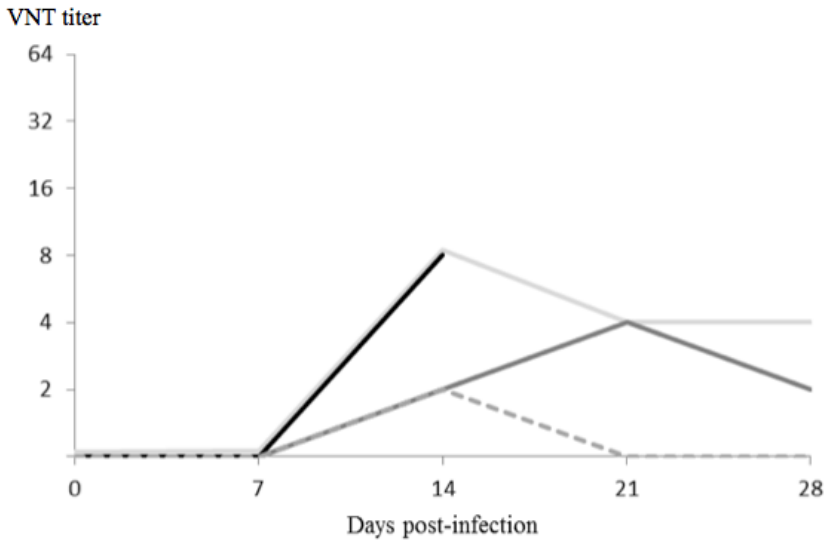

Figure 16: Schmallenberg virus specific antibody titres in pigs after an experimental infection with Schmallenberg virus

Schmallenberg virus (SBV) specific antibody titres as measured by a virus neutralization test (VNT) are shown for pigs subcutaneously inoculated with SBV at day 0 (pig 1 (—), 3 (—) and 4 (—)) and a transmission control pig (pig 5 (dashed line) that were present in the same pen. One inoculated pig (pig 2) and 3 other transmission control pigs had VNT titres <2 at each time point.

end of the experiment at day 28 post-infection, this titre had already dropped below the positive threshold in the remaining piglet that had seroconverted at day 14. The VNT titres of all other piglets remained negative throughout the experiment (Figure 15). All samples from the experimental study were also tested in ELISA and scored negative (data not shown). No SBV specific neutralizing antibodies could be found in the field sera collected from domestic pigs in 2012 – 2013 (Figure 15b). In the SBV infected ewe, anti–SBV antibodies were observed at 9 dpi by VNT and a titre of 128 was obtained at 17 dpi (data not shown).

4.2.5 Discussion

An in vivo infection experiment in which pigs were subcutaneously infected with SBV was performed to evaluate their possible role in the epidemiology of this newly emerged virus. The complete absence of RNAemia upon infection indicates that pigs do not have an obvious role in the SBV epidemiology since SBV is normally transmitted by insects that can only become infectious after the uptake of virus infected blood (Steukers *et al*., 2012). This is further supported by the absence of SBV in nasal swabs and in feces after infection, since this also eliminates a possible spread of the virus by other routes, as was confirmed by the absence of virus transmission to contact animals in our experiment. The absence of RNAemia in pigs is in contrast with the RNAemia observed after infection of one ewe in this experiment and in cows and sheep infected with the same inoculum via the same route of infection described in literature (Wernike *et al*., 2012b, 2013b and 2013c), indicating host dependent properties influencing virus replication upon infection.

During our experiment, one pig developed clinical signs like fever, respiratory problems and apathy at 16 days after SBV infection and died 6 days later. The complete absence of SBV in all samples collected at autopsy and the presence of SBV specific neutralizing antibodies in this pig indicate however that the infection with SBV was not the causative factor for its dead.

The differences between the experimental set–up in this study and the published infection study of pigs with AKAV (Huang *et al*., 2003) make that it is difficult to compare the results obtained for both viruses. While viremia and virus replication in organs could be shown after oronasal infection with AKAV, this was not the case upon an intramuscular infection what is in line with our results upon subcutaneous infection. Besides the difference in inoculation route, also differences in the amount of inoculated virus ($10^{6.5}$ for AKAV vs 2.10^{3} for SBV) and age of the pigs (4 weeks for AKAV vs 12 weeks for SBV) could be determining factors influencing the outcome of the experiments.

The experimental infection resulted in the seroconversion of two out of four pigs by two weeks after infection, but only low levels of neutralizing antibodies were found (VNT titres up to 8) and these titres had already dropped below the limit of positivity by the end of the experiment in the remaining pig that had seroconverted. The obtained neutralizing antibody titres in pigs are clearly lower than those found in the infected ewe (1/128) and those reported for SBV infected cows (Wernike *et al*., 2013b). Also AKAV infection of 4 weeks old pigs resulted in higher neutralizing antibody titres (up to 256 at 14 dpi) (Huang *et al*., 2003), but also here it should be considered that the difference in the age of the pigs at the moment of inoculation could have an influence on the outcome of the induction of the antibody response (4 weeks for AKAV vs 12 weeks for SBV). The positive samples in VNT were not confirmed in ELISA. This is in line with results in sheep that show that the multi species ELISA (IDVet) is less sensitive at early time points post infection (Poskin *et al*., 2014b), and probably

indicates that the first produced neutralizing antibodies have an IgM subtype, which are not detected by the anti–IgG conjugate in the ELISA.

Seen the difference between an experimental one shot subcutaneous SBV inoculation applied by a needle and the natural situation where pigs can be exposed to multiple inoculations by insect vectors, we also tested serum samples from a limited number of indoor housed pigs collected after the emergence of SBV. It seems plausible to assume that these pigs have been in contact with SBV seen (i) the evidence of extensive SBV circulation in Belgium in 2011 (Garigliany *et al*., 2012b; De Regge *et al*., 2012; Méroc *et al*., 2013 and 2015), (ii) the indications of a renewed SBV circulation in 2012 in Belgium and surrounding countries (Bayrou *et al*., 2013; Claine *et al*., 2013a), and (iii) the literature data showing that Culicoides involved in SBV transmission also feed on pigs (Bartsch *et al*., 2009; Ninio *et al*., 2011). Nevertheless, all samples tested negative providing indications that also under natural conditions, no high neutralizing antibody levels that remain detectable for longer time periods are induced upon SBV infection of pigs. The lack of data on the indoor presence and vector activity of Culicoides in pig stables make however that these results should be interpreted with caution.

Altogether the results suggest that pigs do not play an obvious role in the epidemiology of SBV and therefore do not contribute to the spread of the virus among livestock.

4.2.6 Conclusion

This study showed that subcutaneous infection of 12 weeks old pigs with an SBV inoculum which induced a viremia and seroconversion in a sheep, can result in a low and short lasting seroconversion, but without viremia and virus excretion. This indicates that pigs are only weakly susceptible to SBV infection what is further supported by the absence of neutralizing antibodies in a limited number of field collected sera from indoor-housed domestic pigs. Taken together, this study emphasized that pigs do not play an obvious role in the wide and fast expansion of the SBV epidemic. Future experiments evaluating the influence of inoculation dose, virus strain and age of the pigs could further clarify the pathogenesis of SBV in pigs.

CHAPTER 5 : IDENTIFICATION OF A STANDARDIZED SCHMALLENBERG VIRUS EXPERIMENTAL MODEL

5.1 Preamble

The Study 1 showed that the teratogenic effects of SBV were the main consequence of SBV in the Belgian farms, namely in sheep. It was therefore essential to study the process of congenital development defects in lambs. In this respect, experimental infections of pregnant sheep were planned. An experimental model is indeed an excellent tool to study the pathogenesis of a new emerging virus, although the development of a reproducible experimental model is essential and requires standardization. In a related experiment, Martinelle and collaborators (2015) evaluated the outcome of SBV inoculation in sheep by three different routes. This demonstrated that subcutaneous inoculation with the infectious inoculum was most appropriate for SBV. In this complementary experiment, the minimal infectious dose was assessed. The minimum dose was taught to reproduce the most efficiently a natural infection, considering the low quantity of saliva transmitted by Culicoides during blood meal. The potential dose–effect of SBV inoculation in sheep under experimental conditions was also evaluated since Wernike and collaborators (2012b) demonstrated that an inoculation of a 1/100 diluted infectious serum conducted to a longer RNAmia compared to an inoculation of a pure, 1/10 or 1/1000 diluted infectious serum in cattle. In this Study 3, four groups of three ewes were inoculated with 4 successive tenfold dilutions of an SBV infectious inoculum. The outcome on the clinical impact of the inoculation, the RNA, the seroconversion and the presence of SBV in the organs at 12 days post-inoculation were evaluated.

5.2 Study 3: Dose–dependent effect of experimental Schmallenberg virus infection in sheep.

This section is written on the basis of the original article published in the *Veterinary journal*.

ARTICLE 3:

Poskin A., Martinelle L., Mostin L., Van Campe W., Dal Pozzo F., Saegerman C., Cay A.B., De Regge N. Dose–dependent effect of experimental Schmallenberg virus infection in sheep. Vet J, 2014, 201, 419–22.

5.2.1 Abstract

Schmallenberg virus 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 viremia, 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.

5.2.2 Introduction, material and methods, results and discussion

Schmallenberg virus is a 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*., 2012b). 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, VNT and rRT–PCR, were included in the study. Three randomly selected ewes in each of four groups were inoculated subcutaneously in the left axilla with 1mL undiluted, or 1/10, 1/100 or 1/1000 diluted in PBS, SBV infectious bovine serum. The infectious serum was obtained from FLI and had been tested in cattle and sheep (Hoffmann *et al*., 2012; Wernike *et al*., 2012b and 2013). The inoculum contained 2 x 10^3 $TCID_{50}$/mL, as determined by end–point titration on BHK cells (Wernike *et al*., 2012b) 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 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 rRT–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 Annex 1).

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 rRT–PCR for viral RNA in blood (Figure 17). No SBV RNA could be detected in other ewes by rRT–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 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

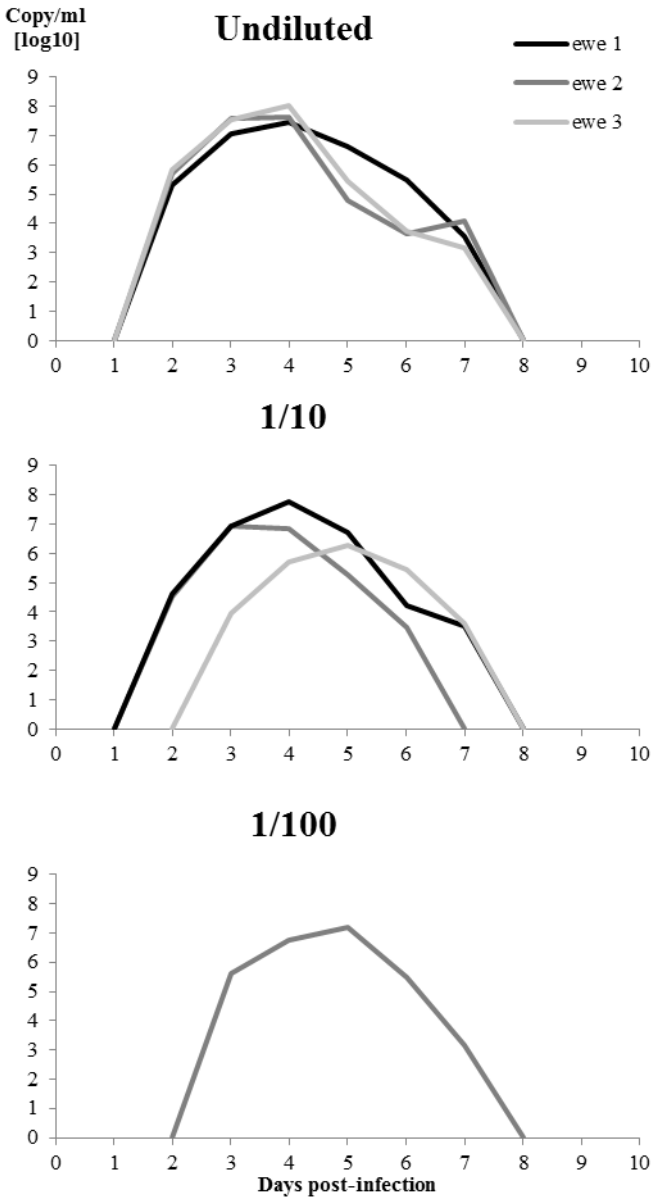

Figure 17: Schmallenberg virus RNA in the blood of sheep after inoculation of different dilutions of infectious inoculum

Detection of Schmallenberg virus (SBV) S segment RNA by rRT–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 rRT–PCR. No SBV RNA was detected at day 0, 9 and 10.

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*., 2013e).

In all sheep that became positive by rRT–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 rRT–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 rRT-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 euthanized at 10 dpi. No gross lesions were observed at post-mortem 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 rRT–PCR for viral RNA in blood (Table V). 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 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 rRT–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). 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

	Pure			1/10			1/100
	Ewe 1	Ewe 2	Ewe 3	Ewe 1	Ewe 2	Ewe 3	Ewe 2
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	4.30×10^6
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	1.30×10^7
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	8.10×10^5
Lung	neg*	neg	neg	neg	neg	6.80×10^4	7.60×10^5

*negative

Table V: Schmallenberg virus S–segment RNA in sheep organs after inoculation of different dilution of infectious inoculum

Schmallenberg virus (SBV) S–segment RNA (copies/g) detected at 10 days post inoculation by rRT–PCR in different organs of ewes subcutaneously inoculated with different doses (undiluted or 1/10, 1/100, 1/1000 dilution) of an SBV infectious serum. All samples from ewes inoculated with a 1/1000 dilution were negative all samples from other organs collected at autopsy were negative.

that were positive by rRT–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*., 2013e). 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.

The presence of SBV specific neutralising antibodies was assessed by VNT (De Regge *et al*., 2013). All ewes that were positive by rRT–PCR for viral RNA in blood seroconverted between 7 and 9 dpi (Figure 18), 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 VNT can detect immunoglobulin (Ig)M antibodies with neutralising capacity, while the ELISA only detects IgG due because it uses an anti–multi–species IgG–HRP 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 rRT–PCR) in the saliva of SBV–infected *Culicoides sonorensis* (Veronesi *et al*., 2013) indicate that this could also be realistic for SBV.

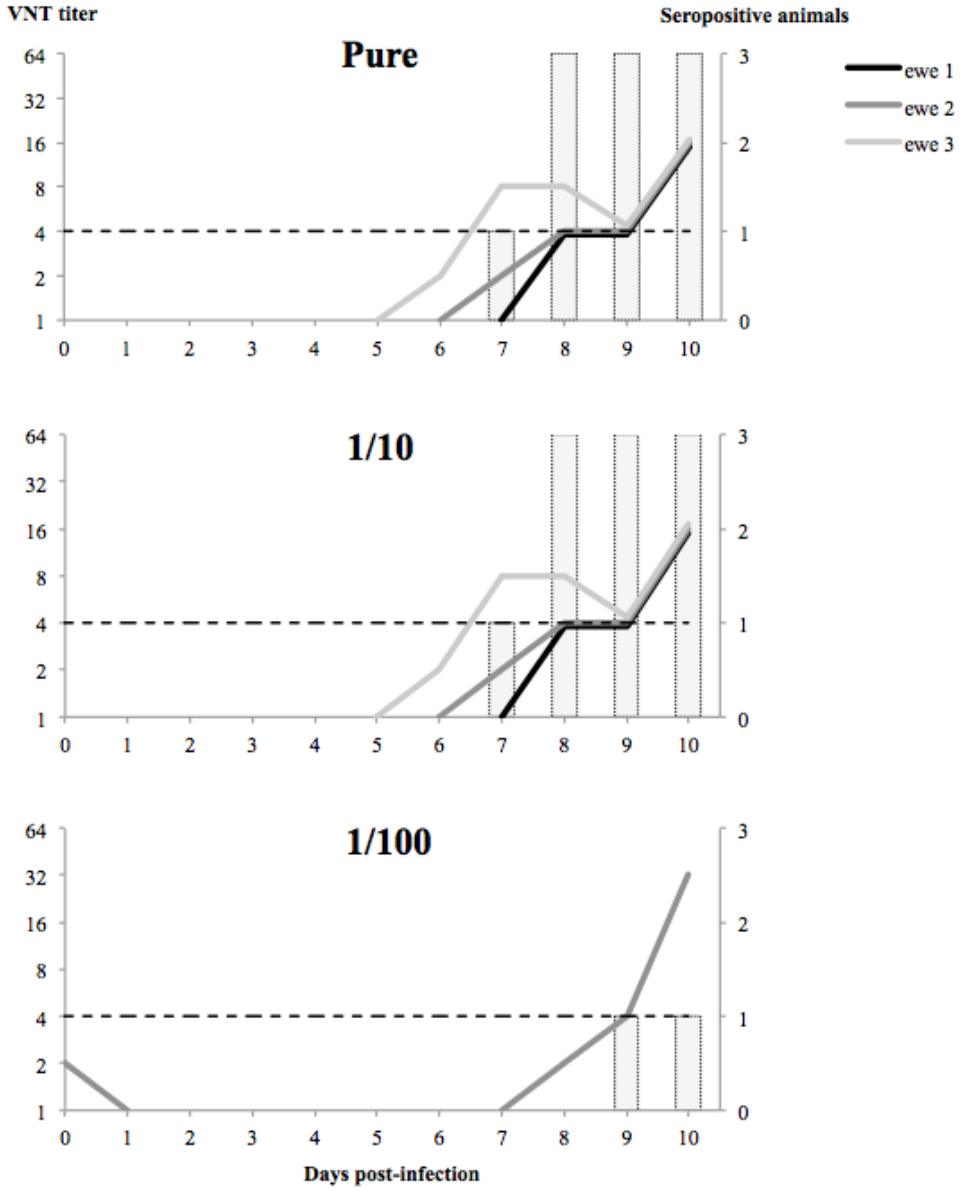

Figure 18: Seroconversion in Schmallenberg virus inoculated sheep after inoculation of different dilution of infectious inoculum

Titres of neutralising anti– Schmallenberg virus (SBV) antibodies measured in serum from four groups, each of three ewes, inoculated subcutaneously at day 0 with undiluted, or 1/10 or 1/100 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. Sera were considered to be positive if the 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.

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 rRT–PCR in blood, or in the amount of viral RNA present in the lymphoid organs.

CHAPTER 6 : INFECTION OF PREGNANT SHEEP WITH SCHMALLENBERG VIRUS

6.1 Preamble

The Study 1 demonstrated that the emergence of SBV led to dramatic congenital repercussions in ruminant livestock. Congenital abnormalities following infection with AKAV, an *Orthobunyavirus* closely related to SBV, demonstrated the relationship between the stage of gestation for infection and the observation of congenital defects. Malformations were observed upon AKAV infection at day 30 and 36 of gestation (Parsonson *et al*., 1977 and 1988). The transplacental infection with SBV remained poorly understood at the time this study was designed. In this respect, an experimental model of SBV infection was developed in preliminary experiments (Study 3) to evaluate the outcome of infection with SBV infectious inoculum in pregnant ewes. To assess the open-window in which an SBV infection must occur to induce congenital defects, this work was conducted in partnership with a Dutch team. The Dutch team inoculated pregnant sheep at day 38 and 45 of gestation, while in this Study 4 the outcome of an inoculation at day 45 and 60 of gestation was chosen. This allowed the covery a susceptible period of gestation for the development of congenital defects as estimated on the basis of AKAV (Martinelle *et al*., 2012). In this respect 17 pregnant ewes were inoculated with an infectious inoculum at day 45 or 60 of gestation, the clinical outcome of the experimental inoculation, the RNAemia and the seroconversion were evaluated in the ewes. The experiment was conducted until the natural birth of the lambs. The clinical outcome of inoculation in living new-born lambs, the RNAemia and the seroconversion in the lambs, and the presence of RNA in lamb organs were studied. Finally, the presence of RNA in the foetal envelopes was evaluated.

6.2 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

This section constitutes the original article published in *PLOS One*.

ARTICLE 4:

Ludovic Martinelle[¶], Antoine Poskin[¶], Fabiana Dal Pozzo, Nick De Regge, Brigitte Cay, Claude Saegerman. 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, 2015, 10(9), e0139375.

[¶] These authors contributed equally to the work

6.2.1 Abstract

Congenital defects (malformations, abortions and stillbirths) proved to be the most deleterious impact of SBV in domestic ruminant livestock. The transplacental transmission of SBV remains however poorly understood. Therefore the outcome of an experimental infection of pregnant ewes with SBV at day 45 and 60 of gestation was evaluated in this study.

Twenty–three "Mourerous" breed ewes were randomly distributed in three groups. Two groups of eight (G45) and nine (G60) ewes were subcutaneously inoculated with 1 ml of

SBV infectious serum at day 45 and 60 of gestation, respectively. In the third group, six ewes were mock–infected with PBS and constituted the control group. The ewes were clinically monitored and blood was sampled every day during two weeks after the inoculation and thereafter once a week. Lambs were born at term via Caesarean section, and immediately after birth a clinical examination was performed on the lambs and their blood was sampled. Finally, the ewe and its lamb(s) were euthanized and necropsied.

All SBV inoculated ewes showed RNAemia, seroconverted and no clinical sign was reported after SBV inoculation. No malformation suggestive of SBV infection, nor RNAemia, nor SBV specific antibody production before the colostrum intake was observed in lambs. Schmallenberg virus RNA positive organs (brainstem, cartilage, lung, prescapularis lymph node and spinal cord) were recovered with rRT–PCR from one (out of 11) and two (out of 10) lambs in G45 and G60, respectively. Also seven (out of 11) lambs from G45 and three (out of 10) lambs from G60 presented SBV RNA in meconium. The extraembryonic structures (amniotic fluid, intercotyledonnary membrane, placentome and umbilical cord) were the most frequently SBV RNA positive organs. The total number of SBV RNA positive extraembryonic structures was significantly higher in G60 (17/36) compared to G45 (6/31).

In conclusion, SBV readily colonized the placenta upon inoculation of pregnant ewes at day 45 and 60 of gestation but did not induce congenital malformations in offspring.

6.2.2 Introduction

In summer 2011, an unspecific clinical syndrome from unknown origin was observed in German adult cattle presenting a febrile disease with milk drop and diarrhoea (Hoffmann *et*

al., 2012). This syndrome was later attributed to a novel *Orthobunyavirus* (family *Bunyaviridae*) named SBV and closely related to AKAV. Few months later, an epizootic of malformations, abortions and stillbirths was observed in calves, lambs and kid goats due to *in utero* SBV transmission (Herder *et al*., 2012).

The day of gestation at the moment of inoculation with the SBV closely related AKAV is known to influence the clinical signs observed in the foetus. Indeed, the placenta needs to be developed and vascularized enough to allow AKAV transmission to the foetus and the infection should not take place after day 50 of gestation to induce congenital malformations (Hashiguchi *et al*., 1979; Parsonson *et al*., 1988).

An early mating season was proven to increase the risk of observation of malformations due to SBV (Luttikholt *et al*., 2014). Reproduction periods are mostly seasonal in sheep and cover July to August or October to November, and SBV is transmitted by Palearctic telmophagous midges of the genus *Culicoides* (Abecia *et al*., 2011; De Regge *et al*., 2012; Veronesi *et al*., 2013; De Regge *et al*., 2014). In this respect, sheep reproduction periods overlap high vector activity season (Takken *et al*., 2008). The identification of the susceptible period for the apparition of malformations due to SBV infection *in utero* is therefore important to unravel. Specific management practice aiming to eliminate contact between sheep and vectors during the susceptible period of gestation could reduce the number of malformed lambs observed during an SBV epidemic.

In another experiment, the impact of infection of pregnant sheep at day 38 and 45 of gestation was evaluated and led to transplacental transmission of SBV. The outcome on congenital malformations was however difficult to evaluate since all ewes were slaughtered only one week after infection, this time–span being too short for the malformation to develop enough and be observed (Stockhofe *et al*., 2013).

In the present experiment, 17 ewes were subcutaneously inoculated with an SBV infectious serum at day 45 (G45) and 60 (G60) of gestation to evaluate the transplacental transmission of SBV at later stage of gestation. Also, the lambs were born at term of the gestation to give the malformations the time to develop *in utero* and be observed at birth.

6.2.3 Material 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 (number of project: 121017–01) on the 11th February 2013. All surgery was performed using xylazin (Paxman®, Virbac, France) and local anaesthesia procaine hydrochloride 4 % (VMD, Belgium), and all efforts were made to minimize suffering.

Animals

Twenty–three "Mourerous" breed ewes of about one year–old and originating from an SBV free area in France were used in this experiment (original sheep flock from the *Alpes–Maritimes* department). The animals were selected after a last serological screening carried out on 08/11/2012. The Mourerous is a middle–size rustic breed from South of France. All ewes were in good clinical condition and in the expected range of physiological parameters

before the start of the experiment (Jackson and Cockcroft, 2002). The ewes tested negative for BTV, Maedi–Visna and were confirmed to be serologically and virologically negative for SBV with ELISA, VNT and rRT–PCR (see below) before and after arrival in BSL 3 facilities of the CODA–CERVA. Insect light/glue traps were hanged to monitor and ensure the absence of *Culicoides* midges throughout the experiment.

Inoculum

A bovine SBV infectious serum provided by the FLI (Riems, Germany) was used for the inoculation. Briefly, the infectious serum originated from a heifer sampled two and three days after inoculation with infectious whole blood obtained from an SBV positive cow. The inoculum contained about 2 x 10^3 $TCID_{50}$/ml (Wernike *et al*., 2012b) and 7.3 x 10^6 RNA copies/ml of SBV S–Segment as determined by a rRT–PCR (see below). The inoculum succeeded to induce SBV RNAemia and SBV specific seroconversion in cattle and sheep in previous experiments (Hoffmann *et al*., 2012; Wernike *et al*., 2012b and 2013e).

Insemination and diagnosis of gestation

The animals were synchronised using 60 mg medroxyprogesterone acetate sponges (Veramix, Zoetis®, Louvain–La–Neuve, Belgium). Twelve days after insertion, the sponges were removed and 500 international units (IU) of pregnant mare serum gonadotrophin (PMSG) were intramuscularly injected to the sheep. Fifty–two hours after sponge removal, an intracervical artificial insemination (AI) was carried out with a double dose of semen. The semen was obtained from four rams ranging from one to six years old: three "Ile–de–France" and one crossbred "Texel" x "Ile–de–France". These rams originated from a Maedi–Visna

free flock. The semen was confirmed to be SBV negative twice, one week apart, before the AI and a sample of the inseminated semen tested negative at SBV rRT–PCR after the AI.

The gestation was diagnosed in ewes with the detection of pregnancy-associated glycoprotein (PAG) with radio immune–assay (RIA) at day 27 of pregnancy (Barbato *et al*., 2009). The gestation was confirmed with ultrasonography at day 40 and 95 of gestation in G45 and at day 55 and 110 of gestation in G60.

Experimental design

The ewes were inoculated subcutaneously in the left axilla with 1 ml of the described inoculum. Eight ewes were inoculated at day 45 of gestation (ewe 1 – 8) and nine ewes were inoculated at day 60 of gestation (ewe 9 – 17). Two control groups, each composed of three animals, were mock–infected with PBS at day 45 (control G45, ewes 18 – 20) and at day 60 of gestation (control G60, ewes 21 – 23), respectively.

A clinical examination was performed and sheep blood was sampled daily during 12 days, then once a week until lambing. Upon the observation of the first signs of lambing, the ewe was anesthetized with intravenous injection of xylazin (Paxman®, Virbac, France) and local injection of procaine hydrochloride 4% (VMD, Belgium), and the lambs were born with a Caesarean section. The ewes were euthanized immediately after lambing with intravenous injection of 5 ml of associated euthanasia drug (T61®, MSD Animal Health BVBA, Belgium) and necropsied. A portion of the following organs was collected during necropsy: prescapular, mesenteric and mediastinic lymph nodes, ovary, lung and spleen. Also, sample of the following extraembryonic structures was collected: amniotic fluid, intercotyledonary membrane, placentome and umbilical cord.

The lambs were nursed immediately after birth and they were given a number referring to their dam (*e.g.*: ewe 1 gave birth to lamb 1; plus a, b or c in case of multiple gestation). Thereafter, the living lambs were evaluated for the presence of neurological defects. The ability to stand and the suckling reflex were particularly investigated. A complete morphologic examination was performed. After the neurologic and morphologic examination, the blood was sampled and the lambs were euthanized with intravenous injection of 1 ml of associated euthanasia drug (T61®, MSD Animal Health BVBA, Belgium). Sample of organs including adrenal gland, brain, cerebellum, brainstem, femoral cartilage, gonads, liver, lung, lymph nodes (prescapular, mesenteric, submandibular and mediastinic), *Musculus Semitendinosus*, spinal cord, spleen and thymus, plus the meconium, were collected from the lambs.

SBV detection by rRT–PCR

The extraction of total RNA from organs, meconium and blood was carried out as previously described (De Regge *et al*., 2013). The presence of the SBV–S segment was detected in a one–step rRT–PCR conducted as previously described (De Regge *et al*., 2013). An RNA standard curve was added to each run of PCR to estimate the number of SBV RNA copies per gram of organ and meconium, and per ml of blood. The standard curve was obtained as previously described (Poskin *et al*., 2014b). Briefly, the RNA standard curve consisted of a ten–fold serial dilution of the RNA transcripts in RNase free water. The dilution series ranging from 3.9 to 3.9×10^7 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 numbers. A linear regression was fitted to the scattered points and was used to calculate the number of copies in the samples of the same run (Annex 1).

Serology

The presence of SBV specific antibodies was assessed by VNT, following the method described by De Regge *et al*. (2013) and using an SBV isolate obtained from brain tissue of a lamb aborted in Belgium in 2011 and passaged four times in Vero cells. Two positive and one negative control were added to each run of VNT. The titre was determined as the reciprocal of the highest serum dilution in which the entire monolayer of Vero cells was still intact. Sera were considered positive if the titre was ≥4 (specificity of 100%) (De Regge *et al*., 2013).

Statistical analysis

The percentage of living lambs (healthy lambs) and the prolificacy rate were compared between G45 and G60 and between infected and control groups with Chi square tests. The mean number of SBV copies/ml of blood recovered over time in each ewe was compared between G45 and G60 with two–way ANOVA with repeated measures. The mean number of SBV copies recovered at the peak of RNAemia in each group was compared between G45 and G60 with Welch test for unequal variance. The total number of SBV RNA positive organs and SBV RNA positive meconium were compared between G45 and G60 with Chi square test.

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

6.2.4 Results

Clinical impact and lambing

No clinical impact was observed in ewes, all the animals stayed in physiological condition, conserved a good appetite and the rectal temperature stayed within the normal range until the end of the experiment.

Sex ratio, prolificacy rate and the percentage of living lambs (healthy lambs) at birth are given in Table VI. The prolificacy rate was not significantly different between G45 and G60 (Chi^2 test=0.1, *P*=0.74; df=1) and between infected and control groups (Chi^2 test=0.008, *P*=0.9; df=1). No significant different was observed in the percentage of living lambs (healthy lambs) between G45 and G60 (Chi^2 test=0.7, *P*=0.4; df=1), and between infected and control groups (Chi^2 test=0.3, *P*=0.6; df=1).

Only one lamb was born three days before the expected date, it was in good health and had the opportunity to drink the colostrum. All other lambs were born at term, and all the living lambs were able to stand up and showed a good suction reflex. No malformation evocative of SBV infection (hydranencephaly–arthrogryposis syndrome, stiff neck, scoliosis, brachygnathism) was observed.

Group	Number of lambs	Sex ratio	Prolificacy rate	Healty lambs (%)
G45	11	0.8	1.5	64
G60	10	1.5	1.1	80
Control G45	4	1	1.3	50
Control G60	3	2	1	67
Total	**28**	**3**	**1.2**	**68**

Table VI: Reproductive performances in Schmallenberg virus inoculated pregnant ewes

The table shows the reproductive performance for ewes inoculated with Schmallenberg virus at day 45 (G45) and 60 (G60) of gestation, or mock–infected at day 45 (Control G45) and day 60 (Control G60) of gestation. The sex ratio was calculated for each group as the number of male lambs divided by the number of female lambs. The prolificacy is defined as the mean number of lambs born per ewe in each group. The percentage of living lambs (healthy lambs) is given and was calculated as the number of living lambs divided by the total number of lambs in each group.

SBV RNA detection

SBV detection in the blood and organs of the ewes

All the inoculated ewes showed an RNAemia. Schmallenberg virus RNA was detectable upon 2 dpi in G45 and G60 and the RNAemia was detected during a maximum period of five days (Figure 19). The mean number of SBV copies/ml of blood recovered over time in each ewe was not statistically different between G45 and G60 (two–way ANOVA for repeated measures; group effect: P = 0.4; group-time interaction: P=0.44). The mean number of SBV copies recovered at the peak of RNAemia in each group was not significantly different between G45 and G60 (Welch test for unequal variance, P=0.83).

No SBV RNA was recovered in organs of ewes from G45. In G60, SBV RNA was detected in the ovaries of ewe 9, the ovaries and the spleen of ewe 11 and the spleen of ewe 16 (Table VII).

No viral RNA was detected in the blood and organs of the control ewes.

SBV detection in the blood and organs of the lambs

No SBV RNA was recovered from the blood of the lambs from the G45. In organs, SBV RNA was detected in the lung of the lamb 4b (Table VII).

No SBV RNA was recovered from the blood of the lambs from G60. In organs, SBV RNA presence was demonstrated in the brainstem and the spinal cord of the lamb 10 and in the femoral joint cartilage, the prescapular lymph node and the *Musculus Semitendinosus* of the lamb 11. Seven and three lambs from G45 and G60, respectively had SBV RNA in

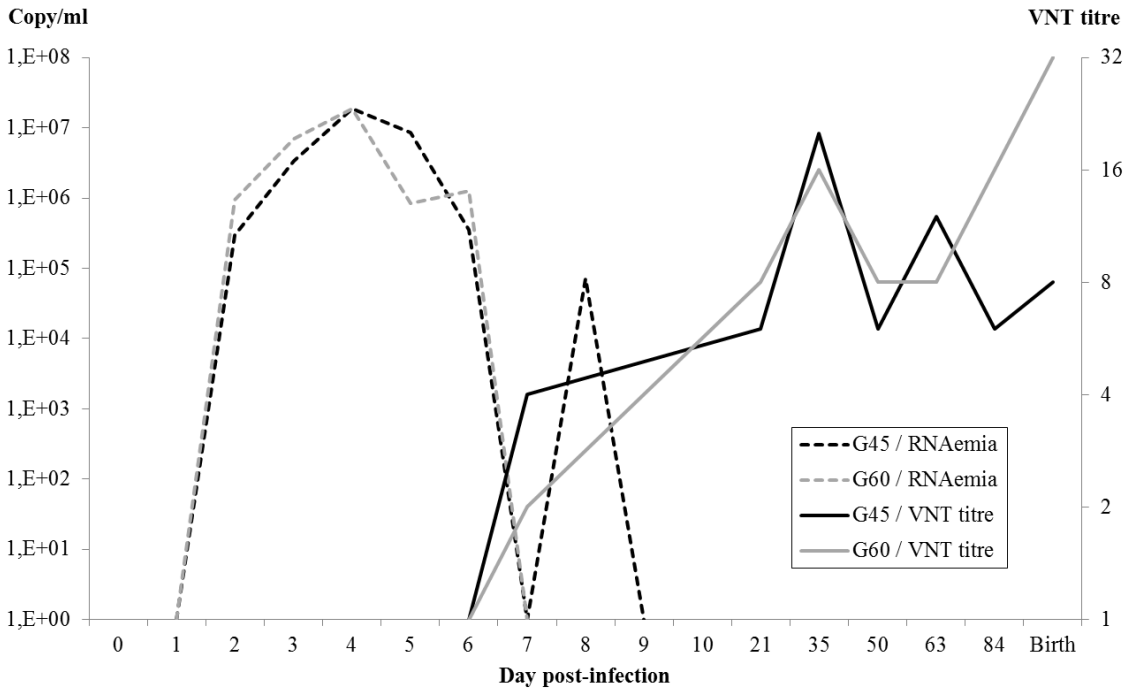

Figure 19: RNAemia and seroconversion in the pregnant ewes inoculated with Schmallenberg virus

The dashed lines indicate the median number of Schmallenberg virus (SBV) RNA copies/ml of blood recovered with rRT–PCR in two groups of pregnant ewes inoculated with SBV at day 45 (G45, dark) and 60 (G60, grey) of gestation. The continuous lines give the median titre of SBV specific antibodies determined with the virus neutralization test (VNT).

	Ewe			Lambs								Placental organs			
	n°	Ovary	Spleen	n°	Brainstem	Cartilage	Lung	Meconium	Muscle	Prescapularis lymph node	Spinal cord	Amniotic fluid	Intercotyledonary membrane	Placentome	Umbilical cord
G45	2	-	-	2	-	-	-	2.5E+05	-	-	-	-	-	-	-
	3	-	-	3a	-	-	-	-	/	/	-	3.2E+04	-	-	-
				3b	/	/	-	3.7E+05	/	/	-	/	/	/	/
				3c	-	-	-	6.5E+05	/	-	-	/	/	/	/
	4	-	-	4a	-	-	-	2E+05	-	-	-	2.9E+04	/	3.6E+07	1.6E+08
				4b	-	-	3.3E+05	4.1E+06	-	-	-	/	/	/	2.9E+05
	6	-	-	6	-	-	-	7.9E+05	-	-	-	-	/	-	-
	7	-	-	7	-	-	-	1.7E+05	-	-	-	-	9.3E+05	-	-
G60	9	10E+06	-	9a	-	-	-	-	-	-	-	-	5E+09	1.2E+10	2.2E+09
				9b	-	-	-	7.5E+07	-	-	-	6E+04	/	/	-
	10	-	-	10	9.6E+05	-	-	-	-	-	7.8E+05	8.2E+04	-	-	-
	11	7.4E+06	4E+06	11	-	4.1E+04	-	1E+05	2E+05	7.2E+04	-	1E+06	/	2.1E+09	1.7E+06
	12	-	-	12	-	-	-	-	-	-	-	-	4E+05	-	-
	13	-	-	13	-	-	-	7.7E+04	-	-	-	5.2E+05	-	-	-
	14	-	-	14	-	-	-	-	-	-	-	-	7.6E+08	5.1E+09	-
	15	-	-	15	-	-	-	-	-	-	-	1.5E+05	1.4E+05	3.1E+07	-
	16	-	2.4E+06	16	-	-	-	-	-	-	-	-	/	-	-
	17	-		17	-	-	-	-	-	-	-	-	-	9.7E+09	1.2E+05

Table VII: Schmallenberg virus RNA in organs collected at birth from ewes, foetal envelope and lambs after inoculation of pregnant ewes with Schmallenberg virus

The table indicates the number of Schmallenberg (SBV) S segment copies/gr of organ and copies/ml of amniotic fluid recovered with rRT–PCR in organs collected in two groups of pregnant ewes inoculated with SBV at day 45 (G45, ewe 1 to 8) and day 60 (G60, ewe 9 to 17) of gestation. The organs were collected in ewes, lambs and placental organs during autopsy, shortly after lambing. (–) no SBV RNA copy was detected at rRT–PCR. (/) no material was collected during autopsy. No SBV RNA was recovered from ewe, lamb and placental organs of ewes 1, 5 and 8. All other organs collected in ewes (lung, mediastinic, mesenteric and prescapularis lymph nodes) and lambs (adrenal glands, brain, cerebellum, gonads, liver, mediastinic and mesenteric lymph node, spleen and thymus) scored negative at rRT–PCR.

meconium. The total number of SBV positive meconium in each group was substantially higher in the G45 (7/11) compared to the G60 (3/10), however not significantly (Chi^2 test=3.38, *P*=0.12; df=1).

SBV detection in extraembryonic structures

The intercotyledonary membrane was the most frequently SBV RNA positive extraembryonic structures in the inoculated ewes (5/10), followed by the amniotic fluid (7/18), the placentomes (6/18) and the umbilical cord (5/18) (Table VII). No statistical difference was observed between G45 and G60 for the number of SBV positive intercotyledonary membrane, amniotic fluid and umbilical cord. The total number of SBV positive extraembryonic structures detected in lambs was however significantly higher in G60 (17/36) compared to G45 (6/31) (Chi^2 test=5.74, *P*=0.017; df=1).

Serology

The first seroconversion was observed at 7 dpi and all the inoculated ewes seroconverted before 21 dpi. Subsequently, the SBV specific antibody titre remained positive until the end of the experiment (Figure 19). All the lambs were seronegative at birth excepted the lamb 13 (that was born three days before the expected date, and that had the opportunity to drink the colostrum), which had a titre of 16 at VNT.

6.2.5 Discussion

Schmallenberg virus RNA positive extraembryonic structures were previously described under natural conditions (Bilk *et al*., 2012). In the present experiment, SBV placental colonization of pregnant ewes was successfully reproduced under experimental conditions upon inoculation of an SBV bovine infectious serum at day 45 and 60 of gestation.

The absence of congenital malformations observed upon SBV inoculation of pregnant ewes at day 45 and 60 of gestation supports the observations made for AKAV by Hashiguchi *et al*. (1979) who estimated that the inoculation should occur between day 28 and 36 of gestation to induce malformations. It supports also the observation made for SBV indicating that the inoculation should occur between day 37 and 42 to induce congenital malformations (Sedda and Rogers, 2013). The absence of congenital malformations could also be related to the natural low propensity of SBV to induce malformations demonstrated under natural conditions (Poskin *et al*., 2015a).

Stockhofe *et al*. (2013) reported 100% of placental colonization (at least one SBV RNA positive placental organ) upon inoculation of pregnant sheep at day 45 of gestation. Therefore it could be expected that more than five out of the eight inoculated ewes in G45 showed SBV RNA positive placenta in the present study (Table VII). The placentas were however analysed only 7 dpi by Stockhofe *et al.* (2013) while this was 105 dpi in the present experiment. It makes thus perfect sense that natural clearance of the virus from the placenta could occur between 7 dpi and 105 dpi. The higher number of SBV RNA positive organs in placentas recovered in G60 compared to G45 supports also this consideration. Indeed, the placentas were analysed at 90 dpi in the G60 while this was only 15 days later at 105 dpi in the G45. Together with SBV natural clearance, the expansion and the vascularization of the

placenta might also contribute to the higher number of SBV RNA positive placental organs in G60 compared to G45 considering that the placenta is reported to be poorly differentiated at day 42 of gestation (Lawn *et al*., 1969; Parsonson *et al*., 1988; Varela *et al*., 2013; EU consortium, 2014). Consequently, the underdevelopment of the placenta at day 45 of gestation compared to the day 60 of gestation could make it a better structure at day 60 of gestation for SBV replication and SBV RNA persistence until birth.

It is intriguing that the lambs were born seronegative at birth despite the passage of SBV through the foetus (as evidenced by the SBV RNA recovery in the meconium of 10 lambs and the organs of three lambs) and considering that AKAV specific antibody production can already be detected at birth after inoculation of AKAV at day 30 and 36 of gestation (Parsonson *et al*., 1988). A strong correlation was demonstrated between the observation of malformations in the foetus and the recovery of precolostral antibody for AKAV (Kurogi *et al*., 1975). This might be the case for SBV as well since recent report showed that 79 to 91% of naturally infected malformed lambs were seropositive before colostrum intake (van Maanen *et al*., 2012; Peperkamp *et al*., 2014). The absence of SBV specific pre–colostral antibodies in the lambs of ewes inoculated at day 45 and 60 of gestation could therefore be logical seen the absence of malformations. A viral load in the organs that would be too low to induce malformations and a subsequent seroconversion could explain this specificity as well. Finally, the absence of immune response could be the consequence of SBV foetal immunotolerance considering that the inoculation occurred before, or at the very beginning, of the period in which the foetus acquired humoral immune competence (day 66 to 100 of gestation) (Silverstein *et al*., 1963). A similar phenomenon was already demonstrated for BVDV (Fulton *et al*., 2009; Brodersen, 2014).

Schmallenberg virus positive CNS is frequently associated with SBV induced malformations in ruminants (Bilk *et al*., 2012; De Regge *et al*., 2013). It remains to be

elucidated why one lamb showed no evidence of congenital malformations despite SBV RNA presence in the CNS. Also it remain to be elucidated why only one SBV RNA positive CNS was recovered from the lambs since 10 lambs showed evidence of SBV passage *in utero* (SBV positive meconium or organ). The BBB was proposed to influence SBV infection outcome in adult ruminant (Varela *et al*., 2013) however this does not seemed to be the case in the present experiment because (i) the BBB is supposed to develop after the inoculation dates (between day 70 and 123 of gestation) (Evans *et al*., 1974); (ii) a delayed transmission (transmission from the placental organs to the foetus after the maturation of the BBB) is highly unlikely considering that an inoculation of SBV at day 45 of gestation induced the infection of the foetus at 7 dpi in another experiment (Stockhofe *et al*., 2013) and (iii) SBV has the capacity to cross a mature BBB since it was already recovered from adult sheep CNS (Wernike *et al*., 2013e).

In the G60, the lamb 10 presented SBV RNA in the muscle. The presence of SBV RNA in the muscle, in the absence of SBV RNA in the CNS, demonstrates that a primary myositis is plausible for SBV and that the malformations due to SBV could be the consequence of a primary myositis as already suggested (Herder *et al*., 2012).

6.2.6 Conclusion

This study demonstrated that the inoculation of pregnant ewes at day 45 or 60 of gestation with an SBV infectious serum induces the placental colonization and the persistence of SBV RNA in different organs of the lambs. The number of SBV RNA positive placental organs recovered at birth increased when SBV infection occurred at day 60 of gestation

compared to day 45 of gestation. The lambs did not present malformations at birth despite one lamb presenting SBV RNA in the CNS and one lamb presenting SBV RNA in the muscle. The lack of malformation observed in the lambs indicates that the infection with SBV must occur earlier than the day 45 of gestation to induce the congenital defects. The reason for the absence of SBV specific antibodies, despite the evidence of SBV passage in the foetus, remains to be clearly elucidated.

CHAPTER 7: PERSISTENCE AND KINETIC OF SCHMALLENBERG VIRUS SPECIFIC ANTIBODIES IN SHEEP

7.1 Preamble

The Study 5 precised the open-window for the development of congenital defects. Interestingly, in the Study 4, the seroconversion appeared to be persistent for at least 6 months upon one single SBV inoculation. It was interesting to evaluate if this persistent seroconversion would further prevent viraemia, and therefore prevent the apparition of congenital defects, the most important consequence of SBV circulation, as outlined in the Study 1. This long period between 2 infections was important to investigate because it mimics the absence of natural virus circulation reported in Europe the two previous years followed by a renewed circulation (ProMed–mail, 20141121.2978286; Wernike *et al*., 2015). In this work the persistence of SBV specific antibodies was studied in 5 ewes infected twice, more than 12 months apart. The presence of RNAemia upon surinfection and the persistence of seroconversion in the absence of surinfection were evaluated. The isotype specific antibody response was also studied.

7.2 Study 5: Persistence of the protective immunity and kinetics of the isotype specific antibody response against the viral nucleocapsid protein after experimental Schmallenberg virus infection of sheep.

This section constitutes the original article accepted for publication in *Veterinary Research.*

ARTICLE 5:

Study 5: Poskin A., Verite S., Comtet L., Van der Stede Y., Cay B., De Regge N. Persistence of the protective immunity and kinetics of the isotype specific antibody response against the viral nucleocapsid protein after experimental Schmallenberg virus infection of sheep. Vet Res, *in press*.

7.2.1 Abstract

Schmallenberg virus is an *Orthobunyavirus* that induces abortion, stillbirths and congenital malformations in ruminants. SBV infection induces a long lasting seroconversion under natural conditions. The persistence of the protective immunity and the isotype specific antibody response upon SBV infection of sheep has however not been studied in detail.

Five sheep were kept in BSL 3 facilities for more than 16 months and subjected to repeated SBV infections (Poskin *et al*., 2014b). Blood was regularly sampled and organs were collected at euthanasia. The presence of SBV RNA in serum and organs was measured with rRT–PCR. The appearance and persistence of neutralizing and SBV N protein isotype specific antibodies was determined with VNT and ELISAs.

Production of neutralizing SBV specific antibodies was first detected around 6 days post primo–inoculation with VNT and correlated with the appearance of SBV N specific IgM antibodies. These IgM antibodies remained present for 2 weeks. SBV N specific IgG antibodies were first detected between 10 and 21 dpi and reached a plateau at 28 dpi. This plateau remained consistently high. No significant decrease in titre was found over a period of

more than one year and the primo SBV infection protected ewes against clinical signs, viremia and virus replication in organs upon challenge infections (second or third inoculation) more than 15 months later

In conclusion, the SBV specific IgM response probably eliminates SBV from the blood and the protective immunity induced by SBV infection protects sheep against reinfection for at least 15 months.

7.2.2 Introduction

Schmallenberg virus SBV is an *Orthobunyavirus* belonging to the family *Bunyaviridae* that emerged in continental Europe in 2011 (Beer *et al*., 2013). It is a vector borne disease of ruminants and transmitted by small hematophagous insects called Culicoides (Koenraadt *et al*., 2014). Shortly upon infection, a viremia develops that lasts four to five days and can coincide with a drop of milk production, diarrhoea and hyperthermia in adult cattle (Hoffmann *et al*., 2012). In sheep, clinical signs were never reported in adult animals under natural conditions and only few clinical signs were described after experimental infection (Beer *et al*., 2013; Wernike *et al*., 2013e). Abortion, stillbirths and malformations can be observed in offspring upon SBV infection of pregnant cattle, sheep and goat (Garigliany *et al*., 2012b).

Orthobunyaviruses have an RNA genome consisting of three segments named according to their size S, M and L. The S–segment encodes the NSs protein and the N protein, which is later associated with the genome in a ribonucleoprotein complex. The M–segment

encodes two glycoproteins that are present in the viral envelope (Gn and Gc) and the NSm protein. The L–segment encodes the RdRp also called L–protein (Elliott and Blakqori, 2011).

Commercial ELISA's have been used to measure SBV specific antibody production and they allowed detecting seroconversion in sheep 10 to 14 dpi under experimental conditions (Wernike *et al*., 2013e; Bréard *et al*., 2013). Despite a good concordance between ELISA and VNT, VNT has been shown to be more sensitive than the commercial ELISA (Loeffen *et al*., 2012; Mansfield *et al*., 2013; Poskin *et al*., 2014a and b).

Schmallenberg VNT reported in literature were conducted with heat–inactivated serum (30 min. at 56°C) (Loeffen *et al*., 2012; De Regge *et al*., 2013; Mansfield *et al*., 2013; van der Heijden *et al*., 2013; Elbers *et al*., 2014a and b; Rodríguez–Prieto *et al*., 2014; Daly *et al*., 2015). Heat–treatment of serum before VNT is a routine practice aiming to inactivate the complement system and is recommended by the OIE for SBV VNT (OIE, 2014).

Schmallenberg virus specific antibodies are known to persist at least 12 to 24 months in cattle after natural infection (Elbers *et al*., 2014a; Méroc *et al*., 2015). Also in sentinel sheep herds it was observed that SBV specific antibodies could last for at least 12 months (Claine *et al*., 2013c). Although these studies show that SBV specific antibodies can last for a long time under natural conditions, one cannot exclude that multiple infections occurred, potentially at distinct moments over time.

Seen the fact that SBV was still circulating in Germany and the Netherlands in 2014 (ProMed–mail, 20141121.2978286; Wernike *et al*., 2015) and the strong epidemiological similarity with AKAV, it is to be expected that SBV will persist in Europe (Hoffmann *et al*., 2012; Poskin *et al*., 2016). It is therefore important to obtain knowledge about the duration of the protective immunity and the development and persistence of the antibody response against this virus.

In this study, five ewes were maintained under experimental conditions during more than one year and subjected to SBV infections. The persistence of the protective immunity, the neutralizing antibody response and the kinetics of the isotype specific antibody response against the SBV N protein were studied and quantified.

7.2.3 Material and methods

Ethical statements

The experiments described hereafter were approved by the Ethical Committee of the IPH–VAR (121017–01).

Animals, housing, inoculum and samples

This study was carried out with five "Mourerous" breed ewes between 14 and 17 months old at the moment of inoculation. All ewes were virologically and serologically negative for SBV at the start, as confirmed by rRT–PCR, VNT and ELISA. The ewes were housed together in a stable inside BSL 3 facilities in which insect traps were used to monitor insects and confirm the absence of *Culicoides* midges throughout the experiment.

The inoculum used in this experiment was an infectious bovine serum containing 2 x 10^3 $TCID_{50}$/mL, as determined by end–point titration on BHK cells that was provided by the FLI (Riems, Greifswald, Germany) (Wernike *et al*., 2012b). The inoculum is known to successfully induce a viremia followed by a seroconversion in sheep upon subcutaneous

inoculation with 1 ml of the described inoculum (Poskin *et al*., 2014b). Each animal was inoculated following the described route and dose at different time points. Four ewes (ewe 1 to 4) were primo-inoculated at the beginning of the experiment (day 0). Ewe 3 and 4 received a second inoculation (booster inoculation) 28 days post primo inoculation. The fifth ewe was kept as a control animal of horizontal and environmental transmission and was not primo inoculated nor received a booster. Thereafter all five ewes received a surinfection (second inoculation in ewes 1 and 2, third inoculation in ewes 3 and 4, first inoculation in ewe 5) more than one year after the primo inoculation (Table VIII).

The five ewes were clinically examined, the rectal temperature was taken and the blood was collected after each inoculation daily during one week, then weekly for one month, and monthly thereafter (Figure 20). The ewe one was kept 98 days longer to allow an indication of seroprotection over a longer period. Serum was prepared from the blood by 15 min centrifugation at 3000 rpm and kept at –80°C until further analysis. All ewes were euthanized 14 days after the challenge and the prescapularis lymph nodes, mesenteric lymph nodes, ovaries and spleen were collected during autopsy and stored at –80°C until further analysis.

SBV RNA detection in serum and organs with rRT–PCR

RNA extraction and rRT–PCR on blood and organs were conducted as previously described (De Regge *et al*., 2013). Briefly, total RNA was extracted from 140µL of serum and eluted in 60µL of elution buffer with the QIAamp Viral RNA Mini Kit following manufacturer's instructions (Qiagen, Hilden, Germany). Before extraction, 100µg of organ was homogenized in 1ml of PBS with Stainless Steel Beads (Qiagen, Hilden, Germany) and shaken two times two minutes at 25Hz with the TissueLyser (Qiagen, Hilden, Germany). The

Ewe	Inoculation	Booster	Challenge	Euthanasia
1	0	-	490	504
2	0	-	392	406
3	0	28	392	406
4	0	28	392	406
5	-	-	392	406

Table VIII: **Experimental design for the study of Schmallenberg virus specific antibody persistence**

The table indicates the day of inoculation, second inoculation (booster) and challenge infection (second inoculation for ewe 1 and 2, third inoculation in ewe 3 and 4, primo inoculation for ewe 5) with a Schmallenberg virus infectious inoculum, and the day of euthanasia for the five ewes used in this study. Ewe 1 and 2 did not receive a booster inoculation. Ewe 5 was not primo–inoculated nor boosted and kept as control until the challenge infection 14 days before euthanasia.

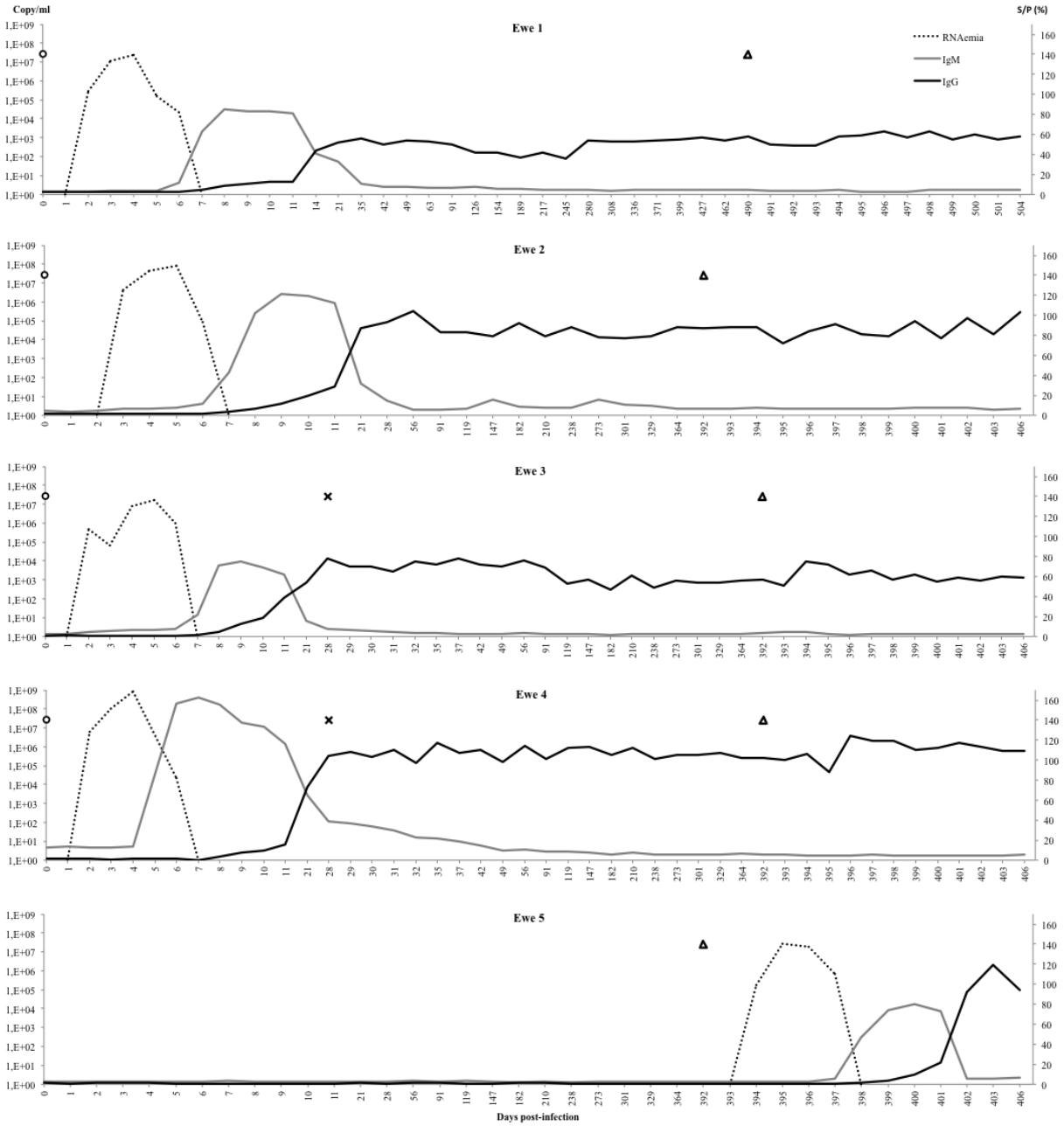

Figure 20: RNAemia and SBV N–specific antibody response to Schmallenberg virus

RNAemia (copy/ml of serum) and isotype specific antibody response (IgM and IgG) against the Schmallenberg virus (SBV)–N protein in five ewes upon inoculation with SBV infectious serum under experimental conditions as measured with rRT–PCR, IgM ELISA and IgG ELISA respectively. The cut–off S/P value for the IgM ELISA was 17%, while for the IgG ELISA the cut–off value was 27%. Empty circles indicate the time points of SBV primo inoculations (ewes 1 to 4), crosses indicate the booster inoculations (ewes 3 and 4) and empty triangles the challenge inoculations (ewes 1 to 5).

homogenate was subsequently centrifuged 5 minutes at 10000 rpm. The total RNA was finally extracted from 100µL of supernatant and eluted in 50µL RNase free water with the RNeasy Mini Kit, following manufacturer's instructions (Qiagen, Hilden, Germany) and measured with the LightCycler 480 Real-Time PCR system (Roche Applied Science, Indianapolis, USA). One negative control of extraction was added to each run of extraction. One positive control and one negative control of PCR amplification were added to each run of rRT-PCR. Cycle threshold values were converted into copy number/ml of serum and copy number/gr of organ using a standard curve consisting of successive ten–fold dilutions of an SBV S–segment RNA transcript that was added to each run of the rRT–PCR (Poskin *et al*., 2014b).

Serology

Virus neutralization test was carried out as previously described (De Regge *et al*., 2013) with the exception that all sera were analysed both without (VNTw/d) and with (VNTd) heat–inactivation of the complement system (30 min at 56°C) before testing. Briefly, successive two-fold dilutions of 50µl of sera, from 1/2 to 1/256 onwards, were made in 50µL of Dulbecco's Modified Eagles Medium (Gibco, Life Technologies, Ghent, Belgium) supplemented by 1000 IU penicillin/ml, 50µg/ml gentamicin (Gibco, Life Technologies, Ghent, Belgium) and 250µg/ml amphotericin B (Gibco, Life Technologies, Ghent, Belgium) (DMEM) in Nunc Edge 96–well plates (Thermo scientific, Waltham, MA, USA). Fifty microliters of virus solution was added to each well and the plate let to incubate one hour at 37°C. Thereafter 100µl of DMEM with 10% foetal calf serum (Gibco, Life Technologies, Ghent, Belgium) and 4 x 10^6 Vero cells/ml were added to each well and the plate let to incubate at 37°C and 5% CO_2. After four days of incubation, the titre was determined as the

reciprocal of the highest serum dilution in which no lysis plaques could be identified in the cell monolayer under the light microscope. Two positive controls and one negative control were added to each run of VNT. Sera were considered to be seronegative if the titre was lower than 4 (specificity 100%) (De Regge *et al*., 2013).

The presence of SBV N specific IgM antibodies was assayed with a capture IgM ELISA. ELISA plates coated with monoclonal anti–ruminant IgM antibodies, recombinant SBV N proteins and monoclonal HRP labelled SBV N specific antibodies were purchased from IdVet (Montpellier, France). Briefly, 10µL of serum and 90µL of dilution buffer were deposed in duplicate on the IgM ELISA plate and let to incubate 45 minutes at 37°C. The wells were washed and 100µl of the recombinant SBV N protein solution was added to one of both replicates. One hundred microliter of the dilution buffer was added to the other replicate. After an incubation of 90 minutes at 37°C, the wells were washed a second time and 100µl of the ready–to–use conjugate was added into the wells and the plate was incubated 30 minutes at room temperature. After washing, 100µl of the tetramethylbenzidine (TMB) substrate solution was added to the wells. After 15 minutes incubation in the dark at room temperature, the reaction was stopped by adding 100µl of the stop solution. One positive and one negative control were added to each IgM ELISA plate. The control samples were defined and tested in preliminary experiments. The positive control sample consisted of pooled sera collected at 10, 11 and 14 dpi from one ewe inoculated with the same inoculum in a different experiment (Poskin *et al*., 2014c). The negative control sample consisted of pooled sera collected from ewes in 2005. The optical density (OD) was read at 450nm with Multiskan Ascent (Thermo Scientific, Waltham, MA, USA). The net OD value was calculated for each sample as (OD value in the replicate with the recombinant SBV N protein solution – OD value in the replicate without the recombinant). The S/P percentage was calculated as (net OD value – OD negative control sample) / (OD positive control sample – OD negative control sample) x 100.

The test was validated only if the ODpos > 0.35 and the ODpos/ODneg > 3. The cut–off value for SBV IgM was calculated based on the obtained values of 25 negative samples from the control ewe (ewe 5) collected before the first inoculation and eight sheep sera collected in 2005. The cut–off value was determined as the mean S/P value of the 33 negative samples + 3 standard deviations. This interval represents 99.6% of the negative samples (Wang *et al*., 2014).

To evaluate the impact of heat–inactivation of serum (30min at 56°C) on SBV N specific IgM detectability by ELISA, a selection of samples was tested in the IgM ELISA described above without (IgM ELISAw/d) and with (IgM ELISAd) heat–inactivation of the complement system.

The presence of SBV N–specific IgG antibodies was measured with the ID Screen SBV Indirect test (IdVet, Montpellier, France) using an anti–multi–species IgG–HRP conjugate following manufacturer's instructions (Bréard *et al*., 2013). The OD was measured as described above. The S/P percentage was calculated as (OD sample – OD negative control) / (OD positive control – OD negative control) x 100. The interpretation prescribed by the kit is as follows: negative if S/P ≤ 50%, doubtful if 50% < S/P ≤ 60% and positive if S/P>60%. We also determined a cut–off value ourselves by testing 50 field-collected sera sampled before 2010. The cut–off value was determined as the mean S/P value of the 50 samples ± 3 x standard deviation. This interval represents 99.6% of the negative samples (Wang *et al*., 2014).

The concordance between the VNTd and the IgG ELISA was determined by dividing the number of samples for which the same qualitative result was obtained in VNTd and the IgG ELISA by the total number of samples analysed in both tests.

Statistical analyses

The differences between the mean time needed to detect seroconversion with VNTw/d and VNTd, and the mean S/P value between IgM ELISAw/d and IgM ELISAd were statistically analysed with two samples paired *t* tests.

A linear mixed model was used (SAS Institute Inc., Cary, NC, USA) to estimate the mean effect of time with relation to the antibody titres and taking into account the correlation of the measurements within each sheep over time. The time effect was put as a fixed effect in the model and a random intercept was allowed meaning that there is an average ELISA titre in the sheep population after infection but that there is variability between the sheep. An autoregressive working correlation matrix (decreasing correlation for further time points) was selected based on the AIC (Aikake Information Criterion) value.

For all statistical tests, *P* values lower than 0.05 were considered to be statistically significant.

7.2.4 Results

Clinical impact

No significant clinical impact was noticed and the rectal temperature stayed within the normal range (38.3°C – 39.9°C) throughout the experiment (data not shown) (Gillespie and Flanders, 2010). Ewes behaved normally, conserved a good appetite and no sign of diarrhoea was noticed until the end of the experiment.

RNAemia

All ewes showed a comparable RNAemia that started on average 2.2 days post primo inoculation (95% CI: 1.6 – 2.8) (Table IX). The RNAemia could be detected with rRT–PCR during a mean period of 4.6 days (95% CI: 3.9 – 5.3). At the peak of RNAemia, the mean number of SBV RNA copies per ml of serum was 2×10^8 (95% CI: $0 – 6.5 \times 10^8$). No viral RNA could be detected in the serum of the four first ewes after the booster and the challenge inoculations. No RNAemia was found in the control ewe before its inoculation at the end of the experiment (Figure 20).

Serology

Schmallenberg virus specific neutralizing antibodies were first detected at 6.2 (95% CI: 5.2 – 7.2) and 7.8 (95% CI: 6.4 – 9.2) days post primo inoculation when determined by VNTw/d and VNTd, respectively. This difference was not significant, although the *P* value of 0.056 suggests that seroconversion is detected earlier when no heat–inactivation of the serum is performed. After seroconversion the titre fluctuated between 4 and 128 and stayed positive until the end of the experiment. No increase in antibody titre was observed after booster or challenge infections. When the serum was analysed without decomplementation, three false positive results (days 8, 10 and 119) were observed for the control ewe before its first inoculation at day 392 (Figure 21). The cut–off value for the SBV N–specific IgM ELISA was calculated to be 17%. The mean period for the first detection of SBV N–specific IgM was 6.6 days (95% CI: 6.1 – 7.1) post primo inoculation (Table IX) and SBV N–specific IgM remained detectable for about two weeks. No production of SBV N–specific IgM was detected after booster and challenge infections in ewes 1 to 4 (Figure 20).

Ewe	RNAemia (first day)	RNAemia (last day)	VNTw/d	VNTd	ELISA IgM	ELISA IgG
1	2	7	8	9	7	14
2	2	7	6	9	7	21
3	2	7	6	6	6	11
4	3	7	6	9	7	21
5	2	6	5*	6	6	10

* 3 low positive results before first inoculation were not taken into account

Table IX: Period of RNAemia and first day of seroconversion after Schmallenberg virus inoculation

Five ewes (ewe 1 to 5) were primo inoculated with Schmallenberg virus (SBV) infectious serum at day 0 of the experiment. The numbers indicate the first and last day of RNAemia and the day of first seropositive result measured with virus neutralisation test without (VNTw/d) and with (VNTd) heat–inactivation of the serum. The first seropositive result measured with ELISA directed against the SBV N protein for IgM (IgM ELISA, cut–off 17%) and IgG (IgG ELISA, cut–off 27%) are also given.

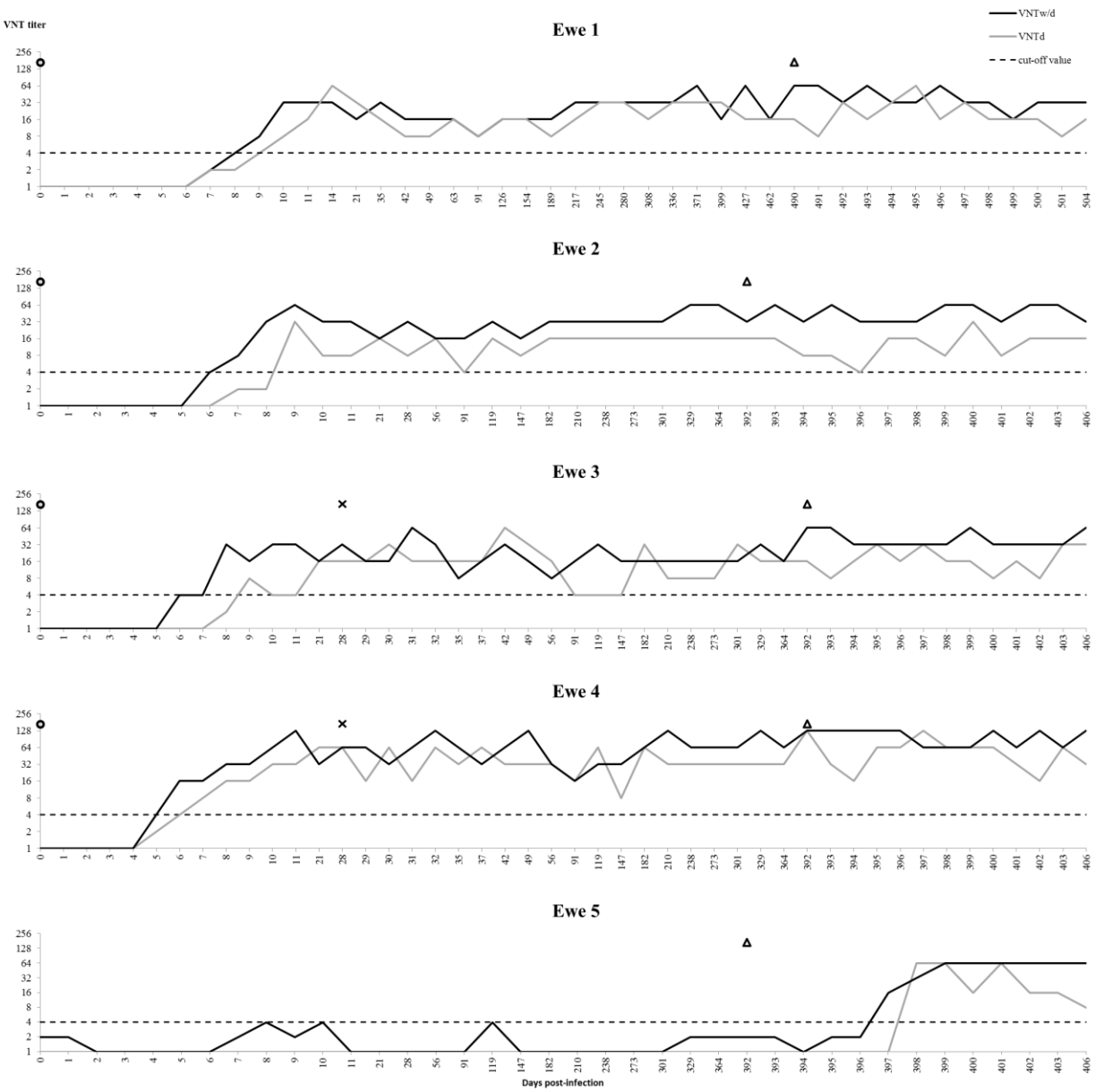

Figure 21: Protective antibody response to Schmallenberg virus inoculation

Schmallenberg virus (SBV) specific neutralizing antibody production in five ewes inoculated with SBV infectious serum as measured with virus neutralization tests (VNT) carried out without (VNTw/d) and with (VNTd) heat–inactivation of the complement system. Open circles indicate the time points of SBV inoculations (ewes 1 to 4), crosses indicate the time points of booster inoculations (ewes 3 and 4) and open triangles indicate the time points of challenge inoculations (ewes 1 to 5).

No significant differences were found between S/P values obtained with SBV N–specific IgM ELISA in sera that were tested without and with heat–inactivation of the complement system (data not shown).

The cut–off value of the SBV N–specific IgG ELISA was determined to be at 27%. The first positive SBV N–specific IgG antibody titre was measured between 10 and 21 days post primo inoculation in all ewes (Table IX). Since blood was only collected weekly after 11 days post primo inoculation, it is not possible to pinpoint the seroconversion date more precisely. The S/P values reached a plateau at 28 dpi. Applying a mixed linear model to the data obtained for ewe 1 to 4 between 28 and 392 dpi predicted an average decrease of 0.0204 % in ELISA titre per day. This estimate was non–significantly different from 0 (P = 0.1734; F value = 3.16), indicating that no significant decrease over time in S/P values was found. Furthermore, no effect was observed after the booster or challenge inoculations with the exception of a small and temporal (1 to 4 days) decrease in the SBV N specific IgG antibodies (Figure 20).

An overall concordance of 92% was found between the VNTd and the IgG ELISA. When the cut–off value prescribed by the manufacturer for the IgG ELISA is used, ewe 1 only became seropositive after the challenge infection, making that the concordance is reduced to 69% in that case.

Necropsy

No lesions were noticed at necropsy in any of the ewes. Schmallenberg virus RNA was only detected in the four organs collected from the control ewe (ewe 5), which was inoculated only once at 14 days before euthanasia: mesenteric lymph nodes (8.7 x 10^8

copies/gr of organ), spleen (2 x 10^8 copies/gr), prescapularis lymph node (1.6 x 10^7 copies/gr) and ovary (3.7 x 10^6 copies/gr).

7.2.5 Discussion

Several studies have shown that natural SBV infection and vaccination induce a protective immunity in cattle and sheep (Elbers *et al*., 2013; Wernike *et al*., 2013c; Rodríguez–Prieto *et al*., 2014; Hechinger *et al*., 2014; Kraatz *et al*., 2015), but the duration of this induced immunity was not studied in detail. This work evaluated the persistence of the protective immunity induced by SBV infection of SBV naïve ewes over a period of more than one year under experimental conditions. This experiment furthermore analysed the kinetics of the isotype specific antibody response against the viral N protein.

It was demonstrated that the immunity that develops after a single SBV infection of sheep protects the animal against clinical signs, RNAemia and virus replication in target organs upon reinfection for at least 15 months. This period probably lasts even longer since the infection was associated with a persistent neutralizing antibody response that did not significantly decrease during that time period. Although not specifically addressed in this study, it seems reasonable to hypothesize that this induced immunity capable of preventing RNAemia upon reinfection will also protect against transplacental virus transmission. Epidemiological data have shown that the massive spread of SBV among ruminants during the first outbreak season, as evidenced by a very high seroprevalence of SBV specific antibodies, correlated with a strongly decreased incidence of reported congenital malformations during the next season despite a renewed virus circulation (Méroc *et al*., 2015).

Another interesting consequence of the observation that no renewed RNAemia could be detected after the booster or challenge inoculations is that the short lasting RNAemia of 4 to 5 days after primo infection is the only time frame in which *Culicoides* midges can take an infectious blood meal and transmit the virus towards new hosts.

The long term protection and the fast and persistent SBV specific antibody response after a primo infection of sheep indicate further that naïve new-born lambs represent on the one hand an appropriate target for a sentinel monitoring system to detect renewed circulation of the virus, but are on the other hand also the most important target population for vaccination. Although the long term protective effect of vaccination still has to be confirmed, it seems the best way to protect livestock against SBV related diseases for a long period of time (Wernike *et al*., 2013c; Anses 2014; Hechinger *et al*., 2014).

Our results show that the decrease of SBV RNA in serum coincides with the appearance of SBV specific neutralizing antibodies as detected by VNT and SBV N–specific IgM antibodies as found in ELISA, suggesting that SBV specific IgM plays an important role in the clearance of the virus from the circulation. The observation that the VNT detects seroconversion around the same time as the SBV N–specific IgM ELISA furthermore strongly suggests that the detection of SBV specific IgM by VNT explains the higher sensitivity of VNT to detect seroconversion than the commercially available ELISA detecting SBV N IgG as was suggested before (Poskin *et al*., 2014b). Since it has been described for other *Orthobunyaviruses* that also glycoproteins Gn and Gc can be the target of a neutralizing antibody response besides the N protein (Akashi and Inaba, 1997), it will be interesting to study the antibody response against both other proteins in the future.

It was observed that seroconversion could be detected earlier by VNT in sera that had not been heat–inactivated. This might be explained by the fact that heat treatment of sera

could result in a (partial) destruction of IgM (Schetters *et al*., 1988; Al–Muzairai *et al*., 2008). The heat–inactivation of the sera had however no effect on the SBV N–specific IgM detection in ELISA. Another possible explanation is that a part of the (early) SBV specific antibodies requires complement factors to fully resort their neutralizing effect as has been shown before for the antibody response against several other viruses (Bartoszcze and Larski, 1978; Sato *et al*., 1987; Takikawa *et al*., 1997; Yoder *et al*., 2004). This seems to be supported by the fact that VNT titres obtained for untreated sera were often higher than those of heat–inactivated sera, also on time points when SBV specific IgM was no longer present. Although omitting heat treatment seems to allow detection of seroconversion earlier by VNT, the occurrence of false positive results in the control ewe in our experiment suggests keeping the heat–inactivation of sera for VNT in place as recommended by the OIE (OIE, 2014).

The decrease of SBV N specific IgM at about two weeks post infection is associated with an increase in SBV N specific IgG. The stable and long lasting (neutralizing) IgG response is in line with VNT results obtained after infection of sheep with the related AKAV (Parsonson *et al*., 1977) and suggests a role of these antibodies in the long-term protection against SBV reinfection. Also the cell–mediated immunity might play a role in the long-term protection, but was not addressed in this study. Its importance has already been suggested based on the lymphohystiocytic infiltration of the white and grey matters in SBV affected foetuses (Herder *et al*., 2012; Hahn *et al*., 2013; Herder *et al*., 2013) and it was suggested to be part of the protective immunity induced by SBV vaccination (Wernike *et al*., 2013c), but its contribution remains to be elucidated.

Our results with the SBV N–specific indirect IgG ELISA highlight that one should be critical when defining the infection status of an animal which has an S/P value close to the cut–off described by the manufacturer. Although the cut–off of this commercial IgG ELISA was chosen based on results of many serum samples and a ROC analysis (Comtet, personal

communication), ewe 1 from this experiment would have been considered as negative or doubtful till the moment of the challenge infection. It seems therefore advisable that doubtful samples are confirmed in the VNTd. ELISA test are however less time consuming and more suitable for testing of a large amount of samples and it would also be interesting to evaluate the sensitivity of competition ELISAs that are currently available since these tend to be more sensitive.

Since IgA is known to be mostly present in mucous secretions and only at low levels in sheep blood (Day and Schultz, 2014), the kinetic of this particular antibody isotype was not investigated. It could be more interesting to test the presence of SBV specific IgA in nasal swabs or milk in the future.

7.2.6 Conclusion

It was shown that a single SBV infection of sheep induces a protective immunity that prevents against clinical signs, RNAemia and virus transmission to organs upon SBV reinfection more than 15 months later. The initial clearance of the virus from the blood coincided with the appearance of SBV specific neutralizing antibodies and SBV N specific IgM around one week post–infection. Between 2 and 3 weeks post–infection, an SBV N specific IgG response appeared that reached a plateau at 4 weeks post–infection and remained detectable for more than one year. It furthermore showed that despite the fact that omitting heat–inactivation of sera allows to detect seroconversion about 1 day earlier, the risk for false positive results suggests to keep decomplementation in place when performing VNT.

PART FOUR : GENERAL DISCUSSION

CHAPTER 8 : EPIDEMIOLOGY OF THE SCHMALLENBERG VIRUS

8.1 Expectations for the future of the epidemic

A large part of the domestic ruminant livestock has been renewed since 2011-2012 due to normal famer's managing practices. Although the seroprevalence in 2015 is unknown, it is to be expected that young animals are seronegative seen the low virus circulation since 2013, the limited lifespan of passive immunity: six months in calves and three to four months in lambs, and the fact that vaccines are currently not used in Belgium (Claine *et al*., 2014; Elbers *et al*., 2014a and b). This together with the indications that SBV is still present in Belgium and the surrounding countries (ProMed–mail, 20141121.2978286; Wernike *et al*., 2015) causes growing concerns for occurrence of a new episode of congenital malformations in the coming months.

If observations from AKAV, a *Culicoides* borne *Orthobunyavirus* causing congenital malformations, are extrapolated to SBV, it is to be expected that SBV presence can be demonstrated regularly with recurrent outbreaks of congenital diseases spared by the time needed for the immunity to decrease in livestock, which can represent a period of several years (Kono *et al*., 2008; CFSPH, 2009).

In three decades, AKAV expanded over four continents: evidence of virus presence were reported in Asia, Oceania, Europe (Cyprus) and East–Africa (Markusfeld and Mayer, 1971; Markusfeld, 1972; Miura *et al*., 1974; Kurogi *et al*., 1975; Della–Porta *et al*., 1976; Kurogi *et al*., 1976; Bak *et al*., 1980; Sellers and Herniman, 1981; Al–Busaidy *et al*., 1987; Taylor and Mellor, 1994; Lee *et al*., 2002; Yamakawa *et al*., 2006). Further spread of SBV can consequently be expected if local *Culicoides* species have the capacity to transmit SBV.

It is interesting to notice that AKAV evolved genetically since its first identification. In this respect, the first emergence of Iriki strain took probably place in 1984 in calves, 25 years after the first isolation of AKAV (Miyazato *et al*., 1989; Kono *et al*., 2008). The Iriki strain affects bovine by an encephalomyelitis in cattle and young calves in Japan and Korea, clinical signs imperviously attributed to AKAV (Kono *et al*., 2008; Oem *et al*., 2012; Kamata *et al*., 2009). SBV has a similar potential for genetic variability (Coupeau *et al*., 2013; Fischer *et al*., 2013; Hulst *et al*., 2013). New genetic and clinic forms of the SBV could consequently emerge in the future.

In conclusion, the most likely situation for SBV in the future is that SBV remain endemic in Belgium for a really long period of time with recurrent outbreaks of clinical signs that could be observed every five or six years, as observed for the AKAV (CFSPH, 2009). Concerning its genetic variability SBV should remain in the differential diagnosis of future epizootic outbreaks of pathologies from unknown origin in endemic zone (new form of the disease) or elsewhere (SBV expansion).

8.2 Preventive measures in case of Schmallenberg virus re–emergence

Vaccination seems an efficient way to prevent SBV infection and its deleterious effects. First vaccines were successfully developed shortly after SBV emergence (Wernike *et al*., 2013c; Hechinger *et al*., 2014; Kraatz *et al*., 2015). The most efficient vaccine proved to be an inactivated SBV preparation in aqueous solution developed by Wernike *et al.* (2013c). Recombinant vaccine lacking the NSs and the NSm proteins constitutes an interesting alternative for vaccination (Kraatz *et al*., 2015). Different efficient commercial vaccines are

available on the market (Hamers *et al*., 2013; Moulin *et al*., 2013). Information on long-term efficacy is however missing, although the Study 5 demonstrated the persistence of seroprotection over 15 months upon on single inoculation. Vaccination should be advised by the authorities even though not mandatory considering the relative low impact of SBV namely in cattle, as highlighted in the Study 1. The farmers should consider vaccination before mating during a season with SBV circulation. The latter implies an intensive monitoring otherwise only detected at the moment of congenital defects when clinical damages and financial consequences are already endured.

Another option would be to protect livestock from *Culicoides*. Extra hygienic measure, drug treatments or specific herd managing practices were tested to protect livestock from *Culicoides* (Zimmer *et al*., 2008a; Zimmer *et al*., 2008b; Calvete *et al*., 2010; Papadopoulos *et al*., 2009; Weiher *et al*., 2014). All led to disappointing or contradictory results and were only able to reduce (and not annihilate) insect pressure, therefore making it useless regarding that low doses of infectious virus are sufficient to transmit the virus (Study 3).

8.3 Strategy for Schmallenberg virus surveillance

Future monitoring and surveillance of SBV and new emerging diseases

In order to have an efficient monitoring and surveillance strategy for SBV and other (new) emerging diseases it is clear that such a system must consist of a combination of passive and active surveillance components. Very often, the purpose of the surveillance as a whole (fit for purpose) will influence the final choice of these components.

Passive surveillance components

Passive surveillance components can be installed in a 'space– and timeliness' way with the aim to provide as soon as possible real time information about SBV (re)–introduction and/or spread of the infection. If early detection of SBV is a choice for veterinary authorities, passive surveillance components such as syndromic surveillance and/or clinical detection may be a proper and cheap choice for surveillance.

Syndromic surveillance (the use of real–time collected data with relation to animal health in general) and clinical notifications can be carried out by veterinary and farmer (organization) networks. Therefore farmers and veterinarians should be encouraged to notify all cases of syndrome that remain undiagnosed in order to allow detection of spatial or timely clusters. Sometimes, syndromic surveillance and notification represents an extra, time consuming task for the veterinarian and therefore it should be more valorised, encouraged and supported by the veterinary authorities. This is particularly the case for new emerging diseases, such as SBV in Belgium, that are not notifiable. Due to the highly suspected underreporting of SBV at Belgium level (Study 1), it could be an interesting opportunity to make new emerging diseases like SBV notifiable diseases in order to get a better follow–up of the epidemic.

In Belgium there is a mandatory notification of all aborted foetuses in cattle with laboratory analysis of samples sent towards accredited laboratories. This abortion protocol can be considered as an early warning syndromic surveillance component. This system allowed a continual follow–up and a large-scale evaluation of SBV impact with rRT–PCR and VNT. Combined with technologies like next–generation sequencing, it is a powerful mechanism for surveillance of new emerging diseases that induce abortions (Rosseel *et al*., 2012) and provides valuable material for scientific studies dealing with new emerging

diseases. However, this protocol is not always followed by the farmers as evidenced in the Study 1 and by Delooz *et al*. (2011), even if it is financially supported by the animal health authorities, because of the potential impact that the detection of a contagious disease could have on their economic activities due to animal movement restriction or sanitary euthanasia. Consequently, it might be an interesting exercise to see if the system could not be adjusted to a less constraining protocol that would encourage farmers to participate. Hereto recent update of the abortion protocol made by the ARSIA gave encouraging increase of the notification rate by the farmers to the authorities (Delooz, personal communication).

Active surveillance components

Besides the passive surveillance components, active surveillance such as a sentinel surveillance system may also be useful to detect new emerging diseases in an early phase. To achieve an early detection of a new disease in a 'space and timeliness' way, a good sentinel system must be adequately sensitive. This can be achieved by applying a proper sample size and an acceptable and realistic design (sero)–prevalence to calculate this sample size.

Sentinel herd surveillance helped to understand the time–course of the epidemic and proved to be an efficient mean for identification of SBV recirculation in Belgium. Furthermore, it provided valuable samples used to increase our fundamental knowledge on SBV and allowed to study antibody persistence into domestic livestock under natural conditions. Close follow–up of sentinel herd surveillance is however time consuming and in the absence of apparent clinical disease and/or delayed immune reaction, samples need to be stored and might only become useful months or years later.

Besides the sampling of animals in different sentinel herds there is also the possibility to define different locations with traps to collect the *Culicoides*/vectors once the vector season starts (vector sentinel system). Detection of SBV in vectors has proven to be an excellent technique for early detection of SBV recirculation (De Regge *et al*., 2015). The short time period in which SBV could be detected in 2012 shows however that it has to be performed on a continuous basis. This makes pathogen monitoring in vectors a labour intensive and expensive surveillance tool. New techniques including mass spectrometry and rRT–PCR–based techniques will contribute to make large–scale pathogen surveillance studies in vectors more easy and less expensive (Cêtre–Sossah *et al*., 2004; Mathieu *et al*., 2011; Kaufmann *et al*., 2012a and b; Mathieu *et al*., 2012). Since global warming and the increasing international transport affect the distribution of *Culicoides* in Europe (Purse *et al*., 2005) and increase the risk for introduction of vector borne diseases, it seems advisable to keep the expertise for vector collection and identification in place.

Cross–sectional studies are preferred and very useful to estimate in global way if a new emerging disease such as SBV circulated or re–circulated again during a season. Cross–sectional studies helped to understand to which extent the virus had spread among the livestock population and to predict the potential impact of future re-emergence. It may be helpful to evaluate the necessity for intervention strategies such as vaccination in case of recirculation. If done on a broad scale, it allows determining regions that can be particularly susceptible to virus circulation or re-emergence during a specific time period in the year. The cross–sectional studies executed for SBV were shown to be useful and cost effective (Méroc *et al*., 2013, 2014 and 2015). The downside of such cross–sectional seroprevalence studies is that they cannot be used to detect the emergence in real–time or early during an epidemic and that they do not allow immediate actions in contrast to sentinel and/or clinical surveillance.

In case of sound knowledge is gathered about the risk factors for a certain emerging disease it might be worthwhile to limit the surveillance resources to only these time periods or places where it makes more sense to detect the disease with sufficient sensitivity (= target surveillance strategy). In this respect, the Study 1 showed that SBV was more frequently confirmed with rRT–PCR in bigger herds compared to smaller herds. A sentinel system for SBV could therefore preferentially be implemented in large farms.

CHAPTER 9 : PATHOGENSESIS AND OVERWINTERING OF THE SCHMALLENBERG VIRUS

9.1 Contribution to the Schmallenberg virus life-cycle

The recovery of SBV RNA in the placenta was made in the presence of a strong seroconversion (Study 4) suggesting that infectious SBV could persist within the placenta, protected from the antibodies and the immune system. The placenta, constitutes an immunosuppressed zone because a foetus is considered an allograft for its dam and different immune suppressive mechanisms prevent the foetus from rejection by the maternal immune system including the placental barrier function, the absorption or blocking of noxious antibodies or the synthesize of nonspecific systemic and local suppressor factor like immunosuppressive proteins (Mehta and Rajput, 1999). The transmission of SBV from the placenta to the adult sheep deserves therefore to be evaluated since placentophagia is a common behaviour in sheep (Krausman *et al*., 1999). This seems however unlikely because the ewe has developed a strong seroprotection during its initial infection (Study 3, 4 and 5), long before eating the placenta, that is protective against virus present in the placenta. Moreover, oro-nasal inoculation of ewes with SBV did not induce SBV transmission into calves or ewes (Wernike *et al*., 2013e; Martinelle *et al*., 2015).

Regarding the low viral quantities recovered from adult sheep and the presence of a strong seroconversion (Study 3, 4 and 5), the SBV RNA detected in these organs by rRT-PCR probably represents residual RNA. It seems however tricky to confirm that SBV RNA recovered from lambs in the Study 4 is only residual SBV RNA and not infectious virus because all lambs were seronegative at birth highlighting the absence of immune response. Hereto the low quantity of material collected during the necropsy, combined with the rather low SBV RNA load in these organs and the successive manipulations for RT-PCR and virus isolation might influenced the results of rRT-PCR. The different freezing-thawing cycles could have altered the quality of SBV RNA. Even if the SBV RNA would represent infectious

virus, the potentially living virus would be eliminated by the colostrum immunity (Study 4), and therefore prevent further virus transmission.

The virus recovered from the amniotic fluid (Study 4) could represent infectious virus regarding its abundance, although it might only be residual virus originating from fluids that enter into the composition of amniotic fluids. This seems very likely since different liquids enter into the composition of the amniotic fluids including the foetal urine, the liquid production originating from the foetal lung and the foetal envelopes (Anderson *et al*., 2013). All these liquids originate from organs in which SBV RNA was identified in the past: SBV was recovered from the lung in lambs and adult sheep (Wernike *et al*. 2013d, Study 3 and 4), from kidney in adult sheep (Wernike *et al*., 2013d) and from the foetal envelopes (Study 4). In this respect, it seems logical that SBV has once transited in one of these organs in the foetus, and was later excreted in their fluid production to accumulate finally in the amniotic fluids.

The high dose of SBV RNA present in the placenta (low Cq values) is an important finding. If infectious virus persists, this means that the ewes, the lambs and the *Culicoides* can be exposed to infectious virus at lambing. Since the reproduction period usually takes place in autumn in sheep, with lambing expected at the end of the winter (Study 1), this constitutes a potential mechanism of SBV overwintering, moreover similar phenomenon could be observed in cattle, in which gestation is longer and the reproduction periods are evenly distributed throughout the year (Study 1). The mean for SBV transmission remain to be clearly elucidated, although the transmission from the placenta directly to the *Culicoides* deserves to be fully evaluated. The *Culicoides* can complete their life-cycle inside cowshed and in the surrounding area (Zimmer *et al*., 2010), the *Culicoides* can consequently enter in contact and eventually feed on the placenta that lies on the floor during or shortly after the birth or on the dung heaps where farmers frequently throw away the placenta. Hereto it is well known that dung and particularly dung heap are preferred *Culicoides* larval habitat (Zimmer *et al*., 2008a

and 2010; Koenraadt *et al*., 2014). This means that *Culicoides* larvae can also enter into contact with infectious SBV. This possibility should be further investigated since the observation of SBV positive nulliparous midges was reported by Larska and colleagues (2013). This means that SBV transmission can happen between the *Culicoides* and its eggs or, by infection during the early life-cycle stages that can occur in the dung heaps (Zimmer *et al*., 2010).

In this thesis, it was also proved that primary myositis is a possible phenomenon to explain the development of malformations, although the frequent recovery of SBV in the CNS suggests that it is not the more likely hypothesis (Hahn *et al*., 2013). Finally, it was evidenced that early IgM production eliminate SBV from the blood and that long-term protection is ensure by IgG production starting around 2 weeks post-infection.

9.2 Contribution to the Schmallenberg virus diagnosis

Different surveillance approaches, including syndromic surveillance, abortion surveillance, sentinel herd and *Culicoides* surveillance as well as cross–sectional epidemiological studies have all proven their utility and complementarity to understand the SBV epidemic. This helped scientists, veterinary authorities and decision makers to increase the knowledge on the virus and should be considered in the future. The choice and/or the combination of different surveillance components will mainly be influenced by the epidemiological situation and/or the aim of the surveillance strategy. This choice can be to detect as fast as possible the disease in order to avoid a big epidemic (reduce transmission), to provide proper intervention strategies if needed (vaccination) and/or to reduce as much as possible the economic impacts and consequences.

The Study 1 showed that even though the SBV impact was considerable in 2011-2012, few evidence of virus renewed virus circulation and few clinical consequences have been observed since. Consequently, it would be advisable for the authorities to keep in place an SBV surveillance program that is not SBV specific, and useful in a more general context of animal welfare. In this respect, it is advisable for the authorities to promote veterinarian surveillance networks and to keep in place the abortus protocol, and include the SBV surveillance in this context.

The low propensity of the farmer to suspected SBV in adult animals was evidenced in the Study 1, therefore inducing SBV underdiagnosis. The short viraemia emphasized in study 2 to 5 further constrains the possibility for a proper diagnosis during to peak of viraemia with rRT-PCR. The veterinarian were the only to (presumably) suspect adequately SBV circulation in Belgium soon enough to allow the authorities to take the appropriate measures, namely the vaccination of naïve animals or the delay of reproduction period. Other measures (detection in *Culicoides*, reporting by the farmers,…), although useful to increase SBV knowledge, seem less indicated for SBV prevention. In this respect, the farmers suspected SBV only when clinical damages were already endured (Study 1). Seroprevalence studies are also not sufficient because the long and persisting seroconversion (Study 5) make it impossible to ensure that SBV-specific antibody production does date from a recent virus circulation. The screening of SBV in the *Culicoides* or in the wild life induces also the identification of renewed virus circulation way too late to allow appropriate measures to be taken. Schmallenberg virus screening in the Culicoides and in wild life should be better achieved in the global context of vector borne diseases surveillance and enhancing of Culicoides knowledge or wild life disease surveillance.

The diagnosis of SBV at country level can be done through the SBV RNA persisting in the foetal envelopes to evidence virus circulation in the pregnant sheep (Study 4). These

foetal envelopes can be obtained by the authorities in the context of the abortus protocol. The study 4 showed that immunity in lambs is not an adequate method to identify SBV circulation in utero, although the limited period for SBV inoculation tested so far requires confirmation on the total gestation span. The amniotic fluids and the meconium constitute an opportunity to identify SBV infection in utero as well. The absence of malformations is however not sufficient to prove the absence of virus infection *in utero*.

9.3 Overwintering process of Schmallenberg virus in Belgium

The recovery of viremic sheep in Germany during winter 2011–2012 proved the persistence of SBV during the winter season (Wernike *et al*., 2013d). Also the increase in seroprevalence observed in Belgium between February and April 2012 suggested SBV circulation during winter (Garigliany *et al*., 2012c). The actual mechanism by which SBV persists during winter stays however poorly understood. Wilson and colleagues (2008) proposed four potential overwintering strategies for BTV, and all seem transposable to SBV.

A first option is that SBV overwinters in adult midges that survive during winter. This has recently been suggested as the most probable overwintering mechanism of BTV in California (Mayo *et al*., 2014) and could be applicable to SBV and Belgian *Culicoides*. In Belgium, it is known that adult midges are able to accomplish their life–cycle and overwinter inside cowsheds and low numbers of *Culicoides* have been caught outdoors all along the winter (Losson *et al*., 2007; Zimmer *et al*., 2010; De Regge *et al*., 2015). Since it has been shown in the Study 2 that only a very low dose is necessary to induce an SBV infection, this low number of persisting *Culicoides* could be enough to perpetuate the infection.

A second potential mechanism is the transovarial transmission of SBV from the midge to its eggs. Such transovarial virus transmission has however never been shown in *Culicoides* for other viruses, including the closely related AKAV (Mellor, 2000; Kono *et al*., 2008). The recent detection of SBV RNA in nulliparous midges seems to support the hypothesis of a potential transovarial transmission, but this should be further examined since age grading was solely done on pigmentation of the abdomen (Larska *et al*., 2013). Furthermore SBV RNA detection in midges is not sufficient to prove the transmission of viable virus (Mellor 2000; Mellor *et al*., 2000) and in the same context, no SBV was detected in a limited set of nulliparous midge collected in Belgium (De Regge *et al*., 2014).

Thirdly, also the persistence of SBV within ruminant hosts during the winter should be considered as an option. SBV RNA was indeed detected in lymph nodes at 44 days post–infection despite rapid seroconversion, and even upon day 105 post-infection in the Study 4 (Wernike *et al*., 2013e). It remains however to be determined whether infectious virus is still present in the lymph nodes and how the virus could be transmitted to a *Culicoides* and perpetuate the SBV life–cycle, this should probably require genetic mutation to putatively evade the persistent immune response emphasized in the Study 5. Similar phenomenon is observed with the Influenza virus or the Hepatitis C virus (van de Sandt *et al*., 2012; Barth, 2015). Although possible on the basis of recent results emphasizing somewhat hypervariability at individual level (Coupeau *et al*., 2013; Fischer *et al*., 2013; Hulst *et al*., 2013), a low genetic evolution at population level is supported by the recent report of a high genetic homology showed between SBV strain sequenced from field collected samples after its first identification in 2011 and its last known emergence in 2014 (Wernike *et al*., 2015).

A fourth overwintering strategy could be the transplacental transmission of SBV from the dam to the foetus and its persistence until birth. SBV RNA persistence was proven at birth of one lamb in Germany under natural conditions and in three lambs out of 21 (Wernike *et al*.,

2014a). It was however unclear if the virus remained infectious in the new-born lamb. The low quantity of remaining virus allow however to hypothesize that this is probably not the case upon inoculation at day 45 and 60 of gestation (Study 4). Also the presence of SBV specific antibody in the colostrum supports that transmission from the dam to the foetus, even if the virus remain alive, has few chances to turn into a productive SBV transmission (Study 4).

Also the potential role of wildlife as a natural reservoir should be further evaluated since some species including red deer, roe deer and wild boar in which virus could have persisted were associated with lower seroprevalence rates during winter 2011–2012 (Linden *et al*., 2012; Claine *et al*., 2013a; Desmecht *et al*., 2013). The Study 2 namely showed that pigs do not contribute to SBV life-cycle, although the difference between wild-board and pigs has to be elucidated.

Since supporting but inconclusive evidence can be found for all options, it remains difficult to clearly pinpoint the overwintering option(s) used by SBV and this should be further examined in the future. The results obtained in this thesis suggest that the most likely scenario is an overwintering mediated by adult Culicoides persisting in the stable.

9.4 Limits of the work

In the Study 1, the control group was infected by SBV; it was consequently difficult to assess the real impact that SBV have in a farm. Also, the impact was clearly higher in sheep, particularly for the congenital defects. It would be necessary to evaluate the influence that the seasonality of sheep had on SBV impact. Sheep reproduction period usually takes places

between the end of the summer and the beginning of autumn, a kown period of SBV circulation in 2011. Most sheep in Belgium were therefore pregnant and naïve at a time with wide virus circulation, which probably contributed to increase the impact of SBV in sheep. Also, the impact in adult animals could have been underestimated by the farmers in cattle and sheep. The clinical signs induced by SBV are difficult to attribute solely to the virus, particularly for the farmers, and even for the veterinarian because they are rather aspecific.

In piglets, the absence or moderate seroconversion observed upon experimental infection necessitates to be confirmed under natural condition. It is unlikely that lot of wild-boars seroconverted and pigs did not. Hereto *Culicoides* could be reluctant or unable to enter pig stables. Also, only limited number of field samples were analysed in the Study 2. It would be necessary to evaluate more blood samples, and more interestingly to analyse blood sampled from domestic pigs bred outside during a period of SBV circulation to further confirm the absence of role for pigs in SBV life-cycle.

In the Study 3, although the viral load required is low, and in the order of magnitude of what could be inoculated by *Culicoides* under natural conditions, the possibility for one single Culicoides to transmit SBV to a sheep should be confirmed by parallel analysis of saliva collected from Culicoides inoculated with the same inoculum that would reach similar $TCID_{50}$ values.

Only a limited number of living lambs were obtain in the Study 4, namely regarding the rate of malformation reported in the Study 1. The reason for this poor rate of living lambs is probably multifactorial, but breed (rustic) and ewes (young ewes) characteristics or stress (e.g. experimental envirronement or multiple manipulations) factors probably contributed. It is also difficult to clearly pinpoint the period for the apparition of malformations although the collaboration conducted with the Dutch team (inoculation of pregnant sheep at day 38 and 45

of gestation) should bring more output. Finally, experimental infection differs from the natural infection and multiple infections might occur under natural condition (against one single inoculation in the Study 4). These could alter the results of the experiments.

The Study 5 was conducted on a limited number of 5 sheep and these sheep could not be maintained until they become seronegative. The experiment can consequently not be extrapolated to a longer period (more than 15 months) without virus circulation. Here again, the immunity induced by a single immunisation with an experimental infectious serum might also differ from a natural and putatively multiple inoculations induced by *Culicoides* under natural conditions.

PART FIVE : CONCLUSIONS AND PERSPECTIVES

CHAPTER 10: GENERAL CONCLUSIONS

This work was implemented in the context of the SBV emergence in Europe. At that time, little was known on SBV impact. In this respect, a case control study was carried out and allowed to precise the impact of SBV in Belgium: between 0.5% and 4% of the calves and between 11% and 19% of the lambs were stillborn, aborted or malformed due to SBV in 2011-2012. The epidemiology of SBV was also poorly understood and pigs were among the potential host of SBV. In this work, an experimental infection of piglets with SBV and SBV ELISA conducted on domestic pigs highlighted the absence of obvious role for the domestic pigs in the SBV life-cycle.

Upon emergence of SBV, all data on pathogenesis were extrapolated from the knowledge gathered from the closely related AKAV. It was therefore essential to increase knowledge on SBV pathogenesis. This work contributed to implement a method dedicated to the study of SBV pathogenesis, through the development and the standardization of a reliable experimental infection model that can faithfully reproduce a natural infection. This model was improved through the study of the minimum infectious dose for an SBV infectious serum. The dose was about 20 $TCID_{50}$.

Due to the high impact that SBV had on congenital defects in foetus lambs, the choice was made to improve the knowledge on the development of malformations *in utero* in collaboration with a research team from Holland. In this respect, pregnant sheep were inoculated at day 45 and 60 of gestation. This work allowed to precise that the likely period for the apparition of malformations in sheep is before the day 45 of gestation and to identify the importance of the foetal envelope in the SBV life-cycle, particularly the placentome. These envelopes were also putatively involved as a mean for SBV overwintering.

In the context of the AKAV, it was suspected that SBV circulation would decrease and subsequent recurrent outbreaks of SBV circulation would be observed afterwards. In this

respect, it was essential to investigate the length of the seroprotection and identify if 2 successive periods of SBV circulation could induce clinical consequence of SBV to be observed in the field. In this work, 2 successive inoculations of SBV carried out 15 months apart demonstrated that ewes are protected against viraemia and clinical symptoms for a long period of time, at least 2 successive years with circulation. This emphasizes that clinical consequences, including the congenital defects, are unlikely to be observed in adult sheep immunized in the year after SBV circulation.

Finally, this thesis demonstrated that SBV outbreak is likely to be recurrent. Therefore, it is advisable for the authorities to support the veterinary networks, that is currently the best mean to identify a renewed SBV circulation soon enough to allow the vaccination of SBV naïve adult animal before mating. Also, SBV diagnosis at birth should preferably be done on foetal envelopes that can stay SBV RNA positives, even when infection occurs in the early gestation.

CHAPTER 11: PERSPECTIVES

Several pathogenesis and genetic aspects of SBV remain unclear and need to be improved, including the factor influencing the teratogenesis of SBV (e.g. virus strain or moment of inoculation) or the mechanism of overwintering. In a recent study, 2 malformed lambs, SBV positive at rRT–PCR, were born from previously immunized ewes in March 2013 and April 2013 in a flock in Namur (Claine *et al*., 2013c). This observation was never reported elsewhere and deserves to be elucidated.

The long–lasting and intermittent excretion of SBV infectious semen in bulls is intriguing and the impact of this phenomenon on the epidemic remains to be clearly determined (Hoffmann *et al*., 2013; Ponsart *et al*., 2014). Hereto, the potential transmission of SBV through artificial insemination must be investigated. Similarly, the recovery of SBV from feces deserves to be fully appreciated as an SBV transmission means (Martinelle *et al*., 2015).

The putative vectors, other than *Culicoides* and mosquitoes, should also be studied (Wernike *et al*., 2014b). In this respect, common European mosquitoes proved to be refractor to SBV multiplication (and therefore transmission) under experimental conditions (Manley *et al*., 2015). The transmission of SBV through rodents is worth investigating since rodents are known to transmit other *Bunyaviruses* (Flint *et al*., 2009) and SBV is known to replicate in mice (Wernike *et al*., 2012a).

The study 2 emphasized an apparent difference between pigs and wild-boars, this has to be clarified. In this respect would be interesting to evaluate the seroprevalence in pigs breed outside during the epidemic.

Experimental infection of pregnant ewes with SBV at day 45 and 60 of gestation was implemented during this thesis and SBV RNA was recovered from the new-born lambs. It

remains to be clearly determined if this virus remains infectious. In the meantime, persistently infected (PI) lambs cannot be omitted. The overwintering process of SBV remains also to be clearly determined (see above). Also, the process for the production of colostrum SBV specific antibodies and its relationship to the development of malformations should be clarified.

The genetic evolution of arboviruses is limited by their obligation for adaptation in two hosts. They show subsequently less adaptation than what can be expected regarding their intrinsic mutation rate (Ebel *et al*., 2004; Parameswaran *et al*., 2012; Davis *et al*., 2012; Forrester *et al*., 2014). A hypervariable hot–spot was however identified at the N terminal part of the SBV M segment corresponding to the putative Gc glycoprotein (Coupeau *et al*., 2013; Fischer *et al*., 2013; Hulst *et al*., 2013). The Gc glycoprotein is a surface protein, which is highly immunogenic, and involved in host–cell attachment for *Orthobunyaviruses* (Akashi and Inaba, 1997; Yoshida and Tsuda, 1998; Kobayashi *et al*, 2007; Fischer *et al*., 2013). A similar hypervariable region was already observed for the closely related AKAV (Kobayashi *et al*, 2007). The hypervariable region has a potential role in immune evasion phenomenon. The latter was suggested in sheep upon SBV inoculation and should be further investigated (Wernike *et al*., 2013e; Fischer *et al*., 2013).

PART SIX : REFERENCES

A

Abecia JA, Forcada F, González–Bulnes A: Pharmaceutical control of reproduction in sheep and goats. Vet Clin North Am Food Anim Pract, 2011, 27, 67–79.

Akashi H and Inaba Y: Antigenic diversity of Akabane virus detected by monoclonal antibodies. Virus Res, 1997, 47, 187–196.

Al–Busaidy S, Hamblin C, Taylor WP: Neutralising antibodies to Akabane virus in free–living wild animals in Africa. Trop Anim Health Prod, 1987, 19, 197–202.

Al–Muzairai IA, Mansour L, Almajed L, Alkanderi N, Alshatti N, Samham M: Heat inactivation can differentiate between IgG and IgM antibodies in the pretransplant cross match. Transplant Proc, 2008, 40, 2198–2199.

Anderson DF, Jonker SS, Louey S, Cheung CY, Brace RA: Regulation of intramembranous absorption and amniotic fluid volume by constituents in fetal sheep urine. Am J Physiol Regul Integr Comp. Physiol, 2013, 305, 506-511.

Andersson BE and Jonasson H: Temperature regulation and environ–mental physiology. In: Swenson MJ and Reece WO (Eds): Dukes' Physiology of Domestics Animals, 11th ed., Cornell University Press, 1993, Ithaca, USA.

Anonymous: Evidence of Schmallenberg virus in Scotland in 2012. Vet Rec, 2013, 172, 629–632.

Anses: Évaluation de risques liés à la diffusion du virus Schmallenberg en France: bilan et perspectives. 2014. Available at www.anses.fr.

Ariza A, Tanner SJ, Walter CT, Dent KC, Shepherd DA, Wu W, Matthews SV, Hiscox JA, Green TJ, Luo M, Elliott RM, Fooks AR, Ashcroft AE, Stonehouse NJ, Ranson NA, Barr JN, Edwards TA: Nucleocapsid protein structures from *Orthobunyaviruses* reveal insight into ribonucleoprotein architecture and RNA polymerization. Nucleic Acids Res, 2013, 41, 5912–5926.

B

Bak UB, Lim CH, Cheong CK, Hwang WS, Cho MR: Outbreaks of Akabane disease of cattle in Korea. Korean J Vet Res, 1980, 20, 65–78.

Balenghien T, Pagès N, Goffredo M, Carpenter S, Augot D, Jacquier E, Talavera S, Monaco F, Depaquit J, Grillet C, Pujols J, Satta G, Kasbari M, Setier–Rio ML, Izzo F, Alkan C, Delécolle JC, Quaglia M, Charrel R, Polci A, Bréard E, Federici V, Cêtre–Sossah C, Garros C: The emergence of Schmallenberg virus across Culicoides communities and ecosystems in Europe. Prev Vet Med, 2014, 116, 360–369.

Balmer S, Vögtlin A, Thür B, Büchi M, Abril C, Houmard M, Danuser J, Schwermer H: Serosurveillance of Schmallenberg virus in Switzerland using bulk tank milk samples. Prev Vet Med, 2014, 116, 370-379.

Barbato O, Sousa NM, Debenedetti A, Canali C, Todini L, Beckers JF: Validation of a new pregnancy–associated glycoprotein radioimmunoassay method for the detection of early pregnancy in ewes. Theriogenology, 2009, 72, 993–1000.

Barry G, Varela M, Ratinier M, Blomström AL, Caporale M, Seehusen F, Hahn K, Schnettler E, Baumgärtner W, Kohl A, Palmarini M: NSs protein of Schmallenberg virus counteracts the antiviral response of the cell by inhibiting its transcriptional machinery. J Gen Virol, 2014, 95, 1640–1646.

Bartoszcze M and Larski Z: Use of complement in seroneutralization test for early detection of antibodies against Aujeszky's disease virus in pigs. Med Dosw Mikrobiol, 1978, 30, 37–41.

Bartsch S, Bauer B, Wiemann A, Clausen PH, Steuber S: Feeding patterns of biting midges of the Culicoides obsoletus and Culicoides pulicaris groups on selected farms in Brandenburg, Germany. Parasitol Res, 2009, 105, 373–380.

Barth H: Hepatitis C virus: Is it time to say goodbye yet? Perspectives and challenges for the next decade. World J Hepatol, 2015, 7, 725-737.

Bayrou C, Garigliany MM, Cassart D, Jolly S, Desmecht D: Schmallenberg virus circulation in Belgium in 2012. Vet Rec, 2013, 172, 296.

Bayrou C, Garigliany MM, Sarlet M, Sartelet A, Cassart D, Desmecht D: Natural intrauterine infection with Schmallenberg virus in malformed newborn calves. Emerg Infect Dis, 2014, 20, 1327–1330.

Beer M, Conraths FJ, van der Poel WH: 'Schmallenberg virus'–a novel orthobunyavirus emerging in Europe. Epidemiol Infect, 2013, 141, 1–8.

Bilk S, Schulze C, Fischer M, Beer M, Hlinak A, Hoffmann B: Organ distribution of Schmallenberg virus RNA in malformed newborns. Vet Microbiol, 2012, 159, 236–238.

Black WC, Bennett KE, Gorrochótegui-Escalante N, Barillas-Mury CV, Fernández-Salas I, de Lourdes Muñoz M, Farfán-Alé JA, Olson KE, Beaty BJ: Flavivirus susceptibility in Aedes aegypti. Arch Med Res, 2002, 33, 379-388.

Blomström AL, Gu Q, Barry G, Wilkie G, Skelton JK, Baird M, McFarlane M, Schnettler E, Elliott RM, Palmarini M, Kohl A: Transcriptome analysis reveals the host response to Schmallenberg virus in bovine cells and antagonistic effects of the NSs protein. BMC Genomics, 2015, 16, 324.

Bréard E, Lara E, Comtet L, Viarouge C, Doceul V, Desprat A, Vitour D, Pozzi N, Cay AB, De Regge N, Pourquier P, Schirrmeier H, Hoffmann B, Beer M, Sailleau C, Zientara S: Validation of a commercially available indirect ELISA using a nucleocapside

recombinant protein for detection of Schmallenberg virus antibodies. PLoS One, 2013, 8, e53446.

Brodersen BW: Bovine viral diarrhea virus infections: manifestations of infection and recent advances in understanding pathogenesis and control. Vet Pathol, 2014, 51, 453–464.

Busquets N, Xavier F, Martín–Folgar R, Lorenzo G, Galindo–Cardiel I, del Val BP, Rivas R, Iglesias J, Rodríguez F, Solanes, D, Domingo M, Brun A: Experimental infection of young adult European breed sheep with Rift Valley fever virus field isolates. Vector Borne Zoonotic Dis, 2010, 10, 689–696.

Byrne K, McWilliam S, Vuocolo T, Gondro C, Cockett NE, Tellam RL: Genomic architecture of histone 3 lysine 27 trimethylation during late ovine skeletal muscle development. Anim Genet, 2014, 45, 427-438.

C

Calvete C, Estrada R, Miranda MA, Del Rio R, Borrás D, Beldron FJ, Martínez A, Calvo AJ, Lucientes J: Protection of livestock against bluetongue virus vector Culicoides imicola using insecticide–treated netting in open areas. Med Vet Entomol, 2010, 24, 169–175.

Cêtre–Sossah C, Baldet T, Delécolle JC, Mathieu B, Perrin A, Grillet C, Albina E: Molecular detection of Culicoides spp. and Culicoides imicola, the principal vector of bluetongue (BT) and African horse sickness (AHS) in Africa and Europe. Vet Res, 2004, 35, 325–337.

CFSPH (Center or Food Security and Public Health, Iowa State University): Akabane Disease. 2009. Available at: www.cfsph.iastate.edu.

Chaintoutis SC, Kiossis E, Giadinis ND, Brozos CN, Sailleau C, Viarouge C, Bréard E, Papanastassopoulou M, Zientara S, Papadopoulos O, Dovas CI: Evidence of Schmallenberg virus circulation in ruminants in Greece. Trop Anim Health Prod, 2014, 46, 251–255.

Claine F, Coupeau D, Wiggers L, Muylkens B, Kirschvink N: Schmallenberg virus among female lambs, Belgium, 2012. Emerg Infect Dis, 2013a, 19, 1115–1117.

Claine F, Coupeau D, Wiggers L, Muylkens B, Kirschvink N: Emergence and time course of spreading of Schmallenberg virus in late summer 2011 in a Belgian naïve sheep flock. In: Proceedings of 7th Epizone Congress, 2013b, Brussels, Belgium.

Claine F, Coupeau D, Wiggers L, Raes M, Muylkens B, Kirschvink N: Evidence for reemergence of Schmallenberg virus during summer and autumn 2012 among naïve female lambs and immunized ewes. In: Proceedings of 7th Epizone Meeting, 2013c, Brussels, Belgium.

Claine F, Coupeau D, Michaux V, Wiggers L, Muylkens B, Kirschvink N: Passive immunity against Schmallenberg virus in lambs born from naturally infected ewes. In: Proceedings of 8th Epizone Meeting, 2014, Copenhagen, Denmark.

Coupeau D, Claine F, Wiggers L, Kirschvink N, Muylkens B: In vivo and in vitro identification of a hypervariable region in Schmallenberg virus. J Gen Virol, 2013, 94, 1168–1174.

D

Daly JM, King B, Tarlinton RA, Gough KC, Maddison BC, Blowey R: Comparison of Schmallenberg virus antibody levels detected in milk and serum from individual cows. BMC Vet Res, 2015, 11, 56.

Davis CT, Ebel GD, Lanciotti RS, Brault AC, Guzman H, Siirin M, Lambert A, Parsons RE, Beasley DW, Novak RJ, Elizondo–Quiroga D, Green EN, Young DS, Stark LM, Drebot MA, Artsob H, Tesh RB, Kramer LD, Barrett AD: Phylogenetic analysis of North American West Nile virus isolates, 2001–2004: evidence for the emergence of a dominant genotype. J Virol, 2005, 86, 8546–8558.

Day MJ and Schultz RD: Veterinary immunology, principles and practice, 2nd ed., CRC Press, 2014, Boca Raton.

De Regge N, Deblauwe I, De Deken R, Vantieghem P, Madder M, Geysen D, Smeets F, Losson B, van den Berg T, Cay B: Detection of Schmallenberg virus in different Culicoides spp. by real–time RT–PCR. Transbound Emerg Dis, 2012, 59, 471–475.

De Regge N and Cay B: Development, validation and evaluation of added diagnostic value of a q(RT)–PCR for the detection of genotype A strains of small ruminant lentiviruses. J Virol Methods, 2013, 194, 250–257.

De Regge N, van den Berg T, Georges L, Cay B: Diagnosis of Schmallenberg virus infection in malformed lambs and calves and first indications for virus clearance in the fetus. Vet Microbiol, 2013, 162, 595–600.

De Regge N, Madder M, Deblauwe I, Losson B, Fassotte C, Demeulemeester J, Smeets F, Tomme M, Cay AB: Schmallenberg virus circulation in *Culicoides* in Belgium in 2012: field validation of a real time RT–PCR approach to assess virus replication and dissemination in midges. PLoS One, 2014, 9, e87005.

De Regge N, De Deken R, Fassotte C, Losson B, Deblauwe I, Madder M, Vantieghem P, Tomme M, Smeets F, Cay AB: Culicoides monitoring in Belgium in 2011: analysis of spatiotemporal abundance, species diversity and Schmallenberg virus detection. Med Vet Entomol, 2015, 29, 263–275.

Della–Porta AJ, Murray MD, Cybinski DH: Congenital bovine epizootic arthrogryposis and hydranencephaly in Australia. Distribution of antibodies to Akabane virus in Australian cattle after the 1974 epizootic. Aust Vet J, 1976, 52, 496–501.

Delooz, L, Czaplicki G, Houtain JY: How to assess the reporting rate of abortions in Walloon region of Belgium? Poster presentation, 6th European Congress of Bovine Health Management, 2011, Liège, Belgium.

Desmecht D, Garigliany M, Beer M, Paternostre J, Volpe S, Linden A: Serological detection of Schmallenberg virus infections in wild boars, Belgium, 2011–2012. In: Proceedings of 7th Epizone Meeting, 2013c, Brussels, Belgium.

Doceul V, Lara E, Sailleau C, Belbis G, Richardson J, Bréard E, Viarouge C, Dominguez, M, Hendrikx P, Calavas D, Desprat A, Languille J, Comtet L, Pourquier P, Eléouët JF, Delmas B, Marianneau P, Vitour D, Zientara S: Epidemiology, molecular virology and diagnostics of Schmallenberg virus, an emerging orthobunyavirus in Europe. Vet Res, 2013, 44, 31.

Dong H, Li P, Elliott RM, Dong C: Structure of Schmallenberg *Orthobunyavirus* nucleoprotein suggests a novel mechanism of genome encapsidation. J Virol, 2013a, 87, 5593–5601.

Dong H, Li P, Böttcher B, Elliott RM, Dong C: Crystal structure of Schmallenberg *Orthobunyavirus* nucleoprotein–RNA complex reveals a novel RNA sequestration mechanism. RNA, 2013b, 19, 1129–1136.

Dudouet C: La production du mouton, 2ème éd., France agricole, 2003, Paris, France

Dunn EF, Pritlove DC, Jin H, Elliott RM: Transcription of a recombinant *Bunyavirus* RNA template by transiently expressed bunyavirus proteins. Virology, 1995, 211, 133–143.

E

Ebel GD, Carricaburu J, Young D, Bernard KA, Kramer LD: Genetic and phenotypic variation of West Nile virus in New York, 2000–2003. Am J Trop Med Hyg, 2004, 71, 493–500.

EFSA (European Food Safety Authority):. "Schmallenberg" virus: analysis of the epidemiological data. Supporting publications, 2012, EN–360. Available at: www.efsa.europa.eu.

EFSA (European Food Safety Authority). Schmallenberg virus: state of art. EFSA Journal, 2014, 12, 3681–3735.

Elbers AR, Meiswinkel R, van Weezep E, van Oldruitenborgh–Oosterbaan MM, Kooi EA: Schmallenberg virus in Culicoides spp. biting midges, the Netherlands, 2011. Emerg Infect Dis, 2013, 19, 106–109.

Elbers AR, Stockhofe N, van der Poel WHM: Schmallenberg Virus Antibodies in Adult Cows and Maternal Antibodies in Calves. Emerg Infect Dis, 2014a, 20, 901–902.

Elbers AR, Stockhofe–Zurwieden N, van der Poel WH: Schmallenberg virus antibody persistence in adult cattle after natural infection and decay of maternal antibodies in calves. BMC Vet Res, 2014b, 10, 103.

Elbers, AR, Meiswinkel R, van Weezep E, Kooi EA, van der Poel WH: Schmallenberg Virus in culicoides biting midges in the Netherlands in 2012. Transbound Emerg Dis, 2015, 62, 339–342.

Elliott RM and Blakqori G: Molecular biology of Orthobunyavirus. In: Plyusnin A and Elliott RM (Eds): Bunyaviridae: Molecular and Cellular Biology. Caister academic press, 2011, Norfolk, United Kingdom.

EU consortium: Schmallenberg virus Technical and scientific studies, final report. 2014, Wagenigen, Holland. Available at www.ec.europa.eu.

Evans CA, Reynolds JM, Reynolds ML, Saunders NR, Segal MB: The development of a blood–brain barrier mechanism in foetal sheep. J Physiol, 1974, 238, 371–386.

F

Faes C, Van der Stede Y, Guis H, Staubach C, Ducheyne E, Hendrickx G, Mintiens K: Factors affecting Bluetongue serotype 8 spread in Northern Europe in 2006: the geographical epidemiology. Prev Vet Med. 2013, 110, 149–158.

Fischer M, Hoffmann B, Goller KV, Höper D, Wernike K, Beer M: A mutation 'hot spot' in the Schmallenberg virus M segment. J Gen Virol, 2013, 94, 1161–1167.

Flint SJ, Enquist LW, Racaniello VR, Skalka AM: Principle of virology, 3rd edition. ASM press, 2009, Washington, USA.

Forrester NL, Coffey LL, Weaver SC: Arboviral bottlenecks and challenges to maintaining diversity and fitness during mosquito transmission. Viruses, 2014, 6, 3991–4004.

Fu H, Leake CJ, Mertens PP, Mellor PS: The barriers to bluetongue virus infection, dissemination and transmission in the vector, *Culicoides variipennis* (Diptera: Ceratopogonidae). Arch Virol, 1999, 144, 747–761.

Fulton RW, Whitley EM, Johnson BJ, Ridpath JF, Kapil S, Burge LJ, Cook BJ, Confer AW: Prevalence of bovine viral diarrhea virus (BVDV) in persistently infected cattle and BVDV subtypes in affected cattle in beef herds in south central United States. Can J Vet Res, 2009, 73, 283–291.

G

Garigliany MM, Hoffmann B, Dive M, Sartelet A, Bayrou C, Cassart D, Beer M, Desmecht D: Schmallenberg virus in calf born at term with porencephaly, Belgium. Emerg Infect Dis, 2012a, 18, 1005–1006.

Garigliany MM, Bayrou C, Kleijnen D, Cassart D, Jolly S, Linden A, Desmecht D: Schmallenberg virus: a new Shamonda/Sathuperi–like virus on the rise in Europe. Antiviral Res, 2012b, 95, 82–87.

Garigliany MM, Bayrou C, Kleijnen D, Cassart D, Desmecht D: Schmallenberg virus in domestic cattle, Belgium, 2012. Emerg Infect Dis, 2012c, 18, 1512–1514.

Garigliany MM, Desmecht D, Bayrou C, Peeters D: No serologic evidence for emerging Schmallenberg virus infection in dogs (Canis domesticus). Vector Borne Zoonotic Dis, 2013, 13, 830–833.

Gerhauser I, Weigand M, Hahn K, Herder V, Wohlsein P, Habierski A, Varela M, Palmarini M, Baumgärtner W: Lack of Schmallenberg virus in ruminant brain tissues archived from 1961 to 2010 in Germany. J Comp Pathol, 2014, 150, 151–154.

Gillespie JR and Flanders FB: Sheep and goat. In: Modern livestock and poultry production, eighth edition. Cengage Learning, 2010, Clifton Park, NY, USA.

Goffredo M, Monaco F, Capelli G, Quaglia M, Federici V, Catalani M, Montarsi F, Polci A, Pinoni C, Calistri P, Savini G: Schmallenberg virus in Italy: a retrospective survey in Culicoides stored during the bluetongue Italian surveillance program. Prev Vet Med, 2013, 111, 230–236.

Goller KV, Höper D, Schirrmeier H, Mettenleiter TC, Beer M: Schmallenberg virus as possible ancestor of Shamonda Virus. Emerg Infect Dis, 2012, 18, 1644–1646.

Grazul-Bilska AT, Borowicz PP, Johnson ML, Minten MA, Bilski JJ, Wroblewski R, Redmer DA, Reynolds LP: Placental development during early pregnancy in sheep: vascular growth and expression of angiogenic factors in maternal placenta. Reproduction, 2010, 140, 165-174.

H

Hahn K, Habierski A, Herder V, Wohlsein P, Peters M, Hansmann F, Baumgärtner W: Schmallenberg virus in central nervous system of ruminants. Emerg Infect Dis, 2013, 19, 154–155.

Hamers C, Brunet S, Hannas Z, Chery R, Murat–Mourgues V, Brunel H, Goutebroze S, Minke J: Demonstration of efficacy against challenge of an inactivated Schmallenberg vaccine in sheep. In: Proceedings of 7th Epizone Congress, 2013, Brussels, Belgium.

Hashiguchi Y, Nanba K, Kumagai T: Congenital abnormalities in newborn lambs following Akabane virus infection in pregnant ewes. Natl Inst Anim Health Q, 1979, 19, 1–11.

Hechinger S, Wernike K, Beer M: Single immunization with an inactivated vaccine protects sheep from Schmallenberg virus infection. Vet Res, 2014, 45, 79.

Hendrickx G, Gilbert M, Staubach C, Elbers A, Mintiens K, Gerbier G, Ducheyne E: A wind density model to quantify the airborne spread of Culicoides species during north–western Europe bluetongue epidemic, 2006. Prev Vet Med, 2008, 87, 162–181.

Hepojoki J, Strandin T, Wang H, Vapalahti O, Vaheri A, Lankinen H: Cytoplasmic tails of hantavirus glycoproteins interact with the nucleocapsid protein. J Gen Virol, 2010, 91, 2341–2350.

Herder V, Wohlsein P, Peters M, Hansmann F, Baumgartner W: Salient lesions in domestic ruminants infected with the emerging so–called Schmallenberg virus in Germany. Vet Pathol, 2012, 49, 588–591.

Herder V, Hansmann F, Wohlsein P, Peters M, Varela M, Palmarini M, Baumgärtner W: Immunophenotyping of inflammatory cells associated with Schmallenberg virus infection of the central nervous system of ruminants. PloS one, 2013, 8, e62939.

Hierholzer JC and Killington RA: Virus isolation and quantification. In: Mahy B and Kangro H: Virology Methods Manual, Academic Press, 1996, London, United Kingdom.

Hoffmann B, Scheuch M, Höper D, Jungblut R, Holsteg M, Schirrmeier H, Eschbaumer M, Goller KV, Wernike K, Fischer M, Breithaupt A, Mettenleiter TC, Beer M: Novel orthobunyavirus in Cattle, Europe, 2011. Emerg Infect Dis, 2012, 18, 469–472.

Hoffmann B, Schulz C, Beer M: First detection of Schmallenberg virus RNA in bovine semen, Germany, 2012. Vet Microbiol, 2013, 167, 289–295.

Huang CC, Huang TS, Deng MC, Jong MH, Lin SY: Natural infection of pigs with Akabane virus. Vet Microbiol, 2003, 94, 1–11.

Hulst M, Kortekaas J, Hakze–van der Honing R, Vastenhouw S, Cornellissen J, van Maanen K, Bossers A, Harders F, Stockhofe N, van der Poel W: Genetic characterization of an atypical Schmallenberg virus isolated from the brain of a malformed lamb. Virus Genes, 2013, 47, 505–514.

Humphries D, Burr P: Schmallenberg virus milk antibody ELISA. Vet Rec, 2012, 171, 511-512.

J

Jackson P and Cockcroft PD: Clinical Examination of Farm Animals. Blackwell Science Ltd., 2002, Oxford, United Kingdom.

K

Kamata H, Inai K, Maeda K, Nishimura T, Arita S, Tsuda T, Sato M: Encephalomyelitis of cattle caused by Akabane virus in southern Japan in 2006. J Comp Pathol, 2009, 140, 187–193.

Kaufmann C, Schaffner F, Ziegler D, Pflüger V, Mathis A: Identification of field–caught Culicoides biting midges using matrix–assisted laser desorption/ionization time of flight mass spectrometry. Parasitology, 2012a, 139, 248–258.

Kaufmann C, Steinmann IC, Hegglin D, Schaffner F, Mathis A: Spatio–temporal occurrence of Culicoides biting midges in the climatic regions of Switzerland, along with large scale species identification by MALDI–TOF mass spectrometry. Parasit Vectors, 2012b, 5, 246.

Kirschvink N, Claine,F, Wiggers L, Muylkens B: Natural infection of a sheep flock with Schmallenberg virus: clinical, serological and virological features. In: Proceedings of the 6th Epizone congress, Schmallenberg Virus Symposium, 2012, Brighton, England.

Kobayashi T, Yanase T, Yamakawa M, Kato T, Yoshida K, Tsuda T: Genetic diversity and reassortments among Akabane virus field isolates. Virus Res, 2007, 130, 162–171.

Koenraadt CJ, Balenghien T, Carpenter S, Ducheyne E, Elbers AR, Fife M, Garros C, Ibáñez–Justicia A, Kampen H, Kormelink RJ, Losson B, van der Poel WH, De Regge N, van Rijn PA, Sanders C, Schaffner F, Sloet van Oldruitenborgh–Oosterbaan MM, Takken W, Werner D, Seelig F: Bluetongue, Schmallenberg – what is next? Culicoides–borne viral diseases in the 21st Century. BMC Vet Res, 2014, 10, 77.

Kono R, Hirata M, Kaji M, Goto Y, Ikeda S, Yanase T, Kato T, Tanaka S, Tsutsui T, Imada T, Yamakawa M: Bovine epizootic encephalomyelitis caused by Akabane virus in southern Japan. BMC Vet Res, 2008, 4, 20.

Kraatz F, Wernike K, Hechinger S, König P, Granzow H, Reimann I, Beer M: Deletion mutants of Schmallenberg virus are avirulent and protect from virus challenge. J Virol, 2015, 89, 1825–1837.

Krausman PR, Sandoval AV, Etchberger RC: Natural history of desert bighorn sheep. In: Valdez R and Krausman PR (eds). Moutain sheep of North-America. The University of Arizona Press, Tucson, Arizona, pp. 160.

Kukkonen SK, Vaheri A, Plyusnin A: Tula hantavirus L protein is a 250 kDa perinuclear membrane–associated protein. J Gen Virol, 2004, 85, 1181–1189.

Kulldorff M: A spatial scan statistic. Commun. Stat. Theory Methods, 1997, 26, 1481–1496.

Kurogi H, Inaba Y, Goto Y, Miura Y, Takahashi H: Serologic evidence for etiologic role of Akabane virus in epizootic abortion–arthrogryposis–hydranencephaly in cattle in Japan, 1972–1974. Arch Virol, 1975, 47, 71–83.

Kurogi H, Inaba Y, Takahashi E, Sato K, Omori T, Miura Y, Goto Y, Fujiwara Y, Hatano Y, Kodama K, Fukuyama S, Sasaki N, Matumoto M: Epizootic congenital arthrogryposis–hydranencephaly syndrome in cattle: isolation of Akabane virus from affected fetuses. Arch Virol, 1976, 51, 67–74.

L

Larska M, Lechowski L, Grochowska M, Zmudzinski JF: Detection of the Schmallenberg virus in nulliparous Culicoides obsoletus/scoticus complex and C. punctatus–the possibility of transovarial virus transmission in the midge population and of a new vector. Vet Microbiol, 2013, 166, 467–473.

Lawn AM, Chiquoine AD, Amoroso EC: The development of the placenta in the sheep and goat: an electron microscope study. J Anat, 1969, 105, 557–578.

Lee JK, Park JS, Choi JH, Park BK, Lee BC, Hwang WS, Kim JH, Jean YH, Haritani M, Yoo HS, Kim DY: Encephalomyelitis associated with akabane virus infection in adult cows. Vet Pathol, 2002, 39, 269–273.

Lievaart-Peterson K, Luttikholt S, Peperkamp K, Van den Brom R, Vellema P: Schmallenberg disease in sheep or goats: Past, present and future. Vet Microbiol, 2015, 181, 147-153.

Linden A, Desmecht D, Volpe R, Wirtgen M, Gregoire F, Pirson J, Paternostre J, Kleijnen D, Schirrmeier H, Beer M, Garigliany MM: Epizootic spread of Schmallenberg virus among wild cervids, Belgium, Fall 2011. Emerg Infect Dis, 2012, 18, 2006–2008.

Loeffen W, Quak S, de Boer–Luijtze E, Hulst M, van der Poel W, Bouwstra R, Maas R: Development of a virus neutralisation test to detect antibodies against Schmallenberg virus and serological results in suspect and infected herds. Acta Vet Scand, 2012, 54, 44.

Losson B, Mignon B, Paternostre J, Madder M, De Deken R, De Deken G, Deblauwe I, Fassotte C, Cors R, Defrance T, Delécolle JC, Baldet T, Haubruge E, Frédéric F, Bortels J, Simonon G: Biting midges overwintering in Belgium. Vet Rec, 2007, 160, 451–452.

Luttikholt S, Veldhuis A, van den Brom R, Moll L, Lievaart–Peterson K, Peperkamp K, van Schaik G, Vellema P: Risk factors for malformations and impact on reproductive performance and mortality rates of Schmallenberg virus in sheep flocks in the Netherlands. PloS one, 2014, 9, e100135.

M

Manley R, Harrup LE, Veronesi E, Stubbins F, Stoner J, Gubbins S, Wilson A, Batten C, Koenraadt CJ, Henstock M, Barber J, Carpenter S: Testing of UK populations of Culex pipiens L. for Schmallenberg virus vector competence and their colonizzation. PLoS One, 2015, 10, e0134453.

Mansfield KL, La Rocca SA, Khatri M, Johnson N, Steinbach F, Fooks AR: Detection of Schmallenberg virus serum neutralising antibodies. J Virol Methods, 2013, 188, 139–144.

Markusfeld O and Mayer E: An arthrogryposis and hydranencephaly syndrome in calves in Israel, 1969/1970; epidemiological and clinical aspects. Refu Vet, 1971, 28, 51–61.

Markusfeld O: An outbreak of arthrogryposis and hydranencephaly syndrome affecting calves in Israel: a retrospective epidemiological study. Adv Exp Med Biol, 1972, 27, 527-537.

Martinelle L, Dal Pozzo F, Kirschvink N, De La Grandière MA, Thiry E, Saegerman C: Le virus Schmallenberg ou l'émergence du premier Orthobunyavirus du sérogroupe Simbu en Europe. Ann Méd Vét, 2012, 156, 7–24.

Martinelle, L, Dal Pozzo F, Gauthier B, Kirschvink N, Saegerman C: Field veterinary survey on clinical and economic impact of Schmallenberg virus in Belgium. Transbound Emerg Dis, 2014, 61, 285–288.

Martinelle L, Poskin A, Dal Pozzo F, Mostin L, van Campe W, Cay AB, De Regge N, Saegerman C: Three different routes of inoculation for experimental infection with Schmallenberg virus in sheep. Transbound Emerg Dis, 2015, doi: 10.1111/tbed.12356. [Epub ahead of print]

Mathieu B, Delécolle JC, Garros C, Balenghien T, Setier–Rio ML, Candolfi E, Cêtre–Sossah C: Simultaneous quantification of the relative abundance of species complex members: Application to Culicoides obsoletus and Culicoides scoticus (Diptera: Ceratopogonidae), potential vectors of bluetongue virus. Vet Parasitol, 2011, 182, 297–306.

Mathieu B, Cêtre–Sossah C, Garros C, Chavernac D, Balenghien T, Carpenter S, Setier–Rio ML, Vignes–Lebbe R, Ung V, Candolfi E, Delécolle JC: Development and validation of IIKC: an interactive identification key for *Culicoides* (Diptera: Ceratopogonidae) females from the Western Palaearctic region. Parasit Vectors, 2012, 5, 137.

Mayo CE, Mullens BA, Reisen WK, Osborne CJ, Gibbs EP, Gardner IA, MacLachlan NJ: Seasonal and interseasonal dynamics of bluetongue virus infection of dairy cattle and Culicoides sonorensis midges in northern California—implications for virus overwintering in temperate zones. PLoS One, 2014, 9, e106975.

McIntosh GH, Baghurst KI, Potter BJ, Hetzel BS: Foetal brain development in the sheep. Neuropathol Appl Neurobiol, 1979, 5, 103-114.

Mee JF: Prevalence and risk factors for dystocia in dairy cattle: a review. Vet J, 2008, 176, 93–101.

Mehta S and Rajput YS: Immunosuppressive activity in buffalo placenta. Indian J Exp Biol, 1999, 37, 959-964.

Mellor PS: Replication of arboviruses in insect vectors. J Comp Pathol, 2000, 123, 231–247

Mellor PS, Boorman J, Baylis M: *Culicoides* biting midges: their role as arbovirus vectors. Annu Rev Entomol, 2000, 45, 307–340.

Méroc E, Faes C, Herr C, Staubach C, Verheyden B, Vanbinst T, Vandenbussche F, Hooyberghs J, Aerts M, De Clercq K, Mintiens K: Establishing the spread of bluetongue virus at the end of the 2006 epidemic in Belgium. Vet Microbiol, 2008, 131, 133–144.

Méroc E, Herr C, Verheyden B, Hooyberghs J, Houdart P, Raemaekers M, Vandenbussche F, De Clercq K, Mintiens K: Bluetongue in Belgium: episode II. Transbound Emerg Dis, 2009, 56, 39–48.

Méroc E, Poskin A, Van Loo H, Quinet C, Van Driessche E, Delooz L, Behaeghel I, Riocreux F, Hooyberghs J, De Regge N, Caij AB, van den Berg T, Van der Stede Y: Large–scale cross–sectional serological survey of Schmallenberg virus in Belgian cattle at the end of the first vector season. Transbound Emerg Dis, 2013, 60, 4–8.

Méroc E, De Regge N, Riocreux F, Caij AB, van den Berg T, Van der Stede Y: Distribution of Schmallenberg virus and seroprevalence in Belgian sheep and goats. Transbound Emerg Dis, 2014, 61, 425–431.

Méroc E, Poskin A, Van Loo H, Van Driessche E, Czaplicki G, Quinet C, Riocreux F, De Regge N, Caij B, van den Berg T, Hooyberghs J, Van der Stede Y: Follow–up of the Schmallenberg virus seroprevalence in Belgian cattle. Transbound Emerg Dis, 2015, 62, e80-84.

Miura Y, Hayashi S, Ishihara T, Inaba Y, Omori T: Neutralizing antibody against Akabane virus in precolostral sera from calves with congenital arthrogryposis–hydranencephaly syndrome. Arch Gesamte Virusforsch, 1974, 46, 377–380.

Miyazato S, Miura Y, Hase M, Kubo M, Goto Y, Kono Y: Encephalitis of cattle caused by Iriki isolate, a new strain belonging to Akabane virus. Nihon Juigaku Zasshi, 1989, 51, 128–136.

Moulin V, Goovaerts D, de Smit H: Development of a vaccine that protects sheep and cattle against challenge with Schmallenberg virus, a novel Orthobunyavirus recently found in Europe. In: Proceedings of 7th Epizone Congress, 2013, Brussels, Belgium.

Muskens, J, Smolenaars AJ, van der Poel WH, Mars MH, van Wuijckhuise L, Holzhauer M, van Weering H, Kock P: Diarrhea and loss of production on Dutch dairy farms caused by the Schmallenberg virus. Tijdschr Diergeneeskd, 2012, 137, 112–115.

N

Ninio C, Augot D, Delecolle JC, Dufour B, Depaquit J: Contribution to the knowledge of Culicoides (Diptera: Ceratopogonidae) host preferences in France. Parasitol Res, 2011, 108, 657–663.

Nobel TA, Klopfer U, Neumann F: Pathology of an arthrogryposis–hydranencephaly syndrome in domestic ruminants in Israel 1969/1970. Refu Vet, 1971, 28, 144–151.

O

Oem JK, Yoon HJ, Kim HR, Roh IS, Lee KH, Lee OS, Bae YC: Genetic and pathogenic characterization of Akabane viruses isolated from cattle with encephalomyelitis in Korea. Vet Microbiol, 2012, 158, 259–266.

OIE (World Organisation for Animal Health): Bunyaviral diseases of animals. In: OIE Terrestrial Manual, 2014. Available at www.oie.int.

Overby AK, Popov VL, Pettersson RF, Neve EP: The cytoplasmic tails of Uukuniemi Virus (Bunyaviridae) G(N) and G(C) glycoproteins are important for intracellular targeting and the budding of virus–like particles. J Virol, 2007, 81, 11381–11391.

P

Papadopoulos E, Bartram D, Carpenter S, Mellor P, Wall R: Efficacy of alphacypermethrin applied to cattle and sheep against the biting midge Culicoides nubeculosus. Vet Parasitol, 2009, 163, 110–114.

Parameswaran P, Charlebois P, Tellez Y, Nunez A, Ryan EM, Malboeuf CM, Levin JZ, Lennon NJ, Balmaseda A, Harris E, Henn MR: Genome–wide patterns of intrahuman dengue virus diversity reveal associations with viral phylogenetic clade and interhost diversity. Virology, 2012, 342, 252–265.

Parsonson IM, Della–Porta AJ, Snowdon WA: Congenital abnormalities in newborn lambs after infection of pregnant sheep with Akabane virus. Infect Immun, 1977, 15, 254–262.

Parsonson IM, Della–Porta AJ, O'Halloran ML: Akabane virus infection in the pregnant ewe. I. Growth of virus in the foetus and the development of the foetal immune response. Vet Microbiol, 1981, 6, 197-207.

Parsonson IM, McPhee DA, Della–Porta AJ, McClure S, McCullagh P: Transmission of Akabane virus from the ewe to the early fetus (32 to 53 days). J Comp Pathol, 1988, 99, 215–227.

Peperkamp NH, Luttikholt SJ, Dijkman R, Vos JH, Junker K, Greijdanus S, Roumen MP, Garderen EV, Meertens N, Maanen CV, Lievaart K, Wuyckhuise LV, Wouda W: Ovine and bovine congenital abnormalities associated with intrauterine infection with Schmallenberg virus. Vet Pathol, 2014, pii: 030098581456023. [Epub ahead of print]

Pioz M, Guis H, Crespin L, Gay E, Calavas D, Durand B, Abrial D, Ducrot C: Why did bluetongue spread the way it did? Environmental factors influencing the velocity of bluetongue virus serotype 8 epizootic wave in France. PLoS One, 2012, 7, e43360.

Plassmeyer ML, Soldan SS, Stachelek KM, Roth SM, Martin–Garcia J, Gonzalez–Scarano F: Mutagenesis of the La Crosse Virus – glycoprotein supports a role for Gc (1066–1087) as the fusion peptide. Virology, 2007, 358, 273–282.

Ponsart C, Pozzi N, Bréard E, Catinot V, Viard G, Sailleau C, Viarouge C, Gouzil J, Beer M, Zientara S, Vitour D: Evidence of excretion of Schmallenberg virus in bull semen. Vet Res, 2014, 45, 37.

Poskin A, Van Campe W, Mostin L, Cay B, De Regge N: Experimental Schmallenberg virus infection of pigs. Vet Microbiol, 2014a, 170, 398–402.

Poskin A, Martinelle L, Mostin L, Van Campe W, Dal Pozzo F, Saegerman C, Cay B, De Regge N: Dose–dependent effect of experimental Schmallenberg virus infection in sheep. Vet J, 2014b, 201, 419–422.

Poskin A, Martinelle L, Dal Pozzo F, Cay B, De Regge N, Saegerman C: Experimental infection of sheep with Schmallenberg virus at days 45 and 60 of pregnancy. In: Proceedings of 7th Epizone Congress, 2014c, Copenhagen, Denmark.

Poskin A, Méroc E, Behaeghel I, Riocreux F, Couche M, Van Loo H, Bertels G, Delooz L, Quinet C, Dispas M, Van der Stede Y: Schmallenberg virus in Belgium: estimation of impact in cattle and sheep herds. Transbound Emerg Dis, 2015a, doi: 10.1111/tbed.12367. [Epub ahead of print]

Poskin A, Verite S, Comtet L, Van der Stede Y, Cay B, De Regge N: Persistence of the protective immunity and kinetics of the isotype specific antibody response against the viral nucleocapsid protein after experimental Schmallenberg virus infection of sheep. Vet Res, 2015b, 46, 119.

Poskin A, Théron L, Hanon JB, Saegerman C, Vervaeke M, Van der Stede Y, Cay B, De Regge N: Reconstruction of the Schmallenberg virus epidemic in Belgium: complementary use of disease surveillance approaches, 2016, 183, 50-61.

ProMED–Mail, 20111223.3665: Schmallenberg virus – Europe: (Belgium) Congenital malformations, ovine. 2011. Available at: www.promedmail.com.

ProMED–Mail, 20120117.1012402: Schmallenberg virus – Europe: update, international impact. 2012. Available at: www.promedmail.com.

ProMED–Mail, 20141121.2978286: Schmallenberg virus – Europe: Netherlands, Germany, bovine. 2014. Available at: www.promedmail.com.

Purse BV, Mellor PS, Rogers DJ, Samuel AR, Mertens PP, Baylis M: Climate change and the recent emergence of bluetongue in Europe. Nat Rev Microbiol, 2005, 3, 171–181.

R

Rasmussen LD, Kristensen B, Kirkeby C, Rasmussen TB, Belsham GJ, Bødker R, Bøtner A: Culicoids as vectors of Schmallenberg virus. Emerg Infect Dis, 2012, 18, 1204–1206.

Reynolds LP, Redmer DA: Growth and microvascular development of the uterus during early pregnancy in ewes. Biol Reprod, 1992, 47, 698-708.

Reynolds LP and Redmer DA: Angiogenesis in the placenta. Biol Reprod, 2001, 64, 1033–1040.

Reynolds LP, Borowicz PP, Caton JS, Vonnahme KA, Luther JS, Buchanan DS, Hafez SA, Grazul-Bilska AT, Redmer DA: Uteroplacental vascular development and placental function: an update. Int J Dev Biol, 2010, 54, 355-366.

Rodríguez–Prieto V, Kukielka D, Mouriño M, Paradell H, Plaja L, Urniza A, Sánchez–Vizcaíno JM: Natural immunity of sheep and lambs against the Schmallenberg virus infection. Transbound Emerg Dis, 2014, doi: 10.1111/tbed.12256. [Epub ahead of print]

Rosseel T, Scheuch M, Höper D, De Regge N, Caij AB, Vandenbussche F, Van Borm S: DNase SISPA–next generation sequencing confirms Schmallenberg virus in Belgian field samples and identifies genetic variation in Europe. PLoS One, 2012, 7, e41967.

S

Saeed M, Li L, Wang H, Weaver SC, Barrett ADT: Phylogeny of the Simbu serogroup of the genus Bunyavirus. J Gen Virol, 2001, 82, 2173–2181.

Saegerman C, Berkvens D, Mellor PS: Bluetongue Epidemiology in the European Union Emerg Infect Dis, 2008, 14, 539–544.

Saegerman C, Martinelle L, Dal Pozzo F, Kirschvink N: Preliminary survey on the impact of Schmallenberg virus on sheep flocks in South of Belgium. Transbound Emerg Dis, 2014, 61, 469–472.

Sailleau C, Boogaerts C, Meyrueix A, Laloy E, Bréard E, Viarouge C, Desprat A, Vitour D, Doceul V, Boucher C, Zientara S, Nicolier A, Grandjean D: Schmallenberg virus infection in dogs, France, 2012. Emerg Infect Dis, 2013, 19, 1896–1898.

Salanueva IJ, Novoa RR, Cabezas P, López-Iglesias C, Carrascosa JL, Elliott RM, Risco C. Polymorphism and structural maturation of bunyamwera virus in Golgi and post-Golgi compartments. J Virol, 2003, 77, 1368–1381.

Sato K, Tanaka Y, Tokuhisa S, Inaba Y, Fushiki T, Matumoto M: Appearance of slow–reacting and complement–requiring neutralizing antibody in cattle infected with Akabane virus. Vet Microbiol, 1987, 14, 183–189.

Schetters T, Van Run–Van Breda J, Van Zon A, Eling W: Selective depletion of immunoglobulin isotypes after decomplementation of mouse sera by heat treatment. J Immunol Methods, 1988, 109, 193–197.

Schmaljohn CS and Nichol ST: Bunyaviridae. In: Knipe DM and Howley PM (Eds): Field virology, 5th ed., Lippincott Williams & Wilkins, 2007, Philapdelphia, USA.

Sedda L and Rogers DJ: The influence of the wind in the Schmallenberg virus outbreak in Europe. Sci Rep, 2013, 3, 3361.

Seehusen F, Hahn K, Herder V, Weigand M, Habierski A, Gerhauser I, Wohlsein P, Peters M, Varela M, Palmarini M, Baumgärtner W: Skeletal muscle hypoplasia represents the only significant lesion in peripheral organs of ruminants infected with Schmallenberg virus during gestation. J Comp Pathol, 2014, 151, 148–152.

Sellers RF and Herniman KAJ: Neutralising antibodies to Akabane virus in ruminants in Cyprus. Trop Anim Health Prod, 1981, 13, 57–60.

Shi X, Kohl A, Li P, Elliott RM: Role of the cytoplasmic tail domains of Bunyamwera orthobunyavirus glycoproteins Gn and Gc in virus assembly and morphogenesis. J Virol, 2007, 81, 10151–10160.

Silverstein AM, Uhr JW, Kraner KL, Lukes RJ: Fetal response to antigenic stimulus. II. Antibody production by the fetal lamb. J Exp Med, 1963, 117, 799–812.

Snippe M, Smeenk L, Goldbach R, Kormelink R: The cytoplasmic domain of tomato spotted wilt virus Gn glycoprotein is required for Golgi localisation and interaction with Gc. Virology, 2007, 363, 272–279.

Steukers L, Bertels G, Cay AB, Nauwynck HJ: Schmallenberg virus: emergence of an Orthobunyavirus among ruminants in Western Europe. Tijdschr Diergeneeskd, 2012, 81, 119–127.

Stockhofe N, Hulst M, Hakze R, Vellema P, Van der Poel W: Transplacental infection of Schmallenberg Virus in the first trimester of gestation is highly efficient in sheep. In: Proceedings of 7th Epizone Congress, 2013, Brussels, Belgium.

St George TD: Effects of the pathogenesis of arbovirus infections in cattle on the efficiencies of vectors. In : Eighth Arbovirus Research Symposium & Fourth Mosquito Control Association of Australia Symposium, Queensland Institute of Medical Research, Queensland, Australia. Available at : espace.library.uq.edu.au (last access 20/12/2015).

Strandin T, Hepojoki J, Vaheri A : Cytoplasmic tails of bunyavirus Gn glycoproteins-Could they act as matrix protein surrogates? Virology, 2013, 437, 73-80.

T

Takikawa N, Kobayashi S, Ide S, Yamane Y, Tanaka Y, Higashihara M, Yamagishi H: Early serodiagnosis of porcine reproductive and respiratory syndrome virus infection of pigs by detection of slow–reacting and complement–requiring neutralizing antibody. J Vet Med Sci, 1997, 59, 31–34.

Takken W, Verhulst N, Scholte EJ, Jacobs F, Jongema Y, van Lammeren R: The phenology and population dynamics of Culicoides spp. in different ecosystems in The Netherlands. Prev Vet Med, 2008, 87, 41–54.

Taylor WP and Mellor PS: The distribution of Akabane virus in the Middle East. Epidemiol Infect, 1994, 113, 175–185.

Tizard JR: Immunity in the Fetus and Newborn. In: Tizard J (Ed.): Veterinary Immunology, 9th ed., Elsevier, 2013, St. Louis, Missouri.

Toussaint JF, Sailleau C, Bréard E, Zientara S, De Clercq K: Bluetongue virus detection by two real–time RT–qPCRs targeting two different genomic segments. J Virol Methods, 2007, 140, 115–123.

V

van den Brom R, Luttikholt SJ, Lievaart–Peterson K, Peperkamp NH, Mars MH, van der Poel WH, Vellema P: Epizootic of ovine congenital malformations associated with Schmallenberg virus infection. Tijdschr Diergeneeskd, 2012, 137, 106–111.

van der Heijden HM, Bouwstra RJ, Mars MH, van der Poel WH, Wellenberg GJ, van Maanen C: Development and validation of an indirect Enzyme–linked immunosorbent assay for the detection of antibodies against Schmallenberg virus in blood samples from ruminants. Res Vet Sci, 2013, 95, 731–735.

van der Poel WH: Diagnostics for Schmallenberg virus. Vet Rec, 2012, 171, 294–295.

van der Poel WH, Cay B, Zientara S, Steinbach F, Valarcher JF, Bøtner A, Mars MH, Hakze-van der Honing R, Schirrmeier H, Beer M. Limited interlaboratory comparison of Schmallenberg virus antibody detection in serum samples. Vet Rec, 2014, 174, 15.

van de Sandt CE, Kreijtz JH, Rimmelzwaan GF: Evasion of influenza A viruses from innate and adaptive immune responses. Viruses, 2012, 4, 1438-1476.

van Maanen C, van der Heijden H, Wellenberg GJ, Witteveen G, Luttikholt S, Bouwstra R, Kooi B, Vellema P, Peperkamp K, Mars J: Schmallenberg virus antibodies in bovine and ovine fetuses. Vet Rec, 2012, 171, 299.

Varela M, Schnettler E, Caporale M, Murgia C, Barry G, McFarlane M, McGregor E, Piras I.M, Shaw A, Lamm C, Janowicz A, Beer M, Glass M, Herder V, Hahn K, Baumgärtner W, Kohl A, Palmarini M: Schmallenberg virus pathogenesis, tropism

and interaction with the innate immune system of the host. PLoS Pathog, 2013, 9, e1003133.

Veldhuis AM, Carp–van Dijken S, van Wuijckhuise L, Witteveen G, van Schaik G: Schmallenberg virus in Dutch dairy herds: potential risk factors for high within–herd seroprevalence and malformations in calves, and its impact on productivity. Vet Microbiol, 2014a, 168, 281–293.

Veldhuis AM, Santman–Berends IM, Gethmann JM, Mars MH, van Wuyckhuise L, Vellema P, Holsteg M, Höreth–Böntgen D, Conraths FJ, van Schaik G: Schmallenberg virus epidemic: impact on milk production, reproductive performance and mortality in dairy cattle in the Netherlands and Kleve district, Germany. Prev Vet Med, 2014b, 116, 412–422.

Veldhuis AM, Mars MH, Roos CA, van Wuyckhuise L, van Schaik G: Two years after the Schmallenberg virus epidemic in the Netherlands: does the virus still circulate? Transbound Emerg Dis, 2015, doi: 10.1111/tbed.12349. [Epub ahead of print]

Veronesi E, Henstock M, Gubbins S, Batten C, Manley R, Barber J, Hoffmann B, Beer M, Attoui H, Mertens PP, Carpenter S: Implicating Culicoides biting midges as vectors of Schmallenberg virus using semi–quantitative RT–PCR. PloS one. 2013, 8, e57747.

W

Walter CT and Barr JN: Recent advances in the molecular and cellular biology of *Bunyaviruses*. J Gen Virol, 2011, 92, 2467–2484.

Wang Q, Ju H, Li Y, Jing Z, Guo L, Zhao Y, Ma B, Gao M, Zhang W, Wang J: Development and evaluation of a competitive ELISA using a monoclonal antibody for antibody detection after goose parvovirus virus–like particles (VLPs) and vaccine immunization in goose sera. J Virol Methods, 2014, 209, 69–75.

Weaver AD: Lameness above the foot. In: Andrews AH, Blowey RW, Boyd H, Eddy RG (Eds): Bovine Medicine: Diseases and Husbandry of Cattle, 2nd ed., Blackwell Publishing, 2004, Oxford, United Kingdom.

Weiher W, Bauer B, Mehlitz D, Nijhof A.M, Clausen P.H: Field trials assessing deltamethrin (Butox®) treatments of sheep against Culicoides species. Parasitol Res, 2014, 113, 2641–2645.

Wensman JJ, Blomqvist G, Hjort M, Holst BS: Presence of antibodies to Schmallenberg virus in a dog in Sweden. J Clin Microbiol, 2013, 51, 2802–2803.

Wernike K, Breithaupt A, Keller M, Hoffmann B, Beer M, Eschbaumer M: Schmallenberg virus infection of adult type I interferon receptor knock–out mice. PLoS One, 2012a, 7, e40380.

Wernike K, Eschbaumer M, Breithaupt A, Hoffmann B, Beer M: Schmallenberg virus challenge models in cattle: infectious serum or culture–grown virus? Vet Res, 2012b, 43, 84.

Wernike K, Hoffmann B, Beer M: Schmallenberg virus. Dev Biol, 2013a, 135, 175–182.

Wernike K, Eschbaumer M, Schirrmeier H, Blohm U, Breithaupt A, Hoffmann B, Beer M: Oral exposure, reinfection and cellular immunity to Schmallenberg virus in cattle. Vet Microbiol, 2013b, 165, 155– 159.

Wernike K, Nikolin VM, Hechinger S, Hoffmann B, Beer M: Inactivated Schmallenberg virus prototype vaccines. Vaccine, 2013c, 31, 3558–3563.

Wernike K, Kohn M, Conraths FJ, Werner D, Kameke D. Hechinger S, Kampen H, Beer M: Transmission of Schmallenberg virus during Winter, Germany. Emerg Infect Dis, 2013d, 19, 1701–1703.

Wernike K, Hoffmann B, Bréard E, Bøtner A, Ponsart C, Zientara S, Lohse L, Pozzi N, Viarouge C, Sarradin P, Leroux–Barc C, Riou M, Laloy E, Breithaupt A, M. Beer: Schmallenberg virus experimental infection of sheep. Vet Microbiol, 2013e, 166, 461–466.

Wernike K, Holsteg M, Schirrmeier H, Hoffmann B, Beer M: Natural infection of pregnant cows with Schmallenberg virus—a follow–up study. PloS one, 2014a, 9, e98223.

Wernike K, Jöst H, Becker N, Schmidt–Chanasit J, Beer M: Lack of evidence for the presence of Schmallenberg virus in mosquitoes in Germany, 2011. Parasit Vectors, 2014b, 7, 402.

Wernike K, Hoffmann B, Conraths FJ, Beer M: Schmallenberg virus recurrence, Germany, 2014. Emerg Infect Dis, 2015, 21, 1202-1204.

Wilson A, Darpel K, Mellor PS. Where does bluetongue virus sleep in the winter? PLoS Biol, 2008, 6, e210.

Wisløff H, Nordvik BS, Sviland S, Tønnessen R: The first documented clinical case of Schmallenberg virus in Norway: fetal malformations in a calf. Vet Rec, 2014, 174, 120.

Y

Yamakawa M, Yanase T, Kato T, Tsuda T: Chronological and geographical variations in the small RNA segment of the teratogenic Akabane virus. Virus Res, 2006, 121, 84–92.

Yanase T, Fukutomi T, Yoshida K, Kato T, Ohashi S, Yamakawa M, Tsuda T: The emergence in Japan of *Sathuperi virus*, a tropical Simbu serogroup virus of the genus *Orthobunyavirus*. Arch Virol, 2004, 149, 1007–1013.

Yanase T, Maeda K, Kato T, Nyuta S, Kamata H, Yamakawa M, Tsuda T: The resurgence of Shamonda virus, an African Simbu group virus of the genus Orthobunyavirus, in Japan. Arch Virol, 2005, 150, 361-369.

Yanase T, Kato T, Aizawa M, Shuto Y, Shirafuji H, Yamakawa M, Tsuda T: Genetic reassortment between Sathuperi and Shamonda viruses of the genus Orthobunyavirus in nature: implications for their genetic relationship to Schmallenberg virus. Arch Virol, 2012, 157, 1611–1616.

Yoder SM, Zhu Y, Ikizler MR, Wright PF: Role of complement in neutralization of respiratory syncytial virus. J Med Virol, 2004, 72, 688–694.

Yoshida K and Tsuda T: Rapid detection of antigenic diversity of Akabane virus isolates by dot immunobinding assay using neutralizing monoclonal antibodies. Clin Diagn Lab Immunol, 1998, 5, 192–198.

Z

Zimmer JY, Haubruge E, Francis F, Bortels J, Simonon G, Losson B, Mignon B, Paternostre J, De Deken R, De Deken G, Deblauwe I, Fassotte C, Cors R, Defrance T: Breeding sites of bluetongue vectors in northern Europe. Vet Rec, 2008a, 162, 131.

Zimmer JY, Haubruge E, Francis F, Bortels J, Joie E, Simonon G, De Deken R, De Deken G, Deblauwe I, Madder M, Fassotte C, Cors R, Defrance T, Saegerman C, Thiry E, Mignon B, Paternostre J, Losson B, Kirschvink N: Distribution of potential bluetongue vectors on Belgium farms. Vet Rec, 2008b, 162, 700.

Zimmer JY, Saegerman C, Losson B, Haubruge, E: Breeding sites of bluetongue virus vectors, Belgium. Emerg Infect Dis, 2010, 16, 575–576.

PART SEVEN : ANNEX

Annex 1: Preparation of a standard curve for the Schmallenberg virus S-segment

This method was use as developed by De Regge *et al*. (2013). Briefly, RNA was extracted from Schmallenberg virus (SBV) infectious serum (Wernike *et al*., 2012) with the RNeasyMini kit (Qiagen) and reverse transcribed with Moloney murine leukaemia virus reverse transcriptase (Life Technologies) and random hexamer primers. A PCR amplification using primers targeting a 839 base pair 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 into the 2.1–TOPO cloning vector (Life Technologies) and transformed into competent *Escherichia coli* TOP10 cells. After multiplication, the plasmids were isolated and linearised with *Bam*HI, followed by in vitro transcription with the TranscriptAid T7 High Yield kit (Thermo Scientific). Any residual plasmid DNA was eliminated by two successive Turbo DNA free treatments (Life Technologies) 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 °C.

The RNA standard curve consisted of a 10–fold serial dilution series of the RNA transcripts in Tris–EDTA (TE) buffer. In each PCR run, a dilution series (ranging from 3.9×10^7 to 3.9 copies/µL) was run together with samples of blood or organ and the standard curve was constructed by plotting the Ct values against the log of the input RNA copy number. A linear regression was used to calculate the number of copies in samples during the same rRT–PCR.

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