A Review on Reliable and Standardized Animal Models to Study the Pathogenesis of Schmallenberg Virus in Ruminant Natural Host Species

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Abstract

In the late summer of 2011, the Netherlands reported a cluster of reduced milk yield, fever, and diarrhea in dairy cattle. In March 2012, congenital malformations appeared, and Schmallenberg virus (SBV) was identified, becoming one of the few orthobunyaviruses distributed in Europe. Initially, little was known about the pathogenesis and epidemiology of these viruses in the European context, so assumptions were largely extrapolated from related viruses and other regions worldwide. To study SBV's pathogenesis and its ability to cross the placental barrier, standardized and repeatable models that mimic clinical signs observed in the field are essential. This review discusses some of the latest experimental designs for infectious disease challenges involving SBV, covering infectious doses, routes of infection, inoculum preparation, and origin. Special attention is given to the placental crossing associated with SBV.

Keywords: Schmallenberg; Culicoides; vector-borne disease; experimental challenge; infection; arboviruses

1. Introduction

Amongst pathogens, RNA viruses were a major source of emerging diseases during the last 30 years [1]. High mutation rate and in case of segmented genome, reassortment are responsible for genetic adaptability and variability of these viruses.

Schmallenberg virus (SBV) was responsible for a major outbreak in Mainland Europe in the past 15 years. This outbreak was singular in several ways: the disease was previously unknown; the emergence still has unexplained aspects; the virus displayed the ability to cross the placental barrier. Moreover, these events confirmed that palearctic endemic *Culicoides* species contribute to the spread of SBV and to the epizootic aspect of the diseases.

Until recent nomenclature changes implemented by the International Committee on Taxonomy of Viruses [2-4] Schmallenberg virus was part of the Bunyaviridae family, genus Orthobunyavirus, grouped within the serogroup Simbu along with at least 27 other virus species. The members of the Simbu serogroup show cross-reactions to the complement fixation test but are distinguished by seroneutralization [5] and by genetic sequence analysis. Yet still part of the Orthobunyavirus genus, SBV is now considered as a species of its own, *Orthobunyavirus schmallenbergense*, along with Akabane virus (*Orthobunyavirus akabaneense*) and 136 other species [6,3]. These belong to the new order Elliovirales (formerly Bunyavirales), family Peribunyaviridae (formerly Bunyaviridae), which comprises seven other genera.

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

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

2. Studying the Pathogenesis and Immune Response of SBV in Natural Ruminant Host Species

Experimental infections of mammalian hosts proved to be a highly valuable tool to study pathogenicity, virulence, pathogenesis, and transplacental infections since the dawn of the study of infectious diseases [7]. Design of *in vivo* models evolved and were usefully complemented with in vitro and in silico approaches to better comprehend the host-pathogen interactions.

Prior to study the pathogenesis of SBV in ruminants, including teratogenic potential, experimental models reproducing the disease had to be found.

To date there are no lab-adapted colonies of Palearctic *Culicoides*. Given the feeding behaviour of *Culicoides*, investigating the most adapted route of inoculation is of prime importance to ensure standardization and repeatability of challenge experiments. Amongst other important pathogenesis factors to consider when designing experimental infections, the origin of the inoculum and its passage history has to be carefully evaluated. Indeed, the number of passages, the cell culture system used to grow the inoculum or by contrast, infectious blood or serum source are central to achieve experimental infection matching virological, clinical, and serological parameters of field infection with wild-type viruses.

2.1. A proper experimental infection needs a proper inoculum

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

1) Safe, meaning it should have been screened for contaminations, adventitial agents or other pathogens [8].
2) Easily available, practical and standardised.
3) Contain a virus the closest from wild-type virus found in the field displaying similar replication and virulence properties.

2.2. Infectious blood versus cell passaged inoculum

An infectious inoculum can be used for several purposes, from the investigation of *in vivo* characteristics of recently discovered viruses [9] to vaccine efficacy requirements or to investigate certain specific aspects of the pathogenesis [10]. In the context of an emerging pathogen with an epizootic potential it is obvious that time is of the essence and since standardization is not critical in the very first steps of *in vivo* characterization using an infectious animal product such as blood or serum could be both faster and more secure to reproduce expected clinical signs and viraemia. Nevertheless, it appears that in most of the most recent experimental infections involving SBV, culture grown inocula were used in most challenges (Table 1). The main reasons to use cell-passaged virus can be summarized as follow:

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

Clinical signs of acute SBV infection in adult cattle are reported to be generally mild and short-lived. These include loss of appetite, hyperthermia, diarrhea, and reduction in milk production

[11]. As a matter of fact, regarding Bluetongue Disease Virus (BTV) – another culicoide-borne virus - it was reported that the inoculation of infectious material from field isolates rarely produce a clinical picture as severe as in natural infection [12]. A hypothesis would be that the *Culicoides* saliva might act as a catalyzer to enhance the ability of BTV to produce severe clinical signs [13]. Moreover, in the field and no matter the care and experience of the farmers there are always some animals that would be more sensitive to viral diseases within the herd due to individual poor immune defenses, genetic variability, nutritional aspects or metabolic status. In an experimental context however the health and sanitary status of every single animal is very strictly controlled and monitored, inducing a bias toward the selection of very healthy and vigorous animals most likely able to face and recover from viral infection.

The milder nature of natural SBV infection in cattle hinders the proper clinical comparison between field and culture-grown inocula. Indeed, in cattle Wernike et al. reported a reduced viral replication of culture-grown SBV when compared to natural host-passaged inoculum [14]. By contrast, one year later the same team concluded to the suitability of both infectious serum and low passage cell culture material for SBV experimental challenges in sheep [15]. Besides the passage history the origin of the isolated virus seems to be of importance as central nervous system originating virus failed to reproduce RNAemia in inoculated animals [15]. Successive serial passages in cell-culture indeed are well known to usually result in decreased virulence. However, regarding SBV Varela et al. reported an increased pathogenicity in a SBV strain passaged 32 times in INF-incompetent sheep CPT-Tert cells, associated with a faster spread of the virus in the brain of suckling mice [16]. SBV was demonstrated to grow efficiently in several cell lines including sheep CPT-Tert, bovine BFAE, human 293T, dog MDCK, hamster BHK-21, BSR, KC and VERO cells [16,17]. Whereas serial passages in CPT-Tert led to the accumulation of a variety of mutations mostly in the M and S segments, the porcine cell line SK-6 proved to be highly susceptible and to allow the genetic stability of SBV throughout

successive passages [18]. Therefore, depending on the cell line used to grow SBV, serial passages can lead to attenuation, increased virulence, or efficient propagation with a low frequency of nucleotide exchanges.

2.3. A matter of doses and routes

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

1) The need for a route that will mimicry the most the behavior of the vector in the wild. Usually, haematophagous arthropods are either telmophagous or solenophagous; depending on the vector species the route might be intradermal (ID), subcutaneous (SC) or intravenous (IV). In experimental infections given the size of the arthropods and the size of their mouthparts the inoculated viral load and volume are usually way higher than the ones inoculated through naturally occurring feeding [19]. Another drawback is the lack of vector saliva components, which can modify the structure and infectivity of *Bunyaviridae* viral particles [20].
2) The need for a route that will ensure the virus to reach blood stream. Quite obviously this is the intravenous route. Since vector saliva components can enhance the infectivity of arboviruses there is a risk that the inoculation of the virus alone or at a distal site from the vector feeding site would result in a failed infection [21]. Therefore, the option to by-pass the skin to readily reach the bloodstream may be relevant.

Several authors used mixed routes to overcome the respective disadvantages of each approach (Table 1). In a previous study, we compared intranasal, intradermal and subcutaneous routes for experimental infections with SBV [22]. Intradermal is an interesting yet underused route: indeed most haematophagous arthropods do not make it through the skin and their mouthparts only allow them to feed intradermally. Most of the cellular and fluid exchanges between the

skin and the blood do occur in the dermis [23]. In addition, there are some evidences suggesting that intradermal inoculation can be more appropriate to reproduces many aspects of natural infection, including clinical disease, viral and immune responses [24]. However, to perform an actual intradermal inoculation the volume to be injected has to be limited, the dermis being mostly composed of a network of collagen fibres. Therefore, it is required to multiply the inoculation sites to reach the desire total inoculum volume and infectious titre. To realize the inoculation itself the most practical tools are the Dermojet® (Akra Dermojet) or a special syringe for intradermal injections (used to perform bovine tuberculosis skin tests as an example). These devices allow usually volumes between 0.1 to 0.4 ml, thus the need of multiple injections to reach the common 1-4 ml inoculation volume used in ruminant infectious challenges experiments (Table 1). Moreover, with both systems the inoculum must be transferred from its original vial to a small tank part of the body of the dermojet or to a special cartridge to be used with the intradermal syringe. This extra step increases the number of handlings, which should be limited especially in the case of BSL3 pathogens. The intranasal route was mostly investigated to test whether a potential direct contamination between sheep could be achieved.

Broadly speaking, several authors reported a direct link between the inoculated viral doses and the onset of clinical signs and viraemia, i.e. the higher the dose the sooner the clinical signs and viral RNA detection [25-27]. In another study we evaluated four 10-fold dilutions of a SBV infectious serum inoculum on ewes [28]. The undiluted original inoculum had a titre of 2×10^3 $TCID_{50}$/mL. It appears there is a critical dose to be inoculated to successfully reproduce field-like virological and immunological parameters, and once this threshold is over there no dose-dependent effect anymore. Indeed, in the productively infected animals no statistical differences between the different inoculation doses were found in the duration or quantity of viral RNA circulating in blood, nor in the amount of viral RNA present in virus positive lymphoid organs.

Inoculation by the bite of Culicoides was reported to be more efficient than intradermal inoculation, especially by delaying the early immune response of the host despite a generally lower inoculated viral dose when compared to needle inoculation [29]. Pharmacological agents contained in Culicoides saliva might affect the host's immune response by anti-proliferative effects on leucocytes [30] or a reduced INF alpha/beta expression, as demonstrated with vesicular stomatitis virus and mosquito saliva [31].

Nonetheless, the use of *Culicoides* to perform experimental challenges remains highly limited by practical constraints: to date besides *C. nubeculosus, C. riethi* and *C. sonorensis* no other *Culicoides* species were successfully established as lab-adapted colonies [32,33], the alternative being insects caught in the wild. In addition, prior to the infectious challenge on the ruminant host the infection of culicoides is particularly tricky given the size of the insect and the exact amount of virus delivered to each ruminant cannot be known.

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

2.4. Beware of contaminations!

RNA viruses experimental challenges history is scarred with incidents of contamination of biological samples. For instance BTV modified live vaccines or inocula were reported to be contaminated in several occurrences [34-40].

So far literature does not report experimental infections with a SBV inoculum that was contaminated by another virus belonging to the same or a different family. Broadly speaking contamination routes are most likely related to i) laboratory contamination during sample preparation or ii) natural multiple infection of the original donor animal [41]. Given the potential dramatic consequences of such contamination incidents, inocula should indeed be

tested for major pathogens affecting the host species used in challenge experiments. Extensive screening could however be considered on a case-by-case basis.

3. Placental crossing and teratogenesis

Peribunyaviridae is a family of viruses able to cross the placental barrier, infect the foetus and potentially cause teratogenic effects in the central nervous system or muskuloskeltetal defects (arthrogryposis) [42]. It is generally considered that the age of the foetus (in gestational age) is a key criterion to determine the extent of the congenital defects as differentiating nervous tissues are important targets for SBV: usually the younger the foetus, the more severe the lesions [42].

However, to colonize the foetus viruses need a way in; therefore it is considered that SBV *in utero* infection can only occur once the first placentomes are established, around day 30 of pregnancy in cattle and slightly earlier in sheep [43-45].

Small ruminants and cow have slightly different definitive placental structure. In 1909 Grosser classified the mammalian placentas according to the number of tissue layers between foetal and maternal blood after implantation. The ruminants were considered to have a syndesmochorial placenta, *i.e.* where the uterine epithelium is removed and the chorion is in contact with the maternal connective tissue [46]. Other authors reclassified it as epitheliochorial since the uterine epithelium subsisted. From these historical and oversimplified categories, the ruminant placenta was later considered to be characterized by the migration of the foetal chorionic binucleate cells and their fusion with the uterine epithelial cells. The ruminant placenta is a mix between the syndesmochorial and epitheliochorial placentation and the uterine epithelium subsists as a fetomaternal syncytium [47]. In small ruminants more than 95 % of the placenta forms a fetomaternal syncytium. In cow multinucleate cells appear only transiently once the uterine epithelium has regrown after implantation. This type of placenta is called synepitheliochorial [48].

At implantation several changes occur: the papillae in the uterine glands immobilize the conceptus and it starts to elongate (cow: 15 days post coitum (dpc); sheep: 13-16 dpc). Subsequently the cells of the trophectoderm and the uterine epithelium get interdigitated, binucleate cells start to be seen. Then binucleate cells start to differentiate and to migrate (cow: 20-22 dpc; sheep: 16-18 dpc). Foetal villi develop in the caruncular areas starting at 24-26 dpc in small ruminants and 28-30 dpc in cow, thus defining the end of the implantation and the start of the placental development [49]. Table 2 summarizes some of the essential events during the prenatal development in cattle and sheep.

In a previous study we challenged pregnant ewes with SBV at 45 and 60 days of pregnancy [50]. We reported the birth of 22 alive lambs amongst none had any anti-SBV neutralizing antibodies prior to colostrum intake.

Thus, the experimental infection took place within the critical timeframe, between 30 and 70 days of pregnancy for sheep (Figure 1, [45]). The prenatal period can be divided into four main periods: i) fertilization; ii) blastogenesis; iii) embryogenesis and iv) fetogenesis [51]. The embryo sprouts and develops tissues and organ structures from the three original germ layers (ecto-, meso-, and endoderm). By the end of the embryogenesis the conceptus became a "miniature" version of the adult animal, displaying all its specific features. Once the organs are differentiated the embryo becomes a fetus [52]. The fetal phase is characterized by a fast growth of the conceptus. In cattle and sheep the fetogenesis starts around 45 and 38 dpc, respectively [53]. Therefore, the critical timeframe for SBV infection overlaps the end of the embryo stage and the beginning of the fetal stage. Moreover, although in ruminants ɣ-globulins are unable to go through the placental barrier from the mother to the foetus it is admitted that sheep fetuses become sequentially and increasingly immunocompetent to a larger variety of antigens throughout the pregnancy [54,55]. The critical timeframe for SBV infection spans over the

course of several important events in immune system development (Table 2). Although the sequence of antigens to be successively and progressively recognized by the fetal ruminant through pregnancy seems to be quite conserved between individuals, these antigens can be recognized starting with a difference of a few days between individuals [56]. This individual variability could explain the findings of malformed lambs that were found either SBV viropositive or vironegative with or without SBV antibodies, suggesting the possibility of an *in utero* clearance of the virus. Moreover, most of the malformed calves that were negative in both SBV antibodies and RTqPCR were born from seropositive mothers [57].

The range of teratogenic lesions, congenital defects and other reproductive disorders caused by SBV is summarized in Table 3, along with malformations and nervous lesions induced by some of the most common viruses inducing such lesions in ruminants.

In our experiments timing of inoculation was optimal to achieve transplacental infection of the fetus regarding data available from the literature, yet no malformations could be seen. No antibodies against the virus used to infect the mothers could be detected as well. These striking results might even question the success of the infection, notwithstanding the positive RNA detection in the mothers. Our report of lesions and serology results similar to another experiment on goats [58], and in the detection of SBV nucleic acids in organs of several lambs and many extraembryonic structures [50] provide support to an actual transplacental infection. In addition, in another study [59] we managed to isolate SBV from fetal envelopes in the animals at birth, thus 90 and 105 days post infection. The very low ratio of precolostral seroconversion in immunocompetent fetuses was also confirmed following the infection of pregnant cattle with SBV [60].

SBV vertical transmission rate seems to be lower when compared for instance to BTV, especially in cattle [61]. The rate of malformations caused by SBV was reported to be about 0.5 % in cattle [62] although the rate of intrauterine infection – based on serological results of the

calves prior to colostrum intake – was reported to be about 28 % [43]. Other authors documented field data about congenital malformations affecting 3 % of the calves but 8-10 % of the lambs in farms at the beginning of the SBV epizootic [63,64]. In Belgium based on a survey targeting farmers we also found an estimated 10 % of malformed sheep in SBV positive flocks [65].

4. Final thoughts and prospects

The number of ruminants used in experimental infections is chosen based on welfare and statistical concerns but also quite unfortunately on economic and practical grounds [7]. We performed our experimental infections with SBV in the BSL2+/BSL3 facilities of Sciensano (Ukkel, Belgium) depending on the phase of the experiment. To date, the Belgian Service of Biosafety and Biotechnology as well as the Belgian law do not assign a particular pathogen class for SBV. Our biosafety measures for SBV were based on the analogy with AKAV, also classified as a class 3 pathogen [66]. Domestic ruminants being herd animals, they need to be housed in groups or at least not individually. Euthanasia methods have to be the most humane as possible and clear end points have to be defined. Given the scarcity of clinical signs caused by SBV in the field and the individual variations in the response to the infection the number of animals to be included has to be chosen very carefully to comply with the Reduction objective (Three Rs concept) but has to be sufficient to limit the risk of not being able to provide useful data in the context of the ongoing scientific investigation. This is particularly difficult for experimental infection of pregnant ruminants with low malformation rates following transplacental transmission.

The most objective parameter to assess a vaccine efficacy against a virus and especially a RNA vector-borne virus is the evaluation of the viral RNA detection by RTqPCR in the host target [67]. SBV virulence was demonstrated to vary depending on the ruminant

host whether it is cattle, sheep or goat. In addition, pregnancy length differs between cattle and small ruminants while the placentation and the development of the fetal envelopes present slight differences [68]. Consequently, to study any of the specific aspects related to a ruminant species there are no other animal models or any alternative able to mimic the natural situation in a proper way [7].

According to our experiments, the subcutaneous route with an inoculum passaged a limited number of times on cell culture seems to represent the best compromise between a high probability to reproduce an infection similar to what happens in the field and logistics concerning the preparation/storage/management of the inoculum. To prevent the loss of viral variability and limit the risks of attenuation, SBV could benefit from an isolation on the highly susceptible SK-6 cell line [18]. Screening for concomitant pathogens should be considered on a case by case basis, if required. The dose should be chosen based on literature data yet no advantage is provided by inoculating a massive viral load.

Since some data from other authors suggest a better reproduction of the diseases with intradermal inoculation, it could be further investigated, especially if more user-friendly devices would be available. A breakthrough would be the successful adaptation of a colony of Palearctic SBV vector *Culicoides* species (*C. obsoletus/scoticus, pulicaris*) to laboratory conditions and subsequent use in infectious challenges.

In conclusion, targeting ruminant host species in experimental infections especially with BSL3 *Culicoides* borne pathogens is very expensive, time consuming, subject to stringent animal welfare constraints and critical sample size analysis to meet optimal statistical requirements. However, ruminant model remains unavoidable to assess the disease impact and to study the pathogenesis of emerging vector-borne viruses.

5. References

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6. Figure Captions

Figure 1 : *In utero* potential SBV induced defects following infection of the pregnant dams along the whole gestation time for cattle (A) and small ruminants (B) [69,45,50,70,60]

7. Table Captions

Table 1. Inocula characteristics used in five recent experimental infection studies on SBV (as searched on PubMed with keywords “experimental infection Schmallenberg”)

Table 2. Key events in sheep and cow embryos/foetuses with particular emphasis on nervous and immune systems. Compiled from [71-73,53,74-76]

Table 3. Summary of some of the most common central nervous and musculoskeletal lesions following in utero infection with bovine virus diarrhea virus (BVDV), SBV, BTV, Akabane virus (AKAV), or Aino virus (AV). Adapted from [77].

8. Tables

Table 1:

Virus	Type of inoculum	Host Species	Cell type	Nb of passages	Inoculation route	Volume (ml)	Titre (TCID50/ml)	Ref
SBV	Cell-passaged	ECE	KC+BHK-21+HmLu-1	1+5+2	Yolk sac	0.2	5x10^2-5x10^6.4	Collins et al., 2018
SBV	Serum (cattle)	Goats	/	/	SC	1	/	Laloy et al., 2017
SBV	Cell-passaged	IFNAR mice	KC+BHK-21	1+1	SC	0.1	10^3	Tauscher et al., 2017
SBV	Cell-passaged	IFNAR mice	KC+BHK-22	1+2	SC	0.1	10^8	Boshra et al., 2017
SBV	Cell-passaged	Sheep	KC+BHK-21	1+1	SC	1	2x10^3	Poskin et al., 2015

Table 2:

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

Table 3:

Lesion	**Definition**	**BVDV**	**SBV**	**BTV**	**AKAV/AV**
Hydranencephaly	Extensive loss of cerebral tissue with replacement by clear fluid	+	+	+	+
Porencephaly	Cystic fluid filled cavities in the brain tissue	+	+	+	+
Hydrocephalus	Dilation of the lateral ventricles by cerebrospinal fluid	+	+	+	-
Microencephaly	Reduced size of the cerebrum	+	+	+	+
Cerebellar hypoplasia	Reduced size of the cerebellum	+	+	+	

Kyphosis	Dorsal vertebral column curvature	-	+	-	-
Lordosis	Ventral vertebral column curvature	-	+	-	-
Scoliosis	Lateral vertebral column curvature	-	+	-	-
Torticollis	Twisted cervical vertebral column curvature	-	+	-	-
Arthrogryposis	Joint contraction of the limbs	-	+	+/-	+