SCIENTIFIC REPORT OF EFSA

Schmallenberg virus: State of Art[1]

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ABSTRACT

This scientific report provides an overview of all research carried out on Schmallenberg virus (SBV), reviewing the current knowledge on SBV regarding genotyping findings, susceptible species, pathogenesis, transmission routes, immunity, seroprevalence, geographical and temporal SBV spread, improved within-herd transmission model, SBV impact assessment and within-herd and regional spread models. Metagenomic analysis identified SBV as a novel orthobunyavirus emerged in 2011 and it has been detected in domestic cattle, sheep, goats and 12 wild species. Seroprevalence studies indicate that SBV has probably spread over the whole of Europe, showing high seroprevalence at national scale, while larger variability is observed at regional scales. Clinical disease frequency is low and experimental infection on pregnant ewes and cows suggest that SBV rarely induces malformations. SBV may be detected from semen with a low frequency though there is no scientific evidence of transmission through insemination. Vector competence studies suggest that Culicoides are likely to be able to transmit SBV but found no evidence that mosquitoes are likely to be able to transmit it. SBV vertical transmission has not yet been identified as a major route. SBV has successfully overwintered, despite lengthy period of minimal vector activity and duration of immunity in cattle lasts for at least one year. A farm-to-farm spread model for SBV shows a rapid spread of infection across the study region and latent period, duration of viraemia, probability of transmission from host to vector and virus replication are sufficient to account for the rapid SBV spread. The between-farm SBV transmission model indicates that the application of movement restrictions has little effect on SBV spread. An impact assessment based on limited data suggests a probable effect of SBV infection on abortion, short gestation, non-return and the number of artificial inseminations required per animal. International trade restrictions by third countries represent the main SBV impact.

KEY WORDS

Schmallenberg virus (SBV), impact assessment, disease spread model

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SUMMARY

This scientific report provides an overview of research carried out on Schmallenberg virus in the different Member States (MS), with special attention given to research co-financed by the European Commission, focusing in particular on three main research lines:

- A review of the current knowledge of SBV regarding:
  - Genetic analysis findings
  - Susceptible species reported
  - Pathogenesis, covering viraemic and susceptible periods
  - Potential transmission routes, discussing horizontal, vertical and vector-borne transmission as well as the ability of each to explain overwintering
  - Duration of immunity
  - Findings from seroprevalence studies conducted in different MS.
- The use of transmission models to evaluate geographical as well as temporal spread of SBV, specifically:
  - a within-farm transmission model using the large scale seroprevalence studies from Belgium and the Netherlands to estimate within-herd transmission parameters
  - a network model describing regional spread and the potential impact of animal movement restrictions SBV spread
  - A modified continental spread model similar to that presented in the previous scientific report but exploring a broader range of possible transmission kernels.
- Summarizing SBV impact assessment carried out in several MS.

Metagenomic analysis of animal material allowed the rapid identification of SBV, a newly discovered orthobunyavirus related to viruses in the Simbu serogroup, as the cause of the new disease that emerged in 2011. The availability of the (almost) complete nucleotide sequence of the SBV genome enabled a PCR test for SBV to be developed and distributed throughout Europe. It also contributes to the establishment of reverse genetic systems (Elliott *et. al.*, 2013; Varela *et. al.*, 2013) that will facilitate further research on SBV molecular biology, pathogenesis and vaccine development. The genome sequencing also highlighted the need for wide-scale sequencing studies on orthobunyaviruses in general as this would have helped to more quickly understand the relationship between SBV and extant Simbu serogroup viruses as well as the origin of SBV.

SBV RNA or antibodies have been detected in domestic cattle, sheep and goats and also in another 12 wild species: Alpacas, Anatolian water buffalo, Elk, Bison, Red deer, Fallow deer, Roe deer, Sika deer, Muntjac, Chamois, Wild boar and Dogs, as well as in 19 zoo species. The seroprevalence studies in cattle, sheep and goats indicate that SBV has probably spread over the whole of Europe. According to the seroprevalence studies conducted at national scale, prevalence at animal and herd levels were in general high, while for the regional studies a larger variability was observed.

The number of herds with SBV confirmed AHS (arthrogryposis hydranencephaly syndrome) cases compared to the level of infection indicated by seroprevalence studies, suggest that the frequency of clinical disease is low. SBV induces malformed calves only in a very limited number of cases, as demonstrated by experimental infection studies on pregnant cows and ewes. Although these resulted in only one malformed calf out of a total of 24 foetuses from a cow inoculated at day 90 of pregnancy, the presence of viral RNA could be demonstrated in the placenta of some ewes. The proportion of positive placenta and foetuses was higher in the group of ewes infected at day 45 of pregnancy compared to the ewes infected at day 38 of pregnancy in one experiment and at day 60 compared to day 45 in the other experiment. From these studies it can be concluded that SBV infection leads only in a very limited number of cases to malformation even when the experimental infection is performed during the susceptible period.

Limited numbers of articles have studied the risks of transmission of these viruses via semen and embryos. Recent data indicate that SBV may be detected in semen samples with a low frequency (<

6 %). However, there is no scientific evidence of transmission through insemination. This is in agreement with epidemiological data, indicating that the vector transmission remains the principal route explaining the dissemination of such viruses. Details are given below.

Phylogenetic relations of SBV with viruses of the Simbu serogroup led to suspicion that SBV was transmitted by *Culicoides*. Following detection of the SBV incursion, vector competence assays were performed on colonized mosquitoes and both colonized and field collected *Culicoides* (Veronesi *et. al.*, 2013b; Balenghien *et. al.*, 2014). These studies confirmed that several *Culicoides* species are likely to be capable of transmitting SBV but provided no evidence that the mosquito species studied are likely to be able to act as vectors. Viral RNA presence was also assessed in field collected *Culicoides* from farms in the affected regions. Studies in Belgium, Netherlands and France (De Regge *et. al.*, 2012; Elbers *et. al.*, 2013a; Balenghien *et. al.*, 2014) also suggest a high probability that *C. obsoletus*, *C. scoticus* and *C. chiopterus* have a role as vectors of SBV in northern Europe. *C. dewulfi*, *C. pulicaris*, *C. nubeculosus* and *C. punctatus* have also been implicated as suspected vectors in Belgium, France or Poland (De Regge *et. al.*, 2012; Larska *et. al.*, 2013; Balenghien *et. al.*, 2014), although quantities of SBV RNA detected were equivocal in defining the level of dissemination that had occurred (Veronesi *et. al.*, 2013b). Studies of *C. imicola* in Sardinia failed to convincingly implicate this species in SBV transmission (Balenghien *et. al.*, 2014). Vector competence studies currently being conducted in Italy will indicate the competence of *C. imicola* for SBV. Taken in their entirety, these studies convincingly implicated a range of widespread and abundant farm-associated *Culicoides* species in the transmission of SBV, including at least the species *C. obsoletus*, *C. scoticus* and *C. chiopterus*.

There is no evidence yet that vertical transmission is a major route of transmission of SBV. SBV has been detected in certain tissues of clinically-affected newborn calves, kids and lambs but neither SBV virus nor RNA has been documented in their blood. There is therefore currently no evidence to suggest that clinically affected newborns represent a viable source of virus for vectors. There is limited evidence for the transmission of SBV to progeny *Culicoides*.

SBV has successfully overwintered, despite lengthy period of minimal vector activity. The mechanism is unknown at present; however vertical transmission in host or vector may play a role. Evidence of persistent infection in the host has not been yet documented.

There are only limited data on duration of immunity in cattle and none on the duration of immunity in sheep. The data for cattle suggest that immunity lasts for at least one year following natural infection. Data on immunity over longer periods is not yet available.

A model for the farm to farm spread of a vector-borne virus parameterized for SBV show a rapid spread of infection across the study region. Changes to four epidemiological parameters (latent period, duration of viraemia, probability of transmission from host to vector and virus replication) are sufficient to account for the rapid SBV spread within and between farms relative to that seen for BTV-8. This suggests that alternative transmission mechanisms (for example, direct transmission or additional vector species) are not necessary to explain the observed patterns of spread of SBV, though they may still play a minor role. The enhanced between-farm transmission of SBV brought about by these four parameters is such that the application of movement restrictions, even a total animal movement ban, would have little effect on the spread of SBV (relative reduction around 4 %).

The ability to estimate impact of Schmallenberg virus was restricted by the limited availability of data; studies conducted reported a probable effect of SBV infection on abortion, shorter gestation, non-return and the number of artificial inseminations required per animal. The principle economic impact of SBV has been felt via international trade restrictions, particularly in live animals and semen. Cattle semen trade has been restricted in several countries, in terms of percentage of total semen trade, most of the trades happens within the EU (2010: 73.4 % and 2011: 82.8 %). For the semen trade outside of the EU (2010: 26.6 % and 2011: 17.2 %), around 60 % of those are trade with countries imposing restrictions, representing for 2010 a 15.1 % of the total EU semen trade and for 2011 10.9 %. A decline between 11 and 26 % of the semen doses have been observed from previous years compared to 2012, as for the pure-bred breeding animals, the export value dropped 20 % in 2012 with respect to 2011 (http://www.adt.de/expla_fr.html).

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

The previous request sent to EFSA with reference SANCO/G2/FR/Ip (2012) 97796 re for technical assistance on Schmallenberg virus (SBV).

The reports issued by EFSA were commended in several occasions by the Commission and the Member States for their quality and timeliness. The Commission would like to convey the appreciation of the work done by EFSA's services and would like to state that the EU needs further support by EFSA in this matter.

The Commission and the Member States recognise the importance for the EU to continue in its transparency policy and EFSA has a major role to play in this respect.

The Commission wishes that EFSA provides regular updates, becoming *de facto* the showcase for the entire world on the evolution of the epidemiological situation on SBV in the EU.

Therefore, in the context of Article 31 of Regulation (EC) No. 178/2002, EFSA has been asked to continue providing scientific assistance to the Commission.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

EFSA is requested to deliver:

1. Continue to collect data through the EFSA Data Collection Framework (DCF) from Member States in a structured manner in coordination with DG SANCO. This should allow for updates of EFSA reports (three times per year) on the description of the epidemiological situation of SBV in the EU. This needs to be done keeping the possibility to use it for further risk assessment. A first update should be produced by 15 November 2012. A second report on 31/5/2013 and a third on 1/12/13.
2. An update of the report on the overall assessment of the impact of this infection on animal health, animal production and animal welfare. The intent would be to fill the data gaps identified in the EFSA May 2012 report and to allow for completing the assessment of the impact, specially the within-herd impact. The report should also take in account the latest scientific findings on SBV, especially studies co-financed by the EU[4] providing a comprehensive report on the state of art of the scientific knowledge. Notably this should track the research initiatives going on in several Member States, with a note of attention for the new data to be provided on the traded commodities and their risk of transmitting the infection. A report should be produced by 1 December 2013.

CONTEXT OF THE SCIENTIFIC OUTPUT

This scientific report provides a state of art summary description of the research conducted on SBV in the different MS. It also provides a re-assessment of SBV spread model parameters, as well as an alternative model to assess potential effect of animal movement restrictions as control measures.

The scientific provides summaries regarding three main research outputs concerning SBV in Europe to answer TOR 2:

- A review of the current knowledge on SBV regarding:
  - Genetic analysis findings
  - Susceptible species reported
  - Pathogenesis
  - Transmission routes
  - Immunity
  - Seroprevalence studies
- Geographical and temporal spread of SBV:
  - Fine tuning within herd transmission parameters
  - A network model to describe regional spread
  - Continental spread model revisited
- Summary of SBV impact assessment carried out in several Member States

[4] Commission Implementing Decision 2012/349/EU

RESEARCH FINDINGS: SCHMALLENBERG STATE OF ART

1. Schmallenberg Virus - Genotyping Findings

1.1. Genetic Analysis of SBV

More than 170 named virus isolates comprise the genus *Orthobunyavirus* in the family *Bunyaviridae*. Distinguishing features of orthobunyaviruses are the pattern of sizes of genomic RNA segments, the pattern of sizes of the structural proteins and the consensus nucleotide sequences at the 3' and 5' termini of the viral RNA segments (Elliott and Blakqori, 2011). Orthobunyaviruses are conveniently divided into 18 serogroups on the basis of complement fixation (CF), neutralisation (NT) and haemagglutination inhibition (HI) assays. While this serological classification of viruses proved convenient, it did produce anomalies due to the propensity for genome segment reassortment, where different serological tests reflected antigenic relationships of proteins encoded by different genome segments e.g. CF is mediated by the S segment-encoded nucleocapsid protein whereas NT and HI antibodies are directed against the M segment encoded glycoproteins. The International Committee for the Taxonomy of Viruses has defined 48 species with the *Orthobunyavirus* genus (Plyusnin *et. al.*, 2012) (In virus taxonomy, a species is described as "a monophyletic group of viruses whose properties can be distinguished from those of other species by multiple criteria"; http://www.ictvonline.org/codeOfVirusClassification.asp). For orthobunyaviruses, species demarcation is based on serological criteria (cross-NT and cross-HI tests), the inability of one species to genetically reassort with another species, and that the amino acid sequences of the nucleocapsid protein of different species differ by more than 10 %, but such classification has to be considered fluid due the general paucity of molecular details of most orthobunyaviruses.

According to the ICTV, a virus belongs to a serogroup if it cross-reacts with members of that group by one or more serological tests (Nichol *et. al.*, 2005). Previous studies of Simbu group viruses have demonstrated extensive cross reactivity through CF tests.

The identification of SBV was based on metagenomic analysis of pooled blood samples from acute infected cattle; comparison of the obtained sequences indicated that the closest relatives were viruses in the Simbu serogroup (Hoffmann *et. al.*, 2012). The Simbu serogroup contains 23 viruses that have been divided among 8 species (Table 1.1), some of which are associated with disease in ruminants, while Oropouche virus causes a severe febrile illness in man. Based on the available sequences in the database at that time, SBV sequences showed 69 % identity with Akabane virus L segment, 71 % identity with Aino virus M segment and 97 % identity with Shamonda virus S segment (Hoffmann *et. al.*, 2012). When sequences of more Simbu group viruses were determined, it was reported that the M segment of the Sathuperi and Douglas orthobunyaviruses displayed higher identity with SBV whereas the S and L segments were closer to Shamonda virus, suggesting that SBV was a reassortant virus between Sathuperi and Shamonda viruses (Yanase *et. al.*, 2012).

Subsequently, near complete genome sequences were determined for Aino, Douglas, Peaton, Sabo, Sango, Sathuperi, Shamonda, Shuni, and Simbu viruses. Phylogenetic analysis of these sequences suggested SBV belongs to the Sathuperi virus species, and further that SBV is an ancestor of Shamonda virus, which in turn is a reassortant containing the S and L segments from SBV and the M segment from an unknown virus (Goller *et. al.*, 2012). In addition, it was shown that anti-SBV serum neutralised Douglas and Sathuperi viruses, but not Shamonda virus. While these studies have further defined the relationship of SBV to other Simbu serogroup viruses, they do not help in identifying the origin of SBV. Future research requires more nucleotide sequence analysis of the remaining Simbu serogroup viruses and of other isolates of these viruses from different geographical locations.

Table 1.1: Simbu serogroup viruses.

Species	Virus	Distribution	Clinical Signs	Principal Arthropod Vector
Akabane	Akabane	Africa, Asia, Australia	+	Mosquitoes, *Culicoides* spp.
	Sabo	Africa		*Culicoides* spp.
	Tinaroo	Australia		*Culicoides* spp.
	Yaba-7	Africa		?
Manzanilla	Manzanilla	S America		?
	Buttonwillow	N America		*Culicoides* spp.
	Ingwavuma	Africa, Asia	+	mosquitoes
	Inini	S America		?
	Mermet	N America		mosquitoes
Oropouche	Oropouche	S America		*Culicoides* spp, mosquitoes
	Facey's Paddock	Australia		?
	Utinga	S America		?
	Utive	S America		?
Sathuperi	Sathuperi	Africa, Asia	+	*Culicoides* spp, mosquitoes
	Douglas	Australia		*Culicoides* spp.
Simbu	Simbu	Africa		mosquitoes
Shamonda	Shamonda	Africa, Asia	+	*Culicoides* spp.
	Peaton	Australia	+	*Culicoides* spp.
	Sango	Africa	+	*Culicoides* spp, mosquitoes
Shuni	Shuni	Africa		*Culicoides* spp. mosquitoes
	Aino	Asia, Australia	+	Mosquitoes, *Culicoides* spp.
	Kaikalur	Asia, Australia		mosquitoes
Thimiri	Thimiri	Africa, Asia		?

It is not known how SBV was introduced into Europe. One hypothesis is that the introduction of SBV, and indeed other viruses such as BTV 8, could be via infected Culicoides transported into Europe. In this regard, detection, isolation and characterisation of Simbu serogroup viruses in other regions in the world are needed to determine whether a virus closely related to SBV circulates in a particular region. This would be a starting point to investigate possible routes of introduction. On the other hand in Turkey, Azkur *et. al.*, (2013) reported that antibodies to SBV were detected in serum samples collected from slaughterhouses between 2006 and 2013, suggesting that SBV (or a similar virus) may have been present before its first detection in Germany. However, these authors used an commercial ELISA which recognise also antibodies against other orthobunyaviruses and they did not further analyse the positive samples in seroneutralisation assay for confirmation. Further characterisation of these samples is needed.

1.2. Evolution of SBV

Bunyaviruses can evolve through two mechanisms, the accumulation of mutations because the viral RNA-dependent RNA polymerase is known to be error-prone as it lacks a proofreading ability, and genome segment re-assortment which results in more dramatic antigenic changes. Studies investigating SBV isolates have reported high levels of variability, especially in the M segment. , but no correlation was found between host and geographical location of the variants found (Hulst *et. al.*, 2013, Rosseel *et. al.*, 2012). Two studies have reported a hypervariable mutation within the coding

sequence for the N-terminus of Gc glycoprotein, and have suggested that this may play a role in immune evasion (Coupeau *et. al.*, 2013, Fischer *et. al.*, 2013).

Two inactivated SBV vaccines have been approved in Europe, Bovilis SBV (MSD Animal Health) and SBVvax (Merial), which are reported to elicit neutralising antibodies within three weeks of inoculation though the duration of immunity has not been determined. The impact of genetic variation within SBV isolates on protection by these two vaccines requires assessment. The biological significance of hypervariable region in Gc (glycoprotein) also requires study, both in ruminants and in vector species.

Reassortment is restricted to closely related bunyaviruses, and even then certain combinations of viruses appear genetically incompatible. The lack of other Simbu serogroup viruses in Europe suggests that reassortment will not be of immediate concern. However, the introduction of another Simbu group virus may give opportunity of reassortment in the future, and reassorted bunyaviruses have been shown to have different vector specificities and virulence properties. Enhanced surveillance is recommended.

2. Susceptible Species

A susceptible species is an animal species that can support replication of an agent. A susceptible species could be an animal species in which infection by a disease agent has been demonstrated by natural cases or by experimental infection that mimics the natural pathways. A reservoir host is one in which an infectious agent normally lives and multiplies and is therefore a common source of infection to other animals (Thrushfield M. 1995)

Regarding SBV and the identification of susceptible species the information available can be summarised in different categories:

1. Animal species where the agent (SBV) and clinical expression (either in adult animals or their offspring) of the disease have been demonstrated either by direct or indirect detection.
    a. Animal species infected naturally: Domestic Cattle, Sheep, Goats
    b. Animal species infected experimentally: Domestic Cattle, Sheep, Goats
2. Animal species where the infectious agent (SBV) has been detected (direct detection of the pathogen): Dog.
3. Animal species where a serological reaction to the agent (SBV) has been demonstrated (indirect detection of the pathogen): Alpacas, Anatolian water buffalo, Elk, Bison, Red deer, Fallow deer, Roe deer, Muntjac, Chamois, Dog

The evidence available regarding species other than domestic cattle, sheep and goats is summarised in Annex B. Other species such as horses and llamas (EFSA, 2013a) have been tested but not confirmed by serological testing.

The three publications related to SBV in domestic dogs present conflicting evidence. One is a case report of a seropositive dog with no clinical signs (Wensman *et. al.*, 2013), a second reports detection of viral RNA by RT-PCR in the cerebellum of an animal showing neurological signs (Saileau *et. al.*, 2013) and the third (Garigliany *et. al.*, 2013) reports the results of testing of a group of animals likely to have been exposed to the virus, but where only one tested inconclusive for SBV specific antibodies. With the available evidence it is impossible to draw definite conclusions regarding the susceptibility of domestic dogs to SBV infection.

Experimental infections in pigs (Poskin *et. al.*, 2014) and poultry (EC, 2014) have been performed and results indicate that virus replication does not occur in these species. Also, a study conducted on South American camelids (SAC) in Germany showed high seroprevalence at animal (62.4 %) and herds (92.4 %) levels, but no SVB-RNA was detected which might be linked to the short-time viraemia. Although 3 malformed SAC crias were reported, SBV infection could not be confirmed to be the cause of malformations.

In addition, the role of wildlife was studied in Germany, France, the Netherlands and United Kingdom (EC, 2014), showing seropositive results for: Moufflons, Roe deer, Fallow deer, Red deer, Sika deer,

Wild boar. Moreover, samples from 38 different species in two zoos in United Kingdom were tested for SBV (using competitive ELISA) for which 19 resulted in seropositive results (Bongo, Babirusa, Banteng, Congo buffalo, European bison, Gaur, Gemsbok, Greater kudu, Grevy's zebra, Moose, Nile lechwe, Nubian goat, Onager, P.S. deer, Reindeer, Roan antelope, Scimitar-horned oryx, Sitatunga and Yak). These studies showed that horses, mice and wild carnivores might not play a role as reservoir in the epidemiology of SBV.

3. Pathogenesis

3.1. Viraemic Period

When the first cases of acute Schmallenberg virus infections were observed, symptoms of milk drop, diarrhoea and fever were reported. Most of these symptoms were observed only during a short period (few days). These field observations of an apparently short viraemic period were confirmed by the first experimental infections conducted by the researchers of FLI (Hoffmann *et. al.*, 2012). In this experiment 2 calves were inoculated with blood samples originating from PCR positive cows for SBV (1 animal subcutaneously and 1 animal intravenously) and one calf with an on KC cell isolated SBV strains. Independent of the inoculation route, the inoculated animals became infected and had positive PCR results from 2–5 days post inoculation and one animal developed fever (a temperature of 40.5 °C) four days post infection.

Shortly after this first experiment, the same research group confirmed these first results after a second experimental infection study (Wernike *et. al.*, 2013a) and nearly the same results were obtained: 2 days after inoculation the animals became PCR-positive and stayed PCR-positive until 6 days post infection.

Following the observations of Poskin *et. al.*, 2014 there is no dose dependent difference in the duration and level of RNAemia after experimental infections of sheep. These authors inoculated different groups of sheep with different dilutions of SBV infectious serum and followed the RNAemia until 10 days post inoculation. In contrast, the inoculation dose had an effect on the number of animals that became infected in each group.

Although a short RNAamic period was observed after experimental inoculation of infectious serum, viral RNA could be detected in lymph nodes, particularly in the mesenteric lymph node, and spleen samples taken at autopsy. This viral RNA could be detected until 44 post-inoculation days of adults' sheep indicating a possible persistence in the lymphoreticular system (Wernike *et. al.*, 2013b). Identical observations have been reported recently in cattle also (Wernike *et. al.*, 2012 and Wernike *et. al.*, 2013a) and sheep (Poskin *et. al.*, 2014, paper accepted). It is not yet known if the presence of viral RNA in the lymphoreticular system plays a role in the pathogenesis of the virus.

In contrast to the high similarity of results obtained after experimental infections of sheep and cattle, a slightly different RNAemia pattern was obtained from field observations. In a study conducted by Claine *et. al.*, (2013) fifty female lambs born in autumn 2011 and January 2012 were investigated by analyzing bimonthly blood samples collected during April–October 2012. During this field trial, the SBV infection was observed by qPCR positive results around mid-July and ended in mid-October 2012 and all of the animals became positive. Against all expectations, ten lambs tested positive in two samplings two weeks apart. This unexpected finding indicates that the duration of viraemia in sheep (assessed as positive RT-qPCR result) may be longer after natural SBV infection in comparison to experimental SBV infection in cattle and sheep. Unfortunately, these results are the only evidence about the viraemic period of SBV infections under field conditions.

3.2. Gestation Susceptible Periods

Experimental infections of pregnant ewes were performed at CVI Lelystad and CODA-CERVA Brussels and of pregnant cows at FLI Isle of Riems (EC, 2014). Inoculations of pregnant goats have been performed at ANSES and LNCR, Maisons-Alfort but these experiments are still on going. The

analyses of all data obtained from these experiments are till now not finished and no published data are available today.

From the CVI experiment preliminary results were presented by N. Stockhofe-Zurwieden during the 7th Epizone meeting (1st-4th of October 2013, Brussels), it was demonstrated that an SBV infection leads to infection of the placenta in most of the inoculated animals and a successive infection of the umbilical cord and the CNS in some of the foetuses (EC, 2014). The proportion of positive placentas was higher in the group of pregnant ewes inoculated at day 45 of pregnancy than in the group inoculated at day 38.

During the same Epizone meeting FLI presented the results from the experimental infection of pregnant cows. Four groups of 6 pregnant cows were inoculated with SBV infectious serum at four different time points during pregnancy (day 60-90-120-150). One month after inoculation the cows were euthanized and samples of mother and foetus analysed. Only one foetus coming from a pregnant cow inoculated at day 90 of pregnancy showed arthrogryposis and torticollis. The first results demonstrated a correlation between positivity found in the placenta and the foetus (EC, 2014).

At CODA-CERVA the experimental infection study was performed on three groups: i) group 1 with 8 ewes that were subcutaneously infected with infectious SBV serum at day 45 of gestation, ii) group 2 with 9 ewes that were infected at day 60 of gestation and iii) control group 3 that was mock inoculated. Ewes were kept till the end of gestation. When signs of birth became apparent, colostrum was collected, the ewes were anesthetized and a caesarean section was performed. The lambs were assessed for malformations or other aberrant clinical signs and their capability to stand up and drink milk was evaluated. After euthanasia, blood and tissue samples were collected for further analysis.

Only one lamb was born before the expected date and was in good health. It was able to drink colostrum from the mother and subsequently showed elevated anti-SBV antibody titters. Considering all groups in the study (control and infected groups) around 37 % of the lambs were dead at birth but showed no abnormalities (EC, 2014). All other lambs were born at term, no malformations were observed and they were able to stand up and showed a good suction reflex. No anti-SBV antibodies were detected in these lambs.

When organ tissues from control ewes and their lambs were tested by PCR for the presence of the SBV-S segment, all samples were negative. In both the groups infected at 45 and 60 days of gestation, maternal tissues like placenta and cotyledons of some ewes were positive. All other organs of the ewes were SBV negative. Statistical analysis on the final results will have to show if there was a statistical difference between the numbers of ewes positive for maternal tissues in both groups. Of all samples tested from the lambs of the ewes infected at 45 days of gestation, only 1 umbilical cord was positive. All other organs were negative. Of all samples tested from the lambs of the ewes infected at day 60 of gestation, 3 were positive in some tissue.

The results obtained from this experimental infection study demonstrate that infection of Mourerous sheep at day 45 and 60 of gestation did not induce malformations in the lambs and that only small amounts of SBV RNA could be found in some of the lambs at birth. Although a statistical results are not yet available, it seems that more positive samples were found in lambs originating from ewes that were infected at day 60 of gestation compared to day 45.

No typical symptoms were reported in sheep after natural infections (Hoffmann *et. al.*, 2012), although Wernike *et. al.*, (2013b) reported one single sheep out of 13 RNAemic animals with clinical signs for several days after an experimental infection.

From these studies it can be concluded that SBV infection leads only in a very limited number of cases to malformation (1 out of 24 fetuses) even when the experimental infection is performed during the period of susceptibility that the virus can reach the foetus (EC, 2014).

It is worth noting that other orthobunyaviruses have been associated with congenital defects in the offspring of ruminants. Just to mention a few, and without being exhaustive, it is known that Cache Valley virus (CVV), a member of the Bunyamwera serogroup, causes malformations in lambs in North America, and has also been associated with a few human cases, one of them fatal (de la Concha-Bermejillo, 2003). Another member of the Bunyamwera serogroup found in the Americas, Main Drain virus, is associated with encephalomyelitis in horses, but also causes congenital malformations in experimentally inoculated pregnant ewes (Edwards *et. al.,* 1997).

4. Transmission Routes

4.1. Vector Transmission: Role and Capacity to Spread the Disease

Formal criteria to recognise a species as a vector have been defined by the World Health Organization (WHO 1961). These are:

1) recovery of virus from wild-caught specimens free from visible blood;
2) demonstration of ability to become infected by feeding on a viraemic vertebrate host or an artificial substitute;
3) demonstration of the ability to transmit biologically by bite;
4) accumulation of field evidence confirming the significant association of the infected arthropods with the appropriate vertebrate population in which disease or infection is occurring.

A given species that fulfils only one of the criteria can be considered a suspected vector. A species that passes the test of natural infection and experimental transmission can be considered a potential vector, whereas a species that fulfils all the conditions can be considered a confirmed vector (WHO 1967).

Initial phylogenetic studies placed SBV in the Simbu serogroup, sharing a close relationship to Sathuperi and Douglas viruses and secondarily to Shamonda virus and included in the same lineage as Akabane virus (Saeed, 2001; Goller, 2012, see section 1). These viruses have been primarily isolated from *Culicoides* (Table 1.1) (Doherty *et. al.*, 1972; St George *et. al.,* 1978; Lee 1979; Cybinski 1984; Blackburn *et. al.*, 1985; Kurogi *et. al.*, 1987; Yanase *et. al.,* 2005) and more rarely from mosquitoes (Dandawate *et. al.*, 1969; Metselaar *et. al.*, 1976). Moreover, it has been demonstrated that *Culicoides* were efficient experimental vectors for Akabane virus (Jennings *et. al.,* 1989), whereas attempts to demonstrate replication of this virus in mosquitoes have so far proved unsuccessful (Kay *et. al.,* 1975).

These findings and the recent emergence of the similarly *Culicoides*-borne bluetongue virus (BTV) in western and northern Europe (Mellor *et. al.,* 2009a), therefore led to immediate suspicion that SBV was transmitted by *Culicoides*. Following detection of the SBV incursion, vector competence assays were performed on colonized mosquitoes and both colonized and field collected *Culicoides* (Veronesi *et. al.,* 2013b; Balenghien *et. al.,* 2014). Virus RNA presence was also assessed in field collected *Culicoides* from farms in the affected regions (De Regge *et. al.*, 2012; Rasmussen *et. al.*, 2012; Elbers *et. al.*, 2013; Elbers *et. al.*, 2013a; Goffredo *et. al.*, 2013; Larska *et. al.*, 2013; Balenghien *et. al.*, 2014). Taken in their entirety, these studies convincingly implicated a range of widespread and abundant farm-associated *Culicoides* species in the transmission of SBV, including at least the species *Culicoides obsoletus*, *Culicoides scoticus* and *Culicoides chiopterus*.

About 1,250 *Culicoides* species are described worldwide and about 120 in Europe. Among these species, the most abundant species in non-Mediterranean Europe are *C. obsoletus* and *C. scoticus*, usually grouped into the Obsoletus complex. This complex dominates *Culicoides* collections in European farms, becoming less abundant or absent in high Scandinavian latitudes, in Mediterranean regions and at high altitudes. The Obsoletus complex is often associated with *Culicoides dewulfi* and *C. chiopterus* which are known to be abundant along the English Channel and the North Sea in France, England, and Netherlands, whereas these species become rare or absent in southern Europe. Finally *Culicoides impunctatus* and *Culicoides newsteadi* are very abundant species respectively in northern Europe, as in Scotland, and in the Mediterranean region.

The transmission of a virus by a biological vector is the process in which virus particles ingested with the blood meal infect the midgut cells, replicate, disseminate throughout the vector, infect the salivary glands and be transmitted via saliva during subsequent blood feeding. A midgut barrier to BTV infection has been described in *C. sonorensis*, which can limit the infection of midgut cells or the dissemination to target organs including salivary glands (Mellor *et. al.,* 2009b). Although salivary barriers have been described for several viruses in different mosquito species, these have yet to be identified in any species of *Culicoides*, suggesting that females with a fully disseminated infection would be able to transmit (Mellor *et. al.,* 2009b). Thus, the recovery of virus from saliva illustrates a transmissible infection; the recovery in head, legs or wings illustrates a disseminated infection, whereas the recovery in a pool of entire insects may indicate an infection limited to the midgut cells. Detection of viral DNA by rt-PCR assay prove the presence of viral genome segments, but not necessary the presence of infectious viral particles. Nevertheless, a comparison of C_t values obtained by a semi-quantitative rt-PCR assay and results of isolation of infectious BTV suggested that C_t values may be used to define if infection could be considered as transmissible (fully disseminated with infectious virus) or subtransmissible (not fully disseminated or without infectious virus) (Veronesi *et. al.,* 2013a). This approach may be applied to SBV infections (Veronesi *et. al.,* 2013b).

Intrathoracic inoculation of *Culex pipiens* and *Aedes albopictus* mosquitoes strongly suggested that SBV can replicate in individuals when introduced directly into the haemocoel, bypassing mid-gut barriers to arbovirus dissemination (Balenghien *et. al.,* 2014). Oral infection, however, did not result in C_t values indicative of full SBV dissemination in either mosquito species (Balenghien *et. al.,* 2014). Experimental studies on vector competence were also conducted in the Netherlands using *An. atroparvus* mosquitoes. The mosquitoes were blood-fed on SBV-infected animals and incubated at 25 °C. For up to five days post-infection SBV S-segment RNA was detectable via PCR from the heads of the insects but not the abdomens. This result therefore probably represents residual contamination after feeding rather than a disseminated infection (EC, 2014). While these results should be interpreted with caution as they utilised inbred colony lines, this study provides preliminary evidence that these mosquitoes may not play a substantial role in transmission of SBV in the field. Systematic studies to characterise biting rates of mosquito species on livestock in Europe, however, would be useful in understanding the role of this group to potentially transmit pathogens such as SBV.

Vector competence studies in *Culicoides nubeculosus* colony lines highlighted the ability of *Culicoides* to replicate SBV to transmission level after intrathoracic inoculation and oral exposure (Veronesi *et. al.,* 2013b; Balenghien *et. al.,* 2014). These studies have indicated low rates of competence of approximately 3 % for *C. nubeculosus* (Veronesi *et. al.,* 2013b; Balenghien *et. al.,* 2014), similar to rates assessed for BTV with this colony line (Veronesi *et. al.,* 2013a). It is important to note, however, that such infection rates have been demonstrated to vary with vector population for BTV-9 (0.4 to 7.4 % for Obsoletus complex from different geographic regions of the United Kingdom) or other *Culicoides*-borne arboviruses (Tabachnick 1996; Carpenter *et. al.,* 2006). *Culicoides nubeculosus* remains rare in light trap collections carried out across Europe suggesting a limited potential role in SBV transmission, but the abundance of this diurnal species may be under-estimated by light traps. Preliminary evidence was also provided that *C. scoticus* is able to replicate SBV to transmissible levels (Balenghien *et. al.,* 2014), albeit using a technique (pledglet feeding with sugar) that is likely to result in virus being transported to the crop rather than the gut (Jennings *et. al.,* 1988).

A detailed study of SBV replication and dissemination in the model species *Culicoides sonorensis* allowed determination of RNA levels in studies carried out on field collected midges that were likely to represent transmissible infections (Veronesi *et. al.,* 2013b). Studies in Belgium, Netherlands and France (De Regge *et. al.,* 2012; Elbers *et. al.,* 2013a; Balenghien *et. al.,* 2014) confirmed the role of *C. obsoletus*, *C. scoticus* and *C. chiopterus* as highly probable vectors of SBV in northern Europe, and especially *C. obsoletus*, which is among the most abundant livestock-associated species in the region (Meiswinkel *et. al.,* 2008; Carpenter *et. al.,* 2009; Venail *et. al.,* 2012) and its apparently ubiquitous distribution on farms across the Palaearctic and Nearctic may facilitate spread of SBV to new regions (Table 4.1). On the contrary, *C. dewulfi*, *C. pulicaris*, *C. nubeculosus* and *Culicoides punctatus* were

implicated as suspected vectors in Belgium, France or Poland (De Regge *et. al.*, 2012; Larska *et. al.*, 2013; Balenghien *et. al.*, 2014), although quantities of SBV RNA detected were equivocal in defining the level of dissemination that had occurred (Veronesi *et. al.*, 2013b).

Studies of *C. imicola* in Sardinia (Table 4.1) failed to convincingly implicate this species in SBV transmission through detection of SBV RNA (Balenghien *et. al.*, 2014), despite its well documented role in transmission of other *Culicoides*-borne arboviruses (Mellor *et. al.*, 2009b) and association with BTV outbreaks in Italy (Goffredo *et. al.*, 2003; Goffredo *et. al.*, 2004). The fact that *C. imicola* dominated the *Culicoides* fauna in Sardinia and especially at outbreak sites where only very limited numbers of the Obsoletus complex were present, however, indicates its probable involvement in transmission of SBV in Sardinia in 2012 (Balenghien *et. al.*, 2014). An absence of pools of *C. imicola* containing significant quantities of SBV RNA may have been due to the time of sampling, thus further screening within the distribution this species would be desirable to identify species involved in SBV transmission in Mediterranean region. The vector competence studies carried out currently in Italy would allow the assessment of the experimental competence of *C. imicola* against SBV.

The detection of RNA in field collected nulliparous females in Poland (Larska *et. al.*, 2013) was not sufficient to challenge the current statement of the absence of vertical transmission in virus/*Culicoides* model (Mellor *et. al.*, 2000), because the presence of viral RNA does not necessarily indicate the presence of infectious virus at a transmissible level (Veronesi *et. al.*, 2013a). Often vertical transmission rates are low (about 4 % for *Aedes*/dengue virus, and about 0.8 % for *Culex*/West Nile virus) and therefore statistically difficult to detect. It might be especially difficult to determine for European *Culicoides* as probable vector species have not been colonized and field collected individuals are difficult to feed on blood.

From 2011 to 2013, SBV has spread across a huge geographic area in Europe at a rate substantially exceeding that of the BTV-8 epidemic which occurred in the same region from 2006 to 2010 (Elbers *et. al.*, 2012; Meroc *et. al.*, 2013a,b). A partial explanation for this phenomenon could be the absence of animal movement restrictions, but this will be discussed in section 7. Additionally, however, it was hypothesised that the vector competence of *Culicoides* for SBV may exceed rates recorded for BTV either in the number of species capable of transmitting the virus or in the proportion of individuals within a species able to act as vectors. This hypothesis receives support from the fact that the related Akabane virus is isolated at a far higher frequency than BTV from *Culicoides* in Australia (St George *et. al.*, 1978), although comparative laboratory-based investigations of susceptibility rates in vector species have not been performed. The review of studies conducted to date found equivocal support for this hypothesis. Indeed, observed SBV infection rates (Table 4.1) were usually greater than those previously recorded during BTV-8 epidemic, but the proportion of *Culicoides* exposed to viraemic hosts within screened populations is unknown and in general the numbers of individuals and sites investigated in initial studies were low (De Regge *et. al.*, 2012; Rasmussen *et. al.*, 2012; Elbers *et. al.*, 2013a).

The most straightforward way to assess the true competence of populations is to carry out infection studies of field-collected *Culicoides* in the laboratory using either viraemic hosts or artificial means of feeding, as conducted for BTV (Jennings *et. al.*, 1988; Carpenter *et. al.*, 2006; Carpenter *et. al.*, 2008). As the timing of animal-based experiments in biosecure containment with population peaks in *Culicoides* is logistically challenging, pledgelet-based blood feeding methods are most commonly employed to assess infection rates. Membrane-based methods, such as those employed to feed colony *Culicoides* and mosquitoes in this study currently result in extremely poor rates of feeding in field collected *Culicoides* from northern Europe (Jennings *et. al.*, 1988; Venter *et. al.*, 2005). As it is known that pledgelet feeding significantly underestimates the proportion of competent *Culicoides* in a population (Venter *et. al.*, 2005) it is therefore vital that standard membrane-based techniques are developed for northern European species. Then, vector competence studies could be systematized with standardised protocols to test different field collected *Culicoides* populations against SBV.

Table 4.1: Published reports of Schmallenberg virus detection from field collected *Culicoides* in Belgium, Denmark, Netherlands, Italy, Poland and France using quantitative detection assays.

Country	Period	Pool constitution[a]	Species	No. midges (pools) tested	No. positive pools	Mean C_t value [min-max]	Minimum infection rate	Reference
Belgium	August to October 2011	25 heads (PF)	Obsoletus complex	688 (34)	5	33.9 [30.7-36.0]	0.73%	De Regge, 2012
			C. obsoletus	283 (32)	3	35.9 [34.9-36.5]	1.06%	
			C. scoticus	240 (27)	0			
			C. dewulfi	181 (20)	2	35.2 [32.2-38.1]	1.10%	
			C. chiopterus	227 (23)	1	28.7	0.44%	
			C. pulicaris	89 (11)	1	37.9	1.12%	
Denmark	October 2011	5 entire females	Obsoletus group	91	2	26.0 [25.0-27.6]	2.20%	Rasmussen, 2012
Netherlands	August to September 2011	10 heads (NF or PF)	Obsoletus complex	2,300 (230)	12	24.6 [19.6-36.0]	0.52%	Elbers, 2013
			C. obsoletus		1	24.6		
			C. scoticus		10	25.0 [19.6-36.0]		
			C. dewulfi	1,300 (130)	0			
			C. chiopterus	1,440 (144)	2	31.6 [27.9-35.4]	0.14%	
			C. punctatus	1,050 (105)	0			
	May to September 2012	50 entire females (PF or GF)	Obsoletus complex	2,100 (42)	2	36.3 [35.0-37.7]	0.10%	Elbers, 2013
			C. dewulfi	1,300 (26)	0			
			C. chiopterus	1,050 (21)	0			
			C. punctatus	1,550 (31)	0			
			C. pulicaris	500 (10)	0			
Italy	September to November 2011	< 50 entire females	Obsoletus complex	1,104	5	29.0 [26.0-33.0]	0.45%	Goffredo, 2013
	May 2012		Obsoletus complex	769	1	27.0	0.13%	
Italy[b]	October to December 2012	< 50 entire females (PF)	*C. imicola*	22,126 (456)	2	36.0 [34.0-38.0]	0.04%	Balenghien, 2014
			C. newsteadi	5,503 (124)				
			Obsoletus complex[d]	131				
			C. pulicaris	72 (13)				
Poland	September/October 2011 and April to October 2012	~ 20 entire females (NP, PF or GF)[c]	Obsoletus complex	~ 3,600 (181)	28	~ 29.8 [17.5-39.4]	0.78%	Larska, 2013
			C. punctatus	~ 2,100 (108)	6	~ 31.4 [23.9-37.2]	0.29%	
France[b]	October 2011	5 entire females	Obsoletus complex[d]	1 734	10	32.9 [23.4-38.2]	0.58%	Balenghien, 2014
			C. obsoletus		8	34.4 [28.3-38.2]		
		< 50 entire females	*C. dewulfi*	1 729 (47)	0			
			C. chiopterus	1 224 (40)	2	32.0 [30.6-33.4]	0.16%	
			C. pulicaris	271 (27)	1	38.3	0.37%	
			C. newsteadi	65 (12)	0			
			C. nubeculosus	43 (7)	1	28.8	2.33%	
			C. lupicaris	24 (9)	0			

(a): PF: parous females; NF: nulliparous females; GF: gravid females
(b): We did not report here results for species for which less than 20 individuals were tested
(c): The number of *Culicoides* per pools was not given precisely, it ranged from 9 to 60 (meanly 20). Blood-fed females were also tested in this study, but we did not report the results here
(d): Individuals of the Obsoletus Complex were tested individually or by pools and then positive individually

4.2. Semen

Different authors reported variable excretion patterns in SBV-infected bulls (Table 4.2). Coincidental detection of SBV-RNA in semen together with early SBV antibodies in the blood was reported (Hoffmann *et. al.*, 2013), although viraemia is very short. From initial field data, SBV-RNA was detected in only 55 semen batches out of 1719 samples tested in seropositive bulls (3 %; ProMed-mail: Schmallenberg virus – Europe, 2012, 76 and 77). When semen samples were strictly selected around seroconversion from period targeted or experimental bulls (Hoffmann *et. al.*, 2013; Ponsart *et. al.*, 2014; Steinrigl *et. al.*, 2013; Van der Poel *et. al.*, 2013), the proportion of positive batches averaged 6 % (72/1118), which has to be considered as an overestimated frequency rate compared to the total number of straws produced in Europe, due to the selection bias. Following experimental infection, the highest SBV RNA concentrations in semen were observed between 4–7 days post infection, but SBV-RNA detection in semen can be independent from SBV viraemia. In this case, viable SBV was only isolated from blood samples and not from semen or genital tissues (Van der Poel *et. al.*, 2013).

Extraction methods influenced sensitivity of detection (Hoffmann *et. al.*, 2013), but trizol has been validated for the efficient extraction of RNA from matrices with a potentially high amount of PCR inhibitors (Vanbinst *et. al.*, 2010; Hoffmann *et. al.*, 2013). As shown in Table 4.2, a large variability has been reported in the excretion of SBV in semen of naturally infected bulls. Positive results were observed in different breeds (Ponsart *et. al.*, 2014). Particular patterns in semen viral RNA were characterized as i) sustained and prolonged SBV genome in consecutive semen batches, up to 2.5 months following seroconversion in rare cases (Hoffmann *et. al.*, 2013; Ponsart *et. al.*, 2014), or ii) single positive semen batch (Hoffmann *et. al.*, 2013; Steinrigl *et. al.*, 2013) or iii) intermittent excretion patterns (Hoffmann *et. al.*, 2013; Van der Poel *et. al.*, 2014) or iv) absence of SBV-RNA in semen (Hoffmann *et. al.*, 2013; Ponsart *et. al.*, 2014). Recent papers demonstrated from few targeted semen batches that SBV RNA-positive bovine semen could contain infectious SBV using the most sensitive experimental transmission model such as subcutaneous injection of positive semen batches in calves (Schulz *et. al.*, 2014) or in IFNAR -/- mice (Ponsart *et. al.*, 2014; Schulz *et. al.*, 2014). However, there is no scientific evidence of transmission through insemination and the risk may be considered as low compared to the principal route of transmission via *Culicoides*. No positive semen batch has been observed in sheep and goats (Table 4.2).

According to Hoffmann *et. al.*, 2013, the rare prolonged SBV-RNA excretion in bovine semen could be explained by the infection of seminal cells, gonadal or testicular tissues or any other tissue in some of these bulls, as it has also been described for bovine herpes virus type 1 (van Oirschot, 1995). This was supported by the results of the SBV-RNA distribution in seminal fractions indicating that SBV-RNA can be detected in seminal cells of semen collected from bulls that showed consecutive positive RTqPCR results together with seroconversion, but not in semen of bulls with only a single SBV positive semen batch (Hoffmann *et. al.*, 2013).

It remains difficult to compare behaviour between SBV and other worldwide Orthobunyaviruses such as Akabane, Aino or Cache Valley viruses in semen, considering the facts that i) limited scientific data are available regarding semen shedding, ii) a low proportion of SBV-seropositive bulls with positive RT-PCR results in semen, iii) the virus detection methods developed for semen need to be highly sensitive to detect RNA viruses (specific extraction protocols have been developed recently for SBV and were unavailable when similar tests were developed for other viruses of the same group).

Controversial data were published for Akabane virus, as no virus was detected using culture in semen collected from viraemic bulls following experimental infection (Parsonson *et. al.*, 1981a; Table 4.2). Gard *et. al.*, (1989) used bull semen naturally infected by viruses of the Simbu serogroup to inoculate sheep. Although four animals showed seroconversion, the possibility of natural infection by vectors could not be ruled out. Intrauterine inoculation of Akabane virus in cattle during artificial insemination did not lead to clinical signs, although most of the animals developed a viraemia. The virus was isolated from a certain number of tissues, including the reproductive system (ovaries, uterus) and the lymph nodes of cows slaughtered up until 7 days after intrauterine inoculation (Parsonson *et. al.*,

1981b). All the pregnant cows gave birth to healthy calves (Parsonson *et. al.*, 1981b). It is important to highlight that results here presented on Akabane were obtained during the decade of 1980s, and much have been developed regarding detection methods and facilities, which might help to elucidate differences in their findings.

Limited numbers of articles have studied the risks of transmission of SBV virus via semen and embryos. Recent data indicate that SBV may be detected from semen samples with a low frequency (< 6 %). However, there is no scientific evidence of transmission through insemination. This is in agreement with epidemiological data, indicating that the vector transmission remains the principal route explaining the dissemination of such viruses (see Section 7).

Table 4.2: Impact of Orthobunyaviruses of the Simbu group on the male genital tract (Akabane=AKAV, Schmallenberg=SBV, VNT=virus neutralisation test, dpi=days post infection, dg=days of gestation, qRT PCR=quantitative reverse transcriptase polymerase chain reaction).

Virus	Reproductive disorders	Country	Species	Reference
AKAV	Experimental infection of 8 bulls and 2 controls; subcutaneous inoculation of 1-2 ml of semen in 10 Hereford cows; viraemia in the 8 bulls (2-9 dpi); no viruses isolated from the semen, no seroconversion of cows	Australia	Cattle	Parsonson *et. al.*, 1981a
	Epidemiological study over 5 years in 29 bulls. Virus isolated from blood and semen. Intravenous inoculation of 12 sheep (3 by bull) followed by serology. 51 episodes of viraemia related to 14 viruses. Seroconversion of sheep inoculated with blood (Aino: 2/2; Akabane: 6/8) or infected semen (Aino: 2/2; Akabane: 6/8). Natural infection of sheep not ruled out.	Australia	Cattle	Gard *et. al.*, 1989
SBV	740 semen batches from 94 SBV-infected and seroconverting/seroconverted bulls. 26 semen batches from 11 bulls reacted positive in the RT-qPCR analyses with Cq-values from 26 to 37.			ProMed-mail: SBV virus - Europe (76)
	-Central Veterinary Institute, The Netherlands: 55 semen samples tested from 8 seroconverting bulls; 3 positive samples using qRT PCR, from 2 different bulls. -ANSES and LNCR, France: 904 semen samples by 160 seropositive bulls; 26 positive samples using qRT PCR, from 2 different bulls.			ProMed-mail: SBV virus - Europe (77)
	-12 extraction methods comparatively validated using a dilution series of SBV-spiked semen. Most sensitive extraction (Trizol® LS Reagent with combined purification of the viral RNA with magnetic beads) and RT-PCR subsequently used with 766 semen batches from 95 field SBV-infected bulls (collected between May and October 2012) to detect SBV-RNA. 29 of 766 semen batches from 11 of 95 SBV-infected bulls positive (C_t 26 to 37). Intermittent virus excretion observed in 2 bulls. - no SBV found in 390 straws batches collected from May to December 2011 from 38 bulls that were SBV seropositive	Germany	Cattle	Hoffmann *et. al.*, 2013
	2 bulls inoculated subcutaneously with viraemic calf serum. Semen collected daily from both animals for 21 days and tested for SBV by qRT–PCR. Bulls necropsied 24 dpi. SBV RNA detected in semen from both bulls (trizol based extraction protocol). The highest SBV RNA concentrations in semen between 4–7 dpi, but low concentrations (C_t values 30–39). Viable SBV only isolated from blood samples and not from semen or genital tissues.	Nether-lands	cattle	Van der Poel et. al.,., 2013

Extraction (Trizol® LS Reagent/Chloroform treatment followed by silica membrane based purification) and RT-qPCR in 164 semen batches from 7 bulls. Positive results in 7 of 164 semen batches from 7 SBV-infected bulls (one single positive batch / bull)	Austria	Cattle	Steinrigl *et. al.*, 2013
6 semen batches from 6 bulls (C_q values: 26.4-36.4) injected subcutaneously to 6 to 9-month-old heifers. 2 SBV infections were confirmed. Confirmation of infectivity from 1 single straw injection (one confirmed infectious batch) with 5 additional cattle. 3 SBV infections were confirmed. 20 SBV RNA positive semen batches from 11 bulls subcutaneously injected into 40 IFNAR -/- mice (4-6 weeks old). Only negative results reported.	Germany	Cattle	Schulz *et. al.*, 2014
7 bulls, 1 to 5 years of age, seroconverted between Sept 2011 and Dec 2012, with semen production including at least 14 ejaculates, collected from 4 weeks before to 4 weeks after the first seropositive sample. Extraction (Trizol® LS Reagent with combined purification of the viral RNA with magnetic beads Trizol based extraction) and qRT-PCR with 146 semen batches from 7 SBV-infected bulls: 29 positive batches from 3 bulls. Semen replicates (each 100 µl) from 1 bull (4 SBV-infected ejaculates) injected subcutaneously into the neck scruff of 3 or 4 adult IFNAR-/- mice. Viraemia and presence of SBV-specific antibodies detected in mice inoculated with highly positive semen batches (C_t values <23).	France	Cattle	Ponsart *et. al.*, 2014 (accepted)
2 bocks inoculated subcutaneously with a SBV isolate (1 ml Vero cell culture 106 TCID50). Semen collected from both animals 1 or 2 times a week (7, 9, 14, 16, 21, 25, 28 dpi) and tested for SBV by qRT–PCR. Bocks necropsied 28 dpi. No SBV RNA detected in semen from both animals.	France	Goat	LNCR, unpubl. data

4.3. Vertical Transmission

Vertical transmission is the passage of an infection from a mother to her embryo or foetus which persists to the point of birth. In the case of SBV, vertical transmission can be considered in both its insect vectors and its ruminant hosts.

No viruses have been shown to be vertically transmitted by colony-reared *Culicoides*, although viral antigens have been detected in their reproductive structures (Mellor, Carpenter & White, 2009a). Two studies provide limited evidence for vertical transmission of viruses by *Culicoides* under field conditions. BTV RNA was detected in pools of larval *Culicoides* in the U.S.A. (White *et. al.*, 2005), although attempts to isolate live virus were not successful. More recently, SBV RNA was detected in nulliparous *Culicoides* in Poland (Larska *et. al.*, 2013). As described in Section 4.1, the significance of this result should remain in doubt until live virus is isolated or a fully disseminated infection is detected in *Culicoides* known to have not taken a bloodmeal. Hence, the evidence for vertical transmission of SBV by *Culicoides* remains very slight.

Vertical transmission of SBV in ruminant hosts would require the offspring of infected mothers to be infected with live virus when born. This could apply to clinically-affected offspring, if they live for a period of time after parturition, or clinically healthy offspring. In both cases, for vertical transmission to be epidemiologically important it must be feasible for the virus to be transmitted from the offspring to other ruminants or *Culicoides*.

Studies have detected SBV RNA in clinically-affected live new-born animals several days after birth. In Belgium, a 7-day old calf with signs of Schmallenberg was euthanized and SBV genomes were detected in CNS samples but not in a variety of other tissues (Garigliany *et. al.*, 2012). In the Netherlands, a 10-day old clinically-affected calf was euthanized and evidence for SBV was detected

in brain tissue by PCR and in brain and spinal cord tissue by immunohistochemistry (Peperkamp *et. al.*, 2012).

These results suggest that vertical transmission of SBV can occur in cattle, although it should be noted that isolation of live SBV from the blood or skin of newborn animals has not yet been reported, which would be a prerequisite for transmission to vectors. Live SBV in the CNS of newborns is unlikely to be transmitted further, indicating that this is likely to be an epidemiological dead-end. SBV has not been reported in healthy newborns of affected mothers.

Pseudo-vertical transmission is the passage of infection from a mother to offspring shortly after birth (Phillips *et. al.*, 2003). It may occur, for example, by the consumption of milk, or from exposure of the offspring to infectious birth tissues such as placenta. SBV has not been reported in milk and, although SBV has been detected in the external placenta and umbilical cord (Bilk *et. al.*, 2012), it is considered unlikely that offspring would become infected with SBV after licking or ingesting these tissues as evidence suggests the virus cannot be transmitted by the oral route (Wernike *et. al.*, 2013a).

In conclusion, there is currently little or no evidence that vertical or pseudo-vertical transmission play an important role in the epidemiology of SBV.

4.4. Mechanism of Overwintering

As discussed elsewhere in this report (section 4.1, 7.3.1) the evidence suggests that SBV is primarily transmitted via the bites of infected *Culicoides*. The duration of viraemia is not clear; although experimental infections suggest duration of only a few days, some field studies have indicated that SBV nucleic acid is present in blood samples for a period in excess of two weeks (Claine *et. al.*, 2013). However, the period of several months between confirmed transmission events is substantially longer than the period for which individual vertebrate hosts are likely to remain infectious, or the period for which adult *Culicoides* are commonly believed to survive. This ability of the virus to "overwinter" – that is, to survive for prolonged periods during lower vector activity and no new hosts appear to be infected, is a characteristic previously observed in other *Culicoides*-borne viruses such as Akabane virus, bluetongue virus and African horse sickness virus.

When contact between the primary vector population and the primary host population is interrupted, there are three ways that a virus can theoretically persist: in the vector population, in the host population, or via an alternative transmission cycle involving one or more novel vector or host populations. Persistence in the vector or host populations may be achieved via horizontal (direct) transmission between individuals, vertical transmission from infected parent to offspring, or persistence in individuals. Because insect vectors are generally infectious for life but relatively short-lived, persistence in individual vectors would require the survival of infected vectors for substantially longer periods than are currently believed to occur in the field (Wilson *et. al.*, 2008).

In the case of SBV, persistence via long-lived adult *Culicoides* has previously been considered for other *Culicoides*-borne viruses and is highly unlikely to be able to account for overwintering periods of longer than three months. However, there are several well-documented reports of SBV infections during the winter (e.g. Davies and Daly 2013; Shaw *et. al.*, 2013; Wernike *et. al.*, 2013a,b). During this period, adult *Culicoides* would normally be expected to be absent or inactive, although entomological surveillance during one of these studies (Wernike *et. al.*, 2013a) confirmed *Culicoides* activity at a very low level despite maximum temperatures of only 9 °C. However, arboviruses require a minimum threshold environmental temperature to replicate to transmissible levels in the insect vector, and although this has not yet been measured directly for SBV it is likely to be between 10 and 14 degrees (see Section 7.1 and Table 7.1). Consequently, these observations could only be explained either as transmission from extremely long-lived *Culicoides* that had completed the extrinsic incubation period during warmer conditions, or via another as-yet-unknown transmission route.

The potential for persistence within the *Culicoides* population via vertical transmission is also likely to be low; as discussed in section 4.3 above, SBV RNA (like BTV RNA) is believed to be occasionally transmitted to offspring, but intact virus has not been detected. Horizontal transmission of virus

between individual *Culicoides* has never been shown and furthermore could not by itself explain the overwintering of SBV during periods of adult vector absence.

Persistence in the population of known ruminant hosts is also possible but appears to be rare. The only evidence for persistent infections with SBV is the shedding of infectious virus in the semen of affected bulls for a prolonged period (section 4.3), but this is very rare. Transplacental transmission to offspring occurs, but live virus has not been demonstrated to be present in blood. Evidence suggests the virus cannot be transmitted by the oral route (Wernike *et. al.*, 2013a).

Regarding the potential for SBV to overwinter via continued transmission in as-yet unrecognised reservoir host species, the evidence for SBV's ability to infect other host species is discussed in section 2 of this report and the specific studies are detailed in Appendix 2. To summarise, there is no strong evidence that any other species plays a substantial role in the epidemiology of SBV, but the limited data available mean that the potential for any of these species, or of another as-yet unidentified species, to act as an SBV reservoir during apparently transmission-free periods cannot be ruled out.

In conclusion, while SBV is able to overwinter, the exact mechanism remains unknown. While the epidemiological data indicate that SBV is capable of surviving in the absence of competent vector activity for prolonged periods, the data available suggest that this is unlikely to occur via the vector population. There is also no strong support for the hypothesis that additional host reservoirs are involved in the persistence of infection, although this cannot be ruled out from the data available. The evidence does indicate that SBV can be transmitted transplacentally and that a limited number of infected bulls may shed infectious virus in semen for prolonged periods, but neither route has yet been shown to result in further transmission.

5. Duration of Immunity

There are limited data on the duration of immunity following SBV infection. To date information is available on the duration of immunity in cattle in both experimental and field settings. However, no information has been published on the duration of immunity in sheep.

In an experimental study of the duration of immunity in cattle, two heifers which had been infected previously, and which were seropositive, could not be reinfected when SBV was injected subcutaneously eight weeks after the previous infection and no SBV replication was detected (Wernike *et. al.*, 2013a). This suggests that the duration of immunity in cattle is at least 56 days.

The results of surveillance in Belgium, where serological surveys were carried out in the winters of 2011/2012 and 2012/2013 (Méroc *et. al.*, 2013a,b,c), also provide information on the duration of immunity in cattle. In particular, the seroprevalence in animals of age between 12 and 24 months sampled in the 2011/2012 survey (87 %, 95 % CI: 84-89) was not significantly (P>0.05) different than that in animals older than 24 months of age sampled in the 2012/2013 survey (85 %, 95 % CI: 82-88 %). This may indicate that the level of antibodies to SBV remains high for at least one year (Méroc *et. al.*, 2013c). However, it should be noted that the same animals were not sampled in both surveys, though the sampling scheme was similar and, hence, results should be comparable between the surveys. It should also be noted that there is not evidence either to refute long term immunity.

6. Seroprevalence Studies

Epidemiological studies to investigate disease prevalence rely on selecting representative samples from the population of interest. In order to ensure precise and unbiased estimates of the population, sufficient samples must be collected to warrant sufficient statistical power (EFSA, 2013b). It is crucial to set the specific objective of the study, to follow the appropriate statistical procedure together with the right use of the methodology (i.e. study design, parameter of the population to be estimated and tested, etc.). Sampling methods vary depending on the rationale of the study and the resources available. In a *systematic sampling* approach subjects from the population frame are selected at regular intervals, once the first case has been randomly chosen. If instead a *random sampling* method is used

each element of the population frame has the same probability of being chosen. A *stratified sampling* design involves the identification of different strata with similar characteristics, and in each of the strata a random sample can be chosen to represent each subpopulation. In the case of *cluster sampling* design groups of elements of the population frame are selected rather than individual elements, such groups are known as clusters. In addition to selecting an appropriate sampling method, sampling frames must be identified. Sampling frames are made of different strata or clusters that form a population. In veterinary medicine commonly used strata/clusters are species, herds/flocks and individual animals.

The validity of a study on disease seroprevalence depends on sample size (sample size calculations should be documented a priori, aiming to achieve specific confidence level with a pre specified power to test the hypothesis of interest), sampling design, sampling frame and the accuracy of the test used.

Several seroprevalence studies have been conducted and published since the emergence of SBV in Europe. Twelve studies were identified for domestic ruminants from eight countries (Appendix A). Their seroprevalence values and confidence intervals were grouped by sampling unit (i.e. animal and herd) and geographical context of the samples taken (i.e. national and regional) and were plotted in Figure 6.1 and Figure 6.2.

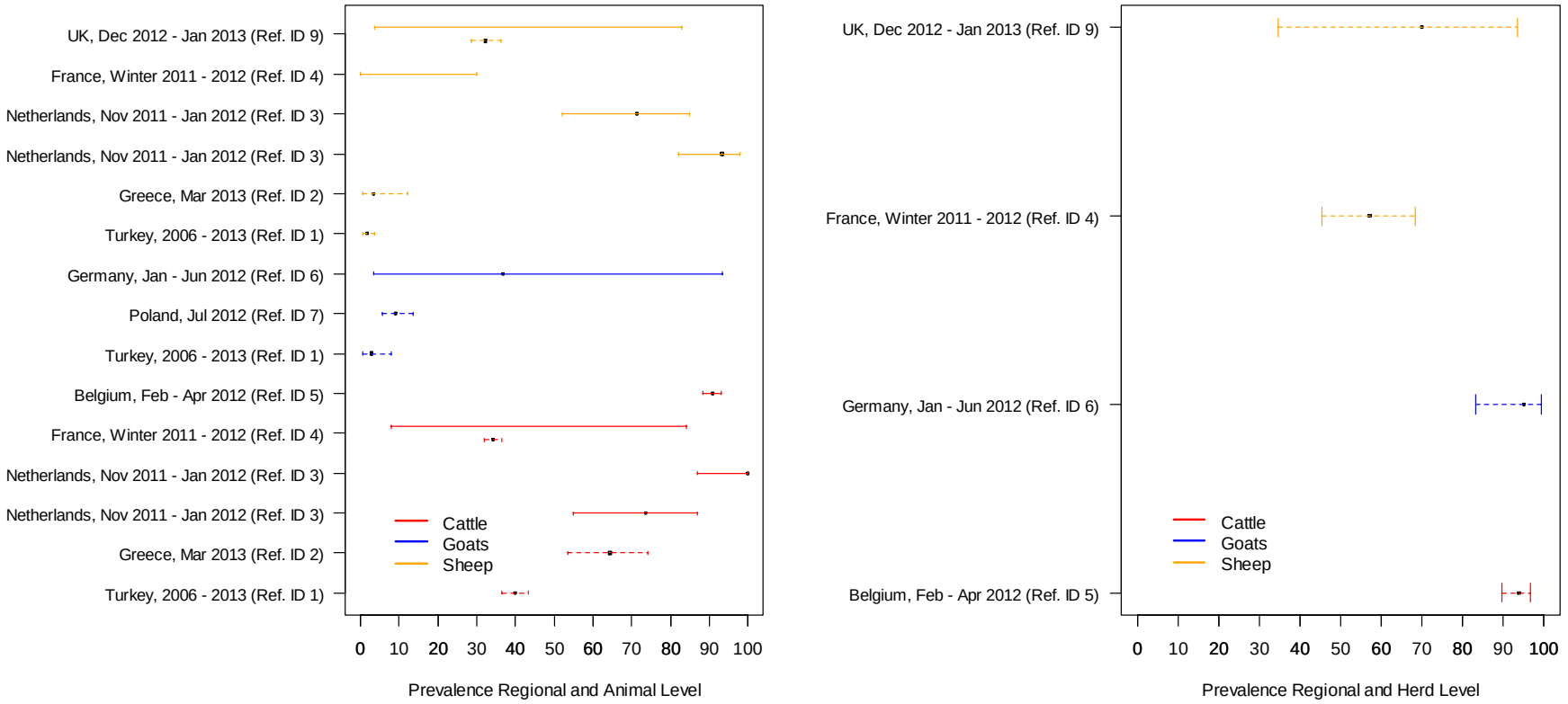

Figure 6.1: Seroprevalence (in percentage, black dots) and 95th Confidence Intervals (bars) for SBV in domestic ruminants (cattle in red, goats in blue and sheep in yellow) grouped by sampling unit and geographical coverage (Regional). Solid lines represent intervals provided by cited authors, dotted lines show exact intervals calculated from data provided in cited papers.

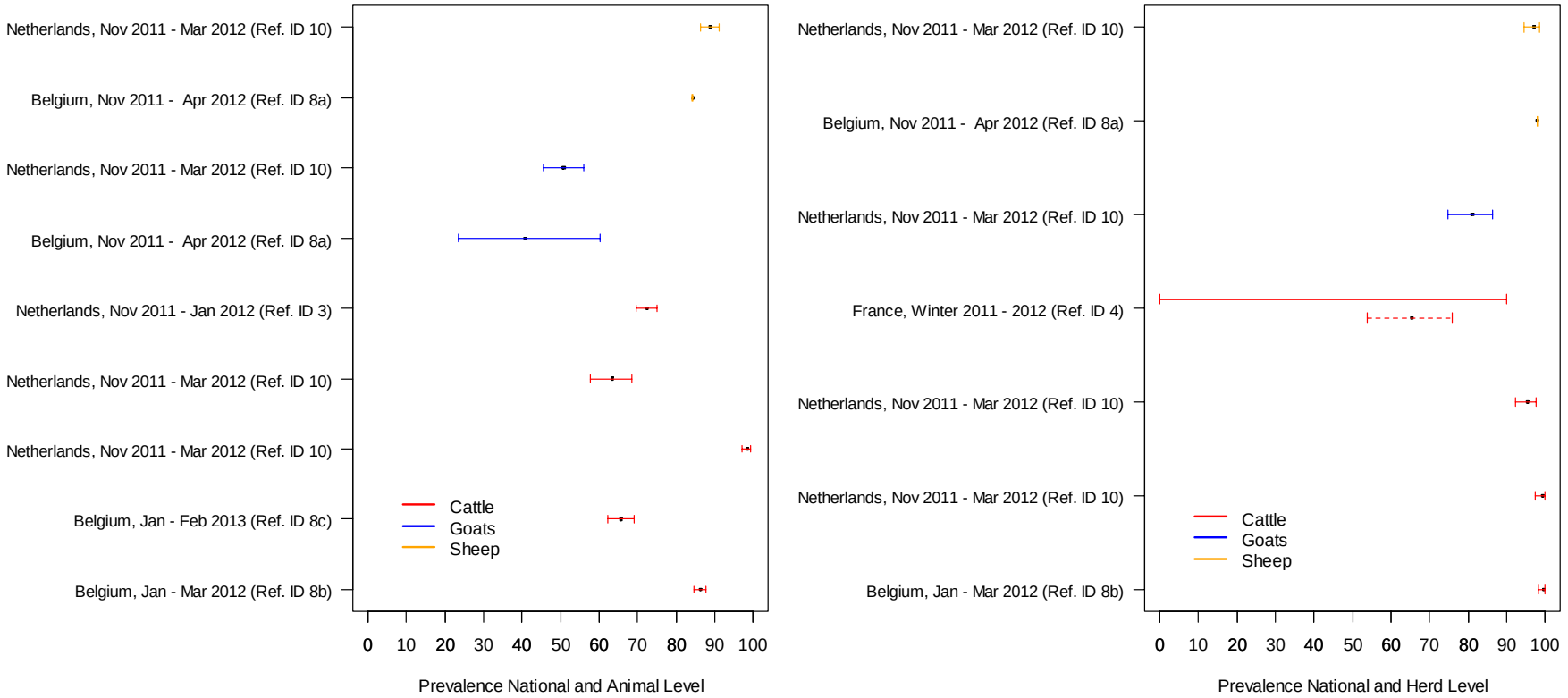

Figure 6.2: Seroprevalence (in percentage, black dots) and 95th Confidence Intervals (bars) for SBV in domestic ruminants (cattle in red, goats in blue and sheep in yellow) grouped by sampling unit and geographical coverage (National). Solid lines represent intervals provided by cited authors, dotted lines show exact intervals calculated from data provided in cited papers.

Seroprevalence values and their confidence intervals are highly variable among studies and comparison of the results obtained are very limited due to the difference between methods used to collect (referring to sampling frame, population frame, sampling design used, sampling unit) and data analysis performed. It is likely that the power achieved by some studies might not be sufficient to support inferences at population level and this could compromise the reliability of the estimation and their interpretations, given samples size and sampling design used in some of the studies. It should be highlighted that for seroprevalence studies conducted at national level, larger variation is observed for animal level seroprevalence estimations compared to the herd level estimations. In five of the National level studies in cattle between herd seroprevalence were estimated above 90 %.

7. Geographical and Temporal Spread of SBV

The spread of SBV has been explored at a range of scales, from within individual holdings to the spread across Europe (Gubbins *et. al.*, 2014a,b). Here we provided summaries of the approaches used and results of these two articles.

7.1. SBV Within Herd Specific Transmission Parameters

7.1.1. Background and approach

Several early studies of SBV transmission within a herd used models parameterised by data on Akabane virus (a related *Culicoides*-borne virus) and Bluetongue virus (BTV) (an unrelated, but well-studied *Culicoides*-borne virus) when exploring scenarios for the spread of SBV (European Food Safety Authority 2012a,b; Bessell *et. al.*, 2013). However, suitable data, notably from seroprevalence surveys (Elbers *et. al.*, 2012; Gache *et. al.*, 2013; Méroc *et. al.*, 2013a,b; Veldhuis *et. al.*, 2013), have become available that allow inferences about the transmission of SBV to be drawn directly.

To draw such inferences a stochastic compartmental model, whose structure is similar to one previously developed for BTV (Gubbins *et. al.*, 2008; Szmaragd *et. al.*, 2009), was developed and fitted to data on the seroprevalence of SBV in cattle and sheep farms in Belgium (Méroc *et. al.*, 2013a,b) and the Netherlands (Veldhuis *et. al.*, 2013). Parameters in the model were estimated using approximate Bayesian computation rejection sampling (Marjoram *et. al.*, 2003; Toni *et. al.*, 2009). This approach generates distributions of parameters for which the within-farm seroprevalences predicted by the model are consistent with those observed in the field. Prior distributions for model parameters (see Fig. 7.1) were generated using data from the published literature. For some parameters data relating to SBV were available, but data for BTV were used instead where this was not the case (Gubbins *et. al.*, 2014a).

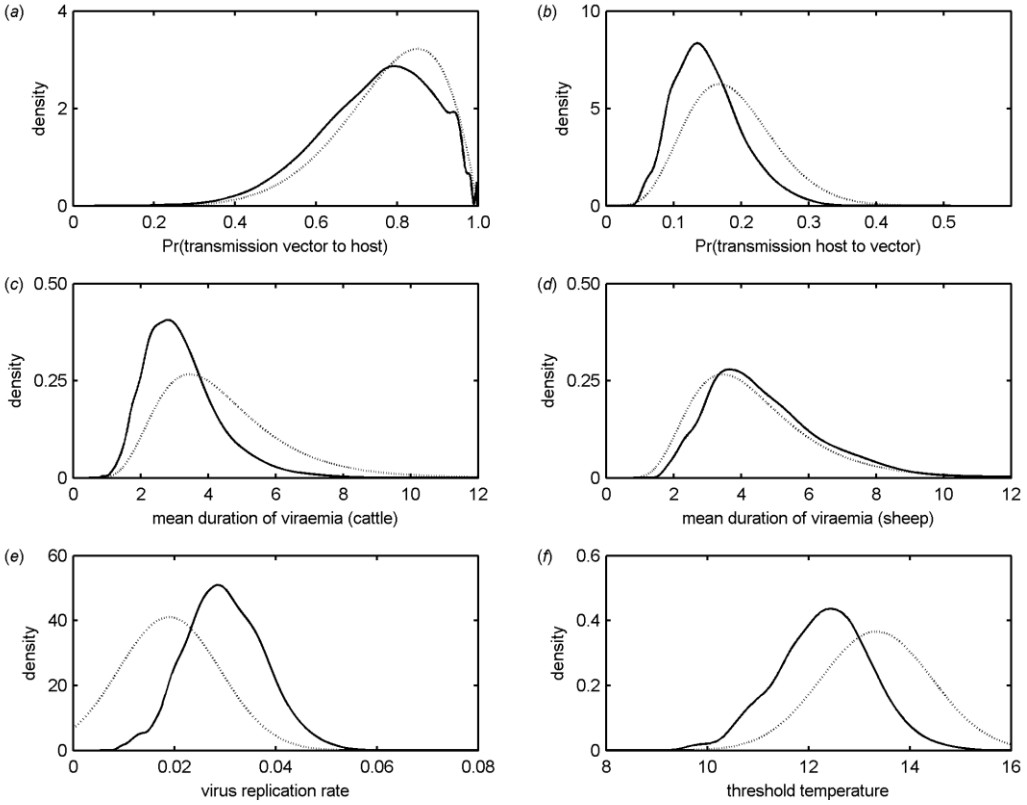

Figure 7.1: Marginal posterior distributions for epidemiological parameters for Schmallenberg virus (SBV): (*a*) probability of transmission from vector to host; (*b*) probability of transmission from host to vector; (*c*,*d*) mean duration of viraemia (days) in (*c*) cattle or (*d*) sheep; (*e*) virus replication rate; and (*f*) threshold temperature (°C) for virus replication. Each figure shows the prior (dotted black line) and posterior (solid black line) densities when the model for the within-farm transmission of SBV was fitted to seroprevalence data for cattle and sheep from Belgium and the Netherlands.

7.1.2. Results

Transmission from vector to host was estimated to be very efficient (posterior median for probability of transmission from vector to host: 0.76) (Fig. 7.1a) and much more so than transmission from host to vector (posterior median for probability of transmission from host to vector: 0.14) (Fig. 7.1b). The mean duration of viraemia was short in both species, but was estimated to be shorter in cattle (approximately three days) than in sheep (approximately four days) (Table 7.1; Figs 7.1c,d). The virus replication rate (above the threshold temperature) was estimated to be approximately 0.03 per day-degree (Table 7.1; Figs 7.1e). Finally, the threshold temperature for virus replication was estimated to 12.3 °C (Table 7.1; Fig. 7.1f).

Table 7.1: Posterior median and 95 % credible intervals (CI) for parameters in the model for the within-farm transmission of Schmallenberg virus (SBV).

parameter	Median	95 % CI
probability of transmission		
vector to host	0.76	(0.46, 0.95)
host to vector	0.14	(0.07, 0.26)
duration of viraemia (cattle)†		
mean (days)	3.04	(1.63, 5.91)
scale parameter	11	(1, 20)
duration of viraemia (sheep)†		
mean (days)	4.37	(2.24, 9.02)
scale parameter	11	(1, 20)
extrinsic incubation period†		
virus replication rate	0.030	(0.016, 0.045)
threshold temperature	12.35	(10.52, 14.02)
scale parameter	6	(2, 35)

† the duration of viraemia in cattle and sheep and the extrinsic incubation period in vectors is assumed to follow a gamma distributions; the scale parameter relates the mean and variance of the distribution, such that variance is equal to the mean squared divided by the scale parameter

The posterior densities (Fig. 7.1) were used to calculate the basic reproduction number (R_0) for SBV in cattle and sheep and its dependence on temperature (Fig. 7.2). For both species, R_0 increases with temperature up to 21 °C, after which it decreases. Moreover, the threshold at R_0=1 is exceeded for temperatures between 13 °C and 34 °C. The basic reproduction number is slightly higher for sheep (Fig. 7.2b) compared with cattle (Fig. 7.2a), which is a consequence of the longer duration of viraemia in this species (Figs 7.1c,d).

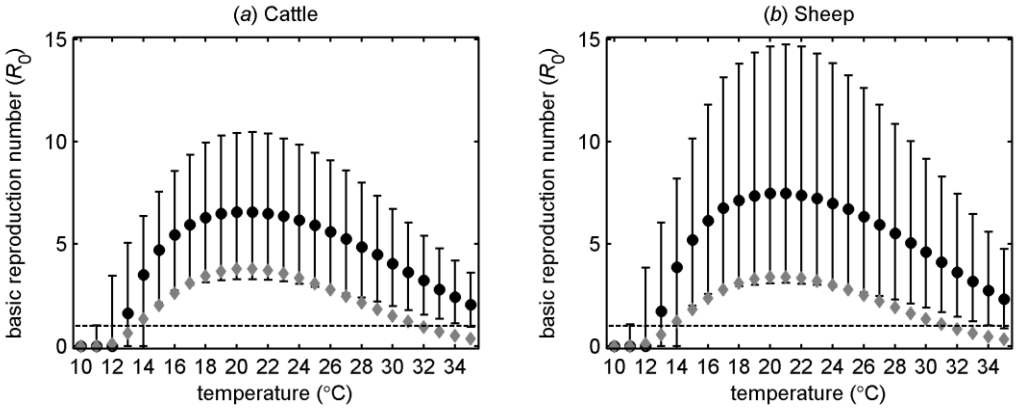

Figure 7.2: Basic reproduction number (R_0) for Schmallenberg virus in (*a*) cattle and (*b*) sheep and its dependence on temperature. Each figure shows the posterior median (circles) and 95 % credible intervals (error bars) for R_0. The black dashed line indicates the threshold at R_0=1. The grey diamonds indicate the median R_0 for Bluetongue virus computed from the uncertainty analysis presented in Gubbins *et. al.*, (2012).

7.1.3. Discussion

In several previous studies, BTV has been used as a proxy when studying SBV (European Food Safety Authority 2012a,b; Bessell *et. al.*, 2013), yet our analysis of within-farm spread has highlighted three key differences between these two viruses. First, the duration of viraemia is much shorter in both cattle and sheep, typically around 3 to 4 days (Table 7.1; Figs 7.1c,d) compared with 16-20 days for BTV (see Gubbins *et. al.*, 2008 and references therein). Despite this much shorter duration of viraemia (and, hence, infectiousness) the within-farm seroprevalence for SBV (Fig. 7.1; see Méroc *et. al.*, 2013a,b;

Veldhuis *et. al.,* 2013) is still typically higher than what was observed at a similar point in the outbreak of BTV serotype 8 (BTV-8) in northern Europe in 2006/7 (Elbers *et. al.,* 2008; Méroc *et. al.,* 2008; van Schaik *et. al.,* 2008). This observation can be accounted for by the second and third differences between SBV and BTV: vector competence and virus replication.

Vector competence was estimated to be 15 % (95 % credible interval (CI): 8-27 %) (Table 7.1), which is slight lower than that estimated for SBV in colony-reared *C. sonorensis*, a North American vector species (19 %; 95 % CI: 14-23 %) (Veronesi *et. al.,* 2013b). This compares with estimates for vector competence for BTV in field-caught *Culicoides* populations of around 1 % (Carpenter *et. al.,* 2006, 2008). In the model, the posterior mean for the peak prevalence of SBV-infected midges was 0.48 % (95 % CI: 5×10^{-4} to 2.64 %), which is consistent with reported prevalence in the field (De Regge *et. al.,* 2012; Elbers *et. al.,* 2013).

In terms of virus replication, SBV is predicted by the model to have a lower threshold temperature for replication (12.3 °C) and to replicate at a faster rate above the threshold (0.03 per day-degree) (Table 7.1) than has been reported for any strain of BTV (Carpenter *et. al.,* 2011). However, there are currently only very limited data on SBV replication in *Culicoides* biting midges, which precludes comparison with our indirect inferences from the transmission model.

Combining the posterior estimates for the individual epidemiological parameters in the basic reproduction number, R_0, shows that, despite the short duration of viraemia, the combination of higher vector competence and faster virus replication result in high values for R_0 (peak R_0 is approximately 6.2 for cattle-only farms and 7.6 for sheep-only farms; Fig. 7.2) and exceeds the threshold at R_0=1 for a wide range of temperatures (13-34 °C) (Fig. 7.2). This contrasts with estimates previously derived for BTV (Gubbins *et. al.,* 2008; 2012) for which the median peak R_0 is lower (3.8 in cattle and 3.4 in sheep) and for which the threshold of R_0=1 is exceeded for a narrower range of temperatures (14-31 °C) (Fig. 7.2).

7.2. SBV Regional Spread: A Network Approach

7.2.1. Approach

To explore the transmission of SBV between farms, we adapted a stochastic model for the spread of BTV between farms in Suffolk and Norfolk, two counties in eastern England (Turner *et. al.,* 2012). This is an area measuring approximately 100 x 100 km, containing over 3000 farms. Transmission between farms is assumed to occur by two mechanisms, animal movements and vector dispersal. Transmission via movements is simulated using recorded animal movements, while transmission via vector dispersal is described by a distance kernel. Rather than describe explicitly the dynamics of infection within a farm, a prevalence curve is constructed for an infected farm based on the time of infection, local temperature and seasonal vector activity.

The between-farm model was adapted to examine the regional spread of SBV by replacing parameter estimates for BTV (Turner *et. al.,* 2012) with those obtained for SBV (Table 7.1). The model also included a shorter latent period (2 days) for SBV (Hoffmann *et. al.,* 2012) compared with BTV. To identify the roles played by differences in parameters between SBV and BTV, simulations were run for seven parameter sets (see summary in Table 7.2). These describe SBV, BTV, and BTV with the following modifications: estimated vector to host transmission rate for SBV (set 2); estimated host to vector transmission rate for SBV (set 3); estimated recovery rate in cattle and sheep for SBV (set 5); and estimated relationship between temperature and extrinsic incubation period (EIP) for SBV (set 6). In addition, we ran a simulation for BTV with the short, 2 day, incubation period for SBV (set 4).

The effect of varying the day of introduction of virus (considering introduction in June, July, August and September) was explored.

Table 7.2: Impact of epidemiological parameters and movement restrictions on predicted regional spread (cumulative number of affected farms and extent of spread in km) of BTV and SBV. An affected farm is counted once only, even if it is infected, recovers and then reinfected. Infection is introduced on day 182, July 1st. Results are for day 365 (31st December). All results are the mean of 100 simulations.

parameter set	Description	movement restrictions				Relative Reduction	
		no		yes			
		no. farms	radius	no. farms	radius	no. farms	radius
BTV	all estimates for BTV	166	23.1	109	9.4	34.3	59.3
set 2	as BTV, except probability of transmission from vector to host for SBV	148	21.6	-	-		
set 3	as BTV, except probability of transmission from host to vector for SBV	1191	34.8	-	-		
set 4	as BTV, except incubation period for SBV	534	28.0	-	-		
set 5	as BTV, except recovery rates in cattle and sheep for SBV	12	8.1	-	-		
set 6	as BTV, except EIP parameters for SBV	819	31.6	-	-		
SBV	all estimates for SBV	3281	50.9	3148	49.1	4.1	3.5

7.2.2. Results

The mean cumulative number of cases and mean cumulative spread on day 365 are given in Table 7.2 for each parameter set. For the two extreme cases (i.e. BTV and SBV), these measures are plotted against time (Fig. 7.3). A small reduction in the probability of transmission from vector to host (from 0.9 [BTV] to 0.76 [SBV]) led to a negligible reduction in the cumulative number of cases and distance spread (Table 7.2; BTV vs. set 2). A large increase in the probability of transmission from host to vector (from 0.01 [BTV] to 0.14 [SBV]) led to a 7.2-fold increase in the cumulative number of cases and 1.5-fold increase in the distance spread (Table 7.2; BTV vs. set 3). The short incubation period of SBV (2 days, compared to 5-7 for BTV) more than tripled the cumulative number of cases and increased the distance spread (Table 7.2; BTV vs. set 4). The short durations of SBV relative to BTV viraemia dramatically reduced the cumulative number of cases and distance spread (Table 7.2; BTV vs. set 5). The relationship between temperature and EIP for SBV led to a five-fold increase in the cumulative cases and 37 % increase in spread relative to BTV (Table 4; BTV vs. set 6). Therefore, most parameters for SBV increase the scale and size of outbreaks (compared to BTV), while one (short viraemia) decreases them. However, the net effect of including all of the SBV parameters together is a 20-fold increase in the cumulative number of cases and doubling of the distance spread (without movement restrictions) (Table 7.2; BTV vs. SBV).

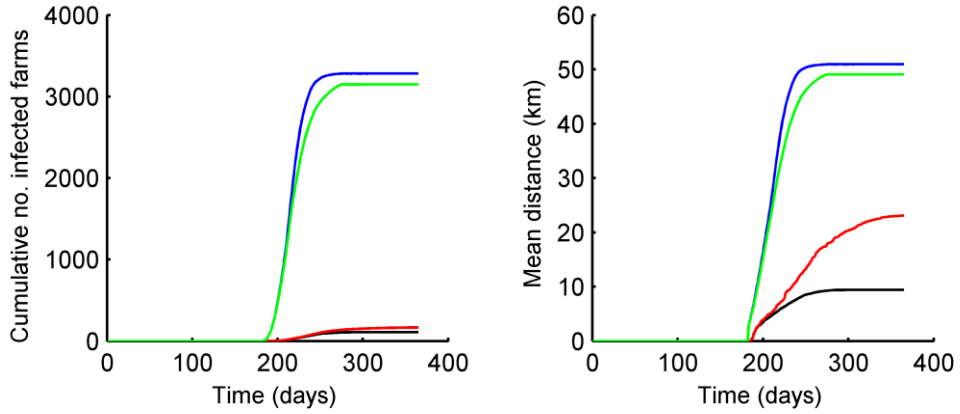

Figure 7.3: Predicted cumulative (*a*) number of cases and (*b*) spread versus time for regional outbreaks of BTV and SBV. Each figure shows results for the regional spread of BTV with no movement restrictions (red line), SBV with no movement restrictions (blue line), BTV with standard movement restrictions imposed during an outbreak in the UK (black line) and SBV with a total movement ban (green line). Each line is the mean of 100 simulations. Infection was introduced on day 182 (i.e. 1 July).

The effect of delaying the introduction of the infection after 1st July reduces the size of the outbreak in terms of number of farms affected by around 20 % with respect to introduction in August, while around 10 % reduction is observed in terms of mean distance (Figure 7.4). As the date of infection approaches 1st September, the size of the outbreak drops dramatically, as there is little time for the infection to spread before cold temperatures reduce vector populations. The effect of introducing the infection earlier (i.e. 1st June) is to achieve saturation (all farms infected) about a month earlier.

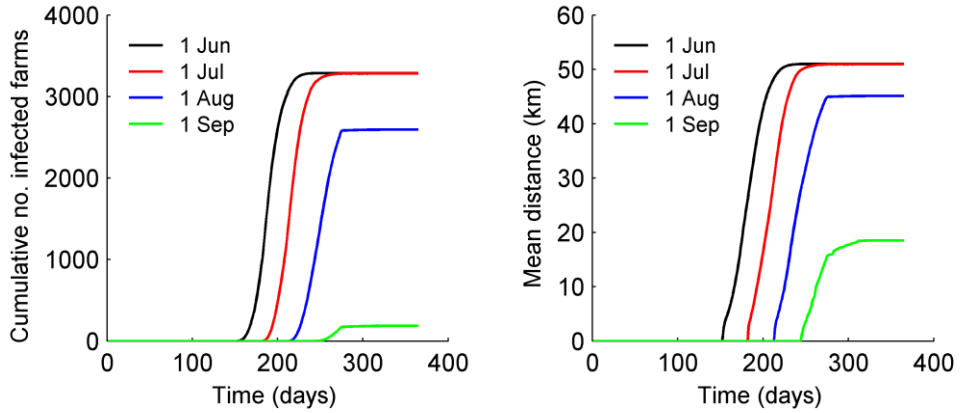

Figure 7.4: Effect of different dates of introduction of infection on predicted cumulative (*a*) number of cases and (*b*) spread versus time for regional outbreak of SBV. Each line is the mean of 100 simulations.

The model suggests that the mean cumulative number of cases for SBV, without any movement restrictions, is over 30 times greater than the number predicted for BTV with standard BTV movement restrictions (Table 7.2 (3281 vs 109); and Fig. 7.3a). These are the conditions under which the two infections spread in the UK: standard movement restrictions were imposed in 2007 when BTV was detected in the UK, but no movement restrictions were imposed to try to control the spread of SBV. As targeted movement restrictions would not be possible for SBV without a widespread surveillance programme, we considered the effect of imposing a total movement ban. The model indicates that such a ban could reduce the mean cumulative number of cases of SBV by only about 4 % (Table 7.2

and Fig. 7.3a) and the distance spread by only 3.5 % (Fig. 7.3b). By contrast, a BTV outbreak appears to be much more sensitive to the effects of movement restrictions. Imposing UK standard movement restrictions (i.e. less stringent than a total ban) achieves a 34 % reduction in cumulative cases and 59 % reduction in the distance spread (Fig. 7.3).

7.2.3. Discussion

Scaling the results for transmission within a farm (section 7.1) to the regional level indicated that the changes to the within-farm transmission parameters (latent period, duration of viraemia, competence and virus replication) are probably sufficient to account for the observed differences in spread between SBV and BTV. Three characteristics of SBV (compared to BTV) increased outbreak size and spread (namely, the greater host to vector transmission rate, the shorter latent period and modified virus replication rate with temperature), while one decreased them (shorter duration of viraemia). The net effect, however, is that SBV is predicted to infect many times more animals, and spread considerably further, than BTV in the same time period.

Our model shows that imposing UK standard movement restrictions shows a considerable reduction of the size and spread of a BTV outbreak. It is difficult to apply such restrictions to SBV outbreaks, because the detection of infected farms would require extensive active surveillance and, therefore, a total movement ban might be a more straightforward approach. However, the model shows that even a total movement ban is expected to have only very minor effect on the final size and spread of an SBV outbreak.

7.3. SBV Continental Spread

The continental-scale spread of SBV was described at NUTS (Nomenclature of Units for Territorial Statistics) level 2 (NUTS2). This was the level at which cases were reported to EFSA by each Member State. Countries included in the model were the 28 EU member states, Switzerland and Norway. Analyses were restricted to infections estimated to have occurred during 2011, so that we can assume a completely naïve population and, hence, do not need to take into account pre-existing immunity to SBV.

7.3.1. Spread between NUTS2 regions

Transmission between regions was modelled using a kernel-based approach, similar to that adopted previously for SBV (European Food Safety Authority 2012b). However, three different shapes for the kernel were considered (fat-tailed, Gaussian and exponential), as well as density-dependent and density-independent formulations (i.e. a total of six forms for the kernel were considered). Parameters were estimated in a Bayesian framework and the fits of the models using different kernels were compared using the deviance information criterion (DIC) (Spiegelhalter *et. al.*, 2002).

The best fit was obtained using a density-dependent fat-tailed kernel (DIC=1175.2). The fit using this kernel was significantly better than for either the density-dependent Gaussian kernel (DIC=1260.5) or the density-dependent exponential kernel (DIC=1218.0). Moreover, the density-dependent kernels provided a significantly better fit than the density-independent kernels. This is consistent with the significant role of vectors in the spread of SBV, as spread by *Culicoides* dispersal is likely to be affected by herd/animal density. Alternative routes of transmission may, of course, still play a role, but those which result in density-independent transmission, such as via the movement of equipment, people, animals and animal products (including semen), are less likely to be the main mechanisms of spread. This conclusion is in accordance with the more detailed analysis of the spread of SBV between farms, which indicated vector dispersal is more important than animal movements (see section 7.2).

7.3.2. Spread within NUTS2 regions

The number of cattle and sheep holdings within a region reporting arthrogryposis hydranencephaly syndrome (AHS) cases in cattle or sheep were described by a Poisson distribution with mean proportional to the species- and region-specific force of infection, the number of holdings keeping the

species in the region and seasonal vector activity. The force of infection was allowed to vary between regions.

The model predicted that the force of infection was markedly higher (>10 times) for sheep than for cattle (Fig. 7.5a), and that there was considerable variation in the force of infection amongst regions. However, species and regional differences will reflect both differences in epidemiology and in case ascertainment. For example, the apparent force of infection for sheep may be much higher than for cattle because the lambing season coincided with the period when SBV was circulating. In addition, sheep flocks tend to be larger than cattle herds and so have a greater chance of having a case.

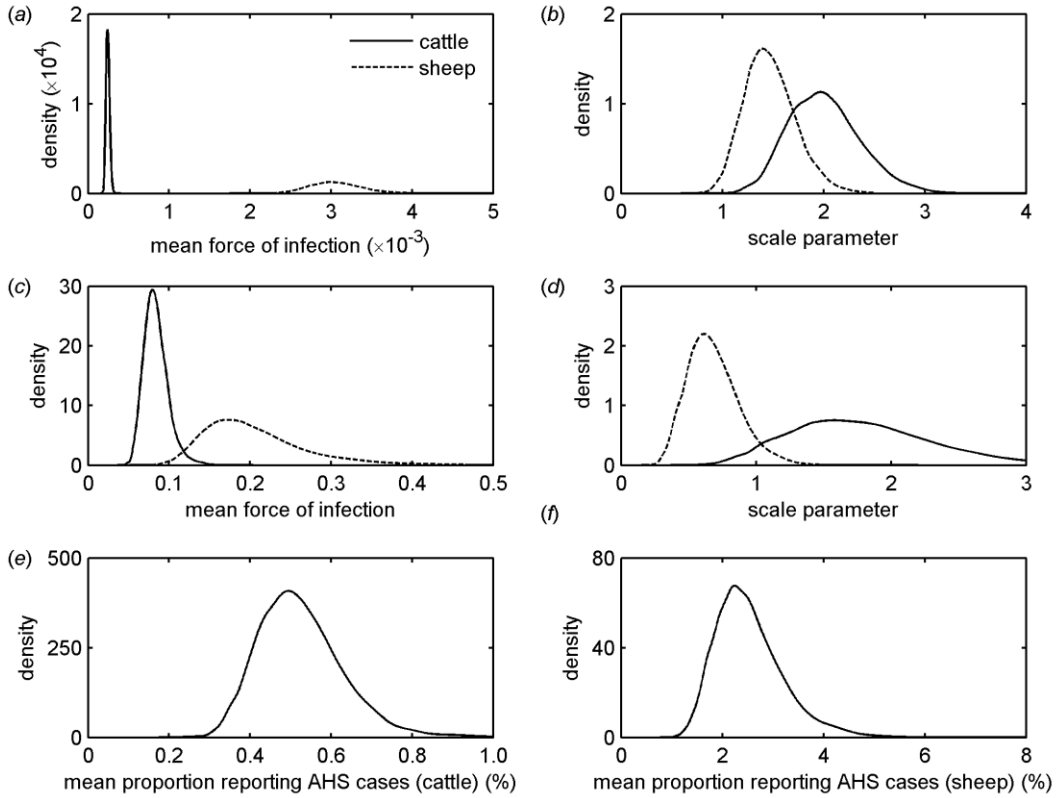

Figure 7.5: Marginal posterior densities for hierarchical parameters in models for the incidence of SBV-affected cattle and sheep holding within NUTS2 regions. (*a*,*b*) Estimated force of infection based on the number of cattle and sheep holdings within a region reporting AHS cases: (*a*) mean and (*b*) scale parameter in hierarchical distribution for cattle (solid line) and sheep (dashed line). (*c*-*f*) Parameter estimates based on the number of cattle and sheep holdings within a region reporting AHS cases and on serological surveys (Belgium and the Netherlands only). (*c*,*d*) Estimated force of infection: (*c*) mean and (*d*) scale parameter in hierarchical distribution for cattle (solid line) and sheep (dashed line). (*e*,*f*) Mean proportion of (*e*) cattle or (*f*) sheep holdings affected by SBV experiencing and reporting AHS cases.

For Belgium and the Netherlands it was possible to adjust the estimates for the force of infection to allow for under-ascertainment, because a second, independent source of data on SBV occurrence is available (in this case data from serological surveys; Méroc *et. al.*, 2013a,b; Veldhuis *et. al.*, 2013).There were still differences in the force of infection between cattle and sheep holdings, though the difference was much smaller (Fig. 7.5c). There were also differences amongst regions in the force of infection for both species. Under-ascertainment of SBV-affected holdings was much higher in cattle compared with sheep farms. We estimated that 0.5 % of affected cattle holdings reported AHS cases (Fig. 7.5e), whereas 2 % of affected sheep holdings reported AHS cases (Fig. 7.5f). Two factors could help explain this difference between species. First, calving tends to occur all year round (at least when

aggregated at a regional level) while lambing tends to be much more strongly seasonal. Second, calves infected *in utero* can clear SBV infection (and so may not be confirmed as SBV cases), while lambs cannot (De Regge *et. al.*, 2013). However, extrapolating these estimates to other regions will be complicated because under-ascertainment of AHS cases in a region will depend on the seasonality of lambing and calving and the time of introduction of SBV, as well as other factors such as farmer willingness to report.

8. Impact Assessment

A considerable level of under ascertainment has been estimated in relation to the SBV epidemic in the EU (EFSA, 2012a), this renders the assessment of the infection impact very difficult. A small number of studies based on active or passive surveillance have now been published which attempt to measure the impact of the disease or provide data likely to be useful for estimating the impact of the disease; these are also discussed below. The impact of the infection can be described as:

Direct impact on adult animals

Clinical signs of acute SBV infection in adult animals include fever and diarrhoea and decreased milk production in low proportion of infected animals. Typically, full recovery occurs within a few days. At herd or regional level reduction on production features such as fertility efficiency has also been demonstrated (Veldhuis, *et. al.*, 2014).

Direct impact on foetuses/newborn animals

The offspring of animals infected with SBV during certain stages of pregnancy are at risk of complications including deformation and abortion. At regional and national level impact has been demonstrated to be relatively low (Afonso *et. al.*, 2014). At a farm level, this impact is highly variable and is likely to depend on the calving or lambing programme used. Farms adopting calving or lambing patterns which result in the at-risk period falling mostly or entirely during the periods of low vector activity (approximately December-March) are likely to experience much lower impact than those adopting calving or lambing patterns which result in the at-risk period coinciding with a period of high vector activity, for example August-September (see Section 4.1).

Indirect impacts

Other consequences of SBV infection which are likely to have had a significant impact include trade restrictions and costs related with treatments or for example expenses related with complications at calving and lambing.

8.1. Studies on impact

8.1.1. Impact on dairy farms

Veldhuis *et. al.*, (2014) report an analysis of various productivity parameters including milk production, reproductive performance and mortality from dairy herds in Germany and the Netherlands during the SBV transmission period compared to a pre-SBV reference period, which shows a probable effect of SBV infection on abortion, shorter gestation, non-return and the number of artificial inseminations required per animal. Overall, average production between August 15th and September 19th 2011 was 0.26kg per day, per cow lower than the same period in 2009-2010. In herds which notified malformations in newborn calves during this period, production was 0.43kg lower.

Veldhuis *et. al.*, (2014) also mention anecdotal reports of reduced fertility in dairy cattle, and Brouwer *et. al.*, (2012) note that the number of samples submitted to the Dutch national abortion monitoring system during the last quarter of 2012 was elevated.

Wernike *et. al.* (2013c) presented the study of a farm located near the city of Schmallenberg closely monitored between May 2011 and January 2012 in the context of tick-borne fever surveillance. The animals in the farm (58 dairy cows, their offspring and two breeding bulls) were kept until the end of the study and no animals were introduced. Every tested animal resulted negative to SBV up to week 37 of 2011 and after week 41 all tested samples were positive, and no abnormalities such as decrease milk yield or diarrhoea were observed, while fever was reported. It was also reported that not premature, stillbirth or birth of malformed calves was observed despite the fact that at the end of September 2011 12 of the tested cows were pregnant between days 75 and 175 of gestation (presumably the critical period).

8.1.2. Impact on beef farms

No data are available for beef farms.

8.1.3. Impact on sheep farms

Dominguez *et. al.*, (2012) report a study of the impact of Schmallenberg virus in France based on lambing records from 362 SBV-positive flocks in 28 districts. In most cases (76 % of flocks for which data were available), the mating period started between early August 2011 and mid-September 2011, during a period when the *Culicoides* vectors of SBV were likely to be active.

This study suggests that 85 % of ewes (34,470) gave birth at full term to only healthy lambs. Of the 15 % of ewes that had lambing problems, 72 % (11 % of the total) gave birth at full term but at least one of their lambs was stillborn, born deformed or died within 12 hours of birth, and the remainder aborted. Furthermore, of the 15 % of ewes that had lambing problems, 12 % (2 % of the total) died within 15 days of delivery.

Extrapolation of these data to estimate the broader impact of SBV across the affected regions is complicated by a lack of data on seroprevalence within the farms in the survey. Moreover the authors recognise that the imputability of SBV virus in the occurrence of the lambing problems, or in the death or deformities reported in lambs was not assessed and could therefore be due to other concurring causes.

Saegerman *et. al.*, (2013) report a preliminary survey comparing 13 positive (by RT-qPCR) flocks with 13 negative flocks (flocks on which no clinical signs consistent with SBV were observed). Several characteristics were present more frequently in the positive flocks, including an increased rate of abortions (6.7 % vs 3.2 %), malformed full-term lambs (10.1 % vs. 2.0 %), and complications in labour (10.1 % vs. 3.4 %).

Another study was conducted in the Netherlands (EC, 2014) to identify and quantify flock level risk factors for malformations in newborn lambs caused by SBV, as well as to describe the effect on mortality and reproductive performance. It was concluded that significantly higher mortality rates before weaning were observed in case flocks, as well as an increase of repeat breeders compared to the lambing period preceding the introduction of SBV. Clinical signs were reported as limited in adult animals. The impact for the entire sheep industry in the Netherlands was reported to be very limited.

8.1.4. Impact on goat farms

No data are available for goat farms.

8.2. Estimation of indirect impact

It is likely that, the principle economic impact of SBV has been felt via international trade restrictions. In particular cattle semen trade has been restricted in several countries, in terms of percentage of total semen trade, most of the trade happen within EU (2010: 73.4 % and 2011: 82.8 %), from the semen trade outside EU (2010: 26.6 % and 2011: 17.2 %), around 60 % of those are trade with those countries imposing restrictions, representing for 2010 a 15.1 % of the total EU semen trade and for

2011 only 10.9 %. In normal years, the EU Member States are exporting between 10 and 12 million doses of bovine semen to third countries. However, in 2012 the trade problems due to the Schmallenberg virus caused a decline to 8.9 million doses (a decline of between 11 and 26 %). Previously, deliveries to Third countries made up for 55 % to 60 % of overall trade, but that figure declined to just over 40 % in 2012. As for the pure-bred breeding animals, the official statistics (EUROSTAT) show that the export value dropped from almost 590 million Euros in 2011 (heifers, cows and other breeding animals) to 475 million Euros in 2012 (a decline of 20 %). The 28 EU Member States sold around 303.000 animals in 2012, thereof 120.000 to Third countries (more than 94 % of the total are heifers; source EXPLA Platform; http://www.adt.de/expla_fr.html).

8.3. Expected future impact of SBV

The impact of the SBV epizootic consists of direct impact (abortion, infertility) and indirect impact (international trade restrictions). The direct impact observed during 2012 resulted from the spread of SBV into a completely naïve host population. It is therefore likely to represent a worst-case scenario which is unlikely to be repeated; if SBV remains endemic in Europe (immunity will continue to be present in a fraction of the host population indefinitely). However, the incidence of SBV infection may vary between years. The duration and amplitude of interannual epidemic cycles will depend on the rate at which susceptible hosts enter the population, which in turn will depend on restocking rates, the level of vaccine use and the durations of immunity following natural infection and vaccination. In the absence of data on several of these variables, it is not possible to estimate the future direct impact of SBV and the extent to which this will vary between years.

In the event that Europe becomes SBV free, the population level of immunity will decline. Subsequent reintroduction of SBV in Europe could then result in an outbreak of similar magnitude to that seen in 2012.

The future indirect impact of SBV will depend on the position adopted by the international community with respect to trade restrictions applied to export from SBV-affected countries.

Farmers have the opportunity to mitigate the direct impact of SBV by vaccinating their animals, or by ensuring that animals are not in the at-risk period of pregnancy during the high vector activity season. Similar strategies have been proposed to deal with other viruses such as Akabane in Australia (http://www.daff.qld.gov.au/animal-industries/animal-health-and-diseases/a-z-list/akabane).

CONCLUSIONS

Metagenomic analysis of animal material allowed the rapid identification of SBV is a newly discovered orthobunyavirus, related to viruses in the Simbu serogroup, as the cause of the new disease that emerged in 2011. The availability of the (almost) complete nucleotide sequence of the SBV genome enabled a genetic test for SBV to be developed and distributed throughout Europe. It also contributes to the establishment of reverse genetic systems that will facilitate further research on SBV molecular biology, pathogenesis and vaccine development. Wide-scale sequencing studies on orthobunyaviruses would have helped to more quickly understand the relationship between SBV and extant Simbu serogroup viruses.

SBV RNA or antibodies have been detected in domestic cattle, sheep and goats and also in another 12 wild species: Alpacas, Anatolian water buffalo, Elk, Bison, Red deer, Fallow deer, Roe deer, Sika deer, Muntjac, Chamois, Wild boar and Dogs, as well as in 19 zoo species. The seroprevalence studies in cattle, sheep and goats indicate that SBV has probably spread over the whole of Europe. According to the seroprevalence studies conducted at national scale, prevalence at animal and herd levels were in general high, while variables between regions within a country.

The number of herds with SBV confirmed AHS (arthrogryposis hydranencephaly syndrome) cases compared to the level of infection indicated by seroprevalence studies, suggest that the frequency of clinical disease is low. Experimental infection studies on pregnant ewes and cows suggest that SBV

rarely induces malformations, although the presence of viral RNA can be demonstrated in the placenta and foetuses of some ewes and cows.

Limited numbers of articles have reported the risks of transmission of orthobunyaviruses via semen and embryos. Recent data indicate that SBV may be detected in semen samples with a low frequency. However, there is no scientific evidence of transmission through insemination. This is in agreement with epidemiological data, which indicate that the vector transmission remains the principal route explaining the dissemination of such viruses, details are given below.

Phylogenetic relations of SBV with viruses of the Simbu serogroup led to suspicion that SBV was transmitted by *Culicoides*. Vector competence studies in *Culicoides nubeculosus* colony lines demonstrated the ability of *Culicoides* to replicate SBV to a transmissible level after intrathoracic inoculation and oral exposure. Studies of field collected *Culicoides* confirmed *C. obsoletus*, *C. scoticus* and *C. chiopterus* as highly probable vectors of SBV in northern Europe. In addition, *C. dewulfi*, *C. pulicaris*, *C. nubeculosus*, *C. imicola* and *C. punctatus* were implicated as suspected vectors. Taken in their entirety, these studies convincingly implicated a range of widespread and abundant farm-associated *Culicoides* species in the transmission of SBV. Studies of vector competence provide preliminary evidence that mosquitoes do not play a substantial role in transmission of SBV in the field.

There is no evidence yet that vertical transmission is a major route of transmission of SBV. SBV has been detected in certain tissues of clinically-affected newborn calves, kids and lambs but neither SBV virus nor RNA has been documented in their blood. Therefore, clinically affected newborns are unlikely to be source of virus for vectors. There is limited evidence for the transmission of SBV to progeny *Culicoides*.

SBV has successfully overwintered, despite lengthy period of minimal vector activity. The mechanism is unknown at present; however vertical transmission in host or vector may play a role. There is no evidence of persistent infection in the host.

There are only limited data on duration of immunity in cattle and none on the duration of immunity in sheep. The data for cattle suggest that immunity lasts for at least one year following natural infection.

A model for the farm to farm spread of a vector borne virus parameterized for SBV show a rapid spread of infection across the study region. Changes to four epidemiological parameters (latent period, duration of viraemia, probability of transmission from host to vector and virus replication) are sufficient to account for the rapid SBV spread within and between farms relative to that seen for BTV-8. This suggests that alternative transmission mechanisms (for example, direct transmission or additional vector species) are not necessary to explain the observed patterns of spread of SBV, though they may still play a minor role. The enhanced between-farm transmission of SBV brought about by these four parameters is such that the application of movement restrictions, even a total animal movement ban, has little effect on the spread of SBV (relative reduction around 4 %).

The ability to estimate impact of Schmallenberg virus was restricted by the limited availability of data; studies conducted reported a probable effect of SBV infection on abortion, short gestation, non-return and the number of artificial inseminations required per animal. The principle economic impact of SBV has been felt via international trade restrictions, particularly in live animals and semen.

RECOMMENDATIONS

Complete genome sequences of Simbu serogroup viruses from multiple geographical locations, in particular, detection, isolation and genetic characterisation of Simbu serogroup viruses, should be obtained to understand relationships between these viruses and to help understand the origin of SBV.

Characterisation of genetic variation within SBV isolates across Europe should be continued and correlated with the ability of serum from vaccinated animals to neutralise these isolates to confirm that available vaccines continue to be efficacious.

Measurement of the duration of immunity in both naturally infected and vaccinated animals should be undertaken to understand the likely continuing impact of the outbreak and inform vaccination strategies.

Membrane-based techniques should be developed for northern European *Culicoides* species, with the aim to standardize vector competence studies of field collected *Culicoides* populations against SBV, this will allow more accurate identification of vector species.

Vertical transmission studies should be undertaken, both in host and vectors, in order to understand overwintering mechanisms and assess risk from trade in live animals.

Studies on vector activities during the winter periods (period of lower temperatures in Europe) should be conducted in order to complement our understating of vector role in SBV epidemics.

Access to data readily available is required to improve assessment of Schmallenberg virus transmission and any other novel diseases entering in Europe:

- Herd size information
- Locations of herds at least at NUTS3 regions
- Monthly animal movements (number of animals) preferably between NUTS3 regions in Europe, but should be available at least for NUTS2

Access to data readily available is required to improve assessment of Schmallenberg virus impact and future novel diseases incursions in Europe:

- Monthly milk production at NUTS3 level
- Mortality in adult female for cattle and sheep due to complication related to delivery in targeted herds
- Number of new born calves and lambs per month, stillbirths, number of abortions for both species preferably at herd level, but at least information at NUTS3 should be available
- Total number of tested herds at NUTS3 level

In order to increase preparedness for new disease threats:

- Development of experimental protocols to facilitate measurement/estimation of transmission parameters involved in transmission and spread models
- Further research on potential control strategies for vector borne diseases

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APPENDICES

Appendix A. Seroprevalence Studies

Ref. ID	Citation	Coverage	Study Design	Sample Units	Laboratory Testing	Prevalence	Strengths and Weaknesses
1	Azkur A.K, Harun Albayrak, Ali Risvanli, Zuleyha Pestil, Emre Ozan, *et. al.*, 2013 Antibodies to Schmallenberg virus in domestic livestock in Turkey Tropical Animal Health and Production DOI 10.1007/s11250-013-0415-2	Turkey (3 regions) 2006 - 2013	Retrospective analysis of serum samples from animals at slaughter	Cattle = 816 Sheep= 307 Goats = 109 Anatolian water buffalo= 130	IDvet ELISA, ID Screen® Schmallenberg virus indirect Positive S/P > 60%	Percentage seropositive Cattle = 325/816 (39.8 %) Sheep = 5/307 (1.6 %) Goats = 3/109 (2.8 %) Anatolian water buffalo = 2/130 (1.5 %)	Wide area coverage, but sample may not be representative No second confirmatory test (VNT or SNT) - cross reactivity may lead to false positives Seroprevalence a simple ratio of positive/tested No consideration of intra-class correlation
2	Chaintoutis SC, Kiossis E, Giadinis Nd, Brozos Cn, Sailleau C, Viarouge C, Bréard E, Papanastassopoulou M, Zientara S, Papadopoulos O, Dovas CI. 2013. Evidence Of Schmallenberg Virus Circulation In Ruminants In Greece. Trop Anim Health Prod. 2013 Jul 19. [Epub Ahead Of Print].	Greece (1 region) March 2013	Outbreak investigation Random sample animals within herd Stratification by origin of animal	Cattle=90 (3 herds) Sheep = 57 (3 herds)	IDvet ELISA, ID Screen® Schmallenberg virus indirect Cut-off not reported SNT confirmatory testing of positives	Within herd seroprevelance Cattle range 30.0 % - 86.7% Sheep 1 positive animal in two flocks	Low sample size - lack of precision Sample may not be representative Second confirmatory test (VNT or SNT) included Herds selected on basis of clinical signs Regional or temporal variability may be due to seroconversion ongoing during the study
3	Elbers ARW, Loeffen WLA, Quak S, de Boer-Luijtze E, van der Spek AN, Bouwstra R, Maas R, Spierenburg MAH, de Kluijver EP, van Schaik G and van der Poel WHM, 2012. Seroprevalence of Schmallenberg Virus Antibodies among Dairy Cattle, the Netherlands, Winter 2011-2012. Emerging Infectious Diseases, 18, 1065-1071. Available from <Go to ISI>://WOS:000306034600005.	Netherlands November 2011–January 2012	Retrospective analysis of serum samples for BTV programme and sentinel herds Random sample of animals with serum samples Stratification by region Expected prevalence = 50% (maximum allowable error ≈3%)	Dairy Cattle =1,123 (489 herds) Sheep flock1=60 Sheep flock2=35 Cattle herd1=34 Cattle herd2=34	VNT Loeffen *et. al.*, (2012) Titers >8 were considered positive - specificity and sensitivity of >99% were estimated with this cut-off	Estimated seroprevalence Dairy cattle 72.5% (95% CI 69.7%–75.1%). Within herd seroprevalence Dairy cattle range 73.5%, 95% CI 55%–87%) - 100%, 95% CI 87%–100%). Sheep range 71.4%, 95% CI 52%–85%) - 93.3%, 95% CI 82%–98%)	Sample size allows estimation at design prevalence Seroprevalence estimated using generalized estimating equations Estimation of intra-class correlation Regional or temporal variability may be due to seroconversion ongoing during the study Herds selected on basis of clinical signs

4	Gache K, Dominguez M, Pelletier C, Petit E, Calavas D, Hendrikx P and Touratier A, 2013. Schmallenberg virus: a seroprevalence survey in cattle and sheep, France, winter 2011–2012. Veterinary Record, 173, 141-141.	France Winter 2011-2012	Retrospective analysis of serum samples for Brucellosis or IBR programme Voluntary samples at holding level, random selection of animals within holding Stratification by outbreak status - No congenital SBV outbreak (Category 1), departments with 1–20 outbreaks (Category 2) and departments with more than 20 outbreaks (Category 3) Expected prevalence = 7%	Sheep = 3007 (77 holdings) Cattle=3252 (78 holdings)	IDvet ELISA, ID Screen® Schmallenberg virus indirect Cut-off not reported	Within herd seroprevalence for herds in Category 3 departments with more than 20 outbreaks Cattle median = 90% Sheep median = 30%	Sample size allows estimation at design prevalence Sample may not be representative Regional or temporal variability may be due to seroconversion ongoing during the study Seroprevalence estimate using simple ratio of positive/tested
4	Gache K, Dominguez M, Pelletier C, Petit E, Calavas D, Hendrikx P and Touratier A, 2013. Schmallenberg virus: a seroprevalence survey in cattle and sheep, France, winter 2011–2012. Veterinary Record, 173, 141-141.	France (1 region) Winter 2011-2012	Retrospective analysis of serum samples from IBR programme Voluntary samples at holding level, random selection of animals within holding Stratification by region	Cattle = 1525 (343 holdings)	IDvet ELISA, ID Screen® Schmallenberg virus indirect Cut-off not reported	Seroprevalence Cattle range 8% - 84%	Sample may not be representative Regional or temporal variability may be due to seroconversion ongoing during the study Seroprevalence estimate using simple ratio of positive/tested No consideration of intra-class correlation
5	Garigliany M-M, Bayrou C, Kleijnen D, Cassart D and Desmecht D, 2012. Schmallenberg Virus in Domestic Cattle, Belgium, 2012. Emerging Infectious Diseases, 18 (6), 1512-1514. Available from <Go to ISI>://WOS:000307989700023.	Belgium February 13–April 22, 2012	Seroprevalence study Random sample of cow/calf pairs	Cow/calf pairs =519 (209 holdings)	IDvet ELISA, ID Screen® Schmallenberg virus indirect Positive S/P > 70%	Apparent seroprevalence adult cows 90.8% (95%, CI 88.3–93.2 Calves born to seropositive cows, 116/471 (24.6% 95% CI 20.7–28.5)	Low sample size - lack of precision Insufficient information on study design Regional or temporal variability may be due to seroconversion ongoing during the study Seroprevalence estimate using simple ratio of positive/tested

6	Helmer C, Eibach R, Tegtmeyer PC, Humann-Ziehank E, Ganter M, 2013. Survey of Schmallenberg virus (SBV) infection in German goat flocks, Epidemiol Infect Mar 18:1-11.	Germany (6 regions) January- June 2012	Seroprevalence study Stratification by region Random sample of herds and animals Expected prevalence = 20%	Adult female goats = 1065 (>1 year) (40 herds)	IDvet ELISA, ID Screen® Schmallenberg virus indirect Positive S/P > 70%	Between herd seroprevalence Goats = 38/40 (95%) Within herd seroprevalence Goats range 3.3% - 93.3% median 36.7%	Sample size allows estimation at design prevalence Within herd sample size requirement result in bias towards larger herds Regional or temporal variability may be due to seroconversion ongoing during the study
7	Kaba J, Czopowicz M, Witkowski L, 2013. Schmallenberg virus antibodies detected in Poland. Transboundary and emerging diseases. 60, 1-3, doi: 10.1111/tbed.12039	Poland (3 regions) 24 - 30 July 2012	Serology Survey Stratification by region Targeted sample based on proximity to Polish border Expected prevalence = 5%	Adult goats = 230	IDvet ELISA, ID Screen® Schmallenberg virus indirect Positive S/P > 60%	Percentage seropositive in region Goats range 2% - 16%	Sample may not be representative No second confirmatory test (VNT or SNT) - cross reactivity may lead to false positives Regional or temporal variability may be due to seroconversion ongoing during the study
8a	Méroc E, De Regge N, Riocreux F, Caij AB, van den Berg T and van der Stede Y, 2013b. Distribution of Schmallenberg Virus and Seroprevalence in Belgian Sheep and Goats. Transboundary and Emerging Diseases, doi: 10.1111/tbed.12050	Belgium November 2011 - April 2012	Retrospective analysis of serum samples from Maedi-Visna and Caprine Arthritis and Encephalitis Programme Voluntary samples Expected prevalence = 90–95% (accepted error of 5%)	Sheep = 1082 (83 herds) Goats =142 (8 herds)	IDvet ELISA, ID Screen® Schmallenberg virus indirect Positive S/P > 60%	Estimated within herd seroprevalence Sheep 84.31% (95% CI: 84.19–84.43) Goats 40.68% (95% CI: 23.57–60.4%) Between herd seroprevalence Sheep 98.03% (95% CI: 97.86–98.18)	Sample size allows estimation at design prevalence Within herd sample size requirement result in bias towards larger herds Seroprevalence estimated using generalized estimating equations Estimation of intra-class correlation

8b	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, 2013a. Large-Scale Cross-Sectional Serological Survey of Schmallenberg Virus in Belgian Cattle at the End of the First Vector Season. Transbound Emerg Dis. doi: 10.1111/tbed.12042.	Belgium 2 January - 7 March 2012	Retrospective analysis of serum samples for BTV and IBR programme Random sample of herds Stratification by region at herd level and by age at animal level	Cattle = 11 635 (422 herds)	IDvet ELISA, ID Screen® Schmallenberg virus indirect Positive S/P > 60%	Estimated within herd seroprevalence Cattle 86.3% (95% CI: 84.75–87.71) Between herd seroprevalence Cattle 99.76% (95% CI: 98.34–99.97)	Sample size allows estimation at design prevalence Seroprevalence estimated using generalized estimating equations Estimation of intra-class correlation Within herd sample size requirement result in bias towards larger herds
8c	Méroc E, Poskin A, Van Loo H, Van Driessche E, Czaplicki G, Quinet C, Riocreux F, Hooyberghs J, De Regge N, Caij AB, van den Berg T, Hooyberghs J, and van der Stede Y, 2013c. Follow-up of the Schmallenberg Virus Seroprevalence in Belgian Cattle. Transbound Emerg Dis. doi: 10.11 11/tbed. 12202	Belgium 1st January – 28th February 2013	Serology survey Stratified by region and age Expected prevalence = 90% (accepted error of 5%)	Cattle = 7130 (188 herds)	IDvet ELISA, ID Screen® Schmallenberg virus indirect Positive S/P > 60%	4470 positive samples (all herds had at least one positive animal) Mean within-herd seroprevalence 65.66% (95% CI: 62.28-69.04)	Sample size allows estimation at design prevalence The doubtful results are considered as positive in the data analysis. Seroprevalence estimated using generalized estimating equations
9	Nanjiani IA, Aitken P, Williams P, 2013. Prevalence of seropositive sheep within flocks where Schmallenberg Virus infection was suspected or confirmed. Veterinary Record doi: 10.1136/vr.101796.	United Kingdom (5 regions) December 2012-January 2013	Voluntary herd selection Survey Voluntary samples Expected prevalence = 10% (Error 4.8-11.7)	Sheep = 594 (10 herds)	IDvet ELISA, ID Screen® Schmallenberg virus indirect Positive S/P > 70%	Percentage seropositive Sheep range 8.5 - 73.3 per cent (95 per cent confidence limits as low as 3.7 per cent, and as high as 82.9 per cent)	Low sample size - lack of precision Sample may not be representative Seroprevalence estimate using simple ratio of positive/tested Regional or temporal variability may be due to seroconversion ongoing during the study

10	Veldhuis AMB, van Schaik G, Vellema P, Elbers ARW, Bouwstra R, van der Heijden HMJF and Mars MH, 2013. Schmallenberg virus epidemic in the Netherlands: Spatiotemporal introduction in 2011 and seroprevalence in ruminants. Preventive Veterinary Medicine, 112, 35-47. Available from http://www.sciencedirect.com/science/article/pii/S0167587713002092.	Netherlands November 2011 - March 2012	Retrospective analysis of serum samples from IBR programme Random sample Expected prevalence = 50% (maximum allowable error ≈8%)	Cattle, non-dairy = 1373 (276 herds)	ELISA (Van der Heijden *et. al.*, 2013) sensitivity of 98.8% (95% confidence interval (CI): 93.3–99.8) and a specificity of 98.8% (95% CI: 97.5–99.6). Positive S/P > 15%	Estimated seroprevalence Non dairy cattle 98.5% Between herd seroprevalence Non dairy cattle 99.3% (95% CI: 97.4–99.9)	Sample size allows estimation at design prevalence Seroprevalence estimated using generalized estimating equations Estimation of intra-class correlation Within herd sample size requirement result in bias towards larger herds
10	Veldhuis AMB, van Schaik G, Vellema P, Elbers ARW, Bouwstra R, van der Heijden HMJF and Mars MH, 2013. Schmallenberg virus epidemic in the Netherlands: Spatiotemporal introduction in 2011 and seroprevalence in ruminants. Preventive Veterinary Medicine, 112, 35-47. Available from http://www.sciencedirect.com/science/article/pii/S0167587713002092.	Netherlands November 2011 - March 2012	Retrospective analysis of serum samples from BTV programme Random sample Stratification by region Expected prevalence = 50% (maximum allowable error ≈8%)	Dairy Cattle = 3066 (247 herds)	ELISA (Van der Heijden *et. al.*, 2013) sensitivity of 98.8% (95% confidence interval (CI): 93.3–99.8) and a specificity of 98.8% (95% CI: 97.5–99.6). Positive S/P > 15%	Estimated seroprevalence Dairy heifers 63.4% Between herd seroprevalence Dairy cattle 95.5% (95% CI: 92.3–97.7)	Sample size allows estimation at design prevalence Seroprevalence estimated using generalized estimating equations Estimation of intra-class correlation Within herd sample size requirement result in bias towards larger herds Regional or temporal variability may be due to seroconversion ongoing during the study
10	Veldhuis AMB, van Schaik G, Vellema P, Elbers ARW, Bouwstra R, van der Heijden HMJF and Mars MH, 2013. Schmallenberg virus epidemic in the Netherlands: Spatiotemporal introduction in 2011 and seroprevalence in ruminants. Preventive Veterinary Medicine, 112, 35-47. Available from http://www.sciencedirect.com/science/article/pii/S0167587713002092.	Netherlands November 2011 - March 2012	Retrospective analysis of serum samples from Brucella melitensis and maedi-visna virus or caprine arthritis encephalitis virus programme Voluntary and random samples Stratification by region Expected prevalence = 30% or higher	Sheep = 2876 (344 herds) Goats = 1553 (185 herds)	ELISA (Van der Heijden *et. al.*, 2013) sensitivity of 98.8% (95% confidence interval (CI): 93.3–99.8) and a specificity of 98.8% (95% CI: 97.5–99.6). Positive S/P > 15%	Estimated seroprevalence Sheep 89.0% Goats 50.8% Between herd seroprevalence Sheep 97.1% (94.7–98.6) Goats 81.1% (95% CI: 74.7–86.5)	Sample size allows estimation at design prevalence Seroprevalence estimated using generalized estimating equations Estimation of intra-class correlation Sample may not be representative

11	Chenais E, Ståhl K, Frössling J, Blomqvist G, Näslund K, Svensson L, Renström L, Mieziewska K, Elvander M, Valarcher JF., 2014. Schmallenberg Virus beyond Latitude 65°N. Transbound Emerg Dis. 2013 Dec 11. doi: 10.1111/tbed.12195. [Epub ahead of print]	Sweden August 2011 – May 2013	Six hundred sera from sheep were collected between 1 August 2011 and 31 March 2012 from 150 herds, and selected among samples originally collected within the Swedish surveillance programme for Maedi/Visna. Bulk milk survey in cattle was conducted before and after vector season of 2012	Sheep = 600 (150 herds) Bulk milk from 641 and 723 dairy farms Rsik based surveillance from 67 herds (30 cattle, 34 sheep, 2 alpaca and 1 goat)	An indirect in-house SBV ELISA was developed (K. Näslund, G. Blomqvist, C. Vernersson, S. Zientara, E. Breard and J. F. Valarcher, in preparation). Commercially available indirect SBV ELISA (ID Screen Schmallenberg virus Milk Indirect ELISA; IDvet, Grabels, France), VNT and RT-PCR	Reported number of positive herds for sheep and cattle per month from November 2012 until May 2013. Reported different levels of S/P ratio for bulk milk after vector season.	The different serological surveys were conducted; each designed to detect a prevalence of SBV infection at approximately 2% and with at least 95% confidence, but not details are given on how it was calculated. Population is hierarchical in nature and variability at herd and animal levels are not mentioned.
12	Steinrigl A, Schiefer P, Schleicher C, Peinhopf W, Wodak E, Bagó Z, Schmoll F, 2014. Rapid spread and association of Schmallenberg virus with ruminant abortions and foetal death in Austria in 2012/2013. Prev Vet Med. DOI: 10.1016/j.prevetmed.2014.03.006	July – December 2012	Samples were collected in the context of national Bluetongue monitoring program, national screening programme for Bovine brucellosis, Enzootic bovine leucosis and Infectious bovine rhinotracheitis, national Brucella melitensis screening programme as well as private commissions and samples link to abortions.	Cattle = 2113 (801 herds), Initially samples for Sheep = 1031 and Goat = 230, from which in total only 830 were used (248 herds)	IDvet ELISA, ID Screen® Schmallenberg virus indirect	Temporal and regional differences were tested. For cattle temporal differences were identified, while for shep and goats both temporal and regional differences were found.	This study is one of the few to analyze the dynamics of SBV spread in cattle for a whole country. The sampling strategy applied for this study cannot exclude bias coming from unbalanced temporal or spatial sampling design, because samples were compiled from different screening programmes in order to allow sufficient monthly coverage.

Appendix B. Other Susceptible Species

Citation	Species found	Clinical signs	Laboratory Testing	Strengths and Weaknesses
Schmallenberg virus 'still circulating in the UK Veterinary Record, 2012 171:140 doi: 10.1136/vr.e5373	Alpacas (*Vicugna pacos*)	None recognised	Detection of antibodies in two animals in a very small flock	The specie is not closely related to sheep or goats, but very little detail about study design used is given
Jack, C, O. Anstaett, J. Adams, R. Noad and J. Brownlie, 2012: Evidence of seroconversion to SBV in camelids. Vet. Rec. 170, 603	Alpacas (*Vicugna pacos*)	None recognised	Detection of antibodies in 2/10. ID Screen Schmallenberg Virus Indirect Elisa (ID.vet).	
Azkur A, Albayrak H, Risvanli A, Pestil Z, Ozan E, Yılmaz O, Tonbak S, Cavunt A, Kadı H, Macun H, Acar D, Özenç E, Alparslan S and Bulut H, 2013. Antibodies to Schmallenberg virus in domestic livestock in Turkey. Tropical Animal Health and Production, 1-4. Available from http://dx.doi.org/10.1007/s11250-013-0415-2.	Anatolian water buffalo	None recognised	Detection of antibodies in 2 out of 130 sampled The sera were screened by indirect ELISA (ID Screen® Schmallenberg virus indirect, IDvet Innovative Diagnostics), following the manufacturer's instructions. Test samples (S), positive (PC) and negative controls (NC) were run on each plate. Optical densities at a wavelength of 450 nm (OD) were determined, and results were calculated using an automated ELISA reader (BIOTEK ELX800). For each sample, the S/P percentage was calculated as follows: (ODsample−ODNC)/(ODPC−ODNC)×100. Samples with an S/P% of ≤50 % were considered negative, 50–60 % doubtful, and >60 % positive, respectively	Wide area and temporal coverage, but sample may not be representative. Description of potential test reaction to other Simbu virus. No detail about study design.

Larska M, Krzysiak M, Smreczak M, Polak MP, Zmudzinski JF, 2013. First detection of Schmallenberg virus in elk (Alces alces) indicating infection of wildlife in Bialowieza National Park in Poland, Vet J. Aug 16. pii: S1090-0233(13)00388-2. doi: 10.1016/j.tvjl.2013.08.013.	Elk (*Alces alces*), bison (*Bison bonasus*)	None recognised	Elk calf (one serum sample tested positive by RT-PCR, negative indirect ELISA). Serum samples collected from free-living bison (n = 60, 22 positive), wild red deer (n = 69, 15 positive), farmed red deer (n = 24, no positive) and fallow deer (n = 16, no positive)	No details are given about the study design, and how sample were collected. Samples collected from eight different locations.
Barlow A, Green P, Banham T, Healy N, 2013. Serological confirmation of SBV infection in wild British deer. Vet Rec. Apr 20;172(16):429. doi: 10.1136/vr.f2438	Red deer (*Cervus elaphus*), fallow deer (*Dama dama*) , roe deer (*Capreolus capreolus*) and muntjac (*Muntiacus reevesi*)	None recognised	Red deer (5 positive out of 7), fallow deer (9 positive and 1 inconclusive out of 16), roe deer (8, all negative) and muntjac (1 inconclusive out of 35) commercially available ELISA (IDScreen Schmallenberg Virus Indirect Antibody ELISA; ID-Vet).	Results of sero-surveillance from a short period from February 20 to March 6, 2012. Small number of samples and no detail about study design.
Chiari M, Sozzi E, Zanoni M, Alborali LG, Lavazza A and Cordioli P, 2014. Serosurvey for Schmallenberg Virus in Alpine Wild Ungulates. Transboundary and Emerging Diseases, http://dx.doi.org/10.1111/tbed.12158.	Red deer (*Cervus elaphus*)and Chamois (*Rupicapra rupicapra*)	None recognised	Samples from several years (2007-2013) were tested, and only samples from 2012-2013 resulted positive (1 out of 6 chamois, 21 out of 52 red deer) All sera tested by serological SBV ELISA kit (ID Screen_ Schmallenberg Virus Competition, Multispecies; IDvet Innovative Diagnostics, Montpellier, France). All sera positive by ELISA were also positive by VNT.	Sampling area with high red deer density, but very restrictive, potential issues with representativity. Small number of samples and no detail about study design.

Linden A, Desmecht D, Volpe R, Wirtgen M, Gregoire F, Pirson J, Paternostre J, Kleijnen D, Schirrmeier H, Beer M and Garigliany M-M, 2012. Epizootic Spread of Schmallenberg Virus among Wild Cervids, Belgium, Fall 2011. Emerging Infectious Diseases, 18, 2006-2008. Available from <Go to ISI>://MEDLINE:23171763.	Red deer(*Cervus elaphus*) and roe deer (*Capreolus capreolus*)	None recognised	Blood samples were collected during post-mortem examination of 313 red deer (seroprevalence 40.5%, 95% CI 31.6%–49.5%) and 211 roe deer (seroprevalence 45.9%, 95% CI 36.5%–55.2%) shot during the 2010 and 2011 hunting seasons. . IgG against the recombinant nucleoprotein of the emerging SBV was detected by using an ELISA kit (ID Screen Schmallenberg Virus Indirect, version 1; ID.vet Innovative Diagnostics, Montpellier, France). Results are expressed as percentages of the reference signal yielded by the positive control serum; serologic status is defined as negative (<60%), doubtful (60%–70%), or positive (>70%). Neutralizing antibodies against SBV were sought as described (*3*) in subsets of roe deer serum (IgG-negative and IgG-positive according to ELISA), and a linear relationship between percentages and reciprocal neutralizing titers was found	Samples were randomly collected during October–December from 35 hunting estates in 4 of the 5 provinces in southern Belgium, limited information regarding study design.

Sailleau, C, Boogaerts, C, Meyrueix, A, Laloy, E, Bréard, E., Viarouge, C., Desprat, A, Vitour, A, Doceul, V, Boucher, C, Zientara, S. and Grandjean, D, 2013. Schmallenberg Virus Infection In Dogs, France, 2012. Emerg. Infect. Dis. 19:11. doi: 10.3201/eid1911.130464.	Dogs (*Canis domesticus*)	Yes	Signs of ataxia, exotropia, a head tilt, and stunted growth were observed in a litter of 5 puppies. Four die at 5 – 6 weeks, blood sample from survivor (age of 3 months) VNT as well as ID vet test were performed to the puppy and the mother, showing negative results for the puppy and positive for the mother. RT-PCR was also performed to the puppy, identifying it as positive	The study used several test techniques to scrutinize the suspected animals adapting the methods in order to cope with the differentiation between ruminants and canines.
Wensman JJ, Blomqvist G, Hjort M, Holst BS, 2013. Presence of antibodies to Schmallenberg virus in a dog in Sweden, J Clin Microbiol. Aug;51(8):2802-3. doi: 10.1128/JCM.00877-13	Dogs (*Canis domesticus*)	None recognised	One positive female dog out of 86 (sample tested with IDvet and confirmed with serum neutralization test)	No details are given on how the animals were selected to be included in this study.
Garigliany MM, Desmecht D, Bayrou C, Peeters D, No Serologic Evidence for Emerging Schmallenberg Virus Infection in Dogs (Canis domesticus). Vector Borne Zoonotic Diseases 13(11), 830-833 doi: 10.1089/vbz.2012.1251.	Dogs (*Canis domesticus*)	None recognised	IDvet results showed all 132 serum samples but one were negative, the last being classified as doubtful (S/P between 60 and 70%). Second generation ELISA confirmed the results from IDvet test.	No details are given on how the animals were selected to be included in this study. The study includes dogs with potentially high level of exposure to SBV, containing three groups according to their exposure.

Laloy Eve, Emmanuel Breard, Corinne Sailleau, Cyril Viarouge, Alexandra Desprat, Stéphan Zientara, François Klein, Jean Hars, Sophie Rossi. 2014. Schmallenberg Virus Infection among Red Deer, France, 2010- 2012**.** Emerg. Infect. Dis, 20, 131-134.	Red deer	No reported	Three tests were used. i-ELISA ID Screen Schmallenberg Virus Indirect, Bicupule; ID Vet (S/P<60%, negative; S/P>70%, positive; and S/P 60–70%, doubtful result), c-ELISA; ELISA ID Screen Schmallenberg Virus Competitive; ID Vet (Positive results by c-ELISA corresponded to a percentage of inhibition (PI) <50, doubtful result if 40>PI≤50, and negative when PI >50) and seroneutralization test (SNT). The two ELISA methods exhibited a 92% match (449/486), larger discrepancies were observed between the ELISA tests and the SNT results, indicating low sensitivity and specificity for the ELISA tests.	The study used several test techniques to scrutinize the samples. No details are given on how the samples were selected. The three tests were only performed in 23% of the samples available due to bacterial contamination or cytotoxicity.