Schmallenberg virus

Technical and scientific studies[1]

Final report
March 2014

[1]Commission Implementing Decision of 27 June 2012, supporting studies on "Schmallenberg" virus by the five-country consortium of veterinary research institutes coordinated by the Central Veterinary Institute in the Netherlands:
Friedrich-Loeffler-Institut (FLI), Germany;
Veterinary and Agrochemical Research Centre (VAR-CODA-CERVA), Belgium;
L'Agence nationale chargée de la sécurité sanitaire de l'alimentation, de l'environnement et du travail (ANSES), France;
Animal Health and Veterinary Laboratories Agency (AHVLA), United Kingdom;
Central Veterinary Research Institute of Wageningen University and Research (CVI), The Netherlands (coordinator).

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Summary

Schmallenberg virus (SBV) emerged in Europe in 2011. First acute infections were detected in cattle in late summer 2011. They induced a short fever period and a marked reduction in milk yield in dairy cattle. In a number of farms, especially in the Netherlands, severe diarrhoea was a first striking clinical observation. The virus was first identified in November 2011 and named after the village in Germany where the first definite samples originated from. The virus was putatively included in the Simbu serogroup of the genus *Bunyavirus* family, genus *Orthobunyavirus*. In December 2011, congenital malformation was reported in new-born lambs in the Netherlands linked to the presence of the virus. Subsequently up to March 2012, Belgium, Germany, United Kingdom, France, Luxembourg, Italy and Spain reported congenital malformations in lambs and calves, and the presence of SBV was confirmed by Polymerase Chain Reaction (RT-PCR) testing.
This was the first time that this virus had been isolated in Europe. Very little information was known of this emerging pathogen, most assumptions were extrapolated from scientific information available on other viruses of the Simbu serogroup. No efficient diagnostic tools were available to assess the actual spread of SBV and its impact on animal health. There were no harmonised rules with regard to the control or notification of SBV.

On 23 January the Agriculture Council requested the European Commission to take action with respect to the SBV outbreak and in February 2012, the European Commission in close collaboration with the Member States identified the priorities and areas for which additional information should be gathered prior to consideration of veterinary legislation addressing the SBV infections. These were in particular the pathogenesis, the epidemiology, the confirmation of the non-zoonotic potential of the virus, and the methods to diagnose the disease in animal samples including their validation.
In March 2012 technical and scientific studies on Schmallenberg virus were started commissioned by the European commission and the involved EU member states according to Commission Implementing Decision of 27 June 2012. A large part of the scientific studies were performed by a five-country consortium (Belgium, Germany, France, United Kingdom and The Netherlands) coordinated by the Netherlands. Within this consortium the objectives as well as the methods of the studies were discussed repeatedly and in a number of cases shifted or adapted based on increased scientific knowledge.

From the SBV technical and scientific studies performed by the consortium coordinated by the Netherlands it can be concluded that Schmallenberg virus primarily infects domestic and wild ruminants and cattle and sheep seem to be the most susceptible species. Schmallenberg virus was introduced in Europe in 2011. After exposure SBV rapidly spread within naive herds, and also throughout winter. Blood samples collected before the first clinical cases of SBV were observed in Europe in 2011 were all tested negative for SBV antibodies. The origin of the virus remains unknown. Certain species of Palearctic *Culicoides* biting midges are the main vectors of SBV. Transovarial SBV-transmission in culicoids has not been observed. In pregnant cattle and sheep, the virus can infect multiple organs of the un-borne foetus and this infrequently leads to malformations. For detection of SBV sensitive RT-PCR assays have been developed and validated and for diagnosis of previous SBV infection reliable virus neutralization tests and ELISAs have been developed and validated. Schmallenberg virus was detected in semen and embryos from SBV-infected cattle and sheep, respectively. A frequency of 0-4% SBV-RNA-positive bovine semen batches was found in the participating countries. Subcutaneous injection of SBV-RNA-positive semen in cattle and mice demonstrated that semen from SBV-infected cattle may contain viable SBV. In-vitro studies

with embryos suggest a negligible risk for SBV-transmission. Whether infectious virus can be transmitted to susceptible cows at service or by insemination is unknown.

As a result of the Schmallenberg virus technical and scientific studies a lot of scientific information of Schmallenberg virus issues has been obtained. Increased insights in SBV topics and related issues also revealed that there are several important topics remaining for which study is recommended. This includes the tracing back of the SBV origin and in relation to that the study on SBV strain variation. A risk analysis of possible ways of introduction may be helpful to avoid new introductions of SBV-like viruses in future. To better understand the role of the arthropod vector in the epidemiology further study of SBV and related Simbu serogroup orthobunyaviruses vector competences will be needed. To elucidate the role of SBV-contaminated gametes in the epidemiology of SBV studies on SBV transmission via artificial insemination are required. To early detect recurrent cases of SBV in ruminants a basic surveillance is recommended and to detect new emerging arthropod borne viruses monitoring of sentinel herds together with midge trapping may be useful.

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Area 1 : Pathogenesis

Main objectives

To determine replication and virus shedding and to assess the virulence of the virus in young and adult animals (in particular in sheep cattle and goat). To determine the dynamics of the virus towards and in fetusus and to determine the pathogenicity of the virus in fetuses at different gestation stages. To study the development of immunity to Schmallenberg virus. This included onset of immunity and estimations of protection of immunity after infection.

Workplan (concise)

Infection experiments were done with all three major target species (cattle, sheep and goat). Experimental infections in pregnant cattle were carried out by partner D (FLI). Inoculations were performed at different gestation stages (around d60, d90, d120 and d150). Inoculation route: 1ml subcutaneous titer: 10E5-10E8. FLI prepared a master stock (1ml aliquots x 600-700) which was used by all partners. The inoculum was bovine serum of 3dpi (assumed to be closest to natural infection).

Experimental infections in sheep were performed by partner NL (CVI) and Be (CODA). Inoculations were performed at different gestation stages (around d20, d40 and d60). Inoculum: bovine serum 3dpi (assumed to be closest to natural infection). Inoculum was provided to partners by FLI.

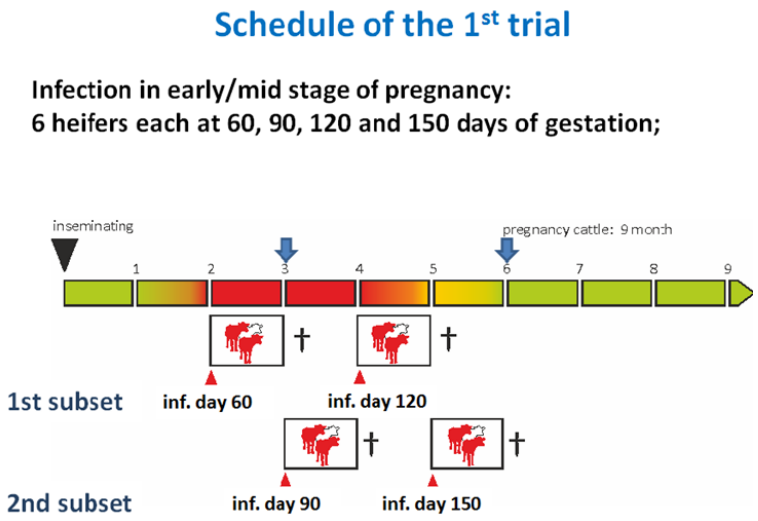
Experimental infections in goats were performed by partner Fr (ANSES/INRA, LNCR). This study involved a large number of animals (>80). Gestation stages at inoculations were harmonized with sheep inoculations as much as possible (gestation stages around d20, d40 and d60).

In the experimental infections, samples for testing immunological parameters were collected and provided to partners.

1.1 Pathogenesis in pregnant animals

1.1.1 Studies of Schmallenberg virus pathogenesis in pregnant cattle

Following Schmallenberg virus (SBV) infection, ruminants have shown clinical pictures which are very similar to AKAV including malformation of lambs, calves and kid goats with the arthrogryposis-hydranencephaly-syndrome (AHS) as the guiding symptom complex.
As for most target species no or only very limited data about duration of viremia, incubation time, virus distribution and shedding were available, the collection of experimental data about the pathogenesis of Schmallenberg virus for pregnant animals and especially for their fetuses was the main target of the study.
To characterize the dynamics of the virus towards and in fetus, experimental infection studies in pregnant cattle at different gestation stages were performed in order to determine the pathogenicity of the virus in fetuses at different gestation stages.



In a first animal trial, 4 groups of 6 pregnant heifers each were subcutaneously inoculated with the FLI standard SBV challenge virus preparation (2x 0.5ml serum pool, Wernike et al., 2012) at different stages of pregnancy. All adult animals became infected and showed comparable titers and duration of viraemia. In the adult animals no clinical disease was recorded, but 3-6 animals of each group showed elevated rectal body temperatures for several days. All animals seroconverted for SBV antibodies.

Experiment I and II: sampling schedule

dam:
- serum/EDTA blood
- maternal placenta: uterus, caruncle
- RES: tonsil, Ln. mesenterialis, spleen; Peyer's patches/intestine

fetus:
- serum/EDTA blood
- fetal placenta: cotyledon, amniotic membrane and fluid, umbilical tissue
- RES: Ln. cervicalis superficialis, spleen
- parenchyma: lung, kidney, liver, cartilage, muscle
- neural tissue: cerebrum, cerebellum, brainstem, spinal cord
  cerebrospinal fluid
  meconium

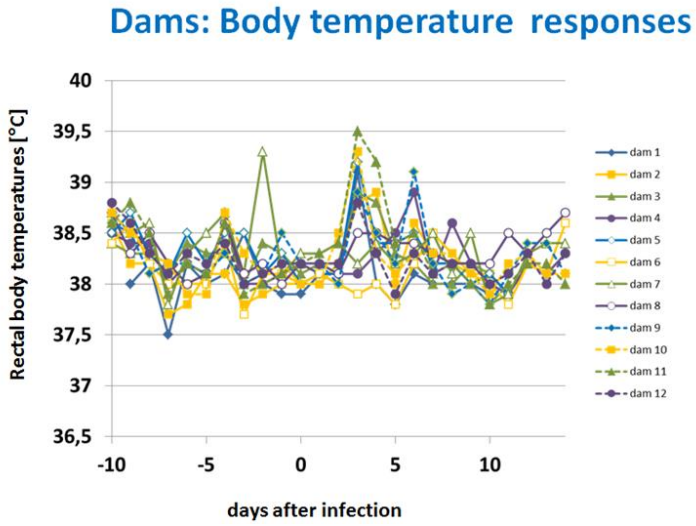
Minimal invasive sampling; fetuses were *in toto* formol-fixed

A broad panel of maternal and fetal tissue and organ samples as well as body fluids was collected at necropsy 6 weeks after infection.
At post mortem SBV RNA was not detected in the circulation of the dams but in the lymphoreticular tissues of each adult animal and in maternal placental tissues of most of the animals independently from the stage of gestation at the time point of infection. An overall correlation between SBV positive maternal/fetal placenta and positivity of the fetus was observed.
Viral genomes in the fetal circulation were detected in 2 out of 6 fetuses whose mothers were infected at d60, in 1/6 at d90, in 3/6 at d120, and in none of the 6 fetuses of the day 150 group. SBV positive fetal parenchyma were found in all groups with exception of the fetuses of the d150 group. 4 fetuses each scored positive in lymphatic tissues in the d60 and d120 groups and one of the fetuses in the d90 and d150 groups. After infection at the time point d120, genome loads were detected in the CNS of 4 animals.

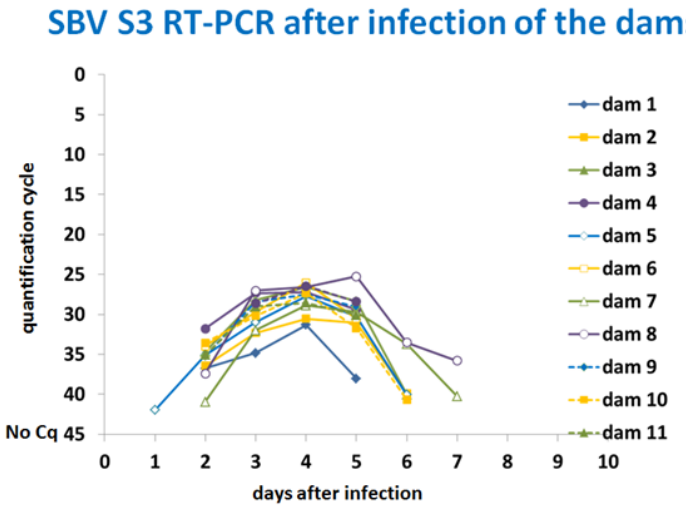
	Circulation	Parenchyma	RES	CNS
Group d60	2/6	1/6	4/6	2/6
Group d90	1/6	1/6	1/6	2/6
Group d120	4/6	4/6	4/6	4/6
Group d150	0/6	0/6	1/6	0/6

Typical malformations with torticollis and arthrogryposis were obvious in only one of the 24 fetuses. Infection of the corresponding dam was carried out at d90 of gestation. The only fetal organ that was found SBV genome positive was the cerebellum of the unborn with a cycle of quantification (cq) value of 36. Therefore, no correlation between viral genome loads and congenital deformity could be established.
In conclusion, no common patterns of infected organs could be identified. Sites and amounts of virus replication were varying to a high degree in the individual fetuses. Infectious virus could not be recovered from the amniotic fluids of the fetuses neither after inoculation on insect cells nor in inoculated Vero cell cultures. Moreover, no histological alterations could be observed in the fetuses and in situ hybridisation with SBV genome probes was not successful

in the progeny. The fetuses were collected at 6 weeks after infection of the dams. Therefore, infection was probably already resolved and infectious SBV was eliminated from the fetuses.
The experimental data confirmed that diaplacental SBV infection in cattle is a very rare event. Observations in field studies underline that less than 10% of the offspring of susceptible SBV antibody naïve dams are found positive for SBV genomes or precolostral antibodies. In summary, it can be stated that the cattle model has turned out to be not really suitable for SBV pathogenesis studies due to the very low numbers of malformed calves after in utero infection of unprotected fetuses. Case control studies suggest an overall percentage of <1% malformed fetuses after SBV infection in cattle.
As a conclusion of our study, the vulnerable phase of SBV infection in pregnant cattle is approximately ranging between day 60 and day 150 of pregnancy. These finding are in accordance with field observations after AKAV infection in cattle. Fetuses are refractory to SBV infection or replication in the later stages of gestation. This study also confirms a very low ratio of precolostral seroconversion in immunocompetent fetuses. After inoculation at d150 of gestation none of the 6 fetuses reacted positive for SBV specific antibodies.
In a subsequent animal experiment, pregnant heifers were infected approximately at d120 of pregnancy (d105-120). Inoculum, infectious dose and route of infection were coincident with the first trial. In order to elucidate pathogenesis at early stages of SBV infection, necropsy was carried out between 10 and 29 days after infection. 2 animals each were sacrificed at d10/11, d14/15, and d28/29.
One of the heifers aborted at day 4 after infection. The fetus could be collected and a nearly complete panel of tissue and body fluid samples was acquired.
One of the heifers was found to be not pregnant at the end of the study.
The residual 10 pregnant animals yielded normally developed, macroscopically inconspicuous fetuses.



Besides raise in body temperatures, no clinical disease was observed in the heifers.
According to the first experiment, all dams were infected at comparable levels with a duration of viremia between 4-6 days.



At necropsy SBV genome was detected in lymphoid tissues of all dams. Uterus and maternal placenta scored positive up to day 15 post infection (p. i.).
Fetal parts of the placenta were also tested positive for SBV genome until day 15 p.i.
Positive RT-PCR results on fetal serum and blood plasma were obtained until day 15 p.i. However, only 1 out of 4 fetuses was still positive until d14/15.
Viral loads in fetal parenchyma were only detected in the aborted fetus (d4 p.i.) and in one unborn at d10 p.i.
After SBV inoculation at d120 of gestation the proportion of infected fetuses was lower than in the first experiment:

Study I:	6 / 6 fetuses RT-PCR positive	5 fetuses ≥ 2 organs positive
Study II:	6 / 11 fetuses RT-PCR positive	5 fetuses ≥ 2 organs positive

In the second study a low percentage of infected fetuses was again confirmed.
Until 4 weeks after infection of the heifers no malformed fetus was detected.
A low in utero transmission rate of SBV to the fetus was evident, even in early stages of infection no relevant genome loads in the developing fetuses were observed. Histological investigations are still in progress.
Abundant virus replication at the maternal/fetal barrage was evident with rapidly decreasing numbers of genome copies in fetal/maternal placenta tissues over the time.

1.1.2 Transplacental infection in sheep in the first trimester of gestation

In the first year after the recognized introduction of Schmallenberg Virus (SBV) into North-West Europe musculoskeletal malformations and pathological changes of the central nervous system as porencephaly, hydranencephaly and hypoplasia of the cerebellum in new-born lambs and calves were the most intriguing clinical features of this infection (1). Therefore, SBV joins the group of other teratogenic, arthropod-borne viruses such as Akabane virus. On the basis of epidemiological studies of the recent SBV outbreaks and the comparison with the pathogenesis of Akabane virus, it is assumed that the teratogenic infection takes place in the first trimester; however, the efficiency of transplacental infection generally and in relation to the gestation time point is unknown. Also, information on the transplacental transfer and the

virus tropism in the uterus and foetus is lacking. Recent studies on central nervous tissue of naturally infected, new born lambs and calves have described a differential distribution of virus and inflammatory cells, if present in the CNS. In an animal study, the early transplacental infection at 5 and 6 weeks of gestation was examined.

Twenty-one SBV sero-negative ewes (Texelaar breed) of primo- or multiparity were acquired from a Dutch herd with a known low incidence of seropositive sheep. The ewes were synchronized by hormone treatment and mated by natural mating. This resulted in 95% of pregnancy. All pregnant ewes were inoculated subcutaneously with 1 ml of SBV viraemic calf serum (provided by, FLI Riems) at either day 38 (n=10, group 1) or day 45 (n=11), group 2) of gestation. Ewes were followed for seven days by clinical observation and repeated serum analysis was done to demonstrate viraemia by PCR analysis. Seven days post inoculation the ewes were euthanized and tissue samples were taken from the reproduction tract, especially several placentomes, and from the fetuses (umbilical cord, skull, including CNS and amnion fluid). All samples were investigated by RT-qPCR for the presence of SBV mRNA.

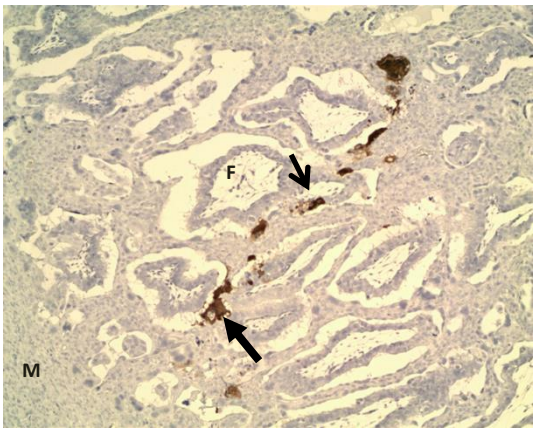


Figure 4a. Placentome of SBV infected ewe, 7 days post infection; SBV antigen detected in the maternal placental epithelium (close arrow) and in the fetal placenta (open arrow), demonstrating the transplacental transfer. Note the focal distribution of virus antigen and the lack of inflammation in the placental tissue. Immunohistochemical staining, low microscopic power field; M = maternal placenta, F = fetal placenta

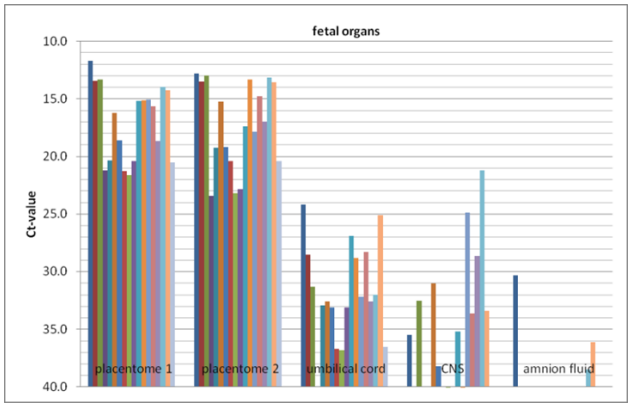


Figure 4b. Detection of viral nucleic acid by PCR assay in fetuses of ewes (infected at 38 days of gestation) at 7 days post inoculation. At this time point no virus was detected in the blood, but a high virus load was observed in the placentomes and dispersion of SBV virus in the fetal tissues, including CNS.
Data show results from two different placentomes, the umbilical cord the CNS and amniotic fluid.

Three days after inoculation of the ewes in all, but one ewe sera were positive in the qPCR (mean PCR ct value: 21,7 (group 1), 20.9 (group 2)) and during necropsy samples were taken from a total of 39 fetuses (n = 20, group 1) and n = 19, group2).

No morphological changes were observed at this early time point after infection in any of the fetuses. Generally, the placental tissue was unchanged and no inflammatory reaction was seen in the placenta. However, by immunohistochemical staining few foci with SBV antigen were found in the maternal and fetal placenta epithelium . In these areas also a focal epithelial necrosis was observed (Figure 4a). PCR analyses revealed that placentomes taken at seven days post infection were positive in all viraemic ewes with a mean ct value of 17.1 in group 1 and 18.9 in group 2. In all ewes at least one fetus contained SBV nucleic acid in either umbilical cord or CNS. In 85% of the fetuses of group 1 the umbilical cord was positive for SBV with a mean PCR ct value of 32.2 and 74% of group 2 with a mean PCR ct value of 31.7. Skull tissue (including CNS) was SBV positive in 55% of the fetuses of group 1 and in

74% of group 2 with a mean PCR ct value of 33.0 and 34.1, respectively. In 15% (group 1 and 11% (group2) of the fetal amnion fluids SBV was detected (Figure 4b.).
From this study it can be concluded, that SBV is able to very efficiently pass the placental barrier and infect the fetus in the first trimester of the gestation. The changes in the placenta are mild and focal and it is expected that these changes do not directly interfere with the functionality of the placenta.

1.1.3 Schmallenberg virus experimental infection in pregnant sheep

Methods:
At CODA-CERVA, 50 SBV seronegative sheep (breed Moureroux) have been synchronized and inseminated. After pregnancy was assessed via echography and blood analyses, only 23 ewes turned out to be pregnant. These were divided in three groups : i) group 1 with 8 ewes that were subcutaneously infected with infectious SBV serum (provided by FLI) 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 (3 at day 45 and 3 at day 60 of gestation) with PBS. Also 4 non-pregnant ewes were kept in the same experimental unit as an environmental control. After SBV inoculation, blood samples and feces were collected each day during the first two weeks and afterwards once each week till the end of the experiment. Ewes were kept till the end of gestation. When signs of birth became apparent, colostrum was collected, the ewes were anesthetized and a caesarian section was performed. Immediately thereafter, ewes were euthanized and autopsied and lung, spleen, ovaries, lymph nodes, cotyledons and placenta and amniotic fluids were collected. The lambs were assessed for malformations or other aberrant clinical signs and their capability to stand up and drink milk was evaluated. Thereafter blood was collected followed by euthanasia during which cerebrum, cerebellum, brain stem, spinal cord, lymph nodes, spleen, kidney, lung, thymus, muscle tissue, cartilage tissue, reproductive organs, umbilical cord and meconium were collected.

Results:
Analysis by CODA-CERVA of the blood samples collected from the pregnant ewes after SBV inoculation at 45 and 60 days of gestation showed that a viremia has occurred in each infected ewe. This viremia was followed by a seroconversion in all infected animals and SBV has been found at day 4 and 5 pi in the feces of some ewes. No virus or antibodies have been detected in the control animals.
Only one lamb was born before the expected date and was in good health. It drunk colostrum from the mother and subsequently showed elevated anti-SBV antibody titers. Few lambs from both the control and infected groups were dead at birth but showed no abnormalities. 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 number 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 the umbilical cord, one in brain tissue and another in cartilage tissue.

The results show 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 analysis has to be performed, 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. Future studies in which ewes are infected at later stages of gestation will have to show if malformations can be reproduced under experimental conditions. It should furthermore also be pointed that the outcome of infection could be influenced by the sheep breed and that till now only one virus isolate, originating from SBV infected calves, has been used in these kind of studies.

1.1.4 Schmallenberg virus experimental infection in goats

Field observations and findings from serological survey studies suggest that goats are generally less prone to SBV infections and its teratogenic effects in pregnant dams than cattle and sheep. However, at present it is still unknown whether this difference is linked to a differential susceptibility of the three ruminant livestock species to SBV infections, or is rather due to different housing conditions or a preference of *Culicoides* biting midges for cattle and sheep. In two experimental infection studies we have assessed the ability of SBV (i) to induce viremia in non-pregnant goats and he-goats, and (ii) to elicit teratogenic effects in pregnant goats at two different times of gestation (day 28/42). In a third experimental infection study (to be completed in June 2014) we are currently assessing the effects SBV infections (prior and concomitant to artificial goat insemination) on the female reproductive physiology, fertility parameters, and fetal development.

Methods:
(Experiment 1 – October/November 2012) A pilot experimental infection study was performed at the INRA-PFIE animal experimental platform using 4 non-pregnant goats and 2 he-goats to test the efficacy of the SBV inoculum (infectious bovine serum pool kindly provided by Dr. Martin Beer, FLI; Wernike et al., 2012) to induce viremia in this ruminant livestock species. To this end, all animals were inoculated with 2 x 0.5 ml infectious serum and monitored during a period of 4 weeks. Whole blood samples were taken daily between days 0 and 7 post infection (p.i.) to determine the onset and duration of viremia by qRT-PCR. Serum samples were collected weekly to test for SBV-specific seroconversion. Semen samples were collected twice weekly from the inoculated he-goats to test for the presence of SBV RNA by qRT-PCR. 4 weeks p.i. all animals were euthanized and necropsies were performed. Genital tract samples (uterus, oviducts, ovaries, and oocytes) were collected from the necropsied goats and subsequently scored for the presence of SBV by qRT-PCR.

(Experiment 2 – November 2013 to March 2014) A total of 29 goats were purchased from French breeders and tested by the LNCR to confirm their sanitary status (seronegativity for SBV, Brucellosis, and Q-Fever). From November 2013, all animals were housed in the insect-proof experimental facilities at the INRA-PFIE. On December 27th 2013 all animals were oestrus synchronised using vaginal sponges, and on January 10th 2014 an artificial insemination (AI) protocol was employed using 2 frozen straws per goat that were obtained from one selected he-goat. Pregnancy was assessed by echography and hormonal profile analyses on day 21 (24/29 animals positive) and confirmed by echography for 14 out of 29 goats at day 42 of gestation. The 14 pregnant dams were distributed into 3 different groups: groups A (5 animals), B (5 animals), and C (4 animals). The dams of group A were inoculated with 2 x 0.5 mL of infectious serum (Wernike et al., 2012) at day 28 of gestation. The dams of group B were inoculated with infectious serum at day 42 of gestation. The dams of groups C

were mock (PBS)-inoculated at day 28 (2 animals) or 42 (2 animals) of gestation. Whole blood samples were taken during 7 days p.i. to determine the onset and duration of viremia in the infected dams. During 2 weeks (4 days before infection until 10 days p.i.), the body temperature was measured in all dams by using rumen temperature boluses. Serum samples were collected weekly during the trial from all dams to test for SBV-specific seroconversion. At days 53 to 56 all animals were euthanized and necropsies were performed. The dams and foetuses were macroscopically scored for pathological lesions and/or foetal abnormalities and a wide array of maternal and foetal tissue samples were collected for downstream analyses including qRT-PCR, histopathology, and immunohistochemistry.

(Experiment 3 – February 2014 to June 2014) A total of 45 goats were purchased and tested by the LNCR to confirm their sanitary status (seronegativity for SBV, Brucellosis, and Q-Fever). Since February 2014, all animals are housed in the insect-proof experimental facilities at the INRA-PFIE. The animals will be distributed into 5 different groups: groups A (10 animals), B (10 animals), C (10 animals), D (10 animals), and E (5 animals). In April 2014 all animals will be oestrus synchronised and artificially inseminated as described earlier (see Experiment 2). 7 days prior to AI, the animals of group A will be inoculated with 2 x 0.5 mL of infectious serum (Wernike et al., 2012). The animals of group B will be inoculated with infectious serum on the same day the AI protocol will be performed. Attempts will be made to infect the animals of group C by AI with SBV (infectious serum)-spiked semen. At the day of the AI, the animals of group D will be inoculated with PBS (mock control). As a control for group C, the animals of group E will be artificially inseminated with non-infectious serum-spiked semen.
Body temperature measurements and whole blood/serum sample collections will be performed exactly as described for Experiment 2. In addition, blood samples will be taken from all goats at various time points during the trial to assess by ELISA protocols the putative effects of SBV on the endocrinological profile of the infected animals. At day 35 of gestation pregnancy will be assessed by echography, and all non-pregnant animals will be euthanized. Tissue samples from the genital tract (uterus, oviducts, ovaries, and oocytes) of the non-pregnant goats will be collected for downstream analyses including qRT-PCR, histopathology, immunohistochemistry, and fertility assays. All pregnant dams will be euthanized and necropsied at days 53-56 of gestation. Sampling of maternal and foetal tissues and downstream analyses will be performed exactly as described for Experiment 2.

Results:
(Experiment 1) All goats and he-goats developed viremia without showing clinical signs of infection. All genital tract samples from 3 goats (ovaries, follicular fluid, cumulus cells and oocytes, uterus) scored negative for the presence of SBV RNA. However, samples of the two ovaries (left and right) from 1 goat showed positive qRT-PCR results. For the two he-goats, a total of 8 semen batches were collected during the course of the experimental infection. However, no SBV RNA was found in all samples processed from fresh sperm or frozen-thawed semen.

(Experiment 2) All inoculated dams from groups A and B developed viremia between days 3 and 5 p.i. and SBV-specific seroconversion from day 14 p.i. In agreement with our observations from Experiment 1, no clinical signs of infection (including elevated body temperatures) could be observed for the SBV-inoculated animal groups. Upon necropsy, no gross lesions could be detected in the maternal carcasses from all groups. All 9 foetuses (1 x 1, 2 x 2, and 1 x 4 foetuses per dam) obtained from the 4 dams of the control group C showed a normal size and morphology. In contrast, among the 11 foetuses (1 x 1, 2 x 2, and 2 x 3 foetuses per dam) obtained from the 5 dams of group A (SBV-inoculation at day 28 of

gestation), we found 3 foetuses from 2 different dams (2/2 and 1/3 foetuses, respectively) showing clear abnormalities with respect to size and morphology. A representative gross pathological finding is shown in figure 5 (upper panel, foetus 1). In addition, we detected slight morphological alterations (haemorrhagic, glossy, and swollen aspect of foetuses) in 2 foetuses (2/2 foetuses) from an additional dam of group A. Similar alterations were observed for 2 foetuses (2/3 foetuses) from 1 of the 5 dams of group B (SBV-inoculation at day 42 of gestation). A representative gross pathological finding is shown in figure 5 (middle panel, foetuses 1 and 2). All the other of the 10 foetuses (1 x 1, 3 x 2, and 1 x 3 foetuses per dam) obtained from the 5 dams of group B showed a normal size and morphology. Further analyses described in the study design are being performed at the present time and will complement our macroscopic findings.

Figure 5. Gross pathological findings representative for some of the foetuses obtained from dams of groups A (SBV-inoculation at day 28 of gestation) and B (SBV-inoculation at day 42 of gestation). Normally developed foetuses from group C (mock control) are shown for comparison. Note the different size and morphology of foetus 1 from animal #046 (group A) compared to normally developed foetuses 2 and 3 from the same dam.

1.2 Pathogenesis in non-pregnant animals

1.2.1 Pathogenesis of different SBV isolates in cattle

To study differences in the pathogenesis caused by different SBV isolates, three 6-months-old Holstein cattle were infected with any of three whole-blood or serum samples obtained from cattle in the field in 2012 or from cattle experimentally infected with a SBV isolate from 2011. The table below (Table 2) gives the origin, C_q values, the preparation of the inocula and the infected animals. All specimens were stored at 4°C until experimental infection. The three cattle were subcutaneously infected at multiple sites in the shoulder and dorsal thorax regions.

Table 2. Animals, origin and preparation of inocula used for SBV infection of cattle.

Cattle (C) no.	Submission/ Identification no.	Origin (federal state)	Inoculum	C_q-value
C2	648/12-1	Hessen	2 ml whole blood[a] + PBS	20
C3	612/12-3 + 612/12-4	Baden-Wuerttemberg	2 ml whole blood[a] + 2 ml whole blood[a]	26 + 22
C4	Challenge-Serum 1 (Cattle no. 20)[b]	North Rhine-Westphalia	2 ml serum	26

[a] containing potassium EDTA and antibiotics; [b] obtained from cattle experimentally infected with SBV; PBS, phosphate buffered saline

Serum and whole-blood samples were collected 2, 3, 4, 5, 6, 7, 10, 12, 14, 17, 21 and 28 days post infection (dpi). The samples were analysed for SBV-RNA and antibodies using real-time RT-PCR (RT-qPCR) and ELISA, respectively. At post-mortem examination at 31 dpi, tissue samples were collected from spleen, liver, lung and from mediastinal, mesenteric and mandibular lymph nodes. Rectal temperature and clinical signs were monitored daily for the duration of the experiment.

SBV-RNA was detected earlier (from 2 to 6 dpi) in C2 and C3 than in C4 (from 3 to 10 dpi). C_q values were similar for serum and whole blood (data of whole blood samples not shown) (Fig. 6). Seroconversion was detected 12 dpi or 14 dpi. Rectal temperature and clinical signs were not observed in any of the cattle. At post-mortem examination, SBV-RNA was found in the spleens and mesenteric lymph nodes of all cattle, in mesenterial and mandibular lymphnodes of two cattle, but not in the livers or lungs (Table 3).

The late onset of the infection in C4 was possibly due to long storage of the challenge serum at 4°C: 6 months compared to approximately 2 to 5 weeks of storage of the whole blood samples. However, no other differences in the pathogenesis of SBV infection were found between the SBV isolates.

Table 3. C_q-values of tissue samples collected at post-mortem examination 31 dpi.

Cattle (C) no.	SBV-S3 RT-qPCR					
	Spleen	Liver	Lung	**Mediastinal lnn.**	**Mesenteric lnn.**	**Mandibular lnn.**
C1	29.74	No C_q	No C_q	37.53	35.60	No C_q
C2	34.29	No C_q	No C_q	35.21	32.77	32.83
C3	31.78	No C_q	No C_q	No C_q	26.85	33.45

lnn., lymphnodes

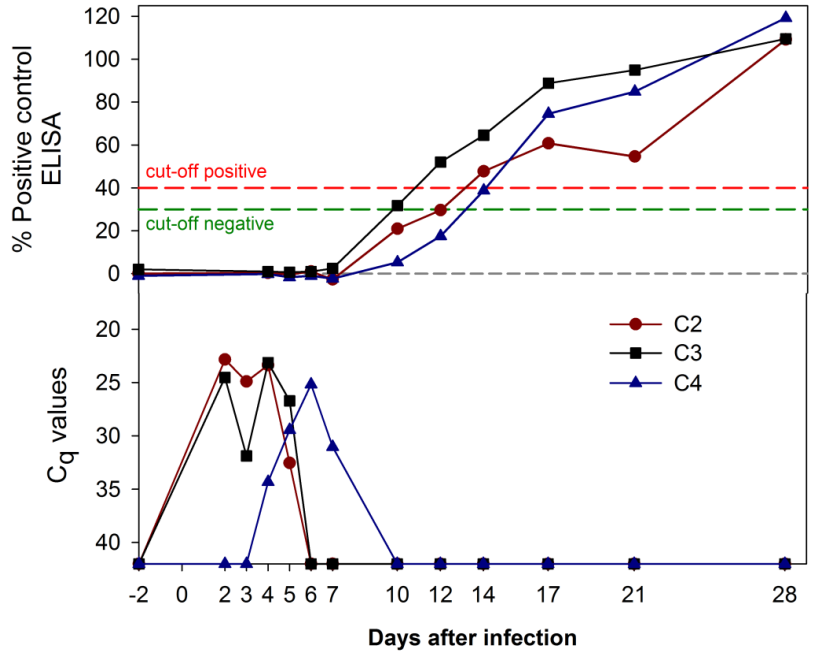


Figure 6. Progression of the amount of SBV-RNA and antibodies in serum of cattle comparatively infected with any one of three different SBV isolates. The ELISA cut-off was set according to the current manufacturers' recommendation.

1.2.2 Pathogenesis of a German SBV containing serum collected from cattle in 2012 in experimentally infected sheep

In the previous section, we reported the experimental infection of 6-month old Holstein calves with SBV isolates from cattle originating from different federal states in Germany. Cattle (C) no. 3 ('TV13/12_R790') was subcutaneously injected with 2 ml of antibiotic treated whole-blood collected from two cattle from Ravensburg (C_q 26) respectively Biberach (C_q 22) in Baden-Wuerttemberg, Germany, in August 2012. Serum collected from this calve at 4 dpi (C_q 22.75) was injected in sheep to investigate whether the serum contains infectious SBV suitable for SBV challenge infection of sheep (and cattle).

Methods:
Five lambs were purchased from local German breeders. After acclimatisation of the animals, serum was collected one week before experimental SBV infection, to proof them free of circulating SBV-RNA and antibodies. Each of the five sheep was inoculated twice with 0.5 ml of the serum of C3 at two locations in the shoulder region. Serum samples were collected for serological and virological analyses daily from 1 to 10 dpi and at, 14, 21, 28 and 29 dpi. Spleens and mesenteric lymph nodes were collected post-mortem at 29 dpi.
Serum samples were tested for SBV RNA and antibodies by ELISA and SBV-S3-specific RT-qPCR (Bilk et al., 2012).

Results:
All five sheep seroconverted between 6 and 10 dpi and remained seropositive until the end of the study at 29 dpi. SBV-RNA was detected in serum at 1 or 2 dpi for a period of 3 to 4 days and in spleen and/or mesenteric lymph nodes of 4 of 5 sheep (Table 4) at 29 dpi. One sheep (S30) was negative for SBV-RNA in the tested tissue samples although the serological and virological results were similar to those of the other sheep.

Table 4. C_q-values of tissue samples collected post-mortem at 29 dpi.

Sheep (S) no.	SBV-S3 RT-qPCR	
	Spleen	**Mesenteric lymph nodes**
S7	38.4	No C_q
S17	No C_q	34.8
S24	33.3	No C_q
S27	32.1	37.2
S30	No C_q	No C_q

Serological and virological results were similar to those of sheep and cattle experimentally infected with other SBV sera in previous studies. Presence of SBV-RNA in spleen and mesenteric lymph nodes is a common finding in most, but not all, SBV-infected ruminants (Wernike et al., 2013). The SBV serum collected from and passaged in cattle has proven to be infectious for sheep and can therefore be used for challenge infections in the future.

Other laboratories (including AHVLA and CVI) also isolated a SBV strain from cattle and carried out an initial characterisation in a sheep model, which confirms that biologically characterised isolates can be used alike.

1.2.3. Influence of inoculation route and inoculation dose on SBV infection in sheep

Methods:
At CODA-CERVA, two experimental infection experiments in sheep (Mourerous) have been performed to address the i) impact of the inoculum route and ii) the impact of the inoculum dose on the outcome of infection. In the first experiment, three groups of three ewes each were infected with an SBV infectious serum (provided by FLI) via either the intranasal, intradermal or subcutaneous route. In the second experiment, four groups of three ewes each were subcutaneously infected with either an undiluted or 1/10, 1/100 or 1/1000 dilution of the SBV infectious serum provided by FLI. In each experiment, blood samples were collected daily and animals were euthanized at 10dpi. At autopsy, samples from brain, lymph nodes, spleen and lung were collected.

Results:
In any of the two experiments, sheep showed clinical signs upon SBV infection. The results showed that both intra-dermal and subcutaneous infections could induce productive SBV infections based on the presence of an RNAemia and seroconversion by the end of the experiment. The intranasal route of infection did not result in a productive infection but virus could however be detected in the feces. The experiment with the different inoculation doses showed that a critical amount of virus has to be administered to induce a productive infection since all animals inoculated with the undiluted and 1/10 diluted serum became RNAemic while only 1 sheep inoculated with the 1/100 dose and none of the sheep inoculated with the 1/1000 dose became RNAemic. Interestingly, when a sufficient amount was administered to induce a productive infection, no statistical difference in the length and height of RNAemia was found between the different inoculum doses. All animals that became RNAemic seroconverted before the end of the experiment. Anti-SBV antibodies could however only be detected by seroneutralisation tests and not with a commercial ELISA, probably because the latter only detected IgG antibodies that are most probably not yet present at those early time points post infection.

1.3 Pathogenesis in seropositive and seronegative animals

Results from in vivo studies and from the field indicate the persistence of SBV in cattle and sheep. However, some animals have declining antibody titres arguing against a persistent infection in all animals.

Methods:
Partner AHVLA infected a group of 12 sheep with the infectious serum provided by FLI in February 2012 and maintained these sheep for one year. At the end of this period, we killed some to test for the detection of SBV persistence. The investigations into this aspect are ongoing. In addition, an immunosuppressive drug (dexamethasone) was appllied in four animals seeking for a reactivation of virus. The final group of 4 animals was re-challenged one year after infection. At that time, these animals had still detectable Ab titres, but in most cases well below their immediate post infection levels.
A commercially available vaccine was bought at the end of September 2013 and 12 sheep were vaccinated as per manufacturer's instruction at the beginning of October 2013. One group of these sheep was challenged at the end of February 2014 another one at the end of March 2014.

Results:
The analysis in this topic is not concluded yet, but indicates the following conclusions: We detect (as described by others alike) SBV in some kind of persistent form in mesenteric Lnn and spleen. The precise location therein remains subject of further analysis.
Unfortunately we could not obtain industry support to obtain vaccine products before marketing making it impossible to analyse the duration of immunity conferred. Our results indicate so far that animals mounting an antibody response are protected up to 4 months after vaccination (analysis will be continued after the end of the project).

Area 2. Epidemiology

Main objectives

To clarify or exclude horizontal transmission. To investigate which arthropod species may be potential vectors for Schmallenberg virus. To clarify if there is a potential risk of transmission of Schmallenberg virus via semen and embryos. To investigate if SBV is excreted in semen or may be transmitted via embryo's. To determine if pigs (and several other species) can play a role in the epidemiology of SBV and represent a trade issue. To obtain an estimate of possible SBV infections in wildlife species.

Workplan (concise)

To elucidate if horizontal transmission of SBV is possible antibody responses in non-inoculated controls were monitored. Inquiries were made on how to assess potential intranasal and contact infections (partner UK, AHVLA).

Retrospective vector studies were done in all five countries of the consortium and by all partners. Retrospective and prospective vector studies were done in The Netherlands, France and Germany.

To investigate the role of semen a large number of batches were tested. The test protocol of choice was the SBV S-segm RT-PCR and processing of samples was essentially according to Vanbinst et al. (J Virol. Meth. 169: 162-168).

To investigate the role of embryo's an experimental infection study in in vitro produced embryos was performed in France (UNCEIA). Virus, embryos and semen for this study were provided by partner institutes in France.

SBV specific antibodies were determined by different partners in several wildlife species and also in horses swine, camelids and mice. An experimental infection study in pigs was performed in Belgium. Infection experiments in other species were performed by different partners: poultry, llamas, alpacas and IFNAR mice (FLI), rabbits, hamsters, IFNAR mice (ANSES, CVI)

2.0.1 Transmission of Schmallenberg virus during winter, Germany

Methods:
The emergence of SBV-infection in sheep was monitored on a farm with 1000 ewes in Mecklenburg-Western Pomerania, Germany in winter 2012/2013. Blood samples were taken from 60 sheep in September 2012 and from additional 15 and 90 sheep on January 10 and in January through February, respectively (Fig. 5). The samples were tested for SBV-RNA and antibodies by SBV-specific real-time RT-PCR and indirect ELISA, respectively.

Results:
Serum samples collected in September and on January 10 from the 75 sheep were all negative for SBV antibodies. However, samples from 4 of 15 sheep that were tested in January revealed PCR-positive results (C_q-values 31.6 to 39.9). Blood from 1 of the 4 sheep was found infectious for two inoculated interferon alpha/beta receptor deficient (IFNAR) mice. Of the 90 additional sheep, 9 were SBV seropositive in January and two showed doubtful ELISA results. Four weeks later, 1 of the 2 latter sheep was tested SBV seropositive, and 1 and 2 sheep, which were previously found seronegative, showed doubtful and positive ELISA results, respectively (Fig. 7).
During the study period, PCR-confirmed SBV cases were also recorded for 52 adult cattle and sheep by the German Animal Disease Reporting System (TSN) in other German federal states of Germany between the 1st of January and 20th of February 2013 (Fig. 8).
This study indicates that SBV transmission occurred in early January at a low level. In the end of February 2013, 13% of the 90 sheep were seropositive, which contrasts within-herd seroprevalence of >90% found in other ruminant herds in 2011 (Loeffen et al., 2012; Wernike et al., 2013c; 2013d). During the sampling period in 2013, a temperatures increase to 5 or 6°C for several consecutive days with a maximum of 9°C was measured (Fig. 9), and vector activity was confirmed by a single *Culicoides* biting midge that was caught in a UV-light trap in the end of January on the study farm.

In conclusion, transmission of SBV by hematophagous insects seems possible, even during the winter in central Europe, if minimum temperatures rise above a certain threshold for several consecutive days (Wernike et al., 2013c).

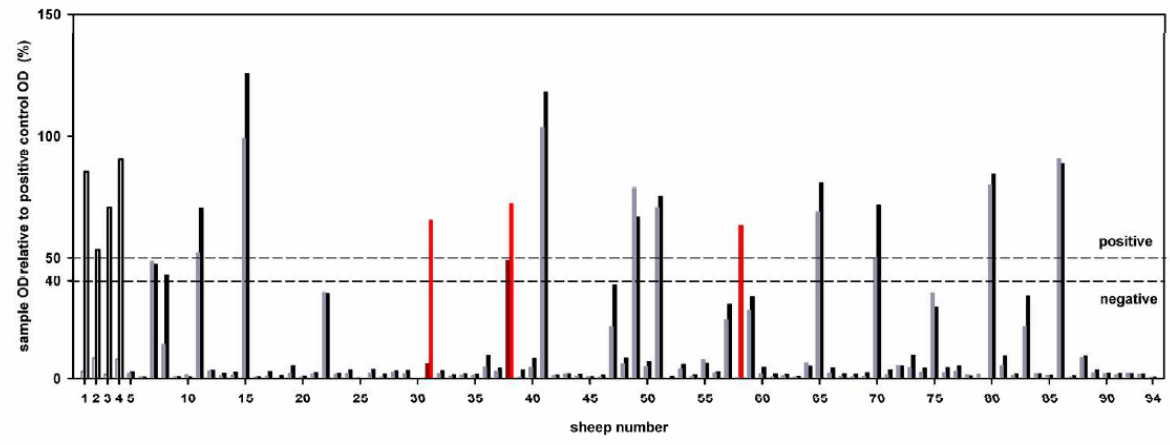


Figure7. Serological results as measured by SBV ELISA. The cut-off values of the ELISA are marked by a dashed line. Blood samples were taken twice four weeks apart. The results of the first sampling are depicted in grey or dark red (S31, S38, and S58, negative or doubtful at this time), of the second one in black or red (S31, S38, and S58, positive at the second sampling).

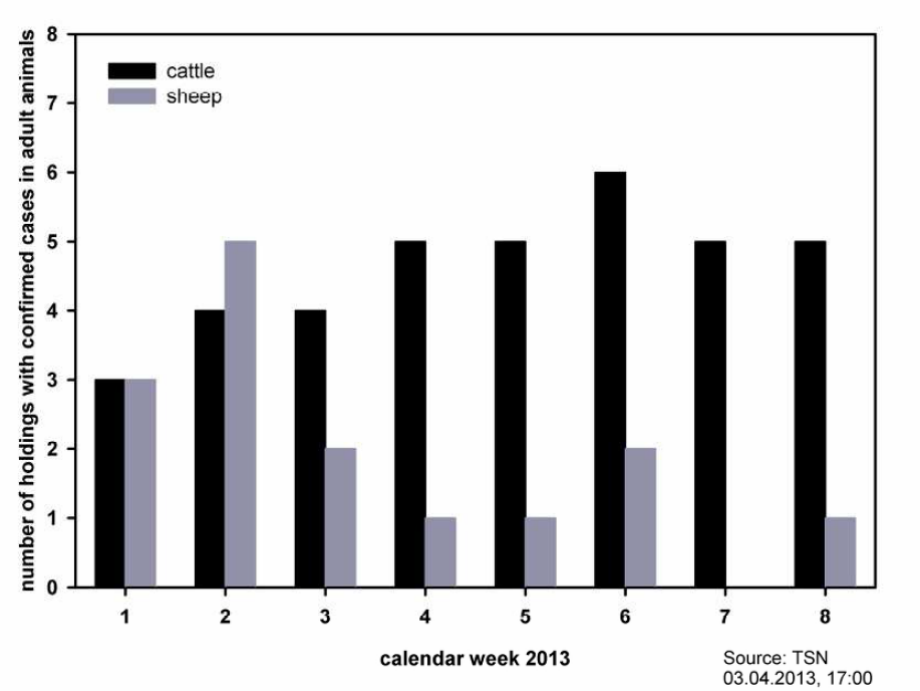


Figure 8. PCR-confirmed SBV cases in adult cattle (black bars) or sheep (grey bars) recorded by the German Animal Disease Reporting System (TSN) between the 1st of January and 20th of February 2013 (Wernike et al., 2013c).

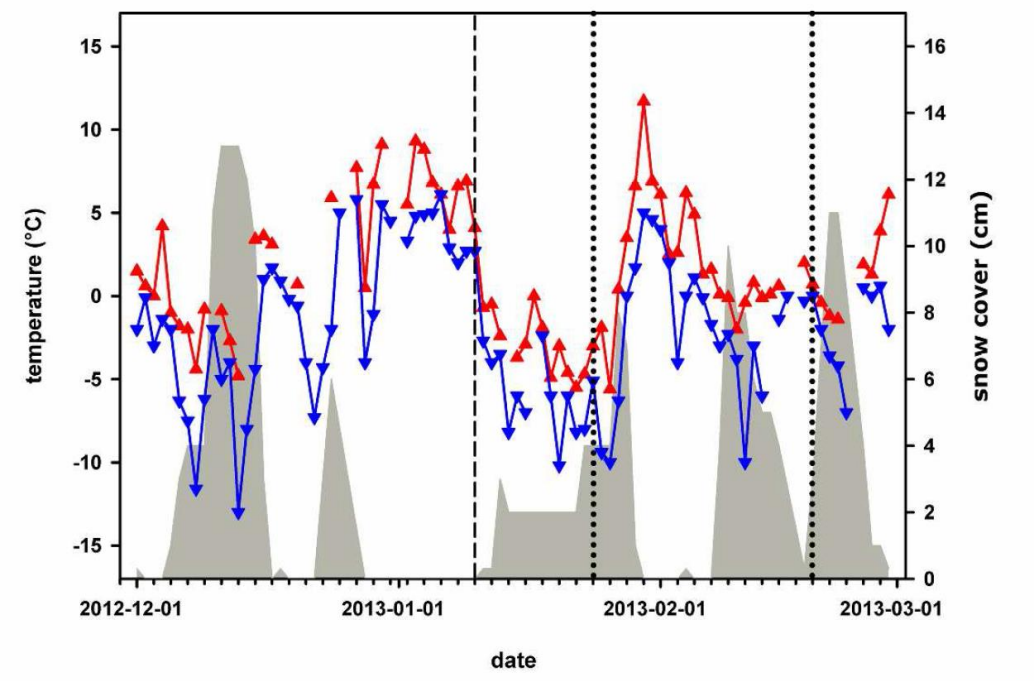


Figure 9. Climate versus sampling data. The maximum temperatures are shown in red and the minimum temperatures in blue. Snow cover is symbolised by a grey area. The dashed line represents the day of the detection of SBV genome in four sheep. Further sampling days are marked by dotted lines (Wernike et al., 2013c).

2.0.2 Dynamics of Schmallenberg virus infection within a cattle herd in Germany, 2011

Methods:
A cattle herd located approximately 9 km from the initial holding near the city of Schmallenberg (Hoffmann et al., 2012) was closely monitored between May 2011 and January 2012 in the context of a tick-borne fever surveillance (Nieder et al., 2012; Wernike et al., 2013d). During the study six cattle were slaughtered or culled, which were all unrelated to SBV-infection, and no animals were introduced from outside. Milk yield and body temperature were monitored in regular intervals. The detection of an increase in body temperature in an animal was followed weekly and, later, bi-weekly blood sampling intervals. Blood samples were taken at several dates (n=58, Fig. 10) from all dairy cows of the farm and analysed for SBV antibodies by a SBV competition ELISA. Samples taken between August and October 2011 were tested by S-segment-specific real-time RT-PCR (Bilk et al., 2012).

Results:
Every sample taken between calendar weeks 18 and 37 tested seronegative by ELISA. PCR-positive results and seroconversion were first detected in calendar weeks 37-40, and from week 41 SBV antibodies were detectable in all tested serum samples (Figs. 10 and 11).
A decrease in milk yield and an increase in body temperature, stillbirth or malformed offspring were reported after SBV-infection of cattle by other groups. In the present study, a decrease of the milk yield after SBV-infection was not observed. The onset of fever was rarely (n=3) associated with SBV-infection and suggests that SBV-infection of all other animals occurred without initial fever. Premature, stillbirth or malformed offspring were not observed, although 12 of the tested pregnant cows were infected with SBV in September 2011 during the period of pregnancy (days 75 to 175 after conception) that is critical for the cause of stillbirth or birth of malformed calves.
In the present study, RNAemia was only detected on a single day in a few cattle (n=6) and not detectable in all other animals, despite continuous sampling. A short viraemia of a few days was also observed after experimental inoculation of cattle with SBV. Seroconversion varied from 4 days to 2 weeks after a PCR-positive result, which is similar to seroconversion recorded in cattle experimentally infected with SBV (8 days to 3 weeks after infection).
In this study, a rapid spread of SBV infection throughout the entire herd (100%) was observed, which confirmed the high within-herd seroprevalence in cattle and sheep herds from affected areas that was reported by other groups. The entire study herd was infected with SBV between September and mid-October – during the main *Culicoides*-vector season in Northern Europe.

After SBV emergence during the first vector season, SBV has obviously spread rapidly and efficiently within naïve herds exposed to SBV. The study results confirm the previous evidence for the first entry of SBV in Europe at the end of the summer in 2011 and allows further insights into SBV epidemiology (Wernike et al., 2013).

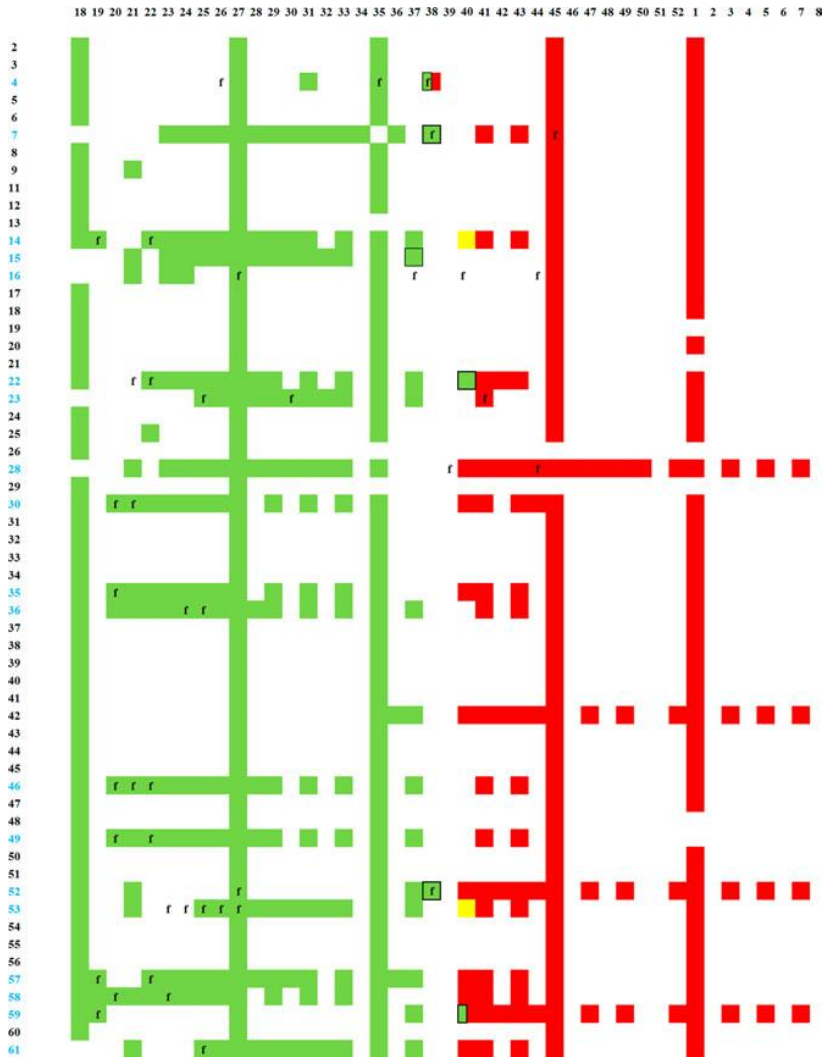


Figure 10. ELISA and real-time RT–PCR results of all dairy cows kept on the monitored farm between calendar week 18 of 2011 and week 8 of 2012. Serum samples tested negative by ELISA are depicted in green, doubtful in yellow, and positive in red. PCR-positive samples are framed in black, body temperatures exceeding 39.5 °C are indicated by 'f ', and the numbers of primiparous animals are depicted in blue.

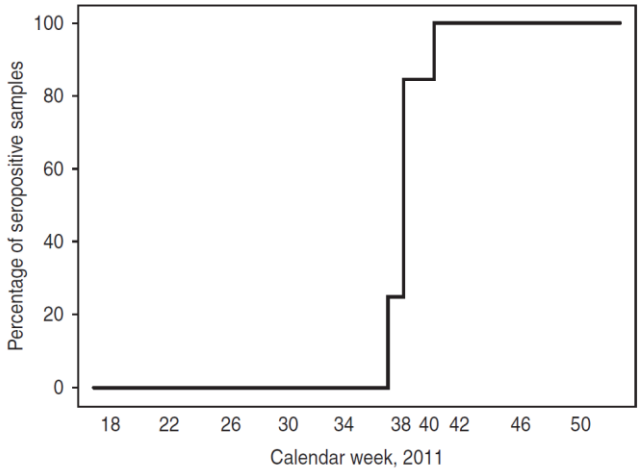


Figure 11. Percentage of samples positive by ELISA during the course of 2011. Doubtful results were considered as seropositive.

2.0.3 Collection of SBV case data in Germany

Case data have been retrieved from the German national animal disease database (Zentrale Tierseuchendatenbank) in the Animal Disease Notification System (Tierseuchennachrichten, TSN) to report these data to EFSA for supranational reporting a joint risk assessment. Routines have been created for the fast convenient extraction of the required data from the database and the need for manual handling of the data minimized. The data were reported to EFSA within the foreseen deadlines. Moreover, data on the re-occurrence of SBV in Germany during the vector-active season in 2012 were analysed, compared to the previous year and the results published (Conraths et al., 2013).
The database has also been evaluated to assess the impact of SBV on cattle, sheep and goat holdings at the level of the German federal states (Table 5). While the proportion of cattle holdings with reported SBV cases is generally low (1.02 % on average; 0.00-2.73 min.-max.), it is much higher in sheep and widely varies between regions in sheep (4.36 % on average; 0.00-23.08). In North Rhine-Westphalia, i.e. in the centre of the epidemic, the proportion of sheep holdings with reported SBV-cases was as high as 12.01 %.

Table 5. SBV-affected cattle sheep and goat holdings

	Cattle farms	SBV in cattle	%	Sheep farms	SBV in sheep	%	Goat holdings	SBV in goats	%
Germany	**144 850**	**1473**	**1,02**	**22 273**	**971**	**4,36**	**11 219**	**50**	**0,45**
Baden-Württemberg	17 991	70	0,39	2 921	39	1,34	2 574	7	0,27
Bavaria	54 731	461	0,84	6 255	49	0,78	3 819	1	0,03
Berlin	10	0	0,00	8	1	12,50	8	0	0,00
Brandenburg	2 572	26	1,01	630	24	3,81	263	0	0,00
Bremen	95	0	0,00	10	0	0,00	9	0	0,00
Hamburg	110	3	2,73	26	6	23,08	11	0	0,00
Hesse	8 623	125	1,45	1 553	141	9,08	761	9	1,18
Mecklenburg-W. Pommerania	2 067	17	0,82	529	14	2,65	136	1	0,74
Lower Saxony	21 093	235	1,11	2 480	147	5,93	884	6	0,68
North Rhine-Westphalia	16 610	294	1,77	2 299	276	12,01	881	14	1,59
Rhineland-Palatinate	5 314	54	1,02	966	40	4,14	424	5	1,18
Saarland	686	1	0,15	148	4	2,70	63	2	3,17
Saxony	3 532	18	0,51	1 275	44	3,45	367	0	0,00
Saxony-Anhalt	1 598	19	1,19	424	23	5,42	144	2	1,39
Schleswig-Holstein	7 943	115	1,45	1 925	110	5,71	537	1	0,19
Thuringia	1 875	35	1,87	824	53	6,43	338	2	0,59
SBV Data as of 14.01.2014; Farms: Destatis Viehhaltung der Betriebe Agrarstrukturerhebung - Fachserie 3 Reihe 2.1.3 - 2010									

2.0.4 Case/Control study in cattle, sheep and goats, Germany

A case/control study has been designed and implemented for cattle and sheep together with veterinary authorities in several German states and the University of Veterinary Medicine in Hanover. With regards to goats, it has been decided that a case/control study in this species (90 holdings) is conducted by the University of Veterinary Medicine in Hanover, Clinic for Small Ruminants, and that the FLI will assist in the evaluation of the data.
For cattle and sheep, it was planned to include at least 30 case and 30 matching control farms per species. Cases were farms with at least one confirmed case of SBV-infection (PCR or virus isolation) along with clinical indication of SBV infections in the holding (birth of malformed offspring with AHS, PCR-confirmed acute infection in adult dams). Controls were holdings without indication of clinical SBV-associated disease. To verify the status control holdings, at least 14 animals per farm were serologically tested (by IFAT or ELISA) to detect a seroprevalence of 20% at the 95% confidence level. All serum samples were collected, sent

to the Institute for Epidemiology of the FLI, registered and passed to the Institute for of Diagnostic Virology of FLI for testing, and the results recorded in a database.
It was originally planned to conduct the study in North Rhine-Westphalia and Lower Saxony, but due to the rapid spread of the disease and the interest of other federal states to contribute to the study, it was decided to include also holdings in Schleswig-Holstein, Rhineland – Palatinate, Hesse, Saxony-Anhalt and Brandenburg. This led also to an adaptation of the planned numbers of case and control holdings.

All farms were visited by veterinarians who sampled the animals as described above and conducted structured interviews with a standard questionnaire. The standard questionnaire, made available as an excel spread sheet that allows automated upload of data into a database, was jointly developed with members of the veterinary services of the federal states participating in the study and with the Clinic for Small Ruminants of the University of Veterinary Medicine in Hanover.
The questionnaire was used to record details on the visited holdings and vets serving it, species of animals kept, number of animals per species, production type, husbandry and farm management practices, hygienic status, treatments; diseases, cleansing and disinfection management, observed clinical symptoms in case herds, fertility, farm contacts, observations on vector abundance, use of repellents and insecticides, potential wildlife disturbances etc. The questionnaire was made available to other partners in the Schmallenberg Response Group jointly convened with EFSA. Interviewers were trained before they did the interviews.
A database was designed and programmed to take up the interview data and the results of diagnostic testing (IT personnel employed through the project). A PhD student was employed for data management and evaluation (employed through the project).
Data were obtained for a total of 108 holdings (50 cattle and 58 sheep holdings; 29 case flocks, 29 control flocks). Herds and flocks with confirmed cases of SBV-infections in malformed fetuses were regarded as case holdings (cattle: 33 case holdings; sheep: 29 case holdings). Herds and flocks without such cases were preliminarily attributed to the control group (cattle: 17 case holdings, 29 control holdings). On each holding, at least 14 animals were blood-sampled and tested for antibodies to SBV by ELISA. Control holdings, in which the number of seropositive animals did not exceed 4 or 28.6%, remained in the control group. Holdings with a higher seroprevalence (10 cattle herds and 13 sheep flocks) were excluded from the study leading to a final study population of 33 cattle case plus 7 cattle control holdings and 29 sheep case and 16 sheep control holdings.
It should be noted that control holdings could mainly be found in the peripheral areas of the SBV-affected region, i.e. in Brandenburg, Rhineland-Palatinate and Schleswig-Holstein (Fig. 12). The study design, emerging problems (difficulties to identify control holdings due to the fast and efficient spread of the infection in the study area) and preliminary results were discussed within the EFSA Schmallenberg Response Group to prepare for joint meta-analysis.

Statistical analysis of the collected data (bivariate and multivariate testing) was conducted to identify potential risk factors using the statistical software "R" (http://www.r-project.org/; version 4.0) and an optimised logistic regression model produced. Selection of the variables included in the optimised model was based on the p value, the Akaike Information Criterion (AIC) and pseudo R^2 values. In a first step, those variables were considered for inclusion in the model that had a p- value ≤ 0.1 in bivariate testing or explained more than 10% of the outcome variable (pseudo $R^2 > 0.1$). The AIC value was then used as an additional indicator of the model quality to rank the explanatory variables with regard to their impact.

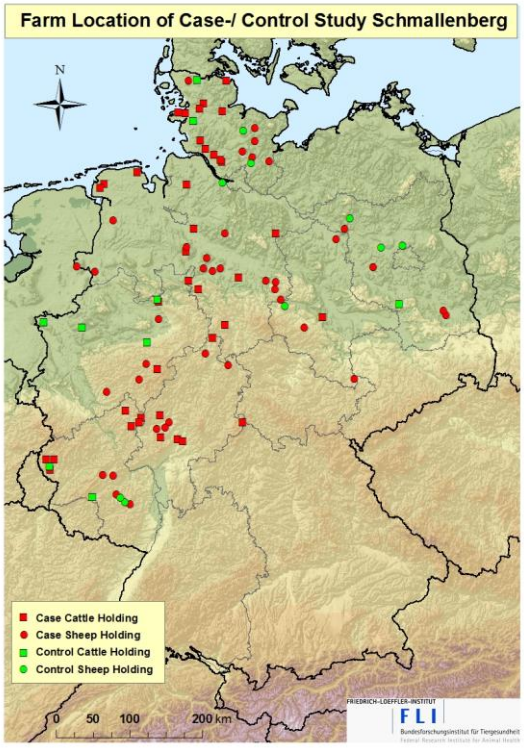


Figure 12. Spatial distribution of case and control holdings.

Results:
Bivariate analysis

Cattle
Bivariate testing indicated statistically significant associations with an Odds Ratio (OR) >1, i.e. potential risk factors) between the following variables and the outcome variable:

- Occurrence of malformations (OR 93.0)
- Animals kept temporarily indoors (OR 32.5)
- Bull kept for a natural mating (OR inf)
- Purchase of animals during the study period (OR 7.2)

The variable "Occurrence of malformations" must be regarded as a clinical expression of the response variable (occurrence of SBV infections, i.e. case holding) and was therefore excluded from further analysis.
Bivariate testing indicated statistically significant associations with an Odds Ratio (OR) < 1, i.e. potential protecting factors) between the following variables and the outcome variable:

- Animals kept indoors permanently (OR 0.03)
- Use of a milking robot recording individual performance (0.13)
- Presence of other factors with a negative impact on reproduction (0.00)
- Presence of migrant sheep flocks in the area (OR 0.11)

"Region" (i.e. the location of the farms relative to the centre of the epidemic) emerged as a potential risk factor for clinically apparent SBV infection due to the heterogeneous spatial distribution of SBV infections in the study region in Germany.

Sheep
Bivariate testing indicated statistically significant associations with an Odds Ratio (OR) >1, i.e. potential risk factors) between the following variables and the outcome variable:

- Abortions observed (OR 22.0)
- Stillbirth (OR inf)
- Occurrence of malformations (OR 202.5)
- Flock regularly visited by a vet (OR 5.8)
- Animal holder assists at the birth and observed increase in abortions and malformations (OR 27.1)
- Abortions/malformations observed in animals new in the flock (OR inf)
- Abortions/malformations observed in older sheep (OR 103.5)
- Abortions/malformations observed in lambs with mobility disorders (OR inf)
- Increased number abortions/malformations as compared to previous periods (OR inf)
- Reproduction disorders in ewes (OR 15.2)

Most of these variables represent expressions of the same phenomenon and must be regarded as a clinical expression of the response variable (occurrence of SBV infections, i.e. case holding). The following variables were therefore excluded from further analysis: abortions observed; stillbirth, occurrence of malformations, animal holder assists at the birth and observed increase in abortions and malformations, Abortions/malformations observed in older sheep, abortions/malformations observed in lambs with mobility disorders, increased number abortions/malformations as compared to previous periods.
Bivariate testing indicated statistically significant associations with an Odds Ratio (OR) < 1, i.e. potential protecting factors) between the following variables and the outcome variable:

- Poultry kept (OR 0.1)
- Mating all year (OR 0.0)
- Keeping hair sheep (0.11)

Multivariate analysis

Cattle
A total of 13 variables with a p value $\leq$ 0.1 or explaining more than 10% of the variance of the outcome variable were included in the full logistic regression model.
The model with the best pseudo R^2 value (0.595) included the variables “animals kept indoors permanently” (protecting factor), “purchase of animals during the study period” (risk factor), “presence of migrant sheep flocks in the area” (risk factor). ROC analysis revealed that this model discriminates reliably between case and control holdings (AUC 0.92).

Sheep
A total of 8 variables with a p value $\leq$ 0.1 or explaining more than 10% of the variance of the outcome variable were included in the full logistic regression model.
The model with the best pseudo R^2 value (0.483) included the variables “poultry kept” (protecting factor) and “reproduction disorders in ewes” (risk factor). ROC analysis revealed that this model discriminates reliably between case and control holdings (AUC 0.86). In another model, the variables “poultry kept” and “flock regularly visited by a vet” (risk factor)

were combined and yielded a pseudo R^2 value of 0.473 and an AUC of 0.86 in ROC analysis. A third model included the potential risk factors "reproduction disorders in ewes" and "flock regularly visited by a vet" and had a slightly lower pseudo R^2 value of 0.390 and an AUC of 0.80 in ROC analysis. The presence of the variable "flock regularly visited by a vet" indicates that monitoring the health status of a flock helped to identify SBV infections. We have currently no plausible biological explanation for the finding that the presence of poultry in a sheep holding represented a statistically significant protective factor by both, bivariate testing and multiple logistic regression analysis.

2.1 Transmission pathways

2.1.1 Horizontal transmission

2.1.1.1 Intranasal inoculation in sheep

Methods:
At the AHVLA, experimental infections in sheep were performed to assess the ability of virus to cause clinical disease in lambs following intranasal inoculation. At first a group of 6 animals received approximately 10^4 TCID50 in 1 ml of UK isolate from a brain sample.
A further group of sheep (n=7) were inoculated intranasally using a well-defined isolate that could be grown to a high titre. While this high titre of inoculation might not represent the situation in the field, it should be noted that SBV is widely present on infected offspring during birth.

Results:
All sheep inoculated, irrespective of route, failed to develop clinical disease and all remained serologically negative. SBV could not be detected by RT-qPCR in any of the sheep (blood) post intranasal infection.

2.1.1.2 Antibody responses in susceptible controls

In none of the experimental infection studies, antibody responses in non-inoculated controls which were kept in contact with inoculated animals, were observed, neither in cattle, nor in sheep or goat. There never have been indications for direct horizontal transmission of SBV.

2.2 Transmission-competent vectors

2.2.1 Retrospective studies

Methods:
Netherlands retrospective study 2011
Culicoides were trapped almost daily throughout September and early October, 2011 at a dairy herd and at two locations in the vicinity of sheep (Elbers et al., 2013). Prior to assay, working under a dissecting microscope and using a scalpel, the head of each midge was separated away from the rest of the abdomen; 10 heads per species were then pooled and assayed for Schmallenberg virus (SBV), whereas the corresponding abdomens (also pooled) were stored away in 70% ethanol. A total of 610 pools (10 heads per pool) were assayed. Only when a pool of 10 heads was found SBV-positive was the corresponding pool of dissected abdomens retrieved and assayed individually.

Germany retrospective study 2011
Biting midges were trapped by OVI (Onderstepoort Veterinary Institute) traps, BG-Sentinel biting midge traps, BG Sentinels and EVS traps at 28 sites in Western Germany within other projects than the SBV project and made available for testing. All midges were pooled in groups of 1-50 individuals, according to species, collection date and site, for Schmallenberg virus (SBV) screening. Additionally, 48 pools containing 673 black flies (Simuliidae), caught in 2011, were screened for the virus without being identified to species prior to testing.
By the end of 2013, realtime RT-PCR (Hoffmann et al. 2012) screening for SBV was conducted on 4,999 biting midges and 633 black flies collected between April and October 2011.

Belgium retrospective study 2011
Culicoides collected at 16 locations (divided over 4 regions) in Belgium with OVI (Onderstepoort Veterinary Institute) traps between July and November 2011 were morphologically identified and physiologically examined. Species specific pools originating from parous females containing maximum 25 heads were prepared. The RNA from these pools was extracted using the MagMAX Total Nucleic Acid isolation kit and the MagMAX Express-24 purification system. Obtained RNA was thereafter subjected to a qRT-PCR detecting the SBV-S segment. In total, 7305 midges divided over 480 pools were screened for the presence of SBV (De Regge et al., 2012).

Results:
Retrospective studies 2011, Netherlands
SBV was detected in several different *Culicoides* species (Elbers et al., 2013): *C. obsoletus sensu stricto, C. scoticus, C. chiopterus*.
Ct values of positive pools in The Netherlands were lower than expected (compared to BTV). From testing individual *Culicoides* specimens in the Netherlands it became clear that prevalence of SBV in midges was 5-10 times higher when compared to BTV detection in *Culicoides* in Europe during 2002-2008. Vector biology was positively influenced by climatological circumstances in 2011 with a prolonged vector season (several weeks due to higher temperatures than normal) and a higher survival rate and increased vector abundance (rain in summer and higher temperatures than normal in autumn) (Elbers et al., 2012).
A field study executed outside this research consortium investigated the presence of SBV in mosquitoes overwintering at 11 ruminant farms in the Netherlands, where between November

2011 and January 2012 SBV circulation had been proven based on the presence of SBV RNA in the brains of malformed newborns (Scholte et al., 2014). No evidence was found for the presence of SBV in hibernating mosquitoes (*Culex, Anopheles,* and *Culiseta spp*.), collected from January to March 2012). It was suggested that mosquitoes do not play an important role, if any, in the persistence of SBV during the winter months in north-western Europe.

Retrospective studies 2011, Belgium
Pools of heads of several *Culicoides* species were found positive in qRT-PCR: Obsoletus complex, *C. dewulfi* and *C. chiopterus,* indicating that these species might play a role in the transmission and spread of SBV. The first SBV positive midges were found at August 23th 2011 in the region of Liège. This represents till now the earliest detection of SBV in Belgium. Depending on time and place, a high percentage (up to 30%) of pools was found SBV positive. If it is considered that each positive pool contained one SBV positive midge, a high infection prevalence of 2.4% was found in Obsoletus complex midges in October in Liège. This high infection prevalence in *Culicoides* helps to explain the fast spread of the virus upon its emergence. No positive pools were found in the south of Belgium in 2011, correlating with a low seroprevalence rate in sheep and cows at the end of the first vector season (end 2011) in that region (De Regge et al, 2012).

Retrospective studies 2011,Germany
Culicoides pools and pools of black flies (*Simuliidae*) from 2011 in Germany tested negative for SBV.

2.2.2 Prospective studies

Methods:
Netherlands prospective study 2012
A total of 130 pools (50 specimens per pool) of *Culicoides* biting midges collected between May and September 2012 in the Netherlands were assayed for SBV (Elbers et al., 2014). The *Culicoides* midges were caught in the same area as where in 2011 a high proportion of *Culicoides* pools tested positive for SBV (Elbers et al., 2013).

Germany prospective studies 2012-2013
Insects were trapped by OVI (Onderstepoort Veterinary Institute) traps, BG-Sentinel biting midge traps, BG Sentinels and EVS traps between April and October 2013 at 38 collection sites in southern and eastern Germany (Fig 13.). Further insect samples collected between in 2012 at 28 additional locations in western Germany within other projects were also made available for this study. Captured insects were pre-sorted and biting midges, in particular specimens of the genus *Culicoides*, were separated according to morphological features.
By the end of 2013, realtime RT-PCR (Hoffmann et al. 2012) screening for SBV was conducted on 5,562 midges and 3 simuliids collected between May and October 2012 at 10 locations in western Germany, respectively, as well as 10,840 midges plus 37 black flies sampled between May and October 2013 at 21 sites in southern and eastern Germany were tested.

Figure 13. Trap stations operated in the Schmallenberg project

Belgian prospective study 2012

Culicoides collected biweekly at 12 locations (divided over 4 regions) in Belgium with OVI (Onderstepoort Veterinary Institute) traps between July and November 2012 were morphologically identified and physiologically examined. Subgenus (*Avaritia, Culicoides, Monoculicoides*) specific pools of maximum 20 whole parous females were prepared. The RNA from these pools was extracted using the MagMAX Total Nucleic Acid isolation kit and the MagMAX Express-24 purification system. Obtained RNA was thereafter subjected to a qRT-PCR detecting the SBV-S segment. In total, 17461 midges divided over 904 pools were screened. Furthermore, 69 pools representing 1359 nulliparous midges caught in May in the region of Antwerp and Gembloux were tested by similar methods to assess a possible transovarial transmission.

Results:

Prospective studies, 2012-2013, Netherlands

Two of a total of 42 pools comprising 50 midges/pool of the Obsoletus Complex from the 2012 collection, tested weak positive (C_t values: 34.96 and 37.66), indicating a relatively low viral load (Elbers et al., 2014). On an individual midge level, the proportion of SBV-infected *Culicoides* of the Obsoletus complex caught in the same area and in a comparable period of the year, was significantly lower in 2012 (0.1% = 1 per 1,050 tested) compared to 2011 (0.56% = 13 per 2,300 tested). As a significantly lower proportion of SBV-infected

Culicoides was observed in 2012, it can be assumed that there was a lower level of circulation of SBV in this area in 2012. The most obvious explanation for the lower level of SBV circulation in the field in 2012 is the fact that just a small fraction of hosts was left susceptible for infection after the massive epidemic in 2011.

Prospective studies, 2012-2013, Germany
Of all pools tested, only two appeared to be weakly positive for SBV with Ct values of 35 and 42.46, respectively. Both pools consisted of Obsoletus Complex *Culicoides* (including *C. dewulfi*) and had been collected at two different locations in the federal state of North-Rhine Westphalia, western Germany, in late August and early September 2012. The pool sizes amounted to 20 and 22 midges per pool, respectively. None of the *Simuliidae* screened for SBV proved positive. In contrast to previous considerations, mosquitoes were not tested for SBV, due to negative results of other working groups (e.g. Scholte et al. 2014) combined with time constraints.

Prospective studies, 2012-2013, Belgium
No SBV could be detected in nulliparous midges caught in May 2012. This provides an indication that transovarial transmission is not likely to occur. This should, however, be further investigated since it was recently reported that SBV RNA was detected in midges considered as nulliparous based on visual inspection in Poland in 2012 (Larska et al,2013).
A renewed but short lived circulation of SBV in parous midges belonging to the subgenus *Avaritia* occured in August 2012 at all four regions. The infection prevalence reached up to 2.86% in the south of Belgium, the region where a lower seroprevalence was found at the end of 2011 than in the rest of the country. The infection prevalences in the other regions where positive pools were found in 2011 were markedly lower (0.4, 0.3, and 0.2% in *Avaritia* in Antwerp, Liège and Gembloux, respectively). No more positive pools were found from September onwards. A frequency analysis of the Ct values obtained for 31 SBV-S segment positive pools of *Avaritia* midges showed a clear bimodal distribution with peaks of Ct values between 21-24 and 33-36. This closely resembles the laboratory results obtained for SBV infection of *C. sonorensis* and implicates indigenous midges belonging to the subgenus *Avaritia* as competent vectors for SBV (De Regge et al, 2014).

2.2.3 Vector competence studies

Methods:
In this study, the group at the Laboratory of Entomology (Wageningen, The Netherlands) in collaboration with CVI (Lelystad, The Netherlands) inoculated five sheep intramuscularly with SBV and let suspected vector insects (*Culicoides* spp. and *An. atroparvus* mosquitoes) feed on the sheep during peak viraemia. *Culicoides nubeculosus* midges were reared according to a protocol developed by Boorman (1974), with minor modifications. The insects were reared at 23°C and 75% RH, and were fed daily on cattle blood through Hemotek FU1 feeders (Hemotek, Accrington, UK) using a Parafilm® membrane (Bemis, Oshkosh, WI, USA). *A. atroparvus* mosquitos were reared under similar conditions according to a protocol developed by the rearing staff at the Laboratory of Entomology of Wageningen University. Wild *Culicoides* spp. midges were caught in May and June 2013 at a horse farm near Renkum, The Netherlands. Insects were 'forced' to feed on the sheep by placing known numbers of insects in small cardboard cages on the inner leg of the sheep. After exposure (i.e. blood feeding), the cardboard cages were detached from the sheep and insects were incubated at 25 °C to allow for the virus to replicate and disseminate within the arthropods. After the

incubation period the insects were killed, their heads separated, pooled and frozen until RNA extraction and PCR amplification. These experiments were carried out in the High Containment Unit (BSL-3) of the Central Veterinary Institute, Lelystad, part of Wageningen UR.

Results:
All five sheep showed the mild clinical symptom of raised body temperature (above 39.5°C) within the first five days after injection with SBV. SBV infection in the sheep could be confirmed by PCR of serum samples. All groups of insects (*An. atroparvus*, *C. nubeculosus*, field-collected *Culicoides* spp.) fed on all five sheep, albeit at different rates. *C. nubeculosus* showed the highest feeding rate (180/260, 71.5%), followed by *An. atroparvus* (18/60, 30.0%) and field-collected *Culicoides* spp. (5/754, 0.6%). In total, 1074 insects were applied to the sheep, of which 203 (18.9%) fed. Head pools of all three insect groups showed positive PCR bands for SBV S-segment fragment, but no individual insect abdomen. Positive pools included five pools of *C. nubeculosus* (30 individual heads in total) and one each from *An. atroparvus* (eight heads) and field-collected *Culicoides* sp. (one head). Positive signals were found in insects having fed on all five sheep between days 1 and 5 p.i.. Sequencing of amplified fragments from these seven positive head pools confirmed the identity of six out of seven samples as SBV S-segment. A BLAST search revealed 96-100% identity with the SBV strain NO/13/04/7678 segment S nucleocapsid protein and non-structural protein genes, partial cds (Access. no.: KF314813.1). We thus demonstrated for the first time a successful host-to-vector transmission of SBV and highlight the role of *An. atroparvus* mosquitoes as potential vectors.

2.3 Role of semen and embryos

2.3.1 RT-PCR testing of semen samples

Methods and results:
CODA-CERVA has tested 40 semen samples from sheep collected between summer and autumn 2011. None of these were tested positive by qRT-PCR. However no clear conclusions can be drawn since not sufficient data about the serological status of the animals was available.
Schmallenberg virus has been detected in bovine semen using RT-PCR in Germany, France, UK and The Netherlands. By December 2012 within these countries 0-4% of recently produced semen batches were SBV positive by RT-PCR:
By December 2012 at CVI a number of 55 semen samples produced in 2012 by 8 seroconverting/viraemic bulls have been analysed using a real-time RT-PCR system developed by FLI and an RNA extraction method developed by CVI. In total 3 samples produced by 2 different bulls tested positive.
By December 2012 LNCR (National Laboratory for sanitary controls in breeding animals) together with ANSES (Fr.), a number of 904 semen samples produced in 2011 and 2012 by 160 seropositive bulls have been analysed using a real-time RT-PCR system developed by FLI and an RNA extraction method developed by LNCR. In total 26 samples produced by 2 different bulls were tested positive for 2 to 3 months.
In Germany frozen semen collected between May 2012 and November 2012 from 95 seroconverted bulls was analysed for SBV-RNA by real-time RT-PCR (RT-qPCR).
A total of 766 semen batches from 95 SBV-infected bulls were obtained from 7 stock-bull breeding centres in Germany in 2012. A total of 29 (3.8% of 766) semen batches from 11 bulls from 3 breeding centres were positive in RT-qPCR analysis (see paragraph 3.2.1 for more details).

2.3.2 Evaluation of transmission risks via embryos

Methods:
Evaluation of transmission risks via embryos were performed using different in vitro models with experimentally (*in vitro* spiking) or naturally infected oocytes or semen (LNCR). Efficiency of sanitary washes recommended by (International Embryo transfer Society (IETS) was tested in each model following *in vitro* maturation, fertilization and culture. Zygotes, embryos, media and washes were tested for SBV RNA using RT PCR.

Results:
Fertilization of i*n vivo* contaminated gametes leaded to produce contaminated zygotes, however D7 embryos following IETS washing protocols were negative regarding SBV RNA. At the contrary, *in vitro* spiked gametes resulted in contaminated embryos and IETS washing procedure was inefficient to remove SBV RNA. This may be explained by higher doses of virus during spiking or different properties of cultured viruses regarding interaction between virus and the embryo zona pellucida. All together, these results suggest that naturally infected gametes (oocytes / semen) may be associated with a negligible risk of transmission through in vitro produced embryos. However, the results using *in vitro* spiking approaches were not in agreement with the *in vivo* contamination models. The mechanisms involved in the

differences observed between both models remain to be explained. Additional data on *in vivo* derived embryos are needed.

2.3.3 Studies on in vitro embryo (from cattle) production and efficiency of IETS washing procedures

Methods:
Evaluation of transmission risks via embryos were performed using different in vitro models with experimentally (*in vitro* spiking) or naturally infected oocytes or semen. Efficiency of sanitary washes recommended by IETS was tested in each model following *in vitro* maturation, fertilization and culture. Zygotes, embryos, media and washes were tested for SBV RNA using RT PCR. Results are summarized in Table 6.

Results:
Fertilization of i*n vivo* contaminated gametes leaded to produce contaminated zygotes, however D7 embryos following IETS washing protocols were negative regarding SBV RNA. At the contrary, *in vitro* spiked gametes resulted in contaminated embryos and IETS washing procedure was inefficient to remove SBV RNA in this case. This may be explained by higher doses of virus during spiking or different properties of cultured viruses regarding interaction between virus and the embryo zona pellucida.

Table 6. *In vitro* produced embryos following in vitro spiking of gametes (oocytes and semen) or issued from naturally infected gametes

***In vitro* produced embryos**	**Semen** **Negative samples**	**Semen** ***In vitro* spiking**	**Semen - *In vivo* contamination**
Oocytes	Control group	Inefficient IETS washing	Positive zygotes
Negative samples			Negative embryos following IETS washing
Oocytes **I*n vitro* spiking**	Inefficient IETS washing	x	x
Oocytes	Positive oocytes	x	x
***In vivo* contamination**	Negative embryos following IVP		

All together, these results suggest that naturally infected gametes (oocytes / semen) may be associated with a negligible risk of transmission through in vitro produced embryos. However, the results using *in vitro* spiking approaches were not in agreement with the *in vivo* contamination models. The mechanisms involved in the differences observed between both models remain to be explained. Additional data on *in vivo* derived embryos are needed.

2.3.4 Experimental infection of bulls to investigate SBV excretion in semen

To study Schmallenberg virus excretion in bovine semen after experimental infection, two bulls were inoculated subcutaneously with a Schmallenberg virus isolate (1ml Vero cell culture 10E6 TCID50). After inoculation (at Day 0), semen was collected daily from both animals for 21 days and samples were tested for Schmallenberg virus by qRT-PCR assay. At 24 days after inoculation both animals were subjected to necropsy and genital organs and lymph nodes draining these organs were also tested for Schmallenberg virus RNA (qRT-PCR). After SBV infection both animals in the study showed viraemia (qRT-PCR) with fever and diarrhoea. Schmallenberg virus RNA could be detected in semen from both animals. Schmallenberg virus RNA was detected with the highest levels in the period of Day 4 to Day 7 after inoculation (Figure 14.), five consecutive days in one bull and at one occasion in the other. Scattered low concentration positives were found in week two and three after inoculation in single amplification runs. Schmallenberg virus RNA was not detected in the genital tissues after necropsy but SBV RNA was detected in the mesenteric and inguinal lymph nodes draining these tissues. SBV RNA concentrations in semen samples were relatively low (Ct-values 30-39). SBV RNA concentrations were higher in whole blood and serum (Day 2 to Day 4; CT-values 27-31) and in the lymph nodes (Day 24; Ct-values 29-39). Schmallenberg virus was only isolated in cell cultures from blood samples at Day 3 after inoculation. Schmallenberg virus could not be isolated in Vero cells from any of the semen samples or the tissue samples.

The observations from this study indicate that Schmallenberg virus RNA can be excreted in bovine semen. This suggests that bovine semen might be contaminated with viable virus. Although viable virus was not detected in semen in this study, in an earlier study by others, infectivity of semen from SBV RNA positive straws was reported after subcutaneous injection in calves, even in specimens with relatively low concentrations of SBV RNA. To exclude that SBV RNA is present in produced semen, highly sensitive RT-PCR-testing of semen from SBV-infected (RNA or antibody positive) bulls is the method of choice (Hoffmann et al., 2013; Schulz et al., 2014). However, at present it is not clear if the detected low concentrations of SBV RNA will be associated with infectious virus. Moreover, even in case of contamination of semen with infectious Schmallenberg virus it still needs to be elucidated if infectious virus can be transmitted to susceptible cows at service or by insemination. A study on Schmallenberg virus infection after insemination with qRT-PCR positive semen straws will be needed to answer this question.

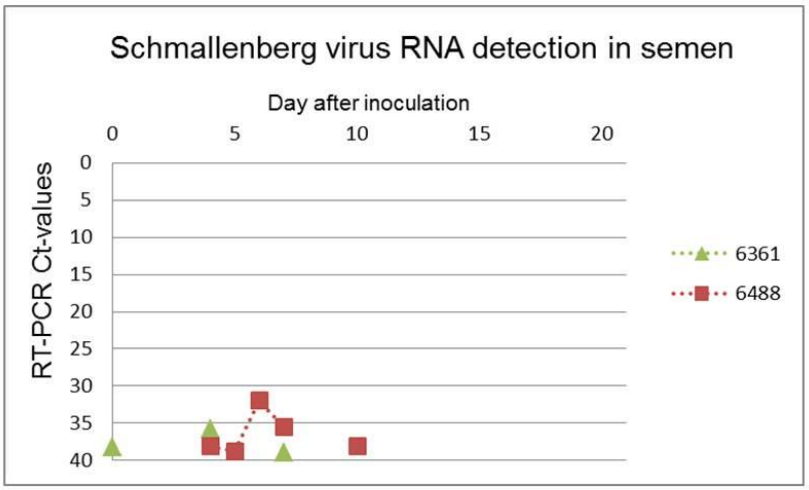


Figure 14. Schmallenberg virus RNA detection (RT-qPCR values) in semen samples collected from two experimentally infected bulls from Day 0 until Day 21 after inoculation. Semen samples with extender (1:10 tris/egg yolk 20%).

2.3.5 Experimental infection of cattle and IFNAR$^{-/-}$ mice with SBV-RNA-positive bovine semen

Methods:
In the previous field study, SBV-RNA was detected in frozen bull semen by real-time RT-PCR (RT-qPCR). Vectorborne transmission by *Culicoides* spp. biting midges is most common, but venereal transmission of SBV might contribute to the spread of this virus to previously unaffected regions. Therefore, the infectivity of SBV-RNA–positive semen was investigated by experimental subcutaneous injection of cattle and interferon α/β receptor–deficient (IFNAR$^{-/-}$) mice.

Experimental infection of cattle with SBV-RNA positive semen, Germany
The straws originating from 6 semen batches (C_q 26.4-36.4) collected from 6 bulls (A-C and E-G) during August and September 2012 (Hoffmann et al. 2013). To increase the probability of infection of injected cattle, 5 straws of semen from one batch from an individual bull were pooled, and 6 cattle (C1-C6) were subcutaneously (s.c.) inoculated, each with a pool from 1 of the 6 bulls. To investigate the infectivity of a single insemination dose (1 straw), 5 cattle (C7-C11) were s.c. injected with single straws from bull F that had been confirmed to contain infectious SBV (by s.c. inoculation of calves). Serum samples were obtained in several days (Figure 15) and tested for SBV-RNA and antibodies by small-segment-specific RT-qPCR, ELISA and neutralization test, respectively. Clinical signs and rectal body temperatures for the injected cattle were monitored daily.

Experimental infection of IFNAR$^{-/-}$ mice with SBV-RNA-positive semen
A total of 20 SBV-RNA-positive semen batches (C_q-values 25.9-36.5) collected from bulls during August to November 2012 (Hoffmann et al., 2013) were subcutaneously injected into

40 IFNAR$^{-/-}$ mice. For each batch, two mice were each injected with half of a semen straw (80-120µl). The mice were monitored clinically and weighed daily. Serum and tissue samples were harvested post-mortem at 22 dpi and tested for SBV antibodies and genome by ELISA and SBV-S3-specific RT-qPCR, respectively.

Results:
SBV infection was confirmed in 5 of 11 injected cattle (C3, C5 and C9-C11). RNAemia (C_q 25.0-29.3) was first detected at 3 to 6 days post-infection (dpi) and persisted for 3 to 4 days. Seroconversion occurred between 8 and 12 dpi (Figure 15). No obvious clinical signs were observed in any of the cattle (Schulz et al., 2014).
In none of the mice, SBV-RNA or antibodies were detected (Schulz et al., 2014).

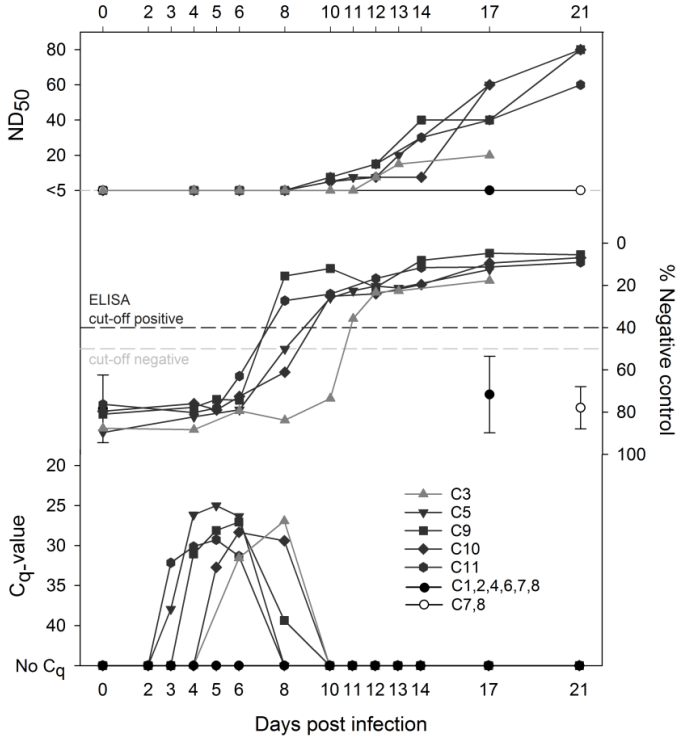


Figure 15. Detection of Schmallenberg virus (SBV) RNA and antibodies in serum of 5 of 11 cattle (C) injected with SBV RNA–positive bovine semen (quantification cycle [Cq] values 26.4–36.4) (Schulz et al., 2014).

We demonstrated that subcutaneous injection of a single insemination dose of SBV-RNA-positive semen can be infectious for cattle, although not every straw of the same batch was infectious. Possible explanations are that infectivity of individual straws does not correlate with the viral RNA load or that the infectivity of one straw is lower than the minimal cattle infectious dose for SBV. IFNAR$^{-/-}$ mice were not infected with SBV, which might be due to a lower susceptibility to SBV infection compared to cattle. Despite the possibility of infective SBV in some semen batches, the actual risk for SBV transmission by insemination of dams remains to be evaluated. To exclude that SBV RNA is present in produced semen, highly sensitive RT-PCR-testing of semen from SBV-infected (RNA or antibody positive) bulls is the method of choice (Hoffmann et al., 2013; Schulz et al., 2014).

Experimental infection of IFNAR$^{-/-}$ mice with SBV-RNA-positive semen, France

Methods:

In France (LNCR), seven bulls, 1 to 5 years of age, with no SBV detectable neutralizing antibodies prior to the beginning of the study were selected with the following criteria: SBV seroconversion observed between September 2011 and December 2012 and the most complete semen production batches including at least 14 ejaculates, collected from 4 weeks before to 4 weeks after the first positive SBV ELISA result. Semen was collected once or twice a week, diluted, conditioned in straws and frozen using classical procedure.

From each ejaculate collected from one month following seroconversion, one straw was tested for the presence of SBV RNA by one-step real time RT-PCR (rtRT-PCR) method by LNCR.

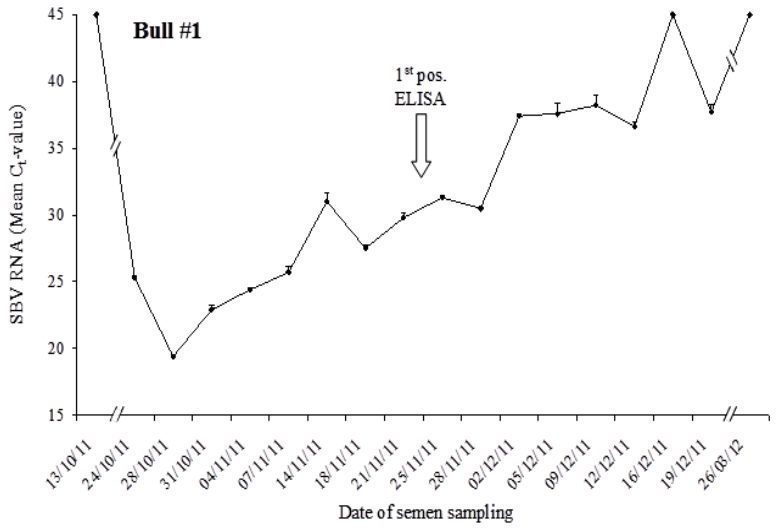
For each bull, the presence of SBV specific antibodies was tested monthly in serum using an indirect ELISA kit.

The presence of infectious SBV in semen was appreciated by inoculating IFNAR$^{-/-}$ mice (ANSES). Three semen replicates (each 100 µl) from one single ejaculate, first batch presenting low C_t value (C_t = 25.4) results were injected into the neck scruff of three adult IFNAR$^{-/-}$ mice. After 4 days, EDTA blood samples were tested by SBV RT-PCR (ANSES).

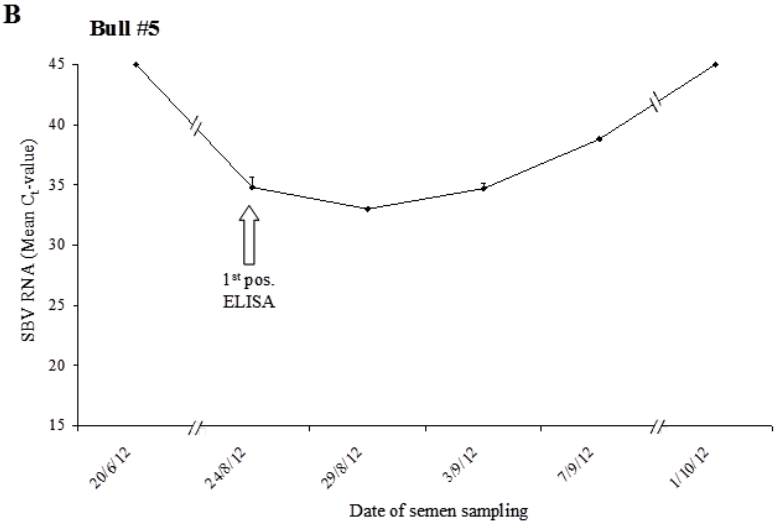
Results:

All the seven bulls presented a SBV seroconversion by ELISA. From 83 ejaculates, no SBV RNA was detected in four bulls (bulls 2, 3, 4, 6) before and following seroconversion. However, three bulls (1,5 and 7) from different breeds (Holstein or Normand) were found SBV RNA positive on more occasions using rtRT-PCR (Figure 16 A, B and C).

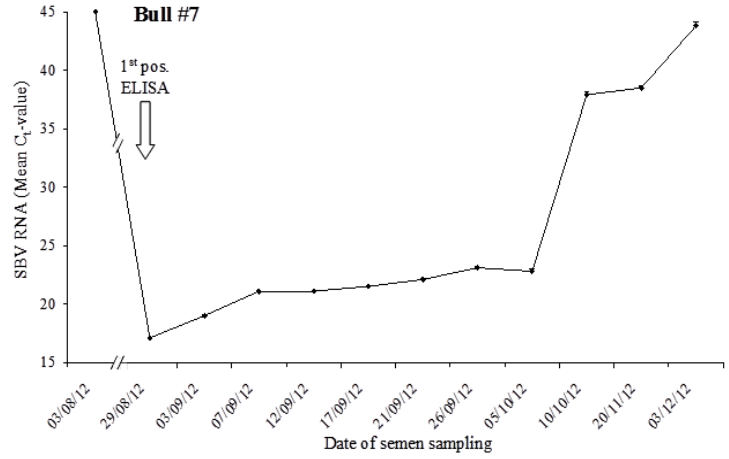
A



B



C



To confirm that bull semen contains infectious SBV and that semen keeps infectivity even after bull seroconversion, we performed additional *in vivo* experimental infections using the IFNAR$^{-/-}$ mice model.

In a first experiment, we showed that 100 µl of semen replicates from one SBV RNA positive ejaculate (bull 7 sampled on 29/08/2012; SBV C_t value =17.1) induced a viremia in three adult mice (Ct-values ranged between 23 to 28.9) and that they seroconverted within 3 weeks following infection.
To improve these results, a second *in vivo* assay were performed, involving four groups of four IFNAR-/- mice inoculated again with the same semen batch as well as semen samples collected before or after this date. Results are summarized in table 7 (see below).
Again, all four mice inoculated with the semen batch from the 29/08/2012 displayed high SBV RNA levels in IFNAR$^{-/-}$ blood 4 days p.i. (Ct-values ranged between 20 to 26) and were SBV ELISA positive 3 weeks p.i. Inoculation of the semen from 07/09/2012 induced low SBV RNA and antibody levels in two out of four mice, while SBV RNA and antibodies remained undetectable in all mice upon inoculation with the semen from 17/09/2012. Interestingly, the semen collected the 24/08/2012 induced positive SBV Ct value in only two mice but without detectable SBV antibody by ELISA. It is noteworthy that all mice with no detectable SBV RNA 4 days p.i. have also no detectable SBV antibody in serum, suggesting

that a viraemia seems to be required to induce a humoral response upon inoculation with naturally SBV RNA positive semen. This is in favor of the presence of a replicative virus in the inoculum.
Finally, positive SBV blood samples collected from mice at day 4 p.i. with semen from 29/08/2012 during experiment 2 were pooled (Inoculum A) and subsequently diluted 1 to 10 or 1 to 100 in MEM medium. Two groups of four mice were inoculated with 100 µl of these two blood preparations (experiment 3). Among all inoculated animals, one mouse was found SBV qRT-PCR and ELISA positive when inoculated with the IFNAR blood diluted 1/10. This indicates that the SBV RT-PCR positive IFNAR-/- blood collected at 4 days p.i. with the semen from 29/08/2012 (experiment 2) and inoculated to new IFNAR-/- mice (experiment 3), contained infectious material at probably low level but sufficient to induce SBV viraemia and seroconversion in at least one mouse.

Table 7. Ct values and seroconversion results in blood mice inoculated with SBV positive bull semen or IFNAR blood.

Inoculum	Exp. N°	Mice N°	C_t inoculum	C_t blood (4 days p.i.)	ELISA results
Bull 7 (24/08/2012)	2	8587	33.6	**36.7**	Neg
		8585		Neg	Neg
		8589		**24.0**	Neg
		8581		Neg	Neg
Bull 7 (29/08/2012)	1	7728	17.1	**28.9**	**Pos**
		8216		**23.5**	**Pos**
		8410		**23.0**	**Pos**
	2	8583	19.7	**21.7**	**Pos**
		8849		**20.0**	**Pos**
		8582		**25.6**	**Pos**
		8588		**26.0**	**Pos**
Bull 7 (07/09/2012)	2	8827	22.3	**31.5**	**Pos**
		8571		Neg	Neg
		8847		Neg	Neg
		8856		**37.0**	**Pos**
Bull 7 (17/09/2012)	2	8621	23.1	Neg	Neg
		8709		Neg	Neg
		8853		Neg	Neg
		8630		Neg	Neg
Inoculum A (1/10)	3	8631	22.0	**22.1**	**Pos**
		8634		Neg	Neg
		8858		Neg	Neg
		8629		Neg	Neg
Inoculum A (1/100)	3	8688	25.0	Neg	Neg
		8682		Neg	Neg
		8848		Neg	Neg
		8625		Neg	Neg

Conclusion:
All together, these results strongly suggest that semen batches collected the 29/08/2012 and the 07/09/2012 contained infectious SBV.

2.4 Determination of the role of other species in the epidemiology of SBV

2.4.1 Experimental infection in pigs

Methods:
An experimental infection study in pigs was performed at CODA-CERVA. Four 12 week old seronegative piglets were subcutaneously infected with 1ml of SBV infectious serum (FLI) and kept into contact with four non-infected piglets to examine direct virus transmission. Throughout the experiment blood, swabs and feces samples were collected and upon euthanasia at 28 dpi different organs (cerebrum, cerebellum, brain stem, lung, liver, iliac lymph nodes, kidney and spleen) were sampled. The presence of SBV RNA was examined by a qRT-PCR detecting the SBV-S segment and the presence of anti-SBV antibodies was assessed by seroneutralisation tests and ELISA.
Furthermore, field collected pig sera from before (n=109) and after (n=106) the SBV outbreak was tested in a seroneutralisation test.

Results:
No clinical impact was observed in the SBV inoculated pigs. Also all collected samples tested negative for SBV in rRT-PCR. Despite the absence of viremia and SBV in organs and, low and short lasting amounts of neutralizing antibodies were found in 2 out of 4 infected piglets. In ELISA, all samples tested negative. This is in line with results in sheep that show that the multi species ELISA (IDVet) is less sensitive at early time points post infection, and probably indicates that the first produced neutralizing antibodies are of the IgM isotype, which are not detected by the anti-IgG conjugate in the ELISA. The limited impact of SBV infection in pigs was further supported by the absence of neutralizing anti-SBV antibodies in field collected sera from indoor housed domestic pigs collected after the SBV emergence. In conclusion, SBV infection of pigs can induce a transient seroconversion but is ineffective in terms of virus replication and transmission indicating that pigs have no obvious role in the SBV epidemiology (Poskin et al, 2014).

2.4.2 Studies of SBV infection in South American camelids

Serological evidence of a previous SBV-infection demonstrated the susceptibility of South American camelids (SAC) to SBV. However, SBV pathogenesis in SAC and their role in the epidemiology of SBV are unknown. SAC become increasingly popular, and their current population is estimated to be 15,000 animals in Germany. Therefore, a field study and an animal trial were conducted. Furthermore, reference material was collected from experimentally infected SAC to validate serological and virological assays for SBV diagnosis in SAC.

German field study of SBV infection in South American camelids

Methods:
To investigate the incidence and clinical outcome of SBV infection in SAC, a field study was carried out in Germany from September 2012 to December 2013. SAC owners, veterinary laboratories and universities were contacted to submit blood and post-mortem samples and to

document clinical signs, malformations and abortions in SAC and their offspring ("cria") in a provided questionnaire.
Blood and post-mortem samples were analysed for SBV-RNA and antibodies using ELISA, neutralisation test and SBV-S3-specific RT-qPCR, respectively. SAC were considered SBV-seropositive or doubtful when at least one serological test result (ELISA or neutralisation test) was positive or doubtful. The serological results analysed by neutralisation test were compared with those determined by ELISA.

Results:
Blood samples from 502 SAC from 66 herds in 11 of 16 federal states were collected in Germany from September 2012 until December 2013. The overall sample size was higher than the sample size required to detect a seroprevalence of 10 to 90% with a confidence level of 95% and an accepted error of 5% (Conraths et al., 2011), and is therefore considered representative for the German SAC population.
SBV antibodies (n=309 seropositive, n=4 doubtful) were found in two-thirds (62.4%) of 502 SAC in 61 of the 66 herds (92.4%). The within-herd seroprevalence ranged from 0 to 100%, and the proportion of seropositive animals by federal state from 31 to 100%.
The age-group was provided for 277 SAC of which 67.1% were found seropositive. Of these SAC, a considerably lower proportion of crias (25.6%) were SBV seropositive compared to 1 to 2-year-old juveniles (78.0%) and >2 year-old adults (73.9%). In contrast to the high SBV seroprevalence, no SBV-RNA was detected in any sample.
Clinical signs were not reported in any SAC tested in this study. A total of three malformed crias were reported, but SBV infection could not be confirmed to be the cause of the malformations in any of the cases. An increase in the number of malformed or aborted crias in Germany was not recorded in this study.
A significantly ($p < 0.01$) higher number of animals was tested seropositive with neutralisation test compared with ELISA and a few animals only tested positive with ELISA.

SBV pathogenesis in South American camelids experimentally infected with SBV

Methods:
Three llamas (*lama glama*) and two alpacas (*Vicugna pacos*) were purchased from breeders in Germany and were tested negative for SBV-RNA and antibodies before their transfer to the high containment facility of the FLI, Isle of Riems. All SAC were experimentally infected with SBV-containing serum well characterised by previous animal trials (Wernike et al., 2012). Clinical signs and fever were monitored daily. Serum was collected at -1, 2 to 7, 9, 11, 14, 17, 21, 28, 40, 47 and 54 dpi, and post-mortem examination was conducted at 62 dpi. Post-mortem samples included spleen, liver, mesenteric and mediastinal lymph nodes, tonsils, cerebrum, cerebellum and medulla oblongata. Serum samples of all SAC were comparatively analysed for SBV antibodies with ELISA and neutralisation test. Serum and post-mortem samples were tested by S-segment-specific RT-qPCR.

Results
No obvious clinical signs were observed in any of the 5 SAC. SBV-RNA was detected from 3 to 7 dpi for a period of 1 to 5 days with peak C_q-values between 24.0 and 41.6 (average C_q 30.2). Seroconversion occurred between 9 and 21 dpi and between 9 and 40 dpi as measured by neutralisation test and ELISA, respectively. A considerable increase of SBV antibody levels below the cut-off values of the ELISA was observed in all animals at 14 dpi compared to -1 dpi; at a time when all except one SAC were seropositive by neutralisation test. The

detection of SBV antibodies in SAC serum was found more sensitive using neutralisation test compared to ELISA, which is in accordance with the results of the field study. At post-mortem examination, at day 62 p.i.,none of the SAC showed gross pathological lesions. SBV-RNA was detected in the spleen of L1 (C_q 39.7), but not in any other tissue sample.

The overall and within-herd SBV seroprevalence found for SAC was lower compared to SBV seroprevalence reported for cattle and sheep, but higher than in goats and Alpine wild ungulates. Interestingly, a lower within-herd seroprevalence was found in SAC in North-Western German federal states - the federal state that recorded a high number of new SBV cases in domestic ruminants between 2011 and 2012. This contrasts the higher seroprevalence in SAC herds found in South-Eastern Germany where most of the SBV cases were reported in 2013. A possible reason might be that SBV antibodies do not persist for a long time in SAC after SBV infection. No SBV-RNA was detected in any sample, which was probably due to a short-time viraemia (as observed in the animal trial) and the period of sample collection - after the peak of SBV infections in 2011.
The low proportion of SBV-infected crias can be explained by the lower number of SBV infections reported during the study period compared to the number of new infections reported in domestic ruminants from 2011 to summer 2012 in Germany.
Serological and virological methods for SBV diagnosis in SAC that were validated with the specimens collected from SAC experimentally infected with SBV were found suitable for SBV diagnosis in SAC. Based on the serological results of the field study and animal trial, we recommend to use ELISA or neutralisation test for herd screening, but a combination of both methods for diagnosis of SBV antibodies in individual SAC.

Similar to SBV-infected ruminants, SBV genome can be detected in SAC for a short time after SBV-infection. The results of the field study and animal trial indicate that SAC are resistant to SB disease, but their role in the epidemiology of SBV has to be further investigated.

2.4.3 Experimental infection in poultry

Experimental Simbuvirus infection of poultry were performed at FLI. 6 groups of 4 chickens each were inoculated with Sabo virus, Simbu virus, Sathuperi virus, Schmallenberg virus (challenge serum), Schmallenberg virus (cell culture propagated virus) and cell culture medium (negative control animals).

None of the chickens showed specific clinical signs. All samples collected on several days post inoculation were SBV genome negative (RT-qPCR). Furthermore, the inoculated chickens produced no virus specific antibodies. In conclusion, also chicken are not susceptible for Simbu, Sabo, Sathuperi and Schmallenberg virus.

2.5 Determination of the role of wildlife (deer, wild boar etc.) in the epidemiology of Schmallenberg virus (SBV)

2.5.1 Wildlife in Germany

Methods:
In 2011 Schmallenberg virus (SBV) was first detected in domestic ruminants i.e. cattle and sheep. Moufflon as a wild sheep is the only free-living wild form of susceptible domestic animals in Germany. To identify native wildlife species in Germany that are infected with SBV, blood samples were collected from following species: (1) Moufflon, (2) further native free-living ruminants (roe deer, fallow deer, red deer, sika deer); (3) wild boar (which belong to the closely related Suidae family within the Artiodactyla order); (4) carnivores (foxes, raccoon dogs, raccoons, marten spp. etc.) as scavenger, which could be in contact with SBV eating aborted fetuses and stillborn and distorted newborn fawns; (5) mice as a potential reservoir for SBV.
The sample collection was selected on the basis of the first detected cases in Germany (11/2011) in periods a) before 2011, b) during the peak of the SBV epizootic (hunting period 2011/2012) and c) current situation (hunting period 2012/2013).
To determine date and place of SBV emergence in wildlife, we analysed historical samples collected from wild ruminants in North Rhine-Westphalia before 2011.
Furthermore, during the hunting season 2013 /2014 (beginning 1st April 2013) the sample collection was limited to North Rhine-Westphalia, the federal state where the first case of SBV infection was detected. To detect possible new SBV infections in autumn 2013 - at a time when maternal immunity has most likely ceased - we focused on samples from subadults < 1 year of age (fawns, calves, lambs and young boars).
SBV antibodies were analyzed with ELISA. Sera with a serological status defined as doubtful or negative were retested by neutralization test. A representative number of samples with positive ELISA-results from each species were confirmed by serum neutralization test. In this section, doubtful results are referred to as ‘positive’.

Results:
A total of 27.7% of 1868 blood samples collected from moufflons (72% of 29), roe deer (42% of 499), fallow deer (36% of 44), red deer (29% of 226), sika deer (25% of 20) and wild boar (21% of 1079) between 2011 and 2013 were positive for SBV antibodies. In contrast, the proportion of seropositive animals was considerable lower in samples collected in North Rhine-Westphalia in 2013/2014 (10.5% of 702: 30% of 10 moufflons, 27% of 229 roe deer, 12% of 43 fallow deer, 0% of 51 red deer, 4% of 369 wild boar) compared to 2011-2013. In the study period 2013/2014, no samples were obtained from sika deer. For subadults tested in autumn 2013 and January 2014, 10 (moufflons, fallow deer, red deer, wild boar) and 1 (red deer), respectively, were found seropositive.
No SBV antibodies were detected in 788 historical samples collected before 2011 and in 281 wild carnivores and 195 mice collected from 2011 to 2012.
For 80 samples that were retested with neutralization test, about twice as much were positive by neutralization test (n=33) but negative or doubtful with ELISA. A disagreement between the two test systems was particularly found for red deer (18 of 19 only positive with neutralization test). These results suggest that seroprevalence given for red deer and wild boar for the study period 2011-2013 might be higher since the samples were only tested with ELISA.

In addition to wild cervids, wild boars were found SBV seropositive indicating that not only ruminants and camelids but also other members of the Artiodactyla order are susceptible to SBV infection. Hence, free-ranging Artiodactyla, but not mice or wild carnivores, might play a role as reservoir in the epidemiology of SBV. During the last three years the rate of seropositive wild ruminants as well as wild boar in North Rhine-Westphalia increased from autumn 2011 to winter 2012/2013 and decreased in summer 2013 indicating that a small number of new SBV infections have occurred in subadults in autumn/winter 2013/2014. Currently, the low level of SBV-specific immunity in wild cervid and wild boar populations in North Rhine-Westphalia is low. Further investigation in the next years would show whether a low level of SBV infection in the population will persist (a low number of new infected young animals each year) or possibly lead to a new peak of SBV infection in wild ruminants and wild boar populations. To compare the spread of SBV in domestic and wild animals, seroprevalence of both populations has to be investigated in the same region.

2.5.2 Wildlife in France

Methods:
ANSES was involved in epidemiological studies performed in France in wild ruminant (deer) but also in a non-ruminant species (dog). Retrospective serological studies performed on sera collected during the SBV spread in France were conducted.

Results:
Our data suggest also that SBV spread very quickly from north-eastern to south-western France between October and December 2011 (800 km). They also put in evidence that SBV spread among red deers and domestic flocks at the level of the department, and highlight the perspective of that red deers could be sentinels of SBV spreading for livestock. We also pinpointed the relevance of new competition ELISA for improving SBV surveillance in wildlife species, even though SNT remained the most reliable assay for SBV antibody detection in red deers.
More interestingly, following neurologic disorders detected in five 15-day-old puppies (Belgian shepherd) from a dog breeding kennel in northwestern France (Orne), we report data suggesting that these puppies were infected with SBV. The presence of specific SBV antibodies in the mother associated with congenital symptoms and the presence of SBV genome in her puppy suggested that these dogs experienced SBV infection. In addition, the fact that the malformed puppies were born in March 2012 and that SBV antibodies were still detectable in the mother in March 2013 suggests that the duration of SBV antibodies in dogs is at least one year.

2.5.3 Wildlife in the Netherlands

Schmallenberg virus antibody screening in wildlife in the Netherlands was focussed on Fallow deer, roe deer and wild boar. High seroprevalences were observed in Fallow deer (42%), roe deer (46%) and wild boar (22%).

Schmallenberg virus antibody prevalences observed in the different countries for the most abundant wildlife species were largely in agreement.

2.5.4 Schmallenberg virus antibody testing in horses and zoos in the United Kingdom

Methods:
Serum samples were collected from horse holdings in regions heavily affected by the initial outbreak in the UK. In total 92 horses were tested using both ELISA and VNT format. SBV-specific antibodies could not be determined in any horse, making an infection of equids unlikely.

Furthermore, serum samples were opportunistically collected two zoos in the UK. In the first case, 180 samples from 01/01/2011 and 01/02/2014 representing 38 different species were tested using a competitive ELISA (Table below) and 29 samples of 17 different species were tested using PRNT for confirmation.

Order	Species- Latin name	Species – common name	Samples (n)	Different individuals
Artiodactyla	*Vicugnapacos*	Alpaca	1	1
	Bison bison	American bison	1	1
	Oryx leucoryx	Arabian oryx	4	4
	Axis axis	Axis deer	1	1
	Camelusbactrianus	Bactrian camel	6	6
	Antilopecervicapra	Blackbuck	1	1
	Tragelaphuseurycerus	Bongo	4	3
	Hydropotesinermis	Chinese water deer	1	1
	Bison bonasus	European bison	9	7
	Damadama	Fallow deer	1	1
	Bosgaurus	Gaur	2	2
	Oryx gazellagazella	Gemsbok	10	6
	Tragelaphusstrepsiceros	Greater kudu	6	3
	Axis / Hyelaphusporcinus	Hog deer	3	2
	Aepycerosmelampus	Impala	2	2
	Lama glama	Llama	3	2
	Alcesalces	Moose	5	3
	Kobus megaceros	Nile lechwe	4	2
	Capra aegagrushircus	Nubian goat	3	3
	Elaphurusdavidianus	Pere David's deer	4	4
	Hexaprotodonliberiensis	Pygmy hippo	3	1
	Potamochoerusporcus	Red river hog	2	2
	Rangifertarandus	Reindeer	1	1
	Giraffacamelopardalisreticulata	Reticulated giraffe	4	3
	Oryx dammah	Scimitar-horned oryx	16	12
	Tragelaphusspekii	Sitatunga	14	10
	Rucervusduvaucelii	Swamp deer	4	4
	Kobus ellipsiprymnus	Waterbuck	2	2
	Capra aegagrushircus	White Windsor goat	1	1
	Bosgrunniens	Yak	21	10
Perissodactyla	*Equusafricanusasinus*	Domestic donkey	2	2
	Equusgrevyi	Grevy's zebra	13	6
	Rhinoceros unicornis	Indian rhinoceros	2	2
	Equushemionus	Onager	8	5
	Equusasinuspoitou	Poitou donkey	2	2
	Equusferusprzewalskii	Przewalski horse	5	2
	Equusferuscaballus	Shetland pony	2	2
	Ceratotheriumsimum	White rhinoceros	4	4

In addition 28 samples from a second zoo representing 16 different species were assayed using the competitive ELISA.
Furthermore, tissue samples of two newborn yak were screened using PCR.

Results:
Positive SBV competitive ELISA results from samples from zoo 1 and zoo 2 are detailed below. The ELISA was positive in samples representing 14 species from zoo 1 and in eight samples from eight individuals representing seven species from zoo 2 (Table 9).

ZOO 1													
Species - common name	**Individual Samples**												
Bongo	X												
European bison	X	X	X										
Gaur	X												
Gemsbok	X												
Greater kudu	X	X	X										
Grevy's zebra	X												
Moose	X	X											
Nile lechwe	X	X											
Nubian goat	X												
Onager	X												
Reindeer	X												
Scimitar-horned oryx	X	X	X	X	X	X	X	X	X	X	X		
Sitatunga	X	X	X	X	X	X	X						
Yak	X	X	X	X	X	X	X	X	X	X			
ZOO 2													
Species - common name	**Individual Sample S/N results**												
Babirusa	X												
Banteng	X												
Bongo	X												
Congo buffalo	X	X											
P.S. deer	X												
Roan antelope	X												
Scimitar-horned oryx	X												

Table 9. Positive SBV competitive ELISA samples both zoos

PRNT was carried out and confirmed the validity of (both positive and negative) ELISA results for deer (hog deer, reindeer), antelope (Greater kudu, blackbuck), bovids (yak, gaur,) giraffes and red river hogs. An ELISA inconclusive white rhinoceros was negative on PRNT, further confirming the notion that the infection is limited to ruminants (and possibly some pig species). Two camelids (Bactrian camel, llama) that showed a negative, but suspicious ELISA results were positive on PRNT.

In addition fetal Yak tissues were confirmed to be SBV positive by PCR demonstrating that this species can be clinically affected too.

Area 3 : Diagnostics

Main objectives

To harmonise the validation of RT-PCR methods for the detection of SBV and to harmonise the validation of SBV serological assays.

Workplan (concise)

All partners of the consortium were using a diagnostic RT-PCR targeting the SBV S-segment. To asses RT-PCR diagnostic procedures in the different labs, an interlaboratory comparison study of RT-PCR diagnostics was performed. In all partner laboratories tests were done using the routine procedure of the lab. Sample processing procedures as well as SBV RNA extraction procedures were included in this comparison study.

Among partners there was no common protocol for validation of serological tests. Based on currently used methods some minimum criteria for validation of SBV serology were listed. A limited inter-laboratory ring trial for serology was organised by CVI.

3.1 Harmonisation and validation of serologic tests

Methods:
For SBV antibody detection laboratories first implemented virus neutralization tests (VNTs) and later on SBV ELISAs. In order to obtain a first impression of laboratory performances in SBV antibody testing in Europe a simple and straightforward inter-laboratory ring trial was organised in June 2012. A set of 10 SBV antibody positive and negative sheep and cattle sera was circulated among eight different veterinary laboratories in 7 countries in Europe. The eight laboratories in the trial all together used 6 different assays for Schmallenberg virus antibody detection. Besides a virus neutralization test (VNT), three laboratories performed an in-house ELISA and six laboratories used one or more commercially available ELISAs. All test results were reported to the organising laboratory for comparison.

Results:
Schmallenberg virus antibody test results by VNT were in agreement for eight participating veterinary laboratories in the ring trial. ELISA assays by these eight laboratories performed well but some test results had to be designated as 'doubtful' by some labs whereas these samples were mainly scored positive by other labs. VNT was more sensitive in detecting SBV specific antibodies than some of the used ELISA assays. Eight veterinary laboratories within the ring trial performed a virus neutralization test (VNT) as well as one or two ELISAs on a set of 10 ruminant serum samples, and swiftly detected SBV antibodies using these assays. VNT was more sensitive in detecting SBV specific antibodies than some of the used ELISA assays. Based on the test results one cattle and one sheep SBV antibody positive serum were selected to serve as reference sera, which now can be supplied to other laboratories on request.

The ring trial gave a good first impression of routine SBV serology in European veterinary laboratories and the results were promising for VNT as well as for ELISAs. VNT results were in agreement between laboratories. For ELISA results there were some discrepancies but in general these assays also performed well. The study gave an impression of the sensitivity and the specificity of SBV antibody detection assays in different laboratories, but it has to be taken into account that a very limited number of samples was tested in this trial. To assess sensitivity and specificity of ELISAs a much larger and more varied set of samples will have to be tested. It can be concluded that Schmallenberg virus antibody detection in the different veterinary laboratories within EU is reliable. Based on the results of the study one SBV antibody positive cattle serum and one SBV antibody positive sheep serum were selected to serve as reference sera, which now can be supplied to other laboratories on request.

3.1.1 Further validation of serologic tests

In extension to the first ring trial comparing ELISA and VNT, further comparisons were carried out at AHVLA, taking ELISAs into account that were not available in 2012. Five ELISA tests from three manufacturers were compared to the PRNT published before.
In the first table (Table 10), dams that had given birth to malformed lambs that were tested positive for Schmallenberg were analysed. It can therefore be assumed that the PRNT results reflect the reference, albeit at fairly low Ab titres at the time of analysis. While some ELISAs clearly struggle to detect the SBV antibodies, one test achieves a perfect match with the PRNT and two further ELISA provide acceptable results.

	A		B		C		D		E		PRNT	
samples	S/P %	result	S/P %	result	S/P %	result	S/P %	result	S/P %	result	titer	result
220	58.3	negative	73.00	positive	30	positive	21.07	negative	66.70	positive	1/16	positive
227	61.3	doubtful	65.47	positive	19.5	positive	19.58	negative	45.28	doubtful	1/16	positive
231	48.6	negative	76.11	positive	14.4	positive	20.83	negative	62.63	positive	1/16	positive
233	57.4	negative	77.10	positive	17.15	positive	24.87	negative	56.84	positive	1/16	positive
247	28.9	negative	64.15	positive	24.6	positive	21.10	negative	58.95	positive	1/16	positive
657	43.5	negative	55.07	doubtful	31.02	positive	26.26	negative	69.96	positive	1/16	positive
232	37.7	negative	57.92	doubtful	25.49	positive	12.30	negative	36.51	negative	1/16	positive

In the second part (Table 11), sheep infected in vivo with the FLI-SBV serum were analysed. After 4 weeks, all result between the PRNT and the ELISA "C" are matching again (important to note that sheep no 5 was resistant to the infection). Here also ELISA "E" is able to detect all samples correctly demonstrating the within 4 weeks after infection animals can securely be serologically detected by both validated VNT and ELISA methods.

	B		C		E		PRNT	
samples	S/P %	result			S/P %	result	titer	result
Day 14								
1	59.83	doubtful			66.38	positive		
2	63.73	positive			89.20	positive		
3	46.45	negative			58.35	positive		
4	80.98	positive			63.85	positive		
5	1.46	negative			1.99	negative		
6	46.47	negative			44.48	doubtful		
7	60.40	positive			65.07	positive		
8	52.21	doubtful			43.60	doubtful		
1	19.43	negative			16.05	negative		
2	46.69	negative			42.83	doubtful		
3	40.86	negative			32.97	negative		
4	82.60	positive			69.46	positive		
Day 21								
1	76.11	positive			42.98	doubtful		
2	75.41	positive			56.97	positive		
3	76.10	positive			86.96	positive		
4	62.77	positive			78.34	positive		
5	0.95	negative			3.13	negative		
6	52.03	doubtful			57.00	positive		
7	53.93	doubtful			72.34	positive		
8	38.48	negative			38.67	negative		
1	22.22	negative			19.64	negative		
2	55.05	doubtful			65.32	positive		
3	62.27	positive			35.79	negative		
4	35.11	negative			71.48	positive		
Day 28								
1	65.51	positive	23.65	positive	113.3	positive	1/64	positive
2	75.15	positive	15.76	positive	138.2	positive	1/32	positive
3	68.7	positive	14.38	positive	94.4	positive	1/64	positive
4	77.25	positive	10.99	positive	188.2	positive	1/128	positive
5	9.32	negative	83.45	negative	10.4	negative	<4	negative
6	72.5	positive	11.97	positive	165	positive	1/128	positive
7	108.2	positive	14.9	positive	189.4	positive	1/64	positive
8	54.5	doubtful	18.99	positive	123.6	positive	1/64	positive
1	33.7	negative	16.46	positive	68.2	positive	1/64	positive
2	79	positive	7.82	positive	134.9	positive	1/64	positive
3	40.7	negative	19.16	positive	57.7	positive	1/64	positive
4	58.3	doubtful	22.9	positive	133.7	positive	1/64	positive

Table 11. Schmallenberg virus: efficacy of current serological tools

The direct and competitive SBV ELISA (ID-VET, France) able to detect SBV antibodies in ruminants, but also in other species, has been evaluated by ANSES/FLI and CODA-SERVA. Many published studies confirm that these ELISA are useful for the SBV sero-diagnosis and disease-surveillance studies in domestic and wild ruminant species in Europe (in goats, sheep and cattle).

3.2 Harmonisation and validation of RT-PCR tests

3.2.1 Validation of diagnostic methods for Schmallenberg virus in bovine semen and field study in Germany

Spiking experiments: Validation of extraction methods
Twelve different manual and automatized extraction procedures were comparatively validated using dilution series of spiked semen, blood samples and minimum essential medium (MEM) as control (Table 12). In a second experiment, 8 SBV-RNA-positive semen batches (C_q 22.9-37.4) collected from 5 bulls in the German field study (see below) were used for the assessment of performance characteristics of 5 of 12 methods (see Table 12) and one additional method – using Trizol® LS Reagent only (method 13).

The highest diagnostic and analytical sensitivity for the extraction of SBV-RNA in semen was found for the Trizol® LS Reagent lysis (method 13) or in combination with a subsequent purification of the viral RNA based on magnetic beads using the MagAttract® Virus Mini M48 Kit at a KingFisher® Flex workstation (method 3). In contrast, the Chelex® 100 kit was proven to be the most unsuitable extraction procedure in this study (see Table 12 and Figures 17 ab and 18). The diagnostic specificity of all tests was 100%.

German field study
Using the significantly most suitable extraction method (#3), frozen semen collected between May 2012 and November 2012 from 95 seroconverted bulls was analysed for SBV-RNA with real-time RT-PCR (RT-qPCR).

A total of 766 semen batches from 95 SBV-infected bulls (56 fresh semen batches from 56 bulls, and 710 straw batches from 94 bulls; from some collection days fresh semen as well as straws were available) were obtained from 7 stock-bull breeding centres in Germany in 2012. A total of 29 (3.8% of 766) semen batches from 11 bulls from 3 breeding centres were positive in RT-qPCR analysis with mean and median Cq-values of 33 ranging from 26 to 37. In 4 of the 11 bulls, SBV-genome positive semen was co-incidentally detected with first SBV-antibodies. Multiple SBV-genome positive results were observed in 6 of the 11 bulls for a period of 3 days to 8 weeks in 2 to 11 consecutively collected semen batches (Fig. 19). In two of the bulls intermittent virus excretion could be observed within 3 and 8 weeks.

<u>Conclusions</u>
This study provides methods for the safe diagnosis of SBV-RNA in bovine semen. How long intermitting virus excretion may persist is currently unclear and will be the topic of further studies. This could have considerable consequences on the trade of semen from bulls in SBV-affected regions. Similar to recommendations for BHV-1 testing in semen, we currently recommend to test two straws of each semen batch of SBV-infected (RNA or antibody positive) bulls in replicates for SBV-RNA to allow a reliable evaluation.
Since the mere presence of RNA does not necessarily imply venereal transmission, subsequent in vivo and in vitro studies of the infectivity of SBV-RNA-positive semen were conducted, which were described in the previous section (Hoffmann et al. 2013).

Table 12. Extraction methods comparatively analysed for diagnostic and analytical sensitivity to detect SBV-RNA in bull semen. Methods 1 to 12 were validated with SBV-spiked samples. Methods 1, 3, 4, 6, 7 and 13 (*) were assessed with SBV-RNA-positive semen samples from the field. Various statistical analyses revealed the highest sensitivity for methods 3 and 13 (Hoffmann et al. 2013).

Method #	Pre-treatment (lysis)	Extraction kit	Extraction method	Sample volume (µl)	Diagnostic sensitivity (%)
*1	ND	MagAttract[b]	KingFisher Flex[h]	100	9.4
2	Trizol[a]	MagAttract[b]	KingFisher Flex[h]	250	ND
*3	Trizol[a]	MagAttract[b] without lysis buffer	KingFisher Flex[h]	250	96.9
*4	ND	RNeasy[c]	manual	200	50.0
5	Trizol[a]	RNeasy[c]	manual	100	ND
*6	Trizol[a]	RNeasy[c]	manual	250	71.9
*7	ND	QIAamp Viral RNA Mini[d]	manual	75	46.9
8	Addition of 75 µl of 1-fold PBS to sample	QIAamp Viral RNA Mini[d]	manual	75	ND
9	ND	Total NA[e]	MagNa Pure[i]	100	ND
10	ND	QIAamp cador Pathogen Mini[f]	manual	200	ND
11	ND	Chelex[g]	manual	10	ND
12	ND	Chelex[g], supernatant extracted with RNeasy[c]	manual	10	ND
*13	ND	Trizol[a]	manual	250	96.9

ND, not done; PBS, phosphate buffered saline; [a] Trizol® LS Reagent, Life Technologies, Darmstadt, Germany; [b] MagAttract® Virus Mini M48 Kit, Qiagen, Hilden, Germany; [c] RNeasy® Mini Kit, Qiagen; [d] QIAamp® Viral RNA Mini Kit, Qiagen; [e] MagNA Pure® LC Total Nucleic Acid Isolation Kit, Roche Diagnostics, Mannheim, Germany; [f] QIAamp® cador® Pathogen Mini Kit, Qiagen; [g] Chelex® 100, Sigma-Aldrich, Steinheim, Germany: Protocol according to Wang et al. (2007); [h] KingFisher® Flex, Thermo Fisher Scientific, Schwerte, Germany; [i] MagNA Pure® LC, Roche Diagnostics.

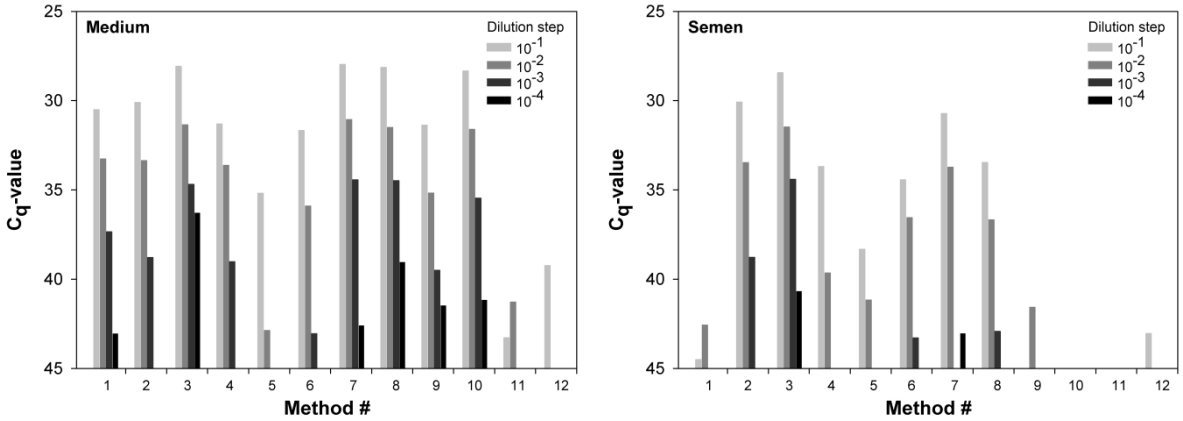


Figure 17ab. RT-qPCR results of the two SBV-spiked and serially diluted matrices medium and semen extracted with 12 different methods in the spiking experiment (see also Table 1). Given C_q-values show means of replicates. Method 3 revealed the highest diagnostic and analytical sensitivity compared to the other methods. PCR results obtained with the matrixes serum (data not shown) and medium were very similar (Hoffmann et al. 2013).

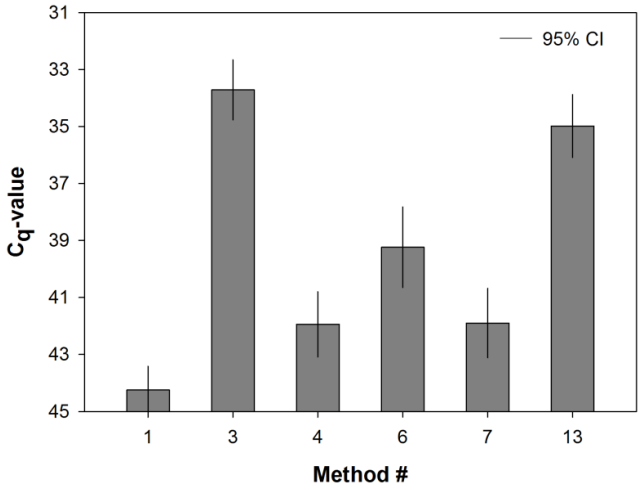


Figure18. Comparison of 6 extraction methods with SBV-RNA-positive semen samples obtained in the field study. Mean C_q-values by method ranged from C_q 33.7 to 44.2. Error bars show 95% confidence interval (CI). Methods 3 and 13 showed a significantly higher number and lower C_q-values compared to the other methods (Hoffmann et al. 2013)..

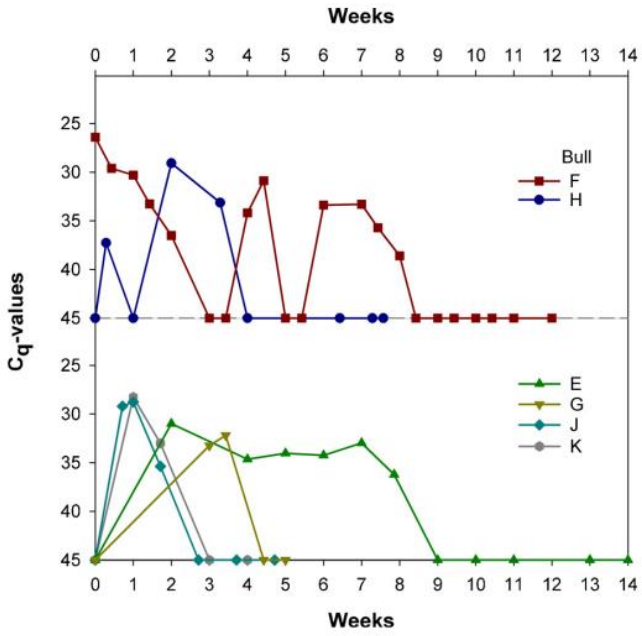


Figure 19. Progression of C_q-values in semen samples of SBV-infected bulls collected between August and October 2012 in Germany and extracted with method 3 as measured by RT-qPCR. Two (F and H) of the six bulls with consecutive SBV-RNA positive semen batches showed intermittent virus excretion (Hoffmann et al. 2013).

3.2.2 RT-PCR methods ring trials with different matrices to assess performance characteristics.

The proficiency of methods for SBV-RNA extraction and detection used in 27 German and 17 European laboratories was evaluated in two method trials. Four of the five European laboratories participated in both of the trials. A set of defined samples was provided by the Friedrich-Loeffler-Institut (FLI), Insel Riems. The specimens included serum, EDTA-treated whole-blood, tissue homogenates, RNA-eluates and bovine semen positive or negative for SBV-RNA. To determine the reference C_q-values at the FLI, serum, whole-blood and tissue homogenates were extracted with QIAamp® Viral RNA Mini kit (Qiagen), and semen samples with the significantly most efficient extraction method described previously (Hoffmann et al., 2013). Briefly, viral RNA in semen was lysed with Trizol® LS Reagent (Invitrogen) and subsequently purified using the MagAttract® Virus Mini M48 kit (Qiagen), which is based on magnetic beads, at a KingFisher® Flex workstation (Thermo Fisher Scientific). SBV-RNA was quantified using small-segment specific real-time RT-PCR (Bilk et al., 2012). All participants received straws of additional semen batches and the corresponding reference C_q-values to allow a validation of the recommended and the in-house RNA extraction methods conducted at their laboratories before the analysis of the specimens of the method trial. Detailed information on the SBV-RNA extraction and PCR-assays used by the different laboratories were recorded. The proficiency of different methods were comparatively analysed with respect to the PCR-results.
The total number of specimens by matrix, their corresponding reference C_q-values and the extraction methods and PCR-protocols recommended by the FLI are given in tables 2 and 3. In the first (S1, B4, P2) and second (R7, T5, P1) method trial 6 specimens contained SBV-RNA loads at the detection limit ($\geq C_q$ 35). For these specimens, positive and doubtful results were considered correct and will be referred to as ‘positive’.

Results:
For the matrices serum, whole-blood, tissue homogenate and RNA-eluate, results were provided by all participants of the first (n=43) and second (n=5) method trial, respectively.
The method trial demonstrated that all extraction and PCR-procedures (100%) were robust to determine C_q-values of SBV-RNA below the detection limit in the matrices serum, whole-blood, tissue homogenate and RNA-eluates generally similar to the reference C_q-values determined by the FLI. A high proportion of laboratories also detected borderline specimens, while the proportion of SBV-RNA-negative samples erroneously determined as positive and doubtful was low (8 of 189 results determined by 4 of 44 laboratories).
Semen samples were tested for SBV-RNA by 18 of 27 national, 16 of 17 European (including the 5 SBV-Consortium laboratories). This study confirmed that the recommended methods have a high diagnostic sensitivity independent from the laboratory (92.7% for all semen samples; 100% for semen batches with C_q-values $\leq$35). Significant differences ($p < 0.05$) were found between means of the C_q-values of all three (P1-P3) semen batches tested in the first method trial and the single semen batches P1 and P2, respectively, when analysed by the recommended compared with other extraction procedures. A similar high proportion of positive results were obtained by the combination of Trizol® LS with any other (94.2%) than the MagAttract® Virus Mini M48 kit. In contrast, the use of other Trizol preparations with and without (72.7%)/or (50.0%) other kits revealed a considerably lower proportion of true positive results.
The highest proportion of false negative results was found for the semen batch (n=7 of 10) with the highest C_q-value (C_q 36.4), which was comparatively analysed in the second method trial. This batch was also tested negative for SBV-RNA by two laboratories that used the

recommended extraction methods. Accordingly, it is important to thoroughly validate extraction methods with standardised reference semen batches to establish alternative extraction and PCR-protocols for a reliable diagnosis of SBV-RNA in bovine semen. The two semen batches negative for SBV-RNA (P5 and P7) were diagnosed as SBV-RNA-negative by all 5 laboratories in the second method trial.

Table. Matrices and C_q-values of samples used for comparative SBV-RNA-testing in 27 national and 16 European laboratories as measured by SBV-S3 RT-qPCR (Bilk et al., 2012) and recommendations given for extraction and PCR-assays by the Friedrich-Loeffler-Institut.

	EDTA-blood (B)	**serum (S)**	**tissue (T) homogenate**	**semen (P)**
number of samples	5	5	5	3 batches
C_q-value of positive samples	22-38, no C_q	27–37, no C_q	15–26, no C_q	30–35
recommended extraction	in-house RNA extraction			Trizol® LS with/ without purification with magnetic beads*
recommended PCR testing	in-house PCR-protocol			
specifications	report one result			

* MagAttract® Virus Mini M48 kit used at a used at a magnetic-bead separator.

Table. Matrices and C_q-values of samples used for comparative SBV-RNA-testing in 5 European laboratories of the SBV-Consortium as measured by SBV-S3 RT-qPCR (Bilk et al., 2012).

	RNA (R)	**serum (S)**	**tissue (T) homogenate**	**semen (P)**
number of samples	16	16	12	6 batches
C_q-value of positive samples	19–36, no C_q	23–32, no C_q	18–35, no C_q	29–36, no C_q
recommended extraction	None	QIAamp viral RNA mini kit and in-house RNA extraction		Trizol® LS with/ without purification with magnetic beads* and in-house RNA extraction
recommended PCR testing	SBV-S3-RT-qPCR and in-house RT-qPCR			
specifications	technical duplicates			2 biological and 2 technical replicates

* MagAttract® Virus Mini M48 kit used at an automated or manual magnetic-bead separator.

The methods used for SBV-RNA diagnosis in all matrices, except semen, were found robust to produce positive results for all clearly SBV-RNA-positive specimens and in a high number of samples with SBV-RNA loads at the detection limit.
In contrast, the application of a suitable extraction method was found critical for a reliable diagnosis of SBV-RNA in bovine semen. The high sensitivity of the recommended, published

extraction methods for SBV-RNA diagnosis in bovine semen were confirmed by the method trial. Although several other extraction procedures in combination with Trizol® LS were found suitable, alternative methods should be thoroughly validated with standardised reference semen batches. Highly sensitive methods that allow the detection of C_q-values below the detection limit of C_q 35 are strongly recommended since infectivity of SBV-RNA-positive semen with C_q-values around the detection limit cannot be excluded. SBV-RNA-positive semen with a C_q-value of 34.2 has been infectious for subcutaneously injected cattle (Schulz et al., 2014).

4 Conclusions

Schmallenberg virus primarily infects domestic and wild ruminants. Cattle and sheep seem to be the most susceptible species. Goats, pigs and camelids seem to be less susceptible. In pregnant cattle and sheep, the virus can infect multiple organs of the un-borne fetus. However, this infection often does not cause major lesions and infrequently leads to malformations. Serological evidence of a previous SBV-infection were found in various wild ruminant species and dogs. Seroprevalence varied among different species. No SBV-infection was found in horses, poultry and in wild mice. Schmallenberg virus did infect immunocompromised (IFNAR -/-) mice.

Certain species of Palearctic *Culicoides* biting midges are the main vectors of SBV. Transovarial SBV-transmission in culicoids has not been observed.

Schmallenberg virus was introduced in Europe in 2011. After exposure SBV rapidly spread within naive herds, and also throughout winter. Blood samples collected before the first clinical cases of SBV were observed in Europe in 2011, were all tested negative for SBV antibodies. The origin of the virus remains unknown.

Schmallenberg virus was detected in semen and embryos from SBV-infected cattle and sheep, respectively. By the end of 2012, a frequency of 0-4% SBV-RNA-positive bovine semen batches was found in the participating countries. Subcutaneous injection of SBV-RNA-positive semen in cattle demonstrated that semen from SBV-infected cattle may contain viable SBV. In vitro studies with embryos suggest a negligible risk for SBV-transmission. Whether infectious virus can be transmitted to susceptible cows at service or by insemination, still needs to be elucidated. Since the role of SBV-contaminated gametes in the epidemiology of SBV is still unknown, additional studies are required.

5 Recommendations

Schmallenberg virus has rapidly spread over Europe reaching prevalences in cattle and sheep of close to 100%. Two years after the virus was first detected, the outbreak seems to be over in the countries first affected. However, since the number of susceptible animals in Europe is rising since 2013, we need to be aware of potential recurrent cases in EU countries and a basic SBV surveillance is recommended.

The role of SBV-contaminated semen in the epidemiology of SBV is still unknown. Therefore additional studies on SBV transmission via artificial insemination are required.

Since it remains unknown how Schmallenberg virus was introduced in Europe the tracing back of the SBV origin and in relation to that studies on strain variation of SBV and other Simbu serogroup viruses is indicated. A risk analysis of possible ways of introduction should be considered, this may be helpful to avoid new introduction of such viruses in future.

To early detect new emerging (arthropod-borne) viruses, monitoring of sentinel herds in European countries together with midge trapping on the same farms to study vector epidemiology would be useful. In parallel methods to detect new and emerging diseases in livestock need to be established and/or maintained across Europe.

The culiciodes vector seems to play a more and more important role in the transmission of Orthobunyaviruses in Europe. To better understand this and also to find out how this is influenced by climate change vector competences of Schmallenberg virus and related Simbu serogroup orthobunyaviruses need further study.

6 Scientific Publications of the studies

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