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Examining bull semen for residues of Schmallenberg virus RNA

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Abstract

Schmallenberg orthobunyavirus (SBV) was initially detected in 2011 in Germany from dairy cattle with fever and decreased milk yield. The virus infection is now established in many parts of the world with recurrent epidemics. SBV is transmitted through midges and transplacental. No direct virus transmission including via breeding has ever been demonstrated. In some bulls, however, the virus is detectable transiently, in low to minute quantities, in semen post-infection. While the infection is considered of low impact for the dairy industry, some SBV-free countries have adopted a zero-risk approach requiring bull semen batches to be tested for SBV RNA residues prior to import. This, in turn, obligates a protocol to enable sensitive detection of SBV RNA in semen samples for export purposes. Here, we describe how we established a now ISO/IEC 17025 accredited protocol that can effectively detect minute quantities of SBV RNA in semen and also its application to monitor bull semen during two outbreaks in the United Kingdom in 2012 and 2016. The data demonstrate that only a small number of bulls temporarily shed low amounts of SBV.

KEYWORDS
bull semen, RT-qPCR, SBV, Schmallenberg virus

1 | INTRODUCTION

In 2011, a novel *Orthobunyavirus*, *Schmallenberg orthobunyavirus* (SBV; generally referred to as Schmallenberg virus), was detected by metagenomic analysis at the Friedrich-Loeffler-Institut (FLI) in blood samples of dairy cows with fever and decreased milk production (Hoffmann et al., 2011). Further investigations confirmed that the virus is genomically distinct but clusters closely to other viruses of the Simbu-serogroup (Goller et al., 2012). Other Simbu viruses such as Akabane virus or Aino virus are widely distributed in Asia, Africa and Australia (Saeed et al., 2001). *Orthobunyaviruses* are enveloped RNA viruses whose genome comprises three molecules of circular negative-sense single-stranded RNA. The first virus in the genus, Bunyamwera virus, was characterized in the 1940s from Uganda and the family Bunyaviridae was established in 1975. Since 2019, the family has been designated Peribunyaviridae in the order Bunyavirales (Hughes et al., 2020).

SBV infects a range of mainly ruminant animal species (EFSA, 2014; Molenaar et al., 2015). In adult ruminants, the virus causes mild disease at most but can cause malformations and death in foetal lambs and calves upon vertical transmission (Afonso et al., 2014; Beer et al., 2013; Wernike, Elbers, et al., 2015). The origin of SBV is unresolved, however, it had been speculated to have come from equatorial Africa, and SBV-like viruses have since been demonstrated there (Blomström et al., 2014; Mathew et al., 2015; Oluwayelu et al., 2018).

SBV is now an established infection in many parts of the world beyond Europe (Yilmaz et al., 2014; Zhai et al., 2018) and recurrent epidemics have been reported every couple of years from Germany, Belgium, Ireland and the United Kingdom (APHA, 2016; Collins et al.,

2017; Dastjerdi & Steinbach, 2021; Delooz et al., 2017; Wernike & Beer, 2020; Wernike, Hoffmann, et al., 2015). In line with the relatively low impact of the virus infection (mainly on sheep breeding), its international spread and the build-up of a herd immunity that sustains some protection for years after an outbreak, the World Organisation for Animal Health (OIE) has suspended advice on SBV. Accordingly, national as well as international activities involving animals (e.g., trade) should not be subject to testing or restrictions.

SBV, however, can be shed transiently in the semen of some bulls post-infection, occasionally until after the bull seroconverted but mostly in low or minute quantities (Hoffmann et al., 2013; Ponsart et al., 2014; Van Der Poel et al., 2014) and no infection via insemination has ever been demonstrated. Accordingly, the OIE had previously concluded that direct transmission of SBV between animals is very unlikely (OIE, 2017). To demonstrate the freedom from SBV in semen batches down to the presence of low levels of SBV RNA, a highly sensitive reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is required. This has become necessary not so much for scientific reasons but to meet import conditions of some countries that, despite a lack of OIE recommendations, adopt a zero-risk approach, requiring tests of virus detection in bulls and semen for trade purposes. Unfortunately, the detection of SBV in semen at this low level is technically challenging to detect minute quantities associated with an assumed risk of the introduction of the virus to a new animal or a disease-free region.

A large variability has been reported in the SBV RNA load in the semen of naturally infected bulls, corresponding to quantitative cycle (Cq) values in RT-qPCR from 17 to 38 (Hoffmann et al., 2013; Ponsart et al., 2014). Thus, in the absence of information on virus transmission through SBV-infected semen, a detailed, sensitive and accredited protocol for detection of SBV RNA in semen samples remains indispensable for some international trade purposes. Earlier publications have shown that the method of RNA extraction was crucial for the success of the subsequent RT-qPCR, pointing towards an advantage of magnetic bead extraction methods, compared to silica columns (Hoffmann et al., 2013; Schulz et al., 2014). However, following that suggestion, some methods and laboratories still performed not as expected in an international ring trial (Schulz et al., 2015; Wernike et al., 2017).

Starting from samples used in the international ring trial (Schulz et al., 2015), we set out to further optimize a real-time RT-qPCR for the detection of SBV RNA in bull semen and accredited this to the International Standards Organisation (ISO)/ the International Electrotechnical Commissions (IEC) 17025. In this context, previous outbreaks of the disease in the United Kingdom (2012 and 2016) were also investigated using this protocol.

2 | MATERIALS AND METHODS

2.1 | Samples and general handling procedures

Two semen straws, each containing 250 μl of semen from every batch of extended bull semen, are required for testing. Semen straws were transported to the laboratory, chilled for overnight delivery or frozen and sent on cardice and, if not processed on the same day in the laboratory, stored at −70°C. The two straws were processed independently for nucleic acid extraction and tested in duplicate in RT-qPCR, that is, a total of four RT-qPCRs for every batch of extended semen. Similar samples (two straws/batch) were obtained from the 2012 and 2016 outbreaks of SBV in a bull stud in the United Kingdom, in both cases following a series of bulls over time. The procedure was conducted in ISO/IEC 17025 accredited laboratories. RNase- and DNase-free filter tips and consumables were used throughout the procedure. To avoid cross-contamination and in line with other molecular assays safe handling procedures, samples were also handled in a sequential order throughout the procedure, and disposable gloves were worn and changed frequently.

Ring trial samples used in this study were kindly organized by FLI, Germany.

2.2 | Nucleic acid extraction

Sterile scissors were used to cut one end of the semen straws while being held over a sterile container, for example, a safe-lock 2-ml microcentrifuge tube (Eppendorf). Subsequently, another end of the straw was cut off allowing the semen to flow into the labelled tubes. An SBV negative semen sample was used as the negative extraction control (NEC).

Inside an MSC II with air exhaust, 800 μl of TRIzol LS Lysis Reagent (Fisher Scientific) was added to each tube containing the semen samples including the NEC tube. All tubes were then capped, vigorously shook or vortexed for 15 s, left on the bench at room temperature for 5 min and centrifuged briefly. A 200-μl volume of Chloroform (Merck Life Science UK) was added to each tube, vortexed vigorously for 15 s and left for 10–20 min at room temperature. The mix was then centrifuged at 12,000 × g in a refrigerated microfuge at +4°C for 10 min. A 300-μl volume of the upper aqueous phase from each tube (sample) was then transferred to a well of a deep-well sample plate (SP; Fisher Scientific).

Next, the beads solution, from the LSI MagVet Universal Isolation Kit or MagMAX™ CORE Nucleic Acid Purification Kit (Thermo Fisher Scientific), was thoroughly vortexed for at least 1 min, and 20 μl were dispensed quickly into each well of the SP. The beads solution was vortexed as required to maintain the beads in suspension. This was followed by the addition of 3-μl Carrier RNA (3 μg, Qiagen) and 200-μl Isopropanol (Merck Life Science UK) to each well, and SP was sealed with a Plate Sealant (Microseal® 'B' Adhesive Seals, Bio-Rad). The content of the plate was mixed for 30–60 s at 1000 rpm on a plate mixer and centrifuged for 5–10 s at 2000 × g before being loaded onto a Kingfisher robot (Thermo Fisher Scientific).

Nucleic acid extraction was continued using the NM-LSI_RRC96 protocol on the robot. Disposable reservoirs (trough) and a multichannel pipettor were used to facilitate dispensing of the kits reagents. A 100-μl volume of elution buffer was dispensed into the required number of wells of a 96 shallow-well plate, matching location of the

samples in the SP and followed by dispensing 600 μl of 80 % ethanol, wash solution 1 and wash solution 2 into three separate deep-well plates (Thermo Fisher Scientific), and the plates were labelled accordingly. The run was then started, which takes approximately 40 min to complete. At the end of the run, the plate containing the elution buffer were recovered for use in the RT-qPCR. If the extracted nucleic acid was not used immediately, the plates were sealed and stored at −80°C.

2.3 | RT-qPCR

The VIROTYPE SBV RT-PCR kit (Indical Bioscience) was used to detect SBV RNA following the kit instructions. The kit contains a combination of primers and fluorescently labelled TaqMan-probes, specifically amplifying part of the S segment of SBV genome (GenBank HE649914) and a β-actin gene for extraction control. The kit PCR mix (20 μl) was dispensed into a 96 well PCR plate. A 5 μl of the isolated RNA or water, as no template control (NTC), were added to the corresponding wells in the plate. Last, the kit SBV positive template was added, the PCR plate was covered firmly with an optical grade sealing film (Bio-Rad) and spun briefly. The real-time RT-qPCR was carried out using Mx3000P and AriaMx thermocyclers (Agilent Technologies) at 45°C for 10 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 56°C for 30 s and 72°C for 30 s. Fluorescence data were acquired at the end of the 56°C annealing step for FAM (SBV), HEX (b-actin) and ROX (passive reference dye).

The validity of the assay procedure was evaluated by the FAM fluorescence signal of the SBV positive control, a Cq value of less than 35 (Cq < 35) and NEC and NTC with no Cq values. A sample was concluded negative if only the HEX fluorescence signal (β-actin) had been measured. A positive result was identified by measurement of a FAM fluorescence signal, that is, sample contains SBV RNA. A sample was concluded positive for SBV RNA when at least two out of the four reactions produced a FAM fluorescence signal. In the absence of a HEX fluorescence signal for a sample, the respective sample nucleic acid was diluted in nuclease-free water (e.g., 1 in 5 dilutions), and the RT-qPCR was repeated. If this diluted nucleic acid failed to produce a HEX signal, the test procedure was repeated entirely.

3 | RESULTS

3.1 | Diagnostic test performance

The analytical sensitivity and specificity of the VIROTYPE® SBV RT-PCR kit were provided by the kit manufacturer, titrating a series of in vitro transcribed RNA (10^6–10^{-1} copies/well) and testing in triplicates in the RT-PCR. The highest Cq value was for the 10^0 copies/well (37.53) and no Cq thereafter. A high correlation between RNA copies and the amplification products was also demonstrated in the range of 10^6 to 10 RNA copies with a correlation coefficient of .999 and an efficiency of 96.6%. Similarly, analytical specificity of the kit was evaluated testing samples positive for 24 serotypes of bluetongue virus, bovine viral diarrhoea virus genotypes 1 and 2, border disease virus, foot and mouth disease virus and epizootic haemorrhagic disease virus, all with a negative outcome.

The diagnostic sensitivity of this protocol was further evaluated by testing samples received from FLI, Germany, as part of two European SBV ring trials. Initially, the diagnostic sensitivity of the kit was assessed using SBV RNA extracted from various matrices (semen, tissues, sera and virus cultures). The Cq values generated by the kit were in concordance with those generated by FLI using the Bilk et al. (2012) protocol and were anonymously described in Schulz et al. (2015; Table 1). In the second ring trial, four known SBV positive semen samples, considered to be of medium and low SBV load, were processed for SBV RNA detection applying the entire protocol. The four samples were correctly identified and with Cq values matching those reported by the organiser (Table 2). Diagnostic specificity of the test was also assessed at our hands using 14 semen samples collected from six bulls in 2010, prior to the incursion of SBV in the United Kingdom in 2011 (McGowan et al., 2018) and the protocol described here. All the samples were tested negative for SBV RNA (data not shown). Using four SBV-RNA positive and one SBV-RNA negative samples and the kit positive and negative controls, the manufacturer demonstrated intra- and inter-assay variation coefficient (VC) between 0.40% and 2.55% for the FAM and HEX signal, respectively. Further to these data, we also analysed Cq values obtained from NEC and the kit positive and negative controls for the tests performed in our laboratory between 2017 and 2020 (Table 3). An overall VC between 3.3% and 7.4% for the β-actin Cq obtained for NEC used in the 98 submissions highlights the robustness of the protocol. Similarly, kit positive and negative controls perform reliably with VCs of 2.2%—5.2% and 3.1%—5.0% respectively.

3.2 | Assessment of SBV in bull semen during the UK 2012 outbreak

The initial outbreak of SBV in 2011/2012 reached UK bull studs in 2012, and we investigated the presence of SBV RNA in the semen of sampled bulls. Of eight bulls of a single bull stud tested for SBV, bull no. 5 tested positive for 18 days with Cq values of 27.6–38.7, while bull 8 tested positive only on one sampling occasion with a Cq value of 38.7 (Table 4).

3.3 | Assessment of SBV in bull semen during the UK 2016 SBV outbreak

The initial outbreak of SBV in 2011/2012 was followed by a re-emergence in 2016. By this time, bulls in the United Kingdom used for semen production were regularly tested for SBV antibodies. Thus, we could follow a cohort of more than 100 bulls from a bull stud over time, identify their seroconversion and analyse a large set of respective semen batches. One hundred and seventy semen samples from semen batches produced between August to December 2016 from more than

TABLE 1 *Schmallenberg orthobunyavirus* (SBV) RNA samples distributed by Friedrich-Loeffler-Institut (FLI) Germany as the organiser of a European SBV ring trial and according to cycle (Cq) values. VIROTYPE® SBV RT-PCR kit (Indical Bioscience) was applied here to test for SBV RNA. SBV Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) used by FLI is as described in Bilk et al. (2012)

RNA source	Sample ID	FLI	APHA
		Mean Cq value of technical duplicates	
Semen	BH 661/12	33.0	34.35
Semen	BH 657/12	32.2	32.3
Cell culture	SBV-REF-RNA 2×10^0	35.7	36.1
Cell culture	SBV-REF-RNA 2×10^1	32.8	32.7
Cell culture	SBV-REF-RNA 2×10^2	29.2	29.2
Cell culture	SBV-REF-RNA 2×10^3	25.8	25.6
Cell culture	SBV-REF-RNA 2×10^4	22.5	22.5
Cell culture	SBV-REF-RNA 2×10^5	19.2	19.1
Tissue	BH 200/12	20.4	20.3
Tissue	BH 254/12	24.8	24.8
Serum	790	24.6	25.1
Serum	668	29.6	30.1
SBV-RNA free	RSB50	No Cq	No Cq
RNase-free water	NTC	No Cq	No Cq

TABLE 2 Semen samples with challenging Cq values (provided by FLI, Germany). The samples were tested applying the entire SBV RNA extraction and SBV RT-qPCR protocol described here. SBV RT-qPCR used by FLI is as described in Bilk et al. (2012)

Sample ID	FLI		APHA
	Biological replicates		Technical replicates averages ($n = 2$)
BH 651/12–31	36.2	36.6	36.2
BH 657/12–100	28.9	28.7	28.9
BH 684/12–01	33.5	32.9	33.5
BH 657/12–151	34.0	33.4	34.0
BH 159/13–01	No Cq	No Cq	No Cq
BH 159/13–02	No Cq	No Cq	No Cq

TABLE 3 Summary of Cq values obtained for the SBV RT-qPCR controls. An SBV-negative semen sample was used as the negative extraction control (NEC). NEC Cq values obtained for the HEX fluorescence signal (β-actin) were analysed. It is of note that eight different real-time PCR machines were used to test samples received under 27 submissions in 2020 and associated controls, overall indicating the robustness of the assay

Year	No. of submissions	NEC Cq ± STDEV[b] [VC[c]]	Neg. SBV control[a] Cq ± STDEV [VC]	Pos. SBV control[a] Cq ± STDEV [VC]
2017	23	29.2 ± 1.17 [4]	27.9 ± 1.39 [5.0]	28.6 ± 1.5 [5.2]
2018	26	31.35 ± 2.33 [7.43]	28.5 ± 1.22 [4.3]	28.8 ± 0.88 [3.1]
2019	22	32.9 ± 1.07 [3.3]	28.9 ± 1.29 [4.5]	29.08 ± 0.65 [2.2]
2020	27	31.5 ± 1.6 [5.1]	29 ± 0.89 [3.1]	28.9 ± 0.88 [3.0]

[a]SBV negative and positive controls provided in the VIROTYPE® SBV RT-PCR kit.
[b]Standard deviation.
[c]Variation coefficient (%).

TABLE 4 Bull semen from a UK bull stud was collected during 2012 SBV outbreak and subsequently tested for SBV RNA using the RT-qPCR protocol described here. The semen of eight bulls was monitored over a prolonged period in 2012 for the appearance of SBV therein. Only two bulls at any given point in time had SBV RNA in their semen and only one (no. 5) over a prolonged period. Days in which the bulls were tested positive for SBV RNA are in bold and underlined

Animals	Cq values	Days in year 2012 from which semen was tested
1	No Cq	5, 13, 27, 32, 255, 258, 265, 276, 311, 321
2	No Cq	241, 244, 248, 251, 255, 262, 265, 268, 272, 276, 300, 304, 307, 311, 321, 325, 335, 339, 342, 346, 352, 355
3	No Cq	261, 304
4	No Cq	268, 277, 284, 285, 332, 336, 339, 344, 352, 353
5	No Cq	148, 241, 251, 255
	34.6	**258**
	35.4	**262**
	35.2	**268**
	27.6	**270**
	32.6	**276**
	No Cq	283, 295, 300, 304, 307, 311, 321, 325, 335, 342, 352, 355,
6	No Cq	255, 258, 262, 270, 276, 304, 307, 311, 321, 335, 353
7	No Cq	225, 268, 270, 276, 279, 283, 289, 292, 295, 300, 304, 307, 311, 318, 321, 325, 328, 332, 335, 354
8	No Cq	247, 250, 251, 255, 257, 261, 265
	38.7	**268**
	No Cq	277, 284, 290, 292, 293, 297, 306, 312, 318, 320, 326, 329, 332, 339, 342

100 bulls were analysed. Semen samples from five bulls tested positive with the Cq values ranging from 33.6 to 38.3 (Table 5).

4 | DISCUSSION

SBV has in recent years established itself as a recurring endemic infection of ruminants in Europe and beyond. The impact of infection is generally (with the exception of newborn lambs in particular) very low. However, previous studies and our own data presented here demonstrate the transient occurrence of SBV RNA in semen (Hoffmann et al., 2013; Ponsart et al., 2014; Van Der Poel et al., 2014).

Semen is composed of cellular (mainly spermatozoa and urogenital epithelial cells) and non-cellular components of seminal fluid. Biochemically, semen is composed of a high amount of DNA and protein and a lower amount of glycogen, as an energy source, and lipids. Seminal glands also add metal ions such as zinc, which protects the sperm chromatin from condensation. In addition, it has to be considered that semen obtained and processed for artificial insemination (AI) is mixed with semen extenders that contain proprietary formulations not restricted to but including the above components. Accordingly, extraction of nucleic acid from this matrix has historically been a challenge, particularly through inhibitory factors that need to be removed by enhanced nucleic acid purification approaches (St-Laurent et al., 1994; Wang et al., 2007; Wiedmann et al., 1993). Further, animals may shed viruses in semen in low quantities (Hoffmann et al., 2013; Ponsart et al., 2014; van der Poel et al., 2014; Wang et al., 2007).

Initially, our detection of SBV RNA was based on the combination of TRIzol reagent (Fisher Scientific) with QIAamp viral RNA mini kit (Qiagen) for extraction of RNA from tissue samples (lymph nodes, spleen, kidney and neuronal tissues). The isolated SBV RNA from these tissues using this combination produced matching Cq values to the reference samples and values provided by the FLI (Table S1). However, this RNA isolation protocol when applied to bull semen produced substantially higher Cq values Table S2) and was thus not deemed fit for purpose.

The proposal that magnetic bead isolation of RNA was key to a successful detection of SBV in semen (Hoffmann et al., 2013; Schulz et al., 2014) led to the investigation of other methods. Not least we considered it prohibitive if all labs would have to invest in the same technology for the automation proposed. Accordingly, different RNA extraction protocols and PCR kits were evaluated with the aim to produce Cq values similar to the results provided by the FLI (Table S3). It became evident that magnetic extraction as such was not the key to success since this was also part of the EZ1-based method. It is highly likely that the benchmark protocol benefits particularly from the combined use of TRIzol LS Lysis Reagent, magnetic isolation of RNA and the addition of isopropanol (Table S4) to precipitate the RNA and achieve a maximal biological sensitivity.

The protocol outlined here reflects successful approaches described elsewhere including by the FLI (Schulz et al., 2014; Wernike et al., 2017). The protocol also benefits from being adaptable to high throughput, as part of the RNA isolation is performed on the Kingfisher robotic workstation with a capacity of processing 96 samples in ~45 min. The simultaneous detection of an internal control (β-actin)

TABLE 5 Testing of bulls from a UK bull stud for SBV RNA in 2016. A series of bulls (> 100) was continuously monitored serologically at the time of the 2016 SBV outbreak in England and batches of semen, taken around the period of SBV seroconversion, were tested to detect traces of SBV RNA by the VIROTYPE® SBV RT-PCR kit. Only five bulls were found to have SBV RNA in their semen, of which two bulls (nos. 3 and 5) shed virus on more than one occasion. Days in which the bulls were tested positive for SBV RNA are in bold and underlined

Bull No	*Days in 2016*	*Cq values*
1.	246, 249	No Cq
	253	36.8
	265, 268, 272, 275	No Cq
2.	**260**	33.60
	278, 285	No Cq
3.	233, 259, 263, 266, 269	No Cq
	293	36.4
	294	35.8
	301	38.07
	304	37.1
	305, 318	No Cq
4.	256, 259, 263, 266, 269	No Cq
	273	35.8
	274	No Cq
	277	No Cq
5.	274, 277, 279	No Cq
	280	No Cq
	284	38.3
	288	No Cq
	291	34.9

ensures the optimal extraction of RNA, demonstrating a removal of PCR inhibitors in individual samples, thereby eliminating the likelihood of false negative results.

Analysing selected semen samples from the 2012 outbreak of SBV in the United Kingdom identified 2/8 bulls (25%) that temporarily had low to minute amounts of SBV in their semen. This data is comparable to the findings from Hoffmann et al. (2013) that found an intermittent shedding in 2/6 (33%) of bulls in Germany and Ponsart et al. (2014) that identified SBV RNA in 3/7 (43%) bulls.

In 2012, as the disease was first emerging, we could only test a small number of bulls. However, in 2016 when tests were routinely applied and the re-emergence of SBV had been noted elsewhere in Europe, a much wider pool of animals were tested. Notably, only a few animals (4.9%) had SBV RNA in their semen, and only two bulls tested positive more than once. This might fit into the general concept of adaptation of viruses over time and the notion that the impact of the second outbreak in 2016 was less severe than in 2012. Specific changes in SBV that might explain this have been described elsewhere (McGowan et al., 2018).

It has been suggested that SBV-positive semen could elicit an SBV infection in cattle and interferon α/β receptor–deficient ($IFNR^{-/-}$) mice, particularly when semen containing relatively high amounts of SBV (Cq < 35) was applied (Ponsart et al., 2014; Schulz et al., 2014). However, it must be pointed out that these results were obtained under special circumstances and contained some ambiguity. First, the experimental setup to inject semen subcutaneously is a very artificial way to apply semen and not one comparable to AI, while no infections could be obtained in applying SBV to mucosal surfaces of sheep or cattle (Schulz et al., 2014; unpublished). Even so, the infections were only moderately effective. In the first attempt, no mice could be infected (Schulz et al., 2014), and on the second approach the SBV RNA positive blood from $IFNR^{-/-}$ mice was not able to transfer the infection to other mice (Ponsart et al., 2014). This does beg the question of whether SBV carrying cells in the blood (e.g.m antigen-presenting cells) were carrying the inoculum around as antigen rather than an infection resulting, which limits the value of the results further.

In calves, only five out of 11 (45.4%) were successfully infected subcutaneously with SBV containing semen (Schulz et al., 2014) and that might for one be a dose effect with some semen in these studies containing significantly higher amounts (> 100-fold greater) than detected here. This is relevant as it refers directly to the sensitivity of the RT-qPCR assay where the lowest amounts detectable are seemingly not able at all to infect animals. As mentioned afore, it is unclear, if not unlikely, whether SBV could infect via the natural insemination route at all, and if so, whether this would have any impact. At this stage in their reproductive cycle, dams are not producing high levels of milk; hence, a drop in production would hardly be noticeable. The short incubation period (≤4 days) combined with the short viraemia (≤6 days) would mean that the infection would be resolved before the placenta was fully established and before an infection of the embryo could occur.

In summary, the occurrence of SBV in bull semen is a phenomenon not occurring regularly and likely mediated by host factors. A prolonged shedding for example, such as reported in occasions before, could not be seen in our cases, neither in 2012 nor in the more extensive study following the 2016 outbreak. For those concerned that such covert infection might introduce the virus into countries currently free or perceived to be free of SBV, the protocol described here is sufficient to safely detect low levels of SBV in bull semen and thus reduce the risks associated with SBV infections in bulls to effectively zero.

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ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. All samples used for this study were collected for clinical purposes under the Veterinary Surgeons Act 1966, the United Kingdom.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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