Molecular Detection of Schmallenberg Virus: An Initiative to Approach Sero-Positive Samples

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ABSTRACT

Schmallenberg virus (SBV) is a pathogen of veterinary importance that was first detected in the European ruminant population in 2011. Since then, the RNA virus has spread to various parts of the world, causing clinical manifestations such as abortions, stillbirths, and congenital malformations in ruminants. There are no molecular surveillance studies for SBV in Malaysia, and this study aimed to perform molecular detection of SBV using the positive SBV serum samples from small ruminants collected from selected farms in Terengganu and Negeri Sembilan in 2019. A molecular technique, reverse transcriptase polymerase chain reaction (RT-PCR) was used to analyze 78 serum samples in comparison to an in-house synthesized positive control of the SBV L segment sequence. None of the serum samples tested were positive for SBV RNA but this does not rule out the absence of SBV in animals. This was due to the fact that an undetectable viraemia period of SBV after a short duration of the active viral infection cycle or the presence of neutralizing antibodies could eliminate the viral particles from the bloodstream over time. It is therefore highly recommended to take samples from acutely infected animals.

Keywords: Schmallenberg virus, RNA virus, molecular detection, RT-PCR, viraemia, acute infection

INTRODUCTION

Schmallenberg virus (SBV) is an enveloped, segmented, negative-sense, single-stranded RNA virus that is classified in the Simbu serogroup of the genus Orthobunyavirus in the family Bunyaviridae (Wernike et al., 2015). According to the report of many epidemiological studies, SBV is known to infect ruminants but is not a zoonosis (OIE 2017). The origin of SBV remains controversial, but few reports of the discovery of SBV-like virus particles from equatorial Africa have been documented, even before the outbreak in Europe. The virus was first isolated in Germany and has currently been reported in many countries in Europe such as the UK, France, Italy, Luxembourg, Spain, Denmark, Estonia, and Switzerland (Lievaart-Peterson et al., 2015; Stavrou et al., 2017). Over time, the livestock trade has meant that SBV has also spread to the rest of the continent (Hoffmann et al., 2012; Zhai et al., 2018). In addition, recurring epidemics have been reported every few years in 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). The SBV outbreak from 2011 to 2012 resulted in estimated direct and indirect costs of approximately €150–€200 million for the European livestock industry. These costs include livestock losses, veterinary expenses, and reduced productivity (EFSA, 2014). In certain infected dairy herds, milk production dropped by as much as 25% in the weeks following infection, primarily due to fever and loss of appetite in the affected animals (Sudhakar et al., 2020). Additionally, studies have shown that SBV infection during pregnancy in sheep led to abortion and increases stillbirth rates within affected herds (Peperkamp et al., 2015).

First, SBV was found to infect a range of ruminant species, followed by wildlife species. It should be noted that only domesticated ruminants show overt clinical SBV symptoms, while other wild ruminants (buffalo, deer, alpaca, bison, mouflon) and other mammalian species (boar, horse, kudu, oryx, zebra) have been described exclusively with indirect application were serological proof (Wernike et al., 2013). Infection with SBV is associated with a short period of viraemia (2-6 days) that causes fever, diarrhea, hyperthermia, and reduced milk production (up to 50%) in cattle. On the other hand, similar clinical symptoms attributed to infected sheep and goats are mostly subclinical (Hoffmann et al., 2012). In addition, seroconversion of SBV antibodies has been reported approximately 1-3 weeks after infection (Hoffmann et al., 2012). In some extreme cases, miscarriages or congenital fetal malformations have been reported in pregnant ruminants in which SBV was transmitted vertically during gestation (Bayrou et al., 2014). Not to forget the detection of SBV RNA in the semen of infected bulls (Hoffman et al., 2013). SBV contains three core genomic segments: S (small), M (medium), and L (large) segments. The nucleoprotein N and nonstructural proteins are encoded by the S segment, while the envelope glycoproteins Gn and Gc are encoded by the M segment. In comparison, the L segment encodes RNA-dependent RNA polymerase (RdRp) (Chowdhary et al., 2012; Elliot 2014). All of these segments were used to detect the presence of viruses by different PCR systems (Bilk et al., 2012; Fischer et al., 2013; Hoffmann et al., 2012). The assay based on S and L segments is considered to be superior in terms of specificity and sensitivity in detecting the viral RNA, while high genetic variations occur in the M segment, facilitating immune escape from the target host (Wernike et al., 2021).

Bunyaviruses consist largely of viruses transmitted by arthropod vectors, with the exception of hantaviruses, which are transmitted by rodents. SBV, like other members of its serogroup, is transmitted by haematophagus insect vectors, Culicoides or mosquitoes (Pawaiya &

Gupta, 2013), which are common in Malaysia. Interestingly, several species of *Culicoides* namely *C. obsoletus*, *C. dewulfi* and *C. chiopterus* were found to be SBV positive by RT-PCR (de regge et al., 2012). However, studies on transmission of SBV by local *Culicoides* species have yet to be conducted in Malaysia. The year-round availability of mosquitoes in the country's tropical climate and the increasing number of livestock in Malaysia could influence the spread of SBV.

Small ruminant production in Malaysia is significantly impacted by high rates of morbidity and mortality, as well as the costs associated with treatment, all of which are exacerbated by the prevalence of diseases (Jesse et al., 2022). The global emergence of SBV expands our understanding of diseases that cause reproductive failure in ruminant livestock production, making it an important differential to consider in the clinical diagnosis of infertility. While SBV has become a significant economic disease in European countries, its potential to cross boundaries raises concerns, especially given Malaysia's heavy reliance on imported breeding stock for its livestock industry.Considering the conditions that may facilitate the spread of SBV in Malaysia, this study aimed to detect the presence of SBV nucleic acid using SBV serum positive samples as part of an early molecular surveillance initiative. Considering the fact that there is a lack of specific knowledge on SBV epidemiology in Malaysia, this study was initiated to detect SBV RNA by RT-PCR in serum from SBV-positive small ruminant flocks for the first time in the states of Negeri Sembilan and Terengganu, Malaysia.

MATERIALS AND METHODS

Ethics statement

The Department of Veterinary Services (DVS), Malaysia and The Institutional Animal Care and Use Committee (IACUC) of Universiti Putra Malaysia (UPM/IACUC/AUP-013/2018) granted the required permission to conduct this study. Blood samples were collected by trained veterinarians by taking animal welfare into consideration and we performed the molecular assay at virology laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia.

Sample Collection

In total, 362 individual sheep (n=132) and goats (n=230) were randomly selected from seven farms located at Negeri Sembilan and Terengganu states of Malaysia. Initial screening on SBV antibodies were conducted by Jesse et al. (2022) by using commercial ID vet® SBV multispecies ELISA test kit (produced by ID vet, Montpellier, France). Out of 362, only 78 samples were shown to be sero-positive against SBV, thus all the sero-positive samples were subjected for molecular detection subsequently in this study. All the SBV positive serum samples were stored in a -20°C freezer prior to nucleic acid extraction.

RNA extraction and RT-PCR assay

The RNA from the serum samples were purified and extracted using the GENEzol^{TM} TriRNA Pure Kit according to the manufacturer's instructions. The concentration of extracted RNA was measured by spectrophotometry using the Nanodrop 1000 spectrophotometer (Thermo Scientific^{TM}). For a biosafety reason, a standard positive control in the molecular diagnosis of SBV was designed based on published SBV genomic L segment sequence available in the NCBI GeneBank (Accession number: KX384874.1) with the length of 1273 base pairs. The respective

sequence manufactured by Integrated DNA Technologies, Inc., Malaysia and was delivered as gBlocks® Gene Fragments, in the form of lyophilized double-stranded DNA.

Prior to RT-PCR, the primers were designed based on published SBV genomic sequence available in NCBI GeneBank (Accession number: KY828195.1). To confirm the primer specificity, the primers were tested against the sequence of positive control gBlocks® Gene Fragments which was synthesized previously. Due to positive amplification (data not shown), the primer set was selected for this experiment. The forward sequence is 5' TGA CAT TCC ATG AGT CTA TG 3' and reverse is 5' GTC GGA TTG TCT CCT GCA AAC 3' targeting a specific region on the SBV L-segment sequence. Then, the extracted RNA was subjected to RT-PCR using MyTaq^{TM} One-Step RT-PCR Kit, following the standard protocol. The following thermal program was applied: 1 cycle of 45°C for 20 min and 95°C for 1 min, followed by 40 cycles of 95°C for 10s, 53°C for 10s, and 72°C for 30s The analytical sensitivity of the assays was determined with 6 serials of 10-fold dilutions using a synthetic positive control of SBV with an initial concentration of 1000 ng/µL down to 0.01 ng/µL.

Analysis of the PCR Products

The PCR products were separated by electrophoresis using 1.5% of agarose gel in 1X TAE buffer solution. For gel analysis, 15µl of the products (samples and positive control) were loaded in each gel slot. To determine the fragment sizes, 1000 bp plus DNA Ladders (Qiagen, Germany, GmbH) was utilized followed by gel visualization using GeneSnap (Synoptics Ltd.), an image acquisition software to capture the electrophoretic gel images. A comparison was then made between the bands produced by the positive control, the negative control, and the samples to determine the presence of the virus DNA.

RESULTS AND DISCUSSION

The analytical sensitivity of PCR in this study was evaluated using 10-fold serial dilution of a synthetic positive control DNA sequence, with concentrations starting from 1000 ng/µL in lane 1 and decreasing sequentially to 0.01 ng/µL in lane 6. Clear PCR amplification bands are visible in lanes 1 through 5, which correspond to concentrations ranging from 1000 ng/µL down to 0.1 ng/µL (Figure 1). However, lane 6, containing 0.01 ng/µL, shows no visible band, indicating that the PCR assay was unable to detect the target sequence at this lowest concentration. Based on these observations, the limit of detection (LOD) for this assay can be defined as 0.1 ng/µL, the lowest concentration at which amplification was reliably observed.A total of 78 individual animal samples of selected farms from Negeri Sembilan and Terengganu were used to screen for SBV in serum. Of the 78 samples collected between March and September 2019, no SBV RNA was detected in any SBV positive serum samples as shown in Figure 2.

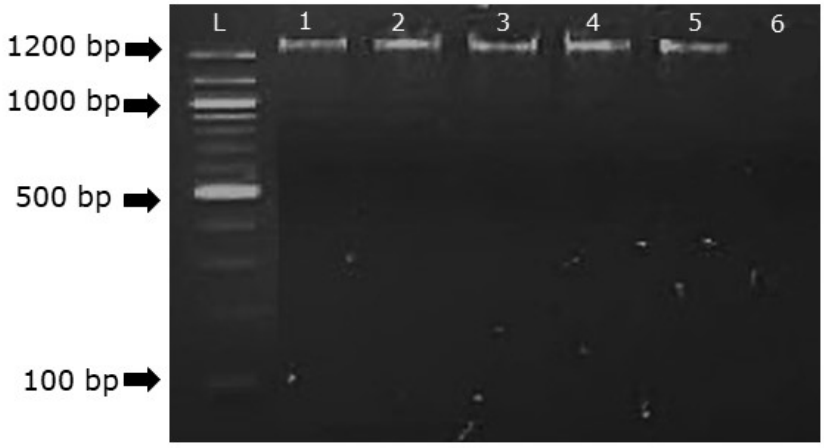

Figure 1 A 1.5% agarose gel electrophoresis of SBV positive control. Bands of PCR product were visualized by ethidium bromide staining. Lane L: 1000 DNA marker. Lanes from right to left corresponded to concentration of positive control from 1000 ng/µL to 0.01 ng/µL. The detection limit was 0.1 ng/µL.

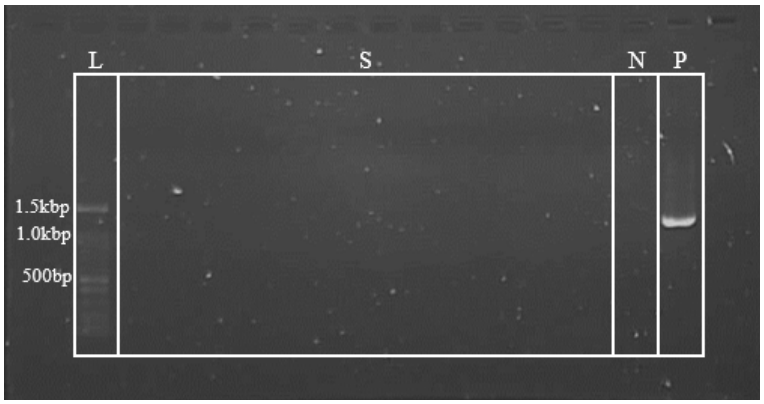

Figure 2 Agarose Gel Electrophoresis Illustration. The figure depicts the RT-PCR products of SBV L-segment with size of 1273 bp amplified using SBV primers. Column L represents the ladder (1.5 kbp), column S represents the RT-PCR products of the samples, whereas columns N and P show the negative and positive controls, respectively.

Based on the current literature, there are no studies on the molecular detection of SBV in Malaysia. Focusing on SBV, lower exposure in the country does not make a reliable laboratory diagnosis available in the country. The preliminary study on the detection of SBV antibodies in small ruminants was carried out by Jesse et al. (2022), while the current study is an ongoing attempt to detect the nucleic acids of SBV in positive SBV serum samples from Negeri Sembilan and Terengganu states in Malaysia. The results obtained from the molecular test showed negative results for SBV in all 78 serum samples. However, this result does not completely rule out the presence of SBV RNA in the animals, as all serum samples tested in this study were previously serologically positive for SBV by ELISA, suggesting that the animal was infected at some point in its life (Jesse et al., 2022). The number of positive cases detected by RT-PCR in the literature is

consistent with this study in the sense that a small number of positive cases are usually detected by RT-PCR in serum samples. A study conducted by Chaintoutis et al. (2014) showed that out of 147 samples that tested positive by ELISA, no sample was positive for RT-PCR. The study by Yilmaz et al. (2014) reveals a similar relationship between the serological and genomic prevalence of SBV exposure. Among 116 aborted foetuses from small and large ruminants, they tested 46 blood samples from lambs and 20 organ samples from sheep using PCR. Of these, only three samples tested positive for RT-PCR: one from an aborted calf and two from aborted lambs. The inability of this study to detect the presence of SBV by RT-PCR assay could be explained by a few possible factors.

First, the duration of viremia in ruminant SBV infection is relatively short, lasting only a few days. In a study of experimentally infecting goats and buck with SBV, viral RNA was detected within three to four days post infection (pi), beginning on days one to three pi, as detected by RT-PCR. In particular, SBV RNA was no longer detectable from day six pi. Furthermore, this viremic phase was not accompanied by clinical symptoms (Laloy et al., 2015). In an experimental challenge in sheep, viral RNA was detectable for three to five days, starting on days one, two and four after inoculation. Similar to the goat study, the majority of sheep with RNAemia showed no clinical signs including fever (Wernike et al., 2013). This proves that viral RNA could be successfully detected in serum samples when obtained during an acute infection. In the current study, the failure to detect viral RNA can be attributed to long-term infection, with the concentration of viral particles in the goats and sheep being low and undetectable by the RT-PCR threshold

Second, however, determining the acute phase of infection in adult animals is difficult in ruminants, especially goats and sheep, which rarely show clinical signs even during the viraemia phase (Wernike et al., 2013; Lievaart-Peterson et al., 2015). Therefore, other samples that have shown a higher concentration of viral RNA can be selected instead of serum samples for more successful molecular detection of SBV. This includes lungs, liver, outer placental fluid, and umbilical cord from aborted, stillborn, or malformed offspring (Bilk et al., 2012; Yilmaz et al., 2014). In addition, spleens and lymph nodes of infected adults proved to be a suitable target region, as viral RNA was detectable up to day 44 post-inoculation (Wernike et al., 2013). Furthermore, seropositive flocks for SBV sampling should be closely monitored for clinical signs such as abortions or congenital malformations that are strongly suggestive of SBV infection.

Next, the absence of viral RNA in the serum is considered to be the cause of elimination of viral particles by neutralizing antibodies in the bloodstream. Experimental infection of goats showed that seroconversion occurred between day seven and day 14 pi and the viral RNA was later undetectable after seroconversion by RT-PCR (Laloy et al., 2015) and similar findings were also demonstrated in sheep (Wernike et al., 2013). This was further supported by the virus neutralization test (VNT) performed concurrently in their studies along with ELISA. In comparison, the presence of antibodies against SBV in this study may have resulted in the virus being neutralized and subsequently the SBV RNA becoming undetectable in the RT-PCR assay. Finally, most ELISAs are unable to accurately discriminate between members of the Simbu serogroup of viruses, so claiming the samples as SBV seropositive required more validation. Because of the similar pattern of transmission, multiple viruses of the same serogroup could be circulating in certain regions, and the virus neutralization test (VNT) is more specific and can discriminate between them. Apart from that, it has been suggested that fetal body fluids would be better suited to test the antibody response of SBV infection.

CONCLUSION

In conclusion, this study could not detect SBV nucleic acid content in all SBV seropositive samples. Thus, no SBV viremia was detected in the analyzed samples. Collecting serum samples from acutely infected animals is strongly recommended since the duration of SBV viraemia is relatively short. Future studies should focus on collecting other infected tissue samples of placenta or umbilical cord of affected foetusinstead of serum samples. However, the successful design and synthesis of an innocuous SBV L segment can be used as a standard positive control in the diagnosis of SBV in Malaysia in the future.

CONFLICT OF INTEREST

Authors have no conflict of interest to declare.

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AUTHOR CONTRIBUTIONS

KNB, JAB, FFAJ and MLMA postulated the experimental design. KNB, AR, BTP, KMI and JMN performed work associated with this study. KNB, JAB and MLMA prepared the manuscript. All authors reviewed the manuscript upon submission.

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