Accepted Manuscript

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PII: S1090-0233(14)00228-7
DOI: http://dx.doi.org/doi:10.1016/j.tvjl.2014.05.031
Reference: YTVJL 4167

To appear in: The Veterinary Journal

Accepted date: 23-5-2014


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Short communication

Dose-dependent effect of experimental Schmallenberg virus infection in sheep

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Abstract

Schmallenberg virus (SBV) is an orthobunyavirus affecting European domestic ruminants. In this study, the dose-dependent effect of experimental infection of sheep with SBV was evaluated. Four groups of three ewes were each inoculated subcutaneously with 1 mL of successive 10-fold dilutions of an SBV infectious serum. The ewes were monitored for 10 days, but no clinical signs were observed. The number of productively infected animals within each group, as evidenced by viraemia, seroconversion and viral RNA in the organs, depended on the inoculated dose, indicating that a critical dose has to be administered to obtain a homogeneous response in infected animals under experimental conditions. In the productively infected animals, no statistical differences between the different inoculation doses were found in the duration or quantity of viral RNA circulating in blood, nor in the amount of viral RNA present in virus positive lymphoid organs.

Keywords: Schmallenberg virus; Sheep; Experimental infection
Schmallenberg virus (SBV) is newly emerged orthobunyavirus transmitted by *Culicoides* spp. (De Regge et al., 2012) that causes abortions, stillbirths and malformations in domestic ruminants (Herder et al., 2012; Hoffman et al., 2012). In a recent study in cattle, subcutaneous inoculation with a 1/100 dilution of an SBV infectious bovine serum induced a longer duration of viral RNA circulating in blood compared to inoculation with undiluted infectious serum (Wernike et al., 2012). The present study was conducted to determine if a similar dose-dependent effect occurs in sheep.

Twelve 1-year-old Mourerous ewes, negative for SBV by ELISA, serum neutralisation test (SNT) and quantitative reverse transcriptase PCR (qRT-PCR), were included in the study. Three randomly selected ewes in each of four groups were inoculated subcutaneously in the left axilla with 1 mL undiluted, or 1/10, 1/100 or 1/1000 diluted, SBV infectious bovine serum in phosphate buffered saline (PBS). The infectious serum was obtained from the Friedrich Loeffler Institute and had been tested in cattle and sheep (Hoffmann et al., 2012; Wernike et al., 2012, 2013). The inoculum contained 2 x 10^{3} 50% tissue culture infectious doses/mL (TCID_{50}/mL), as determined by end-point titration on baby hamster kidney (BHK) cells (Wernike et al., 2012) and was sent to CODA-CERVA on dry ice under appropriate transport conditions. The study was approved by the joined Ethical Committee of the Belgian Scientific Institute of Public Health and CODA-CERVA (project number 121017-01; date of approval 11 February 2013).

During the 10 day period following infection, clinical examinations of ewes were performed daily and blood was collected from the jugular vein. Two ewes, inoculated with the undiluted or the 1/1000 diluted inoculum had rectal temperatures of 40 °C 1 day post-
inoculation (dpi), but the average and median temperatures in the groups stayed in the normal range (38.3-39.9 °C). No other clinical signs were detected throughout the experiment.

The presence of SBV RNA in serum and whole blood was determined by detection of the SBV S segment using a one-step qRT-PCR (De Regge et al., 2013). In case of doubtful results, RNA extracts were retested in a two-step PCR with the same primers, as described previously (De Regge and Cay, 2013). Cycle threshold (Ct) values were converted into S segment copy numbers using an RNA standard curve (see Appendix A: Supplementary material).

All ewes inoculated with the undiluted or 1/10 diluted SBV infectious serum, along with one ewe inoculated with the 1/100 infectious serum, were positive by qRT-PCR for viral RNA in blood (Fig. 1). No SBV RNA could be detected in other ewes by qRT-PCR during the experiment. The number of ewes in each group that were positive for viral RNA in blood decreased significantly as a function of the inoculated dose (Fisher's exact test; n = 12; P = 0.045), providing evidence that a critical dose needs to be administered to induce a homogenous productive infection in sheep. When the Spearman-Karber method was applied to the data (Hierholzer and Killington, 1996), the undiluted serum contained at least $10^{1.83}$ sheep infectious doses per mL.

It would be interesting to see if inoculation of other sheep breeds with SBV would result in similar results, since differences in breed susceptibility have been described for another bunyavirus, Rift Valley fever virus (Busquets et al., 2010). The influence of the inoculum should be considered when planning future experiments in sheep and there is a need to be careful with extrapolation of TCID$_{50}$ values used in this experiment. Previous studies
have shown that the origin of the virus and the way it has been passaged might strongly influence the outcome of an experimental infection, even if high inoculation doses are used (Wernike et al., 2013).

In all sheep that became positive by qRT-PCR for viral RNA in blood, SBV RNA could be detected from 2 to 7 dpi. The duration of detection of viral RNA in blood by qRT-PCR and the SBV copy number at the peak of detection were not significantly different between groups inoculated with undiluted or 1/10 diluted infectious bovine serum (two-sample t tests with unequal variances; \( n = 6; \) \( P = 0.14 \) and 0.26, respectively). The copy number at the peak of detection of viral RNA by qRT-PCR in blood in sheep inoculated with 1/100 diluted infectious serum reached a similar level. Comparable results were obtained when the presence of SBV RNA was determined in whole blood samples (data not shown).

All ewes were euthanased at 10 dpi. No gross lesions were observed at postmortem examination. Portions of cerebrum, cerebellum, brain stem, lung, spleen, left superficial cervical and mesenteric lymph nodes, tonsils and ovary were collected. Virus was detected in the spleen, and the superficial cervical and mesenteric lymph nodes in all seven ewes, and in the lungs of two ewes, that were positive by qRT-PCR for viral RNA in blood (Table 1). There was no significant difference in the SBV RNA copy number in the superficial cervical and mesenteric lymph nodes, or spleen between sheep inoculated with the undiluted and 1/10 diluted infectious serum (two-sample \( t \) test with unequal variances; \( n = 6; \) \( P = 0.30, 0.99 \) and 0.38, respectively). The copy numbers in the three different lymphoid organs of the sheep that were positive by qRT-PCR for viral RNA in blood following inoculation with 1/100 diluted infectious bovine serum reached similar levels.
These observations raise the question of the importance of the lymphatic system in the pathogenesis of SBV in sheep. Interestingly, similar observations were obtained after SBV infection of other sheep breeds (Wernike et al., 2013). However, as little is known about the pathogenicity of orthobunyaviruses of veterinary importance (Doceul et al., 2013), it remains difficult to interpret these data. Further studies quantifying SBV in these lymphatic tissues over time are needed to clarify this issue.

The presence of neutralising anti-SBV antibodies was assessed by SNT (De Regge et al. 2013). All ewes that were positive by qRT-PCR for viral RNA in blood seroconverted between 7 and 9 dpi (Fig. 2), while the other ewes were negative. The number of SBV antibody positive animals by group decreased significantly as a function of the inoculated dose (Fisher's exact test; $n = 12; P = 0.045$). Serum samples collected on the day of euthanasia were also tested by the ID Screen Schmallenberg virus Indirect Multi-Species ELISA (IDVet); all samples were negative. This discrepancy is probably because the SNT can detect immunoglobulin (Ig) M antibodies with neutralising capacity, while the ELISA only detects IgG due because it uses an anti-multi-species IgG-horseradish peroxidase conjugate.

The productively infected animal in the 1/100 dilution group was inoculated with a theoretical dose of, at most, 20 TCID$_{50}$. It seems reasonable to assume that infectious doses of this magnitude can be delivered by SBV-infected Culicoides spp. during feeding. For BTV, another disease transmitted by Culicoides spp., a single midge can transmit 0.32-7.79 TCID$_{50}$ (Fu et al., 1999). Recent reports of Ct values of around 30 for the SBV S segment (obtained using the same qRT-PCR) in the saliva of SBV-infected Culicoides sonorensis (Veronesi et al., 2013) indicate that this could also be realistic for SBV.
In conclusion, this experiment provides evidence that a critical dose needs to be
administered to induce a homogeneous productive infection in sheep. When a sufficient dose
is however administered, no dose dependent effect was observed, either in the duration and
quantity of viral RNA detected by qRT-PCR in blood, or in the amount of viral RNA present
in the lymphoid organs.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other
people or organisations that could inappropriately influence or bias the content of the paper.

Acknowledgements

We warmly thank all the personnel of CODA-CERVA Machelen for taking care of the
animals and the technical personnel of the unit Enzorem who helped with the analysis of the
collected samples, including Celia Thoraval, Dennis Kozlowski, Laurent Rosar, Muriel
Verhoeven, Sophie De Laet, Thibault De Maesschalck and Virginie Colasse. We also thank
Prof. Dr. Martin Beer and Dr Bernd Hoffmann from the Friedrich Loeffler Institute for
providing the inoculum. This study was financially supported by Federal Public Service
Public Health and Safety of the Food Chain and Environment (RF12/6270) and the European
Union, as outlined in Council Decision 2012/349/EU concerning a financial contribution by
the Union for studies on SBV.

Appendix A: Supplementary material

Supplementary data associated with this article can be found, in the online version, at
doi: …
References


De Regge, N., van den Berg, T., Georges, L., Cay B., 2013. Diagnosis of Schmallenberg virus infection in malformed lambs and calves and first indications for virus clearance in the fetus. Veterinary Microbiology 162, 595-600.


Table 1

<table>
<thead>
<tr>
<th></th>
<th>Undiluted</th>
<th>1/10 dilution</th>
<th>1/100 dilution</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Ewe 1</td>
<td>Ewe 2</td>
<td>Ewe 3</td>
</tr>
<tr>
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<td>Neg</td>
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</tr>
<tr>
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</tr>
<tr>
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<tr>
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SBV S segment RNA (copies/g) detected at 10 days post-inoculation by qRT-PCR in different organs of ewes inoculated subcutaneously with different doses (undiluted or 1/10, 1/100, 1/1000 dilution) of an SBV infectious serum. All samples from ewes inoculated with a 1/1000 dilution were negative.

Neg, negative; NA, not available.
**Figure legends**

Fig. 1. Detection of Schmallenberg virus (SBV) S segment RNA by qRT-PCR (copy number/mL) in the blood of sheep (three ewes in each group: ewe 1 ▬, ewe 2 ▬, ewe 3 ▬) inoculated subcutaneously at day 0 with undiluted (a), or 1/10 (b) or 1/100 (c) dilutions, of SBV infectious bovine serum. None of the animals inoculated with a 1/1000 dilution became positive for SBV RNA by quantitative reverse transcriptase PCR.

Fig. 2. Seroconversion in Schmallenberg virus (SBV) inoculated animals. Titres of neutralising anti-SBV antibodies measured in serum from four groups, each of three ewes (ewe 1 ▬, ewe 2 ▬, ewe 3 ▬), inoculated subcutaneously at day 0 with undiluted (a), or 1/10 (b) or 1/100 (c) dilutions, of an SBV infectious serum. None of the animals inoculated with a 1/1000 dilution seroconverted. The dashed line indicates the cut-off value of the serum neutralisation test. Sera were considered to be positive if the titre was ≥ 4 (specificity 100%, De Regge et al., 2013a). The columns (■) represent the cumulative number of ewes which had seroconverted at different days post infection.