Transboundary and Emerging Diseases

SHORT COMMUNICATION

Three Different Routes of Inoculation for Experimental Infection with Schmallenberg Virus in Sheep

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Summary

Schmallenberg virus (SBV) is an emerging *Orthobunyavirus* affecting European domestic ruminants. In this study, three groups of ewes (n = 3) were inoculated with 1 ml of an SBV infectious serum, via the subcutaneous (SC), intradermal (ID) or intranasal (IN) route. The ewes were monitored for 10 days and no clinical signs were reported. IN inoculation failed to generate any detectable RNAemia. SC and ID inoculation induced typical SBV RNAemia and seroconversion upon day 6 post-inoculation in 3/3 and 2/3 sheep, respectively. In all the animals that showed RNAemia, the viral genome could be detected in spleen and mesenteric lymph nodes. Both the SC and ID routes seem suitable to properly reproduce field conditions, as comparable observations were reported regarding RNAemia, seroconversion and viral genome detection in organs.

Introduction

Schmallenberg virus (SBV; family *Bunyaviridae*, genus *Orthobunyavirus*) emerged in Europe in 2011 and is transmitted by *Culicoides* biting midges. It is associated with a mild non-specific syndrome in adult cattle, but most of the clinical impact is related to congenital malformations that can affect ruminant neonates following *in utero* infection (Herder et al., 2012). So far, in most of the experimental infections performed in host species, subcutaneous (SC) inoculation was chosen, but the intravenous (IV) (Hoffmann et al., 2012), oral (PO) and intramuscular (IM) routes were also evaluated (Wernike et al., 2013a,b). As *Culicoides* spp. are 'pool-feeder', that is blood feeding following laceration of the dermis of the host, superficial tissues are in first line of contact with the pathogen. Therefore, intradermal (ID) inoculation of SBV is worth investigating. In addition, intranasal (IN) inoculation has not been tested yet and could provide additional insights for potential SBV contact transmission. Thus, SC, ID and IN inoculations were compared based on their clinical, virological and serological outcomes.

Materials and Methods

Nine 'Mourerous' ewes of about 1 year of age were used. The Mourerous is a middle-size rustic breed from south of France. All were serologically and virologically negative for SBV. Three isolated groups of three ewes were randomly constituted in a BSL3 facility (CODA-CERVA Machelen, Belgium). Each animal was inoculated with 1 ml of the infectious serum via the IN way (ewes IN1 to IN3) in the right nostril, or subcutaneously (SC1 to SC3) in the left axilla or for the last group, intradermally on the left side of the neck (ID1 to ID3). ID inoculation was performed using a Dermojet® device (Akra Dermojet, Pau, France). The

inoculum (graciously provided by Friedrich Loeffler Institute, Riems, Germany) contained about 2×10^3 50% tissue culture infective dose/mL ($TCID_{50}$/mL) and 7.3×10^6 RNA copies/mL of SBV S-segment. This study was approved by the Ethical Committee of the IPH-VAR (Scientific Institute of Public Health – Veterinary and Agrochemical Research Centre, number of project: 121017-01, date of approval: 11 February 2013).

A daily clinical examination was performed associated with blood, faecal and nasal sampling. Ewes were euthanized 10 days post-infection (dpi). The extraction of RNA from organs, faeces and serum, and the detection of SBV S-segment by a one-step reverse-transcription (RT) quantitative PCR (RT-qPCR) were performed as described previously (De Regge et al., 2013). The quantification cycle (Cq) values were converted into copy numbers (Poskin et al., 2014), and results were included only when negative extraction controls and negative and positive amplification controls were satisfactory.

Results and Discussion

No clinical signs were observed that could be related to an acute SBV infection in the inoculated sheep. No viral RNA was detected in the blood, nor in the organs of the animals from the IN group. Inoculation of ID3 did not result in a detectable RNAemia. Failures to reproduce or induce infection were previously described with the same inoculum in calves (Hechinger et al., 2013) and sheep (Wernike et al., 2013b) through SC route. Thus, the lack of infection is most likely not related to the ID route itself and could be related to a random failure of infection or individual factors of resistance. The observed RNAemia (Figs 1a and b), in both SC and ID groups, was comparable in duration (3–5 days) and in line with previous data (Hoffmann et al., 2012; Wernike et al., 2013b; Poskin et al., 2014) following IV and SC inoculations. The mean SBV genome copy numbers detected in the blood of sheep with RNAemia were not significantly different between SC and ID groups through time (two-way ANOVA with repeated measures; group effect: $P = 0.15$; group–time interaction: $P = 0.41$). In contrast, at viraemic peak, maximum copy number was significantly higher in the ID group versus SC group (Welch's test with unequal variance; $P = 0.026$).

The sporadic low-level detection of SBV genome in nasal swabs (Figs 1c and d) is highly unlikely to play a role in the epidemiology of the disease. As direct IN inoculation failed to cause RNAemia, an intermittent and low-level nasal

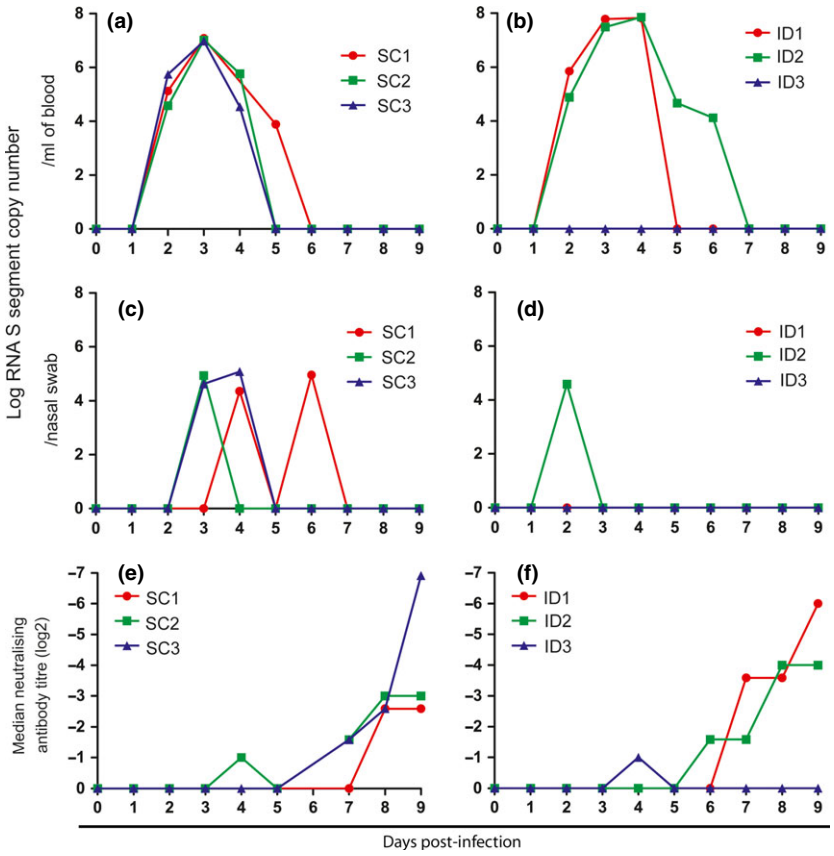

Fig. 1. Reverse-transcriptase quantitative PCR results in blood (a and b) and nasal swabs (c and d) for SC and ID groups. In panels (c) and (d), no sheep had positive detection in both nostril the same day; thus, right or left nostril origin is not specified. Panels (e) and (f) show the neutralizing antibody titres per mL in SC and ID groups, respectively. ID: Intradermal and SC: Subcutaneous. [Colour figure can be viewed at wileyonlinelibrary.com].

shedding should be of very limited impact on the further spread of the virus. SBV genome was found in faeces of animals of all the three groups (Fig. 2), but only sporadically and inconstantly in animals of group IN. No SBV RNA was found in faeces of ewe ID3 that had no detectable viral RNAemia. Mean and maximum viral RNA copy numbers and duration of detection in faeces were not significantly different between SC and ID groups (Welch's test with unequal variance; $P = 0.13$, 0.12 and 0.48, respectively). In contrast with previous work (Wernike et al., 2013a), SBV genome remained detectable in faecal samples of SC and ID groups 1–2 days after the end of the positive detection in blood. The origin of the faecal positive detection, especially in IN group as no RNAemia could be detected, remains debatable. However, a gastrointestinal origin cannot be ruled out. Indeed, positive detection in IN group might be a consequence of a partial swallowing of the inoculum. Positive faeces in SC and ID groups might be related to infected cell loss or infected secretions in the gastrointestinal tract, further detected in faeces. Infected cells could reach gastrointestinal lumen through epithelium renewal or intestinal micro-bleeding caused by chronic parasitic infestation, or virus could be transferred from the blood to the bile and then to faeces. In addition, Wernike et al. (2013b) reported diarrhoea in a sheep following SBV infection, which could lead to a transient increase in intestinal permeability. Moreover, the 1–2 days delay between blood and negative faeces detection might be caused by the transit inertia, as mean ingesta retention time in sheep is about 48 h (Hadjigeorgiou et al., 2003). Other *Bunyaviridae* like hantaviruses are well known to be detected in faeces of infected animals, possibly longer than in the blood (Voutilainen et al., 2015).

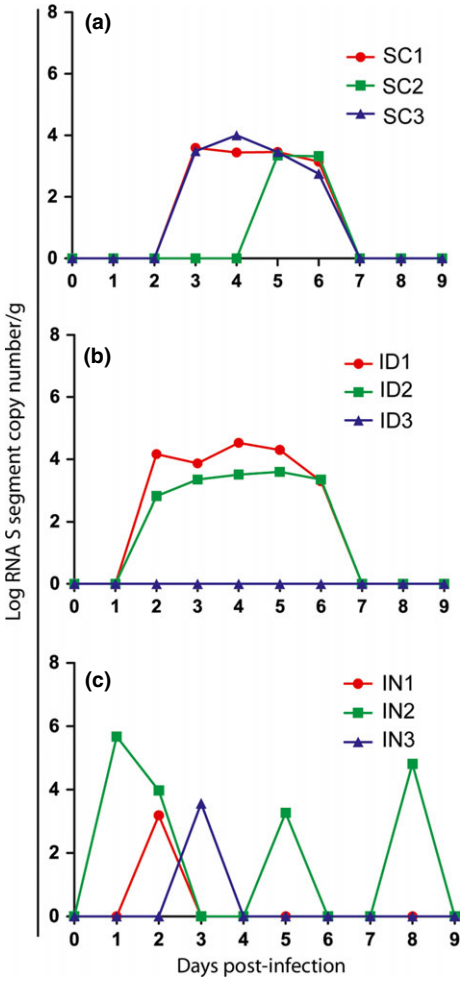

Fig. 2. Reverse-transcriptase quantitative PCR in faeces of subcutaneously (a), intradermally (b) and intranasally (c) inoculated animals. [Colour figure can be viewed at wileyonlinelibrary.com].

The presence of neutralizing anti-SBV antibodies was assessed by seroneutralization (SNT, De Regge et al., 2013). Only ewes that had RNAemia seroconverted within the duration of the experiment. Seroconversion occurred between 6 and 8 dpi in SC and ID groups (Figs 1e and f). As previously reported, the decrease of SBV RNA in the blood correlated with seroconversion (Poskin et al., 2014). However, as the animals were only tested for 10 days, a later seroconversion might have been possible in ewes without RNAemia.

At post-mortem examination, no particular gross lesions were reported. No SBV genome could be detected in the central nervous system or genital tract. Within the same ani-

Table 1. Log copy number of SBV S-segment by weight (g) of organ for each ewe

Organ	SC1	SC2	SC3	ID1	ID2	ID3	Mean by organ[a]
Lung	0.00	5.37	0.00	5.91	0.00	0.00	5.64 (0.38)
Spleen	6.46	6.70	6.72	7.69	7.44	0.00	7.00 (0.53)
Mesenteric lymph node	6.69	7.30	6.75	7.91	7.51	0.00	7.23 (0.52)
Submandibular lymph node	7.20	5.87	4.74	0.00	0.00	0.00	5.94 (1.23)
Superficial cervical lymph node	5.54	0.00	0.00	7.09	0.00	0.00	6.32 (1.10)
Mean by route[a]		6.3 (0.81)			7.26 (0.72)		

[a]Means were calculated only considering positive organs. Standard deviation is in curved brackets.

mal, SBV RNA detection in serum was systematically associated with detection in spleen and mesenteric lymph nodes.

Table 1 shows the log of copy number of SBV RNA in each positive tested organ for each animal. Considering animals that showed RNAemia, more organs were detected positive in SC group (11/15) when compared to ID group (6/10), however, this difference was not significant (Fisher's exact test for count data = 1.79; $P = 0.67$). Comparing organs that were detected positive in both ID and SC groups (lung, spleen, mesenteric and superficial cervical lymph nodes), SBV RNA copy numbers were significantly higher in organs of ID group (two-way ANOVA with repeated measures; $P = 0.03$).

Conclusion

IN inoculation failed to generate any detectable RNAemia. In both SC- and ID-inoculated animals, viral RNA could be detected in faeces 1–2 days longer than in the blood. The cause of this prolonged faecal viral detection is currently uncertain but not uncommon in *Bunyaviridae* and it might be a consequence of the digestion time in sheep. Both of the SC and ID routes seem to properly reproduce field conditions, as similar outcomes for parameters such as RNAemia, seroconversion and viral genome detection in organs were found. SBV RNA copy number was significantly higher in organs of the ID group, but this result has to be interpreted with circumspection as the sample size was small and one ewe had no RNAemia at all. However, to be properly performed, ID inoculation requires the use of a regular syringe, with more care while injecting and a high risk to go SC if the animal is not perfectly calm, or specific devices such as syringe tuberculin pistol or jet injectors (like the Dermojet® used in the current study) with the constraint of a limited volume to be injected per shot. Therefore, SC inoculation might be easier to deal with from a practical point of view.

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