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Development and validation of an indirect Enzyme-linked Immunosorbent Assay for the detection of antibodies against Schmallenberg virus in blood samples from ruminants

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ABSTRACT

To detect Schmallenberg virus (SBV) infections in ruminants and to perform SBV epidemiological studies a cost-effective serological test is required. For these purposes an indirect whole virus Enzyme-linked Immunosorbent Assay (ELISA) for detection of SBV specific antibodies in ruminant blood samples was developed. Schmallenberg virus antigen was produced by propagation on Vero cells, partly purified and coated onto ELISA plates. The indirect ELISA procedure included the subsequent incubation of diluted samples, protein-G-HRP conjugate and TMB substrate solution. Net Optical Densities (OD) values were calculated and expressed as a sample to positive percentage (S/P%) by comparison of the average net OD with the OD of the positive control. Validation of this assay was performed using 633 samples from SBV-free sheep, goats and cattle, and 141 samples from SBV suspect ruminants. The diagnostic specificity was 98.8%. Test results of 86 ruminant serum samples using both the SBV-ELISA and an SBV virus neutralization test (VNT), designated as the gold standard serological test for SBV, showed good correlation: at an S/P cut-off of 15% only one VNT positive sample tested negative in the SBV ELISA. The diagnostic sensitivity of the ELISA, relative to the VNT, was 98.8% (95% CI: 93.3–100.0%). The ELISA showed a high repeatability (cv = 6.5%) and reproducibility (100% agreement). It was concluded that this ELISA is a suitable test method for the detection of SBV antibodies in sera from cows, sheep and, possibly, goats.

1. Introduction

In September 2011 a new *Orthobunyavirus* was identified in dairy cows showing clinical signs of fever, milk drop and diarrhoea in North-West Germany and Eastern regions of the Netherlands (Hoffmann et al., 2012; Muskens et al., 2012). This *Orthobunyavirus* was tentatively named Schmallenberg virus (SBV). From November 2011 on, SBV was found to be the causative agent in outbreaks of abortion and congenital malformations in newborn calves, lambs and goat kids (Elbers et al., 2012; Hoffmann et al., 2012; Muskens et al., 2012; van Maanen et al., 2012).

Schmallenberg virus was classified in the Simbu serogroup of the genus *Orthobunyavirus* of the family *Orthobunyaviridae*. The virus has an M RNA segment very similar to that of Sathuperi virus whereas the S and L segments are closely related to those of Shamonda virus (Garigliany et al., 2012; Goller et al., 2012; Yanase et al., 2012). The S segment encodes for the nucleocapsid protein (N) and a non-structural protein (NSs), the M segment encodes for two glycoproteins (Gn and Gc) and a second non-structural protein (NSm), whereas the L segment encodes for an RNA polymerase (L) (Schmaljohn and Nichol, 2007). In general, antibodies against N-proteins of viruses within the Simbu serogroup *Orthobunyavirus* cross-react, while antibodies against Gn and Gc are species specific (McPhee and Della-Porta, 1988) or even strain specific (Akashi and Inaba, 1997; Akashi et al., 1997).

For diagnostic purposes an SBV specific qPCR has been developed (Hoffmann et al., 2012) which is now used in many laboratories for SBV RNA detection in blood, serum and tissue samples from ruminants. For the detection of SBV specific antibodies firstly a virus neutralisation test (VNT) was developed (Loeffen et al., 2012). However, for large scale epidemiological studies, an SBV-ELISA would be a more practical and more cost-effective tool. Furthermore, in order to find serological evidence of the presence of precursor viruses in the 2011 ruminant population using old serum panels, a whole virus ELISA will probably be more suitable than a VNT as such assay is more likely to detect antibodies against the

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N protein of all viruses within the Simbu serogroup, while an SBV VNT would probably only detect antibodies directed against SBV and Sathuperi virus glycoproteins.

For other orthobunyaviruses of the Simbu serogroup, ELISAs have been described (Blacksell et al., 1997), in particular for Akabane virus (Ide et al., 1989; Tsuda et al., 2004; Ungar-Waron et al., 1989). Recently, an SBV ELISA based on recombinant protein has become commercially available (Bréard et al., 2013). In the present study an indirect whole virus SBV ELISA was developed and validated as a practical, sensitive and specific assay for detection of SBV antibodies in ruminants.

2. Materials and methods

2.1. SBV culture

African green monkey kidney cells (Vero cells) were grown in roller bottles (Corning, Amsterdam, the Netherlands) using 100 mL DMEM (Gibco, Bleiswijk, the Netherlands) with 5% foetal calf serum (Pan Biotech, Aidenbach, Germany) and 1% antibiotic–antimycotic (100×; Gibco 15240). The roller bottles were seeded with 5 mL 1.0–2.0 × 10^5 cells/mL and incubated (Incudrive D-I, Schuett-Biotec, Göttingen, Germany) at 36–38 °C running at a speed of 1 revolution per 4–5 min and grown to a 70–90% confluence.

The SBV isolate used for virus culture originated from a serum sample of a dairy cow which was tested positive by qPCR (Loeffen et al., 2012). Later on this serum sample was also screened for other viruses by microarray (EPIZONE DNA-chip) and besides SBV no other viruses were detected (unpublished observations). After five virus culture passages, the SBV isolate was titrated using Vero cells. The virus titre of the SBV stock was $10^{6.4}$ $TCID_{50}$/mL. After titration, the SBV stock was divided in smaller portions and stored at −80 °C.

Cells and SBV-stocks were routinely screened for Mycoplasma-contamination by PCR and for Bovine Virus Diarrhoea virus by culture and Immuno Peroxidase Monolayer Assay and were always negative.

2.2. Production of SBV ELISA antigen

For the production of SBV antigens, SBV was propagated on Vero cells. Before inoculation, the cell culture medium was removed from the roller bottle and the monolayer of Vero cells was carefully washed thrice with 25 mL of sterile PBS. The roller bottle was then inoculated with 5 mL SBV seeding stock in 20 mL of maintenance medium consisting of DMEM with 1% antibiotic–antimycotic, without foetal calf serum. For production of control antigen, 25 mL of the maintenance medium was added to the roller bottles. After a 1 h incubation at 36–38 °C at a speed of 1 revolution per 2–3 min, 75 mL of maintenance medium was added to each roller bottle. Subsequently, the roller bottles were incubated at 36–38 °C (1 revolution per 2–3 min) until more than 80% of the cells showed cytopathogenic effect (usually after 2–3 days of incubation). During this up to 3 days incubation the roller bottles were inspected microscopically twice a day.

Roller bottles with either SBV virus or cell control culture underwent three freeze/thawing cycles. Subsequently, 0.05% beta-propiolactone (Sigma–Aldrich, Zwijndrecht, the Netherlands) was added and the roller bottles were incubated for 2 h at room temperature. The culture fluids were clarified by low-speed centrifugation at 3800*g* for 10 min. The cell pellets were discarded and the supernatant was aliquoted in 40 mL tubes and centrifuged at 75,600*g* (Avanti J-26 XP with JA25.50 rotor, Beckman, Woerden, the Netherlands) for 2 h at 15 °C. The supernatant was carefully discarded and the pellets were resuspended in 1.12 mL of a 0.01 M Tris–hydrochloride buffer pH 7.6 containing 0.15 M NaCl and 0.003 M EDTA (Obijeski et al., 1976) and subsequently stored at −20 °C until used. Each lot of SBV antigen was titrated using ELISA with optimal dilutions ranging from 1/150 to 1/175. For the corresponding cell control antigen the same dilution was used.

2.3. Serum samples

Negative serum panels consisted of samples from sheep (n = 88) and goats (n = 157) collected in 2004 or 2005 and from cattle (n = 264) obtained in 2002. There was no evidence of SBV infection in Europe before 2011. All serum samples had been stored at −20 °C until used for this study. Additional samples from Scottish sheep (n = 100), were kindly provided by Dr. K. Willoughby, Moredun Research Institute, Scotland as well as sheep (n = 12) and goat (n = 12) samples which were kindly provided by Dr. C. Ritchie, Scottish Agricultural College, Scotland. Since no clinical signs of SBV were reported in Scotland before 2012 (Garigliany et al., 2012), the Scottish samples were also considered to be negative for SBV antibodies.

Positive sera (n = 86) originated from herds in which SBV clinical signs (malformed foetuses) were observed. These signs had been notified to the Dutch Food and Consumer Safety Authority, while the malformed foetuses were submitted to the Animal Health Service (GD) for post mortem examination, and from mothers of these foetuses (21 cows, 63 sheep and 2 goats), blood samples were taken for antibody detection using the SBV virus neutralization test (VNT) (Loeffen et al., 2012). Another positive serum panel (n = 22) was collected from 16 cows of 11 herds which had shown clinical signs of fever and diarrhoe in September 2011 and which were re-sampled during the first quarter of 2012, from three dams that had delivered malformed foetuses which tested positive in SBV PCR, and from three goats with malformed foetuses which were not confirmed by PCR. The third sample panel (n = 33) was randomly collected at a single flock in which sheep showed mild clinical symptoms (fever and diarrhoea) of SBV infection.

2.4. SBV-ELISA

An indirect ELISA was developed by performing checkerboard titrations of ELISA reagents (antigen, conjugate) with well-defined ruminant serum samples. After further optimization (SBV isolate, cells, plastics, coating buffer and ELISA buffer, incubation temperature, blocking) the following protocol was implemented for the indirect SBV ELISA:

SBV antigen or cell control antigen were diluted 1/150–1/175 in 0.05 M carbonate/bicarbonate buffer pH 9.6 and 100 μL per well added to polystyrene ELISA-plates (PolySorp, Nunc, Roskilde Denmark). The plate was incubated for 14–16 h at 4 °C. Serum samples were diluted 1/40 in sample buffer, consisting of PBS pH 7.2 with 5% foetal calf serum and 0.05% tween 80. In each plate positive and negative control samples were included in duplicate, as well as positive control samples from sheep, goats and cattle and a conjugate blank (only buffer).

After washing the ELISA-plate three times using ultrapure water containing 0.05% tween 80, 100 μL of the diluted sample was added to a well coated with virus antigen and to a well coated with cell control antigen. The ELISA-plate was incubated for 1 h at 20–24 °C. After another washing, 100 μL/well protein-G-HRP conjugate (31499, Thermo Scientific, Rockford, USA), diluted 1/12,000 in PBS pH 7.2 with 0.05% tween 80, was added, and the ELISA-plate was incubated for 1 h at 20–24 °C. After further washing, 100 μL/well ready-to-use TMB-substrate solution (Idexx, Schiphol, the Netherlands) was added and the ELISA-plate incubated at 20–24 °C for 15 min. Then 50 μL/well of 2 M sulphuric acid was then