Short Communication

Evaluation of two commercial enzyme-linked immunosorbent assays for detection of bovine viral diarrhoea virus in serum and skin biopsies of cattle

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Abstract

AIM: To assess the ability of two commercial bovine viral diarrhoea (BVD) virus (BVDV) antigen-capture enzyme-linked immunosorbent assays (ELISAs) to detect virus in serum and skin biopsies.

METHODS: Thirty cattle persistently infected (PI) with BVDV were identified using routine diagnostic laboratory testing. Additional ear-notch skin biopsies and blood samples were collected from these animals to confirm the diagnosis, and from 246 cohorts, to determine their BVDV status. Skin biopsies were soaked overnight in buffer and the eluate collected. All sera and eluates were tested using two commercially available ELISAs for detecting BVDV antigen, and a subsample of positive and negative sera was tested using a polymerase chain reaction (PCR) test. A study was also performed to ascertain the risk of cross-contamination occurring during the collection and processing of skin biopsies.

RESULTS: Both serum and skin samples tested using either ELISA resulted in the detection of all cattle identified as PI and no non-infected cattle were incorrectly classified as infected using either method. Agreement between all assays (ELISAs, whether performed on serum or skin, and PCR) was 100%. No cross-contamination of skin samples between animals was evident using routine biopsy methods.

CONCLUSIONS: Viraemic cattle infected with BVDV were accurately identified using either of the two commercial ELISAs evaluated on either serum or skin samples.

CLINICAL RELEVANCE: Either skin biopsies or serum samples can be collected from cattle to determine their BVDV status. This should overcome problems in accurately identifying the infection status of young calves in which colostral antibodies might interfere with the antigen-capture ELISA.

KEY WORDS: Cattle, bovine viral diarrhoea virus, BVD, diagnosis, skin, serum, antigen-capture ELISA, PCR

Introduction

Bovine viral diarrhoea virus is an important pathogen of cattle worldwide (Lindberg 2003) and is thought to be responsible for a large proportion of abortions in cattle in New Zealand (Thobok-

we and Heuer 2004). Infection can also cause embryonic loss, stillbirth and congenital defects (Grooms 2004). PI cattle are a source of infection to other calves and older animals, which can result in acute disease in those animals. PI cattle themselves often develop terminal mucosal disease (Brownlie et al 1984).

Control efforts have been successful in a number of countries around the world (reviewed by Lindberg 2003) and are contingent on the detection of BVDV-infected animals. Most control attempts rely on the identification and then elimination of PI cattle from the herd. PI cattle are recognised as the most important source of virus, shedding virus throughout their life, and are a source of re-infection within a herd. Diagnostic tests used to detect these infected individuals include virus isolation, immunohistochemistry (Njaa et al 2000; Grooms and Keilen 2002), antigen-capture ELISAs, and traditional and real-time PCR (RT-PCR) (Mahlum et al 2002; Saliki and Dubovi 2004).

Virus isolation and immunohistochemistry are time-consuming and laborious, and results may be difficult to interpret (Cornish et al 2005), whereas ELISA technology lends itself to large-scale testing of a greater number of samples. Some commercial antigen-capture ELISAs have been validated overseas for the detection of virus in ear-notch skin biopsies of cattle (Cornish et al 2005). In calves <6 months of age, colostral antibodies obtained from their own or other dams may have interfered with the detection of virus in serum (Zimmer et al 2004), whereas detection of virus in ear-notch samples was not interfered with (Cornish et al 2005). Hence, the ear-notch technique may allow detection of PI calves earlier in their life than would be feasible using serum alone.

Isolates of BVDV in New Zealand have been classified as being of Type 1 only, but showed greater genetic diversity in preliminary studies (Vilcek et al 1998) than isolates found in Australia (Mahony et al 2005). Re-confirmation of the ability of ELISAs to detect types of BVDV common in New Zealand is necessary. The present study was aimed at assessing the ability of two BVDV antigen-capture ELISAs to detect BVDV in serum and skin biopsies of cattle. ELISA-positive sera and a subsample of ELISA-negative sera were then tested using RT-PCR, and the results compared.

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Ag Antigen
BVD Bovine viral diarrhoea
BVDV Bovine viral diarrhoea virus
ELISA Enzyme-linked immunosorbent assay
FAM 6-Carboxyl-fluorescein
OD Optical density
PCR Polymerase-chain reaction
PI Persistently infected
RT-PCR Real-time polymerase chain reaction
S–N Sample minus negative
S/P Sample-to-positive
TG-ROC Two-graph receiver operating characteristics
**Materials and methods**

Veterinarians that submitted blood samples from cattle that tested BVDV antigen-positive and antibody-negative by ELISAs during routine diagnostic testing were contacted and invited to submit additional samples to confirm the diagnosis of PI animals. Additional samples included serum and ear-notch skin biopsies collected from BVDV-positive animals and other herd-mates, on a later occasion. Skin samples were collected from the caudal ear margin, most commonly using an ear-marking punch. Sample size varied from 4 x 4 to 10 x 10 mm. Some samples were collected surgically by incision using a sterile scalpel blade. Skin samples were stored individually in labelled sterile pottles and both blood and skin samples were received in the laboratory within 24 h of collection. Serum was separated by centrifugation on the day of reception, and tested that day or stored frozen at –20°C until analysis at a later date.

Cattle (n=276) were sampled from 20 herds, distributed from Northland in the North Island to Canterbury in the South Island, and ranged in age from 6 months to 4 years; most of the cattle (n=271) were <18 months old. Breeds included Friesian (n=8), Friesian x Jersey cross (n=5), mixed-breed (n=4), Angus (n=2), and Charolais (n=1). Selection of the additional animals to sample was left to the discretion of the submitting veterinarian in 19/20 herds. Generally, any other sick cattle of interest from the same mob were sampled, as well as two or three healthy cattle. From these samples, 29 PI and 47 BVDV-antigen-negative cattle were identified. From the 20th herd, comprising 9-month-old calves, all the herd-mates were sampled (n=200). One additional PI calf was identified, and 199 were BVDV-antigen-negative.

Fresh skin samples were either processed immediately, or frozen at –20°C and batch-processed in groups. This involved trimming to the margin, most commonly using an ear-marking punch. Sample size varied from 4 x 4 to 10 x 10 mm. Some samples were collected surgically by incision using a sterile scalpel blade. Skin samples were prepared individually in labelled sterile pottles and both blood and skin samples were received in the laboratory within 24 h of collection. Serum was separated by centrifugation on the day of reception, and tested that day or stored frozen at –20°C until analysis at a later date.

Antigen (Ag) ELISA

Detection of BVDV antigen in the serum samples and the matched skin eluates from each animal was performed using two kits available commercially, Herdchek BVDV antigen/serum plus test kit (IDEXX Ag-ELISA; IDEXX Scandinavia, Österbybruk, Sweden), and ELISA BVD/MD antigen mix screening test (Pourquier Ag-ELISA; Institut Pourquier, Montpellier, France), according to the manufacturers’ instructions.

Cross-contamination study

A small study to determine whether cross contamination of skin samples between animals occurred in the laboratory was conducted. After sampling skin from an antigen-positive animal, any antigen-negative skin samples were then sampled with no cleansing, chemical disinfection, or sterilisation by flaming of the scalpel blade. Eluates from the soaked samples were then processed and tested as described above.

Antibody ELISA

Detection of antibody to BVDV was carried out using a kit available commercially (ELISA BVD/MD/BD P80 Antibodies Screening Test; Institut Pourquier) on all the BVDV antigen-positive samples, according to the manufacturer’s instructions.

**Extraction of RNA**

Viral genomic RNA was extracted from 150 µl serum, using a commercial viral RNA isolation kit (Nucleospin RNA Virus, Catalogue No. 740 956.50; Macherey-Nagel, Düren, Germany). A final volume of 50 µl RNA in molecular biology-grade water was eluted and stored at –20°C until used.

**RT-PCR**

To detect BVDV RNA, forward and reverse primers and an hydrolysis probe were designed around the 5’ untranslated conserved region of the genome, based on previous reports (Hamel et al 1995; Weinstock et al 2001), and homology analysis on available sequences on the GenBank database (Table 1). Using these primers, an amplicon of 153 base pairs was routinely created when tested on known BVDV-positive serum samples. For PCR, 1.2 µl 20 µM BVD-UTRFor primer, 0.8 µl 20 µM BVD-UTRRev primer, and 1 µl 10 µM BVD-FAM (amplicon-specific 6-carboxyl-fluorescein (FAM)-labelled hydrolysis probe) per reaction were used with the reaction mix components of the Quantitect Probe RT-PCR kit (Catalogue No. 204443; Qiagen, Hilden, Germany). Magnesium chloride was present in the reaction at a concentration of 1.25 mM. Reverse transcriptase RT-PCR was performed using aRotor-Gene 2000 real-time cycler (Corbett Research, Sydney, Australia). Appropriate positive and negative controls were included with each RT-PCR run. The resulting products were sequenced and confirmed to be of the target sequence. Amplification was performed following a PCR programme consisting of a cDNA production step of 50°C for 30 min followed by 15 min at 95°C, to activate the DNA polymerase activity, DNA amplicon production and detection were accomplished using 50 cycles of denaturation at 95°C for 20 sec, annealing and amplification at 60°C for 60 sec, and detection at 510 nm on the FAM channel. A total of 55 sera, including 30 antigen ELISA-positive sera and 25 antigen ELISA-negative sera chosen randomly, were tested using PCR.

**Two-graph receiver operating characteristics (TG-ROC)**

TG-ROC analysis was carried out as described previously (Reichel and Pfeiffer 2002), after log-transformation of the optical density (OD) of the sample-to-positive (S/P) OD ratios (for the Pourquier Ag-ELISA), and of the corrected OD of the sample minus the negative (S–N) OD (for the IDEXX Ag-ELISA).

Cut-off thresholds (for parametric and non-parametric distributions) were selected by the software where sensitivity and specificity were ≥90% (for details see Reichel and Pfeiffer 2002).

Table 1. Details of the primers and probe used in a study evaluating two commercial enzyme-linked immunosorbent assays for detection of bovine viral diarrhea virus (BVDV) in serum and skin biopsies of cattle. The genomic positions of the sequences are based on BVDV Type 1 (Strain KS86-1npc) sequence, GenBank Accession Number 28071147.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Genome location</th>
</tr>
</thead>
<tbody>
<tr>
<td>BVD-UTRFor</td>
<td>TAG CCA TGC CCT TAG TAG GA</td>
<td>pos. 104 to 123</td>
</tr>
<tr>
<td>BVD-UTRRev</td>
<td>TGG GCA TGC CCT CGT CCA</td>
<td>pos. 240 to 257</td>
</tr>
<tr>
<td>BVD-FAM</td>
<td>FAM-GTA GCA ACA GTG GTG</td>
<td>pos. 141 to 162</td>
</tr>
</tbody>
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FAM = 6-carboxyl-fluorescein; pos = position of the oligonucleotide sequence (in nucleotides) on the BVDV sequence.
Results

A total of 276 paired sera and ear-notch samples (30 positive (PI) and 246 negative) were tested using both ELISAs. There was complete agreement in the classification in every case between results for serum and skin samples in each ELISA and between both ELISAs. All 30 samples positive using the BVDV antigen ELISA were negative in the BVDV antibody ELISA. The cross-contamination study demonstrated there was no cross contamination of samples in the laboratory, whether cleansing was used or not.

RT-PCR results were positive for all 30 of the samples positive using the BVDV antigen ELISA and negative for a subset of 25 serum samples negative to that test. The average number of PCR cycles for the positive samples was 31.5 (range 26.2–43.3).

Cut-off values for the ELISAs were determined using TG-ROC analysis. For the Pourquier Ag-ELISA, these values were close to the cut-off threshold suggested by the manufacturer (30% S/P ratio), being 29% and 36% (skin, parametric and non-parametric) and 28% (serum, non-parametric), respectively. The only exception was the parametric cut-off determined for serum samples, which was lower than that recommended by the manufacturer, at an S/P ratio of 20%. For the IDEXX Ag-ELISA, cut-off thresholds determined by the TG-ROC analysis were all higher than the value suggested by the manufacturer (S–N >0.3), being 0.42 and 0.59 for serum (parametric and non-parametric) and 0.49 and 0.74 for skin, respectively (Figures 1 to 4).

The means of the S/P ratios (Pourquier Ag-ELISA) and ODs (IDEXX Ag-ELISA) for negative and positive sample populations, respectively, were similar when the results for the assay performed on serum or skin eluates were compared. An exception was the S/P ratios for the positive population in the Pourquier Ag-ELISA, for which values for skin samples were more than twice the value for serum samples (191% vs 93%).

Discussion

This study demonstrated complete agreement between two BVDV antigen-capture ELISAs conducted on serum samples and skin biopsies, and suggests skin biopsies could be used to accurately identify PI calves, in the same way serum samples can be used. This might make BVDV testing of calves easier and cheaper, since the farmer could collect the samples while ear-marking. It might also be used in cases where maternal antibodies ingested...
in colostrum may have interfered with BVDV antigen in serum from calves <6 months old (Cornish et al 2005). As cross contamination of samples was not shown to occur in a small laboratory study, lack of sterilisation of sampling equipment by collectors should not compromise the accuracy of results.

The ELISA results also showed complete agreement with the results of RT-PCR performed on sera from all BVDV antigen-positive samples, as well as complete agreement with a subset of negative samples. This provides further confidence in the results of the two assays.

The S/P ratios for skin samples assayed using the Pourquier Ag-ELISA were more than double those for serum samples from the same animals, suggesting viral antigen may have been present in skin eluates in higher concentrations than in serum; this is the subject of further evaluation at present.

Cut-off thresholds for ELISAs determined using TG-ROC analyses differed from those recommended by the assay manufacturers; for the IDEXX Ag-ELISA, cut-off values were substantially higher for both skin and serum, whilst for the Pourquier Ag-ELISA, a lower cut-off value was determined for serum samples. These optimised cut-off thresholds might be specific to PI animals, as selected in this study, and should be used when testing for PI animals using serum or skin samples.

This study confirmed the two ELISAs tested could accurately identify BVDV antigen present in the skin and serum of BVDV-infected cattle in New Zealand.

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