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Transient elimination of circulating bovine viral diarrhoea virus by colostral antibodies in persistently infected calves: a pitfall for BVDV-eradication programs?

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ABSTRACT

Infections with bovine viral diarrhoea virus (BVDV) cause substantial economic losses to cattle industries. Rapid detection of persistently BVDV infected (PI) calves is of utmost importance for the efficacy of BVDV control programs. Blood and ear skin biopsy samples are conveniently used for early mass screening of newborns. However, little is known about the impact of colostral antibodies on the outcome of relevant analyses. Here, we rigorously tested a series of samples obtained from five colostrum-fed PI calves from birth until they reached the status of seronegativity for NS3-specific antibodies. We comparatively quantified virus loads in blood samples and dried skin biopsies as detected with BVDV-NS3-, -Erns-capture ELISA and RT-qPCR. Monitoring of NS3-positive leukocytes was done with flow cytometry. Within seven days after colostrum intake, BVDV infected leukocytes disappeared for a three- to eight-week period. Immediately after colostrum ingestion, detectable Erns antigen levels dropped 10–100-fold in biopsy samples and in sera detection of Erns failed for one to two weeks. Virus demonstration in biopsy samples with a NS3-antigen-ELISA failed until days 90–158 after birth. Specific antibodies against BVDV also impaired the detection of viral RNA in leukocytes and blood. Mean RNA levels of the five calves were reduced in sera 2.500-fold and in leukocytes 400-fold, the lowest values were at week three of live. In contrast, levels of measurable viral RNA in biopsy samples remained constant during the observation period.

1. Introduction

The bovine viral diarrhoea virus (BVDV) causes high economic losses due to reproductive, enteric and respiratory diseases in cattle. Persistently infected (PI) animals are the major source of virus for transmission. PI animals originate in fetal infection within the first four months of gestation before immunocompetence of the foetus develops (Coria and McClurkin, 1978; McClurkin et al., 1984; Moennig and Liess, 1995). Because PI animals shed large amounts of BVDV throughout their lives (Coria and McClurkin, 1978; Houe, 1995) early detection and elimination of these individuals is a key requirement for BVDV eradication in control programs. To date, most common methods for detecting PI calves are RT-PCR assays and antigen capture ELISAs. Virus isolation and immunohistochemistry are laborious and rarely applied (Edmondson et al., 2007; Fulton et al., 2009; Hilbe et al., 2007).

Maternal antibodies in PI calves can interfere with diagnostic testing. This may even lead to false-negative results, a phenomenon known as "colostral diagnostic gap". BVDV isolation from blood samples of PI calves is inhibited by maternal neutralizing antibodies for several weeks (Brock et al., 1998; Meyling and Jensen, 1988; Palfi et al., 1993; Zimmer et al., 2004). Testing blood leukocytes with NS3-capture ELISA can produce false-negative results in the first months of life of PI calves (Brinkhof et al., 1996;

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Zimmer et al., 2004). This colostral diagnostic gap is less pronounced when capture ELISAs for the soluble BVDV-Erns in blood sera are used (Greiser-Wilke et al., 2003; Sandvik, 2005). In contrast, BVDV-specific PCR with blood samples is believed to be a reliable method for testing PI animals also in the presence of colostral antibodies (Gaede et al., 2003; Rossmanith et al., 2001).

In PI animals high amounts of BVDV are found in hair glands, hair follicles and in epidermal cells and skin biopsy samples can be used for BVDV diagnostics with immunohistochemistry (Thür et al., 1996a,b). For antigen-capture ELISAs with skin samples, the detection of secreted soluble BVDV-Erns (Rumenapf et al., 1993) seems to be most suitable (Cornish et al., 2005; Huchzermeier et al., 2004). Indeed, when Holmquist tested dried ear biopsy samples collected from eleven PI calves that had received maternal antibodies with an Erns ELISA, all samples reacted positive for Erns while serum samples collected at the same time were negative (Kühne et al., 2005). In terms of virus detection PCR is described as an appropriate method for BVDV screening using fresh or desiccated ear samples (Fux, 2007; Kennedy et al., 2006). By now millions of ear biopsy samples collected by animal tagging were tested with different methods in several European BVDV control programs (AU, CH, I, D) (Oettl et al., 2010; Presi et al., 2011; Tavella et al., 2008). Importantly, a significant proportion of false-negative diagnosis of BVDV-PI calves was noticed. However, the reasons for diagnostic failure are commonly unknown, and, to our knowledge, there are no data available from follow-up studies monitoring the development of virus loads in skin biopsy samples.

In this study we tested the duration and the degree of the impact of colostrum-derived antibodies on the BVDV detection in PI calves. We clearly demonstrate various reasons for false-negative results when BVDV loads are measured in blood samples or biopsy samples with NS3- and Erns-antigen capture ELISAs, or in virus infected cells with flow cytometry or with real-time RT-PCR. Highly consistent virus detection required the use of dried ear biopsy samples and analysis of viral RNA with an appropriate RT-PCR.

2. Materials and methods

2.1. Persistently BVDV-infected calves, colostrum feeding and sample collection

Five BVDV-naive Brown Swiss heifers in early pregnancy had accidentally contact with a BVDV-1 PI animal on a common pasture. Because of seroconversion we expected an intrauterine infection and the development of PI calves. Heifers were housed in a quarantine unit until where they gave birth to five healthy BVDV PI calves (A, B, C, D and E). Births were spontaneous at the expected time. The calves were clinically healthy over the whole observation period. We offered repeatedly re-warmed colostrum to the calves of the respective cow ad libitum. The intake volumes in the first 24 h of life were 4.0 (A), 6.3 (B), 9.6 (C), 7.5 (D) and 4.3 (E) litres, respectively. Until 72 h of life the calves had drunk 12.4 (A), 16.7 (B), 24.1 (C), 20.5 (D) and 15.8 (E) litres of colostrum. EDTA blood, serum and a skin biopsy samples were collected immediately after birth. Subsequently blood samples were collected daily, skin biopsy samples the following ten days every third day. From days 10 to 28 days of age all samples were collected twice a week and after that time once weekly. Native and EDTA stabilized blood samples were collected by puncture of the jugular vein. Skin biopsy samples were collected applying a 6-mm biopsy punch lateral at the neck.

2.2. Sample preparation

After blood clotting sera were prepared by centrifugation and stored in aliquots at −80 °C. Leukocyte pellets were obtained from EDTA blood following haemolysis in ice-cold ammonium chloride (8.29 g/L NH_4Cl, 1.0 g/L $KHCO_3$, 1 mM EDTA). For PCR analysis cells were washed in PBS (centrifugation 5 min, 500 × *g*) and stored in aliquots of 5×10^6 cells at −80 °C. For BVDV-NS3 detection 50 μl whole blood aliquots were haemolysed in 96-well plates and leukocytes were washed in PBS before immediate immunostaining.

The skin biopsy samples were divided into four aliquots within 1 h after collection. To mimic the storage conditions in ear tag sampling devices, biopsies were sealed in 2-ml vials with hygroscopic molecular sieve (Merck, Darmstadt, Germany, no. 105734). Dried samples were stored at +4 °C until testing.

2.3. Real-time RT-PCR

RNA isolation for real-time RT-PCR was performed with RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany) for biopsy samples, NucleoSpin Virus Core Kit (Macherey-Nagel, Düren, Germany) for serum and High Pure Viral RNA Kit (Roche, Penzberg, Germany) for leukocytes according to the manufacturers' instructions, respectively.

Dried skin biopsies were homogenized in 300 μl RLT buffer (part of RNeasy Mini Kit) with a 5-mm steel bead in 2-ml vials using a mixer mill MM300 (Retsch, Haan, Germany, 2.5 min at 25 Hz). Subsequently 500 μl proteinase K solutions (part of RNeasy Mini Kit) were added for 10 min at 55 °C. After centrifugation (500 × *g*, 2 min), total supernatants were completely used for RNA isolation. The NucleoSpin Kit for RNA extraction from 100 μl sera was processed with a workstation (Hamilton Robotics, 100 μl elution volume), all other extractions were performed manually (50 μl elution volume).

The 5′ untranslated region of the BVDV-RNA was detected using a real-time RT-PCR protocol designed by Hoffmann et al. (2006). For the one-step RT-PCR the QuantiTect™ Probe RT-PCR Kit (Qiagen, Hilden, Germany) was used. Five microlitres of RNA template were added to 12.5 μl Master Mix, 5.25 μl RNase-free water, 0.25 μl RT Mix and 2 μl primer-probe-mix. The following temperature profile was chosen: reverse transcription 50 °C for 30 min, reverse transcriptase inactivation and polymerase activation 95 °C for 15 min, 42 cycles of denaturation for 30 s at 95 °C, annealing for 45 s at 57 °C and elongation for 45 s at 68 °C. A Stratagene MX3005P was used for fluorescence-measurement (Agilent Technologies, Waldbronn, Germany). A log10 dilution series of in vitro transcribed