Detection of Bovine Viral Diarrhoea Virus Infected Cattle – Testing Tissue Samples Derived from Ear Tagging Using an E\textsuperscript{rns} Capture ELISA

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Summary
A new diagnostic approach testing tissue samples derived from cattle ear tagging for bovine viral diarrhea virus (BVDV) antigen in a commercially available antigen capture enzyme-linked immunosorbent assay (ACE) was developed. To validate this method, 99 positive and 469 negative samples were tested. With those samples the assay yielded a sensitivity of 100% and specificity of ≥99.6%. Serum and ear tissue samples from 11 persistently infected (PI) BVDV calves were tested. While serum samples were negative after intake of colostrum, the ear tissue samples could be detected positive for BVDV all the time. Testing multiple samples derived from the same ear from PI cattle yielded positive results and low variation. Using cattle ear tags combining the ear tag application with sampling of a small ear tissue plug and testing those tissue samples with an ACE could be a reliable and economic way of BVDV testing.

Introduction
Bovine viral diarrhea (BVD) is a viral disease causing high economic losses in the dairy and beef industries worldwide. Estimates of losses because of BVD range between 10 and 40 million US$ per million calvings (Houe, 2003). Others have calculated the annual costs for an average cow herd of 50 being infected with BVDV with 2421 US$ (Chi et al., 2002). Because of the high economic impact of BVDV, several countries have implemented BVDV eradication or control programmes (Greiser-Wilk et al., 2003).

Direct contact with persistently infected (PI) animals is the most important method of transmission of the infection (Houe, 1995). Therefore, identification and elimination of PI animals is important for eradication and control programmes. Some control programmes such as those in Sweden, Norway and Finland rely on surveillance of the BVDV-negative status by screening milk and bulk milk samples for antibodies to BVDV, using an indirect antibody enzyme-linked immunosorbent assay (ELISA) (Niskanen et al., 1991; Niskanen, 1993; Bitsch and Ronsholt, 1995; Lindberg and Alenius, 1999).

Another approach is to test five to 10 animals per cattle herd between 6 and 24 months of age for BVDV antibodies in order to understand if there is a PI animal causing seroconversion in the offspring (Houe et al., 1995). However, BVDV antibody tests can be helpful to determine the BVDV status of cattle herds, but to eliminate the source of infection, the identification of PI animals with BVDV detection assays are necessary.

As BVDV is highly infectious and calves are often traded as early as 2–8 weeks of age, PI calves should be identified and removed as soon as possible. Therefore, a reliable detection of PI in young cattle is extremely important. A concern is the detectability of BVDV antigen in young calves under the influence of collostral antibodies. Colostrum may contain virus neutralizing antibodies with titres higher than 1:500 000. Those antibodies may interfere with BVDV antigen detection (G. Wolf, unpublished observation, 2004).

There are several methods available for the detection of BVDV. For testing whole blood or peripheral blood leucocytes (PBL) in the past often NS2/3 antigen capture ELISA (ACEs) were used (Fenton et al., 1991; Shannon et al., 1991; Gottschalk et al., 1992; Mignon et al., 1992; Frey et al., 1996). Fluorescence-activated cell sorting (FACS) from PBL (G. Wolf and G. Rademacher, unpublished observation, 1992) and virus isolation (VI) (Anonymous, 2004) are commonly used as well. Recently, polymerase chain reaction has been used for BVDV detection in blood, milk, tissue samples and fetal fluids (Radwan et al., 1995; Drew et al., 1999; Renshaw et al., 2000; Stokstad et al., 2003).

New BVDV ACEs detecting E\textsuperscript{rns}, a pestivirus structural protein which is secreted extracellularly during virus replication can be used for testing serum, plasma and whole blood samples (Saliki et al., 1997, 2000). In disease control programmes E\textsuperscript{rns} ACEs allow user friendly and high throughput testing. Enzyme-labelled methods are useful to detect BVDV antigen in tissue sections (Wilhelmsen et al., 1991). For \textit{in vivo} diagnosis of persistent BVDV infection, skin tissue has been shown to be a specimen (Thur et al., 1996; Anonymous, 2004). Therefore, for the detection of BVDV, ear tissue samples are commonly tested in the USA and Canada by immunohistochemistry (IHC) and ACE (Cornish et al., 2005). In Switzerland skin tissue samples are collected by biopsy under local anaesthesia and tested for BVDV using IHC (Thur et al., 1996). For collecting tissue samples, in North America ear notchers are commonly used which cut pieces of ear tissue of about 10 × 5-mm size. Such ear tissue sample sizes collected \textit{in vivo} are not well accepted in many countries for animal welfare reasons. In Europe cattle ear tags with integrated tissue
Testing Ear Tissue Samples for BVDV

Materials and Methods

Ear tissue samples from 19 cattle from Bavaria were collected from cattle submitted for pathological examination to the Department of Pathology of the Bavarian State Laboratory in Oberschleissheim (samples were provided by Dr Schrott). Those cattle were tested persistently BVDV infected by VI from tissue. Eighteen more ear notch tissue samples from cattle previously detected as BVDV positive by FACS in PBL at the Animal Health Service Laboratory in Thuringia were obtained.

Serum and ear notch tissue samples from 11 calves born persistently BVDV infected were collected before colostrum intake and for a period of 5 weeks after (G. Wolf, 2004, unpublished data).

Ear tissue samples were obtained from 82 preselected cattle from Tirol, Austria. Serum samples from 51 cattle were characterized positive and 31 negative previously as part of a BVDV control programme in a commercially available BVDV antigen serum ELISA (Chekit II; Dr Bommeli AG, Bern, Switzerland) at the laboratory AGES, Veterinary Institute, Innsbruck, Austria.

Ear tissue samples from 231 cattle from cattle herds in Thuringia participating in a BVDV control program and being certified BVDV free since 1999 and 207 ear tissue samples from Swedish dairy farms, certified BVDV free by a compulsory BVDV control program were obtained by applying ear tags with attached tissue samplers.

A commercially available ACE, HerdChek BVDV Ag/ Serum Plus (IDEXX Scandinavia, Österbybruk, Sweden) for the detection of E\textsuperscript{HSV} virus protein (E\textsuperscript{HSV} ACE) was used to test serum according to the manufacturer's instructions. Ear tissue samples of 2- to 3-mm diameter were soaked overnight at 2–8°C in 150 µl soaking buffer in vials for immunoanalysis (Sarstedt, Nürenbrecht, Germany). Fifty microlitre of this soaking buffer were used as diagnostic specimen and incubated in the microtitre wells of the assay in a 2 h short-sample incubation protocol or overnight. The remaining soaking buffer was separated and kept frozen. The presence or absence of BVDV antigen was determined by calculating the corrected OD value (S - N). Result interpretation was performed according to the manufacturer's instruction for serum with S - N values ≤0.3 considered negative and S - N >0.3 considered positive. Ear tissue samples with S - N values ≤0.2 were considered negative, S - N values >0.2 but ≤0.3 were considered suspect and >0.3 were positive for BVDV antigen.

Virus isolation was performed as described in the 2004 edition of the OIE Manual for Diagnostic Tests and Vaccines (Anonymous, 2004). Briefly, after an intensive washing separated tissue cells were inoculated in primary fetal bovine lung cells. After 3–5 days these cells were tested by FACS for BVDV.

The FACS for testing BVDV was performed as previously described (Wolf and Rademacher, 1992; Beer et al., 1997).

A commercially available ear tag tissue sampler combination (Typifix; Agrobiogen GmbH, Thalmannsdorf, Germany) was used for collecting the 2- to 3-mm diameter size ear tissue samples (Fig. 1). A small number of 3-mm diameter ear tissue samples were collected using the FlexoPLUS Geno Tissue-Sampling Ear Tags (Caisley, Bocholt, Germany). Both systems have a hollow tip on the male part of the ear tag. By applying the ear tag, a tissue sample is punched into a sampling device, which is attached to the ear tag. A major advantage of those systems is the ear tagging and tissue sampling in one step without contamination of the sample or the sampling tool. The Typifix device preserves the tissue samples by desiccation.

Results

For detectability evaluation, ear tissue samples of 88 BVDV-positive cattle from Bavaria, Thuringia and Tirol were obtained using the Typifix ear tag tissue sampler and tested in the E\textsuperscript{HSV} ACE in the short-sample incubation protocol of the assay. All samples were detected positive. The S - N values were between 0.7 and 4.0 with a mean of 2.4 (Fig. 2).

For assay specificity evaluation 469 BVDV-negative samples were tested in the E\textsuperscript{HSV} ACE in the short-sample incubation protocol. A total of 467 samples were negative, one sample reacted suspect and one sample was strong positive. As the two cows with the non-negative results were slaughtered already, retesting of these samples was not possible.

The mean S - N value of the negative population was 0.02 ± 0.007. This population mean was four times the calculated SD value from the positive cut off threshold of 0.3 (Fig. 3).

A total of 113 samples were tested in the overnight sample incubation protocol of the test. All samples were detected negative as well yielding a mean S - N value of 0.011 ± 0.006. This population mean was 48 times the calculated SD value from the positive cut off (Fig. 3).

![Fig. 1. Ear tissue sample in sampling device (Typifix). The sampling device is attached to the ear tag. During ear tagging a small tissue plug is punched into the sampling device.](image-url)
Detection of BVDV from ear tissue samples from young calves

To test the influence of maternal antibodies on the detection of BVDV antigen from ear tissue samples, 11 PI calves were kept at loose house facilities and tested for BVDV in serum and ear tissue before receiving colostrum and up to 5 weeks after. Ear tissue samples were collected using Typifix ear tag tissue samplers, stored at room temperature, and tested around day 7 after sampling.

Testing soaking buffer from ear tissue samples of those calves in the Erns ACE all samples showed some influence of colostral antibodies, but were detected clearly positive on all collection dates (Fig. 4).

Serum samples of those calves were collected on days 1 or 2, respectively, on day 4 and 35 post-partum after colostrum intake and tested in the Erns ACE. After intake of colostrum all serum samples were tested negative in the Erns ACE, 1 or 2 days post-partum. At day 4 serum samples of four calves could be detected BVDV-positive but serum samples from seven of the 11 PI calves were negative. At day 35 serum samples from all calves were detected positive (data not shown).

Reproducibility

From ear tissue of eight cattle up to 10 samples were collected from each animal using the Typifix ear tag tissue sampler. In the short-sample incubation protocol of the Erns ACE the samples of seven animals showed a very high degree of reproducibility with 0.06–0.12 SD. Only one sample showed a higher SD of 0.53. One sample reacted low positive twice and one was suspected (SD 0.12). This sample and four more were tested in the overnight assay incubation protocol. While no difference in signal was found for the medium and strong-positive samples, the weak-positive samples revealed a higher signal (SD 0.04 and 0.33; Fig. 5).

Results testing ear tissue samples obtained with devices from different suppliers

Samples from 13 cattle with a BVDV PI status characterized previously were obtained using two commercially available ear tag tissue sampler combinations (Typifix and FlexoPLUS) and incubated in soaking buffer. The samples were tested 1 day after sampling in the short and in the overnight sample
incubation protocol of the assay. All positive samples were detected positive from both devices and both sample incubation protocols. One sample reacted stronger positive in the overnight sample incubation protocol (Fig. 6).

Discussion

The commercially available ACE for detection of the Eems glycoprotein used in this study demonstrates high sensitivity and specificity to detect BVDV in serum and blood samples and a broad detection of BVDV I and II strains (R. Toomilk, G. Holmquist, C. Schroeder and A. Ballagi, unpublished data, 2001). We validated this ACE for ear tissue samples using characterized samples from Bavaria, Thuringia, Austria and Sweden.

Testing ear tissue samples from negative-cattle populations a narrow distribution of negative test results and the high specificity of this method could be demonstrated. The population mean was four times the SD value from the positive cut off in the short-sample incubation protocol and 48 times in the overnight sample incubation protocol of the test. However, in the overnight test protocol a subset of samples were tested by an experienced laboratory. The short-sample incubation protocol summarizes data from different negative populations tested in different laboratories.

A summary of all samples tested are shown in Table 1. All 99 ear tissue samples positive in one of the reference methods were detected correctly in the Eems ACE (88 positive samples and 11 samples from PI calves). A total of 467 of the 469 negative samples were tested negative in the Eems ACE. One sample reacted suspect and one positive. Those samples derived from cattle with a BVDV status determined with serum in another commercially available BVDV antigen capture ELISA (Chekit II). Retesting of the two cattle, reacting suspect and positive on ear tissue was not possible because those animals were removed from the herd. Therefore, the true status of those cattle could not be confirmed. Based on those samples, the Eems ACE ear tissue testing protocol demonstrated a sensitivity of 100% and a specificity of ≥99.6%. In serum samples collected from PI calves after receiving colostrum and tested with the Eems ACE colostral antibodies interfered with BVDV antigen detection. Testing ear tissue samples from these calves in the Eems ACE, S–N values decreased in the first 2 weeks after colostrum intake compared with pre-colostral values but PI calves could always be detected positive.

Other authors reported a minor influence of colostral antibodies on BVDV Ag detection from ear notch tissue samples compared with blood samples too (E. J. Dubovi, personal communication, Braun et al., 1999; Brodersen, 2004; C. Schroeder, H. Nieper, C. Veit, W. Obritzhauser, G. Obritzhauser, S. Kuhne, G. Brem and F. Ehrensperger, unpublished observation, 2005). This could potentially allow to test calves of any age, regardless, if they received passive antibodies to BVDV via colostrum. Saliki and Dubovi (2004) recommended IHC or ACE from ear notch tissue samples to test cattle of all age for BVDV. However, further studies with different BVDV strains are necessary to determine if testing ear tissue samples for BVDV is a reliable method to detect PI cattle under influence of colostral antibodies.

Testing multiple samples from the same ear of PI animals in the Eems ACE yielded strong positive results and low variation. Only one sample yielded a somewhat higher degree of
Table 1. Summary comparing results obtained with Eiren ACE ear tissue samples versus other bovine viral diarrhoea virus antigen detection methods performed from blood or tissue samples

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<tr>
<th>Reference methods</th>
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variation. Ear tissue samples of one PI animal reacted weak positive twice and suspect once. Poor sample quality could be a possible reason, as some ears were derived from cattle that had died in the field and were sent for necropsy. Some cattle ears where kept at room temperature unprepared for up to 1 week before stored frozen. The weak-positive ear tissue sample revealed a higher signal in the overnight sample incubation protocol of the test.

Most samples for this study were obtained using the Typifix ear tag tissue sampler combination. The Typifix device is cutting and punching the samples easily and contains a desiccant which helps to preserve the tissue sample in the sampling device for 2 weeks and longer at room temperature (data not shown). Comparing results from ear tissue samples from cattle with characterized PI status obtained with Typifix and FlexoPLUS Geno ear tags, the results were very similar. Also here, the overnight sample incubation protocol showed a stronger signal for weak-positive samples. The overnight sample incubation protocol had no impact on negative samples. Therefore, suspected samples are recommended to be retested in the overnight sample incubation protocol of the assay so as to obtain most sensitive results.

After soaking ear tissue samples in ELISA buffer DNA can still be isolated from those samples and can be used for genetic analysis like marker assisted selection and traceability testing (C. Schroeder et al., unpublished observation, 2005).

The development of modern technology, combining ear tag application with sampling of an 2- to 3-mm size ear tissue plug and testing in a highly sensitive ELISA system for BVDV detection could be a reliable and economic way of BVDV eradication. The advantage of such combined ear tag tissue sampler systems is a simple and inexpensive way of obtaining diagnostic specimen. The labelled sampling device, attached to the ear tag helps sample traceability and minimize sample mix up. As the puncher does not get in contact with the tissue itself, there is no contamination of the sampling tool or samples. Collecting 2- to 3-mm diameter ear tissue samples while applying the ear tag should comply with animal welfare regulations in Europe.

References


viremic with bovine viral diarrhoea virus – a comparison with BVD virus isolation from buffy coat cells in bovine kidney cells. Zentralbl. Veterinarmed. B 39, 467–472.


