Evaluation of diagnostic methods to distinguish between calves persistently and transiently infected with bovine viral diarrhoea virus in respect to the presence of maternal antibodies

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Abstract

Two issues concerning virus detection and identification of persistently infected (PI) cattle were analysed in the study: 1) interference by maternal antibodies and 2) discrimination between PI and transiently infected (TI) animals. Antigen ELISA and RT-PCR based methods were compared using serum samples from natural and experimental PI and TI calves. RT-PCR and real-time RT-PCR using primers within 5'UTR region were more sensitive in detecting PI animals than E\textsuperscript{NS1} and NS3 antigen capture ELISAs, and they were not influenced by the presence of colostral antibodies in serum or by bovine viral diarrhoea virus genotype. The serum samples with Ct values ≤ 29.10 (corresponding to 10\textsuperscript{4.87} viral RNA copies/µL) identified PI animals with 100% probability, while all samples with Ct values > 32.06 (corresponding to viral RNA load below 10\textsuperscript{4} copies/µL) indicated TI status. The samples with Ct values between 29.10 and 32.06 (17.2% of PI and 11.5% of TI) should be considered as PI suspect and retested.

Key words: cattle, bovine viral diarrhoea virus, persistent infection, transient infection, ELISA, RT-PCR.

Introduction

Bovine viral diarrhoea virus (BVDV), a ruminant pestivirus within the Flaviviridae family, is one of the most important pathogens of cattle with significant economic impact on dairy and beef production worldwide (12). BVDV infection can be manifested by a variety of clinical signs including gastrointestinal disorders, respiratory problems, reduced milk production, mucosal disease, and immunosuppression leading to secondary infections (2). BVDV can spread horizontally, usually by a direct contact with persistently infected (PI) animals. The acute or transient infection, which is usually subclinical, lasts up to two weeks before the animal clears out the virus and becomes immune for many months or even for a life (Fig. 1A). Vertical infection of the foetus during the early pregnancy, before it reaches immunocompetency may result in persistent infection of newborn calves (5). PI animals shed virus in high concentrations throughout their entire life without mounting an antibody response to the infecting virus (22). Most PI animals develop mucosal disease after superinfection with a homologous cytopathic BVDV strain and die within 24 months of age (6). Successful eradication requires the detection and elimination of persistently infected animals (PIs) prior to breeding season (28). Currently, methods for detecting PI animals include antigen capture ELISAs and RT-PCR on pooled samples, followed by testing of individual samples using an antigen capture ELISA (9). However, this strategy has been shown to be not completely reliable for the identification of PI calves (14). The presence of colostral antibodies in serum samples from young animals (Fig. 1B) can interfere with the antigen capture ELISA and may lead to false negative results (4, 11, 18, 28). Ear-notch samples instead of serum have been proposed as samples for the antigen capture ELISA to avoid false negative results in seropositive PI animals (8). In contrast, the RT-PCR assay is not influenced by the presence of maternal antibodies but has the disadvantage of being more expensive and laborious.
monitoring and were confirmed negative 3 weeks later on the second testing by real-time RT-PCR test confirming the TI status. The remaining 43 real-time RT-PCR positive serum samples came from 3 to 5 months old calves experimentally infected with BVDV-1a as described previously (17). Samples were collected between 2nd and 28th d post-infection. Serum samples from 50 animals free of BVDV infection and antibodies were used for the specificity evaluation.

**RNA extraction and conventional RT-PCR.**

Total RNA was extracted from 500 μL serum samples using TRI Reagent (Sigma-Aldrich, USA), according to the manufacturer’s instructions. The RNA was eluted in 20 μL of DEPC water and stored at −70°C until testing. RT-PCR was carried out using a commercial kit (Titan One Tube RT–PCR System, Roche Biochemicals, Germany) following the manufacturer’s protocol and panpestivirus primers 324F/326R flanking the 5’ untranslated region (5’UTR) were used (27). The final volume of RT-PCR mixture was 25 μL comprising: 14 μL of RNase-free water; 5 μL of reaction buffer; 0.5 μL of dNTP mixture (10 mM); 1 μL of each primer (10 μM); 1.25 μL of DTT solution (100 mM); 0.5 μL of enzyme mix, and 2 μL of RNA template. Amplification was performed in 40 cycles of denaturation at 94°C for 15 s, annealing at 53°C for 30 s, and extension at 68°C for 30 s. The reaction products were evaluated in ethidium bromide-stained 1.5% agarose gel and the size of the amplicons was compared with the molecular weight standard.

**Real-time RT-PCR.**

In-house real-time RT-PCR was performed using AgPath-ID kit reagents (Life Technologies, USA) according to the manufacturer’s protocol in the StepOnePlus real-time PCR system (Life Technologies, Republic of Singapore) with primers and probes designed by Baxi et al. (3). The primers: Pesti-F (5’-CTAGCCATGCCCCGTTAG-3’) and Pesti-R (5’-CGTCGAACCTAGTGACGACT-3’) allow the detection of both genotypes of BVDV, namely BVDV-1 and BVDV-2, while the probes BVDV-1: 5’TAGCAGAGTTGGAATTTATGAGGC-3’ and BVDV-2: 5’TAGCAGGATGAAGTGTGCTG-3’ are genotype-specific. The probes were produced and labelled with fluorescent reporter FAM and VIC at the 5’ ends and a quencher BHQ-1 at the 3’ ends (Metabion International, Germany). Ten-fold serial dilutions of in vitro transcribed 5’UTR RNA from Polish BVDV-1d isolate, cloned in the plasmid were included as the quantity standards as described previously (16, 17).

**Antigen ELISA.**

The presence of BVDV antigen was tested with two enzyme-linked immunosorbent assay (ELISA) kits. EIA-based Ag ELISA (HerdChek Ag/ Serum Plus test kit, IDEXX, Switzerland) was used for the detection of BVDV specific antigen. All samples with optical density (O.D.) values equal to, or above 0.3 were considered positive according to the manufacturer’s guidelines. The second antigen ELISA was NS3-based Ag ELISA (SERELISA BVD p80 Ag

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**Material and Methods**

**Persistently and transiently infected calves.**

Serum samples were collected from 29 calves 6 month aged from 12 different herds in Poland. Persistent infection of these animals with BVDV was confirmed by two consecutive positive results using conventional RT-PCR assay performed on serum samples collected 2–3 weeks apart. Samples from the first collection were used. Additionally, serum samples from 52 animals transiently infected with BVDV were used. Nine of these samples were collected during the herd

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**Fig. 1.** Persistent (A) and transient (acute) (B) BVDV infections presented schematically

The purpose of the study was to compare and to evaluate the reliability of commonly used diagnostic methods to detect BVDV PI cattle in the presence of maternal antibodies. The reliability of selected diagnostic methods was evaluated in order to reduce the risk of spreading the virus in the herd through the effective detection of PI animals at an early age, thus avoiding the diagnostic gap due to the presence of maternal antibodies.
Mono Indirect, SYNBIOTIC, France) used for the detection of BVDV p80 (non-structural NS3 protein). The test was performed with ten-fold diluted serum samples. The samples with O.D. values above cut-off values corresponding to ≥70%, and ≤40% competition of the positive control O.D. value were considered positive and negative, respectively.

**Antibody ELISA.** A commercial indirect ELISA was used for the detection of BVDV specific E\textsuperscript{ns}–antibodies (HerdCheck BVDV Ab, IDEXX, Switzerland). The 10-fold prediluted serum samples were tested and those with corrected O.D. values ≥0.3 were classified as positive. In a blocking ELISA referred to as NS3 Ab ELISA (SERELISA BVD p80 Ab Mono Blocking, SYNBIOTICS, France), the samples with O.D. values corresponding to ≥50%, and <30% competition of the positive control O.D. value were considered positive and negative, respectively. All ELISA tests were performed according to the protocols provided by the manufacturers and the O.D. values were read at 450 nm wavelength.

**Virus neutralisation test (VNT).** Two-fold serial dilutions (from 1:5 up to 1:320) of serum samples heat-inactivated at 57°C for 30 min were loaded in duplicate rows of 96-well microtiter plates. One hundred to 300 TCID\textsubscript{50} of cytopathic BVDV-1a Singer strain and 50 μL of media was added to each well and the plates were incubated for 1 h at 37°C. One hundred microlitres of bovine turbinate (BT) cells suspension (3 × 10\textsuperscript{3}/mL) were added and the plates were incubated for 4–5 d at 37°C in an atmosphere of 5% CO\textsubscript{2}. The cells were observed for cytopathic effect (CPE) and the antibody titres were determined as the reciprocal of the highest serum dilution, which neutralised the virus in at least 50% of wells (1 well).

**Virus genotyping.** The 5′UTR amplicons were purified and sequenced in both directions using the same primers as for the conventional RT-PCR using Big Dye Terminator v3.1 Cycle Sequencing kit (Life Technologies, Canada) on a 3730XL Genetic Analyzer (Applied Biosystems, USA). The alignments were performed using ClustalW from MEGA software version 5.05 (the Biodense Institute, USA) with reference sequences of BVDV-1 subgenotypes retrieved from GenBank as described elsewhere (15).

**Statistical analysis.** The distribution of continuous variables was assessed by the normality and probability plots as well as the Shapiro-Wilk test. Continuous variables were presented as mean or median with the 95% interquartile range and dichotomous variables as percentages. Comparison between the groups was performed by unpaired t or Mann-Whitney tests. Continuous variables among the two groups (PI and TI) in correlation to the presence or the absence of detectable antibodies were compared by Anova or Kruskal-Wallis test, when appropriate. Categorical variables were compared using the chi square test. Correlations were analysed by Pearson or Spearman test, when appropriate. A two-tailed P-value < 0.05 was considered as the level of statistical significance. Receiver operating characteristic (ROC) analysis was used to calculate the optimal cut-off for the real-time RT-PCR and for antigen ELISA that would be able to distinguish between PI and TI animals. The PI/TI status provided reference variable, while Ct value, copy number, and E\textsuperscript{ns} Ag ELISA O.D. value were considered as classification variables. Persistent infection variable (PI = 1, TI = 0) was considered the reference variable, while Ct value, log viral RNA copy number, and Ag ELISA O.D. value were the classification variables. The areas under curve (AUC), which indicated the probability of correct random ranking of positive sample by the classifier were presented below the graph. ROC curves were created by plotting the fraction of true positives out of all positives (specificity) versus the fraction of false positives out of the true negatives (specificity), at various threshold settings. Statistical analyses were performed using STATA v.11 (Stata Corp., USA).

**Results**

**Distinction between PI and TI calves.** The Ct values and corresponding viral loads expressed as the copy number per microlitre (calculated using BVDV-1d standard) established for PI and TI groups differed significantly (P < 0.0001) (Table 1). The means and confidence intervals presented in Table 1 refer to the values obtained for the samples positive in the corresponding test. The percentage of positive and O.D. values in E\textsuperscript{ns} Ag ELISA was also significantly different (P = 0.001) between the two groups. The E\textsuperscript{ns} Ag ELISA detected 26 out of 29 PI animals, while only one PI animal was detected by NS3 Ag ELISA. Transient BVDV infection was also detected in over a half of calves using E\textsuperscript{ns} Ag ELISA. However, the O.D. values of this ELISA were significantly related (P = 0.004) to the type of infection (persistent vs. transient).

NS3 Ab ELISA failed to identify 87.5% (14/16) and 25% (3/12) of antibodies found by E\textsuperscript{ns} Ab ELISA in PI and TI animals, respectively. No correlation between seropositivity and the age of PI animals was found (P = 0.11), although seropositive PIIs constituted 100% of calves under the age of one month and 57% of calves below the age of 6 months. Neutralising antibodies were detected only in one PI animal with the titre of 40. On the other hand, VNT showed higher sensitivity in detecting antibodies in TI animals when compared with E\textsuperscript{ns} and NS3 Ab ELISA results (16 positive samples versus 12 and 9, respectively). Therefore, the differences observed for two groups in VNT results were statistically significant (P = 0.004).

Influence of the presence of ELISA detectable antibodies on the Ct values and virus load in the real-time RT-PCR, with respect to the type of infection is presented in Figs 2A and 2B. Data is presented as box plots with medians and interquartile range.
Table 1. Positive results for serum samples collected from BVDV PI and TI cattle using different diagnostic methods

<table>
<thead>
<tr>
<th>Method</th>
<th>PI</th>
<th>TI</th>
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<tbody>
<tr>
<td></td>
<td>29/29 (100%)</td>
<td>nd</td>
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<tr>
<td><strong>RT-PCR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Mean Ct value (95% CI)</td>
<td>29/29 (100%)</td>
<td>52/52 (100%)</td>
</tr>
<tr>
<td>• Mean log copy number/μL</td>
<td>26.70 (25.65–27.74)</td>
<td>35.44 (34.76–36.11)</td>
</tr>
<tr>
<td>• Mean Ct value (95% CI)</td>
<td>5.59 (5.27–5.90)</td>
<td>2.97 (2.77–3.18)</td>
</tr>
<tr>
<td><strong>E™ Ag ELISA</strong></td>
<td>26/29 (89.7%)</td>
<td>28/52 (53.8%)</td>
</tr>
<tr>
<td>• Mean O.D. (95% CI)</td>
<td>3.07 (2.63–3.52)</td>
<td>0.77 (0.48–1.05)</td>
</tr>
<tr>
<td><strong>NS3 Ag ELISA</strong></td>
<td>1/29 (3.4%)</td>
<td>nd</td>
</tr>
<tr>
<td><strong>E™ Ab ELISA</strong></td>
<td>16/29 (55.2%)</td>
<td>12/52 (23.1%)</td>
</tr>
<tr>
<td>• Mean O.D. (95%)</td>
<td>0.55 (0.36–0.74)</td>
<td>0.23 (0.11–0.34)</td>
</tr>
<tr>
<td><strong>NS3 Ab ELISA</strong></td>
<td>2/29 (6.9%)</td>
<td>9/52 (17.3%)</td>
</tr>
<tr>
<td><strong>VNT</strong></td>
<td>1/29 (3.4%)</td>
<td>16/52 (28.9%)</td>
</tr>
<tr>
<td>• Mean titre (95% CI)</td>
<td>40</td>
<td>98.88 (24.2–173.6)</td>
</tr>
</tbody>
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*conventional RT-PCR using the 5'UTR primers; †not done; ‡confidence interval

Statistically significant differences are indicated by brackets and corresponding P-values are included.

No differences of Ct values and virus load were found between seropositive and seronegative PI animals (P = 0.45); whereas, the difference between the mean Ct value for seronegative TI (35.13; 95% CI: 34.31–35.96) and seropositive TI animals (36.44; 95% CI: 35.50–37.11) was statistically significant (P = 0.0291), and the variance between the means was below zero (P = 0.05). The viral load correlated with the presence or absence of antibodies in TI animals; however, the relationship was not strong (P = 0.03). The antigen ELISA results were antibody-independent for PI animals, while the difference in the variances of seronegative and seropositive TI animals was highly significant (P = 0.002) (Fig. 2C). Highly significant differences were observed between seropositive and seronegative PI and TI animals (P < 0.00001) when all the methods directed at virus detection were compared.

The agreement between real-time RT-PCR results and E™ Ag ELISA was only 53.8%. However, PI animals were detected by E™ Ag ELISA (89.7%) more frequently than TIs (53.85%). The O.D. values were independent from the Ct values and viral copy numbers (Spearman $\rho_s = -0.4$ and $\rho_s = 0.4$; P = 0.002).

**Influence of BVDV subtype on the PI detection.**

Nucleotide sequencing of amplified 5'UTR fragment of viruses detected in PI animals resulted in approximately 288 nucleotide long sequences, which were genotyped by comparison to the reference sequences retrieved from GenBank (NCBI, USA). All viruses represented BVDV type 1 and were subgrouped into BVDV-1b (n = 11), BVDV-1d (n = 9) and BVDV-1f (n = 9) subtypes. Two out of nine samples collected from field TI cases were successfully sequenced and the viruses were classified as BVDV-1d and BVDV-1f subtypes. No correlations between Ct ($\rho_s = 0.32; P = 0.1$), viral load values ($\rho_s = -0.33; P = 0.09$), and BVDV subtypes were found.

Fig. 2. Threshold cycle (Ct) values in real-time RT-PCR (A), viral load expressed as log value of number of copies/μL calculated using BVDV-1 RNA standard (B) and BVDV antigen ELISA O.D. values (C) in serum samples collected from PI and TI cattle in respect to the antibody absence (Ab-) or antibody presence (Ab+)
Similarly, O.D. values in E\textsuperscript{M} Ag ELISA ($\rho_S=0.34$; $P = 0.53$) and in E\textsuperscript{m} Ab ELISA ($\rho_S = 0.44$; $P = 0.1$) were independent from virus genotype.

![Fig. 3. Receiver operating characteristic (ROC) curves illustrating the variation in the sensitivity and specificity of real-time RT-PCR and antigen ELISA with the different threshold values for the discrimination between PI and TI.

Optimal cut off to detect PI animals. Real-time RT-PCR, with the area under the ROC curve (AUC) for the Ct values and viral RNA copies/μL equal to 0.995, gave the highest probability of classifying a PI animal correctly, while AUC for E\textsuperscript{m} Ag ELISA OD values was 0.91 (Fig. 3). The optimal Ct cut off value for PI/TI differentiation by real-time RT-PCR established by ROC analysis Ct ranged between 29.01 and 32.06 enabling the detection of single PI animals with the sensitivity and the specificity ranging between 82.8%–100%, and 100%–88.5%, respectively. The cut off value of Ct, which was considered the best, detecting 96.3% of PI animals, was 30.68, with 93.1% sensitivity and 98.1% specificity. For the future interpretations of real-time RT-PCR results, the serum samples with the Ct values ≤ 29.10 ($n_{PI} = 24$) would most likely (AUC = 1.0) indicate PI animals, the samples with Ct values > 29.10 and ≤ 32.05 ($n_{PI} = 5$; $n_{TI} = 6$) should be considered suspect, while all samples with Ct values > 32.06 ($n_{TI} = 46$) would most probably indicate TI animals. Such thresholds enabled the detection of 82.8% of PIs at the first testing, while 17.2% of PI animals with doubtful results required retesting. The cut off value set at 10\textsuperscript{4} copies of viral RNA per 1 μL was found to be optimal in order to detect 100% PI with 88.5% specificity. If the objective was to distinguish PIs and TIs, and to decrease the number of TI animals misdiagnosed as PIs, a ‘grey zone’ category for suspects would be necessary. Therefore, the number of viral copies/μL ≥ 10\textsuperscript{4.87} (74,943 copies/μL) should be interpreted as PI infection, the values ≥ 10\textsuperscript{3} and < 10\textsuperscript{4.87} as suspect, and all samples containing less than 10\textsuperscript{4} copies of viral RNA/μL would suggest TI animals. The E\textsuperscript{m} Ag ELISA was found suitable to distinguish between PI and TI animals with the optimal O.D. cut off value of 3.11, which enabled correct classification of 92.2% samples and 88% sensitivity and 94.2% specificity with respect to PI detection. Independently to the ROC optimised cut off value and the presence of antibodies, the sensitivity of PI detection did not reach 100%.

Discussion

Eradication of bovine viral diarrhoea virus is based on the identification and elimination of PI animals. The rapid identification and removal of such animals minimises the risk of BVDV transmission to the herdmates, particularly pregnant females. The loads of virus shed by the PIs into the environment are so high that even short contact with such animals may lead to infection in the herd (20). BVDV persistent infection is observed in 1%–2% of cattle population (13, 18, 19, 24). In Poland, prevalence of PI animals is similar to other geographic regions of Europe. In the late 1990s*, 0.9% of bulls in artificial insemination centres in Poland were persistently infected with BVDV (23), while at the moment all the bulls at the stations are free from the virus after introducing the compulsory testing.

A failure to detect PI animals may have serious consequences for the health of the cattle and epidemiological status of the herd. The BVDV infections cause also socio-economical problems due to the financial losses and reduced trust of farmers in veterinary service. The importance of reducing the risk of false positive PIs was reviewed extensively by Laureyns et al. (18). Most populations with endemic BVDV infections are characterised by a high seroprevalence, which is reflected by the presence of virus specific antibodies in thecolostrum transferred to the newborns after parturition. Maternal antibodies can mask the presence of the virus in the blood of persistently infected individuals (1, 4, 7) therefore, until recently, BVDV testing was performed in calves after maternal antibodies declined, usually in animals older than 3 months (28). The antibody titre depends on the concentration of antibodies incolostrum and thecolostrum intake by calves. While maternal antibodies would disappear from most of young calves by 90 d of age, in some cases antibodies detectable by Ab ELISA and VNT are still present even one year after birth (7, 11). BVDV antibodies are detected in blood of the newborns even before thecolostrum intake, despite the fact that such particles do not technically penetrate bovine placenta (11). Ear notch samples collected during calf tagging were recently evaluated as an alternative to blood or serum sampling. However, this method, successful in some countries (25), failed to work in others due to a lack of sensitivity of the test used (8, 11) or the errors in the sample collection and labelling. The antigen capture ELISA, may fail to detect PI animals in ear notch samples, and the age of calves tested by this method is advised to be one month of age. The possibility of even further simplifying
BVDV PI identification by testing hair samples was recently explored; however, the results varied with respect to the number of hair tested (25). The development of a test, which identifies PI animals regardless of the presence of maternal antibodies, and transient infection in the herd should be a priority when planning BVDV eradication programme. In the study, the impact of passive immunity on PI detection by E\textsuperscript{rns} Ag ELISA was evident, and it has been also observed by others (11). Nevertheless, the superiority of the methods based on RT-PCR detection of viral RNA was confirmed (10, 14). Using RT-PCR in both conventional and real-time formats allowed the detection of BVDV PI animals regardless of age, including antigen capture ELISA test negative animals. Distinction between PI and TI animals was also possible by real-time RT-PCR using cut-off Ct values, and it eliminated the need for retesting the animal. The number of animals, which needed retesting was limited to 17.2% of PI and 11.5% of TIs, which gave Ct values between 29.10 and 32.06 corresponding to 10\textsuperscript{10} and 10\textsuperscript{8.7} copies/μL. Similar observation was made recently by Belgian researchers (13). Their study, however, did not include measurement of the impact of colostral antibodies. In countries like Poland where the BVDV seroprevalence is high (23), and the cattle production is still rather extensive or semi-industrial, maternal immunity is an important consideration when establishing a testing method for BVDV control programmes. Our results confirm also the finding of Zimmer et al. (28), who demonstrated that conventional RT-PCR identifies PI animals more accurately than antigen capture ELISAs in the presence of antibodies circulating in the blood of a PI calf. They, however, encountered the problem of false positive results because of the inability to differentiate persistent from acute BVDV infection.

High sensitivity of real-time RT-PCR (16) allows the testing of pooled samples (13) while reducing the costs of testing with respect to Ag ELISA on an individual sample basis (21). However, problems such as sample quality, cross-contamination (13), sensitivity and specificity of the tests (11), and the need for validation of the methods under individual laboratory conditions should be also considered when selecting RT-PCR-based method.

In conclusion, the eradication of BVDV from the areas of high prevalence demands efficient removal of PI animals as the main reservoirs of the pathogen. In the study, viral RNA detection methods showed superiority in identifying PI animals in the presence of maternal antibodies. Quantitative results of real-time RT-PCR enabled distinction between PI and TI animals by implementing optimised cut off values.

References


