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Comparison of bovine viral diarrhea virus detection methods: Results of an international proficiency trial

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ABSTRACT

Bovine viral diarrhea virus (BVDV), one of the most important infectious cattle diseases globally, is being combated in multiple countries. The main source for virus transmission within herds and especially to unaffected cattle farms are life-long persistently infected (PI), immunotolerant animals. Therefore, the early identification of PI calves is a major pillar of disease control programs. In addition, rapid and reliable virus identification is necessary to confirm the causative agent in acute clinical cases. Here, we initiated an international interlaboratory proficiency trial in order to evaluate BVDV detection methods.

Four ear notch samples and four sera were provided to the participating veterinary diagnostic laboratories (n = 40). Two of the ear notches and two sera contained BVDV and two ear notches and one serum were negative for pestiviruses. The remaining serum was positive for the ovine border disease virus (BDV). The sample panel was analyzed by an E^{RNS}-based ELISA for antigen detection, diverse real-time RT-PCR (RT-qPCR) assays and/or virus isolation. Occasionally, additional typing of the virus strains was performed by sequencing or specific antibody staining of the obtained cell culture isolates. While the antigen ELISA allowed reliable BVDV diagnostics, infectious virus could be isolated only in just under half of the attempts (43.33%). RT-qPCR enabled the sensitive detection of pestiviruses, though an impact of the extraction method on the resulting quantification cycle values was observed. In general, subsequent typing of the detected virus strains is required to differentiate BVDV from BDV infections.

In conclusion, for BVDV identification in clinical cases or in the context of disease control, RT-qPCR methods or E^{RNS} antigen ELISAs should be preferentially used.

1. Introduction

Bovine viral diarrhea virus (BVDV) is a highly contagious and economically significant pathogen that poses a substantial threat to cattle populations around the world. It belongs to the genus *Pestivirus* within the family *Flaviviridae* and exists in the distinct species *Pestivirus bovis* (commonly known as BVDV-1), *Pestivirus tauri* (BVDV-2) and *Pestivirus brazilense* (BVDV-3 or HoBi-like pestivirus) (ICTV, 2023; Postler et al., 2023). Although BVDV is predominantly a pathogen of cattle, interspecies transmission to sheep can occur. In addition, infections of cattle with another representative of classical pestiviruses, the ovine border disease virus (BDV), have been described (Braun et al., 2019).

BVDV infections in cattle can lead to a wide range of clinical manifestations from mild or subclinical forms to severe and life-threatening diseases (Lanyon et al., 2014). Clinical presentations of BDV infections are indistinguishable from those induced by BVDV (Braun et al., 2019). The complex nature of BVDV, with its ability to establish life-long persistent infections when fetuses are infected in a critical phase during their development (Brock, 2003), makes accurate and timely diagnosis crucial for the management and control of the disease. BVDV diagnostics might be further complicated by a considerable biological and antigenic diversity of the virus strains, necessitating the development of accurate, reliable and broad-range diagnostic methods. Besides nasal and oral swabs from clinically diseased animals, the most important sample matrices for BVDV diagnostics are blood samples and ear tissue. The latter proved especially beneficial in eradication programs, which have been implemented in several countries worldwide (Moennig and Becher, 2018). The centerpiece of the so-called “Swiss approach” of BVD control is the detection of persistently infected (PI) animals as early as possible by testing every newborn calf during the first days of life. The vast majority of calves are tested through ear notch samples taken during the tagging process (Schweizer et al., 2021; Wernike et al.,

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2017a). Therefore, ear notch and serum samples were selected as matrices for the interlaboratory comparison of BVDV detection systems described hereinafter.

2. Methods

The proficiency trial sample panel that was sent to each participating laboratory consisted of four ear notch samples and four sera. The ear notches were obtained from PI calves euthanized according to the German BVD regulation (sample IDs BVD-A-11/23 and BVD-A-13/23) or from BVDV negative cattle (BVD-A-12/23 and BVD-A-14/23). The sample BVD-A-11/23 contained a BVDV subtype 1b strain and the sample BVD-A-13/23 a BVDV-1d strain (subtyping based on 5′ UTR sequencing (Wernike et al., 2017b)). The samples were provided in duplicate, one ear notch in a collection tube of a Caisley Tissue sampling Cattle Tag (Caisley International GmbH, Bocholt, Germany) and the second ear notch in a collection tube of an Allflex Tissue Sampling Tag (Allflex Group Germany GmbH, Bad Bentheim, Germany). In addition, a serum containing BVDV-1d (BVD-A-15/23), a serum with BVDV-2c (BVD-A-18/23), a BDV-positive serum (BVD-A-16/23) and a pestivirus-negative cattle serum (BVD-A-17/23) were provided.

The participants were asked to analyze the samples with the methods and test systems routinely used in their laboratory. A total of 40 veterinary diagnostic laboratories from 10 countries (Austria, Denmark, France, Finland, Germany, Ireland, Italy, Lithuania, the Netherlands, Switzerland) participated. They investigated the samples by ELISA for antigen detection, real-time RT-PCR (RT-qPCR), virus isolation and/or typing by sequencing of the original sample or staining of the obtained cell culture isolates.

The sensitivities and specificities as mentioned in the Results section were calculated by using the free statistical calculator MedCalc (MedCalc Software, Ostend, Belgium).

3. Results

3.1. Reliable detection of BVDV by E^{RNS}-based antigen ELISA

The proficiency trial sample panel was analyzed in 29 approaches in 28 laboratories by a detection system for viral antigen. The ear notch samples were tested 23 times and the sera 29 times and, in all cases, the E^{RNS}-based IDEXX BVDV Ag/Serum Plus Test (IDEXX, Liebefeld, Switzerland) was applied. In one laboratory, both sample incubation protocols included in the manufacturer's instructions were used, i.e. the short and the overnight incubation protocol. No other antigen ELISA system was used.

The status of the pestivirus-negative and of the BVDV-positive samples was consistently identified correctly by the antigen ELISA by all participants that applied this test (Fig. 1). The BDV-positive serum (BVD-A-16/23) was assessed inconclusive once and negative in the remaining 28 approaches. When considering "inconclusive" as "negative" for the calculation of the diagnostic specificity, an overall value of 100.00% (95% confidence interval [CI]: 96.52% to 100.00%) results for the sensitivity and specificity, respectively. When considering "inconclusive" as "positive", values of 100.00% (95% CI: 96.52% to 100.00%) and 99.04% (95% CI: 94.76% to 99.98%) were calculated for the sensitivity and specificity of the ELISA-based BVDV detection, respectively.

3.2. Commercial RT-qPCR kits allow for the sensitive detection of BVDV and BDV genomes

Alternatively, or in addition to the E^{RNS}-based antigen ELISA, RT-qPCRs were performed by 39 participants. To be used for PCR analysis, viral RNA was either extracted using diverse commercially available purification kits or, for the ear notch samples, direct lysis buffers (ADIAPURE™ TLB [Bio-X Diagnostics S.A., Rochefort, Belgium], virotype Tissue Lysis Reagent [INDICAL BIOSCIENCE GmbH, Leipzig, Germany], Direct lysis buffer [Innovative Diagnostics, Grabels, France], or not further specified lysis buffers).

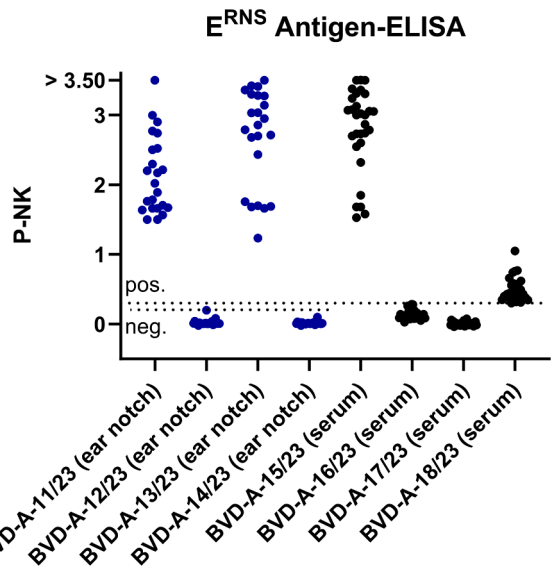

Fig. 1. Results of a commercial BVDV E^{RNS} antigen ELISA (IDEXX BVDV Ag/Serum Plus Test). The corrected optical density values (P-NK) determined for each ring trial sample are shown by blue dots for the ear notch samples and by black dots for the sera. The cut-off values of the test for ear notches and sera, respectively, are indicated by dashed lines.

PCR amplifications were carried out with either published (Gaede et al., 2005; Hoffmann et al., 2006; McGoldrick et al., 1999; Wernike et al., 2017b; Willoughby et al., 2006) or unpublished in-house assays or one of the following commercial RT-qPCR kits (sorted alphabetically by manufacturer):

- ADIAVET™ BVDV REAL TIME (Bio-X Diagnostics S.A., Rochefort, Belgium), used in 8 laboratories.
- ADIAVET™ BVDV FAST TIME (Bio-X Diagnostics S.A., Rochefort, Belgium), 1 laboratory.
- virellaBVDV 2.0 real-time PCR Kit (gerbion GmbH & Co. KG, Kornwestheim, Germany), 1 laboratory.
- RealPCR BVDV RNA Mix (IDEXX Europe B.V., Hoofddorp, the Netherlands), 2 laboratories.
- virotype BVDV RT-PCR Kit (INDICAL BIOSCIENCE GmbH, Leipzig, Germany), 16 laboratories.
- virotype BVDV 2.0 RT-PCR Kit (INDICAL BIOSCIENCE GmbH, Leipzig, Germany), 5 laboratories.
- ID Gene™ BVDV/BDV Triplex (Innovative Diagnostics, Grabels, France), 3 laboratories.
- VetMAX™ BVDV 4ALL Kit (Thermo Fisher Scientific, Waltham, USA), 2 laboratories.

The ear notch samples were tested by 50 extraction/RT-qPCR combinations, while for the sera 52 result sets had been generated. The pestivirus-negative samples were correctly assessed regardless of the applied extraction method and PCR assay (Fig. 2). The BVDV-positive samples were likewise in most cases tested correctly positive, with only two exceptions. Both false-negative results were generated for the ear notch sample BVD-A-13/23 following treatment with a direct lysis buffer, once combined with the virotype BVDV RT-PCR Kit and once with the ID Gene™ BVDV/BDV Triplex test. In a further case, no Cq-value was measured for the sample BVD-A-18/23, but in the results sheet the assessment "positive" was entered. The BDV-positive sample (BVD-A-16/23) tested consistently positive (Fig. 2). When considering the BDV-positive sample as "negative" for the calculation of the diagnostic specificity, overall values of 98.83% (95% CI: 96.61% to 99.76%) and 74.51% (95% CI: 67.95% to 80.34%) result for the sensitivity and specificity of BVDV detection, respectively. However, if one considers

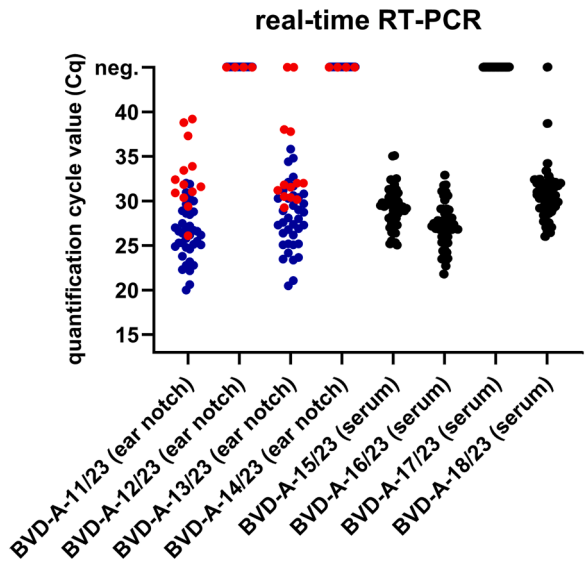

Fig. 2. Results of the RT-qPCRs performed in the participating laboratories. Quantification cycle (Cq)-values produced from the serum samples are shown in black. From the ear notch samples, viral RNA was either extracted by direct lysis buffer or commercial purification kits and subsequently tested by diverse RT-qPCR assays. The resulting Cq-values are shown separately according to the extraction methods (red for direct lysis buffer and blue for commercial purification kits).

that most of the applied published in-house PCR assays are panpesti-PCRs (Gaede et al., 2005; Hoffmann et al., 2006; McGoldrick et al., 1999; Wernike et al., 2017b; Willoughby et al., 2006; WOAH, 2015) and that the commercial RT-qPCR tests also appear to contain broadly reacting PCRs, it could make sense to include the BDV-positive sample as "positive" in the calculations. Then, values of 98.83% (95% CI: 96.61% to 99.76%) and 100.00% (95% CI: 97.60% to 100.00%) result for the diagnostic sensitivity and specificity, respectively.

When taking a closer look at the results generated from the ear notch samples, a clear effect of the nucleic acid extraction method can be observed. The Cq-values measured following treatment with a direct lysis buffer tend to be higher then after RNA extraction using commercially available column- or magnetic bead-based purification kits (Fig. 2).

In five laboratories, sequencing-based subtyping of the detected viruses was performed, in two cases only the sera were analyzed. The viruses present in the ear notches were typed correctly as being BVDV-1b and BVDV-1d, respectively, in each reported approach. In the sample BVD-A-15/23, BVDV-1d was identified three times and BVDV-1 (without further subtyping) twice. Similarly, subtype BVDV-2c was identified three times in the sample BVD-A-18/23 and BVDV-2 without further subtyping twice. For the sample BVD-A-16/23, BDV was indicated twice in the results sheet, BDV-3 another two times, and in the remaining case no sequences could be generated.

3.3. *Virus isolation in cell culture is less sensitive than E^{RNS} antigen ELISA or RT-qPCR*

Eighteen laboratories attempted virus isolation in cell culture and indicated methodological details in the results sheet. Ear notch samples were analyzed by four participants. The sera were investigated in each laboratory that performed virus isolation (n = 18). Cell lines used include bovine (e.g., BEL, EKANAEP, KLu-R, KOP-R, MDBK, primary calf testicular cells) and ovine cells (SFT). For the virus-negative samples, only correct negative results were submitted. Infectious virus was isolated from the positive samples in just under half of the attempts (Table 1). The isolated virus strains were occasionally typed by immunofluorescence or peroxidase staining using monoclonal antibodies (Table 1).

Table 1
Number of attempts to isolate infectious virus by using cell culture methods and number of positive, doubtful and negative results, respectively, per ring trial sample.

sample ID (sample material; status)	number of investigations	positive	doubtful	negative	typing
BVD-A-11/23 (ear notch; pestivirus-positive, BVDV-1b)	4	4	0	0	2x BVDV-1, 2x no typing
BVD-A-12/23 (ear notch; pestivirus-negative)	4	0	0	4	–
BVD-A-13/23 (ear notch; pestivirus-positive, BVDV-1d)	4	2	0	2	1x BVDV-1, 1x no typing
BVD-A-14/23 (ear notch; pestivirus-negative)	4	0	0	4	–
BVD-A-15/23 (serum; pestivirus-positive, BVDV-1d)	18	2	1	15	2x no typing
BVD-A-16/23 (serum; pestivirus-positive, BDV)	18	16	0	2	2x BDV, 1x BVDV-1, 2x BDV or BVDV-1, 11x no typing
BVD-A-17/23 (serum; pestivirus-negative)	18	0	0	18	
BVD-A-18/23 (serum; pestivirus-positive, BVDV-2c)	18	2	1	15	2x no typing

In some cases, the isolated viruses were typed by staining methods.

Similar to the PCR analyses diagnostic sensitivity and specificity of BVDV detection by virus isolation was calculated for two different assumptions, the BDV-positive sample was considered "negative" or "positive". The calculated characteristics are: a) BDV = negative and doubtful = negative: sensitivity of 22.73% (95% CI: 11.47% to 37.84%) and specificity of 61.90% (95% CI: 45.64% to 76.43%); b) BDV = negative and doubtful = positive: sensitivity of 27.27% (95% CI: 14.96% to 42.79%) and specificity of 61.90% (95% CI: 45.64% to 76.43%); c) BDV = positive and doubtful = negative: sensitivity of 43.33% (95% CI: 30.59% to 56.76%) and specificity of 100.00% (95% CI: 86.77% to 100.00%); d) BDV = positive and doubtful = positive: sensitivity of 46.67% (95% CI: 33.67% to 60.00%) and specificity of 100.00% (95% CI: 86.77% to 100.00%).

4. Discussion

Although BVDV is highly contagious and very common in the ruminant populations worldwide, its eradication can be achieved at herd or even at regional or national level, as has been demonstrated by the progress of control programs in several countries (Moennig and Becher, 2018; Wernike et al., 2017a). To eliminate an infectious agent from its target population, the fast and reliable identification of the pathogen is

crucial. An essential component of quality control in the field of diagnostics are ring trials, or proficiency testing, as they aid in demonstrating the competency of the laboratory and allow for an independent assessment of the diagnostic procedures used (FAO, 2015). Therefore, we carried out a ring trial for BVDV detection methods applied in veterinary diagnostic laboratories in multiple countries with and without national BVD control programs in place. The sample panel contained local German BVDV strains, which might influence the transferability of the results to regions, where virus strains of other subtypes are circulating. The highly diverse pestivirus BVDV exists in numerous subtypes (ICTV, 2023; Vilcek et al., 2001) and this diversity poses particular challenges on disease diagnostics. Therefore, it is important to validate the test systems in place in a given region especially with the virus strains that occur locally.

The participants of this ring trial applied RT-qPCRs for viral genome detection, an ELISA for E^{RNS}-antigen detection and different cell culture methods for BVDV isolation. Based in our sample panel, the used antigen ELISA showed nearly optimal diagnostic sensitivity and specificity. However, a BDV-positive sample included in the trial tested negative in all laboratories using the E^{RNS}-ELISA. But previous studies have shown that other BDV strains were detected in clinical samples by this particular ELISA assay and also by other commercial BVDV antigen ELISAs (Bouzalas et al., 2023; Marco et al., 2011; Rosamilia et al., 2014). When RT-PCR methods are used, BDV can also interfere with BVDV diagnostics, as has been demonstrated exemplarily in this ring trial. BDV and BVDV are closely related pestiviruses and share genetic and antigenic characteristics (ICTV, 2023) and, therefore, cross-reactivity is an issue in virological, but also in serological diagnostics (Wernike and Beer, 2022). When cattle and sheep are kept together, BVDV and BDV can be transmitted between their main target species (Braun et al., 2013; Braun et al., 2019; Huser et al., 2021). Consequently, BDV has to be expected in cattle in mixed holdings and detection is therefore beneficial to avoid any further adaption of such strains to cattle. This is especially important since BDV PI cattle have been reported previously (Braun et al., 2019). However, at the moment, disease eradication programs are usually restricted to BVDV. Thus, differentiation of both viruses might be necessary and can be achieved by either molecular detection methods (e.g., discriminating RT-qPCRs or sequence analysis) or differential staining of virus isolates using species-specific monoclonal antibodies directed against the major pestiviral glycoproteins (Marco et al., 2011; WOAH, 2015).

Isolation of the virus in cell culture is required in order to perform differentiating staining. Virus isolation is a classical method for BVDV diagnostics, provides valuable information about the viability and characteristics of the virus and enables studies on the behavior in vitro and in vivo, thereby supporting BVDV research and vaccine development. However, regarding disease diagnostics, major obstacles of virus isolation by cell culture methods are the time requirements, strong dependence on sample quality and poor sensitivity. In addition, when blood samples of young calves are tested, the negative influence of maternal antibodies is another aggravating factor (WOAH, 2015). Maternal antibodies strongly inhibit virus growth in cell culture, and have likewise an inhibitory effect on viral antigen detection by ELISA and, to a much lesser extent, on genome detection (Fux and Wolf, 2012; Hilbe et al., 2007; Laureyns et al., 2010; WOAH, 2015). Hence, skin tissues - like ear notches - from calves that ingested colostrum of their antibody-positive mothers should be preferentially used to avoid the influence of maternal antibodies. With this in mind, the vast majority of calves is tested through ear notches in countries implementing the so-called “Swiss approach” of BVD control (Schweizer et al., 2021; Wernike et al., 2017a), i.e. direct virus diagnostics without serological pre-screening of herds. Overall, virus isolation is not suitable for standard BVDV diagnostics in eradication programs and it is therefore not reasonable to classify this method as a prerequisite for official case confirmations, as recommended in the EU Commission delegated regulation (EU) 2020/689. Hence, this EU requirement needs to be reconsidered as soon as possible and adapted to the recommendations of the World Organisation for Animal Health (WOAH). According to the WOAH Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, RNA detection assays are particularly useful because they are rapid and have very high sensitivity (WOAH, 2015). Especially in cases of acute, transient infections, when the virus levels are low, virus isolation in cell culture is not usually practical to undertake, while RT-qPCR assays offer several advantages including high sensitivity (WOAH, 2015). The same holds true for confirmation that an abortion, stillbirth or perinatal death is caused by BVDV. Though virus may be isolated by cell culture methods from fetal tissue in some cases, emphasis should be placed on the detection of viral antigen by ELISA or RNA by RT-qPCR (WOAH, 2015).

To be used for PCR diagnostics, it is required to extract nucleic acids from the clinical samples. When taking ear notches as sample matrix, direct lysis buffers are frequently applied as an easy-to-use, rapid and cost-effective approach. However, in this proficiency trial they tend to produce higher Cq-values then after RNA extraction by commercially available column- or magnetic bead-based purification kits. This is in line with a previous BVDV ring trial, at which only German laboratories participated and where false-negative results were likewise a very rare event, but when they occurred they had been most likely related to the RNA extraction protocol (Wernike and Beer, 2019). Therefore, thorough validation of the nucleic acid extraction method and its compatibility with the PCR assay in place in that particular laboratory is highly recommended before the usage in routine diagnostics.

5. Conclusion

BVDV is a significant threat to cattle populations, and the accurate and timely diagnosis of infections is essential for effective disease management and control. In this interlaboratory proficiency trial, RT-qPCR, antigen ELISA and virus isolation were compared regarding their key diagnostic characteristics. Virus isolation in cell culture provides valuable information about the viability of the virus and allows for in-depth analysis of the isolated strains. However, this time-consuming and insensitive technique is not very useful for routine testing in the context of disease eradication, where a large number of samples needs to be tested within short periods of times. Hence, for pathogen identification in clinically diseased animals or in the context of disease control programs, the application of RT-qPCRs or E^{RNS} antigen ELISAs is highly recommended because of the much higher diagnostic sensitivity of these methods. When applying RT-qPCR tests, the nucleic acid extraction method is of particular importance and should be thoroughly validated in every laboratory in the context of its testing method before the usage in routine diagnostics. Another important issue in BVDV diagnostics is cross-reactivity with the related BDV, as shown in this ring trial exemplarily for a selected BDV strain and diverse RT-qPCR assays. Therefore, subsequent typing of detected virus strains is recommended to differentiate BVDV from BDV infections, in order to improve the understanding of the extent of BDV infections in bovine populations and to avoid culling measures when BVDV control programs are in place.

CRediT authorship contribution statement

Wernike Kerstin: Conceptualization, Formal analysis, Methodology, Visualization, Writing – original draft. **Beer Martin:** Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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