



## Diagnostics in the context of an eradication program: Results of the German bovine viral diarrhoea proficiency trial



Kerstin Wernike\*, Martin Beer

Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Südufer 10, 17493 Greifswald - Insel Riems, Germany

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### ABSTRACT

Bovine viral diarrhoea (BVD), one of the most important infectious diseases in cattle, causes major economic losses and significant impact on animal welfare worldwide. The major source for virus spread is persistently infected, immunotolerant calves and, therefore, their early identification is of utmost importance for disease prevention. Here, a ring trial was initiated to control the performance of diagnostic tests used in German regional laboratories in charge of the diagnostics within the country's BVD control program. A panel of five ear notch and five serum samples was provided for virological analysis. By an antigen ELISA, which was applied 26 times, the status of every sample was correctly identified in any case. In addition, a total of 54 real-time RT-PCR result sets was generated and also in most cases correctly classified. In addition to the virological test panel, a set of six sera and four milk samples was sent to the participating laboratories to be analyzed by serological methods. With serum neutralization tests, an excellent diagnostic sensitivity was achieved. However, one serum and both milk samples - positive for BVDV antibodies - repeatedly tested false negative by some of the used ELISA kits. All negative serum and milk samples were correctly identified by every commercial antibody ELISA. In conclusion, the BVDV proficiency test demonstrated that the used antigen/genome test systems allowed predominantly reliable diagnostics, while for four of the applied nine antibody ELISA kits adjustments are recommended.

### 1. Introduction

Bovine viral diarrhoea virus (BVDV), a pestivirus within the family *Flaviviridae*, is endemic in cattle populations worldwide and causes major economic losses and significant impact on animal welfare (Houe, 2003; Lindberg et al., 2006; Richter et al., 2017). BVDV isolates are classified into the two distinct biotypes cytopathogenic (cp) and non-cytopathogenic (ncp), and the viruses exist in two species, BVDV-1 and BVDV-2. BVDV is closely related to the other two "classical" pestiviruses, Border disease virus (BDV) and classical swine fever virus. BVDV is also related to so-called "atypical" pestiviruses (Fauquet et al., 2005), among them HoBi-like viruses (syn. BVDV-3), which were originally isolated from fetal calf serum (FCS), likewise infect cattle and could interfere with BVDV diagnostics (Bauermann et al., 2017; Decaro et al., 2012; Mosena et al., 2017; Schirmer et al., 2004).

A BVDV infection of naïve cattle results in most cases in clinically inapparent infections or leads to mild to moderate unspecific symptoms, but severe acute forms characterized by hemorrhagic syndromes and mucosal disease-like lesions may also be seen, mainly associated with virulent BVDV type 2 strains (Brodersen, 2014; Ridpath, 2005). However, an infection of naïve pregnant cows may - with respect to the

phase of gestation - result in abortion, stillbirth, teratogenic effects or, when the infection occurs during the first trimester, in the birth of immunotolerant, persistently infected (PI) animals (Baker, 1995). PI animals are unable to develop a specific immunity against the virus strain they are infected with and, therefore, shed enormous amounts of BVDV throughout their lives, becoming to the major source of spread and perpetuation of the virus within cattle populations (Moennig and Becher, 2018). When PI animals are superinfected with a cp-strain that is antigenically homologous to the persisting ncp-strain or a cp-biotype arises from mutations of the ncp-BVDV already circulating in the animal, the inevitably fatal mucosal disease (MD) develops (Lanyon et al., 2014a).

Due to their pivotal role in the spread of the disease, PI animals are the major target of BVDV eradication programs, which have been implemented in several countries (Moennig and Becher, 2015, 2018; Moennig et al., 2005; Stahl and Alenius, 2012; Wernike et al., 2017a). In Germany, a nationwide, mandatory control program is in force since 2011. Its centerpiece is the detection of PI animals as early as possible and their removal from the cattle population (Wernike et al., 2017a). The vast majority of animals are tested through ear notch samples taken during the tagging procedure, which has to be done for every calf in the

\* Corresponding author.

E-mail address: [kerstin.wernike@fli.de](mailto:kerstin.wernike@fli.de) (K. Wernike).

European Union. In addition, blood samples are investigated, primarily for the investigation of imported cattle or for confirmatory testing, but the use of this sample material from newborn calves is very limited due to the strong inhibitory effect of maternal antibodies (Fux and Wolf, 2012; Hilbe et al., 2007; Laureyns et al., 2010; Schirmer, 2014).

As indicated in the German BVD regulation, diagnostics has to be carried out with test systems prescribed in the Official Collection of Test Methods for BVD (Friedrich-Loeffler-Institut, 2017). Commercial tests for BVD diagnostics require the official approval of the Friedrich-Loeffler-Institut, Germany's Federal Research Institute for Animal Health, as licensing and batch release are part of the quality assurance scheme for diagnostic systems used for notifiable and reportable animal diseases (according to § 11 Abs. 2 TierGesG).

Another very important component of quality assurance is the independent assessment of the quality of results produced in diagnostic laboratories, which may be achieved by participating to interlaboratory proficiency trials. Through ring tests, the quality of analytical results can be monitored, and assays that might need improvement can be identified (FAO, 2015). In this work, the results of a proficiency trial, based on a panel of standardized samples for BVDV virus/antigen/genome or BVDV antibody detection are described.

## 2. Materials and methods

A total of five ear notch samples and five bovine serum samples were prepared and sent to the participating laboratories for the detection of virus, viral antigen or viral genome; furthermore six sera and four milk samples were provided for serological analyses.

The virological panel included four ear notch samples obtained from PI calves (labelled A-11 to A-14), one BVDV-negative bovine ear tissue sample (A-15), three BVDV-1 positive sera (A-16, A-17, A-20), one fetal calf serum (Biochrom GmbH, Berlin, Germany) containing BVDV-3 RNA (A-19), and one pestivirus-negative cattle serum (A-18). The ear notch samples were provided in duplicate, one sample in a collection tube of an Allflex Tissue Sampling Tag (Allflex Group Germany GmbH, Bad Bentheim, Germany), and the second one in the collection tube of a Caisley Tissue sampling Cattle Tag (Caisley International GmbH, Bocholt, Germany).

The serological panel comprised two pools of field-collected BVDV antibody positive sera (S-1 and S-2), one serum sample of an animal previously infected with the BVDV-1 strain NADL (S-4), one serum sample of a previously BVDV-2-infected animal (S-5), and two bovine sera (S-3 and S-6) and two bulk milk samples negative for antibodies against pestiviruses (S-8 and S-10). In addition, two BVDV antibody positive milk samples of individual animals were included; the corresponding sera were tested for BVDV-specific antibodies by a standard microneutralization test (Friedrich-Loeffler-Institut, 2017) against BVDV-1 strain NADL and neutralizing titers of 1/453 (S-7) and 1/80 (S-9) were calculated.

Thirty-four German regional diagnostic laboratories and five manufacturers of test kits licensed in Germany participated in the proficiency test. They were asked to analyze the samples with test systems routinely used in their institution.

The diagnostic accuracy of the obtained results was calculated by the statistical calculator MedCalc (MedCalc Software, Ostend, Belgium; [https://www.medcalc.net/statisticaltests/diagnostic\\_test.php](https://www.medcalc.net/statisticaltests/diagnostic_test.php)), taking the sensitivity and specificity into account. The sensitivity represents the ability to identify a positive sample correctly and is defined as the proportion of true positive results in a set of positive cases (calculated as: true positive results/true positives + false negatives), while the specificity of a test is its ability to determine the negative cases correctly (calculated as: true negative results/true negatives + false positives) (Šimundić, 2009).

Test results that were considered "doubtful" by a participant were regarded as correct for the calculation of the diagnostic accuracy, since it was assumed that the necessary clarifications or follow-up analyses

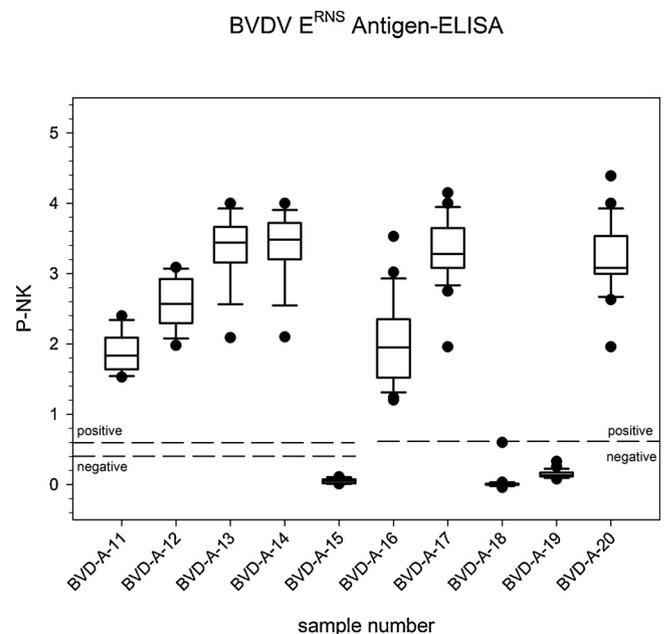


Fig. 1. Results of a commercially available BVDV antigen ELISA (BVDV Ag/Serum Plus, IDEXX). The corrected OD values (P-NK) determined for each individual sample are shown as box plots, each outlier is depicted by a dot. The cut-off values of the test are indicated by dashed lines.

would have been performed in practice.

## 3. Results

### 3.1. Virological analysis: reliable detection of viral antigen

The sample panel aimed for virological analyses was investigated by detection systems for BVDV antigen or pestiviral RNA. Viral antigen detection was carried out by a total of 26 participants, and all of them used the BVDV Ag/Serum Plus test (IDEXX, Liebefeld, Switzerland). The ear notch samples were tested by 14 laboratories, while sera were analyzed in 25 laboratories. In all cases, the BVDV-status of the samples was correctly identified (diagnostic accuracy: 100%) and the BVDV-3 RNA containing fetal calf serum tested consistently negative (Fig. 1).

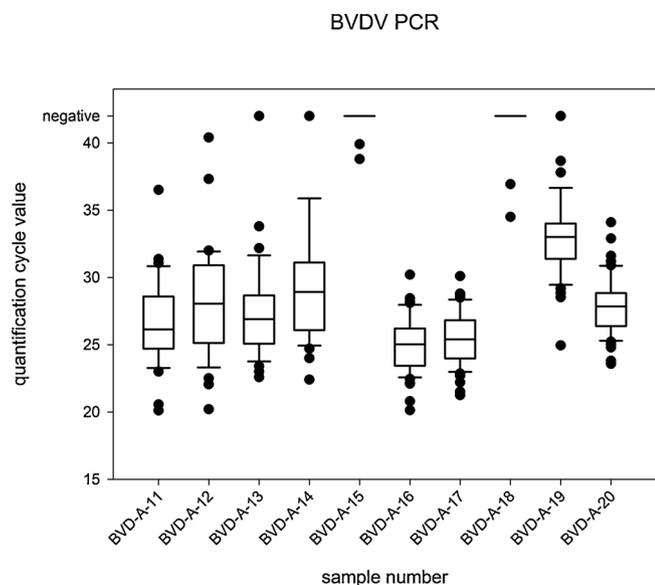
In addition, or alternatively to the antigen ELISA, the samples were analyzed by real-time RT-PCR. While for the ear notch samples a total of 36 result sets (corresponding to 180 individual results as five ear notch samples were tested 36 times) had been generated, the sera were also tested by 52 extraction/real-time RT-PCR combinations (52 x five sera = 260 individual results). To be examined by PCR, viral RNA was either extracted using commercially available purification kits (ear notch samples: n = 22; serum: n = 51) or one of the following direct lysis buffers (ear notch samples: n = 14; serum: n = 1): virotype® Tissue Lysis Reagent (Qiagen, Leipzig, Germany; ear notch samples: n = 9), ADIAPURE™ TLB buffer (BioX diagnostics, Rochefort, Belgium; ear notch samples: n = 2, serum: n = 1) and RealPCR Rapid Lysis Buffer (IDEXX, Liebefeld, Switzerland; ear notch samples: n = 1). For the remaining two sample preparations the manufacturer of the lysis buffer was not indicated in the ring trial results sheet. PCR amplifications were carried out with seven commercial kits licensed for the German market (List of certified products pursuant to section 11 subsection 2 Animal Health Act (Friedrich-Loeffler-Institut, 2019)). In addition, published (Gaede et al., 2005; Hoffmann et al., 2006; Wernike et al., 2017b) or unpublished in-house assays were used.

The status of every sample of the set was correctly identified in 31 (ear notch) and 51 cases (serum), respectively (Table 1), with moderate variations of the obtained quantification cycle (Cq) values (Fig. 2). In two laboratories, incorrect results were produced for one or more

**Table 1**

Results of different BVDV real-time RT-PCR systems. BVDV-1 or BVDV-2 positive samples are printed in bold. Sample A-19 represents a fetal calf serum containing BVDV-3 (syn. HoBi-like virus) RNA.

test system		A-11	A-12	A-13	A-14	A-15	A-16	A-17	A-18	A-19	A-20
ADIAVET BVD/MD, BioX	no. tests	4	4	4	4	4	7	7	7	7	7
	no. positive	4	4	3	2	0	7	7	0	6	7
virellaBVDV 2.0 real time RT-PCR Kit, gerbion	no. tests	1	1	1	1	1	1	1	1	1	1
	no. positive	1	1	1	1	0	1	1	0	0	1
RealPCR BVDV RNA Test, IDEXX	no. tests	4	4	4	4	4	6	6	6	6	6
	no. positive	4	4	4	4	0	6	6	0	6	6
	no. doubtful	0	0	0	0	1	0	0	0	0	0
ID Gene BVD/BD Triplex, ID.vet	no. tests	3	3	3	3	3	3	3	3	3	3
	no. positive	3	3	3	3	0	3	3	0	3	3
ViroReal Kit BVD Virus, Ingenetix	no. tests	1	1	1	1	1	1	1	1	1	1
	no. positive	1	1	1	1	0	1	1	0	1	1
LSI VetMAX BVDV 4ALL, Life technologies	no. tests	4	4	4	4	4	5	5	5	5	5
	no. positive	4	4	4	3	0	5	5	0	5	5
virotype BVDV RT-PCR Kit, QIAGEN	no. tests	15	15	15	15	15	21	21	21	21	21
	no. positive	15	15	15	15	0	21	21	0	20	21
	no. doubtful	0	0	0	0	1	0	0	1	1	0
in-house	no. tests	4	4	4	4	4	8	8	8	8	8
	no. positive	4	4	4	4	0	8	8	1	7	8



**Fig. 2.** Quantification cycle (Cq) values produced by the ring trial participants using different real-time PCR systems. The Cq values determined for each individual sample are shown as box plots, each outlier is depicted by a dot.

samples of the set, resulting in a total number of four incorrect results among 180 individual results for ear notch samples and one false-positive result for a negative serum (sample A-18, in-house PCR). The negative ear notch sample A-15 was classified as doubtful twice (Cq 38.8 and 39.9), both results were produced in the same laboratory from the identical RNA preparation and were correlated to an inadequate opening of the sealed container usually not used in the respective lab.

The BVDV positive ear notch sample A-14 was classified as negative by one participant, and in this laboratory two different real-time systems were applied using the identical RNA preparation (extraction: in-house lysis buffer combined with column based commercial purification kit). In one participating laboratory the samples A-13 and A-14 were considered negative, viral RNA was produced by a tissue lysis buffer (ADIAPURE™ TLB buffer, BioX diagnostics, Rochefort, Belgium) and tested by a real-time PCR system by the same manufacturer (Table 1). The serum sample A-18, which was negative for pestivirus RNA, was falsely assessed positive or doubtful in two cases (Cq 34.5 and 36.93; Fig. 2), one of the laboratories remarked in the results sheet that they suspect cross-contamination and asked for a follow-up sample.

Taken together, the diagnostic accuracy of the RT-PCR tests reached 98.73% for the BVDV positive or negative ear notch and serum samples.

Sample A-19 (HoBi-like virus RNA) tested repeatedly positive by six of the applied seven commercial real-time RT-PCR kits and most in-house real-time PCRs (Table 1), while this sample scored negative by the seventh commercial RT-PCR kit, which was applied once.

### 3.2. Serological analysis: application of a variety of test systems leads to mostly correct results

The provided serological panel was investigated by the participants using a variety of commercially available ELISA test systems and in the cell-culture based serum neutralization test. The antibody detection was carried out by nine different ELISAs, all licensed for the German market (Table 2); from the serum samples, 46 result sets were produced (corresponding to 276 individual results), and the milk samples were analyzed 37 times (= 148 individual results). BVDV antibody negative sera and milk samples scored correctly negative in every trial. The sera BVD-S-1 and BVD-S-2, which consisted of pooled field-collected BVDV antibody positive sera, tested positive by every applied ELISA system in each laboratory that used the respective test. However, the sera BVD-S-4 and BVD-S-5 and both BVDV antibody positive milk samples tested occasionally negative by one or more of the applied ELISAs (Table 2). The diagnostic accuracy of the commercial ELISAs reached 96.36%.

When an individual sample's status was inconsistently assessed correct/incorrect, no correlation could be seen to the used kit batch (data not shown). In the instructions of most of the ELISA kits two different initial incubation protocols are given (short incubation vs. overnight incubation). When testing the milk samples by the BVDV Ab total kit (IDEXX, Liebefeld, Switzerland) both incubation protocols were applied seven times. Sample S-7 tested correctly positive in 12 cases (short protocol: n = 5; overnight incubation: n = 7), once doubtful (short protocol), and once false negative (short protocol), while sample S-9 tested correctly positive in five cases (short protocol: n = 1; overnight incubation: n = 4), three times doubtful (short protocol: n = 1; overnight incubation: n = 2), and false negative in six cases (short protocol: n = 5; overnight incubation: n = 1). The BVDV p80AB test (IDEXX, Liebefeld, Switzerland) was applied using each of the indicated protocols once, serum sample S-4 tested false negative in both cases. When tested by the PrioCHECK® BVDV Ab kit (ThermoFisher Scientific, Darmstadt, Germany) sample S-4 scored correctly positive in one case (short protocol) and false negative in the second case (overnight incubation). The Svanovir BVDV Ab biphasis test (Boehringer Ingelheim Svanova, Uppsala, Sweden) was applied

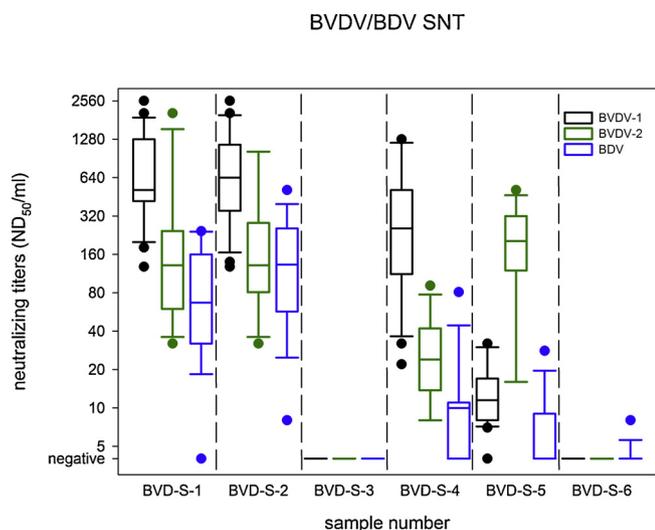
**Table 2**

Results of commercially available BVDV antibody ELISAs. BVDV antibody positive samples are printed in bold and results divergent from the defined sample status are shaded in grey.

test system		S-1	S-2	S-3	S-4	S-5	S-6	S-7	S-8	S-9	S-10
ID Screen BVDV p80 Ab competition, ID.vet	no. tests	<b>11</b>	<b>11</b>	11	<b>11</b>	<b>11</b>	11	<b>9</b>	9	<b>9</b>	9
	no. positive	<b>11</b>	<b>11</b>	0	<b>10</b>	<b>11</b>	0	<b>9</b>	0	<b>9</b>	0
	no. doubtful	<b>0</b>	<b>0</b>	0	<b>1</b>	<b>0</b>	0	<b>0</b>	0	<b>0</b>	0
BVDV Ab total, IDEXX	no. tests	<b>19</b>	<b>19</b>	19	<b>19</b>	<b>19</b>	19	<b>14</b>	14	<b>14</b>	14
	no. positive	<b>19</b>	<b>19</b>	0	<b>19</b>	<b>19</b>	0	<b>12</b>	0	<b>5</b>	0
	no. doubtful	<b>0</b>	<b>0</b>	0	<b>0</b>	<b>0</b>	0	<b>1</b>	0	<b>3</b>	0
BVDV p80AB, IDEXX	no. tests	<b>2</b>	<b>2</b>	2	<b>2</b>	<b>2</b>	2	<b>2</b>	2	<b>2</b>	2
	no. positive	<b>2</b>	<b>2</b>	0	<b>0</b>	<b>2</b>	0	<b>2</b>	0	<b>2</b>	0
PrioCHECK® BVDV Ab, ThermoFisher Scientific	no. tests	<b>2</b>	<b>2</b>	2	<b>2</b>	<b>2</b>	2	<b>2</b>	2	<b>2</b>	2
	no. positive	<b>2</b>	<b>2</b>	0	<b>1</b>	<b>2</b>	0	<b>2</b>	0	<b>2</b>	0
Svanovir BVDV Ab Screening, SVANOVA	no. tests	<b>2</b>	<b>2</b>	2	<b>2</b>	<b>2</b>	2	<b>2</b>	2	<b>2</b>	2
	no. positive	<b>2</b>	<b>2</b>	0	<b>2</b>	<b>2</b>	0	<b>2</b>	0	<b>2</b>	0
Svanovir BVDV Ab biphasisch, SVANOVA	no. tests	<b>7</b>	<b>7</b>	7	<b>7</b>	<b>7</b>	7	<b>7</b>	7	<b>7</b>	7
	no. positive	<b>7</b>	<b>7</b>	0	<b>6</b>	<b>6</b>	0	<b>7</b>	0	<b>3</b>	0
Svanovir BVDV p80 AB, SVANOVA	no. tests	<b>1</b>	<b>1</b>	1	<b>1</b>	<b>1</b>	1	<b>1</b>	1	<b>1</b>	1
	no. positive	<b>1</b>	<b>1</b>	0	<b>1</b>	<b>1</b>	0	<b>1</b>	0	<b>1</b>	0
Monoscreen AB ELISA BVDV (E0)/blocking, BioX	no. tests	<b>1</b>	<b>1</b>	1	<b>1</b>	<b>1</b>	1				
	no. positive	<b>1</b>	<b>1</b>	0	<b>1</b>	<b>1</b>	0				
Serelisa BVD/MD AB Mono Blocking, Zoetis	no. tests	<b>1</b>	<b>1</b>	1	<b>1</b>	<b>1</b>	1				
	no. positive	<b>1</b>	<b>1</b>	0	<b>0</b>	<b>1</b>	0				

seven times for both matrices, serum (short protocol: n = 5; overnight incubation: n = 2) and milk (short protocol: n = 4; overnight incubation: n = 3). The serum samples S-4 and S-5 scored correctly positive six times and false negative in one case (short protocol). The milk sample S-9 tested positive three times (short protocol: n = 1; overnight incubation: n = 2) and false negative in four cases (short protocol: n = 3; overnight incubation: n = 1).

In addition to the ELISA tests, the sera were analyzed by serum neutralization tests for which diverse BVDV-1, BVDV-2 and/or BDV strains were used. In total, the sera were tested against a BVDV-1 strain 22 times, against a BVDV-2 strain 14 times and against BDV 15 times. In general, the BVDV antibody status of the sera was correctly identified; however, the resulting titers differed markedly between the participating laboratories (Fig. 3).



**Fig. 3.** Results of the serum neutralization tests (SNT) against BVDV-1 (black), BVDV-2 (green), and BDV (blue) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

#### 4. Discussion

Interlaboratory proficiency tests are one of the most important essential parts of quality control measures and serve to demonstrate the competency of the laboratory and of the applied methods to accreditation bodies, customers and other regulatory bodies (FAO, 2015). Especially for those animal diseases that are regionally or internationally regulated reliable diagnostics is indispensable for the success of implemented control measures. Here, the diagnostic capacity was assessed for an agent for which eradication programs have been implemented in several European countries (Bachofen et al., 2013; Moennig and Becher, 2018; Stahl and Alenius, 2012).

In contrast to the Scandinavian model, which was based on large-scale bulk milk serology combined with a strict non-vaccination policy to preselect farms with an elevated risk for the presence of PI animals (Lindberg and Alenius, 1999; Stahl and Alenius, 2012), the German BVDV control strategy is based on the direct viral antigen or genome testing of every animal without serological pre-screening (Wernike et al., 2017a). Therefore, in terms of diagnostic systems, the main focus is on virological rather than serological methods. Accordingly, all samples were correctly classified by antigen ELISA in every laboratory that used this test and also by real-time PCR the submitted results were mostly correct. Only four incorrect results were produced among 180 individual results for ear notch samples and one false-positive result for a BVDV-negative serum was obtained. The false negative PCR results that were rarely produced from BVDV-positive ear notch samples are most likely related to the RNA extraction protocol, which is not part of the batch release system, since the identical PCR assays combined with different nucleic acid extraction systems yielded correct results. In one laboratory, negative results were produced for two samples and rather high Cq-values were obtained for the other two BVDV-positive ear tissue samples. In another case of an incorrect result, an in-house lysis buffer combined with a column based commercial purification kit was used. The possible influence of the extraction protocol was discussed with the ring trial participants and a new sample set was provided and retested. All samples were correctly identified in those attempts.

In contrast, the reasons for the failure of the serological diagnostics, which was sporadically observed, could not be identified with certainty, since no correlation could be seen to the used ELISA system or batch

and, when a system was applied several times, in most cases incorrect results were produced using both recommended incubation protocols. However, differences in the diagnostic sensitivity of commercially available BVDV antibody ELISAs have been already described (Hanon et al., 2017).

Due to the high sensitivity and, despite cross-reactivity which occurs between both BVDV species (Ridpath et al., 2010), the possibility to differentiate between antibodies induced by BVDV-1 and -2, neutralization tests are considered as gold standard for serological diagnostics.

Nevertheless, ELISA systems are more frequently used because they are convenient, enable high throughput testing and do not require time-consuming cell culture systems. As sample matrices, both serum and milk are commonly used, since the detection of BVDV-specific antibodies in serum or milk samples is an efficient way to identify the exposure of individual animals or of the cattle herd to the virus (Houe et al., 2006; Lanyon et al., 2014a, b). To screen for the introduction of PI animals into previously not affected herds, bulk milk serology (in non-vaccinated herds) or spot-testing of young stock (calves > 6 months of age to avoid the influence of maternal antibodies) may be used (Booth and Brownlie, 2016; Houe, 1992, 1994; Pritchard, 2001; Valle et al., 2001). However, for both matrices varying and occasionally unsatisfactory sensitivities were previously observed, whereby generally anti-NS3 (syn. p80) ELISAs were less sensitive compared to total antibody indirect ELISAs (Hanon et al., 2017). The latter could not be confirmed in the present study, as some p80-based ELISAs provided an excellent sensitivity, while by individual total antibody ELISAs not every positive sample could be correctly identified. This contradiction could be explained by the ring trial sample panel, since the lower diagnostic sensitivity was previously related to a reduced detection rate of positive samples from vaccinated animals (Hanon et al., 2017) and in the present proficiency test panel only samples of field-infected animals were included.

In conclusion, the ring trial demonstrated that the available antigen and genome test systems allow predominantly reliable BVDV diagnostics in Germany, while for a few of the applied antibody ELISA kits adjustments are recommended. Only recently, the German BVD regulation was modified to meet the new challenges of the now well-advanced control program (Wernike et al., 2017a). Among others, the amendments included the permission of first serological tools in addition to the primary testing of ear tissue samples. Therefore, serological methods gain in importance and based on the ring trial results, but also on previous experiences, the batch release requirements are currently adjusted by demanding a higher analytical sensitivity to pass the process in order to address the need of sensitive and reliable serological tools for the further progress of the BVDV control program.

### Ethical statement

Ear tissue samples and sera of BVDV positive animals were obtained from cattle slaughtered in the context of the German mandatory BVD control program and as prescribed in the BVD regulation (Verordnung zum Schutz der Rinder vor einer Infektion mit dem Bovinen Virusdiarrhoe-Virus; <http://www.gesetze-im-internet.de/bvdvv/>). Further serum and milk samples were taken by the responsible farm veterinarians in the context of the health monitoring program of the respective farms.

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### Declaration of Competing Interest

None.

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