Investigation of the potential for sera from cattle persistently infected with bovine viral diarrhea virus to generate false-negative antibody ELISA results in pooled serum from seropositive and seronegative cattle

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Abstract. We investigated the potential for viremic sera from cattle persistently infected with bovine viral diarrhea virus to create false-negative antibody results when testing pools of 10 sera by indirect or blocking ELISAs. Seronegative viremic sera (n = 23) were each added to a series of artificially constructed pools containing various percentages (0–90%) of antibody-positive sera, and the resulting pools were assayed for antibody. In all 23 cases, a negative antibody result was obtained in the pool containing no seropositive sera. In contrast, all pools containing ≥10% seropositive serum, representing a single seropositive animal in a pool of 10 samples, returned a positive result in both antibody ELISAs. We concluded that the likelihood of a false-negative antibody result occurring as a result of the presence of serum from a viremic animal was low, and therefore did not preclude the use of pooled sera for serosurveillance.

Key words: Antibody; bovine viral diarrhea; ELISA; false negative; pooled serum; viremic sera.

Bovine viral diarrhea virus (BVDV), the cause of BVD, is an economically important pestivirus of cattle.3,4,14,15 As a consequence, many countries, particularly in Europe,13 have completed or are currently engaged in regional or national eradication programs (EU Thematic network on BVD control position paper, 2006. Available at: https://www.afbini.gov.uk/articles/final-report-bvdv-control-europe). In Ireland, a voluntary eradication program was initiated in 2012,3 leading to a compulsory national program, supported by legislation, in 2013 (https://www.agriculture.gov.ie/media/migration/legislation/statutoryinstruments2017/S130BovineViralDiarrhoeaRegulations3100117.pdf). This program is based on individual tissue tag testing of all newborn calves, using samples collected when inserting official identity tags that have been modified for this purpose. This program has seen the prevalence of calves considered to be persistently infected (PI) with BVDV fall from 0.66% in 2013 to 0.10% in 2017, and 0.04% in the first 3 mo of 2018 (http://animal-healthireland.ie/?page_id=229), which is a historically low level. The program in Ireland is coordinated by Animal Health Ireland (www.animalhealthireland.ie) and overseen by a cross-industry BVD Implementation Group (BVDIG), which in turn receives technical advice from a BVD Technical Working Group (BVDTWG), drawn from experts from academia, government, industry, and veterinary practice.

As the program in Ireland moves toward the goal of eradication by 2020, the BVDIG, supported by the BVDTWG, is considering the introduction of alternative, serology-based surveillance methods for use following eradication. Such methods include the use of antibody check (spot) testing of young stock or first-lactation management groups. This approach is essentially the Scandinavian model of eradication, and typically consists of screening 5–10 homebred, non-vaccinated animals from each separately managed group for evidence of antibodies to BVD, based on the principle that the presence of a PI animal in an established management group will result in a seroprevalence above a set design prevalence within that group.6,9,10 In Ireland, the BVDTWG has recommended that check testing should consist of sampling 10 young stock from each management group (CHECK10) with a cut-point of 2 positive test results to achieve herd-level sensitivity (HSe) and specificity (HSp) of 99.5% and 100%, respectively. HSe and HSp were estimated using HerdAcc7 based on a cohort size of 50 animals, a...
were identified and designated VS1–VS23. To ensure that this approach is as cost-effective as possible, the BVDTWG has also suggested that sera from each management group (young stock or first lactation) be pooled, as opposed to testing samples individually. This approach has been used previously in Norway11 and is supported by an Australian study using both experimental and field sera.9 Pilot studies in Ireland (unpublished) have also shown encouraging results, supported by an extensive study using a range of commercial indirect and competitive ELISA kits.

However, a study on bulk tank milk (BTM) samples using an indirect ELISA kit reported that the presence of milk from a PI animal affected the outcome of the BTM antibody result, including the generation of negative results, with the most significant drop in ELISA optical density values seen when 5–10% of the milk in the sample came from a PI cow.12 This effect was presumed to be the result of competitive binding of antibodies to soluble antigen or virus particles in the milk rather than to antigens immobilized on the ELISA plates.

Check tests are normally conducted either in young stock (>9-mo-old, to avoid detection of maternally derived antibodies) or in first-lactation animals. Where persistent infection has been present in such a group for a period of weeks to months, it is expected that the seroprevalence in the non-PI animals will be high, reflecting the efficiency with which PI animals transmit infection. However, the possibility exists that the presence of serum from a PI animal in a pool of sera containing one or more seropositive animals could interfere and generate a false-negative antibody result and consequently cause an infected herd to be wrongly categorized as non-infected.

A study using a single viremic serum and an indirect ELISA kit did not find evidence of an influence on the antibody results for pooled sera, but recommended a more rigorous study be carried out.6 Our primary objective was therefore to test the hypothesis that the inclusion of viremic sera in pools of 10 sera with a variable seroprevalence (10–90%) does not lead to the incorrect serologic categorization of that pool and, consequently, the herd.

At the time of designing the study (May 2017), details of serum samples submitted to the BVD National Reference Laboratory (NRL) of the Department of Agriculture, Food and the Marine were retrieved and analyzed to identify those samples that satisfied the following criteria: 1) were born prior to 2016, and therefore seropositive because of exposure rather than maternally derived antibodies. The NS and PS pools were used to create 10 secondary pools of 180 µL with increasing seroprevalence to simulate pooled check tests containing an increasing proportion of seropositive-to-seronegative serum (Table 1). To each of these in turn, 20 µL of VS1 was added and allowed to stand at 2–8°C for at least 1 h prior to subsequent ELISA testing, with the same procedure in turn followed for VS2–VS23.

All samples were tested for BVDV antibodies and antigen using the kits described below according to the manufacturers’ instructions, and results were interpreted according to the criteria provided for individual serum samples: indirect ELISA (BVD total Ab test, IDEXX Europe); sample-to-positive control (S/P) ratio values <0.2 were considered negative, ≥0.3 positive, and intermediate values inconclusive; blocking ELISA (SVANOVIR BVDV p80-Ah, Boehringer Ingelheim Svanova, Uppsala, Sweden; samples with a % blocking of ≤45% were considered negative, and samples with % blocking >45% were considered positive); antigen ELISA (BVDV Ag/serum plus, IDEXX Europe; samples with a sample-to-noise (S/N) ratio value >0.3 were considered positive, and values ≤0.3 were considered negative).

For each ELISA kit, analysis of variance (ANOVA) was carried out to test for the effect of %PS on the observed mean test values obtained for VS1–VS23. Where the %PS proved to be significant, the pairwise differences were assessed using the Tukey studentized range test. A probability level of 5% was assumed throughout. When the assumptions for the ANOVA were not met, an appropriate transformation of the test values, assessed using Box-Cox analysis, was used. When subjected to confirmatory testing at the NRL, the

Table 1. Volumes (µL) of sera from PS and NS pools and individual VS used to simulate the presence of VS in pools of 10 sera of varying seroprevalence (0–90%).

<table>
<thead>
<tr>
<th>% seropositive sera in pool</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>70</th>
<th>80</th>
<th>90</th>
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<tbody>
<tr>
<td>Volume PS (µL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Volume NS</td>
<td>180</td>
<td>160</td>
<td>140</td>
<td>120</td>
<td>100</td>
<td>80</td>
<td>60</td>
<td>40</td>
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<td>0</td>
</tr>
<tr>
<td>Total PS+NS</td>
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<td>180</td>
<td>180</td>
<td>180</td>
<td>180</td>
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<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
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<td>20</td>
</tr>
<tr>
<td>Total PS+NS+VS1</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
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NS = antibody-negative sera; PS = virus-negative, antibody-positive sera; VS = viremic sera.
low potential for PI sera to cause false-negatives in seropools

maximum, minimum, and mean S/N ratio values of the VS were 3.30, 5.10, and 3.66, respectively.

Significant differences in S/P ratio values were recorded when VS1–VS23 were tested in pools containing various levels of pooled PS (Table 2, Fig. 1). For all 23 VS, the pool containing 0% PS tested negative. Thereafter, all pools containing ≥10% PS tested positive, with the mean value (min., max.) obtained with a pool containing 10% PS of 0.760 (0.414, 0.884). Based on the results from the ANOVA, the differences in mean test values obtained from pools with different %PS were significant (p < 0.001; Table 2). Mean values increased before reaching a plateau at 70% PS.

Significant differences in mean %blocking values were recorded when VS1–VS23 were tested in pools containing various levels of pooled PS (Table 3, Fig. 2). For all 23 VS, the pool containing 0% PS tested negative. Thereafter, all pools containing ≥10% PS tested positive, with the mean value (min., max.) obtained with a pool containing 10% PS of 80.7% (70.9–84.9%). The residuals from the ANOVA based on the raw data did not meet the assumptions of homogeneous variance and normality. Box-Cox analysis was used to identify an appropriate transformation of the test values. In order to carry out the Box-Cox analysis, negative results were set to 0.01 (slightly lower than the lowest value of the results > 0). Box-Cox analysis resulted in using a transformation of the test result to the power 1.25 in order to meet the assumptions of an ANOVA. Differences in mean %blocking in pools with various %PS were significant (Table 3). A plateau in the %blocking value generated was obtained at 40–50% PS.

Significant differences in mean S/N ratio values were recorded when VS1–VS23 were tested in pools containing various levels of pooled PS (Table 4, Fig. 3). For all 23 VS, the pool containing 0% PS tested positive. Thereafter, the number of sera testing positive decreased as the %PS increased, with 9, 2, 1, and 1 VS testing positive in pools containing 10, 20, 30, and 40% PS, respectively. All sera tested negative in the presence of ≥50% PS. The residuals from the ANOVA based on the raw data did not meet the
assumptions of homogeneous variance and normality. Box-Cox analysis was used to identify an appropriate transformation of the test values. In order to carry out the analysis, negative results were set to 0.0001 (slightly lower than the lowest value of the results > 0). Box-Cox analyses resulted in using a transformation of the test result to the power 0.25 in order to meet the assumptions of an ANOVA. No significant differences in mean S/N ratio values for the 23 VS were detected at ≥70% PS (Table 4).

Our results expand on previous findings on the potential impact of the presence of VS on the detection of antibody responses in pooled serum samples. Even in pools containing a low proportion (10%) of seropositive serum, none of the pools containing any of the 23 VS generated false seronegative results when tested with either indirect or blocking antibody ELISAs. Based on these findings, we conclude that concern over the impact of the presence of serum from a BVD PI animal does not preclude the use of pooled serum samples for serosurveillance using check tests of 10 samples per management group, even when only a single seropositive animal is present in the sampled group. Further confidence is provided by the proposal to use a cutoff point of 2 positive animals (i.e., at least 20% seropositivity) and a design prevalence of 50% (i.e., 50% seropositivity) in the context of surveillance in the Irish eradication program.

Our serologic results are in contrast to those reported previously for BTM. These differences may be attributable to differences in the type and level of immunoglobulin found in serum when compared to milk, making serum less susceptible to interference by viral antigen. Although it could be expected that antibody affinity and avidity would be more perfectly adapted to specific viruses within outbreaks, there was no evidence of this tendency across the 23 virus strains used.

The feasibility of testing pools of ear notch samples by antigen ELISA was investigated previously and found only to have an acceptable Se and Sp when no more than 2 samples per pool were included. Although not the primary purpose of our study, the findings do show that VS, when comprising only 10% of the pool, can generate positive results in the absence of antibody, but when only 10% of the pool comprised seropositive serum, the majority of the samples return a false-negative result by antigen ELISA. In the context of surveillance check tests, and given that BVDV PI animals typically generate high within-herd seroprevalences, we conclude that testing pooled serum by antigen ELISA to identify PIs is unlikely to be reliable or cost-effective.

Declarations of conflicting interests

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References


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<th>50</th>
<th>60</th>
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<th>90</th>
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<td>1.77</td>
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<td>0.25</td>
<td>0.22</td>
<td>0.14</td>
<td>0.11</td>
</tr>
<tr>
<td>Min</td>
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<td>0.01</td>
<td>0.00</td>
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<td>0.01</td>
<td>0.00</td>
<td>0.00</td>
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<td>0.37b</td>
<td>0.16c</td>
<td>0.08d</td>
<td>0.05d,e</td>
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<td>0.03f</td>
<td>0.02f</td>
<td>0.01g</td>
<td>0.01g</td>
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Table 4. Maximum, minimum, and mean S/N ratio values and number of positive results (S/N 0.3) by antigen ELISA when VS1–VS23 were tested in pools containing various percentages of pooled BVDV-seropositive serum (%PS).

BVDV = bovine viral diarrhea virus; PS = virus-negative, antibody-positive sera; S/N = signal-to-noise ratio; VS = viremic sera. Superscript letters indicate the results of subsequent Tukey test, with those mean S/N ratio values not sharing a common letter being found to be significantly different from each other.

Figure 3. Boxplots of sample-to-noise (S/N) ratio values obtained by antigen ELISA when viremic sera (VS1–VS23) were tested in pools containing various percentages (0–90%) of pooled bovine viral diarrhea virus (BVDV)-seropositive serum (%PS).
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