Milk as a diagnostic sample for the identification of bovine viral diarrhoea (BVD) infected dairy herds using a commercially available antibody ELISA

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Milk as a diagnostic sample for the identification of bovine viral diarrhoea (BVD) infected dairy herds using a commercially available antibody ELISA

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Abstract

Objective The aims of this study were to evaluate a commercially available enzyme-linked immunosorbent assay (ELISA) for the detection of bovine viral diarrhoea virus (BVDV) specific antibodies in individual milk compared to individual serum, and in bulk milk samples compared to within-herd antibody prevalence and bulk milk quantitative reverse transcription polymerase chain reaction (qRT-PCR) results.

Procedure Paired individual serum and individual milk samples were collected from 125 lactating cows and tested by ELISA and results compared. Ninety-six bulk milk samples were tested by the same ELISA, and the within-herd antibody prevalence was calculated based on milk ELISA results for 25 individual cows in each herd. Additionally, 167 bulk milk samples were tested for BVDV-specific antibodies by ELISA and for the presence of BVDV by qRT-PCR to establish the correlation between antibody result and virus presence.

Results Excellent agreement was observed between individual milk and serum results (Kappa = 0.865). The ELISA was observed to detect BVDV-specific antibodies in individual milk samples with relative sensitivity 96.6% and specificity 89.2%. The bulk milk samples revealed a strong \( r^2 = 0.95 \) relationship between the ELISA result and the within-herd antibody prevalence. The proportion of herds that tested positive by bulk milk qRT-PCR increased as bulk milk antibody S/P ratio increased.

Conclusion Commercially available ELISA testing of individual and bulk milk samples is an appropriate alternative to serum testing with good test performance in these samples. Determining a threshold for the detection of herds containing active BVD infection by testing bulk milk is a novel use for an antibody ELISA kit, and provides more practically relevant test results.
Keywords  bovine viral diarrhoea (BVD), bulk milk, enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR)

Abbreviations  BVD, bovine viral diarrhoea; BVDV, bovine viral diarrhoea virus; DSe, diagnostic sensitivity; DSp, diagnostic specificity; ELISA, enzyme-linked immunosorbent assay; PI, persistent infection; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; S/P, sample-to-positive; TG-ROC, two graph receiver operating characteristic
Introduction

Bovine viral diarrhoea (BVD) is caused by a Pestivirus (BVDV) of the family Flaviviridae.\(^1\) While acute BVDV infection in non-pregnant, immunocompetent animals generally results in no or mild clinical disease,\(^2,3\) infection during pregnancy can lead to a variety of reproductive disorders including failure to conceive, abortion, still birth, congenital deformity and the birth of calves that are specifically immunotolerant to BVDV and persistently infected (PI).\(^2,4,5\) Furthermore, acute BVDV infection has been associated with immunosuppression, causing an increase in the occurrence of other infections such as mastitis and respiratory disease in infected animals or herds.\(^6-8\) The combined immunosuppressive and reproductive effects of BVDV infection cause significant financial losses\(^9-11\) and have made BVD one of the most important viral pathogens of cattle worldwide.\(^12\) In response, many control or eradication programs are in place around the world, with particular progress having been made in Scandinavia and continental Europe.\(^13-15\) Control programs largely rely on the identification and eradication of PI animals which spread and maintain BVDV infection in cattle populations. Many strategies exist to achieve this goal, but it is commonly necessary to stratify herds into those that are likely and unlikely to contain a PI individual, and, later, to monitor participating herds to ensure there is no reintroduction of infection.\(^15-17\) Both these aims can be achieved by testing for the presence of BVDV-specific antibodies, with high or increasing antibody prevalence (that is, a majority of animals having been exposed to BVDV), indicating recent or current BVDV infection.\(^15\) Several diagnostic tests for the detection of BVDV-specific antibodies are available, and include: agarose gel immunodiffusion (AGID), enzyme-linked immunosorbent assay (ELISA) or virus neutralisation test (VNT). The strengths, weaknesses and applications of these tests have been recently reviewed\(^18\), with the ELISA commonly used as it is convenient,
inexpensive, suited to high throughput testing and does not require cell culture. Antibody
ELISAs can be used on individual or pooled serum or milk samples.
Following the use of antibody testing methods to identify herds likely to contain a PI
individual, antigen or virus detection methods can be applied to locate the PI individual(s)
that are maintaining the infection. While immunohistochemistry or virus isolation can be
used for this purpose, antigen ELISAs and reverse transcriptase polymerase chain reaction
(RT-PCR) are more common. Antigen ELISAs can be performed on individual serum, milk
or ear notch samples, while RT-PCR can be applied to either individual or pooled samples,
with PCR on a bulk milk sample having been shown to return a positive result when two of
800 milking cows in a herd were PI.19
A commercially available antibody ELISA was recently evaluated for its ability to detect
BVDV-specific antibodies in serum samples, compared with VNT and AGID testing
methods20. The present study aimed to evaluate the same antibody ELISA for use on both
individual and bulk milk samples, by: comparing the result of individual milk antibody
testing to individual serum antibody testing; by comparing the result of antibody testing of a
bulk milk sample to the within-herd antibody prevalence, and; by comparing the result of
bulk milk testing for antibody to a test for presence of BVD virus (PCR) for the herd.

Materials and Methods

Individual serum and individual milk samples were collected from 125 lactating cows from
five Australian dairy herds (twenty-five cows per herd). Both milk and serum samples were
tested by a commercially available ELISA (IDEXX BVDV Total Ab Test, IDEXX
Laboratories, Rydalmere, NSW, Australia) for the presence of BVDV-specific antibodies,
 according to manufacturer’s instructions (using the short incubation protocol). Samples were
considered positive when the sample-to-positive (S/P) ratio exceeded 0.3, as per the manufacturer’s recommended threshold for serum and individual milk.

The level of agreement between milk and serum sample results was assessed by calculation of a Kappa value using WinEpiscope 2.0 (deBlas et al., http://www.clive.ed.ac.uk/winepiscope/). The diagnostic sensitivity (DSe) and diagnostic specificity (DSP) of the ELISA for detection of specific antibodies in individual milk relative to the serum ELISA was calculated using MedCalc Version 12.7.4 (MedCalc Software, Ostend, Belgium).

The serum data was divided into bands based on the S/P ratio of each sample (band width, 0.2 S/P ratio). Mean serum and milk S/P ratios were calculated for each band and a linear regression line fitted using Microsoft Excel 2007 (Microsoft Inc., Redmont, WA).

Additional individual milk samples were collected non-selectively from twenty-five lactating cows in each of 96 Australian dairy herds. A bulk tank milk sample was also collected from each herd. All milk samples - individual and bulk - were tested for the presence of BVDV specific antibodies using the IDEXX ELISA, performed to the manufacturer’s specifications using the short incubation protocol for all samples.

The within-herd prevalence of each herd was calculated as the proportion of twenty-five cows that tested positive for antibodies against BVDV in the individual milk sample, using the ELISA manufacturer’s recommended threshold for positivity for individual milks of an S/P ratio of 0.3 or greater.

The within-herd prevalence data was divided into bands (band width: 10%), the mean bulk milk S/P ratio and mean within-herd prevalence calculated for each band and a logistic regression line fitted using Microsoft Excel 2007 (Microsoft Inc., Redmont, WA).
Further bulk milk samples were obtained from 167 Australian dairy herds. Samples were
predominantly collected for routine herd testing. All samples were tested for the presence of
BVDV specific antibodies using the ELISA, as per the manufacturer’s recommendations
using the short incubation protocol. They were also tested for the presence of BVD virus by
quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) as previously
described,\textsuperscript{19} with a threshold cycle time (ct) <36 in a protocol of 45 cycles considered
positive (to discount transient infections, non-specific reactions or contamination) indicating
the presence of active BVDV infection in the herd. This PCR protocol was demonstrated by
Hill et al. (2010)\textsuperscript{19} as having an analytical sensitivity of 1:1,000, and a capability to detect
one or two PI individuals contributing to a bulk milk in herds of up to 275 (one PI) or 800
two PIs) cows. Repeat PCR testing after removal of all PI individuals returned negative
results. A PCR bulk milk negative herd was also tested by alternative assays to the individual
cow level with all negative results, thus giving confidence in the diagnostic specificity.

A two graph receiver operating characteristic (TG-ROC) analysis (CDMT, Free University of
Berlin, Germany) was performed on the bulk milk antibody ELISA results, using qRT-PCR
results as the reference standard to determine the threshold S/P ratio for detecting herds with
PCR positive bulk milk results at which both DSe and DSp were equal, as well as
intermediate thresholds such that DSe or DSp attained values of 90%. The threshold required
to achieve 100% DSe and 100% DSp were also calculated. The observed DSe and DSp at
each threshold value were calculated using MedCalc Version 12.7.4 (MedCalc Software,
Ostend, Belgium).

The proportion of herds testing PCR positive at varying bulk milk antibody S/P ratios were
calculated in Microsoft Excel 2007 (Microsoft Inc., Redmont, WA).
Results

Comparison of serum and milk ELISA at the individual level

Using the manufacturer’s threshold for positivity of an S/P ratio > 0.3, a comparison of 125 individual milk and serum results showed 94.4% (95% CI: 90.4 – 98.4%) concordance, with 85 (68.0% [95% CI: 59.8 – 76.2%]) cows testing positive by both serum and milk, and 33 (26.4% [95% CI: 18.7 – 34.1%]) testing negative by both samples. Three individuals (2.4% [95% CI: 0 – 5.1%]) tested positive by serum and negative by milk, while a further four (3.2% [95% CI: 0.1 – 6.3%]) cows tested negative by serum and positive by milk (Table 1).

This resulted in a high level of agreement (Kappa) of 0.865 (95% CI: 0.689 – 1.040), an observed DSe of 96.6% (95% CI: 90.4 – 99.3%) and DSp of 89.2% (95% CI: 74.6 – 96.9%), for individual milks relative to serum testing.

A strong, positive, linear relationship ($r^2= 0.93; \text{slope}= 0.92$) between mean milk S/P ratio and mean serum S/P ratio was observed (Figure 1).

Bulk milk ELISA testing

Ninety-three (96.9% [95% CI: 93.9 – 99.9%]) of the ninety-six dairy herds studied showed some level of exposure to BVDV with at least one (of twenty-five) cows testing positive for BVDV specific antibodies in an individual milk sample tested by ELISA. The observed within-herd prevalence ranged from 0% to 100%. Using the manufacturer’s recommended cut-off threshold for bulk milk (S/P ratio>0.2), the ELISA was able to detect herds with a within-herd prevalence >0, with a DSe and DSp of 93.6% (95% CI: 86.5 – 97.6%) and 100.0% (95% CI: 30.5 - 100.0%), respectively.
A strong, positive, logarithmic relationship was observed between within-herd prevalence of exposure (antibody) and bulk milk ELISA result ($r^2$: 0.9511) (Figure 2).

**Bulk milk antibody ELISA versus PCR**

Sixteen South Australian dairy herds (9.6% [95% CI: 4.4 – 14.8%]) out of 167 tested positive for the presence of BVDV when tested by qRT-PCR. The corresponding S/P ratios of these PCR-positive bulk milk samples, when tested for BVDV specific antibodies by ELISA, varied from 0.28 to 1.56, suggestive of within-herd antibody prevalence ranging from <10% to 100%.

An optimal threshold for bulk milk ELISA result interpretation (for the detection of virus-positive herds) was determined by TG-ROC analysis as an S/P ratio >0.79, such that the theoretical DSe/DSp=64.0%, with intermediate range thresholds of S/P ratio >0.37 and >0.98 to give DSe=90% and DSp=90% respectively (Table 2). In order to achieve 100% DSe or 100% DSp respectively, thresholds for the bulk milk S/P ratio of 0.28 and 1.75 respectively, must be used (Table 2).

An increasing trend was observed, with the proportion of herds that tested positive by bulk milk qRT-PCR increasing with rising bulk milk antibody S/P ratio (Figure 3). At very high antibody levels (bulk milk S/P ratio>1.4), 40.0% (95% CI: 31.4 – 48.6%) of the herds in this study tested positive for BVDV by bulk milk qRT-PCR.

**Discussion**

The data presented here indicate that individual milk samples are an appropriate alternative to serum samples for the detection of antibodies specific to BVDV when using a commercially available ELISA (IDEXX BVDV Total Ab Test, IDEXX Laboratories, Rydalmere, NSW, 2065).
Australia). This assay achieved 96.6% (95% CI: 90.4 – 99.3%) and 89.2% (95% CI: 74.6 – 96.9%) DSe and DSp, respectively, relative to serum testing, when used to test milk samples obtained from individual cows.

The high levels of agreement (kappa: 0.865 [95% CI: 0.689 - 1.040]) and concordance (94.4% [95% CI: 90.4 – 98.4%]) observed between milk and serum samples were supported by a strong, linear relationship ($r^2 = 0.93$) between the S/P ratios of milk and serum samples. Furthermore, the slope of the observed linear relationship (slope = 0.92) is close to one, suggesting that interpretation of milk S/P ratios may be carried out in a similar manner to serum S/P ratios. The lack of any observable plateau in either milk or serum S/P ratios suggests that this relationship hold true, even at very high antibody levels, such as those observed shortly after seroconversion.

As such, individual milk samples offer a convenient and animal welfare-friendly alternative to blood collection and serum testing. In particular, collection of milk samples can be undertaken by dairy farm workers, minimising veterinary costs associated with testing individual cattle for BVDV specific antibodies.

Having established that this ELISA performs well on individual milk samples, such samples were used to assess the within-herd prevalence of BVDV-specific antibodies in ninety-six dairy herds. The observed within-herd prevalence ranged from 0% to 100%, with 96.9% (95% CI: 93.9 – 99.9%) of herds showing within-herd prevalence >0. This is in line with reported levels of infection and exposure with BVDV in Australia\textsuperscript{21,22}. As a result, in areas of endemic BVDV infection (such as Australia), large proportions of herds are likely to return a positive result when a diagnostic test is calibrated to detect herds with within-herd prevalence >0. This ELISA applied to bulk milk samples was shown to be capable of detecting herds with a within-herd antibody prevalence >0 with DSe and DSp of 93.6% (95% CI: 86.5 – 100%).
97.6\%) and 100.0\% (95% CI: 30.5 \textendash 100.0\%), respectively, at the manufacturer’s cut-off threshold of an S/P ratio of \(>0.2\).

The strong correlation of bulk milk result to within-herd prevalence observed here \(r^2: 0.9511\) and recently by Eiras \textit{et al.},\(^{23}\) allows for a quick assessment of herd immunity (and possibly the need, or not, for vaccination), as well as for monitoring antibody levels within a herd through regular testing of bulk milk by ELISA. This also might suggest that a cut-off threshold may not be necessary at all when testing bulk milk samples, with a direct estimate of the within-herd prevalence being able to be obtained from a bulk milk antibody ELISA result. This alternate interpretation of bulk milk antibody ELISA results may allow differentiation within the cohort of herds that would test positive under traditional interpretation. An increase in the S/P ratio, indicative of an increase in the proportion of cows that have been exposed to BVDV (and hence are antibody positive), may signal the introduction of a new BVDV infection into the herd. Importantly, bulk milk antibody testing may warn of infection in the herd despite a negative bulk milk RT-PCR result as a result of the infective source (normally, a PI animal) not contributing to the bulk milk tank (e.g. a bull, heifer or dry cow). This is, practically, a far more relevant application of this test when applied to bulk milk samples than detection of herds with within-herd antibody prevalence \(>0\).

Indeed, if a cut-off threshold was to be applied to bulk milk testing for BVDV antibodies, a threshold suitable for detecting herds containing a PI individual may be of greater practical value than a threshold for detecting the presence of low levels of within-herd antibody prevalence.

TG-ROC analysis of bulk milk antibody ELISA results against PCR positivity determined a threshold for an S/P ratio of 0.79 to suit this purpose while optimising both, DSe and DSp. Interestingly, this is the same threshold for this ELISA proposed by Eiras \textit{et al.}\(^{23}\) to identify...
herds with 65% within-herd prevalence and, therefore, suspected of harbouring active infection. However, Eiras et al.\textsuperscript{23} observed higher DSe (89%) at this threshold than observed in the present study (64%). This is primarily due to 5.7% (95% CI: 1.6 – 9.8%) of qRT-PCR positive (actively infected) herds showing a bulk milk antibody ELISA result below the 0.79 threshold in the present study (as compared to 2.3% observed by Eiras et al.\textsuperscript{23}), and only 16.1% (95% CI: 9.7 – 22.5%) qRT-PCR positive herds with S/P ratio greater than 0.79 (as opposed to 33.4% observed by Eiras et al.\textsuperscript{23}). However, the true prevalence of actively infected herds in the present study is likely higher than revealed by bulk milk PCR, as bulk milk testing will not detect infection in non-milking cohorts.

Indeed, the lower antibody levels observed in virus-positive bulk milk samples here are lower than those observed by Eiras et al.\textsuperscript{23} In the Eiras et al. study, the presence of BVDV in herds with antibody seroprevalence as low as 26.1% was observed, while in the present study, the lowest antibody ELISA results observed in a qRT-PCR positive herd was 0.28, suggestive of a within-herd prevalence below 10%.

This spread of bulk milk antibody results in qRT-PCR positive herds (with S/P ratios ranging from 0.28 to 1.56) demonstrates that there is no “safe” threshold for the identification of herds containing BVDV infection, at which acceptable DSe and DSP can, both, be achieved. At maximum DSe (below an S/P ratio of 0.27) DSP was just ~30%. However, in most applications DSe is the important driver, as the identification of all herds potentially containing a PI is likely to be most beneficial.

Therefore, bulk milk antibody testing may be a valuable first step in identifying herds that contain a PI individual. When a bulk milk sample returns an antibody S/P ratio \( \geq 0.28 \), follow up testing initially with bulk milk RT-PCR seems always warranted. Meanwhile, herds with an S/P ratio <0.28 may be assumed uninfected (although follow up bulk milk antibody testing at a later time point is valuable to ensure herd has not undergone recent virus incursion). All
herds with an S/P ratio >0.98 (the upper 90% intermediate range threshold) should be treated as suspect, even when bulk milk RT-PCR returns a negative result. These herds are highly likely to be infected.

The DSp of this bulk milk ELISA test will always be limited by herds with high within-herd antibody prevalence due to recent (but not current) infection, such as herds that have recently experienced the phenomenon of self-clearance. These herds will continue to test positive by bulk milk antibody ELISA for some time, despite not currently containing a PI individual. Despite these limitations, this novel application of a bulk milk antibody ELISA threshold for detection of herds likely to contain a PI individual provides a valuable tool in the ongoing attempts to control and eradicate BVDV, with it having particular value for routine monitoring.

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References


Tables

Table 1

Results of testing 125 Australian dairy cows for presence of specific antibodies against bovine viral diarrhoea virus (BVDV) in individual serum and milk samples by commercially available enzyme-linked immunosorbent assay (ELISA) using the manufacturer’s recommended cut-off thresholds (Positive = sample-to-positive ratio >0.3). Kappa: 0.865 (95% CI: 0.689 – 1.040).

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<td>Total</td>
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DSe\textsuperscript{a} = 96.6% (95% CI: 90.4 – 99.3%)

DSp\textsuperscript{b} = 89.2% (95% CI: 74.6 – 96.9%)

\textsuperscript{a}Diagnostic sensitivity

\textsuperscript{b}Diagnostic specificity
The observed diagnostic sensitivity (DSe) and specificity (DSp) (with 95% confidence intervals) of a commercially available bulk milk bovine viral diarrhoea virus (BVDV) antibody enzyme linked immunosorbent assay (ELISA\textsuperscript{a}) for detection of herds returning a positive result when tested by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), and thus assumed to be actively infected with BVDV, when using thresholds determined by two-graph receiver operating characteristic (TG-ROC) analysis to meet various criteria.

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<th>DSp\textsuperscript{c} (%)</th>
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\textsuperscript{a}Diagnostic sensitivity

\textsuperscript{b}Confidence interval

\textsuperscript{c}Diagnostic specificity
Legend of Figures

Figure 1. The relationship between the mean sample-to-positive (S/P) ratio of individual milk samples and mean S/P ratio of individual serum samples from 125 lactating dairy cows, when tested by commercially available enzyme-linked immunosorbent assay (ELISA) for the presence of specific antibodies against bovine viral diarrhoea virus (BVDV). Error bars = 95% confidence intervals. $r^2 = 0.9254$.

Figure 2. The relationship between the mean S/P ratio of 96 bulk milk samples tested by commercially available enzyme-linked immunosorbent assay (ELISA) for the presence bovine viral diarrhoea virus (BVDV) specific antibodies and the mean within-herd prevalence of BVDV specific antibodies, as determined by testing individual milk samples from twenty-five non-selectively sampled cows. Error bars = 95% confidence intervals. S/P = sample-to-positive. $r^2 = 0.9511$.

Figure 3

The proportion of herds testing positive for bovine viral diarrhoea virus (BVDV) in bulk milk by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) at varying levels of within-herd antibody prevalence, evidenced by the sample-to-positive (S/P) ratio achieved by testing the same bulk milk sample by commercially available enzyme-linked immunosorbent assay (ELISA) for detection of BVDV specific antibodies.