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Detection of cows carrying a persistently BVDV ("pestivirus") infected foetus

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Abstract

Persistently infected (PI) calves carried by non-PI dams are pivotal for the maintenance of BVD virus (‘bovine pestivirus”) in cattle populations and are hitherto undetectable before they are born. Female cattle are often traded when they are pregnant and the possibility of buying in a PI-carrying (“Trojan”) dam presents an appreciable biosecurity risk for the purchaser.

Up until now, detection of a PI calf has to solely rely on post-natal diagnosis and detection of BVD virus in the calf, often by testing ear notches with antigen capture ELISA or PCR.

The present study created BVD PI calves, followed and monitored their dams throughout gestation and developed a testing protocol, based on antibody-detection in the dam that can differentiate the PI-carrying dam from her non-PI carrying counterparts.

Executive summary

Infection with BVDV during early gestation can result in a variety of pregnancy outcomes including abortion, stillbirth and the birth of neurologically affected or immunotolerant, persistently infected (PI) calves. PI animals play a pivotal role in the epidemiology of the disease, being the source of infection in most outbreaks. Seventeen pregnant heifers were infected with BVDV via exposure to a PI cow at approximately day 69-90 of gestation. Heifers were followed throughout gestation and serum, nasal, saliva and vaginal swabs collected weekly, as well as ear notch samples every four weeks from 7 days prior to exposure until 6 weeks post-calving. Samples were analysed by antibody and antigen enzyme-linked immunosorbent assay (ELISA), agarose gel immunodiffusion (AGID) and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). All heifers seroconverted within 21 (AGID) to 28 days (ELISA) post-exposure, and sub-positive levels of BVDV antigen were detected in 5 (of 17) heifers on days 7 to14 post-exposure. While the starting hypothesis of the trial was aimed at the possibility of detecting BVD virus or viral antigen in the dam's blood or secretions, none was detected in any samples collected after seroconversion was complete.

However, heifers carrying PI foetuses were observed to have significantly higher antibody levels by ELISA than heifers carrying non-PI calves consistently from day 77 post-exposure (days 146 to 167 of gestation) onwards, while AGID showed no differences between these groups. Heifers carrying calves with neurological deficits tended to exhibit lower antibody levels by ELISA than other heifers, but this difference was not significant.

There is potential for the antibody difference between heifers carrying PI foetuses and those carrying non-PI foetuses to be used as a method of pre-natal diagnosis, with an observed diagnostic sensitivity of 100% and specificity of ≥70%. The full description of the method was published and a copy of the manuscript is appended.

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1 Background

Bovine viral diarrhoea virus (BVDV) infection has been shown to have significant economic impacts on infected herds. Losses may stem primarily from an increase in the incidence and severity of secondary disease, such as Bovine Respiratory Disease (BRD) in feedlots, due to an immunosuppressive effect (Brackenbury et al., 2003) and from reproductive losses, including reduced fertility, foetal death and subsequent abortion, stillbirth, or neurological deformations in the developing foetus (Grooms, 2004). There may also be calves that are immunotolerant to BVDV and as a consequence persistently infected (PI) with the virus (Brownlie et al., 1987). In general, PI calves are thought to result from infection prior to 120 days gestation (Brownlie et al., 1987), while infection after this time tends to produce calves that are seropositive at birth (Grooms, 2004). Abortions, or calves with neurological deformities such as hydrocephalus and cerebellar hypoplasia (Trautwein et al., 1986) generally occur following infection between 90 to 150 days of gestation (Grooms, 2004; Trautwein et al., 1986). There is some variation in the timing at which a particular outcome will result as illustrated by a report of a PI calf born with a seropositive twin (Schoder et al., 2004). Infection during the third trimester of pregnancy generally does not affect the foetus. Persistently infected animals are epidemiologically important as a primary source of infection with a persistently high viral excretion rate. Pre-natal detection of a cow or heifer carrying a PI calf would enable this potential source of infection to be removed from the herd or avoided at the point of entry. This would enhance biosecurity to protect naïve herds and assist in eliminating the infection from an already infected herd. Brownlie *et al.* (1998), Lindberg *et al.* (2001) and Stokstad *et al.* (2003) have demonstrated that females carrying a PI foetus have higher levels of BVDV-specific antibodies than females carrying non-PI foetuses. However, attempts to use this difference for diagnostic purposes met with limited success; generally, diagnostic specificity was compromised in order to achieve acceptable diagnostic sensititivity. On rare occasions, however virus detection in the dam's blood has also been reported and would, if repeated, provide more specific, individual dam diagnostic possibilities. Due to the highly infectious nature of PI calves, the ability to accurately differentiate females carrying a PI calf from those carrying a non-PI calf could have important implications in BVDV control. The serological antibody profiles of females that experience abortion or stillbirth or deliver calves with neurological deficits following acute BVDV infection during gestation have not been reported.

2 Project objectives

The aims of this study were to investigate the serological antibody profile in heifers **with varying gestational outcomes following infection during early gestation, and to explore** opportunities for pre-natal diagnosis of PI in the foetus by antibody, antigen and/or virus detection in the dam during gestation.

3 Methodology

3.1 Animals

Twenty-three Angus and Angus cross heifers were oestrus synchronised and artificially inseminated (AI), then naturally mated for approximately three weeks (one cycle). Seventeen heifers were confirmed pregnant by per rectal ultrasound 90 days

post-AI (approximately day 69 of gestation for heifers that conceived to natural mating). The heifers were confirmed naïve to BVDV by negative results in both antibody (Ab) and antigen (Ag) enzyme-linked immunosorbent assay (ELISA) and AGID. These heifers formed the experimental group.
A two year old dairy cow, PI with BVDV 1c was sourced locally (status confirmed by consecutive positive results on Ag ELISA and negative results of Ab ELISA and strain confirmed by sequencing (results not shown)).

3.2 Infection with BVDV

The pregnant heifers were naturally acutely infected with BVDV 1c under experimental conditions via exposure to and co-mingling with the PI cow from days 90 to 118 post-AI (day 90 post-AI = day 0 post-exposure). Co-mingling was conducted at a density of 24 m^2/animal. On day 22 post-exposure, nasal mucous was transferred from the PI cow to the experimental heifers by nasal application of a rag to the PI cow and then to each experimental heifer, with reapplication to the PI cow in between each heifer to ensure all heifers became infected.

3.3 Gestational outcomes

The gestational outcome of each heifer was recorded as one of the following: abortion, neonatal calf death, live healthy calf, live PI calf, or live calf with neurological deficits. A heifer was considered to have aborted if: a) an abortion was observed (and fetus recovered), b) the heifer was found not pregnant by subsequent *per* rectal palpation, or c) the heifer failed to calve (and was subsequently found not pregnant). A neonatal calf death was recorded if the calf was found dead following parturition. A live calf was considered PI when: either, pre-colostral samples returned a positive result by Ag ELISA, or serum collected at 14 days of age returned a positive result by qRT-PCR; and, Ab ELISA returned a negative result on pre-colostral serum (where available). A live calf was considered to have a neurological deficit if clinical neurological signs were apparent. Remaining live calves were considered healthy.

3.4 Sample collection and storage

Serum samples, and nasal, vaginal and saliva swabs were collected from each heifer on days -7, 0, 5, 7, 9, 14, 21, 25 and 28, post-exposure, then weekly until six weeks post-calving. Swab samples were collected by application and repetitive stroking of a rayon tipped swab over the mucosal surface. In addition, ear notch samples were collected from each heifer every four weeks from day -7 post-exposure until six weeks post-calving using an Allflex Tissue Sampling Unit (Allflex Australia Pty Ltd. Capalaba, Queensland, www.allflex.com.au). All the samples were stored at -80°C until processing. At processing, all the ear notches were soaked in 250µL IDEXX ear notch tissue soaking buffer (IDEXX Laboratories Inc. Rydalmere, NSW) for 24 +/- 1 hour at room temperature. Nasal, vaginal and saliva swabs were processed by soaking of the swab tip in 1mL IDEXX ear notch tissue soaking buffer for 24+/- 1 hours at room temperature. After soaking, ear notches and swab tips were removed from the supernatant and both supernatant and sample were stored separately at -80°C until testing.

3.5 Testing for BVDV specific antibodies

3.5.1 ELISA

Serum samples were tested for the presence of BVDV specific antibodies using commercially available Ab ELISA (IDEXX BVDV Total Ab ELISA, IDEXX Laboratories Inc. Rydalmere, NSW), performed according to manufacturer's instructions.
Avidity of antibodies was measured by the addition of an extra incubation and wash step following the 90 minute sample incubation and wash: one duplicate was incubated with approximately 300µL wash solution at room temperature for 5 minutes, while the corresponding duplicate was incubated with 100µL 8M urea at 37°C for 5 minutes. Both duplicates were washed, before continuing with the conjugate incubation specified by the standard ELISA procedure. Avidity for a particular sample was calculated as: $\frac{\frac{S}{P}ratio\ with\ urea\ treatment}{\frac{S}{P}ratio\ without\ urea\ treatment} * 100\%$.

3.5.2 AGID

Weekly serum samples from the first twelve weeks post-exposure, and fortnightly throughout the remainder of the study were also tested for the presence of Pestivirus specific antibodies by agarose gel immunodiffusion (AGID), using C24V BVDV reference strain as antigen. An AGID score of 1, 2, 3 or 3+ was considered positive. A 3+ result was recorded as 4 for statistical analysis and a negative result was recorded as 0. All AGID testing was performed by the New South Wales Department of Primary Industries (Elizabeth Macarthur Agricultural Institute, Menangle, NSW).

3.6 Testing for BVD virus and specific antigen

Selected serum samples, and swab and ear notch supernatants were tested for BVD virus by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), as previously described(Hill et al., 2007). In addition, all serum samples and selected ear notch supernatants were tested for BVDV specific antigen by commercially available Ag ELISA (IDEXX BVDV Serum/Ag Plus ELISA, IDEXX Laboratories Inc. Rydalmere, NSW), as per the manufacturer's instructions. Selected swab supernatant samples were also tested by Ag ELISA, with 50µL supernatant incubated with 50µL detection antibodies, as is the manufacturer's recommended protocol for ear notch supernatants. Results were expressed as corrected optical density (OD), with an OD > 0.3 considered positive.

3.7 Statistical analyses

Differences in antibody levels between heifers carrying PI calves and those carrying non-PI calves were assessed using a two-tailed student's t-test, with a p-value <0.05 considered significant, for both ELISA and AGID results.
The observed diagnostic sensitivity (DSe)($\frac{\#\ heifers\ carrying\ PI\ calves\ that\ tested\ positive}{\#\ heifers\ carrying\ PI\ calves}$), and observed diagnostic specificity (DSp)($\frac{\#\ heifers\ carrying\ non-PI\ calves\ that\ tested\ negative}{\#\ heifers\ carrying\ non-PI\ calves}$) were calculated for diagnosis of calf PI status by Ab ELISA at each of eight different positivity thresholds (0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 S/P ratio) at each time point using MedCalc for Windows version 12.7.7 (MedCalc Software, Ostend, Belgium).

The Youden Statistic was also calculated at each combination of threshold and time point as: DSe + DSe – 100%. The Youden Statistic, DSe and DSp for diagnosis of calf PI status by AGID were similarly calculated at a positivity threshold of >3 (i.e. a 3+ result was considered positive). Approximate week of gestation of time points was back-calculated from day of calving, with heifers assumed to have calved at 40 weeks gestation. Heifers that aborted (n=4) were excluded from this analysis.

Results and discussion

4.1 Results

4.1.1 Gestational outcomes

One abortion was observed on day 253 post-AI, and the foetus recovered and found to be PI. Additionally, one heifer was found not pregnant by palpation at day 222 post-AI (following confirmation of pregnancy by palpation at day 133 post-AI) and two heifers failed to calve (despite confirmation of pregnancy by palpation at day 222 post-AI) and were subsequently found not pregnant. Of the heifers that carried their pregnancies to term, three delivered live, calves with neurological deficits, three delivered live PI calves, six delivered apparently healthy calves and there was one neonatal calf death. Gestational outcomes are summarised in Table 1. One of the healthy calves had apparent cloudiness and impaired vision in both eyes at birth, however the cloudiness receded and vision appeared to improve over the first four months of life. The calf which died in the neonatal period was observed to have superior brachygnathism (relative prognathism) with no other abnormalities revealed on post-mortem examination.
The calves with neurological deficits (n=3) exhibited a range of clinical signs. The most severely affected calf was laterally recumbent with muscular tremors and a weakened suck reflex, and was euthanised soon after birth. The two less severely affected calves were bright and alert, and were unable to stand at birth but were able to stand unaided at 24 hours and 10 days of age, respectively. As the ability of these calves to stand and walk developed, ataxia and wide-based stances became apparent. These calves were euthanized at approximately 4 months of age. Post-mortem examination revealed cephalic dysplasia, hydrocephalus, and cerebellar aplasia in all three clinically affected calves.

4.1.2 Seroconversion

Antibodies were detected in one (5.9%), eight (47.1%), 16 (94.1%) and 17 (100%) of heifers by days 14, 21, 25 and 28 post-exposure, respectively by ELISA and in all heifers by AGID on day 21 after exposure (Figure 1 + 5).

4.1.3 BVDV specific antibodies in heifers following acute infection

4.1.3.1 ELISA

Antibody levels (S/P ratio) continued to rise in all heifers throughout gestation, until day 175 post-exposure (the last sampling time point before the birth of the first calf) (Figure 2). Antibody levels began to decline at around the time of calving, with a steeper decline observed in heifers that had delivered PI calves than those that delivered non-PI calves (Figure 3). A rapid decline in antibody levels post-calving was observed in heifers that delivered a calf with neurological deficits (Figure 3),

however, this decline did not result in those heifers exhibiting significantly lower antibody levels than heifers that delivered healthy calves.
The mean antibody levels were significantly higher in heifers carrying a PI calf, than those carrying non-PI calves at days 28 and 63 post-exposure, and consistently from day 77 post-exposure throughout gestation, and until seven weeks post-calving ($p<0.05$). By eight weeks post-calving, antibody levels in cows that carried PI calves had returned to levels that were not significantly different from cows that carried non-PI calves.
The avidity of BVDV specific antibodies increased steadily from days 28 to 168 post-exposure, with no significant differences between heifers with different gestational outcomes (Figure 4).

4.1.3.2 AGID

When tested by AGID, the mean result of all heifers continued to rise from 2.17 (95% CI: 1.79 – 2.56) at day 21 post-exposure (the first time-point at which positive results were observed) to 3.47 (95% CI: 3.09 – 3.85) at 42 and 49 days post-exposure. The mean AGID score of all heifers then declined until day 77 post-exposure, before remaining between 2.9 and 3.2 for the duration of the trial (that is, until day 252 post-exposure) (Figure 5). No significant differences were observed between mean AGID score of heifers that delivered a PI calf and heifers that delivered a non-PI calf.

4.1.4 Detection of BVD virus/antigen in heifers following acute infection

Five heifers showed sub-positive peaks in Ag ELISA results on days 7, 9 or 14 post-exposure, with one heifer returning a weak positive result on day 9 post-exposure (Figure 6). Two of the heifers exhibiting antigen peaks delivered live PI calves, while an additional two aborted (one of which was observed to abort a PI foetus). The fifth heifer exhibiting an antigen peak delivered a healthy calf. A serum pool containing contributions from all seventeen heifers of day 9 post-exposure returned a positive qRT-PCR result.

No positive qRT-PCR or Ag ELISA results were observed for any sample from any heifer after day 21 post-exposure.

4.1.5 Application of antibody levels for pre-natal diagnosis of persistent infection

4.1.5.1 ELISA

When using antibody levels for pre-natal diagnosis of foetal PI, 100% DSe for the detection of heifers carrying PI calves was observed for approximately the last 24 weeks of gestation when the threshold for positivity (that is, the threshold at which an Ab ELISA result is considered positive) was set at 0.6 S/P ratio. Conversely, 100% DSp was observed at 28 of the 29 timepoints prior to calving when the positivity threshold was set at 2.0 S/P ratio. Declining antibody levels observed in the two weeks prior to calving resulted in a decrease in DSe at these timepoints when thresholds ≥ 1.6 S/P ratio were applied. In general, DSe and DSp ≥ 80% were observed simultaneously for limited time periods at any given threshold, the longest period being approximately 11 weeks (from weeks 28 to 38 of gestation, inclusive) at a threshold of 1.6 S/P ratio (Table 2). The corresponding Youden statistics are shown in Table 3. A Youden statistic of 100%, signifying simultaneous 100% DSe and 100% DSp, was achieved on only five occasions.

Figure 6 shows the highest threshold at which 100% DSe was observed for each timepoint from approximately 16 to 38 weeks gestation, and the corresponding DSp. The highest threshold at which 100% DSe was achieved was observed to increase as gestation progressed. The DSp observed at these thresholds was variable (20% - 80%) in earlier gestation (< 21 weeks). After approximately 21 weeks gestation, DSp ≥ 0.7 was observed and maintained for the remainder of gestation.

Heifers which aborted were excluded from this analysis. However, an observed abortion was recorded in one heifer, and the foetus recovered and found to be PI. This heifer, prior to the abortion event at approximately week 36 of gestation, returned positive results at the thresholds presented in Figure 7 at 4 (19.0%) out of 21 sampling time points (weeks 18, 19, 20 and 34 of gestation).

4.1.5.2 AGID

When considering an AGID score of 3+ to be indicative of a heifer carrying a PI foetus, DSe was lower than that observed when using the ELISA, with one (of three) PI-carrying heifers returning a positive (3+) result on only one occasion. The remaining two heifers returned strong positive results consistently from week 23 of gestation onwards. Similarly, DSp was lower when using the AGID than when using Ab ELISA as nine of ten heifers returned a positive (3+) result on at least one timepoint and several maintained 3+ results until the late stages (up until week 38) of gestation, producing "false positive" results.

4.2 Discussion

In this trial, acute BVDV infection was established in the experimental heifer group following exposure to a PI cow at day 90 post-AI, with positive antigen results observed in some (n=5) heifers, and seroconversion evident in all (n=17). The timing of the (albeit small) antigen peak at between days 7 and 14 (majority at day 9) post-exposure is consistent with previous literature (Raizman et al., 2011). Similarly, seroconversion was observed between 14 and 28 days post-exposure by ELISA and at day 21 by AGID, which is consistent with the timing of seroconversion observed by Raizman *et al.* (2011) and Tsuboi *et al.* (2013). The majority of the heifers in this trial (15 out of 17) seroconverted within five days of each other by ELISA (days 21 to 25 post-exposure), and all seroconverted at the same timepoint (day 21 post-exposure) when measured by AGID. This may suggest that the heifers contracted the infection within a tight time frame (potentially within a few days following initial exposure). This is not surprising, given the high BVDV challenge from the PI animal, and the high stocking density during the co-mingling. Incidence of infection has previously been reported to be as high as 96% in 6 months in herds of barn housed cattle containing a PI individual (Houe et al., 1993).

The results of the present study are consistent with the findings of previous reports (Brownlie et al., 1998; Lindberg et al., 2001; Stokstad et al., 2003) that females carrying a PI foetus have significantly higher levels of BVDV-specific antibodies compared to females carrying a non-PI foetus. In this study, the difference between the heifers carrying PI foetuses and those carrying non-PI foetuses (when tested by ELISA) was statistically significant from 77 days post-exposure (146 to 167 days gestation) onwards. This agrees with results observed by Stokstad *et al.* (2003) where a statistically significant difference in antibody levels was observed from day 135 gestation (approximately day 54 to 61 days post-infection) onwards.

The AGID results did not replicate this finding, and no significant differences were observed between heifers carrying a PI calf or not.

In contrast to previous studies, the antibody levels of non-PI carrying heifers continued to rise throughout gestation when measured by Ab ELISA. Stokstad *et al.* (2003) observed consistent, low antibody levels in females that were carrying non-PI foetuses, while the present study demonstrated a continual rise in Ab ELISA results in all heifers until calving, with those carrying PI foetuses rising faster than those carrying non-PI foetuses. Mean AGID results remained consistently high throughout gestation. The present study showed a rapid rise in antibody levels immediately following seroconversion when measured by both Ab ELISA and AGID, with the ELISA results remaining at that high level and the AGID results decreasing slightly after the acute period. Lindberg *et al.* (2001) also failed to demonstrate a continuing rise in Ab ELISA levels over the course of gestation in females carrying non-PI foetuses, although the time since acute infection in those females was unknown. The continuing increase in Ab ELISA results following acute infection in those heifers carrying non-PI foetuses observed in the present study is more in line with titres rising for at least 10 to 12 weeks post-infection (Brownlie et al., 1987) to reach high antibody levels consistent with serological evidence of 'recent infection' (Lanyon et al., 2013). It is important to note that the heifers carrying non-PI foetuses represent the cohort from which it would be most difficult to distinguish dams carrying a PI foetus: the highest antibody levels in an animal (other than when carrying a PI foetus) are expected to occur shortly after acute infection (Brownlie et al., 1987). It is at this time that an individual could generate sufficiently high antibody readings to produce a false positive result with regards to identifying a heifer carrying a PI foetus. In particular, a recently seroconverted female carrying a non-PI foetus may be indistinguishable from a female in early- to mid-gestation carrying a PI foetus. Indeed, this was observed to be the case when diagnosis using AGID was attempted with several heifers returning 3+ results several months after seroconversion (and well into their pregnancies) despite carrying non-PI calves. In fact, the results of the present study suggest 3+ AGID results may persist much further than the 1-3 months previously suggested (Kirkland and MacKintosh, 2006; McGowan and Kirkland, 1991). By Ab ELISA, the lower thresholds utilised to achieve DSe in early- to mid-gestation are likely to result in lower DSp than that observed in the current study. This agrees with observations by Lindberg *et al.* (2001) that only poor DSp was achieved earlier in gestation (likely due to this very reason). As such, a high threshold (for example, 1.6 S/P ratio) combined with testing only in the last 12 weeks of gestation may be the most practical application. Any pregnant female returning an antibody result over 1.6 S/P ratio should be treated with suspicion.

In the current study, heifers carrying foetuses with neurological deficits (n=3) tended to have lower Ab ELISA results than heifers carrying non-affected calves, however, this difference was not statistically significant; this is the first report of such a finding and further research in a study with a larger number of animals may add clarity to this finding. It may be hypothesised that the induction of cellular apoptosis in the developing foetus (in turn resulting in neurological deformation) may accelerate the clearance of the virus from the dam-foetus unit. This might reduce the effective duration of viraemia and, in turn, the magnitude of the immune response of the heifer to infection. It should be noted that the heifer in this study that was carrying the foetus with the most severe neurological deficit also had the lowest antibody levels throughout gestation, and could have resulted in an artificially low mean antibody results for this group.

For other diseases of cattle, such as *Neospora caninum*, measurement of antibody avidity has allowed differentiation of recent and chronic exposure to the pathogen

(Bjorkman et al., 1999). A female carrying a PI foetus may be subject to continued immunogenic stimulus due to the excretion of BVDV by the foetus. Hypothetically, this ongoing stimulus could mimic the condition of chronic exposure and, hence, high avidity of specific antibodies. However, this study has shown this not to be the case, with no differences in antibody avidity observed between heifers with different gestational outcomes. Antibody avidity was observed to increase over time, consistent with the immune response maturing following infection (Bjorkman et al., 1999).

Non-PI females carrying PI foetuses, such as those induced in this study, have the potential to introduce BVDV infection into BVDV-free herds. Although the dam herself poses no infectious risk, the birth of the PI calf in a naïve herd could result in an epidemic BVD outbreak with significant financial impact. As such, the simple, accurate detection of females carrying PI foetuses could have important implications for BVDV control and prevention. The results of this study demonstrate that serological antibody levels in the dam as measured by Ab ELISA can be used for the diagnosis of foetal PI, while AGID testing is less successful. For the Ab ELISA, positivity thresholds at different stages of gestation were set such that 100% (95% CI: 30.5 – 100.0%) DSe was achieved (that is, all three heifers carrying PI foetuses returned results above the positivity threshold), as maximum (100%) DSe is crucial to ensure PI foetuses do not go undetected. At the set thresholds, DSp was maintained at ≥ 70% from approximately week 21 of gestation onwards. By comparison, Stokstad *et al.* (2003)(Stokstad et al., 2003) reported DSe with a 95% confidence interval of 79 – 100% from day 204 (approximately week 29) of gestation onwards, but did not report DSp. Lindberg *et al.* (2001) achieved DSe ≥ 90% during the 7th to 9th months (approximately 28 weeks onwards) of gestation, with a maximum corresponding DSp of 67% (and minimum of 37%). Finally, Brownlie *et al.* (1998) applied a positivity threshold determined under experimental conditions to a field BVDV outbreak at approximately 180 days (approximately 25 weeks) gestation and achieved an observed DSe and DSp of 73% and 82%, respectively. The present study applied a threshold for diagnosis earlier in gestation (21 weeks) and achieved higher DSp than previous studies, without compromising 100% (95% CI: 30.5 – 100.0%) DSe.

In order to maximise DSp, without compromising DSe, the positivity threshold applied in this study increases as gestation progresses. However, the application of a variable threshold is reliant on accurate knowledge of foetal age. This may not always be feasible in practical, on-farm scenarios. A single threshold throughout gestation may be more achievable. A low threshold will maintain DSe but is likely to compromise DSp, particularly in later gestation, while a high threshold may decrease DSe in earlier gestation.

An apparent limitation, in this study, was the failure to reliably detect a heifer that aborted a PI foetus. Adjustment of the positivity thresholds to ensure detection of this heifer would drastically compromise DSp. However, as the non-viable calf aborted by this heifer was PI, it poses an, albeit lessened, infectious risk. Such infectious material may be sufficient to induce acute infection in animals that are in contact with it (and, in turn, produce a live PI calf).
Therefore, while antibody levels may be a valuable indicator of calf status, it would be preferable to utilise a combination of antibody and antigen or virus detection methods for accurate diagnosis of foetal PI. Unfortunately, this study provided no evidence that BVD virus or specific antigen can be detected in maternal serum, swab supernatants or ear notch supernatants by either Ag ELISA or qRT-PCR. If the virus excreted by the PI foetus does pass the placenta and cross into maternal circulation, the present study suggests it may do so at such low levels that it is undetectable by

Ag ELISA and qRT-PCR. Fux and Wolf (2013) demonstrated, not only that antigen detection by ELISA suffers from interference by colostrum-derived BVDV-specific antibodies in young PI calves, but that qRT-PCR also suffers a drop in signal in the presence of colostrum-derived antibodies. As such, it may not be entirely unexpected that any low levels of virus in maternal circulation would be masked from detection by the presence of the high levels of BVDV-specific antibodies that have been demonstrated in our heifers.

5 Success in achieving objectives

The present trial has, albeit in a small number of experimentally infected animals, developed an understanding of the antibody profiles of cows carrying a BVDV ("bovine pestivirus") persistently infected calf. While no virus antigen (which might have been used for the easy early detection of a PI calf *in* utero) was ever detectable in the dam, the antibody responses in those cows carrying a PI calf versus those that did not, provide a possible interpretation that can lead to the pre-natal identification of PI calves. This now allows identification of individual "Trojan" dams, with high diagnostic sensitivity and moderate specificity. If AGID test results alone were relied on, identification of at-risk pregnancies would be achieved only at the herd level, and not for individuals. Further, the AGID results observed in this study confirm that individual 'Trojan' dams will not necessarily return 3 or 3+ AGID results throughout pregnancy as previously believed (compromised sensitivity), nor are 'Trojan' dams the only females that could maintain such high results until the final weeks of gestation (compromised specificity).

6 Impact on meat and livestock industry

As BVDV infection gains greater recognition in the red meat industries, it is crucial that the tools to allow control and prevention of BVDV infection and associated disease are readily available. Pre-natal diagnosis of PI in the developing foetus by simple, rapid, inexpensive and non-invasive methods would enable this potential source of infection to be removed from the herd or avoided at the point of entry. This would enhance biosecurity to protect naïve herds and assist in eliminating the infection from an already infected herd. This study has contributed to the understanding of the differences between females carrying PI foetuses and those carrying non-PI foetuses, and the potential application for diagnostic purposes.

7 Conclusions and recommendations - Section

In conclusion, levels of BVDV-specific antibodies are significantly higher in heifers carrying a PI foetus than those carrying a non-PI foetus. This antibody difference may be used to gain an indication of the likelihood of a heifer carrying a PI pre-natally, however, this method may have some practical limitations.

The samples collected in this study were selected with industry relevance (including ease and safety of sample collection, ease of preparation and cost) in mind. Historically, peripheral blood lymphocyte preparations have been utilised for antigen detection by ELISA (Gottschalk et al., 1992) with recent advancements allowing serum to become the preferred sample. Similarly, transport and storage media can be utilised (Fulton et al., 2009) and may help to preserve viral RNA on swab

samples. These methods, while originally ruled out for ease of preparation and cost reasons may present more viable options for antigen detection in future studies on cattle carrying BVDV PI foetuses. In the meantime, the antibody profiling as described here already provides highly sensitive means to identify individual “Trojan” dams, necessitating only the quarantining of pregnant dams with very high antibody levels by ELISA, instead of the whole mob. Furthermore, this antibody testing allows result interpretation under specific circumstances that require risk assessment of individual females, rather than herd level assessment (for example, when pregnant animals of unknown history are introduced into a herd).

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9 Appendices

9.1 Appendix 1: Tables

Table 1. Summary of the gestational outcomes of seventeen heifers following acute infection with bovine viral diarrhoea virus (BVDV) on day 69 – 90 of gestation.

Pregnancy outcome	Number of heifers	Percentage of heifers (95% CI)
Abortion, observed	1*	23.5 (3.4 – 43.7)
Abortion, unobserved (found empty)	3	
Neonatal calf death	1	5.9 (0 – 17.1)
Live, calf with neurological deficit	3	17.6 (0 – 35.8)
Live, PI calf**	3	17.6 (0 – 35.8)
Live, healthy calf	6	35.3 (12.6 – 58.0)

*foetus recovered and shown to be PI

**PI = persistently BVDV infected

Table 2. The diagnostic sensitivity (DSe) and specificity (DSp) for diagnosis of foetal persistent bovine viral diarrhoea virus (BVDV) infection (PI) by detection of specific antibodies by enzyme linked immunosorbent assay (ELISA) in the dam (n=3 carrying PI, n=10 carrying non-PI) from 24 weeks before calving, at each of eight sample-to-positive (S/P) ratio thresholds for positivity. Grey shading: DSe or DSp = 100%.

		Weeks before calving																							
		-24	-23	-22	-21	-20	-19	-18	-17	-16	-15	-14	-13	-12	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0
S/P Ratio Threshold	DSe (%)																									
	0.6	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
	0.8	67	67	33	67	67	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
	1.0	33	67	33	67	67	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
	1.2	0	33	33	33	33	67	67	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
	1.4	0	0	0	0	0	33	33	33	67	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
	1.6	0	0	0	0	0	0	33	33	33	33	33	33	100	100	100	100	100	100	100	100	100	100	100	67	67
	1.8	0	0	0	0	0	0	0	0	33	33	0	33	33	33	67	33	100	100	100	100	67	100	67	67	33
	2.0	0	0	0	0	0	0	0	0	0	0	0	33	33	33	33	33	67	100	100	33	67	67	67	33	33
	DSp (%)																									
	0.6	80	70	30	20	30	20	20	20	20	10	10	10	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.8	80	80	70	70	60	50	50	40	40	20	30	20	20	20	20	20	10	0	0	0	0	0	10	10	10
	1.0	100	100	100	90	90	90	70	60	60	40	40	40	30	20	30	20	20	20	20	20	20	20	20	20	20
	1.2	100	100	100	100	100	90	90	100	70	90	70	60	50	40	40	30	40	20	30	20	20	20	20	20	50
	1.4	100	100	100	100	100	100	100	100	100	90	90	90	80	80	70	60	50	60	70	70	80	60	60	70	90
	1.6	100	100	100	100	100	100	100	100	100	100	100	100	100	90	90	100	90	80	90	80	80	80	80	80	90
	1.8	100	100	100	100	100	100	100	100	100	100	100	100	100	100	90	100	90	90	90	90	100	90	90	90	90
	2.0	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	90	100	100	100

Table 3. The Youden Statistic (%)(J = DSe + DSe – 100%) for diagnosis of foetal persistent bovine viral diarrhoea virus (BVDV) infection (PI) by detection of specific antibodies by enzyme linked immunosorbent assay (ELISA) in the dam (n=3 carrying PI, n=10 carrying non-PI) from 24 weeks before calving, at each of eight sample-to-positive (S/P) ratio thresholds for positivity. Grey shading: highest in column.

Youden Statistic (%)		Weeks before calving																							
		-24	**-23**	**-22**	**-21**	**-20**	**-19**	**-18**	**-17**	**-16**	**-15**	**-14**	**-13**	**-12**	**-11**	**-10**	**-9**	**-8**	**-7**	**-6**	**-5**	**-4**	**-3**	**-2**	**-1**	**0**
S/P Ratio Threshold	**0.6**	80	70	30	20	30	20	20	20	20	10	10	10	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.8	47	47	33	37	27	50	50	40	40	20	30	20	20	20	20	20	10	0	0	0	0	0	10	10	10
	1	33	67	33	57	57	90	70	60	60	40	40	40	30	20	30	20	20	20	20	20	20	20	20	20	20
	1.2	0	33	33	33	33	57	57	100	70	90	70	60	50	40	40	30	40	20	30	20	20	20	20	20	50
	1.4	0	0	0	0	0	33	33	33	67	90	90	90	80	80	70	60	50	60	70	70	80	60	60	70	90
	1.6	0	0	0	0	0	0	33	33	33	33	33	33	100	90	90	100	90	80	90	80	80	80	80	47	57
	1.8	0	0	0	0	0	0	0	0	33	33	0	33	33	33	57	33	90	90	90	90	67	90	57	57	23
	2	0	0	0	0	0	0	0	0	0	0	0	33	33	33	33	33	67	100	100	33	67	57	67	33	33

9.2 Appendix 2: Figures

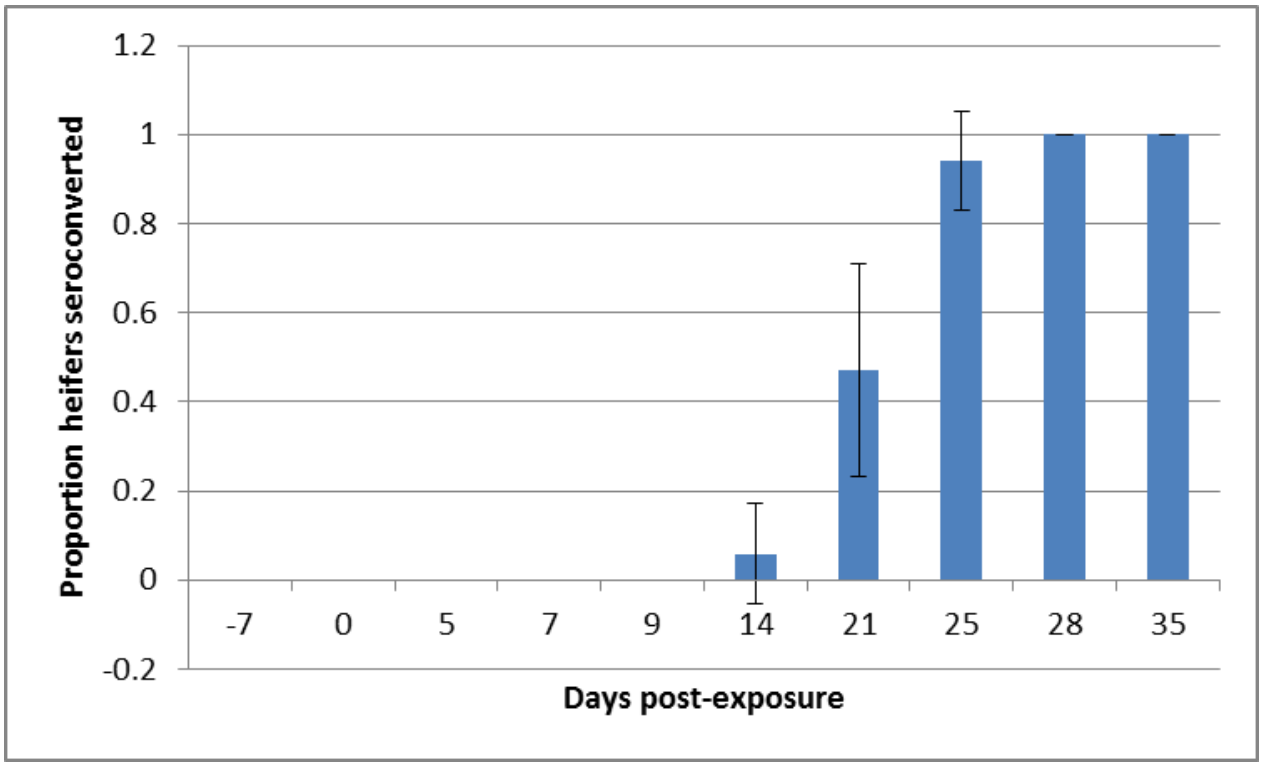


Figure 1. Timing of seroconversion of seventeen heifers following exposure to bovine viral diarrhoea virus (BVDV) by co-mingling with a persistently BVDV infected cow from days 0 to 28 post-exposure at a density of 24 m^2/animal. Error bars show 95% confidence intervals.

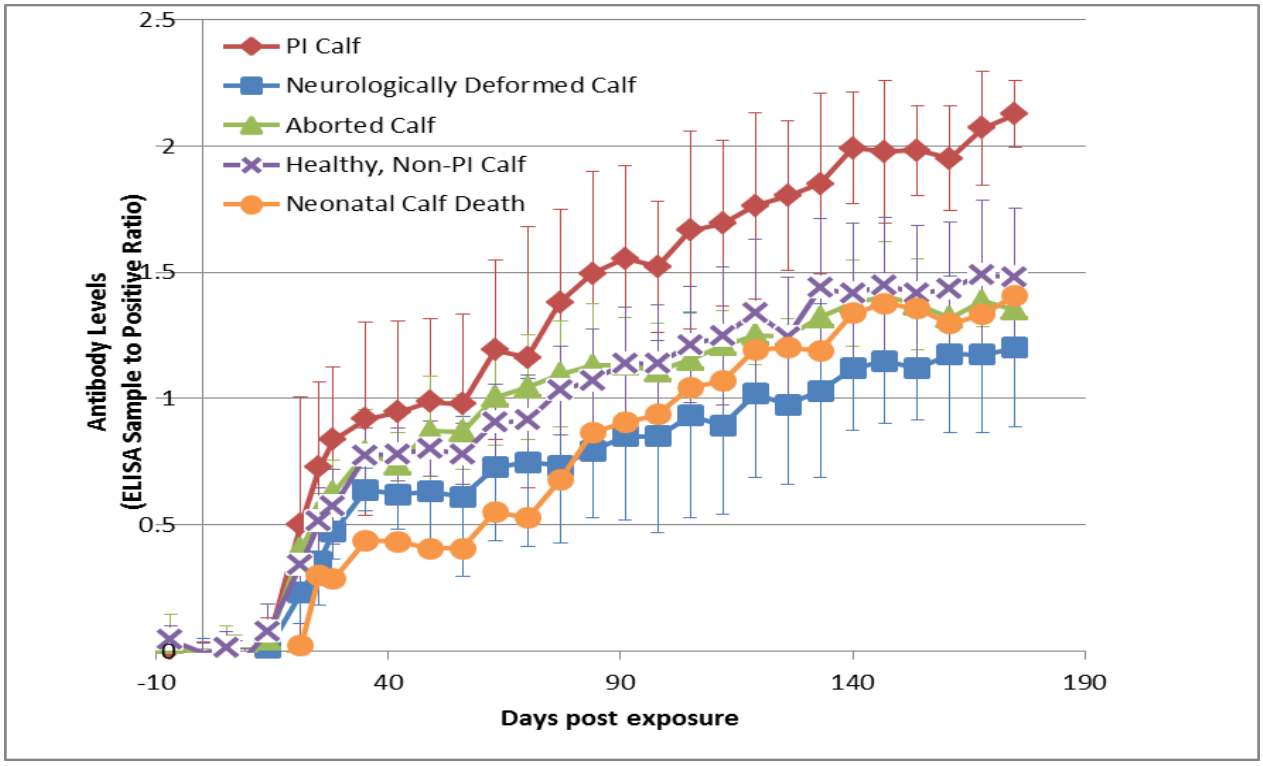


Figure 2. The antibody levels over gestation in seventeen heifers with varying gestational outcomes following acute infection with bovine viral diarrhoea virus (BVDV) on day 69 – 90 of gestation (day 0 post-exposure). Gestational outcomes were classified as: abortion (n=4), neonatal calf death (n=1), live calf with neurological deficit (n=3), live persistently BVDV infected (PI) calf (n=3), healthy calf (n=6), with the first live calf born on day 179 post-exposure. Antibody levels were measured using a commercially available enzyme-linked immunosorbent assay (ELISA), and expressed as a sample to positive (S/P) ratio. Error bars show 95% confidence intervals.

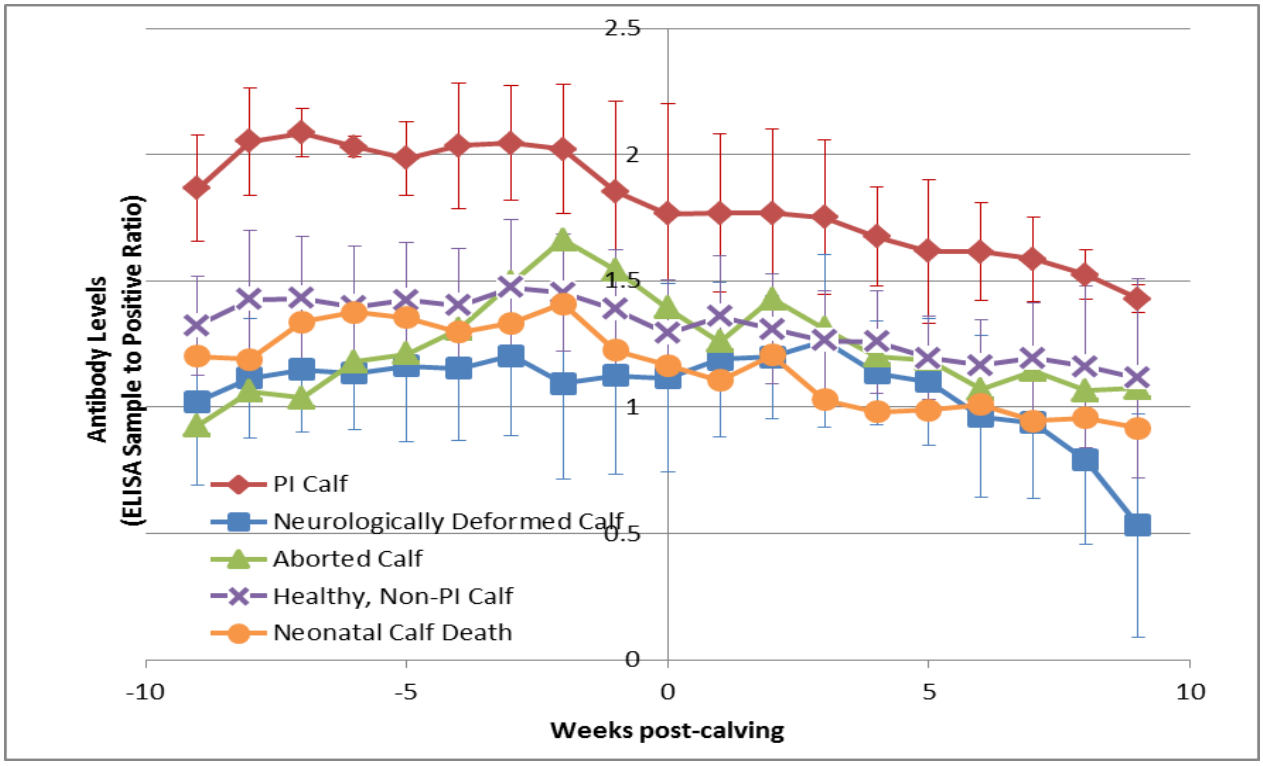


Figure 3. The antibody levels nine week pre- and post-calving in seventeen heifers with varying gestational outcomes following acute infection with bovine viral diarrhoea virus (BVDV) on day 69 – 90 of gestation (day 0 post-exposure). Gestational outcomes were classified as: observed abortion (n=1), neonatal calf death (n=1), live calf with neurological deficit (n=3), live persistently BVDV infected (PI) calf (n=3), healthy calf (n=6). Antibody levels were measured using a commercially available enzyme-linked immunosorbent assay (ELISA), and expressed as a sample to positive (S/P) ratio. Error bars show 95% confidence intervals. (Note: unobserved abortions not included as day of abortion unknown.)

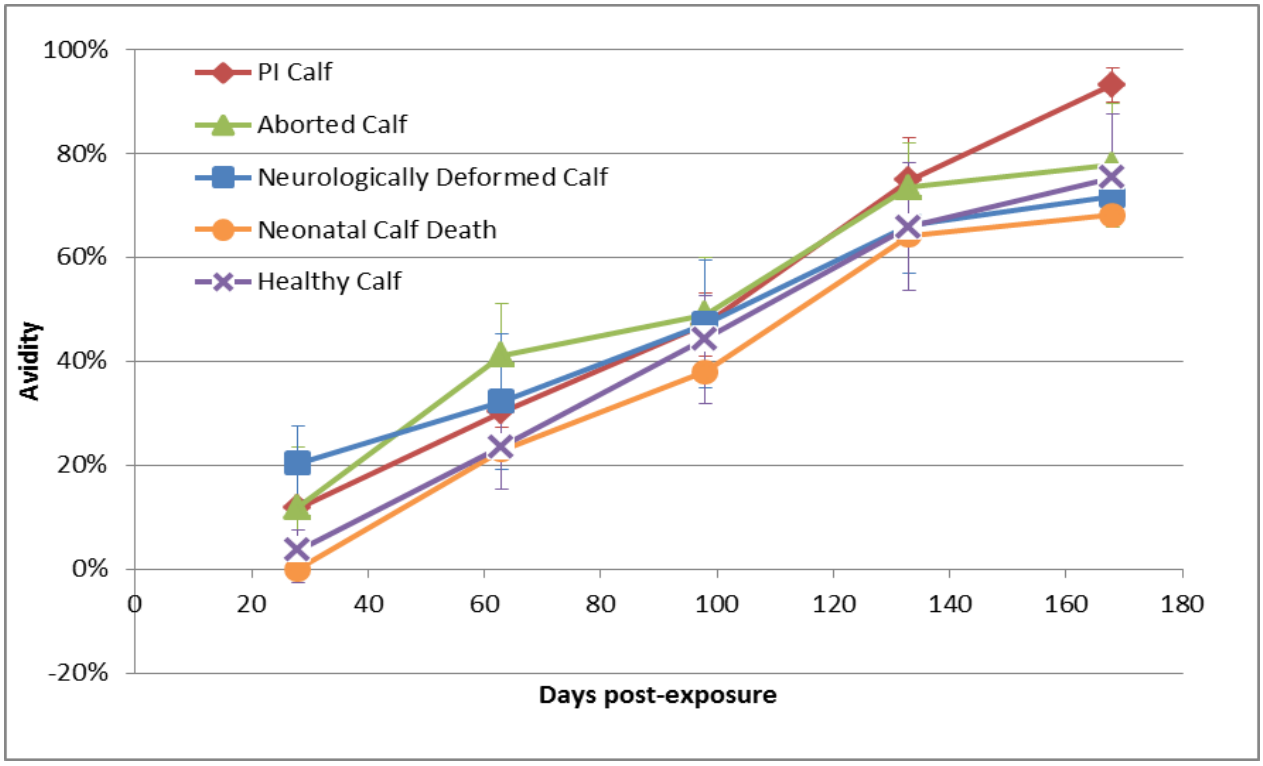


Figure 4. The avidity of antibodies in seventeen heifers with varying gestational outcomes following acute infection with bovine viral diarrhoea virus (BVDV) on day 69 – 90 of gestation (day 0 post-exposure). Gestational outcomes were classified as: abortion (n=4), neonatal calf death (n=1), live calf with neurological deficits (n=3), live persistently BVDV infected (PI) calf (n=3), healthy calf (n=6). Antibody avidity was measured by calculating the ratio (x100%) of the results (sample to positive (S/P) ratios) of urea treated to non-urea treated replicates tested by enzyme-linked immunosorbent assay (ELISA). Error bars show 95% confidence intervals.

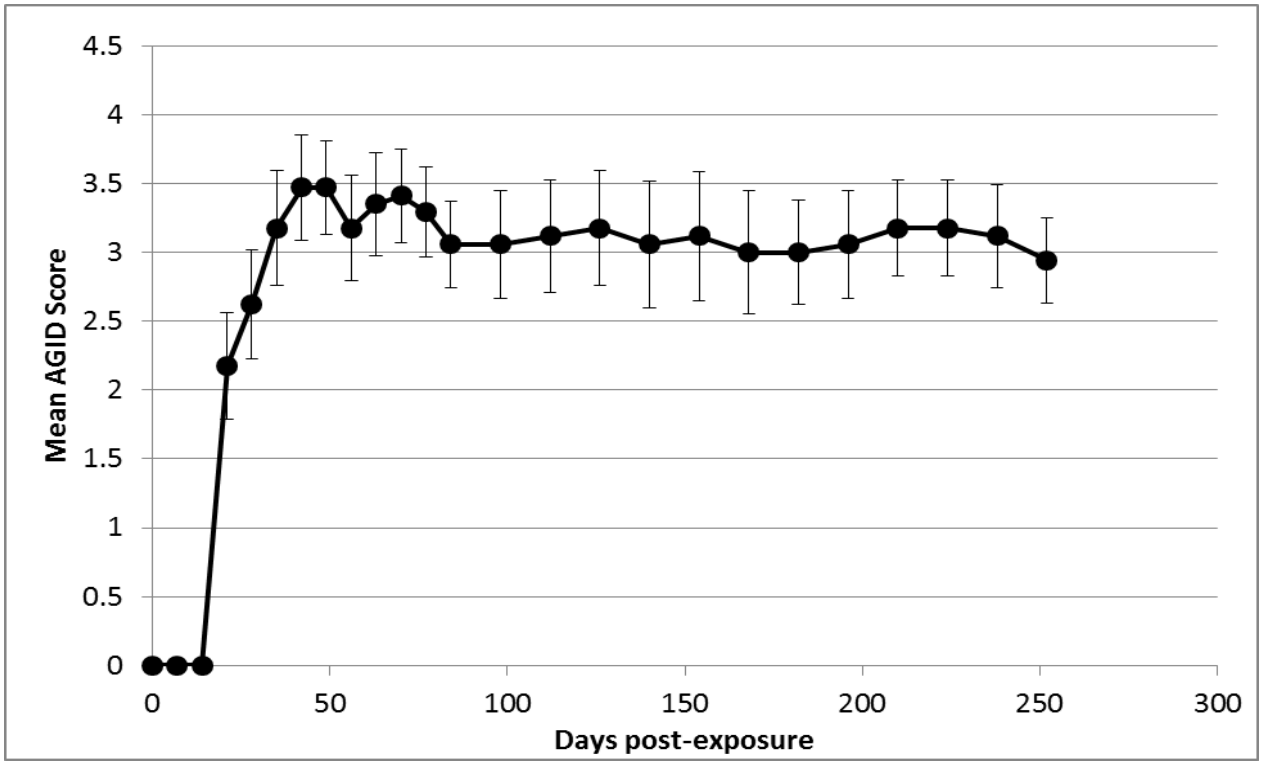
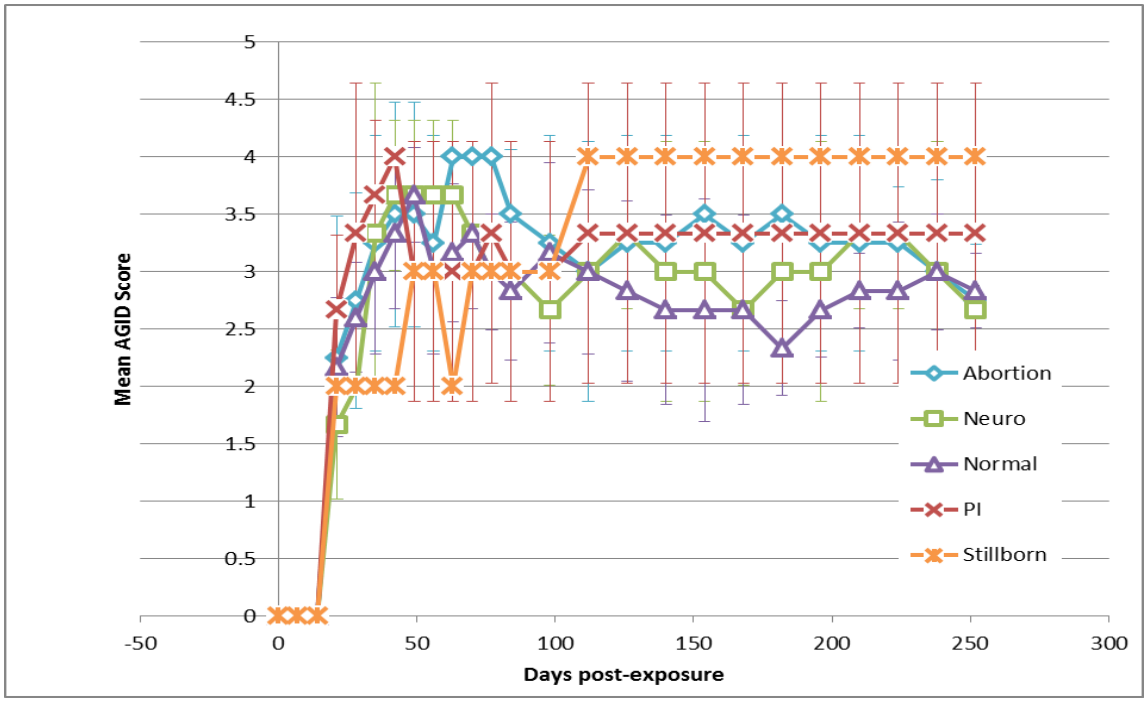


Figure 5. The mean agarose gel immunodiffusion (AGID) score in seventeen heifers following acute infection with bovine viral diarrhoea virus (BVDV). Error bars show 95% confidence intervals; a (above) without and b (below) with gestational outcomes (no significant differences were observed in AGID responses between dams with differing gestational outcomes).



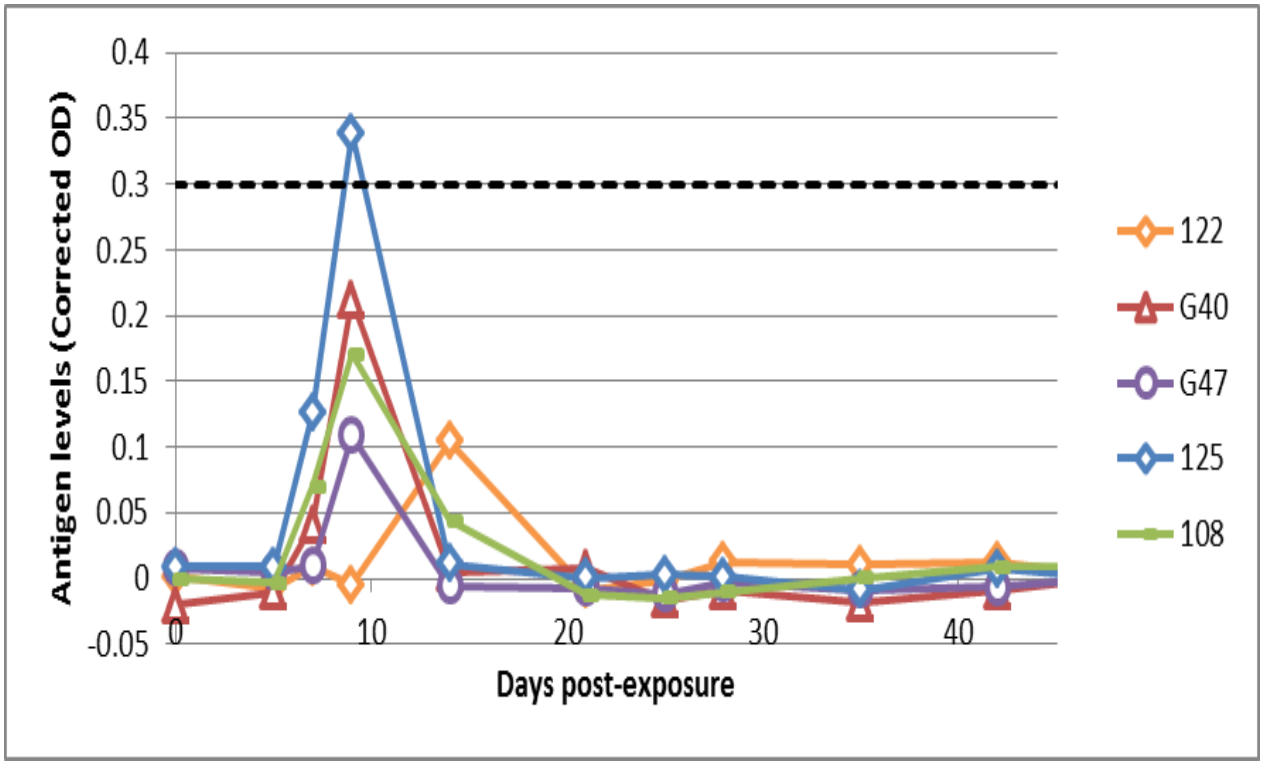


Figure 6. The antigen levels in five heifers that exhibited an antigen peak following acute infection with bovine viral diarrhoea virus (BVDV) on day 69 – 90 of gestation (day 0 post-exposure). Antigen levels were measured by commercially available enzyme-linked immunosorbent assay (ELISA) and expressed as corrected optical density (OD). Dotted line represents the manufacturer's recommended threshold for positivity.

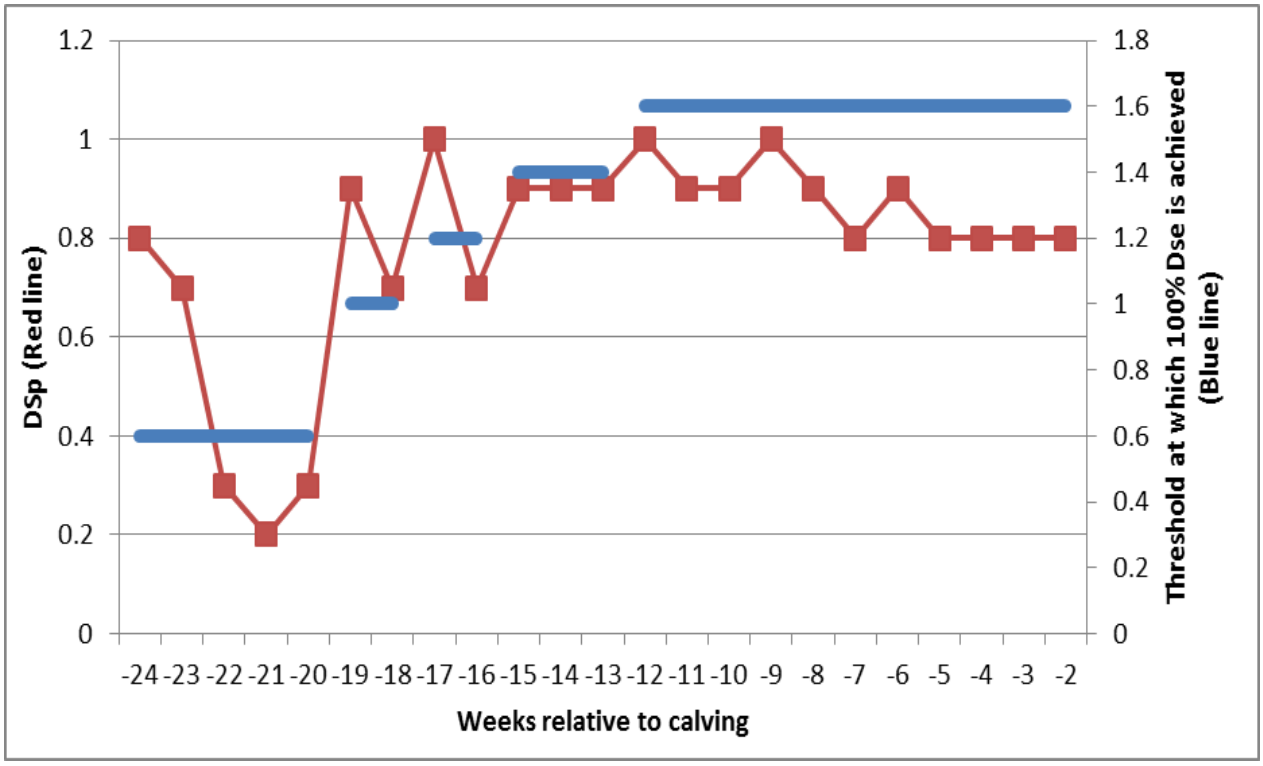


Figure 7. The maximal positivity thresholds (blue line) at which 100% diagnostic sensitivity (DSe) is achieved for diagnosis of persistent bovine viral diarrhoea virus (BVDV) infection in a foetus by testing serum from the dam by commercially available antibody enzyme-linked immunosorbent assay (ELISA) at a particular stage of gestation, when the result (expressed as a sample-to-positive (S/P) ratio) is deemed positive when it exceeds the threshold, and; the corresponding diagnostic specificity (DSp)(red line) observed at that threshold.

Appendix 3: Manuscript

Pre-treatment of serum samples to reduce interference of colostrum-derived specific antibodies with detection of bovine viral diarrhoea virus (BVDV) antigen by ELISA in young calves

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Abstract

The antigen enzyme-linked immunosorbent assay (ELISA) is the preferred method of diagnosis of persistently bovine viral diarrhoea virus infected (BVDV PI) individuals for use in control and mitigation programs and it is rapid and inexpensive. However, colostrum-derived antibodies may interfere with antigen detection in young PI calves. This study aimed to assess serum pre-treatment methods for reducing the 'diagnostic gap'. Dilution series showed that antibody levels equivalent to those observed in colostrum-fed calves eliminated all antigen signal when present in a serum sample. Serum was treated with ethylenediaminetetraacetic acid (EDTA) pH 4.5 – 7.5, boiled, centrifuged and the supernatant was recovered and tested by antigen ELISA. Following treatment, sera returned negative antibody ELISA results. Antigen signal recovery in excess of 90% was achieved when pH was 4 (+/- 0.5). When applied to samples from three PI calves, antigen signal improved such that the 'diagnostic gap' was eliminated in all calves. When applied to samples from non-PI calves that had been subject to acute fetal infection, 98.6% of samples returned negative antigen ELISA results at two or more days of age, while four (of seven) non-PI calves returned false-positive antigen results on the day of birth. Pre-treatment methods may represent a substantial opportunity to improve diagnosis of young, colostrum-fed PI calves.

Keywords Colostrum-derived antibody; Diagnostic gap; Heat; Treatment;

Pestivirus

Introduction

Infection of cattle populations with bovine viral diarrhoea virus (BVDV) causes significant financial impacts due to clinical disease (Brownlie et al., 1987), reproductive disease (Grooms, 2004) and immunosuppression (Brackenbury et al., 2003). Accurate diagnostic testing allows for control and mitigation of disease-associated losses, primarily through the identification and eradication of persistently infected (PI) cattle that arise following acute infection of the pregnant dam, and subsequent fetal infection, during the first 120 days of gestation (Brownlie et al., 1987). These PI individuals shed copious amounts of virus from birth and throughout their lifetimes (Brownlie et al., 1987) and are the primary transmission risk. A variety of diagnostic methods are available for diagnosis of PI, and have recently been reviewed (Lanyon et al., 2013). Of particular relevance to control and mitigation programs where large numbers of animals may require testing is the antigen enzyme-linked immunosorbent assay (ELISA). The ELISA is a simple, rapid, inexpensive testing method that does not require specialised equipment and is well suited to large scale testing. However, the antigen ELISA has one major flaw: colostrum-derived BVDV specific antibodies interfere with detection of antigen in serum from very young PI calves (Fux and Wolf, 2013). As a result, alternative testing strategies are often employed in this cohort. One such strategy is to delay testing until calves are a minimum of twelve weeks of age (Mars and Van Maanen, 2005). However, due to the highly infectious nature of PI calves this delay may result in substantial viral spread and is far from ideal. Another testing strategy is to utilise an alternative diagnostic methods, particularly reverse transcription polymerase chain reaction (RT-PCR) which is not subject to the same interference (Fux and Wolf, 2013). However, RT-PCR is more expensive, labour intensive and time consuming than antigen ELISA. Finally, serum samples may be replaced by ear notch samples in young calves. Although ear notch samples certainly suffer a lower level of interference by colostrum-derived antibodies (Kuhne et al., 2005; Fux and Wolf, 2013), there is still some debate regarding the sensitivity of diagnosis of PI using ear notches (Lanyon et al., 2013). As such, an opportunity to reduce the interference of colostrum-derived antibodies with detection of antigen in serum from young PI calves (and eliminate the 'diagnostic gap') would be of substantial benefit to BVDV control and mitigation efforts. Therefore, this study aimed to assess the ability of serum pre-treatment methods to reduce serum antibody levels and, therefore, increase signal in the antigen ELISA, and the diagnostic relevance of such methods in young, colostrum-fed calves.

Methods

Characterisation of the interference effect: dilution series

Serum collected from a locally-sourced cow previously confirmed PI with BVDV Type 1c was serially diluted in either sample diluent (IDEXX Laboratories Inc., Rydalmere, NSW) or pooled serum from seventeen antibody positive cows previously infected with BVDV Type 1c under experimental conditions. Similarly, either sample diluent or pooled antibody positive serum was serially diluted in serum from the PI cows. That is, serial dilution was conducted in both directions, resulting in samples ranging from neat PI serum to neat diluent or pooled antibody positive serum.

All dilutions were then tested for the presence of BVDV-specific antigen and antibody by ELISA, according to the manufacturer's instructions (IDEXX BVDV Total Ab ELISA; IDEXX BVDV Serum/Ag Plus ELISA; IDEXX Laboratories Inc., Rydalmere, NSW). Antibody ELISA results were expressed as a sample-to-positive (S/P) ratio. Antigen results were expressed as a corrected optical density (OD).

Proof of concept: treatment of laboratory created samples

Three samples were experimentally created as per Table 1. Sample treatment methods were adapted from More and Copeman (1991). An aliquot of 100µL of each sample was treated by: a) boiling for 7 minutes, or; b) addition of an equal volume (100µL) of 0.1M ethylenediaminetetraacetic acid disodium salt dihydrate (Na_2EDTA; Sigma-Aldrich Co., Castle Hill, NSW), pH 4.5, 5.5, 6.5 and 7.5 (+/- 0.1) followed by boiling for 7 minutes. All treated samples were then centrifuged at 16,000 RCF for 7 minutes and supernatant recovered. All supernatants, along with the untreated samples, were tested for BVDV specific-antigen by ELISA (IDEXX BVDV Ag/Serum Plus; IDEXX Laboratories Inc., Rydalmere, NSW). The treated and untreated negative control was also tested for BVDV specific-antibodies by ELISA (IDEXX BVDV Total Ab; IDEXX Laboratories Inc., Rydalmere, NSW). Signal recovery was calculated as:

$$\frac{Signal\ recovered\ by\ treatment}{Signal\ lost\ by\ addition\ of\ antibodies} = \frac{(Corrected\ OD\ treated\ experimental\ sample)-(Corrected\ OD\ untreated\ experimental\ sample)}{(Corrected\ OD\ untreated\ positive\ control)-(Corrected\ OD\ untreated\ experimental\ sample)}.$$

Preliminary application: treatment of serum samples from young, colostrum-fed PI calves and their non-PI herdmates

Serum samples were collected from three PI and seven non-PI calves every two days from the day of birth (predominantly prior to colostrum ingestion) until 14 days of age, and weekly until five weeks of age as part of a previous research trial. All sera were treated by addition of an equal volume 0.1M ethylenediaminetetraacetic acid disodium salt dihydrate (Na_2EDTA; Sigma-Aldrich Co., Castle Hill, NSW), pH 5 (+/- 0.5), followed by boiling for 7 minutes. Samples were then centrifuged at 16,000 RCF for 7 minutes and supernatant recovered. Supernatants and untreated samples were tested by both antibody and antigen ELISA (IDEXX BVDV Total Ab ELISA; IDEXX BVDV Serum/Ag Plus ELISA; IDEXX Laboratories Inc., Rydalmere, NSW).

Results

Characterisation of the interference effect: dilution series

As seen in Figure 1, neat PI serum returned a very strong positive antigen result (corrected OD 3.4) and a negative antibody result (<0.3 S/P ratio). When diluted in sample diluent (antibody negative), the PI serum maintained an antigen ELISA corrected OD ≥3.3 until a dilution of 1:3, PI serum:sample diluent, before decreasing to produce a negative antigen result (corrected OD <0.3) at a dilution of 1:127, PI serum:sample diluent. When diluted in pooled antibody positive serum, the antigen

signal decreased rapidly maintaining a corrected OD ≥3.3 until a dilution of only 127:1, PI serum:pooled seropositive serum and returning a negative antigen result from a dilution of 3:1, PI serum:pooled seropositive serum onwards.

Proof of concept: treatment of laboratory created samples
All sample treatments produced an increase in antigen signal in the experimental sample and a decrease in signal in the positive control (Figure 2). Treatment with 0.1M Na_2EDTA pH 4.5 or 5.5 resulted in the highest signal recovery of 95% and 93% respectively, while resulting in only small decreases in the signal of the positive control. The negative control sample tested negative for antigen (corrected OD <0.1) regardless of treatment, and tested positive for antibodies when untreated (S/P ratio = 1.4) but negative for antibodies (S/P ratio <0.1) after any treatment.

Preliminary application: treatment of serum samples from young, colostrum-fed PI calves and their non-PI herdmates
When untreated, the antigen ELISA returned a negative result until between 6 days (#07) and 4 weeks (#03) of age. Following treatment, all three PI calves returned a strong positive antigen ELISA result (corrected OD > 1.8) at all timepoints. When untreated, all (n=7) non-PI calves returned negative antigen ELISA results (corrected OD <0.3) at all timepoints. Four (of seven) non-PI calves returned positive antigen results (corrected OD >0.3) on the day of birth with the corrected OD ranging from 0.37 to 0.91. Six (of seven) non-PI calves returned negative antigen results following treatment (corrected OD <0.3) from 2 days of age onwards. The seventh calf returned a very low antigen result of 0.397 at 4 days of age, but returned negative results at all other timepoints. Overall, 98.6% of samples from non-PI calves returned negative antigen ELISA results after treatment at 2 or more days of age.

Prior to treatment, all calves (n=10) returned positive antibody ELISA results (S/P ratio >0.3) following colostrum ingestion. After treatment, the three PI calves returned negative results at all timepoints, while 97.4% of samples collected from non-PI calves returned negative antibody results following treatment. The maximum observed antibody level in a non-PI calf following treatment was an S/P ratio of 0.45 at 10 days of age.

Discussion

The dilution series in this study clearly demonstrated that the presence of BVDV-specific antibodies in a serum sample can eliminate the antigen detection signal when the same sample is tested by antigen ELISA. Antibody titres up to $10^{4.8}$ have been observed in young PI calves following colostrum ingestion (Fux and Wolf, 2013) can produce antibody ELISA results as high as an S/P ratio of 2 in the first week of life (Lanyon *et al.* unpublished data). The results of this study show that equivalent antibody levels are sufficient to eliminate an antigen signal of corrected OD > 3. Even levels of specific antibodies as low as 0.3 to 0.5 S/P ratio were observed to produce a three-fold decrease in antigen signal. These results support existing literature detailing the diagnostic gap observed during which antigen ELISAs fail to detect colostrum-fed PI calves (Fux and Wolf, 2013; Lanyon et al. Unpublished

Data). From the dilution series data, a sample consisting of 50% pooled antibody positive cow serum and 50% PI cow serum was identified as producing test results that mimic those observed in young, colostrum-fed PI calves. As such, the proof of concept experiment used this 50/50 mixture as the experimental sample (Table 1).

The results of the proof of concept experiment demonstrate that all methods of treatment that were applied produced an increase in antigen signal in the experimental sample, without resulting in any false positive results on the negative control sample. The negative control (antibody positive) sample returned negative antibody ELISA results following all treatments. This suggests the specific antibodies were successfully removed from the samples resulting in a reduction of the observed antibody interference. The positive control sample returned a positive antigen result untreated and following all treatment methods, although signal was decreased. Maximum signal recovery of > 90% was achieved by treatment of the experimental sample using EDTA pH 4.5 – 5.5. These treatments also resulted in the smallest decrease in signal in the positive control sample. As such, treatment with EDTA pH 5 (+/- 0.5) was applied to samples collected from colostrum-fed PI and non-PI calves.

When untreated, the diagnostic gap for detection of the PI calves following colostrum ingestion extended until up to five weeks of age. Fux and Wolf (2013) observed a diagnostic gap for up to two weeks of age. Following treatment, all (n=3) PI calves included in this study returned a strong positive antigen result (correct OD > 1.8) at all timepoints following colostrum ingestion. Treatment appeared to eliminate specific antibodies from the samples with all PI calves returning negative antibody results at all timepoints following treatment. The non-PI calves in this study returned negative antigen results at all timepoints prior to treatment, and on 98.6% (69/70) of samples collected after 2 days of age. The one positive result returned was low (0.397). This may suggest an adjustment in the positivity threshold would be appropriate for use on treated samples. Treated samples collected from non-PI calves on the day of birth returned higher antigen results than when untreated. This may be associated with the *in utero* BVDV infection that these particular calves underwent. Further research will establish the diagnostic sensitivity and specificity for detection of young, colostrum-fed PI calves by testing of pre-treated serum samples.

The data presented here provide evidence that pre-treatment of serum sample could present an opportunity to eliminate the diagnostic gap for detection of PI calves, without the need for specialised sample collected protocols or alternative testing methods. At present, ear notch samples are the preferred method for detection of PI calves by antigen ELISA (Kuhne et al., 2005; Fux and Wolf, 2013). However, this variation to sample collection protocols can be cumbersome and inconvenient, and is a more invasive method than blood collection. When serum samples are submitted for diagnostic testing, RT-PCR is generally necessary to avoid the interference effect of colostrum-derived antibodies (Fux and Wolf, 2013). However, RT-PCR is more expensive, labour intensive and time consuming than antigen ELISA and requires specialised equipment. As such, an opportunity to treat serum samples such that the simple, rapid and inexpensive antigen ELISA can be reliably applied to young, colostrum-fed calves represents a significant advance in the field of BVDV diagnosis.

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Tables

Table 1. Composition of three samples experimentally created to mimic the bovine viral diarrhoea virus (BVDV) specific antigen and antibody content of serum collected under varying biological situations.

Sample ID	Biological Equivalent	Sample Composition	Antibody Content	Antigen Content
Negative Control	Non-PI individual	100% Pooled Antibody Positive Serum	Positive	Negative
Experimental Sample	PI calf following colostrum ingestion	50% PI Serum; 50% Pooled Antibody Positive Serum	Positive	Positive
Positive Control	Adult PI individual	50% PI Serum; 50% Sample Diluent	Negative	Positive

Figures

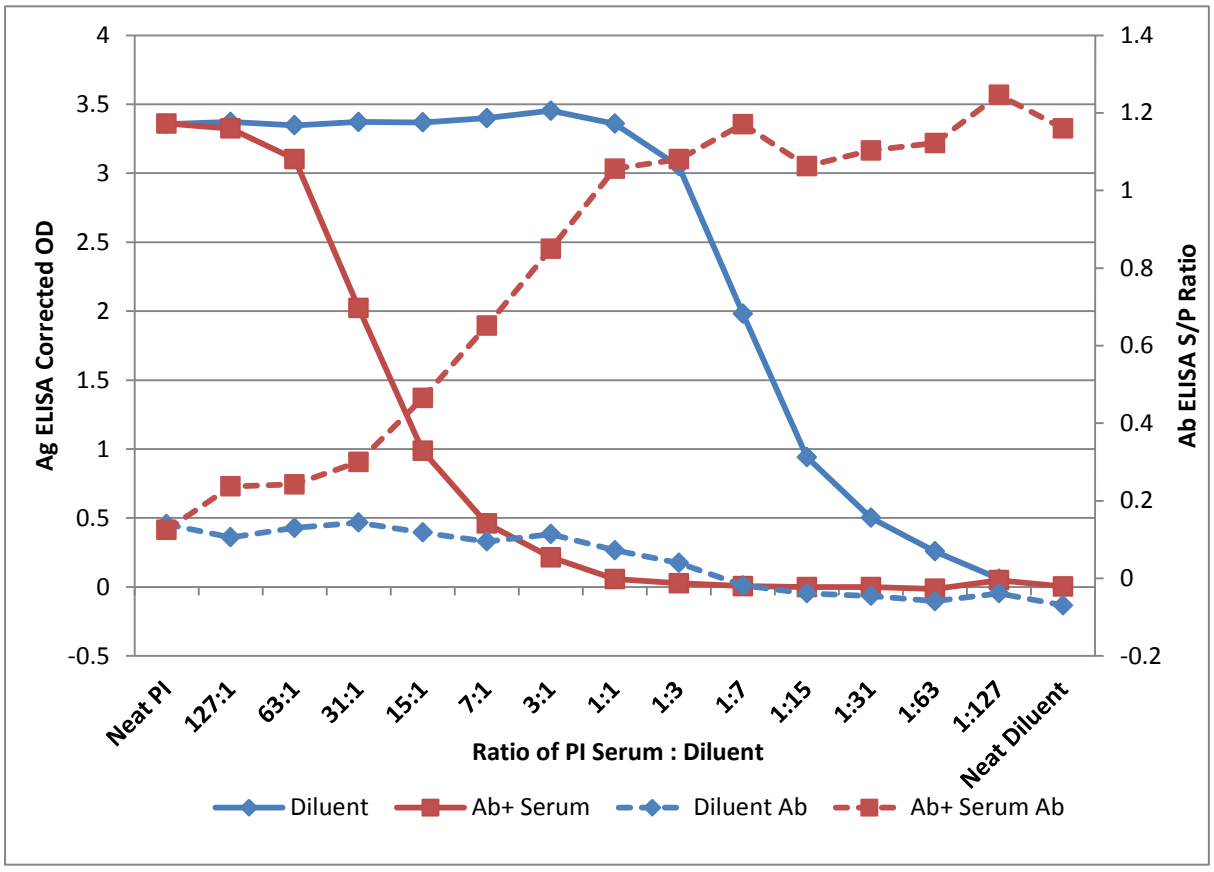

Figure 1. The levels of bovine viral diarrhoea virus (BVDV) specific antibodies (dotted lines) and antigen (solid lines) as measured by ELISA (IDEXX BVDV Total Ab ELISA; IDEXX BVDV Serum/Ag Plus ELISA; IDEXX Laboratories Inc., Rydalmere, NSW) in serum from a persistently BVDV infected (PI) cow when serially diluted in either sample diluent (IDEXX Laboratories Inc., Rydalmere, NSW) (blue lines) or pooled serum from seventeen antibody positive cows previously infected with BVDV under experimental conditions (red lines).

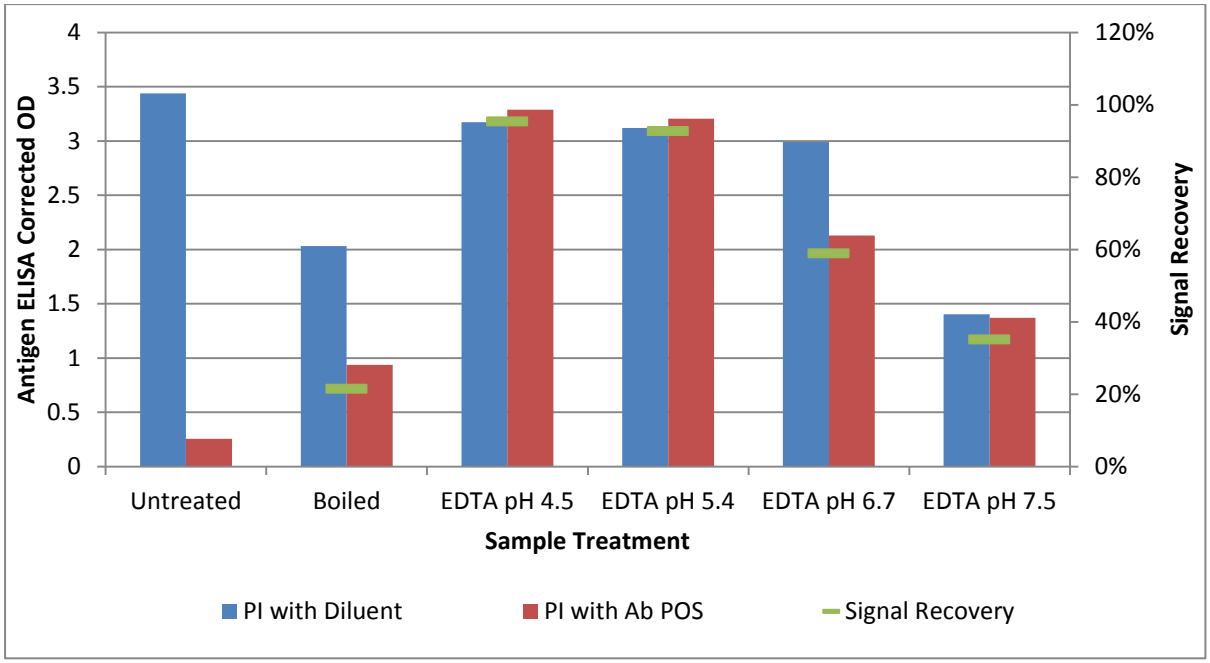

Figure 2. The detectable levels of bovine viral diarrhoea virus (BVDV) specific antigen as measured by ELISA (IDEXX BVDV Serum/Ag Plus ELISA; IDEXX Laboratories Inc., Rydalmere, NSW) and the signal recovery in positive control (blue) and experimental sample (red) when treated: a) by boiling for 7 minutes, or b) by addition of an equal volume of 0.1M Na_2EDTA (Sigma-Aldrich Co., Castle Hill, NSW) at pH of 4.5, 5.5, 6.5 or 7.5 prior to boiling for 7 minutes. All samples were centrifuged and supernatant recovered and tested.

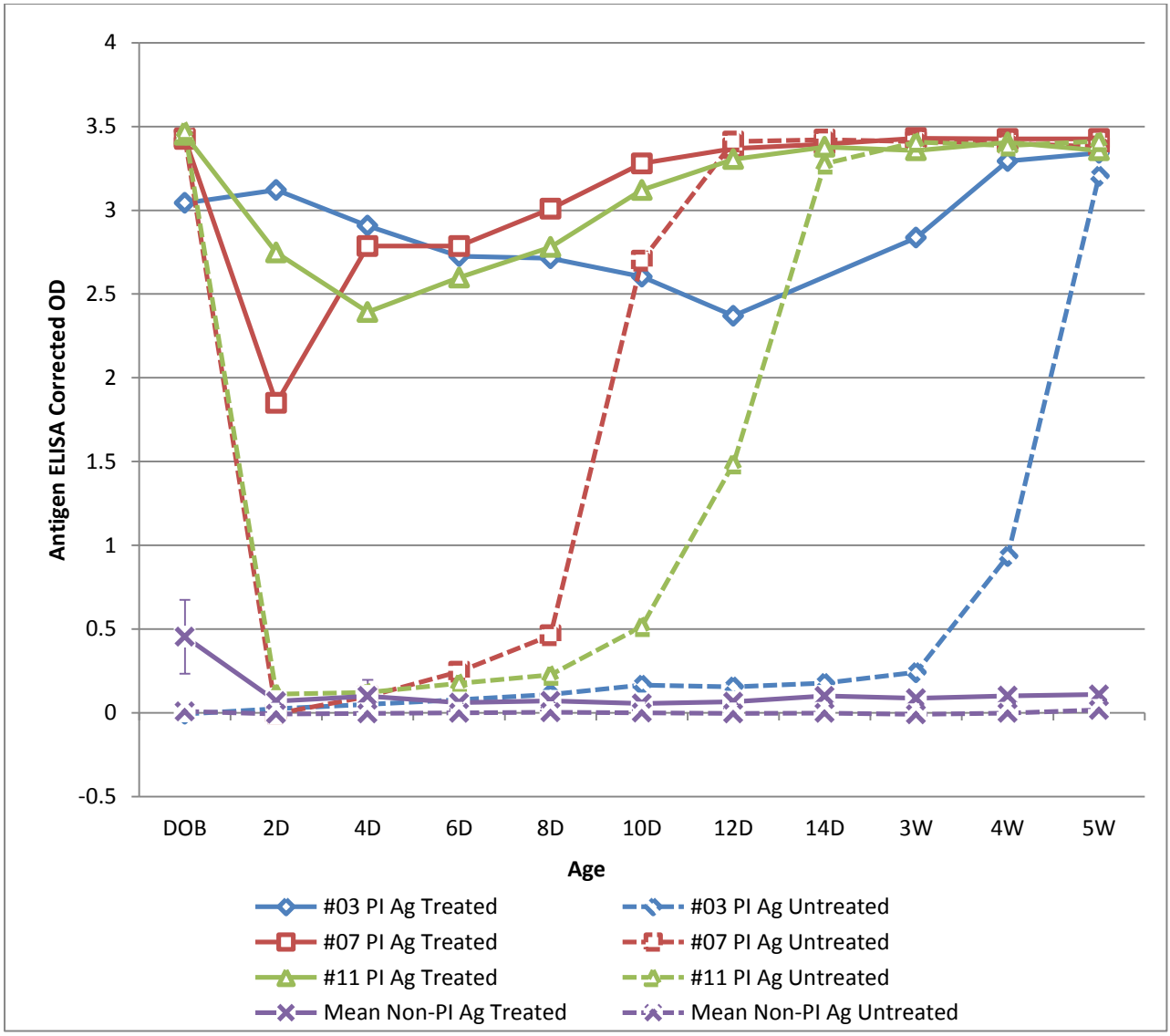

Figure 3. The detectable levels of bovine viral diarrhoea virus (BVDV) specific antigen as measured by ELISA (IDEXX BVDV Serum/Ag Plus ELISA; IDEXX Laboratories Inc., Rydalmere, NSW) in serum from three colostrum-fed persistently BVDV infected (PI) calves (#03, #07 and #11) and the mean levels in seven colostrum-fed non-PI calves from day of birth to 5 weeks of age when untreated (dotted lines) or treated by addition of an equal volume of 0.1M Na_2EDTA (Sigma-Aldrich Co., Castle Hill, NSW) at pH 5 (+/- 0.5) prior to boiling for 7 minutes, centrifugation at 16,000 RCF for 7 minutes and recovery of the supernatant for testing (solid lines). Error bars indicate 95% confidence intervals.