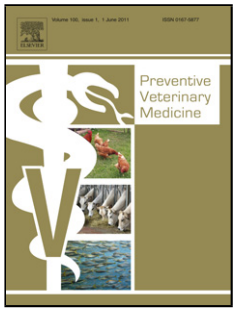

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PII: S0167-5877(16)30042-3
DOI: http://dx.doi.org/doi:10.1016/j.prevetmed.2016.01.025
Reference: PREVET 3973

To appear in: *PREVET*

Received date: 28-7-2015
Revised date: 27-12-2015
Accepted date: 28-1-2016

Please cite this article as: Hay, K.E., Ambrose, R.C.K., Morton, J.M., Horwood, P.F., Gravel, J.L., Waldron, S., Commins, M.A., Fowler, E.V., Clements, A.C.A., Barnes, T.S., Mahony, T.J., Effects of exposure to Bovine viral diarrhoea virus 1 on risk of bovine respiratory disease in Australian feedlot cattle.Preventive Veterinary Medicine http://dx.doi.org/10.1016/j.prevetmed.2016.01.025

Effects of exposure to *Bovine viral diarrhoea virus 1* on risk of bovine respiratory disease in Australian feedlot cattle

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Highlights:

- The prevalence of BVDV-1 PI animals entering Australian feedlots was 0.24%.
- BVDV-1 was detected by qPCR in 59% of feedlot pens.
- BVDV-1 in the pen modestly increased the risk of BRD amongst animals in the pen.
- A single qPCR test was of moderate use in differentiating PI from TI animals.

Abstract

Viruses play a key role in the complex aetiology of bovine respiratory disease (BRD). *Bovine viral diarrhoea virus 1* (BVDV-1) is widespread in Australia and has been shown to contribute to BRD occurrence. As part of a prospective longitudinal study on BRD, effects of exposure to BVDV-1 on risk of BRD in Australian feedlot cattle were investigated. A total of 35,160 animals were enrolled at induction (when animals were identified and characteristics recorded), held in feedlot pens with other cattle (cohorts) and monitored for occurrence of BRD over the first 50 days following induction. Biological samples collected from all animals were tested to determine which animals were persistently infected (PI) with BVDV-1. Data obtained from the Australian National Livestock Identification System database were used to determine which groups of animals that were together at the farm of origin and at 28 days prior to induction (and were enrolled in the study) contained a PI animal and hence to identify animals that had probably been exposed to a PI animal prior to induction. Multi-level Bayesian logistic regression models were fitted to estimate the effects of exposure to BVDV-1 on the risk of occurrence of BRD.

Although only a total of 85 study animals (0.24%) were identified as being PI with BVDV-1, BVDV-1 was detected on quantitative polymerase chain reaction in 59% of cohorts. The PI animals were at moderately increased risk of BRD (OR 1.9; 95% credible interval 1.0 to 3.2). Exposure to BVDV-1 in the cohort was also associated with a moderately increased risk of BRD (OR 1.7; 95% credible interval 1.0 to 3.2) regardless of whether or not a PI animal was identified within the cohort. Additional analyses indicated that a single quantitative real-time PCR test is only moderately useful for distinguishing PI animals from transiently infected animals.

The results of the study suggest that removal of PI animals and/or vaccination, both before feedlot entry, would reduce the impact of BVDV-1 on BRD risk in cattle in Australian feedlots. Economic assessment of these strategies under Australian conditions is required.

Key words:

Bovine respiratory disease, Bovine viral diarrhoea virus 1, feedlot cattle, persistent infection, qPCR.

Introduction

Bovine respiratory disease (BRD) is the major cause of clinical disease and death in feedlot cattle worldwide (Edwards, 2010) and in Australian feedlots it has been estimated to cause more than 70% of clinical disease cases and 50% of deaths (Sackett et al., 2006). This multifactorial disease may occur when there is a combination of susceptible animals, infectious agents and stressors. Specific pathogens are not required for development of BRD; it can develop as a result of infections with various combinations of viruses and bacteria. Bovine viral diarrhoea viruses (BVDV) belong to the pestivirus genus within the *Flavivirus* family and have frequently been associated with BRD. Exposure to BVDV has been associated with increased risk of BRD in feedlot cattle populations (Martin and Bohac, 1986; Martin et al., 1990; Dunn et al., 1995; O'Connor et al., 2001b), and BVDV has been regularly isolated from the lungs of cattle that have died from pneumonia (Gagea et al., 2006; Booker et al., 2008b; Fulton et al., 2009a).

There are two recognised species of BVDV that infect cattle, BVDV-1 and BVDV-2. Seroprevalence and virus detection studies have shown that BVDV-1 is common in cattle populations worldwide (Martin et al., 1989; O'Connor et al., 2001a; Ridpath, 2010b; Morton et al., 2013). Reported seroprevalences for BVDV-1 at feedlot entry have ranged from 20-68% (Martin and Bohac, 1986; Dunn et al., 1995; O'Connor et al., 2001a; Fulton et al.,

2002a). Molecular phylogenetic reconstructions have been used to group BVDV-1 into at least 11 genotypes, BVDV-1a to BVDV-1k (Becher et al., 1999; Vilcek et al., 2001). While six additional genotypes have been proposed, further studies are required to clarify their relationship to the other BVDV-1 genotypes (Booth et al., 2013). In comparison, only two BVDV-2 genotypes have been proposed (Becher et al., 1999). While the biological differences between these genotypes are unknown, BVDV-1b is the genotype most frequently associated with BRD in the United States (Fulton et al., 2002b). Only BVDV-1 has been identified in Australia, with the majority of these viruses being classified within the BVDV-1c genotype (Mahony et al., 2005). These viruses remain genetically distinct from genotypes identified in North America and Europe (Ridpath, 2010b; Ridpath et al., 2010).

In a review of the role of BVDV in BRD, Ridpath (2010a) examined the contributions of persistent and transient infections and synergism with other respiratory pathogens. Infection in immunologically competent animals results in cattle becoming transiently infected (TI) with BVDV-1. Infection of bovine foetuses between 28 and 125 days of gestation may result in the birth of immunotolerant persistently infected (PI) animals which continually shed infectious virus into the environment for life (Ridpath, 2010a). Infection of bovine foetuses at a later stage of gestation may result in the birth of apparently normal calves. However, such congenitally infected calves may perform poorly and be at increased risk of disease compared to calves not exposed to BVDV in-utero (Torres, 2014).

Quantitative real time polymerase chain reaction (qPCR) techniques have excellent analytical sensitivity and specificity for detecting BVDV (Bhudevi and Weinstock, 2001) and the cycling threshold (Ct) values obtained from qPCR analyses are related to the amount of viral RNA present in the processed sample. In a review of diagnostic options, Lanyon et al (2014) noted that qPCR analysis was suitable for the detection of a single PI animal contributing to a pooled test of up to 50 samples. However, relatively low numbers of virus shed during

transient infection may also be detected (Bhudevi and Weinstock, 2003). The duration of viraemia due to transient BVDV-1 infection is usually less than 15 days (Fulton, 2013), so repeated sampling and qPCR testing is recommended after a minimum of 28 days to differentiate PI from TI animals (Lanyon et al., 2014). Because qPCR techniques enable the amount of viral nucleic acid present in biological samples to be quantified and because the concentrations of BVDV-1 in discharges shed by TI animals may be lower compared to PI animals, it has been suggested that qPCR may assist in distinguishing between the two (Lanyon et al., 2014). Following infection with BVDV-1, TI animals become seropositive within two to three weeks, while PI animals remain seronegative to antigenically homologous BVDV-1 strains (Lanyon et al., 2014). In populations exposed to a single genotype, serological data may assist in identifying PI animals when used in combination with qPCR testing.

Because PI animals continuously shed large amounts of the virus, they are the major source of BVDV-1 to in-contact animals, (Ridpath, 2010a). BVDV-1 is transmitted through direct contact with infected animals, and to non-contact animals via aerosol (over short distances), and via fomites (Mars et al., 1999; Lindberg et al., 2004; Ridpath, 2010a). With transient infections, mild clinical signs may ensue after an incubation period of 5-7 (Fulton, 2013). Clinical signs of BRD associated with BVDV-1 infection may develop due to immunosuppression and biological synergism with other infectious agents (Ridpath, 2010a). Although PI animals are the major reservoir for maintenance and transmission of BVDV-1 in cattle populations transmission of BVDV-1 and BVDV-2 from TI animals may also occur. TI animals can shed BVDV-1 and BVDV-2 in nasal secretions (Nickell et al., 2011), and ongoing infection in cattle herds in the absence of PI animals has been documented (Moen et al., 2005). Experimental transmission of BVDV-1a has been demonstrated from apparently recovered and antibody positive animals 98 days post challenge (Collins et al., 2009).

The prevalence of persistent infection in animals arriving at feedlots is low, with reported estimates ranging from less than 0.1% to 0.4%, (Taylor et al., 1995; Loneragan et al., 2005; Fulton et al., 2006). Despite the low prevalence of persistent infection, and the tendency for PI animals to cluster within arrival groups, a small number of PI animals in feedlot settings may result in animals in a large proportion of pens being exposed to BVDV, especially if adjacent pens are considered exposed (Loneragan et al., 2005; Fulton et al., 2009b). Hence, the identification and removal of PI animals has been advocated as a BRD control strategy in cattle populations (Fulton et al., 2009b).

Although seroprevalence studies indicate that BVDV-1 is widespread in Australian cattle populations (Dunn et al., 1995; Durham and Paine, 1997; Taylor et al., 2006), and seroconversion to BVDV-1 has been associated with BRD in Australian feedlot cattle at the animal level (Dunn et al., 1995), there have been no detailed investigations into effects of exposure to PI animals on the occurrence of BRD in Australian feedlot populations. Results from North American studies investigating the impact of the presence of PI animals within feedlots on BRD risk have been variable (Loneragan et al., 2005; O'Connor et al., 2005; Booker et al., 2008a). While it is plausible that prior exposure to BVDV-1 would ameliorate the adverse effect of exposure to PI animals, none of these studies were able to assess the effects of prior exposure to the virus.

Hypotheses and aims

The following *a priori* hypotheses were formulated based on published literature and plausible biological pathways:

a) The risk of BRD is increased in cohorts (feedlot pens) in which BVDV-1 is infecting animals compared to cohorts in which BVDV-1 is not being transmitted;

b) The risk of BRD is increased if a PI animal is present in the cohort compared to cohorts in which BVDV-1 is being transmitted but no PI animal is present, because in-contact animals in the same pen as PI animals are probably exposed to higher viral loads than animals in pens without PI animals;

c) The effects of exposure to BVDV-1 in feedlot pens vary depending on prior exposure to BVDV-1 PI animals. Animals from the same farm as a PI animal may have been exposed in-utero; such congenitally infected animals may be at increased risk of BRD even if not PI. Animals exposed to BVDV-1 PI animals at a later stage are at reduced risk of BRD compared to animals not previously exposed to BVDV-1 PI animals, provided exposure occurred a sufficient time (at least 4 weeks) prior to exposure in a feedlot pen.

d) Animals with sufficiently high antibody concentrations at induction are protected from increases in BRD risk due to exposure to BVDV-1 at the feedlot

e) PI animals have lower Ct readings in qPCR analyses than TI animals, and hence a single Ct value may be of use in discriminating between PI and TI animals.

The objectives of the current study were to determine the prevalence of persistent infection in animals arriving at Australian feedlots, and test the hypotheses outlined above.

Materials and methods

Study design and study population

The current study used data collected as part of the National Bovine Respiratory Disease Initiative (NBRDI), which was a nationwide prospective longitudinal study conducted in Australia to evaluate many possible risk factors for BRD in feedlot cattle (Hay et al., 2014). Cattle were inducted (i.e. identified and animal characteristics recorded electronically), and enrolled in stable cohorts where a cohort consisted of all animals placed and held together in

a feedlot pen following induction. A total of 35,160 animals were inducted into study cohorts from March 2009 to December 2011, of which 35,131 animals had sufficient data for inclusion in the analyses (i.e. project population).

Each animal was monitored from induction until it left the cohort for any reason; the induction date was designated 'day 0' and time at risk began the day after induction into a cohort. As previously reported, data from the Australian National Livestock Identification System database were accessed and used to determine each animal's location, and hence its 'group' at time points of interest prior to induction (Hay et al., 2014). Days prior to day 0 were identified using negative values. Each cohort consisted of one of more 'group-13s' where a group-13 consisted of all animals that were together on day -13 (i.e. 13 days before induction) that then went into the same cohort. The study population had a nested hierarchical structure such that animals were clustered within 1,077 group-13s which were clustered within 170 cohorts, which were clustered within 14 feedlots. Group defined at day -28 (i.e. group-28) and group of origin (comprising animals born on the same farm and that went into the same cohort) were also used to derive a variable describing prior exposure to PI animals.

The BRD case definition was based on clinical signs indicating respiratory system involvement as recorded by feedlot staff in computerised hospital records. The case definition comprised diagnoses of "pneumonia", "respiratory", "BRD" and "IBR" (infectious bovine rhinotracheitis) (Hay et al., 2014). Blood samples were collected from all study animals at induction and again at 'follow-up' sampling, which was scheduled at approximately 42 days after induction. At induction nasal swabs were also collected from all animals. In addition, blood samples and nasal swabs were requested from all animals that were hospitalised due to respiratory signs, and necropsy samples (lung and tracheal tissue) were requested from any animals suspected of having died of BRD. Sample handling, processing and storage have

been described elsewhere (ref, this issue). Data received from the feedlots and laboratory were cross-checked, and samples were verified as belonging to the animal indicated if they could be linked to an individual animal listed in both laboratory and feedlot files.

A subset of the project population (N=7,314), consisting of randomly selected animals enrolled in a nested case-control study (ref, this issue) had individual paired antibody serology results for BVDV-1 and three other viruses commonly implicated in BRD: *Bovine herpesvirus 1* (BoHV-1), *Bovine respiratory syncytial virus* (BRSV) and *Bovine parainfluenza virus 3* (BPIV-3). Serum samples were tested using an indirect multiplex ELISA (BIOX K 284 ELISA®) test. ELISA results were output as optical densities on a continuous scale and then categorised on a six-point scale (from 0 to 5) using an algorithm provided by the manufacturer.

BVDV-1 vaccination

Pestigard®, an inactivated BVDV-1 vaccine registered to reduce reproductive losses due to BVDV-1, was the only commercially available BVDV-1 vaccine in Australia at the time of the study. As part of the NBRDI, farmers ('vendors') who supplied groups of 20 or more study animals to participating feedlots were surveyed. Questionnaires were sent to the vendors of approximately 75% of study animals. Responses were received from the vendors of 10,731 animals and prior vaccination status was determined for the 8,580 animals (about 24% of the total study population) that were born on the vendor's farm or purchased by the vendor before they were 10 months of age and so very unlikely to have been vaccinated before purchase (ref, under review). Questionnaire responses indicated that only about 12% of animals had received one or more doses of Pestigard® at any time during their lives (with the first dose administered at least two weeks before induction for all those vaccinated); of those, about 69% had received two doses. Thus, although the prior vaccination status was

unknown for the majority of the population, extrapolation of results from the vendor questionnaire indicated few study animals had received the recommended vaccinations (two initial doses four to six weeks apart and an annual booster) against BVDV-1.

Identification of PI animals

PI animals were identified by pooled and individual testing of samples collected from each animal (Figure 1) using a previously described qPCR assay (Horwood and Mahony, 2011). Upon receipt of samples at the laboratory, aliquots (10µl) of the serum sample from each animal in the same cohort and from the same time period (i.e. induction or follow-up) were pooled with up to 23 other samples for subsequent extraction and qPCR analyses. Each pooled test result was classified as either negative or positive (any Ct value). All animals with a sample in at least one negative pool were classified as not being PI animals. All animals without a serum sample in an induction or follow-up pool that tested negative were identified and their samples were tested individually. For individual testing, Ct values below 35 were deemed positive, while for tests with borderline values (i.e. Ct values between 35 and 40), only those where the plot of the fluorescent signal against cycle number was consistent with the expected sigmoid shape of a positive test were classified as positive. Tests with Ct values above 40 were classified as negative. Induction samples were tested first and serum samples were tested in preference to nasal swabs. However if serum from an animal for a required time point was unavailable or unsuitable for testing, the nasal swab was tested instead. Animals with a negative induction sample were classified as not being PI animals. For animals with a positive induction sample, a second consistent test result was required, preferably collected at follow-up (Figure 1), but with a minimum interval of 28 days between sampling. For animals without an induction sample, or with a positive induction sample but without a second sample collected after at least 28 days, other test results and data were considered as detailed below and illustrated in Figure 1.

As part of the NBRDI, samples collected from animals which met the BRD case definition at first hospital examination and at post mortem from animals suspected of having died from BRD were also tested using qPCR analyses. Hospital samples comprised serum and nasal swabs. Sampling frames stratified by cohort and the time of diagnosis (ten intervals) were constructed and up to two animals per cohort per time interval were selected for testing. Deaths were attributed to BRD when the reported reason for death directly involved the respiratory system. Where multiple tissue samples were received from the same animal, these were pooled and a single qPCR test performed for that animal. These results were compiled, cross-checked and used to allocate PI statuses. If potential PI animals did not have adequate follow-up samples for the second animal-level test, hospital serum samples were tested as indicated. Animals with any negative qPCR tests from hospital or necropsy samples were classified as not being PI.

Serological results from the case-control study (ref, this issue) for BVDV-1 were compiled and cross-checked against the BVDV-1 qPCR results. Additional ELISA tests were performed using samples from animals suspected of being PI animals and/or animals from the same group-28 as a potential PI animal, as indicated. Assuming animals in the same group-28 as a PI animal were commingled, we expected they would be highly likely to be seropositive at induction. If a potential PI animal did not have a sufficient sample for a follow-up test and was concurrently seronegative and qPCR positive at induction, while some or all animals from the same group-28 were highly seropositive, it was classified at being a PI animal (Figure 1). Animals that were determined not to be PI animals but had a positive animal-level qPCR test were classified as TI animals.

Assessment of Ct value for differentiating PI from TI animals

To assess whether a single qPCR test result was useful in discriminating between TI and PI animals, Ct results were compared between animals classified as TI and those classified as PI.

Because the main aim was to identify PI animals, not all TI animals in the population were individually identified. The majority of animals with a single positive individual qPCR result that were not classified as PI animals were classified as TI animals because they had an additional sample that tested negative in either animal-level or pooled qPCR tests, or because they were concurrently seropositive or seroincreased. For samples collected at induction returning positive Ct results, receiver operating characteristic (ROC) curve analysis was performed and interval likelihood ratios were calculated using MedCalc® (Version 15.11.4). ROC curve analysis was used to assess the overall ability of a single positive qPCR test result to discriminate between PI and TI animals while interval likelihood ratios were used to compare the probability of being a PI animal with the probability of being a TI animal for Ct values within each of four intervals. The interval likelihood ratios can be used in combination with the pre-test probability that a qPCR positive animal is a PI animal to determine the probability that an animal with a Ct value in a given range is a PI animal. Given the short duration of transient infection, for animals with no recent history of mixing, the pre-test probability that a qPCR positive animal is a PI animal would be high. Alternatively, animals immunologically naïve to BVDV-1 which have been recently mixed with a PI animal would be expected to have a lower pre-test probability. Assuming pre-test probabilities of 0.9 and 0.3 respectively for these two scenarios, the probabilities that an animal with a Ct value in a given range is a PI animal were calculated.

Genotyping

Genotyping techniques, as detailed in a previous Australian study conducted at the same laboratory (Mahony et al., 2005), were applied by sequencing PCR amplicons from total nucleic acid extracts from necropsy and hospital samples from animals from 27 cohorts from 10 feedlots that had tested positive to BVDV-1 by qPCR.

Exposure variables and causal diagram

A series of exposure variables were derived to describe exposure of animals to BVDV-1. Exposure variables of interest included the animal-level BVDV-1 PI status ('BVDV PI animal': yes, no) and the cohort-level BVDV-1 status ('BVDV in cohort': yes, no), which was a binary variable indicating whether BVDV-1 had been detected using qPCR analysis in any sample (whether tested in a pool or individually) from any animals in the cohort (i.e. one or more TI and/or PI animals were present in the cohort). A further, more refined variable ('BVDV PI animal in cohort': PI, TI but no PI, no BVDV) differentiated between whether or not a PI animal had been identified in the cohorts in which BVDV-1 had been detected by qPCR. If a PI animal was not identified in the cohort, positive qPCR tests were assumed to be due to at least one TI animal. To evaluate the effects of the timing of exposure to a BVDV-1 PI animal, the group of origin and group-28 of identified BVDV-1 PI animals were determined and a categorical variable was derived ('BVDV PI exposure history'). Thus, study animals were classified by the timing of their first known exposure to a BVDV-1 PI study animal (i.e. group of origin, group-28 or cohort), and whether or not BVDV-1 was detected (using qPCR) in any animal in the cohort. Because animals in the same group went into the same cohort, they were assumed to have commingled in those groups prior to feedlot entry.

Causal diagrams were used to inform analyses of data from the NBRDI (Hay et al., 2014). In causal diagrams, direct effects are indicated by arrows connecting the exposure directly with the outcome. Indirect effects refer to pathways through intermediate variables and total effects are the combination of direct and indirect effects (Dohoo et al., 2009). The postulated causal diagram illustrated in Figure 2 contains covariates relevant to analyses in the current study. Covariates measured in the full study population are shown in black, while those measured only in the nested case-control population are shown in grey. Covariates measured in the full study population consisted of the number of animals inducted into the cohort

(<200, ≥200), whether the water troughs could be accessed by animals in adjoining pens (Shared pen water: yes, no) and mixing history. 'Mixing history' was a composite animal-level variable that described commingling prior to day -27 (yes, no), between days -27 and -13 (yes, no) and the number of group-13s combined to form a cohort (1, 2 or 3, 4 to 9, 10 or more).

Several serological variables were also used in subset analyses. In the case-control study, animals in categories 0 or 1 at induction (on a scale from 0 to 5) were at increased risk of BRD compared to animals in category 2 and above (ref, this issue). In the current study, animals in categories 0 and 1 were classified as 'seronegative' while those in categories 2 to 5 were classified as 'seropositive' for the 'BVDV induction serostatus' variable. 'BVDV seroincrease' categorised animals that seroincreased by at least two categories as 'yes', while those that did not seroincrease or were initially high (i.e. category 4 or 5) were categorised as 'no'. A further variable, 'number of viruses seroincreased', describing the number of the four viruses investigated that each animal seroincreased for between induction and follow-up (ref, this issue), was included in the causal diagram because BVDV-1 is believed to act synergistically with other viruses (Ridpath, 2010a). Animals were selected for the case-control study in two 'selection batches' and cut-points for the ELISA tests varied slightly between plates with four different batch numbers ('test batches') (ref, this issue). Test batch and selection batch were included as covariates in analyses which also included serological variables.

Statistical analyses

The total effects of each exposure variable of interest were assessed using the full study population. In addition, the total and direct effects of prior exposure to BVDV-1 ('BVDV PI exposure history') were assessed and compared using the nested case-control study population subset because serological variables were intervening variables between the

exposure and BRD. Inclusion of the animal-level induction serology variable would be expected to adjust for prior exposure to BVDV-1, whether through natural exposure or prior vaccination with Pestigard®. Further, assuming antibody concentrations at induction reflect immunity against BVDV-1, we expected that there would be interactions between animal-level induction serostatus and 'BVDV PI exposure history'. Hence, a model was fitted to test these interaction terms.

The unit of analysis was the individual animal, and the outcome of interest was the binary variable, development of BRD during the first 50 days following induction. Microsoft Excel® (2010), Microsoft Access® (2010) and Stata® (version 12) software packages were used for data management and preliminary analyses and MLwiN® (version 2.27) was used to fit final multilevel logistic regression models. The DAGitty® software (Textor et al., 2011) was used to reproduce the causal diagram and determine appropriate sets of covariates to adequately control confounding, and to remove pathways via intervening variables when estimating direct effects. Four or three-level logistic regression models were fitted for each exposure of interest, with random effects of feedlot, cohort nested within feedlot and group-13 nested within cohort (excluded from subset analyses), using second-order penalised quasi-likelihood methods to produce starting values for the second model, implemented using Bayesian Markov chain Monte Carlo (MCMC) methods. The MLwiN® default Metropolis Hastings sampling methods and flat Gaussian prior distributions were used, and convergence was assessed by inspecting diagnostic trajectory plots and summary statistics (Browne, 2012). Because models contained cohort-level variables, hierarchical centring at the cohort level and orthogonalisation were applied to improve convergence (Browne, 2012). After a burn-in of 500 iterations, MCMC chains were run for between 20,000 iterations and 200,000 iterations to obtain final posterior parameter estimates of mean odds ratios (ORs) and 95% credible intervals.

Clustering of PI animals within groups of origin, group-28s, cohorts and feedlots was assessed by estimating the intraclass correlation coefficient for each cluster variable. Null random effects models were fitted using Stata's *xtmelogit* command to determine the variance for each cluster variable and by using the latent variable threshold approach, the animal level variance was assumed to be $\pi^2/3$ or 3.29 (Snijders and Bosker, 2012). The within-cluster intraclass correlation coefficients were calculated as the sums of the proportions of total variances accounted for collectively by feedlot, cohort, and group-28, or feedlot, cohort, group-28 and group of origin.

Results

PIs identified

Of the 131 animals with positive induction qPCR results (Figure 1), 33 returned negative follow-up (N=30) or hospital (N=3) sample qPCRs. From the remaining 98 animals with a positive induction sample and one animal with a positive hospital sample (without an induction sample), seven were classified as not being PI animals because of a positive ELISA antibody result (N=1) or serological profiles in common group-28 animals being consistent with the animal having transient rather than persistent infection (N=6), and no PI status was allocated for seven. A total of 85 PI animals were identified, giving an animal-level apparent prevalence of 0.24% (85/35,138; 95% CI 0.20% to 0.30%). Of the PI animals, 76 were identified based on two positive qPCR tests, a positive induction sample and one other. Of these 76 animals, 67 had a positive follow-up sample and the interval between sampling was a minimum of 35 days. A further seven had a second positive qPCR test derived from hospital samples; six of these animals also had paired serology results indicating they were seronegative at induction and follow-up (i.e. at least 35 days between samples), and one was concurrently seronegative and qPCR positive on day 22 at the time of hospital sample collection. Two animals returned positive qPCR results on induction samples and necropsy

samples collected on days 21 (Ct values: 27 and 23) and 148 (Ct values; 31 and 33). A further nine animals were classified as PI animals in the absence of a second positive qPCR test. Eight animals with single positive Ct values (28 to 36) from induction samples were concurrently seronegative while seroprevalences in animals in the same group-28 were high. One animal with a positive induction sample (Ct 29), but without a sufficient follow-up sample for individual testing, was classified as a PI animal because it was the only animal to contribute a sample to positive induction and follow-up plates that did not have a negative test result.

BVDV-1 distribution by group and cohort

For 84 of the 85 identified PI animals, the groups of origin were determined; there were 73 separate groups of origin from 72 source farms. The majority of these groups of origin contained a single PI animal, with two PI animals in each of five groups and three PI animals in each of three groups of origin. Commingling of groups over time was commonly observed in the population; there were a total of 5,883 identified groups of origin but only 1,274 group-28s. The 85 identified PI animals were distributed amongst 67 (5.3%, 95% CI: 4.2 to 6.7%) of the group-28s; a single PI animal was identified in 55 group-28s, two in seven group-28s, three in four group-28s and four in one group-28. PI animals were highly clustered within groups of origin (intraclass correlation coefficient: 0.70, 95% CI: 0.53 to 0.83). A high level of clustering in group-28s was also observed (intraclass correlation coefficient: 0.50, 95% CI: 0.38 to 0.63), but this was markedly reduced when clustering within groups of origin was included in the same model (intraclass correlation coefficient: 0.22, 95% CI: 0.14 to 0.34). Clustering was not observed in cohorts or feedlots if the group-28 and/or group of origin were included in the random effects model. Although the animal-level prevalence of persistent infection was very low, 54 (32%, 95% CI 25.2 to 39.2%) of the 170 cohorts, from 12 of the 14 feedlots, contained at least one PI animal. A single PI animal was identified in 35

cohorts, two PI animals were identified in each of 10 cohorts, three PI animals were identified in each of six cohorts and four PI animals were identified in each of three cohorts. BVDV-1 was detected in qPCR tests (both pooled and animal-level) in many cohorts in which no PI animals were identified, presumably because of transient infection. BVDV-1 was detected in at least one animal from 47 such cohorts (47% of the 101 cohorts with any positive BVDV-1 qPCR test and 28% of all 170 cohorts). For 22 animals, no PI status could be allocated.

Associated antibody results

Of the 85 identified PI animals, 31 had a least one ELISA result. Of these, 29 were seronegative. One further animal (Ct values 30 and 29) was seropositive at induction (category 5) and remained so at follow-up (category 4). The final animal (Ct values 29 and 28) seroconverted between induction and follow up (i.e. moved from category 0 to 5). Both of these animals were categorised as PI animals as they had paired positive qPCR test results; the intervals between testing were 55 days and 43 days, respectively.

At least one animal seroincreased for BVDV-1 in 51 (74%) of the 69 cohorts in which BVDV-1 had not been identified in any qPCR analyses. If seroincrease between induction and follow up in any study animal had been used to define presence of BVDV-1, then BVDV-1 would have been considered as being present in 142 of the 161 (88%) cohorts that contributed animals to the case-control study, compared with 101 of 170 (59%) cohorts in which BVDV-1 was detected on qPCR.

qPCR results

The distributions of animal-level qPCR results by the time of sampling and over BVDV-1 PI status are illustrated in Figure 3. Overall, Ct values for PI animals ranged from 23 to 37 with a median of 29 and interquartile range from 28 to 31; values were similar regardless of the type of sample or when it was collected. All 85 PI animals had a positive qPCR result from a

sample collected at induction (78 sera samples and 7 nasal swabs); the second samples included 67 follow-up sera samples, one hospital serum sample, six hospital nasal swabs and two necropsy samples. Of the 85 animals identified as PI animals, 15 (18%) had at least one Ct value above 33, but all except one had a second value below 33 and seven animals were concurrently seronegative. With a median Ct value of 37 (interquartile range: 34 to 38), the qPCR values for samples collected from TI animals at induction (N=75) were generally higher, but the range of values (26 to 40) overlapped those of PI animals considerably. Further, 10 of 75 (13%) TI animals with a positive serum sample collected at induction returned a Ct value below 33.

Overall, a single qPCR performed on a sample collected at induction had good ability to discriminate between PI and TI animals. The area under the ROC curve was 0.94 (95% CI: 0.90 to 0.97). The interval likelihood ratios are reported in Table 2. Animals with Ct values in the range 20 to <29 (interval likelihood ratio 14.6; 95% CI: 3.6 to 58.6) and 29 to <33 (interval likelihood ratio 4.6; 95% CI: 2.3 to 9.2), were more likely to be PI animals than TI animals whereas animals with Ct values in the ranges 33 to <36 and 36 to 40 were much less likely to be PI animals than TI animals. Assuming a pre-test probability of 0.90, a qPCR value less than 33 would indicate that the probability that the animal is a PI animal is very high (0.98 or higher, Table 2). Alternatively, assuming a pre-test probability of 0.30, the probability that an animal with a Ct value less than 29 is a PI animal would be more modest (0.86; Table 2). Results were comparable when the two animals that seroconverted were instead classified as TI rather than PI animals.

Of animals which met the BRD case definition, 66% (4,086/6,200) had nasal swab samples suitable for testing and collected at first hospital examination. Of these, a total of 759 nasal swabs were tested using qPCR to detect BVDV-1, and 27 (3.6%) returned positive results. Six were classified as PI animals and 21 (Ct values ranging from 23 to 39, with a median of

35 and interquartile range: 33 to 37) were determined to be TI animals. Of the 239 animals in which death was attributed to BRD, necropsy samples were received and tested from 126 animals. Both lung and tracheal tissue was received from 113 animals, ten animals had only tracheal samples and three animals had only lung tissue samples. Of these 126 animals, 39 (31%) had positive qPCR results for BVDV-1 of which 37 were determined to be TI rather than PI animals. The Ct values ranged from 21 to 38 with a median value of 33 and an interquartile range from 29 to 35. Genotyping on a total of 34 isolates determined that all were BVDV-1c (further results not shown).

Risk of BRD

The animal-level distributions of derived exposure variables and estimated odds ratios used to measure their effects on the occurrence of BRD by day 50 are shown in Table 1. At 27.1% (23/85), the 50-day cumulative incidence of BRD in PI animals was much higher than the 17.6% (6,171/35,034) observed in non-PI animals. Animals persistently infected with BVDV-1 were at moderately increased risk of developing BRD compared to animals that were not persistently infected (OR 1.9, 95% credible interval: 1.0 to 3.2).

About two thirds (66%) of animals were in cohorts in which BVDV-1 was detected in at least one pooled or individual qPCR test; these animals were at moderately increased risk of BRD (OR: 1.7, 95% credible interval: 1.1 to 2.5, Table 1) compared to animals in cohorts where BVDV-1 had been not been detected by qPCR testing. This association was further explored by separating animals in cohorts where BVDV-1 had been detected depending on whether or not a PI animal had been identified in their cohort; 39% of all animals were in the same cohort as a PI animal and 27% were in cohorts in which transient but not persistent infection had been detected. Compared to animals in cohorts in which BVDV-1 was not detected by qPCR in any animal, animals in cohorts with identified PI animals were at moderately increased risk of BRD (OR: 1.6, 95% credible interval: 0.9 to 2.5, Table 1) as were animals in

cohorts in which transient but not persistent infection with BVDV-1 had been detected (OR: 1.9, 95% credible interval: 1.1 to 3.0, Table 1).

The variable, 'BVDV PI exposure history', further classified animals based on the timing of their first known exposure to a BVDV-1 PI animal, with other categories as described for the 'BVDV PI animal in cohort' variable. Approximately 4% of study animals were from the same group of origin as an identified BVDV-1 PI animal, while a further 5% were in the same group-28 (but different group of origin) as a PI animal and 30% were not known to have been exposed before day -27 and in the same cohort as a PI animal (Table 1). Compared to animals in cohorts in which BVDV-1 was not detected by qPCR in any animal, point estimates indicated elevated risk for all other categories, although not all 95% credible intervals excluded one. The lowest point estimate was for animals in the same group-28 as a PI animal (OR: 1.3, 95% credible interval: 0.8 to 2.0) and the highest point estimate was for animals in cohorts in which BVDV-1 was detected but no PI animal was identified (OR: 1.8: 95% credible interval: 1.1 to 2.8). The effect estimates obtained from models fitted in the case-control subset indicated consistent but stronger effects across categories (Table 1). Although effect estimates were imprecise, animals in the same group of origin as a PI animal were at the highest risk of BRD at the feedlot after adjusting for the serological variables (OR: 2.3, 95% credible interval: 1.2 to 4.1). Results were consistent when PI animals were excluded from the model.

The Wald p-value for interaction between animal induction serostatus and 'BVDV PI exposure history' was 0.57 but amongst animals with no history of exposure to BVDV-1 in their original group or group-28, there was evidence that being seropositive at induction was protective. For animals in cohorts with identified PI animals, the estimated odds ratio for being seropositive (compared to being seronegative) was 0.7 (95% credible interval: 0.6 to 0.9) and in cohorts in which BVDV-1 was detected but no PI animals were identified, the

estimated odds ratio was 0.8 (95% credible interval: 0.6 to 1.0). However, similar protective effects were evident in cohorts where BVDV-1 was not detected (odds ratio 0.7; 95% credible interval: 0.5 to 1.0).

Discussion

Cattle in the study population were commonly exposed to BVDV-1 during their time on feed at participating feedlots. PI animals were identified in 32% of study cohorts, while BVDV-1, in the absence of PI animals, was detected by qPCR testing in a further 28% of cohorts. Presence of BVDV-1 (any positive qPCR test) in the cohort was associated with moderately increased risk of BRD, whether or not a PI animal was identified in the cohort. BVDV-1 was detected by qPCR in 4% of nasal swabs tested that were collected from BRD cases and 31% of necropsy samples from animals that died from BRD. Consistent with results obtained in a previous study conducted at the same laboratory, genotyping indicated that all isolates were BVDV-1c (Mahony et al., 2005).

In the current study, we identified the PI animals in a large population comprising 35,160 animals in 170 cohorts from source farms located throughout the cattle-producing regions of Australia. The 85 identified PI animals comprised 0.24% of the study population. This prevalence was slightly lower than that reported in some North American studies with reported prevalences at arrival at feedlots of 0.3% (Loneragan et al., 2005) and 0.4% (Fulton et al., 2006) in calves aged approximately six months. Another study reported a prevalence of 0.55% in calves aged two to four months in beef cattle herds (Fulton et al., 2009c). The lower prevalence in the NBRDI study population is not unexpected because cattle entering Australian feedlots are on average older than those entering North American feedlots (Horwood et al., 2014) and prevalence of persistent infection declines with increasing age as PI animals within a population are progressively removed due to increased susceptibility to

other diseases and greater likelihood of culling due to poor performance. In Australia the exact age of cattle entering feedlots is usually unknown but animals enter at a range of weights and ages (Gaughin and Sullivan, 2014). In the NBRDI population, the median induction weight was 438 kg (interquartile range: 408 to 466kg) (Hay et al, in press) indicating that they were on average considerably older than animals in the North American studies. We have identified a high level of clustering of PI animals by group of origin. Importantly, even though the animal-level prevalence was very low and PI animals tended to cluster within groups sourced from a small number of farms, 66% of animals were in cohorts where BVDV-1 was detected.

As expected, exposure to BVDV-1 in the cohort was associated with increased risk of BRD. However, contrary to our hypothesis, animals in cohorts in which BVDV-1 was detected but without an identified PI animal were not at reduced risk compared to animals in cohorts with an identified PI animal. Previous studies investigating the association between exposure to PI animals and BRD in populations of feedlot cattle have reported no effect (Elam et al., 2008) and increased risk (Loneragan et al., 2005) when PI animals in adjoining pens were included in the classification of exposed along with PI animals in the animal's own pen. O'Connor et al. (2005) reported that, amongst animals from multiple sources, mean BRD incidence was similar in pens with PI calves and pens in which no PI animals were identified. Transmission of BVDV-1 from animals in adjoining pens and failure to account for initial immune status could explain some differences in observed effects of exposure to PI animals between previous studies. In a recent review, Fulton (2013) noted the importance of including a large number of pens and PI cattle in research studies; this could result in more generalisable results given variability in virulence of the virus and animal-level variation in immune status at induction.

Some degree of misclassification of BVDV-1 exposure statuses in the cohort probably occurred in the current study. Serological results indicated that prior exposure to BVDV-1 was common and that exposure to BVDV-1 in the cohort was occurring more commonly than indicated by the qPCR results, with at least one animal seroincreasing to BVDV-1 in 74% of the cohorts in which BVDV-1 had not been identified in any qPCR analyses. This is a consequence of sampling animals at only two time points. This may explain why no significant interaction was observed between BVDV-1 PI exposure category and BVDV-1 serostatus at induction. Amongst animals with no history of exposure to BVDV-1 in their original group or group-28, estimated protective effects of BVDV-1 serostatus at induction were similar in cohorts where BVDV-1 had been detected by qPCR analyses and cohorts where no BVDV-1 was detected. Misclassification errors could also have influenced the classification of each animal's prior PI exposure history; there were probably considerable misclassification errors in both directions because animals may have been exposed to PI animals prior to feedlot entry but the PI animal was not in their group at the feedlot, and animals may have been at the same location as PI animals without being in contact with the PI animal. These misclassification errors would be expected to have been non-differential with respect to BRD occurrence, and if so, any bias would be towards the null effect. So the true differences in risk due to both presence of BVDV-1 in the cohort and exposure to BVDV-1 before feedlot entry are probably larger than those observed in the current study. In this study we have utilised knowledge of prior group structure to investigate the effects of probable prior exposure to BVDV-1 at different time points. We have also used this information in combination with qPCR testing on pooled and individual samples and serological testing to assist in the classification of PI animals. Immune responses resulting from PI animal exposure to a immunologically heterologous strain of BVDV-1 have been reported (Collen et al., 2000). However, because identified Australian strains of BVDV-1

appear to be antigenically similar to each other (Mahony et al., 2005), it is unlikely that PI animals would be seropositive to a different strain, but this assumption may not be generalisable to other populations. By identifying study animals which were in the same groups of origin and group-28s as PI animals and probably in contact with these PI animals, we have investigated the effects of the timing of exposure to PI animals. For a subset of the population we have been able to separately estimate direct effects after accounting for the measured serum antibody levels for BVDV-1 and three other viruses. We postulated that exposure to BVDV-1 prior to day-27 would be expected to result in higher BVDV-1 antibody concentrations at induction compared to animals not exposed prior to day-27. A better clinical outcome for these animals compared to animals naïve with respect to antigenically similar strains of BVDV-1 would be generally expected. All categories of animals classified as exposed to BVDV-1 were at increased risk of BRD, with similar increases in risk for animals exposed to a PI animal in the group-28, in the cohort (without known exposure prior to day -27), or exposed to BVDV-1 but without an identified PI animal in their cohort after accounting for serological measures. However, our results showed that animals in the same group of origin as PI animals were probably at further increased risk of BRD after adjusting for serological variables. This is consistent with the hypothesis that congenitally infected non-PI animals suffer long-term health problems. Such calves may perform poorly and be at increased risk of disease (Taylor and Rodwell, 2001; Torres, 2014; Graham et al., 2015). While this relationship could be confounded by other unknown management-related factors, it is biologically plausible that long-term impairment to the immune system may result from congenital infection with BVDV-1 even where the animal does not become PI.

Results of the current study indicate that ongoing transmission of BVDV-1 in the absence of identified PI animals in the pen was commonly occurring. Possible sources of infection to animals in pens with no PI animals included direct contact with PI animals in adjoining pens,

indirect contact with secretions from a PI animal via fomites including shared pen water, or transmission from other in-contact TI animals in the same pen. If transmission from TI animals is possible under feedlot conditions it is likely that widespread infection in susceptible feedlot populations would result despite removal of PI animals soon after arrival and testing at the feedlot. To obtain larger reductions in the impact of BVDV-1 in feedlots, PI animals could be identified and removed at the farm before cattle are shipped to the feedlot. However, unless frequency of vaccination against BVDV-1 in Australia increases from that observed in our study, routine removal of PI animals at source farms combined with biosecurity measures may ultimately create large populations of BVDV-1 susceptible animals on those farms. Therefore, cattle entering feedlots from farms using routine PI elimination from all cattle should be vaccinated before feedlot entry. Alternatively, PIs could be removed only from cattle going to feedlots, or all incoming cattle could be vaccinated before feedlot entry, regardless of farm and cattle history. Economic assessment of these strategies is required.

The results from the current study indicate that the Ct value from a single qPCR test is only moderately useful for discriminating between PI animals and TI animals. Where the pre-test probability that a qPCR positive animal is a PI is high, a Ct value less than 33 would indicate a high probability of a PI animal but higher Ct values would still indicate moderate probabilities. While the median and interquartile range limits of qPCR Ct values for the TI animals was higher than that for PI animals, there was considerable overlap in Ct values between TI and PI animals. Timing of sampling may be an important source of this overlap. The concentration of virus shed by a PI animal would be expected to remain relatively high; hence the determined Ct value would reflect this. In comparison Ct values for TI animals could be influenced by many factors, such as the type of sample, timing of sample collection relative to time of infection, BVDV-1 strain, BVDV-1 exposure status, and immunological

status. Hence, Ct values would be expected to fluctuate more widely with lower values at the time of peak viraemia followed by a progressive increase in Ct values until the virus is cleared. In the current study, animals with transient infection often returned low Ct values, especially when samples were collected in the hospital crush when first diagnosed with BRD which may correspond to peak viraemia.

Conclusions

In this study we have described the prevalence of persistent infection in animals entering Australian feedlots (0.24%), and have demonstrated that, despite a very low prevalence and marked clustering of PI animals by group of origin, exposure to BVDV-1 within cohorts was common. Our findings indicate that BVDV-1 can contribute to the pathogenesis of BRD. Importantly, we have established that the presence of BVDV-1 within a cohort moderately increases risk of BRD whether or not a PI animal is present in the cohort. Removal of PI animals and/or vaccination, both before feedlot entry, would be expected to reduce the impact of BVDV-1 on BRD risk. Economic assessment of these strategies under Australian conditions is required.

Acknowledgements

This study was supported by grant B.FLT.0224 from Meat and Livestock Australia with matching funds provided by the Australian Government.

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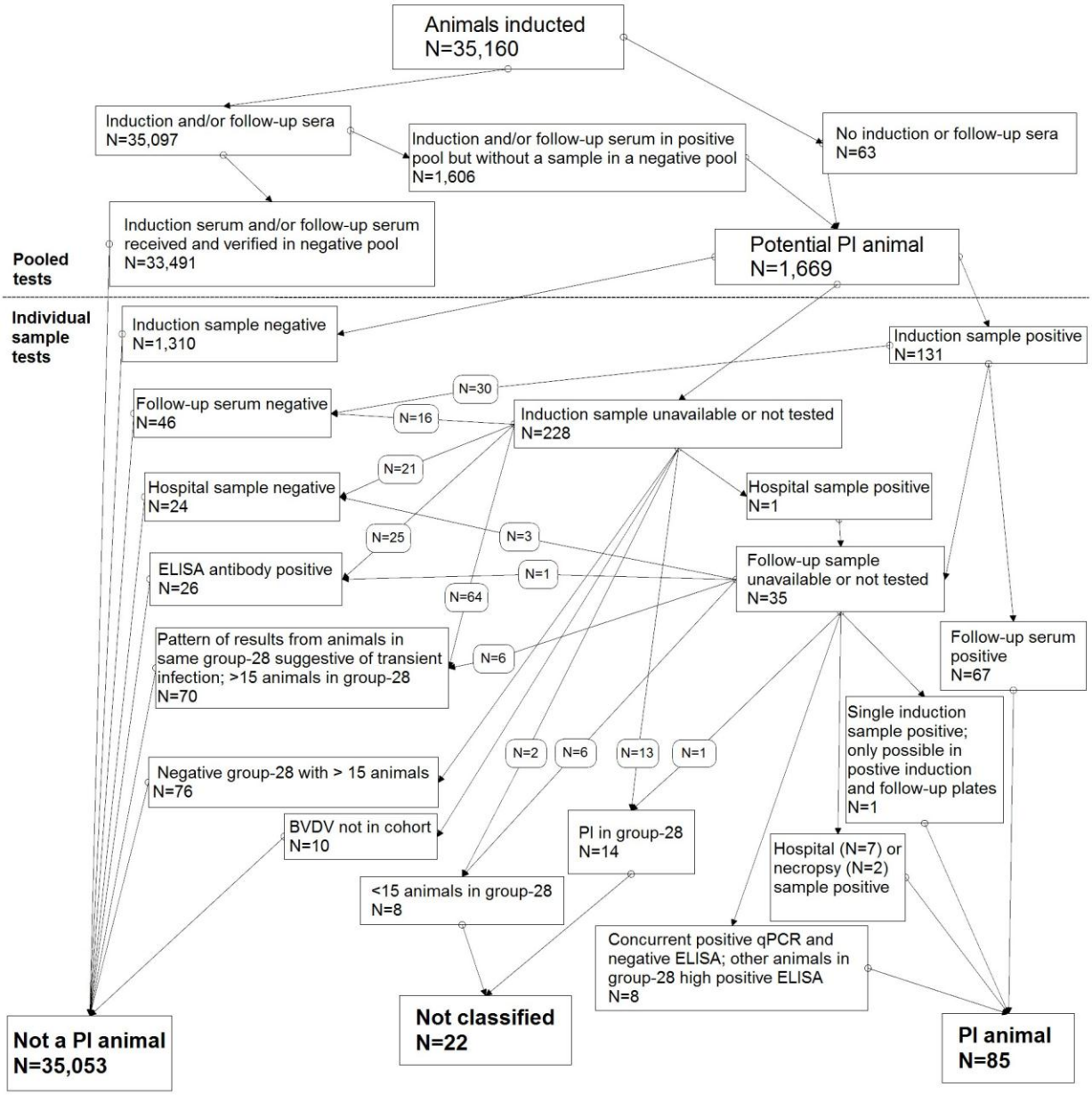

Figure 1: Schematic representation of the classification of animals based on their *Bovine viral diarrhoea virus 1* persistently infected (PI) status.

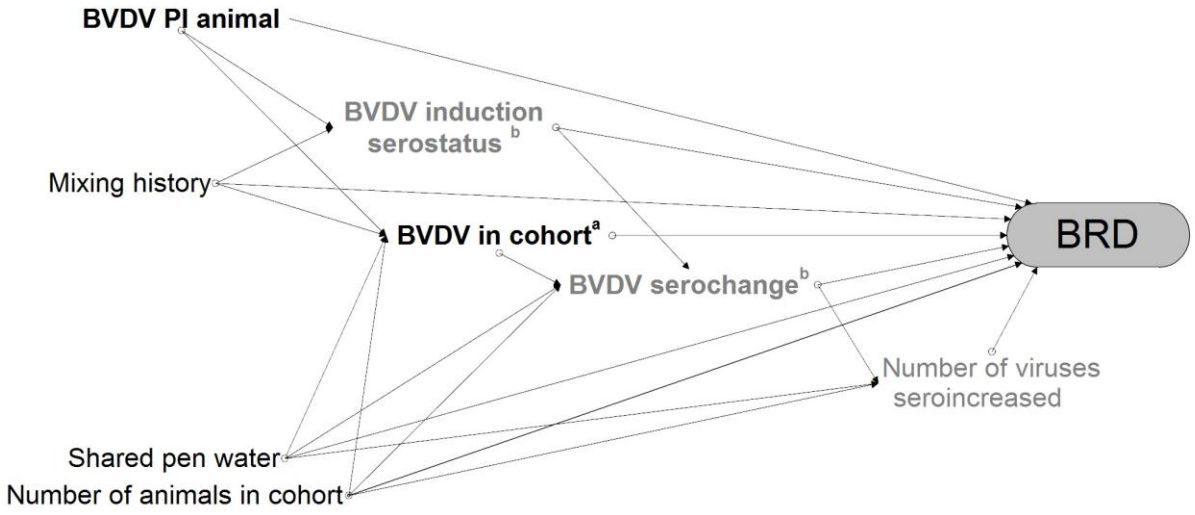

Figure 2: Causal diagram showing postulated causal pathways linking *Bovine viral diarrhoea virus 1* (BVDV) with bovine respiratory disease (BRD) including the role of BVDV persistently infected (PI) animals.

[a] The exposure variables BVDV PI exposure history and BVDV PI animal in cohort were substituted for BVDV in cohort for some analyses

[b]Serological variables (grey) were only included in separate subset analyses using the nested case-control study population (see text for explanation).

Bolding indicates variables of interest in analyses; other covariates are included because they are potential confounders and/or intervening variables for bolded variables.

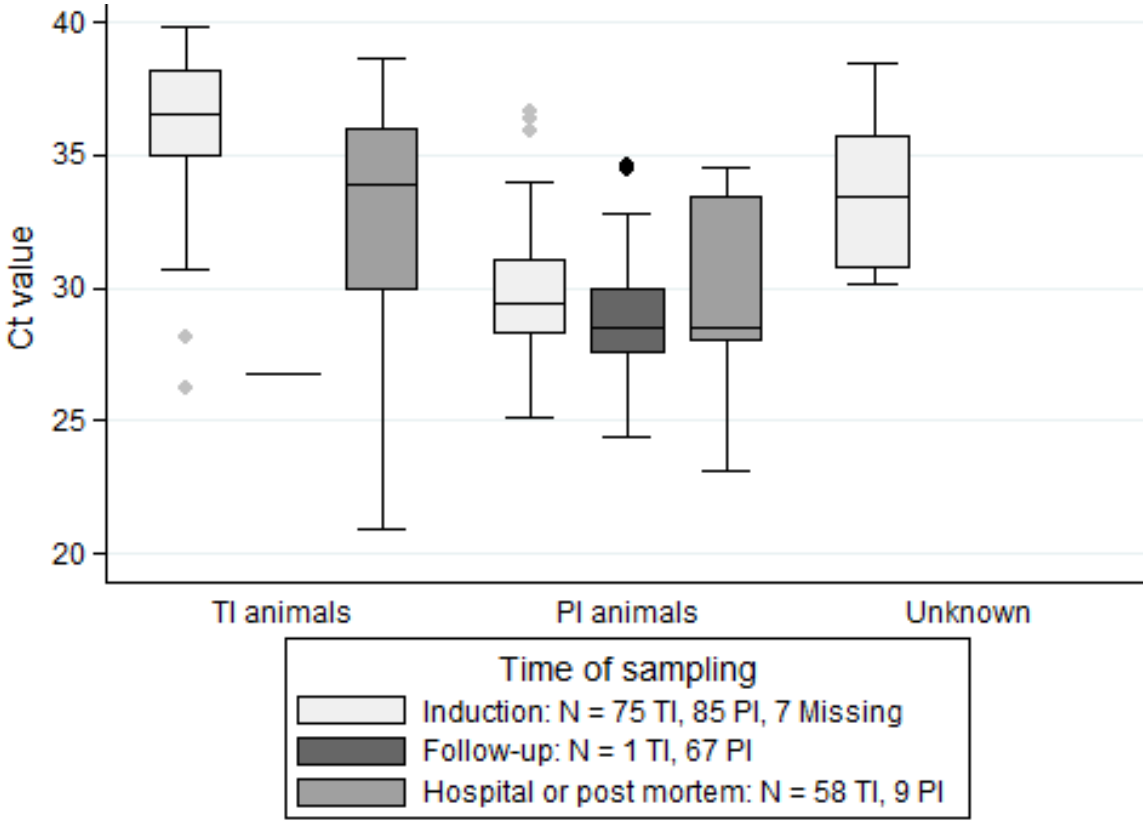

Figure 3: Boxplots displaying distributions of individual quantitative real-time PCR tests expressed as cycling threshold (Ct) test results stratified by time of sampling and *Bovine viral diarrhoea virus 1* persistently infected (PI) or transiently infected (TI) status. Animals with unknown (missing) status had a single sample collected at induction which tested positive, but did not have sufficient information to classify as PI or TI.

Table 1: Distribution of variables describing exposure to bovine viral diarrhoea virus 1 and estimated odds ratios for their total and selected direct effects on the occurrence of BRD by day 50[a]

Variable & category	Number of animals (% of animals)	Crude BRD 50-day incidence risk (%)	Adjusted odds ratio	95% credible interval
BVDV PI animal[b]				
No	35,034 (99.8)	17.6	Ref. cat.	
Yes	85 (0.2)	27.1	1.9	1.0 to 3.2
BVDV in cohort[c]				
No	11,896 (33.9)	8.7	Ref. cat.	
Yes	23,235 (66.1)	22.2	1.7	1.1 to 2.5
BVDV PI animal in cohort[c]				
No	11,896 (33.9)	8.7	Ref. cat.	
PI animal in cohort	13,579 (38.7)	22.5	1.6	(0.9 to 2.5)
TI but no PI animal in cohort	9,656 (27.5)	21.9	1.9	(1.1 to 3.0)
BVDV PI exposure history[c,d]				
Total effect using full study population				
No BVDV in cohort	11,896 (33.9)	8.7	Ref cat	
PI animal in original group	1,352 (3.8)	19.1	1.7	1.0 to 2.7
PI animal in group-28	1,846 (5.3)	15.9	1.3	0.8 to 2.0
PI animal in cohort	10,391 (29.6)	24.1	1.5	0.9 to 2.3
TI but no PI animal in cohort	9,656 (27.5)	21.9	1.8	1.1 to 2.8
Total effects using nested case-control study population[e]				
No BVDV in cohort	1,916 (26.2)		Ref. cat.	
PI animal in original group	252 (3.5)		2.1	1.1 to 3.7
PI animal in group-28	357 (4.9)		1.5	0.8 to 2.7
PI animal in cohort	2,594 (35.5)		1.8	1.1 to 3.0
TI but no PI animal in cohort	2,195 (30.0)		2.2	1.3 to 3.6
Direct effects using nested case-control study population[f]				
No BVDV in cohort			Ref. cat.	
PI animal in original group			2.3	1.2 to 4.1
PI animal in group-28			1.5	0.8 to 2.6
PI animal in cohort			1.7	1.0 to 2.8
TI but no PI animal in			2.0	1.2 to 3.2

cohort				

[a] BVDV in variable names indicates bovine viral diarrhoea virus 1. Multi-level Bayesian logistic models based on causal diagram (Figure 2) with N observations

[b] Model had no additional covariates; N=35,119; 4 level

[c] Covariates: number of animals in cohort, shared pen water, mixing history, BVDV PI animal; N=34,719; 4 level.

[d] BVDV PI exposure history described the timing of each animal's first known exposure to a BVDV PI animal; animals in cohorts without any identified BVDV PI animals were also separated into those in cohorts in which BVDV-1 was/was not detected by qPCR

[e] Covariates: number of animals in cohort, shared pen water, mixing history, BVDV PI animal, test batch, selection batch; N=7,310; 3 level.

[f] Covariates: BVDV induction serology, BVDV seroincrease, number of viruses seroincreased for, number of animals in cohort, shared pen water, mixing history, BVDV PI animal, test batch, selection batch; N = 6,717; 3 level.

Table 2: Interval likelihood ratios for the cycling threshold (Ct) range for bovine viral diarrhoea virus 1 (BVDV-1) on quantitative real-time PCR of biological samples collected at induction

Ct interval	PI	TI	Likelihood ratio	95% CI	Post-test probability that a qPCR positive animal is PI[a]	Post-test probability that a qPCR positive animal is PI[b]
20 - <29	33	2	14.56	3.62 to 58.63	0.99	0.86
29 - <33	42	8	4.63	2.33 to 9.23	0.98	0.66
33 - <36	8	17	0.42	0.19 to 0.91	0.79	0.15
36 - 40	2	48	0.04	0.01 to 0.15	0.26	0.02
Total	85	75				

[a] Assuming a pre-test probability of 0.90 that an animal is persistently infected (PI) rather than transiently infected (TI) with BVDV-1

[b] Assuming a pre-test probability of 0.30 that an animal is PI rather than transiently infected TI with BVDV-1