The Role of Infectious Pathogens on Reproductive Loss in New Zealand Beef Cattle

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Juan Manuel Sanhueza

Institute of Veterinary, Animal and Biomedical Sciences
Massey University
Palmerston North, New Zealand

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Institute of Veterinary, Animal and Biomedical Sciences

Massey University

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Abstract

The economic viability of pasture based beef breeding systems in New Zealand relies to a large extent on herd reproductive performance. During the last decade however, there has been little or no improvement of the generally poor performance of approximately 80% weaned calves from mated heifers and cows. Roughly half of the losses are attributable to conception failure, and the other half to foetal loss or poor calf survival to weaning. Two epidemiological investigations were designed to address some selected causes of these losses. They are reported in the two thesis chapters. The dissertation provides insights about the role of five infectious pathogens on reproductive loss in New Zealand beef breeding herds.

The first chapter addresses the role of *Campylobacter foetus subspecies venerealis* (*Cfv*) on losses from mating to pregnancy diagnosis. Specifically, this study was conducted to assess the accuracy of a commercial real time PCR (RT-PCR) on detecting *Cfv* from bull prepucial scrapings. Anecdotal evidence suggested an increased number of bulls testing positive without apparent relationship with fertility loss in cows served by these bulls. Therefore, bulls from herds with relatively low and high pregnancy rates were sampled and RT-PCR tested about 4-5 months after the end of the mating period. The percentage of RT-PCR positive bulls was related to the pregnancy rate of different age mobs of cows (15 month heifers, 27 month heifers and mixed age cows) mated to these bulls. As *Cfv* is a known cause of extensive reproductive loss in cattle, more RT-PCR positive bulls were expected in low-fertility than high fertility cow mobs if RT-PCR was truly detecting *Cfv*. No significant association was observed in this study suggesting either a lack of specificity of the RT-PCR, an absence or low prevalence of virulent *Cfv* in the population, or the presence of non-virulent *Cfv*. Evidence from previous studies, including a large number of attempts to isolate *Cfv* from high risk mating bulls, strongly suggests that virulent *Cfv* may not be present at any significant rate in the beef cattle population of New Zealand.

As a consequence of our study results, the test was withdrawn from the market.

The second chapter describes a matched case control study addressing the possibly causal association between rates of foetal loss and four endemic pathogens known to potentially cause abortion in cattle: Bovine viral diarrhoea virus (BVDV), *Neospora caninum* (*N. caninum*), *Leptospira borgpetersenii serovar Hardjo* (*L. hardjo*) and *Leptospira interrogans serovar Pomona* (*L. pomona*). Antibody titres were associated with aborting and non-aborting cows from the same herd at a series of titre cut offs.

The risk of foetal loss was significantly increased ($p \leq 0.05$) when cows were seropositive to *N. caninum*, *L. hardjo* and BVD. For *L. pomona* an increased risk of abortion was only observed in non-vaccinated cows. Using published procedures for estimating the 'population attributable fraction' (PAF) from case-control data, it was estimated that at least 15% of the abortions in the beef cattle population was attributable to these pathogens, hence at least 15% of lost foeti could prevented if the pathogens could be controlled. Based on the way cows were classified as cases and controls, exposed and non-exposed, we suspected that non-differential misclassification bias was present in the data. Such bias was likely to arise due to titre decay over time or classifying cows delivering stillborn calves or cows that lost a live calf after parturition as aborting. This would have skewed the PAF towards null. Hence, an unbiased PAF would likely be higher than 15%.

The results of this dissertation suggest that virulent *Cfv* is currently either absent or does not constitute a reproductive problem in beef breeding herds, and that the four investigated pathogens all contributed to foetal loss in the beef breeding herds of our study. Since close to 100% of all beef herds are infected with one or more of these pathogens, the results are relevant for all beef producers in New Zealand.

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CHAPTER 1

Campylobacter foetus venerealis and a real time PCR test: To use or not to use

1.1 Abstract

AIMS: *Campylobacter foetus subspecies venerealis* (*Cfv*) is the causal agent of bovine genital campylobacteriosis, a venereal disease that is asymptomatic in bulls but responsible for reproductive wastage in female cattle. In New Zealand, a commercial real-time PCR test was used to identify the DNA of this agent in preputial scrapings, but concerns were raised about the specificity of the test following anecdotal reports of high prevalences of test-positive bulls with no apparent relationship to reproductive performance. The objective of this study was to examine associations between real-time PCR test results from beef breeding bulls and pregnancy rates in beef herds using these bulls.

METHODS: Veterinarians from four veterinary practices selected beef cattle herds with relatively high and low pregnancy rates subsequent to mating during December 2008 to February 2009. Preputial scrapings were collected from 222 bulls which were distributed among 68 mobs of breeding females (15 or 27 month old heifers or mixed age cows) on 31 farms. Samples were tested for the presence of DNA from (*Cfv*) using the real-time PCR under consideration. Bivariate and multivariable analyses were used to test for the relationship between pregnancy rates in each mob and the percentage of real-time PCR test-positive bulls in each mob. The effects of possible confounders which included length of mating period, cow-age, average body condition score of cows, cow-bull ratio, and correlation of pregnancy rates within farm were considered in the analyses.

RESULTS: Sixty-four (28.8%) of 222 bulls tested positive, 130 (58.6%) tested negative,

and 28 (12.6%) returned an inconclusive result to the real-time PCR. The percentage of bulls testing real-time PCR positive in these mobs was not significantly associated with pregnancy rates after controlling for the effects of cow age, cow body condition score, cow-bull ratio, length of the mating period and farm on pregnancy rates.

CONCLUSION: Real-time PCR test results were not associated with pregnancy rates, suggesting that the real-time PCR was non-specific for detecting virulent (*Cfv*). This study adds to a growing body of evidence which indicates that virulent Cfv strains are either absent from or prevalent at very low levels of endemicity among beef breeding herds in New Zealand.

CLINICAL SIGNIFICANCE: This real-time PCR test should not be used for detection of virulent (*Cfv*) in bulls or for investigations of cases of low conception rates in cattle.

1.2 Introduction

Campylobacter foetus subspecies venerealis (*Cfv*) is the causal agent of bovine genital campylobacteriosis, which is characterised by reproductive wastage in female cattle due either to conception failure or early embryonic loss which may result in an increased number of services per conception (Plastridge 1951, Hoerlein et al. 1964, Clark et al. 1972, Smibert 1978). The disease is venearally transmitted during mating and bulls can carry the bacterium without showing any significant inflammatory or immune response, harbouring *Cfv* in preputial crypts where low oxygen tension and other environmental conditions favour its survival (Samuelson & Winter 1966, Vasquez et al. 1983).

When the disease is introduced into a herd, first-service conception rates may be as low as 15% to 45% (Guay 1967). In a north Queensland beef herd under field challenge conditions, unvaccinated controls had a significantly lower pregnancy rate of 55% (95% CI = 45% - 65%) than vaccinated cows 76% (95% CI = 69% - 82%) (Allan & Mutch 1971). Another investigation in Colorado reported a pregnancy rate of 5% (95% CI = 0% - 15%) after 3 cycles of 21 days in challenged unvaccinated heifers and a rate of 90% (95% CI = 79% - 100%) in non-vaccinated heifers mated with non-infected bulls (Hoerlein et al. 1965). In chronically infected beef herds in Australia, conception rates were found to be between 65% and 75% (Hum 1996).

Isolation by culture and identification of the agent are the World Organisation for Animal Health (OIE) prescribed procedures for international trade requirements (Anonymous 2010). However, isolation of the organism from samples collected in the field is difficult due its fastidious nutritional requirements for culture and low survival rates in transport media (Clark & Dufty 1978, Garcia et al. 1983, Monke et al. 2002). The need for an accurate diagnostic techniques other than culturing led to the development of an alternative method based on real time PCR technology (McMillen et al. 2006). In New Zealand, a real-time PCR test for the detection *Cfv* in bull preputial scrapings became commercially available but a high frequency of real-time PCR positive scrapings from bulls in beef breeding herds with no manifestations of reproductive wastage raised concerns about the specificity of the test. Anecdotal reports suggested that positive real-time PCR test outcomes were poorly correlated with observed pregnancy rates but data were not available to support this hypothesis since the test had only been applied to bulls from herds with poor reproductive performance. The objective of this study was to evaluate associations between pregnancy rates in beef breeding herds and the percentage of real-time PCR positive bulls used for breeding in those herds.

1.3 Materials and Methods

1.3.1 Sample selection

Four veterinary practices who serviced beef cattle herds in the districts of Rangitikei, Marlborough, Southland, and Queenstown-Lakes participated in the study. Farms with beef breeding herds were purposively selected by the practitioners on the basis of pregnancy rates (number pregnant / number mated) to ensure inclusion of herds with relatively high ($\geq 90\%$) and relatively low ($< 90\%$) pregnancy rates. All bulls which could be traced back to the mob they serviced during the mating season 2008-09, and which had not received any antibiotic treatment after mating were eligible for inclusion in the study.

1.3.2 Real time PCR

A commercial real-time PCR based on the methods described by McMillen et al. (2006) was used by Gribbles Veterinary Pathology Ltd., Palmerston North, New Zealand to test preputial scraping samples for the presence of *Cfv*. The laboratory was unaware of pregnancy rates for the mobs served by the sampled bulls. Samples were collected between April and September of 2009 using a bull rasper device (Tricamper) according to the procedure described by McMillen et al. (2006).

1.3.3 Recorded variables

All herd, mob (15 month heifers, 27 month heifers and mixed age cows) and bull related data were entered into a two page questionnaire by the participant veterinarians. Herd related variables were: name of veterinary practice, farm area (hectares), terrain for mating (flat, moderate, steep), co-grazing with sheep (yes/no) or deer (yes/no), use of a service ability test (SAT) (yes/no), and if yes what type of mount animal was used for the SAT.

Mob related variables were: number of cows mated, number of cows pregnant at pregnancy testing, body condition score (BCS), number of bulls used for mating, and duration of the mating period.

Bull variables included: real-time PCR test result, age, breed, and *Cfv* vaccination status.

1.3.4 Categorisation of bull related variables

The percentage of real-time PCR positive results in each mob was calculated from individual bull rest results.

Bull age was categorised as follows: if $\geq$80% of the bulls used in a mob were $\leq$4 years old, bull age was categorised as 'Young'; if $\geq$80% were $>$4 years old, then bull age was categorised as 'Old'; in all other instances where ages were known, bull age was categorised as 'Mixed'. If $\geq$30% of the bulls used in a mob were of unknown age, bull age was categorised as 'Unknown'.

Bull breed was categorised as follows: if $\geq$70% of the bulls used were 'Angus', bull breed was categorised as 'Angus' and as 'Other' for all other breeds.

1.3.5 Statistical analysis

Descriptive statistics were used to examine the percentage of test-positive bulls in each mob (exposure), pregnancy rates of mobs (outcome) and other recorded study variables. Logistic regression was used to assess bivariate associations between the response variable pregnancy (yes / no) and the independent variables recorded including the percentage of real-time PCR positive results. Inconclusive real-time PCR results were observed in 12.6% of the samples and all these samples were classified as negatives in the analysis.

Associations between continuous variables such as length of mating period and pregnancy rate were evaluated linearly and curvilinearly. The percentage of real-time PCR positive bulls and other variables significant at $p<0.2$ were selected for inclusion in a multivariable logistic regression model. Backward elimination was performed and variables were retained in the model when their p-value was smaller or equal to 0.05 using the likelihood ratio test. Real-time PCR test result was included in the model regardless of its statistical significance. Variables excluded initially were added to the multivariable model one by one to evaluate their significance in the presence of other variables. Outliers were investigated using Pearson residuals and influential observations were detected using Cook's distances and removed to assess changes in the real-time PCR test estimate and standard error. Finally a generalised linear mixed model (GLMM) was developed adding herd as a random effect variable to adjust for the correlation of the pregnancy outcome of cow within herd. Penalised Quasi-Likelihood was used to estimate the coefficients of each parameter in the model. Receiver Operating Characteristic (ROC) curves were plotted for models both with and without the percentage of real-time PCR positive bulls as a predictor. The area under the curve (AUC) was used as an indicator for the ability of the model, including and excluding real-time PCR test results, to predict pregnancy. Analyses were performed using Rv2.13.1 (R Development Core Team, 2011; R Foundation for Statistical Computing, Vienna, Austria).

1.4 Results

A total of 224 preputial scraping samples were submitted, of which two were unsuitable for testing by real-time PCR because of gross contamination and bacterial overgrowth. Overall, 64/222 (28.8%, 95% CI = 22.9% - 34.8%) bulls tested positive, 28/222 (12.6%, 95% CI = 8.2% - 17.0%) returned an inconclusive result, and 130/222 (58.6%, 95% CI = 52.1% - 65.0%) were negative.

Five tested bulls could not be matched to the mob they had served, and information about mob pregnancy rate was not available for six more bulls. Also twenty eight bulls PCR-tested served more than one mob (27 served two mobs and 1 served three mobs); therefore, their PCR results were duplicated or triplicated accordingly for analysis purposes. The data set for analysis comprised 240 real-time PCR results from 211 bulls and associated pregnancy rates from 68 mobs of breeding females in 31 herds.

The mean time from mating to sampling was 4.8 months (minimum (Min) = 2.2 months, maximum (Max) = 7.4 months). Percentages of real-time PCR test-positives in samples collected during April-May, June-July, and August- September were 24.1% (95% CI = 12.7% - 35.5%), 34.4% (95% CI = 26.0% - 42.8%), and 26.7% (95% CI = 16.7% - 36.7%) respectively.

Median pregnancy rates of 88.2% (1^{st} quartile (1^{st}Q) = 71.3% - 3^{rd} quartile (3^{rd}Q) 92.1%), 94.0% (1^{st}Q = 87.2% - 3^{rd}Q = 98.3%), and 90.3% (1^{st}Q = 85.4% - 3^{rd}Q = 94.3%) were recorded for 15 month heifers, 27 month heifers and mixed age cows, respectively. Descriptive statistics for overall pregnancy rate, real-time PCR positive percentage, cow-bull ratio, mating period and farm area variables are presented in Table 1.1.

Table 1.1: Descriptive statistics (Median, 1^{st} Quartile (1^{st}Q), 3^{rd} Quartile (3^{rd}Q), Minimum value (Min) and Maximum value (Max) for continuous variables.

Variables	Median	1^{st}Q	3^{rd}Q	Min	Max
Pregnancy rate (%)	90.4	85.3	96.1	37.7	100
Real-time PCR positives (%)	25.0	0.0	50.0	0.0	100
Cow-bull ratio	29.8	21.3	38.8	4.0	63.3
Mating period (days)	63.0	53.8	68.0	31	120
Area (hectares)	1735	629	6275	276	20000

Information about age was available for 186/224 (83%) bulls. The median bull age was

4 years (1^{st}Q = 3 years - 3^{rd}Q = 5 years; Min = 2 years - Max = 10 years, respectively). Breed was recorded for 220 bulls of which 111 (51%) were Angus, 62 (28%) were Hereford and 47 (21%) were other breeds. When *Cfv* vaccination was performed, all bulls within a herd were vaccinated. A total of 25 bulls (11.3%) from 5 mobs and 3 herds had been vaccinated against *Cfv* and these comprised all the bulls in these particular herds. These bull level variables were aggregated into mob level variables; their frequencies along with other recorded mob variables are presented in Table 1.2.

Table 1.2: Categorical variables and relative frequencies of their levels as a percentage (%).

Variable	**Levels**	**n mobs**	**Frequency %**
Mob	15 months	11	16.2
	27 Months	26	38.2
	Mixed age cows	31	45.6
Breed	Angus	37	54.4
	Other	30	44.1
	Not recorded	1	1.5
Age	Young	28	41.2
	Mix	20	29.4
	Old	11	16.2
	Not recorded	9	13.2
Body condition score	Normal	38	55.9
	Above normal	27	39.7
	Below normal	1	1.5
	Not recorded	2	2.9
Cfv vaccination	Yes	5	7.4
	No	63	92.6
Veterinary practice	A	12	17.6
	B	11	16.2
	C	25	36.8
	D	20	29.4
Terrain for mating	Flat	12	17.6
	Moderate	36	52.9
	Steep	12	17.6
	Not recorded	8	11.8
Mount animal	Virgin heifer	9	13.2
	Empty cow	39	57.4
	Unknown	20	29.4
Co-grazing sheep	Yes	63	92.6
	No	5	7.4
Co-grazing deer	Yes	12	17.6
	No	56	82.4

1.4.1 Multivariable analysis

Mob real-time PCR positive percentage was not significantly associated with the pregnancy rate (p = 0.81) in the multivariable model. Predicted values for pregnancy rate from this model were plotted against real-time PCR positive percentage in each mob (Figure 1.1). The horizontal Loess trendline suggests that no relationship existed in these data.

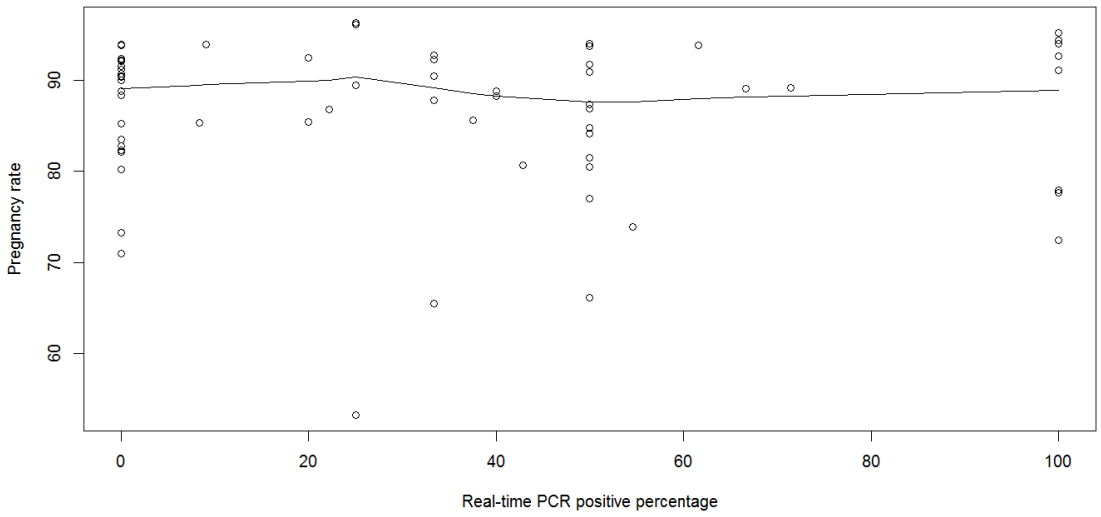


Figure 1.1: Pregnancy rate predicted values from Generalised Linear Mixed Model and real-time PCR positive percentage. The LOESS line (local regression) represents a smoothed trend line.

Independent of other predictor variables in the model, the percentage of real-time PCR positives was unrelated to pregnancy rate (OR = 1). Pregnancy was significantly associated with mob, where mixed age cows and 27 month old heifers were about 3 times more likely to be pregnant than 15 month old heifers. Cows in mobs with a BCS of 'normal' were about half as likely to be pregnant as cows in mobs with a BCS of 'above normal' (OR = 0.5). Cow-bull ratio showed a significant (p = 0.05) weak positive association with pregnancy rate (OR = 1.02). This means that for every unit of increase in the cow-bull ratio the odds of pregnancy increased linearly by 1.02. Mating period was negatively but not statistically significantly associated with pregnancy rate (p = 0.21). Odds ratios and 95% confidence intervals for variables in the final multivariable model are shown in Table 1.3.

Receiver Operating Characteristic (ROC) curves for models with (Model 1) and without real-time PCR (Model 2) had almost identical AUCs (0.7046 for Model 1 and 0.7048 for Model 2). The ROC curves for both models displayed in Figure 2 graphically illustrate

the absence of any effect on pregnancy rate from real-time PCR test results.

Table 1.3: Risk factors included in the Generalised Linear Mixed Model and thier association with pregnancy rate.

Risk factor	Scale/levels	OR (95% CI)	p-value
Intercept	-	3.6 (1.4 - 9.6)	<0.02
RT-PCR (%)	Continuous	1.0 (0.4 - 2.2)	0.81
Mob	15 months	-	-
	27 months	3.5 (1.7 - 7.1)	0.002
	Mixed age	3.3 (1.7 - 6.4)	0.001
Body condition score	Above normal	-	-
	Normal	0.5 (0.3 - 0.9)	0.03
	Below normal	2.9 (0.1 - 115.7)	0.57
	Unknown	1.0 (0.1 - 6.9)	0.97
Cow-bull ratio	Continuous	1.0 (1.0 - 1.0)[a]	0.05
Mating period (days)	Continuous	1.0 (1.0 - 1.0)[b]	0.21

[a] 1.022 (1.001 - 1.044), approximated to 1.0 in table. [b] 0.991 (0.979 - 1.002), approximated to 1.0 in table.

The comparison of ROC curves of a model with RT-PCR (Model 1) and without RT-PCR (Model 2) showed that the AUC was almost identical (0.7046 vs. 0.7048) (Figure 1.2). Therefore, RT-PCR test did not add any value to pregnancy prediction over the one explained by the mob structure, BCS, cow-bull ratio, mating period, and herd.

1.5 Discussion

The main objective of this study was to evaluate the validity of a real-time PCR test for detecting virulent *Cfv* in bulls in beef herds in New Zealand. The adverse effect of infection with *Cfv* on pregnancy rates is well established (Hoerlein et al. 1964, 1965, Guay 1967, Allan & Mutch 1971) and a negative association between rates of real-time PCR positive breeding bulls and pregnancy rates would be expected if the real-time PCR was reliably detecting the presence of virulent *Cfv*. However, no such relationship was found in this study.

The study was sufficiently large to detect a difference of approximately 5% in pregnancy rates between herds mated by real-time PCR negative and positive bulls with 80% power

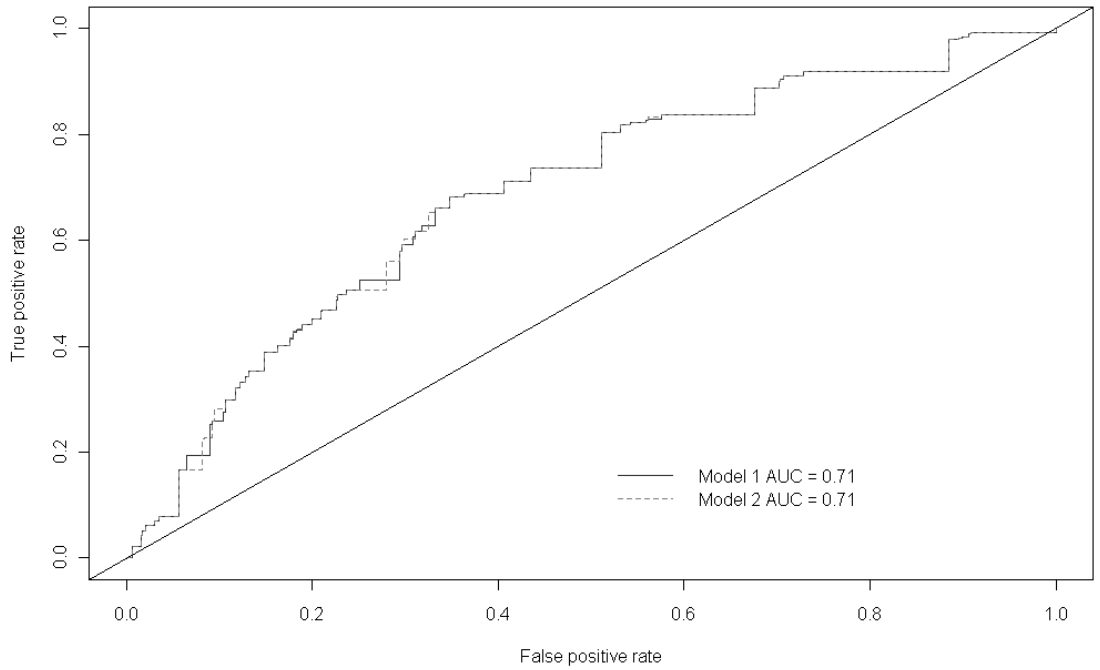


Figure 1.2: Predictive ability of models with (model 1) and without (model 2) Real-time PCR as a predictor. The diagonal line represents a prediction based on pure chance; hence lines above it indicate better predictability than expected by chance.

and 95% confidence. Real-time PCR tests of preputial scrapings of beef breeding bulls were found to be equally likely to be positive in mobs with high and low pregnancy rates (Figure 1.1) and the percentage of real-time PCR positive breeding bulls did not improve the predictive ability of the multivariable model (Figure 1.2).

In our study 28.8% (95% CI = 22.9% - 34.8%) of the bulls were positive to the real-time PCR but there was no detectable effect on pregnancy rates. This finding might suggest the presence of a non-virulent strain but this seems highly unlikely given that *Cfv*, despite many attempts, has not been cultured in New Zealand since 1993 (Loveridge & Gardner 1993, McFadden et al. 2005*a*). Furthermore, it is known that this real-time PCR methodology can produce false positive results to samples containing *Campylobacter hyointestinalis* (Spence et al. 2011). A plausible explanation for this finding is that the parA gene, specific to *Cfv* and targeted by the real-time PCR, is believed to be located in a transferrable genomic island that may be shared among *Campylobacter* species (Abril et al. 2010, Gorkiewicz et al. 2010, Spence et al. 2011). Thus the cumulative evidence suggests that the real-time PCR is non-specific in detecting virulent *Cfv* and it would seem

that virulent *Cfv* is either absent in New Zealand beef cattle or endemic at a very low level.

We considered the possibility that the time frame between mating and sampling may have biased in our results. It has been shown that bulls can harbour *Cfv* for long periods, in some cases for years after artificial exposure (Dufty et al. 1975). In the present study the mean time for sampling was 4.8 months after the end of the mating period thereby producing an opportunity for infection clearance due to natural and/or therapeutic means. Veterinarians were advised not to sample bulls that had received antimicrobial treatment; hence treatment was unlikely to account for a change in the *Cfv* infection status. There was no evidence in our data of any significant variation of the real-time PCR positive percentage over time. Moreover, considering the venereal nature of the disease, it is unlikely that negative bulls at the end of mating would have contracted the disease after this time, hence the observation of real-time PCR positive rates in high fertility mobs is not explained by differences in the time of sampling.

In the study population, 27 month old heifers and mixed age cows had a significantly higher pregnancy rate than 15 month old heifers. Although, a lower pregnancy rate has been previously observed in first year mated heifers compared with heifers mated at around two years old and mixed age cows (McFadden et al. 2005*b*), this difference was not as marked as in this study, perhaps due to its smaller size or the different method for selection of participant herds. The main interest of our investigation was not to estimate the pregnancy rate of each mob, but to control for their effects on the association between real-time PCR positive percentage and pregnancy rate.

Other significant variables in the model were BCS, and cow-bull ratio. Mobs with an average BCS of 'above normal' were 2 times as likely of being pregnant as mobs with a an average BCS of 'normal'. Only one mob was recorded with a BCS of 'below normal', hence the low number of observations in this group did not allow making any valid comparisons. The result agrees in part with previously published evidence that have observed a positive correlation between high BCS, at either mating or pregnancy diagnosis, and pregnancy rates (Rae et al. 1993, Morris et al. 2006). The observed difference may have been greater if there had been more mobs with an average BCS of 'below normal'. A weak increase in the odds of pregnancy was also observed for each unit of increase in the cow-bull ratio. This supports published evidence that have observed a minimal effect of cow-bull ratio on fertility, at least at ratios up to 60:1 (Petherick 2005). A previous

investigation in New Zealand comprising 1,560 mobs in 863 beef herds, found cow-bull ratio to be non-related to the calculated conception rate (CCR: pregnancy rate standardised by the mating period) although a decrease in CCR was observed for groups with a cow-bull ratio greater than 55 (McFadden et al. 2005*b*). In the present study, the median cow-bull ratio was 30 with only one mob with a ratio greater than 55, therefore making any comparisons with the later study inappropriate.

Mating period is in general expected to be correlated positively with pregnancy rate since an increase in the days available for mating will increase the cumulative probability of pregnancy. Nevertheless our data showed no evidence to support this statement as no significant association was observed between mating period and pregnancy rate.

1.6 Conclusion

In our study of 222 bulls of 31 beef breeding herds in New Zealand real-time PCR *Cfv* test results from bulls were not related to pregnancy rates. This and evidence from other investigations suggest that the high percentage of real-time PCR positive results is best explained by poor specificity of the test. Failure to isolate the organism on multiple occasions suggests that *Cfv* may be either absent, present at very low levels of endemicity or prevalent as a low- or non-virulent strain. The latter is only a hypothetical consideration as the presence of low-/non-virulent strains has not been reported to date.

CHAPTER 2

Contribution of Leptospira, Neospora, and BVDV to Fetal Loss of Beef Cattle in New Zealand

2.1 Abstract

The profitability of beef breeding farms in New Zealand depends principally on optimal reproductive performance. The aim of this study was to estimate the impact of four major pathogens, Bovine Viral Diarrhoea Virus (BVDV), *Neospora caninum* (*N. caninum*), *Leptospira borgpetersenii* serovar *Hardjo* (*L. hardjo*), and *Leptospira interrogans* serovar *Pomona* (*L. pomona*), on rates of fetal loss in commercial beef breeding herds. Herds reporting abortions were recruited, and a blood sample from aborting cows (cases) was collected. Controls were normally calving cows from the same herd, similar in age and breed to the cases. At least one control was selected from each herd contributing cases. Samples were tested using ELISA for detection of antibodies against BVDV and *N. caninum*, and Microscopic Agglutination Test (MAT) for detection of antibody against *L. hardjo* and *L. pomona*. A sensitivity analysis was conducted to evaluate the relationship between abortion and sample seroprevalence at various test cut-off titers using conditional logistic regression. The cut-off titer with the strongest association with abortions was included in the multivariate model. A significant increased risk of abortion was found for animals seropositive to BVDV (Odds Ratio (OR) = 2.05; 95% confidence interval (95% CI) = 1.01 - 4.19), *N. caninum* (OR = 3.64; 95% CI = 1.38 - 9.60), *L. hardjo* (OR = 1.88; 95% CI = 1.04 - 3.42), and *L. pomona* in non-vaccinated cows (OR = 14.57, 95% CI =

1.69 - 125.65) at the ELISA titers of ≥1 and ≥30, and MAT titers of ≥1:384 and ≥1:768 for a positive sample, respectively.

Vaccination did not affect ORs for *L. Hardjo* or BVDV and no herd vaccinated against *N. caninum*. Approximately 14.2% of all fetal loss in the beef breeding cattle population in New Zealand may be attributable to BVDV (3.5%), *N. caninum* (3.1%), *L. hardjo* (4.9%), and *L. pomona* (3.9%).

2.2 Introduction

The profitability of New Zealand's pasture based beef breeding industry is largely depending on successful reproductive management and performance. Pregnancy rates, calving percentage and weaning performance have remained stagnant during the past decade. Little is known about the causes of this stagnation, findings from a report suggested a 20% of reproductive losses between mating and weaning, half of these losses were due to conception failure and the other half due to events that occurred between conception and weaning (Heuer 2009*a*). Beef cattle are farmed in extensive grazing systems, often with little observation or intervention. It is difficult to describe and quantify the nature and causes of fetal and calf losses under such conditions. Instead, reproductive loss is usually quantified as a failure to wean a calf from a cow diagnosed pregnant and wintered (wet-dry cows). Although abortion is a component of the wet-dry percentage, its incidence has not been reported.

Bovine Viral Diarrhoea Virus (BVDV) is a highly infectious agent that causes infertility, early embryonic death, and abortions in cattle (Grooms 2004). In New Zealand studies have found that seropositivity to BVDV is highly prevalent and widespread, with a cow estimate of 50% in 65% of all beef breeding herds (Perez et al. 1994). However the importance of BVDV as a cause of abortion in beef cattle has not been assessed.

Neospora caninum (*N. caninum*), formerly misidentified as *Toxoplasma gondii*, was initially described as associated with abortion in cattle in the late 80s (Dubey et al. 1989, Thilsted & Dubey 1989). *N. caninum* is now recognised as an important cause of abortions worldwide (Anderson et al. 2000, Koiwai, Hamaoka, Haritani, Shimizu, Tsutsui, Eto & Yamane 2005, Givens 2006, Ståhl et al. 2006). In New Zealand, *N. caninum* has

been reported as the most frequent pathogen associated with abortions in dairy cattle, and seropositive cows had a substantially higher abortion risk (Relative Risk = 23.6) than seronegative cows (Thornton et al. 1991, Thobokwe & Heuer 2004, Weston et al. 2005). Little information was available about the impact of *N. caninum* on fetal loss in beef breeding herds.

The effect of *Leptospira borgpetersenii* serovar *Hardjo* (*L. hardjo*) on abortions was often controversial as many studies failed to show an association between seropositivity to this pathogen and abortion (Carter et al. 1982, Dixon 1983, Elder et al. 1985, Chappel et al. 1989) in contrast to many other that did associate *L. hardjo* as a cause of abortions (Ellis & Michna 1977, Ellis et al. 1978, 1982). In New Zealand *L. hardjo* is widely distributed among beef breeding cattle with a cow seroprevalence estimate of about 50% (Dreyfus et al. 2011), nevertheless neither its association with abortions nor the percentage of reproductive loss attributed to *L. hardjo* have been assessed in beef breeding herds of New Zealand.

Leptospira interrogans serovar *Pomona* (*L. pomona*) is a well recognised cause of abortion storms in cattle (Givens 2006). Early studies in the 50 already highlighted its role on pregnancy loss (Teigland 1956, Gillespie & Kenzy 1958); nonetheless its importance decreased with the years probably because of vaccination (Grooms & Bolin 2005). Seroprevalence to *L. pomona* in New Zealand was estimated in 25% (Dreyfus et al. 2011), but there is no readily available information about its association with abortion or the percentage of reproductive loss attributed to it in New Zealand beef cattle population.

The aim of this matched case-control study was to evaluate the association between abortion and seropositivity to BVDV, *N. caninum*, *L. hardjo*, and *L. pomona* in pastoral beef breeding herds. These data were then used to approximate the relative impact of these pathogens on abortion rates in beef cattle of New Zealand using published methods for estimating the population attributable fraction from case-control studies.

2.3 Materials and Methods

2.3.1 Study design

A m:n matched case-control study was designed to investigate the association between abortions in beef cattle and antibody titers against BVDV, *N. caninum*, *L. hardjo*, and *L. pomona*. Eligible farms were selected from those that reported an abortion event in a mail survey that captured information about reproductive performance and the incidence of Paratuberculosis and Leptospira. In addition, Landcorp[1] farms participated in the study where and when abortions were observed in the 2009/2010 reproduction season. In order to recruit farms, owners or managers were contacted by phone followed by a mailing out of detailed information about the study. Veterinarians were contracted to collect samples from animals in these and other herds of their client farmers experiencing abortion events during the 2009/2010 season. Samples from cases and controls were collected after the calving period of a herd was completed.

Cases and controls were identified by herd owners and/or mangers. A case was defined as a cow diagnosed pregnant by ultrasound at the end of mating in fall 2009, not having a calf at foot at the time of expected calving and absence of signs for udder development. Controls were cows pregnant by ultrasound with a suckling calf at foot, similar in age and breed as the cases, and from the same herd as the cases. At least one control was selected for each herd contributing cases.

In addition to collecting blood samples, veterinarians completed a two page questionnaire recording the case or control status, age, breed, body condition score, date and type of vaccinations of the sampled cows, calving rate, abortion rate, weaning rate from the 2009/2010 season, and herd of origin.

2.3.2 Sample management and testing

A single blood sample per cow was collected and sent to the laboratory within 24 hours for testing. If this was not achievable, samples were kept in a cool, dry place (10 °C) away from direct sunlight and from contact with ice. Sera were separated from blood no later than 48 hours after sampling and stored at -20 °C until testing.

[1] Landcorp Ltd. is owned by the government of New Zealand and operates 110 pastoral livestock farms.

The Pourquier ELISA BVD/MD/BD p80 antibody screening test kit was used to quantify antibodies against BVDV. When specific antibodies to BVDV were present in the sera, they formed bovine antibody p80 complexes with the p80 protein supplied on the wells of the microplate. This complex 'masked' p80 protein sites that were targeted by other monoclonal antibodies coupled with the enzyme peroxidase that was added into the wells. With the addition of an enzyme substrate, a coloured compound was formed. The intensity of the colour was a measure for the inverse of the rate of anti-p80 antibodies present in the sample. The percentage of inhibition (PI%) for each sample was calculated as:

$$PI\% = \frac{OD_{Sample}}{OD_{NegativeControl}} \times 100 \tag{2.1}$$

Where OD_{Sample} was the optical Density of the sample and $OD_{NegativeControl}$ was the optical density of the negative control.

For *N. caninum*, IDEXX Checkit antibody Test Kit was used. Inactivated antigen present in microtiter plates was binding any specific antibodies to *N. caninum* present in the sera. A peroxidase anti-ruminant IgG binding to the ruminant antibodies *N. caninum* antigen complex was then added. After the addition of substrate, a coloured compound was formed. The degree of colour was directly proportional to the amount of *N. caninum* antibodies present in the sample. The sample/positive control ratio (S/P) was calculated as:

$$S/P = \frac{(OD_{Sample} - OD_{NegativeControl})}{(OD_{PositiveControl} - OD_{NegativeControl})} \times 100 \tag{2.2}$$

Where OD_{Sample} was the optical density of the sample, $OD_{NegativeControl}$ was the optical density of the negative control, and $OD_{PositiveControl}$ was the optical density of the positive control.

Microscopic Agglutination Test (MAT) was used to test for leptospira antibodies. Eight (two-fold) serum dilutions were used from 1:24 to 1:3072. The final titer of a sample was the last dilution agglutinating at least 50% of antigen.

BVDV and *N. caninum* testing was carried out by a commercial laboratory (NZVP[2]), while MAT was performed at the EpiLab, Hopkirk Institute, Massey University.

[2]NZVP: New Zealand Veterinary Pathology Ltd., Palmerston North, New Zealand.

2.3.3 Data analysis

Sample seroprevalence

For BVDV, the commercial ELISA cut-off titer of ≤40 PI% for a positive sample was used to calculate the proportion of seropositive cows. ELISA values >40 PI% were considered negative. For *N. caninum*, the commercial ELISA cut-off titer of ≥40 S/P ratio defining a positive sample was used to calculate the proportion of seropositive cows; an ELISA S/P ratio <40 was considered negative. For Leptospira, a MAT titer ≥1:48 was used as cut-off for seropositive cows; MAT titer values <1:48 were considered negative. For BVDV, *L. hardjo* and *L. pomona* only non-vaccinated cows were used to generate an estimate of sample seroprevalence. A herd was considered positive when at least one cow was seropositive to *N. caninum*, *L. hardjo* or *L. pomona*. For BVDV, a herd was considered seropositive when at least 30% of the cows were seropositive.

Matched analysis

Matched (conditional) logistic regression was performed using a Cox proportional hazards model. The likelihood of the conditional logistic model is identical to the likelihood function for a stratified Cox model (Allison 1999). The PHREG function in SAS with ties handled using the discrete method was used. All cases were given 1 as event time value and all controls a 2 for being censored (Allison 1999). The variable herd was included in the model as a stratum variable.

Sensitivity analysis

Consisted in a bivariate analysis between the outcome abortion (Yes / No) and several cut-off values for seropositivity that were created within the range of test results. For the BVDV ELISA, titer cut-offs for a positive sample were defined as ≤1, ≤2, ≤3, ≤4, ≤5, ≤10, ≤20, ≤30, ≤40, ≤50, ≤60, ≤70, and ≤80 PI%; for *N. caninum* ELISA S/P ratio titer cut-offs for a positive sample were defined as ≥0, ≥5, ≥10, ≥15, ≥20, ≥30, ≥40, and ≥50; and for *L. hardjo* and *L. pomona*, the MAT titer cut-offs were ≥1:24, ≥1:48, ≥1:96, ≥1:192, ≥1:384, ≥1:768, ≥1:1536, and ≥1:3072 (Figure2.2).

Multivariable matched analysis

The titer cut-offs resulting in the strongest association with abortion in the sensitivity analysis were selected for inclusion in the multivariable conditional model. Even though cases and controls were similar in age and breed by sampling design, these variables were added to the model in order to adjust for possible confounding introduced by matching for these traits (Dohoo et al. 2009).

The BCS was excluded from the multivariable model because a high BCS at sampling was a likely consequence, not a precursor of abortion, thus BCS was by definition not a confounder.

Two way interactions between exposure to each pathogen, and also between pathogen exposures and vaccination were explored in the multivariable model. Significance was assessed using Wald Chi square statistics with p-values <0.05 considered significant.

Population attributable fraction

Adjusted odds ratios from the multivariable conditional model were used to estimate the adjusted population attributable fraction (PAF) employing the following formula:

$$PAF = p_{e^+c^+} \times \frac{(RR-1)}{RR} \tag{2.3}$$

Where p_c is the proportion of exposed cases at a selected cut-off titer, and RR the adjusted relative risk, in our study approximated by the adjusted OR (Rockhill et al. 1998, Jewell 2004). The PAF for *L. pomona* was adjusted for the proportion of non-vaccinated cows in the population which has been observed at around 75% in New Zealand (Heuer 2009*b*).

Confidence intervals for the PAF were calculated using the formula described in Steenland & Armstrong (2006), where the variance of the natural logarithm of 1 minus PAF (Var[ln(1 − PAF)]) equals to:

$$\frac{PAF^2}{(1-PAF)^2} \times [\frac{Var[ln(RR)]}{(RR-1)^2} + \frac{2}{n_{e^+c^+} \times (RR-1)} + \frac{n_{e^-c^+}}{(n_{e^+c^+} \times n_{c^+})}] \tag{2.4}$$

Where n_{c^+} is the number of cases, $n_{e^+c^+}$ is the number of exposed cases, and $n_{e^-c^+}$ is the number of nonexposed cases.

The total PAFs for the four pathogens in the study were calculated according to the formula in Steenland & Armstrong (2006):

$$PAF_{Total} = 1 - [(1 - PAF1) \times (1 - PAF2) \times (1 - PAF3) \times (1 - PAF4)] \quad (2.5)$$

2.4 Results

2.4.1 Data description

Samples were taken from 814 cows in 45 beef herds in Gisborne (n = 6), Hawke's Bay (n = 2), Manawatu-Wanganui (n = 20), Northland (n = 2), Waikato (n = 5), Wellington (n = 1), Canterbury (n = 4), Marlborough (n = 3), and Southland (n = 2). From the 45 sampled herds, 17 were farms from the original list of mail survey farmers reporting abortion rates that was provided to each veterinary practice; 10 were Landcorp farms, and 19 farms were clients from veterinary practices added to the study by veterinarians contracted for collecting samples.

Ninety percent of the samples were taken up to 84 days after calving, with a median time of 68 days (1st quartile (Q1) = 50 days - 3rd quartile (Q3) = 76 days) and a maximum of 153 days.

Data to calculate calving rates (n progeny/n wintered) were available from 36/45 (80%) herds. These 36 herds had a median calving rate of 89.7% (Q1 = 83.0% - Q3 = 93.8%). The number of aborting cows was available from 31/45 (69%) herds, and in these herds the abortion rate was 3.0% (Q1 = 1.1% - Q3 = 4.5%).

From the total number of samples received (814), 379 (47%) were from aborting cows (cases), and 435 (53%) from normally calving cows (controls). Most herds (98%) provided between 2 to 12 cases and between 4 to 11 controls. Ratios ranged from 0.2 to 1.8 cases to controls, with a median of 1. One farm with a high abortion rate provided 52 cases and 20 controls (Table 2.1).

For BVDV and *N. caninum*, 812 samples were tested. Two clusters of titers were observed for BVDV ELISA PI%: one with values of ELISA PI% $\leq$40 comprising 64.8% (95% CI 61.5% - 68.1%) of the observations (seropositives), and another with values of ELISA

Table 2.1: Number and frequencies of herds by the ratio of cases to controls.

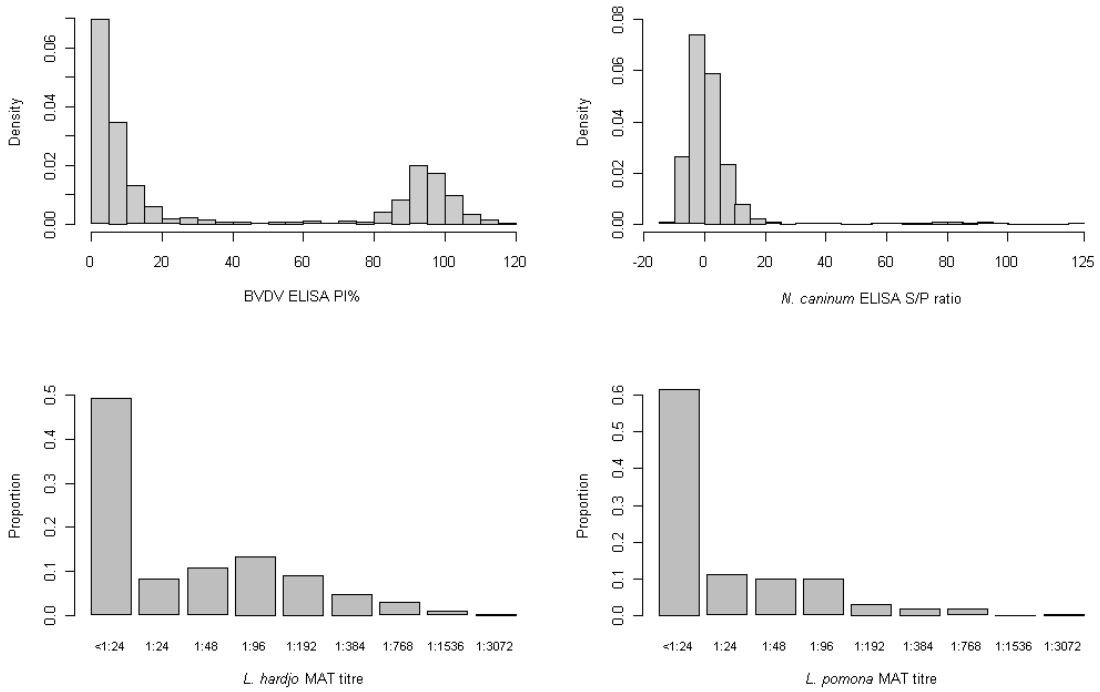
Cases:Controls	Number of herds	Frequency
52 : 20	1	2%
12 : 10	2	4%
12 : 9	1	2%
10 : 10	16	36%
10 : 9	1	2%
9 : 10	3	7%
9 : 5	1	2%
8 : 11	1	2%
8 : 8	2	4%
7 : 10	1	2%
7 : 7	1	2%
6 : 10	1	2%
5 : 5	1	2%
4 : 10	1	2%
4 : 9	1	2%
3 : 11	1	2%
3 : 10	5	11%
2 : 10	3	7%
2 : 9	1	2%
2 : 4	1	2%
Total	45	100%

PI% $>$80 comprising 32.5% (95% CI 29.3% - 35.7%) of the observations whereas only 2.7% were in titer range $>$40 and $\leq$80. For *N. caninum*, 97.3% (95% CI 96.2% - 98.4%) of the observations had values of ELISA S/P ratio $<$40 (seronegatives). MAT titers in 781 samples tested for *L. hardjo* and *L. pomona* showed a 49.2% (95% CI 45.7% - 52.7%) and a 61.5% (95% CI 58.1% - 64.9%) of the observations with titers $<$1:24, respectively. Figure2.1 shows the distribution of titers to BVDV, *N. caninum*, *L. hardjo* and *L. pomona* in the tested cows.

2.4.2 Sample seroprevalence

BVDV had the highest sample seroprevalence at the cow level, while at the herd level BVDV, *L hardjo* and *L. pomona* were highest. Although a low proportion of cows were seropositive to *N. caninum* (2.7%), they were well spread across herds with a herd sample seroprevalence of 24.4%. Table2.2 shows cow and herd sample seroprevalence to BVDV, *N. caninum*, *L. hardjo*, and *L. pomona*.

Figure 2.1: Densities of BVDV and *N. caninum* test titer results in a continuous scale and proportion of samples at each MAT titer cut-off results for *L. hardjo* and *L. pomona*.



2.4.3 Sensitivity analysis

The strongest crude association between *N. caninum* seropositivity and abortions was observed at the cut-off of $\geq$30 S/P ratio for positive sample. Regardless of significance, no point estimate showed a protective OR across the different cut-offs assessed. For *L. hardjo* the strongest association was observed at the MAT titer of $\geq$1:384 for a positive sample; while for *L. pomona* the strongest association was observed at the MAT titer of $\geq$1:768 for a positive sample; after this point confidence intervals became extremely wide due to lack of observations in the exposed non-aborting group. BVDV seropositivity showed a protective association with abortions at cut-off of $\leq$40 ELISA PI% for a positive sample; increased risk of abortions was only observed at low cut-off titers, being the cut-off of $\leq$1 ELISA PI% the strongest. Figure2.2 shows crude matched odds ratio of abortion and 95% confidence intervals by each titer cut-off in the study population.

Table 2.2: Percentage of sample-seroprevalence in cows and herds non-vaccinated against either BVDV or leptospira at the cut-offs of ≤40 for BVDV, ≥40 for *N. caninum*, and ≥1:48 for *L. hardjo* and *L. pomona*. Herds with at least 1 animal seropositive were considered as seropositive, except for BVDV where a herd was considered seropositive when 30% or more cows were seropositive.

Agent	n cows	Seropositive cows (95% CI)[a]	n herds	Seropositive herds (95% CI)[a]
BVDV	521	71.4% (67.5% - 75.3%)	32	81.3% (67.7% - 94.8%)
N. caninum	812	2.7% (1.6% - 3.8%)	45	24.4% (11.9% - 37.0%)
L. hardjo	338	44.7% (39.4% - 50.0%)	21	85.7% (70.7% - 100.0%)
L. pomona	338	19.2% (15.0% - 23.4%)	21	66.7% (46.5% - 86.8%)

[a] 95% confidence inteval.

2.4.4 Multivariable conditional model

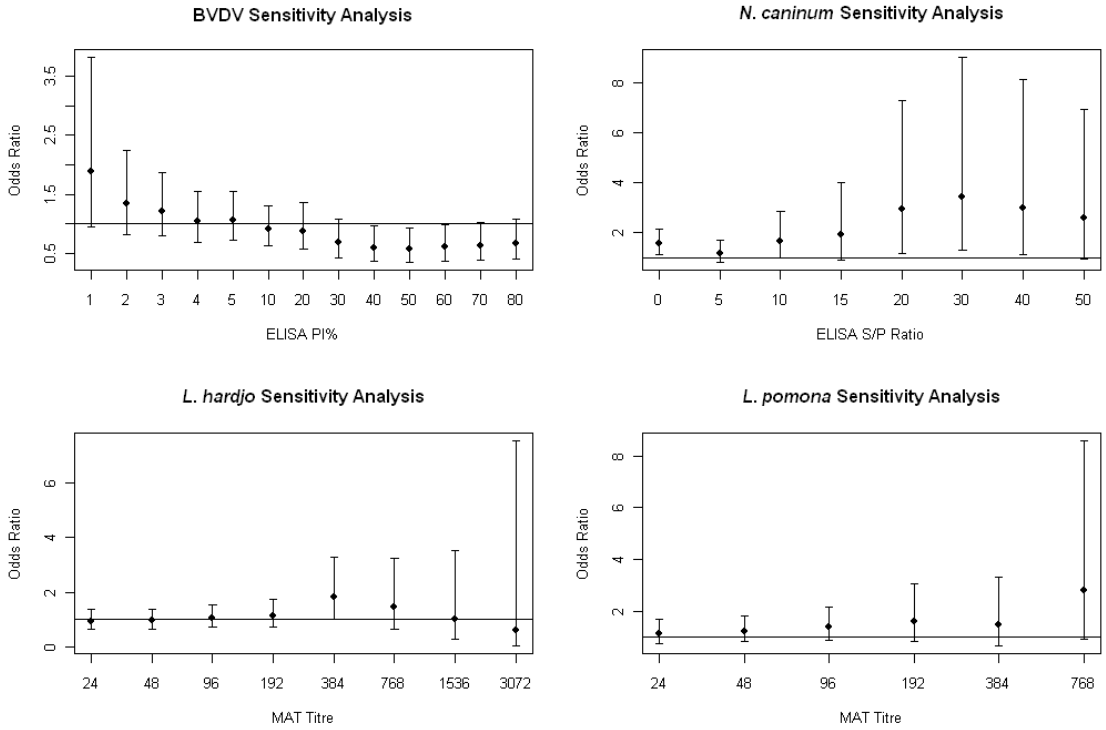
Increased risk of abortion was observed in cows seropositive to BVDV, *N. caninum*, and *L. hardjo*. A significant interaction was observed between *L. pomona* and vaccination against leptospira; aborting cows that were not vaccinated against leptosira were 14.57 times more likely to be seropositive to *L. pomona* than non-aborting cows (controls) non-vaccinated against leptospira. Significant interactions were not observed neither between BVDV / *L. hardjo* seropositivity and vaccination against BVDV / leptospira respectively, nor between the four pathogens under investigation. No animal was vaccinated against *N. caninum*. Table2.3 shows the adjusted OR and 95% confidence intervals for the four pathogens in study.

When BCS was included in the model, aborting cows were about 2.6 times more likely to have a BCS of 3 or more than normally calving cows. As increased BCS was a likely consequence of abortion, it was excluded from the multivariable conditional model.

2.4.5 Population attributable fraction (PAF)

All four pathogens under investigation had similar PAF estimates between 3.1% and 4.9%. It was estimated that approximately 14.2% of abortion cases in New Zealand beef breeding herds may be attributable to BVDV(3.5%) *N. caninum*(3.1%), *L. hardjo*(4.9%) and *L. pomona*(3.5%) (Table2.4).

Figure 2.2: Sensitivity analysis showing the association and 95% confidence intervals between abortions and seropositivity at each test cut-off from the bivariate analysis.



2.5 Discussion

2.5.1 Association with abortion and sample-seroprevalence

Significant associations with abortion were found for all four pathogens in study.

Aborting cows were 3.5 times more likely to be seropositive to *N. caninum* than normally calving cows. This result agrees with several other investigations that have observed an increased risk of abortions in dairy cattle seropositive to *N. caninum* (Koiwai, Hamaoka, Haritani, Shimizu, Tsutsui, Eto & Yamane 2005, Weston et al. 2005, Ståhl et al. 2006, Brickell et al. 2010). However, this the first time that *N. caninum* was related to fetal loss in New Zealand beef breeding herds.

Cow sample seroprevalence of *N. caninum* at the cow level was 2.7% in this study, which is not different to the 2.8% previously reported in New Zealand beef cattle (Tennent-Brown et al. 2000), and similar to the ones estimated in Japan and Sweden, but in general lower compared with other countries in beef cattle populations (Koiwai, Hamaoka, Har-

Table 2.3: Multivariable model associations between abortion and seropositivity to BVDV ($\leq$1 PI%), *N. caninum* ($\geq$30 S/P ratio), *L. hardjo* ($\geq$1:384 MAT) and *L. pomona* ($\geq$1:768 MAT).

Agent	Levels	OR (95% CI)[a]	p-value
BVDV	Positive	2.05 (1.01 - 4.19)	0.05
	Negative	-	
N. caninum	Positive	3.64 (1.38 - 9.6)	0.01
	Negative	-	
L. hardjo	Positive	1.88 (1.04 - 3.42)	0.04
	Negative	-	
L. pomona non-vaccinated	Positive	14.57 (1.69 - 125.65)	0.01
	Negative	-	
L. pomona vaccinated	Positive	0.67 (0.15 - 3.01)	0.60
	Negative	-	
Age	$>$ 2 years old	0.6 (0.31 - 1.14)	0.12
	$\leq$2 years old	-	
Breed	Angus	0.72 (0.29 - 1.79)	0.48
	Other	-	

[a] Adjusted odds ratio and 95% confidence interval from multivariable model.

itani, Shimizu, Tsutsui, Eto & Yamane 2005, Dubey et al. 2007, Loobuyck et al. 2009). Seroprevalence estimates in New Zealand beef cattle populations for *N. caninum* are in general lower than the 6.75% reported in New Zealand dairy cattle (Reichel 1998) in non-aborting cows. This finding agrees to some extent with the published evidence that shows a higher seroprevalence of *N. caninum* in dairy cattle compared to beef cattle (Koiwai, Hamaoka, Haritani, Shimizu, Tsutsui, Eto & Yamane 2005, Dubey et al. 2007). At the herd level, the 24.4% sample seroprevalence observed in this study is in general lower than the one reported by Bartels et al. (2006) in Germany 46% (95%CI = 41% - 51%),

Table 2.4: Population attributable fraction and 95% confidence interval (95% CI) for each individual pathogen and total pregnancy loss attributed to them. The adjusted OR from the multivariable model and the proportion of exposed cases (Pc) at each individual test cut-off with the strongest association with abortion were used to calculate the PAFs.

Pathogen	OR and 95% CI[a]	p_{e+c+}[b]	PAF% (95% CI)[c]	Total PAF%[d]
BVDV	2.05 (1.01 - 4.19)	0.069	3.5% (0.2% - 6.8%)	14.2%
N.caninum	3.64 (1.38 - 9.6)	0.042	3.1% (0.8% - 5.3%)	
L. hardjo	1.88 (1.04 - 3.42)	0.104	4.9% (0.5% - 9.1%)	
L. pomona non-vaccinated	14.57 (1.69 - 125.65)	0.038	3.5% (0.7% - 6.3%)	

[a] Adjusted odds ratio and 95% confidence interval from multivariable model. [b] Proportion of exposed cases at the cut-off with the strongest association with abortion. [c] Population attributable fraction percentage and 95% confidence interval. [d] Percentage of pregnancy loss that may be attributed to exposure to BVDV, *N. caninum*, *L. hardjo*, and *L. pomona* in the population of beef breeding herds.

The Netherlands 41% (95%CI = 31% - 50%) and Spain 61% (95%CI = 50% - 62%).

BVDV showed a marginally non-significant (p = 0.22) protective OR of 0.72 against abortions at the commercial cut-off of $\leq$40 ELISA PI% (result not showed). The reason why cows seronegative to the commercial cut-off (ELISA PI% >40) had a higher risk of abortion than seropositive cows cannot be explained. In a highly seroprevalent population to BVDV, a high percentage of cows exposed prior breeding/pregnancy can be expected. Given that BVDV antibody titers generally persist for years after both natural and artificial exposure (Fredriksen et al. 1999), this may effectively protect the population of pregnant cattle from new infections and their consequences. Nevertheless, this does not explain the reason why in our study cows seronegative to the commercial cut-off at the time of sampling (2-3 months after calving) had a significantly higher risk of abortions. Positive effects of BVDV on reproductive performance (fertility/pregnancy) have been observed in previous studies measuring antibodies. For example, Muñoz–Zanzi, C. A. and Thurmond, M. C. and Hietala, S. K. (2004) observed that cows infected with BVDV at 5-6 months of age had a significant shorter interval to first artificial insemination compared to cows that were not infected at that age. Ståhl et al. (2006) found a protective association (HR = 0.61) between abortion and seropositivity to BVDV; although this association was marginally significant (p = 0.11). In the present study seroconversion was not measured over time; therefore it was not known when case and control cows became infected. Nevertheless, we believe that high BVDV antibody (ELISA PI% $\leq$1) reflected a more recent exposure, and this was associated with a significant (p = 0.05) increased risk of abortion (OR = 2.05).

BVDV was the most seroprevalent pathogen at the cow level (71.4%) and the second most seroprevalent at herd level (81.3%). This result agrees with previously published studies that have found high seroprevalence to BVDV worldwide (Reinhardt et al. 1990, Houe et al. 1995, Guarino et al. 2008, Segura-Correa et al. 2010), and supports the hypothesis that BVDV antibodies are widespread in the New Zealand beef (Perez et al. 1994) and dairy cattle population (Thobokwe & Heuer 2004, Compton et al. 2006).

L. hardjo: The role of *L. hardjo* as a causative agent of abortions in cattle has been controversial. Experimental evidence has shown that *L. hardjo* can affect pregnancy and cause abortions (Ellis & Michna 1977, Thiermann 1982). Other studies, however, did not find an association between this pathogen and abortions (Carter et al. 1982, Elder et al. 1985,

Chappel et al. 1989, Bartels et al. 1999, Davison et al. 1999, Guitian et al. 1999). Ellis et al. (1982) observed great variability in MAT titers after abortion caused by *L. hardjo*, and even found that 22.8% of dams aborting infected fetuses had no detectable antibodies ($<$1:10), which may contribute to the lack of association between abortion and serological response to *L. hardjo* observed in some studies. In the present study *L. hardjo* was significantly associated with abortions at the MAT titer cut-off of $\geq$1:384 for a positive sample. No significant interaction with vaccination against leptospira was observed.

At the cow level, sample seroprevalence to *L. hardjo* was approximately twice as high as to *L. pomona* at the cut-off of $\geq$1:48 MAT titer. Whereas at the herd level, both leptospira serovars were well distributed among the sampled herds with a 85.7% and 66.7% of the herds seropositives to *L. hardjo* and *L. pomona* respectively. A recent study in New Zealand comprising 2,308 animals in 116 beef cattle herds estimated a seroprevalence in cows of 50% (95% CI = 48% - 52%) to *L. hardjo*, and 25% (95% CI = 23% - 27%) to *L. pomona*; while 92% (95% CI = 85% - 96%) and 72% (95% = CI 63% - 80%) of the herds had at least 1 cow with a MAT titer of $\geq$1:48 or higher for *L. hardjo* and *L. pomona* respectively (Dreyfus et al. 2011).

L. pomona: Association between *L. pomona* and abortion was only observed in non-vaccinated cows; aborting cows were 14.57 times more likely to be seropositive to *L. pomona* than normally calving cows. The reason why *L. Pomona* and not *L. hardjo* appeared to interact with the vaccination status against leptospira cannot be easily explained. All leptospira vaccines currently registered in New Zealand for use in cattle include at least these two serovars. *L. pomona* was reported to cause sporadic infections associated with abortion storms, while *L. hardjo* infection may be characterised by a persistent exposure inducing relatively high titers and sporadically causing abortion events in the beef cattle population (Grooms 2004, Givens 2006). If the cattle population is more naturally exposed, and consequently better immunised against *L. hardjo* than *L. pomona*, vaccination may have relatively little additional effect on preventing abortion due to *L. hardjo*. The titer distribution shown in Figure2.1, and the general sample seroprevalence appear to support this diversion in endemicity between *L. hardjo* and *L. pomona*.

Body condition score was associated with abortion; aborted cows were 2.6 times more likely of being in a BCS of 3 or higher than normally calving cows. This result may be explained by the energy demands for pregnancy and lactation: aborting cows did not

require feed energy for fetal growth and milk production, thus were likely to gain more weight than non-aborting cows. Future economic evaluations of abortions in beef cattle (e.g. partial budget) may take this finding into account.

2.5.2 Possible sources of bias

In the generally extensively managed pasture based beef breeding herds in New Zealand; abortions are not usually being observed and monitored. They tend to be accepted as an unavoidable reproductive loss from conception to weaning. Our case definition for abortion aimed to minimise misclassification bias. However, there might be a small proportion of sampled cases that did not have a calf at foot or have poorly developed udders for reasons other than an abortion (stillborn calves, unobserved loss of a calf). Such misclassification of cases was likely to be independent of exposure to any of the four pathogens. This type of bias was therefore regarded as being 'non-differential', thus the observed associations might have been underestimated (biased towards the null hypothesis).

Another important bias present was the interval between exposure, abortion, and blood sampling. It would have been ideal to take the samples closer to the abortion episode, but this was found to be unfeasible under the extensive rearing conditions of beef cattle. The 2-3 months sampling delay certainly allowed sufficient time for the development of titers. But it could have led to the inclusion of non-aborting cows that were infected after calving and developed titers greater than the cut-off. Also, it could have allowed some of the aborting cows to decrease titers below the cut-offs. For example, antibody titers against *N. caninum* were observed to decay rapidly after abortion (Conrad et al. 1993). On the other hand, antibody titers to natural exposure of BVDV and Leptospira persisted for years (Mackintosh et al. 1980, Fredriksen et al. 1999). Exposure misclassification was likely to be independent of abortion status, thus misclassification was again 'non-differential'. The combined effect of such reasons for misclassification might therefore have to some extent reduced (but certainly not increased) the total observed impact of the infectious pathogens under study on abortion rates.

2.5.3 Population attributable fraction (PAF)

The PAF is defined as the proportion of disease risk in the population that can be attributed to being exposed to a risk factor or set of factors (Rockhill et al. 1998). If it is implied that the relationship between exposure and outcome was causal, the PAF constitutes an important measure for population based decisions such as public health interventions (vaccination, screening, awareness campaigns). A causal interpretation appears plausible for BVDV, *N. caninum*, *L. hardjo* and *L. pomona* as several studies, including this one, have shown evidence of association with abortions in cattle (Elder et al. 1985, Thilsted & Dubey 1989, Thobokwe & Heuer 2004, Givens 2006). Although cases and controls in this study were not chosen randomly from the population of cases and controls, the similarity between sample-seroprevalence observed here and the seroprevalence of *L. hardjo* and *L. pomona* reported in studies designed to estimate seroprevalence suggests that they may be representative of the beef breeding cattle population of New Zealand.

To our knowledge this is the first study to estimate the proportion of abortions in the beef cattle population that may be attributable to BVDV (3.5%), *N. caninum* (3.1%), *L. hardjo* (4.9%) and *L. pomona* (3.5%) exposure in New Zealand beef cattle population. These estimates appear to be lower than published observations in dairy cattle for *N. caninum* (Davison et al. 1999, Mainar-Jaime et al. 1999, Garcia-Vazquez et al. 2005, Koiwai, Hamaoka, Haritani, Shimizu, Kimura & Yamane 2005) and BVDV (Rufenacht et al. 2001). One may speculate whether the difference was due to specific conditions of beef breeding herds in New Zealand or caused by misclassification abortion and/or exposure or both. In any case our PAF estimates were likely to be conservative and the impact of infectious pathogens on rates of fetal loss in beef cattle may in reality be higher than reported here. Cost and benefits will have to be evaluated for any valid inferences about profitable interventions for beef cattle breeders.

2.6 Conclusion

This study quantifies the impact of four pathogens on fetal loss rates in New Zealand beef breeding herds. Fetal loss rates were associated with exposure to BVDV (3.5%), *N. caninum* (3.1%), *L. hardjo* (4.9%), and *L. pomona* in non-vaccinated herds (3.5%). We

concluded that at least 14.2% of all abortions in beef breeding cows may be prevented if exposure to these pathogens could be controlled.

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