Epidemiological studies of enteropathogens of newborn calves in New Zealand dairy farms

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ABSTRACT

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This PhD thesis comprises an overview of the literature pertinent to the principles of calf rearing in dairy farms, and the major infectious and non-infectious causes of neonatal calf diarrhoea (Chapter 1 and 2), followed by accounts of four epidemiological studies of neonatal calf diarrhoea in New Zealand dairy farms (Chapters 3-6). The first study assessed the utility of halofuginone lactate for the prevention of cryptosporidiosis in the presence of co-infection (Chapter 3), and the following two studies investigated the prevalence of the common enteropathogens of calves, and risk factors for neonatal calf diarrhoea in New Zealand dairy farms. The final study describes a molecular analysis of *Cryptosporidium* parasites isolated from calves, and a genetic comparison with human *C. parvum* clinical isolates collected by diagnostic laboratories in the same regions.

The results of the first study highlight the limitations of the use of halofuginone lactate for the prevention of cryptosporidiosis of calves in the presence of co-infection. The prevalence study provides epidemiologically robust estimates of the national prevalence of calf enteropathogens in dairy farms and the risk factor analysis identified a number of potential risk factors for neonatal calf diarrhoea pertaining to infection status, colostrum management, infrastructure and even human resource management. Finally, the molecular analysis of *Cryptosporidium* indicate that *C. parvum* is the predominant species cycling in newborn calves in New Zealand. The significant genetic similarities between human and bovine *C. parvum* observed in this project support the model considering young calves as amplifiers of potentially zoonotic *C. parvum* in New Zealand.

This project provides new data on the prevalence of the enteropathogens of newborn calves and the risk factors for neonatal calf diarrhoea in dairy farms, which can be used by the New Zealand industry to target interventions aimed at improving animal health, welfare and productivity. This PhD project represents the first large scale epidemiological study of neonatal calf diarrhoea performed in New Zealand and to the author's knowledge, one of the most comprehensive national studies, worldwide.

GENERAL PREFACE

The initial objective of this PhD project was to study the epidemiology, molecular epidemiology and control of cryptosporidiosis in newborn calves in New Zealand. The first study aimed at evaluating the efficacy of halofuginone lactate for disease prevention. The study was performed on a dairy farm in Taranaki, which had been selected in view of the presence of cryptosporidiosis and an absence of rotavirus, and *Salmonella* among winter calves before the spring calving season. However, faecal specimens submitted for analysis at the beginning of the study tested positive also for rotavirus, *Salmonella* and *Giardia* spp. This result, initially considered a drawback, allowed a study of the efficacy of halofuginone lactate in the presence of co-infections with other enteropathogens, which has been previously poorly characterised. Furthermore, this diagnosis prompted an expansion of the scope of this PhD project, to a more comprehensive study of neonatal calf diarrhoea, including studies of the aetiology and risk factor for this important condition.

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LIST OF ABBREVIATIONS

BRV	Bovine rotavirus
BCV	Bovine coronavirus
K99	Enterotoxigenic *E. Coli* K99
C. parvum	*Cryptosporidium parvum*
spp	Species
HL	Halofuginone lactate
KM	Kaplan–Meier analysis
ANOVA	Analysis of variance
EHEC	Enterohaemorrhagic *E. Coli*
EPEC	Enteropathogenic *E. Coli*
EIEC	Enteroinvasive *E. Coli*
DAEC	Diffusely adherent *E. Coli*
ETEC	Enterotoxigenic *E. Coli*
stx	Shiga toxin
bp	Base-pairs
CI	Confidence interval
GP60	Glycoprotein (or 60-kDA glycoprotein)
HSP70	70 kDa Heat Shock Protein gene
IVABS	Institute of Veterinary, Animal and Biomedical Sciences
MU	Massey University
OPG	Oocysts per gram of faeces
TON	Total oocysts number
PCR	polymerase chain reaction
RFLP	Restriction fragment length polymorphism
UV	Ultra violet
XLD	Xylose Lysine-dehoxycolate
18S rRNA	Small subunit 18S ribosomal RNA
PRU	Protozoa Research Unit
NZDB	New Zealand *Cryptosporidium* sequence database
LR	logistic regression
SNP	Standardised national prevalence
OR	Odds ratio

LIST OF PUBLICATIONS

Almawly J., Prattley D., French N.P., Lopez-Villalobos N., Hedgespeth B., Grinberg, A., 2013. Utility of halofuginone lactate for the prevention of natural cryptosporidiosis of calves, in the presence of co-infection with rotavirus and *Salmonella* Typhimurium. Vet. Parasitol. 197, 59-67.

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Chapter 1

1. INTRODUCTION

The dairy sector is the largest contributor to the New Zealand annual agricultural output. In 2011, the dairy industry contributed $13.2 billion in export revenue (Ministry of Agriculture and Forestry, New Zealand; http: //www.mpi. govt.nz/ agriculture/statistics-forecasting/ international-trade.aspx. accessed 16 July 2012). Morbidity and mortality significantly reduce the income from cattle operations. Diarrhoeal disease, in particular neonatal calf diarrhoea (NCD), which in this thesis refers to diarrhoea manifesting in the first month of life, is one of the major causes worldwide of morbidity and mortality in cattle (Uhde et al., 2008; Bartels et al., 2010; Izzo et al., 2011; Uetake, 2013). NCD is associated with economic losses due to mortality, poor growth and the costs of prevention and treatment. Furthermore, NCD represents a significant animal welfare problem which challenges farmers at the start of the milking season, arguably one of the busiest periods of the year.

The initial objective of this PhD project was to study the epidemiology, molecular epidemiology and control of cryptosporidiosis of newborn calves in New Zealand dairy farms. The first study was conducted during the 2010 winter-spring calving season, with the aim of evaluating the efficacy of a reduced dosing regimen of halofuginone lactate (HL) for the prevention of neonatal cryptosporidiosis (Chapter 3). The study was performed on a farm in Taranaki selected due to the presence of cryptosporidiosis, and no laboratory evidence for the presence of bovine group A rotavirus (BRV), bovine coronavirus (BCV) and *Salmonella* spp. in the preceding autumn (Grinberg, personal communication, 2012). However, a number of faecal specimens submitted for analysis at the beginning of the study tested positive for *Cryptosporidium parvum* (*C. parvum*), BRV, *Salmonella* spp. and *Giardia* spp. Initially, these findings were disappointing, as the study required the recruitment of animals only infected with cryptosporidiosis. However, a literature search indicated that the efficacy and safety of HL in the presence of co-infections was poorly understood, in spite of the common occurrence of co-infections in the field (Izzo et al., 2011; Uhde et al., 2008; Millemann, 2009). Therefore, the study was completed as planned, in order to collect much needed data on the utility of HL in the presence of co-infections (Chapter 3). Further literature searches indicated that there were few

studies on the occurrence of the common enteropathogens of newborn calves available in New Zealand. Most of the published studied and analysed data were obtained from clinical cases, and there was very scarce information about the prevalence of the enteropathogens in the population. This prompted a re-assessment of the objectives of the PhD project, to include broader research questions on the multifactorial aetiology of NCD.

This thesis provides an overview of the literature on the relevant aspects of NCD and the major enteric pathogens involved in this disease (Chapter 2), which should provide the necessary background in order to understand the significance of the research. As stated above, Chapter 3 describes a randomised controlled field trial of the utility of two dosage regimens of HL for the prevention of natural cryptosporidiosis of calves in the presence of co-infection with BRV and *Salmonella* Typhimurium. Chapter 4 reports a nation-wide prevalence study of the major enteropathogens of newborn calves performed on a representative sample of 97 dairy farms. In total, 1283 faecal specimens were collected from newborn calves during the 2011 winter calving season, and analysed for the presence of BRV, BCV, *Cryptosporidium* spp, *Giardia* spp., *Salmonella* spp, and enterotoxigenic *E. Coli* K99 (K99). A questionnaire delivered to the farmers on the day of the sampling elicited data on farm demographics and management. NCD is considered a multifactorial disease, the severity of which may be determined by complex interactions between the infectious agents and animal husbandry practices. Risk factors for NCD existing overseas, where dairy farms manage year-round calving systems, may not be applicable in New Zealand, where most dairy farms manage synchronised calvings, concentrated mainly in the winter and spring. The objective of the study reported in Chapter 5 was to identify the risk factors for NCD in New Zealand, using the laboratory results and the responses to the questionnaire generated in the previous study (Chapter 4). Collectively, Chapters 4 and 5 represent the most comprehensive epidemiological study of NCD performed in New Zealand and to the candidate's knowledge - one of the largest studies worldwide.

Chapter 6 presents a molecular epidemiological study of *Cryptosporidium* parasites isolated from newborn calves on New Zealand dairy farms. The study was necessary in view of the multiple recent international reports of non-parvum parasites phenotypically identical to *C. parvum*, but of uncertain pathogenicity and zoonotic

impact. Therefore, the motivation for this study was two fold: to enhance our understanding of both the potential role of *C. parvum* in NCD, and of cattle as natural amplifiers of potentially zoonotic *C. parvum*. In this study, the *Cryptosporidium* parasites collected in the previous study (Chapter 4) were genotyped to define species and subtypes, and genetically compared with parasites collected by New Zealand human diagnostic laboratories between 2003 and 2010. A total of 84 *Cryptosporidium* isolates from calves were genotyped by analysis of the 18s ribosomal RNA (18s rRNA), 70 kDA heat shock protein (HSP70), and gp60 genes. This study represents the first nation-wide molecular epidemiological study of *Cryptosporidium* parasites circulating in New Zealand cattle, and one of the most comprehensive national surveys worldwide.

The four research-based chapters presented in this thesis have been published (Chapter 3) or prepared for submission to refereed journals (Chapters 4-6). In this thesis, the papers are presented as published or prepared for submission, except for minor modifications necessary to include additional detail or links between the chapters. This format resulted in some inevitable duplication, especially in the introduction sections of each chapter.

Chapter 2

2. Neonatal calf diarrhoea: a literature review

2.1. The principles of rearing newborn dairy calves, with particular reference to the situation in New Zealand

Calf rearing represents an important component of dairy production systems, as calves' growth and development affects the body size of mature cows (Macdonald et al., 2005; Shamay et al., 2005). The first month of life is a critical phase in the life cycle of dairy cattle, characterised by high morbidity and mortality, as neonatal calves are weak and susceptible to diseases (Curtis et al., 1993; Curtis et al., 1988a; Perez et al., 1990; Svensson et al., 2003). In this period, young calves need intensive care and good quality feeding, hygiene and housing (Perez et al., 1990; Pettersson et al., 2001; Vermunt et al., 1995).

Ideally, calf rearing methods should facilitate peak growth rates at the lowest cost (Fariña et al., 2013). The New Zealand dairy production system is characterised by a concentrated calving pattern occurring mostly in late winter (Grosshans et al., 1997; Macmillan et al., 1990). This seasonal pattern allows animal feeding to be based on pasture grazed *in situ*, and decreases the cost of production (Macdonald, 1999; Verkerk, 2003). Compared to cattle-rearing countries with year-round calving, in which there is a continuous presence of young calves on farms, New Zealand's calving pattern might influence the epidemiology of the diseases in newborn calves, including NCD, as a sharp increase in the numbers of immunologically-naive calves during the calving season, and their virtual absence between the seasons, might influence the prevalence of the different causative agents (Grinberg et al., 2005).

Good colostrum management is vital for ensuring good calf health. It is essential to allow newborn calves to ingest 2-3 litres of high quality colostrum during the first 6-12 hours of life, as the high amount of immunoglobulins in colostrum provide a primary line of defence against infections (Godden, 2008; Jaster, 2005; Morin et al., 1997; Wesselink et al., 1999). These immunoglobulins act locally in the small intestine where they act against microorganisms. Calves are born agammaglobulinaemic, as immunoglobulins are not transported through the placenta

(Foelker, 1994). To compensate, in ruminants colostral immunoglobulins are also absorbed systemically, providing passive immunity against infectious agents to which the dam was exposed or against which she was vaccinated. The small intestinal mucosa is absorptive to the intact immunoglobulins for the first 24-36 hours following calving. This permeability decreases from around 6 hours after birth, so the calf should ingest a sufficient amount of immunoglobulins as soon as possible after birth (Jochims et al., 1994; Staley and Bush, 1985; Wesselink et al., 1999; Xu, 1996).The absorption of immunoglobulins is affected by the amount of colostrum consumed and by the concentration of immunoglobulins in the colostrum (Vermunt et al., 1995). At birth, the newborn is transferred from a microbiologically-sterile uterine environment to a highly contaminated farm environment. Thus, any factor that hinders or delays colostrum intake and immunoglobulin absorption will increase the calves' susceptibility to intestinal or systemic infections (Perino et al., 1993). Such infections may cause pneumonia, diarrhoea, and/or other illnesses that are generally debilitating or fatal for very young calves. According to Mellor et al. (2004), several factors can hinder acquisition of immunoglobulins, such as; competition between calves, teat damage, the birth of a weak calf, malformed udders and low colostrum availability in the udder either due to maternal underfeeding or udder infection. Further, Petrie (1984) reported the leakage of first colostrum (of the highest immunoglobulin concentration) in high-producing dairy cows just before birth, which may result in calves ingesting lower quality colostrum, providing less protection than first colostrum.

As the rumen of newborn calves is not completely developed till 4 to 8 weeks of age (Otterby and Linn, 1981), all nutrient requirements must be provided to neonatal calves in liquid form, as whole milk, stored colostrum or reconstituted milk replacer. The young calf can suckle fresh milk directly from the cow's udder or from artificial nipples, or drink it from a bucket. Provided that it is properly stored, colostrum is a satisfactory feed for young calves (Holmes, 2002). In order to reduce daily work-load, young calves may be fed *ad libitum*, where a milk container is filled with an adequate amount of milk to feed calves for 2 or 3 days (Holmes, 2002; Wise and LaMaster, 1968). Further, a range of milk powder types can be used as milk replacement (Heinrichs et al., 1995; Muller et al., 1974). Skim milk powder is not adequate, however, as a complete feed for young calves, as it does not contain

sufficient fat, and absorption of lipo-soluble vitamins is reduced (Jaster et al., 1990; Jenkins and Bona, 1987).

In New Zealand, The Code of Welfare for Dairy Cattle 1999 addresses the necessity for every newborn calf to obtain colostrum from its dam or any recently calved cow as soon as possible after birth, or within the first six hours (Anonymous, 2010a). It has been reported, however, that around 50% of neonatal calves in New Zealand may not receive sufficient quantity of colostrum from their dams, even when the cow and calf are kept together for up to 24 hours (Vermunt et al., 1995; Wesselink et al., 1999). Therefore, it has been recommended that dairy calves be removed from their mothers after 6 hours and fed colostrum individually, to be sure that they receive an adequate amount of colostrum (Wesselink et al., 1999). Calves' feeding systems include: drinking from a bucket, suckling directly from the udder or suckling from an artificial teat. Milk and milk substitute are, however relatively costly (Holmes, 2002; Vermunt, 1994), so in some dairy farms young calves are fed with controlled rate of feeds each day, for instance; 4 to 5 litres milk, or 0.6 to 0.8 kg milk replacer powder per calf daily (Holmes, 2002; Lorenz et al., 2011b; Vermunt, 1994). Although feeding requirements can be provided to young calves in several ways, an essential phase of all systems is the training of the young calves to feed, which is labour intensive and requires dedicated personnel.

As stated previously, young calves may be fed *ad libitum*, where they are allowed to drink milk as much as they want without restriction (Drackley, 2008; Marshall and Smith, 1970). In such situations, Friesian neonatal calves may drink about 7 to 8 litres/calf of milk per day, and they can reach their target weaning weights early, comparing to calves on restricted feeding (Holmes, 2002). Further, neonatal calves can be fed by suckling directly on cows, at the rate of 2 to 4 calves per cow. This system needs little labour and produces healthy and well-grown calves at 8 to 10 weeks of age (Holmes, 2002; Vermunt, 1994). Well-grown calves can be weaned at 5 to 7 weeks old and then fed on concentrates and pasture. Economically, early weaning of young calves can decrease the total cost of feeds and save labour. Young calves usually begin to eat a small quantity of pasture and other solid feeds at around a few days of age, which is important for the development of the rumen. Calves should therefore be allowed to access clean, leafy pasture at all times in order to develop their rumen's capacity (Drackley, 2008). High quality cereal grains as

concentrates can also be fed as a substitute for milk or milk powder to calves not less than four weeks old (Macdonald, 1999).

Unstable, cold, wet and stormy weather is common in New Zealand during late winter and spring, and exposure of newborn calves to these conditions could cause direct mortality and morbidity, or increase their susceptibility to infection, particularly in weaker calves. Therefore, although young calves may be born on paddocks, they should be promptly transferred to a suitable shelter, with barrier against wind and rain. In addition, fresh air, good ventilation and sunlight should be provided generously, to minimise the possibility of respiratory infection (Anderson, 1975). It has also been recommended that good bedding material be provided, and removed periodically**,** followed by washing and disinfection of the barns and between calves' pens, in order to prevent the build-up of infection (Macdonald, 1999).

As mentioned previously, in the present thesis NCD is defined as diarrhoea occurring during the first month of life. In humans, diarrhoea is defined as "passage of three or more loose or liquid stools per day" (World Health Organization. Available: http://www.who.int/mediacentre/ factsheets/ fs33 0/ en/. Accessed 15 January 2014). Also, it is defined as soft or watery faeces lasting for 2 or more days, possibly in combination with impaired general condition or weight loss (Svensson et al., 2003). Overall, diarrhoea is a clinical sign of a disease that can have several causes, in which the intestine fails to absorb fluids, or secretion into the intestinal lumen is increased (Foster and Smith, 2009). Loss of body fluids by diarrhoea can cause dehydration, and the loss of essential electrolytes produces an alteration in body biochemical balances of the newborn (Foster and Smith, 2009). Together with pneumonia and navel infection, NCD is among the most commonly recognised health problem of calves in modern dairy farming (Busato et al., 1997; Lorenz et al., 2011a; Lundborg et al., 2005; Walker et al., 1998; Wells et al., 1997). Knowledge of the infectious agents that cause diarrhoea is essential from the viewpoint of control and prevention (Millemann, 2009). Although infectious organisms may be the main cause of damage to the intestine, in calves the severity of the diarrhoea is often determined by electrolyte imbalance and dehydration, widely believed to be the result of complex interactions between the infectious agents and environmental and husbandry risk factors.

2.2. The economic impact of neonatal calf diarrhoea

NCD is a costly condition for farmers. The direct costs include the costs of labour necessary to treat the affected calves, veterinary services, drugs and laboratory diagnosis (De Graaf et al., 1999; Walker et al., 1998), and those linked to increased calf mortality. Indirect costs are due to reduced weight gain and, later in life, reduced productivity (Virtala et al., 1996a; Waltner-Toews et al., 1986c; Wittum et al., 1993). Additionally, the cost may include the expected loss of genetic potential with a decreased capability to improve herd productivity (Anderson et al., 2003; Uhde et al., 2008; Virtala et al., 1996b; Wittum et al., 1993). NCD also represents a major animal welfare problem that needs to be addressed by farmers (Fraser, 1989; Moran, 2002).

The costs associated with NCD have been estimated at US$30 per calf per year in Switzerland (Busato et al., 1997), US$70 in France (Bendali et al., 1999a) and US$71 per breeding cow, per year in Australia (Gunn, 2003). In the USA, the cost associated with prevention of mortality from gastrointestinal disease in young calves was estimated at US$250 million per year (Frank and Kaneene, 1993).

2.3. Aetiology of neonatal calf diarrhoea: risk factors

It is commonly accepted that the severity of disease is determined by complex interactions between the agent, the host's susceptibility and the environment. Therefore, NCD can be considered a multifactorial disease triggered by the presence of risk factors. Knowledge of the risk factors for NCD can assist the formulation of effective disease mitigation strategies. Potential risk factors for NCD can be categorised into farm-level factors associated with animal husbandry, farm management and infrastructure, and calf-level factors, such as infection with specific enteropathogens, the status of the calf's immune system, and its sex and breed (Bartels et al., 2010; Bendali et al., 1999b; Clement et al., 1995). In addition, risk factors could be categorised into non-infectious and infectious. The results of risk factor studies for NCD are, however, difficult to generalise across countries, due to the significant differences which exist among environments, and among host and pathogen populations.

2.3.1. Non-infectious risk factors for NCD

Non-infectious forms of calf diarrhoea directly attributed to dietary factors are considered common in dairy calves. This type of nutritional diarrhoea is usually attributed to poor quality feed or wrong feeding method. Waltner-Toews et al. (1986a) reported an increased risk of NCD when milk replacer was used, which could be due to poor preparation practice. According to some authors, calves should be fed under constant conditions, as sudden changes in the feeding program, such as overfeeding or change of milk replacer type can affect digestion, resulting in diarrhoea (Drackley, 2008; Woodford et al., 1987). Sudden change from whole milk to milk replacer or use of poor quality milk replacers is also commonly implicated in nutritional diarrhoea. Feeding newborn calves with unsold 'waste' milk (eg. mastitic milk or milk containing antibiotics) is a common economically-convenient practice. However, it is believed that the quality of waste milk varies according to the health status of the contributing cows (Holmes, 2002; Stiles et al., 1974; Vermunt, 1994), so feeding waste milk was regarded as a possible cause of diarrhoea in young calves. Dietary diarrhoea has been described as being white in colour, due to the transit of poorly digested milk throughout the gastrointestinal tract (Andrews et al., 2008). Affected calves may develop profuse forms, but otherwise appear normal, and the mildly affected animals require only minimal treatment (Vermunt, 1994). According to some authors, if the affected calf is still alert and active, no treatment is needed, but if the calf is depressed, it should be nursed and treated as calves can easily develop infectious diarrhoea (Vermunt, 1994).

Husbandry practices define the farm's environment and may influence the prevalence and severity of NCD in different ways. For instance, pathogens' burden, multiplication and transmission intensity rises when calves are overcrowded, especially when diseased calves are not isolated from the healthy animals. Organisms of high pathogen load may overcome maternal immunity, resulting in an increase in disease incidence (Bazeley, 2003; Curtis et al., 1988b). Furthermore, wet, poor bedding may facilitate the build-up of pathogens with similar results, as most pathogens are likely to remain viable longer in moist, warm environments (Frank and Kaneene, 1993; Perez et al., 1990). Mixing different age groups of calves or mixing calves originating from different sources may also increase the transmission

of *Salmonella*, K99, *Cryptosporidium*, BRV and BCV from healthy carriers to susceptible calves (Bazeley, 2003; Holland, 1990).

Other reported non-infectious risk factors include the presence of slatted concrete floors versus other floor types; housing of calves in free-stalls compared with tie-stalls; the importation of calves from other farms; birth of calves in the winter compared with other seasons; a high percentage of heifers in the herd; poor cleaning of feeding tools; and the provision of inadequate shelter to calves (Waltner-Toews et al., 1986b; Quigley et al., 1995; Gulliksen et al., 2009a). Although the use of individual calf housing systems is not common in New Zealand, it has been reported that calf diarrhoea significantly decreased in calves housed individually during the first week of life compared to calves kept in groups (Olsson et al., 1993; Perez et al., 1990; Waltner-Toews et al., 1986a). On the other hand, young calves in group pens showed better social behaviour and started eating concentrate earlier compared to calves kept in individual pens (Pettersson et al., 2001).

It is important noting that in many cases the distinction between non-infectious and infectious risk factors of NCD is unwarranted. For instance, diarrhoea due to failure of passive immunoglobulin transfer (with consequent hypogammaglobulinaemia) could be considered 'nutritional', as several husbandry factors can affect passive immunoglobulin transfer, including the time of first colostrum intake and the quality and quantity of the administered colostrum (Vermunt, 1994; Wesselink et al., 1999). Yet, in many cases, failure of passive transfer affects the immune system, predisposing the calf to infectious forms of diarrhoea.

2.3.2. Infectious risk factors for NCD

Several studies reported K99, BRV, BCV, *Salmonella* and *Cryptosporidium* spp. as the commonest enteropathogens found in newborn calves (Bartels et al., 2010; Bendali et al., 1999a; Izzo et al., 2011; Reynolds et al., 1986; Tzipori, 1981). Serological surveys have shown that antibodies against these pathogens are widely distributed in cattle populations. However, microbiological investigations of diarrhoea cases in neonatal calves have revealed a multifactorial causality, with more than one infectious agent often identified in affected calves. Moreover, several studies have found co-infections at a higher frequency than mono-infections with single pathogens (Izzo et al., 2011; Uhde et al., 2008; Millemann, 2009). Authors

have argued that co-infections cause severe forms of diarrhoea, and that additional managerial and nutritional contributing factors may increase its severity (Bendali, 1999a). Due to the complexity of its aetiology, the severity of NCD can vary not only from farm to farm, but also on the same farm between seasons or years, (Tzipori, 1981). Infections with *C. parvum,* BRV, BCV, K99 and *Salmonella* spp., inadequate colostrum intake and lack of dam vaccination against calf enteropathogens have been defined as significant risk factors for NCD overseas (Frank and Kaneene, 1993; Fayer et al., 2000a; Bazeley, 2003; Trotz-Williams et al., 2007a; Trotz-Williams et al., 2007b; Gulliksen et al., 2009a).

2.3.2.1. Infections with *Cryptosporidium* parasites

The genus *Cryptosporidium* belongs to the phylum Apicomplexa and comprises protozoan parasites that parasitise humans and animals. C*ryptosporidium* are obligatory intracellular protozoa that produce encysted oocysts that are shed in the faeces of infected hosts (Tzipori and Ward, 2002). More than 20 species have been recorded within the genus, based on morphology, host specificity and genetic makeup (Fayer and Santín, 2009; Plutzer and Karanis, 2009; Xiao, 2010). Of these, *C. parvum, C. bovis, C. andersoni* (Fayer et al., 2007; Santín et al., 2008), *C. ryanae* (formerly the 'deer-like' genotype) (Fayer et al., 2008), *C. ubiquitum* (formerly the 'cervine genotype') (Fayer et al., 2010b), and *C. hominis* (Abeywardena et al., 2012; Chen and Huang, 2012; Kang'ethe et al., 2012) have been isolated from cattle. The gastric species *C. andersoni* produces oval oocysts and is predominantly found in juvenile or adult cattle. Conversely, *C. parvum, C. bovis, C. ubiquitum C. ryanae* and *C. hominis* produce round, phenotypically similar oocysts, and are mostly observed in pre-weaned and weaned calves (Fayer and Xiao, 2008; Rzezutka and Kaupke, 2013; Silverlås et al., 2010a). The clinical significance and zoonotic potential of *C. bovis, C. ryanae, C. ubiquitum* and *C. hominis* found in calves is not well understood. Conversely, *C. parvum* is considered a frank pathogen of calves and among the most important causative agents of calf diarrhoea during the first month of life, worldwide (De Graaf et al., 1999; Fayer and Xiao, 2008; Foster and Smith, 2009; O'Handley et al., 1999; Wyatt et al., 2010; Xiao, 2010). *C. parvum* is also one of the most common species infecting humans, and newborn calves are considered amplifiers of this potentially zoonotic species in nature (Fayer, 2010; Fayer and Xiao, 2008; Santín et al., 2008; Wielinga et al., 2008; Xiao, 2010).

2.3.2.1.1. Pathogenesis and Clinical signs of *Cryptosporidium* infection in calves

C. parvum infections in calves vary in severity from subclinical to a severe diarrhoeic disease (Thompson et al., 2008). The first documented report of *Cryptosporidium* infection in calves was published in the 1970s (Panciera et al., 1971). The first report of calf diarrhoea in which *Cryptosporidium* was found as the single agent was reported in 1980 (Tzipori, 1981). At that time, the aetiological role of *C. parvum* as a pathogen of calves was debated due to the frequent finding of co-infections with other enteropathogens and the mild histo-pathological lesions reported (De Graaf et al., 1999). The aetiological role of *C. parvum* as a frank pathogen of young calves was subsequently corroborated by the results of several experimental infections and observational studies (Grinberg et al., 2002; Moore et al., 2003; Moore and Zeman, 1991; O'Handley et al., 1999; Sevinc et al., 2003; Tzipori et al., 1980). Infection with *Cryptosporidium* usually occurs through the ingestion of infectious oocysts from the environment (Thompson et al., 2008; Tzipori and Ward, 2002)**.** Once ingested, the oocysts are exposed to gastric acid and bile salts, leading to excystation and the release of sporozoites in the intestinal tract. The sporozoites invade the intestinal epithelium cells, particularly in the ileum, and multiply asexually and sexually. The cycle culminates with the production of millions of environmentally resilient oocysts that are excreted with the faeces (Dillingham et al., 2002; Tzipori and Ward, 2002). Invasion and colonisation of the epithelial surface by the sporozoites result in villous atrophy and loss of microvillus brush border, causing disruption of epithelial junctions, which results in an increase in epithelial permeability (Foster and Smith, 2009). Consequently, there is a reduced intestinal surface area and impairment in nutrient and electrolyte transport, leading to a malabsorptive diarrhoea (Foster and Smith, 2009; Tzipori and Ward, 2002).

In newborn calves, infections with *C. parvum* are frequently acquired perinatally from a highly contaminated farm environment, and the clinical signs of cryptosporidiosis are commonly observed between five days and three weeks of age (Izzo et al., 2011; Uhde et al., 2008; O'Handley et al., 1999). The role of adult animals in the dissemination of the infection is not well understood, as adult subclinical animals usually shed low numbers of oocysts (Faubert and Litvinsky, 2000). Clinical signs vary from subclinical infections to profuse, usually self-limiting diarrhoea lasting a number of days to up to three weeks (Grinberg et al., 2002;

O'Handley et al., 1999). Young calves can become dehydrated and depressed, but the severity and duration of the illness is variable, perhaps depending on the presence of co-infections with other viral, bacterial or parasitic agents (De Graaf et al., 1999). Newborn calves affected by severe forms of cryptosporidiosis may need several weeks to recover, and this could negatively affect weight gain and increase treatment costs (De Graaf et al., 1999; Fayer and Xiao, 2008).

2.3.2.1.2. Epidemiology of *Cryptosporidium* infection in neonatal calves

Since the first documented report of *Cryptosporidium* infection in calves by Panciera et al. (1971), several prevalence studies based on different detection methods with different levels of sensitivity have been reported, with highly variable results (Fayer and Xiao, 2008). Most studies were cross-sectional and measured point prevalence, wherein one sample is collected per individual animal in a herd under study (Bartels et al., 2010; Castro-Hermida et al., 2002a; Enemark et al., 2002; Grinberg et al., 2005; Izzo et al., 2011). A number of longitudinal studies, where numerous samples were collected from each animal, measured the cumulative incidence of infection over time (Bendali et al., 1999a; Castro-Hermida et al., 2002b; Uga et al., 2000). Bendali, et al., (1999a) investigated patterns of diarrhoea in beef calves in France, and reported that *Cryptosporidium* was less frequent (15.6 %) compared to other agents. It was detected in calves from five to 13 days of age, and was equally distributed between diarrheic and non-diarrheic calves. Conversely, in a longitudinal prevalence study of *C. parvum* infection in calves in Japan, Uga et al. (2000) reported a cumulative incidence of positive calves reaching 80% by day 15 of life. Point prevalence surveys may underestimate the incidence of infection in a population, because the patent period is short and could be missed. On the other hand, cross-sectional studies allow the sampling of a larger number of farms as they require only one visit per farm. Therefore, cross-sectional studies are more suitable for the study of farm-level prevalence than longitudinal studies.

A higher rate of *C. parvum* infection has been consistently reported in calves less than four weeks of age, compared to older animals (Castro-Hermida et al., 2006; Saha et al., 2006; Starkey et al., 2006a). De la Fuente et al. (1998) estimated the prevalence of *C. parvum* among diarrheic dairy calves in Spain to be 52%. In Switzerland, the prevalence estimate was 55% in diarrheic calves (Uhde et al., 2008),

while in Sweden, it was estimated to be 11% (Björkman et al., 2003). Conversely, in Australia, Izzo et al. (2011) identified BRV as the most common enteropathogen in diarrheic calves (79.9%), followed by *C. parvum* (58.5%). In Canada, the prevalence among neonatal calves was 40.6% (Trotz-Williams et al., 2005). In United States, the prevalence ranged from 8.7 to 33.7% (Fayer et al., 2000b; Starkey et al., 2005). At farm level, the prevalence of *C. parvum* in Canada was 76% (Trotz-Williams et al., 2005). In Europe, the farm level prevalence ranged from 42 to 75% (Snodgrass et al., 1986; Uhde et al., 2008). In Australia, it was 62% (Izzo et al., 2011).

In 2005, a new species phenotypically identical to *C. parvum* and named *C. bovis* was described in calves (Fayer et al., 2005). Whereas *C. parvum* is a well-known pathogen of cattle, no clinical infections with *C. bovis* are reported in the scientific literature. Furthermore, *C. parvum* is widely considered an important zoonotic agent, but the zoonotic impact of *C. bovis* is not understood. *C. bovis* is highly prevalent in young cattle (Fayer et al., 2007; Feng et al., 2007). As said, the parasite is phenotypically similar and easily confused with *C. parvum*. Therefore, the differentiation between *C. parvum* and the non-pathogenic *C. bovis* could be required for the investigation of severe diarrhoea outbreaks or for source tracking of human infection. In such cases, molecular analysis should be performed. Studies from overseas using genotyping tools indicated that *C. parvum* was the most common *Cryptosporidium* species of newborn calves, whereas *C. bovis* was more prevalent in post weaned calves (Geurden et al., 2006; Santín et al., 2008; Starkey et al., 2006b).

Studying the prevalence of *Cryptosporidium* parasites in cattle populations is important both from animal and public health standpoints. Humans can contract cryptosporidiosis through several transmission routes, including the human-to-human route (Abd El Kader et al., 2012; Fayer, 2004; Leitch et al., 2011), the zoonotic route (Budu-Amoako et al., 2012; Caccio et al., 2005; Chalmers and Giles, 2010; Xiao, 2010), or through ingestion of contaminated food or water (Fayer et al., 2004; Mac Kenzie et al., 1994; Monge et al.,1996; Ortega et al., 1997). The relative contribution of these transmission routes to the epidemiology of cryptosporidiosis is not completely clear due to the absence of tools for phenotypic differentiation of the *Cryptosporidium* taxa. Several molecular markers have been developed to distinguish between *Cryptosporidium* species and subtypes (Cacciò et al., 2005; Sulaiman et al., 2005; Xiao, 2010; Xiao et al., 2004), and these tools have been

extensively applied in retrospective molecular studies. In New Zealand, epidemiological studies of *Cryptosporidium* infection in newborn calves have been conducted in some regions, but knowledge of the farm-level prevalence and the *Cryptosporidium* species cycling in calves is scarce. In 2003, Learmonth et al. reported the rate of *Cryptosporidium* in 7% of faecal specimens from cows (n=354) and young calves (n=304) on 36 dairy herds in the Waikato region (Learmonth et al., 2003). However, the calves' ages and the farm-level prevalence were not stated. Two years later, Grinberg et al. (2005) reported an epidemiological study conducted in the Manawatu region in 2002 using 185 newborn calves from 24 dairy farms. Infections with *C. parvum* were reported in 33/156 (21.1%) young calves from 10 (41.6%) farms. However, the molecular characterisation of the parasites was not performed. Recently, Abeywardena et al. (2012) undertook a molecular epidemiological investigation of *Cryptosporidium* in pre- and post-weaned calves (n=180) from eight farms in Canterbury. The results indicated the presence of *C. parvum* in three DNA samples from two farms, and *C. hominis* in 12 samples from five farms (Abeywardena et al., 2012). This result was surprising as *C. hominis* is considered anthroponotic and has been only sporadically described in cattle. One of the difficulties in carrying out large-scale prevalence studies in New Zealand is the presence of short and concentrated calving seasons, which hinders the sampling of a large number of farms with a wide geographical reach if limited personnel are available. Chapter 4 reports a large-scale prevalence study in young calves in New Zealand dairy farms.

2.3.2.1.3. Diagnosis and control of *Cryptosporidium* infection in neonatal calves

Clinical diagnosis of *Cryptosporidium* infections in cattle has been principally based on detection of oocysts in faeces. Several chemical staining techniques such as Modified Ziehl-Neelsen (MZN) and immunofluorescence assays (IFA) have been reported (Fayer and Xiao, 2008; Grinberg et al., 2002; Holland, 1990; Johnston et al., 2003). However, among the microscopy techniques, IFA has provided improved sensitivity and specificity compared to other conventional staining techniques (Arrowood and Sterling, 1989; Quilez et al., 1996), particularly for detecting low numbers of oocysts, in the samples. Therefore, it has been recommended that prevalence investigations should benefit from immunofluorescence methods, as many asymptomatically infected animals may shed low numbers of oocysts (Quilez et al.,

1996). Furthermore, detection of *Cryptosporidium* antigens by enzyme-linked immunosorbent assays (ELISA) has also been documented as a diagnostic method for cryptosporidiosis (Fayer and Xiao, 2008; Grinberg et al., 2002; Holland, 1990). This method also showed less sensitivity and specificity, however, when compared with IFA. This could be due to the presence of soluble *Cryptosporidium* antigens ingested from the environment, rather than antigens from cycling parasites (Johnston et al., 2003; Van Zijll et al., 2010). Overall, IFA test for *Cryptosporidium* is in a way considered the ‘‘gold standard’’ (Chalmers et al., 2011; Van Zijll et al., 2010), as it detects entire oocysts rather than soluble antigens or naked DNA. Since oocysts might not be detectable in clinical samples in all cases, especially in the prepatent or the end of the patent period, PCR-based techniques might be more sensitive (Fayer and Xiao, 2008; Thompson et al., 2008), but these techniques are relatively expensive, and their ability to differentiate infections from naked *Cryptosporidium* DNA is not well understood.

The control of bovine cryptosporidiosis on farms is problematic, for a number of reasons. Unlike other coccidian parasites, *C. parvum* does not require particular environmental conditions to become infectious, as its oocysts are excreted sporulated and fully infectious (Smith et al., 2005). Calves can excrete hundreds of millions of oocysts in the faeces (Grinberg et al., 2002; Naciri et al., 1999), and considering there is a high probability of infection with a dose as low as 50 oocysts (Moore et al., 2003), an infected calf could produce enough oocysts to infect thousands of new animals. A number of compounds, such as halofuginone lactate (HL) (De Waele et al., 2010; Jarvie et al., 2005; Klein, 2008; Lefay et al., 2001; Trotz-Williams et al., 2011), paromomycin sulphate (Fayer and Ellis, 1993; Grinberg et al., 2002), nitazoxanide (Ollivett et al., 2009; Schnyder et al., 2009) and decoquinate (Lallemond et al., 2006; Moore et al., 2003) have been tested for the prevention of cryptosporidium infection in calves, with variable results. HL is a synthetic derivative of a quinazolinone alkaloid with cryptosporidiostatic activity, but its mode of action is poorly characterised. In naturally and experimentally infected calves, the oral administration of 60 μg/kg HL for seven consecutive days from the first day of life delays the onset of oocyst shedding, reduces the number of oocysts excreted, and lowers the severity of diarrhoea (Jarvie et al., 2005; Joachim et al., 2003; Klein, 2008; Lefay et al., 2001; Trotz-Williams et al., 2011; Villacorta et al., 1991). A

formulation containing HL (Halocur, Intervet Ltd., Republic of Ireland) is currently the only prescription drug registered for the prevention of cryptosporidiosis of calves in several countries, including New Zealand. The use of HL has a number of limitations, including a substantial market price and a narrow therapeutic index, with toxicity observable at approximately twice the recommended dose (Villacorta et al., 1991, Naciri et al., 1993 and Trotz-Williams et al., 2005; http://www.msd- animal-health. co.uk/ products_public/halocur/090_ product_ datasheet.aspx, accessed 15 July 2012). Furthermore, notwithstanding the frequent occurrence of co-infections with other enteropathogens in the field (De la Fuente et al., 1999; Naciri et al., 1999; Tzipori et al., 1980), the utility of HL in the presence of such co-infections is not well understood as, with some exceptions (Klein, 2008 and Lefay et al., 2001), most anti-*Cryptosporidium* efficacy studies of HL did not analyse or take into account the presence of co-infections. Co-infections might modify the anti-*Cryptosporidium* effect of HL in various ways. The increased fluid content and intestinal motility determined by the presence of the co-infecting pathogens may reduce the activity of HL by dilution, or by reducing the transit time of the drug in the intestinal tract. Furthermore, enteric infection with BRV or other agents may cause exfoliation of infected cells, altering cellular function (Ramig, 2004), potentially enhancing the toxicity of HL via systemic absorption. Chapter 3 reports a randomised-controlled field study of HL performed on a farm co-infected with BRV and *Salmonella* Typhimurium.

Vaccination has been also suggested for the prevention of calf cryptosporidiosis. As *C. parvum* infections are acquired perinatally, vaccination of calves does not seem a feasible option. Nonetheless, a vaccine against *C. parvum* would complement the already available range of vaccines against neonatal enteropathogens administered to cows during the last trimester of pregnancy, which include BRV, BCV and K99. Further, the use of a vaccine would prevent the development of drug resistance compared to the use of drugs (although such resistance has not been reported), and would be more environmentally friendly. Experimental vaccination trials against *C. parvum* have been performed using whole oocysts, subunit vaccines, or DNA vaccines (Burton et al., 2011; Harp and Goff, 1995, 1998; Jenkins et al., 2004; Perryman et al., 1999). For example, young calves fed colostrum obtained from dams vaccinated a few weeks before parturition using *C. parvum* oocysts showed partial

improvements in the clinical outcomes, but vaccination did not eliminate oocyst shedding (Fayer et al., 1989). In an active vaccination trial where newborn calves received an oral preparation of lyophilised inactivated *C. parvum* oocysts, a partial protection against experimental *C. parvum* infection was observed (Harp and Goff, 1995), with reduction of the diarrhoea and oocyst shedding periods in the vaccinated animals. However, the same vaccine failed to induce protection when tested on a large dairy farm with severe endemic *C. parvum* infection (Harp et al., 1996). To date, no effective commercial vaccine against *C. parvum* infection in calves is available, the main reason being the difficulty in *in vitro* cultivation of the parasite.

2.3.2.2. Infections with BRV and BCV in neonatal calves

Numerous viruses can be found in cases of NCD. However, the most common endemic viruses recognised as causes of diarrhoea in calves worldwide are BRV and BCV (García et al., 2000; Holland, 1990; Gumusova et al., 2007; Snodgrass et al., 1986; Vermunt, 2000). Typically, BRV causes diarrhoea in 5 to 14 day-old calves, and calves older than four weeks are rarely infected. However, subclinical infection and shedding have been reported in older cows (Bulgin et al., 1989). BCV is also recognised as a cause of severe diarrhoea, particularly in newborn calves (in adult cows it causes 'winter dysentery', also characterised by severe diarrhoea) (Clark, 1993). BRV and BCV infections are acquired through the oral route, by ingestion of contaminated food and water. As adult ruminants may become asymptomatically infected, they have an important role as reservoirs of infection within the herd (Durham et al., 1979).

2.3.2.2.1. Infections with BRV in neonatal calves

Rotaviruses are classified as a distinctive genus of the family Reoviridae. BRV has been repeatedly reported as a significant causative agent of viral gastroenteritis in humans, as well as animals (Cho et al., 2013; Holland, 1990; Parashar et al., 1998). Rotavirus is non-enveloped and has a double-stranded RNA genome comprising 11 gene segments surrounded with a double-shelled protein capsid (Howe et al., 2008; Parashar et al., 1998). Serologically, BRV is classified into seven serogroups (A though G) containing viruses that have cross-reacting group antigens detectable by serological tests such as ELISA (Burns et al., 1989; Parashar et al., 1998; Pedley et al., 1986). Groups A, B, and C have been recognised in both humans and animals,

and other groups have been found only in animals (Holland, 1990; Parashar et al., 1998). Group A BRV has been clearly recognised as a cause of diarrhoeal disease in calves (Badaracco et al., 2012; Dash et al., 2011; Holland, 1990). Within Group A, several genotypes have been recognised, and of these, G6 and G10 are widely identified as the chief causes of neonatal calf diarrhoea in New Zealand (Garaicoechea et al., 2006; Howe et al., 2008; Lucchelli et al., 1992).

2.3.2.2.1.1. **Pathogenesis and clinical signs of BRV infection in neonatal calves**

Rotaviruses can survive in faecal material and remain a source of infection to susceptible animals for long periods of time (Dhama et al., 2009). Once in the gastrointestinal tract, the virus invades the enterocytes lining the small intestine. The enterocytes covering the villous surface of the upper small intestine are the first cells to be infected (Foster and Smith, 2009). In an early phase, the epithelial cells covering the villi form a layer of columnar cells. During the course of the infection, large numbers of viral antigens can be observed in the cytoplasm of the infected cells, concomitantly with the onset of diarrhoea, and the infection causes exfoliation of the infected cells altering mucosal function (Ramig, 2004). Subsequently, the villi become atrophic with rapid production of immature, undifferentiated cells in the crypts, which are unable to perform efficient absorptive and digestive functions (Foster and Smith, 2009; Holland, 1990). This leads to malabsorption and maldigestion. For example, lactose, which is the main carbohydrate of milk and the principal nutrient of neonatal calves, passes through the intestine undigested and unabsorbed, inducing osmotic diarrhoea (Holland, 1990).

Usually, the incubation period of BRV infection in neonatal calves ranges from 24 to 48 hours, probably depending on the immunity status and the viral dose ingested (Dhama et al., 2009). Clinical signs in experimentally infected calves include diarrhoea and depression, which persists for 2 - 4 days (Bridger, 1994). Natural infections are characterised by sudden onset of diarrhoea rapidly spreading among young calves. The clinical signs of BRV infection include mild depression, salivation and watery diarrhoea. It is usually a non-febrile, non-viremic disease, unless complicated by secondary pathogens (Dhama et al., 2009). A mortality rate of 5–20% has been reported (Dhama et al., 2009). However, the mortality rate could increase among calves that do not receive an adequate amount of high quality colostrum.

2.3.2.2.1.2. Epidemiology of BRV infection in neonatal calves

Epidemiological studies indicate that BRV infections are widespread in nature and usually affect young calves between 1 day and 3 weeks of age (Bendali et al., 1999a; Broor et al., 2003; García et al., 2000; Holland, 1990; Malik et al., 1995). Different sampling strategies and diagnostic methods may result in considerable differences in the reported prevalence of BRV infection. In fact, the prevalence of BRV in different faecal samples from different countries varies as following (in brackets); in USA (44%) (Chinsangaram et al., 1995), Italy (90%) (Castrucci et al., 1988), Britain (42%) (Reynolds et al., 1986), Canada (26.4%) (Hussein et al., 1995), Australia (79.9%) (Izzo et al., 2011), Spain (42%) (De la Fuente et al., 1998), France (45.1%) (Vende et al., 1999), Japan (16.7%) (Fukai et al., 1998), Turkey (41.2%) (Gumusova et al., 2007), Argentina (30%) (Badaracco et al., 2012) and Brazil (17%) (Alfieri et al., 2006).

In New Zealand, an estimate suggested that the direct costs of BRV outbreaks in calves could be as high as NZ$6,000 per farm per season, and Group A G6 and G10 are the main BRV genotypes isolated from diarrhoeic calves (Howe et al., 2008). However, except in small scale studies (Belton, 1995; Burgess and Simpson, 1976; Schroeder et al., 1983), no prevalence studies of BRV in New Zealand dairy herds have been published, and the impact of these viruses on animal health is poorly understood.

2.3.2.2.2. Infection with BCV in neonatal calves

BCV infection was first reported by Mebus et al. (1973). BCV is recognised as a enteric pathogen worldwide, causing infections of the upper respiratory and lower intestinal tracts of young calves (Clark, 1993). In the enteric form, BCV affects calves in the same age-group as BRV, and the clinical signs are also very similar (Holland, 1990; Gumusova et al., 2007). Coronaviruses are a genus of the family coronaviridae, which are large, enveloped viruses (Murphy, 1999). In general, the genus is classified into three genetically-distinctive groups (group 1, 2, and 3). BCV belongs to group 2, and comprise particles containing a nonsegmented, single-stranded, positive sense RNA genome. The virus diameter ranges from 80 to 160 nm, with an average of 120 nm (Clark, 1993; De Vries et al., 1997). It has five main structural proteins encoded by the genomic RNA: a spike glycoprotein, a

transmembrane protein, a nucleocapsid protein, a hemagglutinin-esterase protein, and a small membrane protein (Chouljenko et al., 2001; Clark, 1993; Spaan et al., 1988).

The spike glycoprotein and hemagglutinin-esterase protein of BCV have receptor binding and detachment functions which target receptors of erythrocytes and the susceptible cells (De Groot, 2006; Schultze et al., 1991). These proteins have been determined as the major hemagglutinin of BCV. The variation in host-range and tissue tropism of coronaviruses is largely attributed to variations in the spike glycoprotein that mediates viral attachment to susceptible cells (Schultze et al., 1991).

2.3.2.2.2.1. Pathogenesis and clinical signs of BCV infection in neonatal calves

Newborn calves acquire BCV infection through the oral route. In the gastro-intestinal tract, the virus invades cells in the small intestine, then spreads to the large intestine (Clark, 1993; Hirsh et al., 2004). The pathogenesis of BCV infection is similar to that of BRV: virus replication occurs in the epithelial cells of the villi, which slough off and are replaced with immature cells from the crypts. The replacement with immature cells follows a loss of digestive and absorptive capacity (Foster and Smith, 2009), osmotic imbalance and raise in the amount of intestinal fluid (Foster and Smith, 2009). The decrease in digestive and absorptive capacities leads to diarrhoea, with loss of water and electrolytes. It has been suggested that since BCV infects both the small and large intestine, BCV diarrhoea may be longer in duration than the diarrhoea caused by BRV and *C. parvum* (Clark, 1993).

The clinical signs of BCV infection are similar to those described for BRV. The severity of BCV enteritis is believed to vary with the immunological status of the calf and the viral dose ingested. Diarrhoea develops about 48 hours after infection and continues for 3-6 days (García et al., 2000; Hirsh et al., 2004). Diarrheic calves are often dull and anorexic in the acute phase of infection, and if diarrhoea is severe, they may also become dehydrated (Clark, 1993). Diarrhoea seems to develop more quickly and to become more severe in very young or colostrum deprived calves (Clark, 1993).

2.3.2.2.2.2. Epidemiology of BCV infection in neonatal calves

BCV infections are associated with diarrhoea in newborn calves, winter dysentery (WD) in adult cattle, and respiratory tract infections in calves and cattle (Clark, 1993; Gumusova et al., 2007; White et al., 1989). The infected calves shed BCV in the faeces and the nasal discharge (Reynolds et al., 1985). As most enveloped viruses, outside the host BCV is considered sensitive to inactivation. In some countries infection occurs mostly in the winter, where animals are usually housed inside, due to increased animal contact and stress. Furthermore, the virus survives better at low temperatures and high humidity (Clark, 1993; Saif, 1990). Investigations at the molecular level showed that BCV may differ between outbreaks, both spatially and temporally, demonstrating new introductions in each outbreak (Liu et al., 2006). Although this virus is considered by some a significant cause of diarrhoea in young calves (Abraham et al., 1992; Reynolds et al., 1986), some epidemiological studies have reported viral shedding in the faeces of clinically healthy cows (Collins et al., 1987; Crouch et al., 1985), as well as healthy young calves (De Rycke et al., 1986), which may indicate that BCV has the ability to infect calves without causing clinical signs.

BCV infections have been reported in several countries, with a higher prevalence in adult dairy cows (Clark, 1993; Hägglund et al., 2006; Paton et al., 1998; Vijgen et al., 2006). In neonatal calves, reported calf-level prevalence rates in diarrheic calves in Europe (Uhde et al., 2008; Snodgrass et al., 1986), North America (Marsolais et al., 1978), Africa (Abraham et al., 1992) and Australia (Izzo et al., 2011), ranged from 7.8% to 53%. Also in this case, the variation in prevalence between the countries may have been due to the existence of real differences among environments and host or pathogen populations, or reflect the use of different sampling strategies and diagnostic methods.

In New Zealand, according to a limited number of publications (Durham et al., 1979; Vermunt, 2000) as well as personal communications with veterinary pathologists (Gibson, 2012; Tisdall, 2012), BCV infection is considered uncommon in calves, and was found in ~2% of faecal specimens analysed by diagnostic laboratories. Furthermore, the virus is believed to be of minor importance as an enteric pathogen in neonatal calves (Vermunt, 2000).

2.3.2.2.3. Diagnosis of and control of BRV and BCV infection on farms

Accurate diagnosis of infections is necessary for the optimal management of infected animals as well as the prevention of transmission to other animals. Because of the similarities in the clinical signs caused by the enteropathogens, laboratory diagnosis is very important (Millemann, 2009). Several diagnostic methods for BRV and BCV are available. For example, electron microscopy is used to identify BRV and BCV particles. However, the necessity for costly equipment and highly skilled personnel limits its use. Other techniques have also been used, such as immunodiffusion indirect immunofluorescence, complement fixation, fluorescent antibody staining and hemagglutination tests (Millemann, 2009). The ELISA platform is versatile and cost effective and amenable for the production of self-contained polyvalent kits of relatively low cost and long shelf-life. At present, ELISA tests are widely used for diagnosis of BRV and BCV infection in diagnostic laboratories in New Zealand and overseas (Holland, 1990; Izzo, et al., 2011).

Due to the complex nature of NCD, absolute prevention or control of this condition is difficult. Enhancing non-specific and/or specific immunity is, however, a fundamental in preventing viral associated NCD (Plaza et al., 2009; Radostits, 1975; Saif et al., 1983). Under natural conditions, the calf's immune system may be capable of an active response against the infection, but this is delayed and usually ineffective. Therefore, immunity against BRV and BCV in calves depends on the acquisition of antibodies through colostrum. The colostral antibodies form the most important protection line and the newborn calf must receive an adequate amount of colostrum (Plaza et al., 2009). This passive immunity depends upon the infectious agents to which cows were previously exposed to and may last for approximately one or two weeks. It is capable of reducing the severity, rather than completely eliminating, infection and diarrhoea (Quigley, 2002). Since antibacterial drugs are ineffective against BRV and BCV infections, vaccination of mothers against these pathogens is the most common protection option on the market. Vaccines are commercially available for immunisation of pregnant cows and heifers in order to prevent diarrhoea in their calves, by increasing the quantity of colostrum antibodies against these viruses (Radostits, 1991; Saif and Fernandez, 1996; Snodgrass, 1983). The high quantity of colostrum antibodies of vaccinated calves may confer a protection period

of approximately two weeks, particularly if colostrum is fed within the first 12 hours of birth (Gonzalez et al., 2010; Quigley, 2002; Saif and Smith, 1985).

2.3.2.3. Infection with K99

Escherichia coli bacteria are the principal facultative anaerobe of mammalian colonic flora. The organism usually colonises the intestinal tract of newborn animals within a few hours after birth, and persists harmlessly within the intestinal tract. However, in the immunosuppressed or when the integrity of the intestinal wall is violated, pathogenic or opportunistic *E. coli* strains can cause infection (Nataro and Kaper, 1998). *Escherichia coli* are among the most common causes of diarrhoea in animals and humans. According to their clinical presentation and pathogenicity, diarrhoeagenic *E. coli* have been categorised into six main distinct pathotypes, namely enterohaemorrhagic (EHEC), enteropathogenic (EPEC), enteroinvasive (EIEC), enteroaggregative (EAEC), diffusely adherent (DAEC) and enterotoxigenic (ETEC) (Campos et al., 2004; Kaper et al., 2004; Nataro and Kaper, 1998; Spano et al., 2008). Two additional types have been recently reported: Adherent Invasive *E. coli* (AIEC), which is believed to be related to Crohn's disease but is not associated with diarrhoeal illness (Croxen and Finlay, 2010), and the Shiga Toxin producing Enteroaggregative *E. coli* (STEAEC), which was linked to an *E. coli* outbreak in 2011 in Germany (Clements et al., 2012).

EHEC strains produce Shiga-like toxins are associated with severe bloody diarrhoea and haemolytic uraemic syndrome in humans (Caprioli et al., 2005; Takeda, 1997). These strains rarely cause disease in animals, but cattle are considered important natural reservoirs and one of the main sources of human infections with EHEC O157 (Caprioli et al., 2005). EPEC strains principally cause severe diarrhoea in children younger than six months of age (Levine and Edelman, 1984). EPEC pathotypes/ strains can be further divided into two subclasses: typical (tEPEC) and atypical (aEPEC). The tEPEC strains are characterised by the presence of bundle forming pilli (bfp) and are typically detected in child diarrhoea cases, while aEPEC without bfp are usually recovered from bovine, but also from humans (Trabulsi et al., 2002). EIEC pathotypes cause dysentery in humans and are highly related in their phenotypic and virulence characteristics to *Shigella* spp. (Kopecko et al., 1985; Makino and Yoshikawa, 1988). EIEC infections are associated with profuse

diarrhoea and fever, and have been reported in several countries, with higher frequency in developing countries (Echeverria et al., 1992; Nguyen et al., 2005; Sethabutr et al., 1993; Taylor et al., 1988). EAEC strains seem to be important causes of paediatric diarrhoea in developing countries, with possible fatal outcome in untreated patients, whereas the infections are usually mild and self-limiting in the industrialised world. However, severe EAEC infection in HIV-positive adults and travellers has been reported in developed countries (Boll and McCormick, 2012; Estrada-Garcia and Navarro-Garcia, 2012; Harrington et al., 2006; Saha et al., 2013). Infections with DAEC strains are characterised by diffuse adherence on epithelial cells, associated with diarrhoea in multiple age groups, with a considerably higher detection frequency in cases of childhood diarrhoea in children over 12 months of age (Germani et al., 1996; Gióon et al., 1991; Scaletsky et al., 2002; Spano et al., 2008). Among the *E. coli* pathotypes, ETEC strains are considered among the most important causes of diarrhoea in infants under 5 years of age in developing nations (Qadri et al., 2005; Walker et al., 2007). ETEC strains are also among the most common causes of travellers' diarrhoea, and it has been suggested that this pathotype is commonly imported to the developed world (Black, 1990; Castelli and Carosi, 1995). In animals, ETEC infections are most common in neonatal pigs and calves (Nagy and Fekete, 2005), with strain host-specificity determined by the expression of specific colonisation factors (Nataro and Kaper, 1998).

In order to cause diarrhoea in calves, ETEC strains must express virulence (colonisation) factors that have a significant role in pathogenesis. ETEC colonisation factors show remarkable species specificity, and are different from those of human ETEC strains. In the past, the most common factors have been nominated K88, K99, and 987P, but these are now being recognized by an "F", such as the F4, F5, and F6, rather than K88, K99, and 987P respectively (Gaastra and De Graaf, 1982). Due to the specificity of these virulence factors, bovine ETEC strains typically do not infect humans, contrary to other pathogenic *E. coli*, such as the O157: H7 strain which is found in cows and causes severe infection in humans (Caprioli et al., 2005). The most widely known virulence factor expressed in calf ETEC is the K99 (or F5) surface antigen (Holland, 1990; Myers and Guinee, 1976). It is well documented that K99 strains are causative agents of watery diarrhoea, most commonly in 1-5 day-old calves (Acres, 1985; Bendali et al., 1999a; Bazeley, 2003; Foster and Smith, 2009).

The capacity of $K99^+$ ETEC strains to adhere to the epithelium of the small intestinal is mediated by the K99 antigen, which acts as an adhesine, and is age-dependent. Francis et al. (1989) observed that the K99 antigen is expressed at pH level > 6.5, and that the distal part of the small intestine is the preferred place of colonisation with K99-positive ETEC. K99-mediated attachment to the enterocyte enables K99 ETEC to colonise the ileum, multiply and spread in the small intestine (Runnels et al., 1980). The second stage of pathogenesis is the production of heat stable enterotoxin (Runnels et al., 1980). Runnels et al. (1980) reported changes in the intestinal cell receptors for K99 antigen in neonatal animals more than 5 days of age, which determine a decreased susceptibility of calves to K99 infections after the first week of age. Another factor determining the decreased susceptibility of calves to K99 infections after the first week of age is the mounting of the immune response as a result of the perinatal infection (Qadri et al., 2005).

2.3.2.3.1. Pathogenesis and Clinical signs of K99 infections in neonatal calves

Most of the knowledge about the pathogenesis of ETEC infections was acquired in the 1980s and 1990s. Usually, newborn calves acquire a $K99^+$ ETEC infection shortly after birth through the faecal-oral route. Once ingested, the bacterium colonises and multiplies in the small intestine (Foster and Smith, 2009). Attachment of K99 to the intestinal epithelial cells is vital, as it allows the microorganism to overcome the normal peristaltic cleansing action of the small intestine. Colonisation starts at the ileol-caecal connection within 3 hours of infection, and progresses to involve up to 60% of the small intestine 16 hours post-infection (Pearson and Logan, 1979). After attachment, the bacteria multiply forming micro-colonies that may cover the villi surface (Pearson and Logan, 1979). Once K99 reaches the ileum, both a heat-stabile enterotoxin (STa) and the adhesion antigen are expressed, induced by the increase in pH level (Foster and Smith, 2009).

As stated above, ETEC attachment induces the bacteria to release STa, which binds to guanylyl cyclase-C (GCC), an enzyme localised in the brush border membrane, concentrated in the ileum portion of the intestine (Sack, 1975). This binding of STa toxin to GCC increases the production of cyclic guanylyl monophosphate (cGMP) within the epithelial cells, with a consequent reduction in absorption of electrolytes, leading to chloride secretion (Sack, 1975). Change in chloride secretion osmotically

draws water into the intestinal lumen, causing osmotic diarrhoea (Foster and Smith, 2009).

In infected calves, the onset of clinical signs of K99 infections can be seen within 24 hours of birth, and most infections are observed in calves < 5 days of age (Bazeley, 2003; Foster and Smith, 2009). Disease is characterised by profuse, watery diarrhoea (Holland, 1990). Affected neonates quickly become dehydrated, losing up to 12% of their body weight within the first 6 hours of infection (Tzipori et al., 1981). Consequently, the affected calves exhibit depression, weakness, subnormal body temperature and tachycardia (Holland, 1990). Co-infections with other enteropathogens may increase the severity and duration of the illness (Holland, 1990).

2.3.2.3.2. Epidemiology of K99 infections in neonatal calves

Several epidemiological investigations determining the prevalence of K99 in calves have been conducted overseas. De Graaf (1999) estimated the prevalence of K99 in diarrhoeic newborn calves in Belgium at 4%. In Switzerland, Uhde (2008) found 6% prevalence of this agent in diarrhoeic calves. In Australia, Izzo et al. (2011) identified a prevalence of 17.4% among diarrhoeic calves, and in Spain it was 11.9% (De la Fuente et al., 1998). Only a limited number of studies of individual enteropathogens have been published In New Zealand (Belton, 1995; Grinberg et al., 2005; Vermunt, 2000) and no estimates of K99 prevalence were retrieved in the literature.

2.3.2.3.3. Diagnosis and control of K99 infection in neonatal calves

Because *E. coli* is ubiquitous in the intestinal tract of mammals and can be isolated from every faecal specimen, diagnosis of K99 infection requires the detection of strains carrying the K99 antigen from the faeces of the affected calves. The definitive diagnosis of K99 ETEC infection relies on the demonstration of this fimbrial antigen in the *E. coli* strain isolated from the faeces (Holland, 1990). Briefly, following isolation of an *E. coli* from a faecal specimen, suspected K99 positive colonies are grown on specific selective media that enable expression of the K99 antigen (Acres, 1985), and this can be identified by agglutination with specific antisera. In order to circumvent the isolation phase ELISA tests for the diagnosis of the K99 antigen directly from faeces have been developed using monospecific fimbrial antisera

(Nagy and Fekete, 1999). Thanks to their versatility and the possibility of production of polyvalent kits covering BRV, BCV and *Cryptosporidium* antigens (Izzo et al., 2011; Jäkel, 1995; Schneider et al., 1984), numerous commercial ELISA kits for detection of K99 antigen are currently available, and to the author's knowledge ELISA kit technology has replaced bacterial isolation methods for K99 in most New Zealand diagnostic laboratories.

Under natural conditions, adequate colostral immunoglobulin transfer may reduce the severity of K99 diarrhoea. However, calves may remain susceptible to K99 infection within the first few days of life, even if adequately fed colostrum after birth. Therefore, specific immunising agents against the K99 antigen are often necessary (Radostits, 1991; Snodgrass et al., 1982). As in the case of BRV and BCV, passive immunity obtained by vaccinating pregnant cows during the last trimester of pregnancy with K99 antigen can be obtained. Vaccines are usually commercially prepared using inactivated K99 pili antigen, and are administered subcutaneously to healthy pregnant cows at two doses, the first six to seven weeks before parturition, and the second dose three weeks later (Nagy, 1980; Nagy and Fekete, 2005; Walker et al., 2007). This approach decreases the severity of the infection and diarrhoea but it does not completely prevent the clinical disease (Nagy, 1980; Snodgrass et al., 1982). Following the same rationale, an alternative passive immunisation via oral administration of specific antibodies obtained from hyperimmune chicken egg yolk IgY or bovine colostrum has been reported. This strategy, which has been explored during the past two decades, could represent a new, promising, economically convenient approach to the prevention of gastrointestinal infections in neonatal calves (Ikemori et al., 1992; Mine and Kovacs-Nolan, 2002; Vega et al., 2011). In a trial conducted by Ikemori et al (1992), the protective results of egg yolk IgY obtained from chickens immunised with heat-extracted K99 antigens were estimated in an experimental calf model of K99-induced diarrhoea. In this experiment, the results showed that young calves fed milk containing egg yolk powder with specific IgY had temporary diarrhoea, 100% calf survival and better body weight gain, compared to the control calves that suffered from severe diarrhoea and died within 72 hours after infection.

2.3.2.4. Infection with *Salmonella* spp.

Salmonellosis is an infectious disease of humans and animals caused by bacteria belonging to the genus *Salmonella*. *Salmonella* spp. cause diarrhoeal and systemic infections in humans. The organisms are commonly found in subclinically infected farm animals, leading to contamination of animal products (meat, milk and eggs). Other sources of human infection are fruits and vegetables irrigated or fertilised with contaminated faecal wastes (Berger et al., 2010; Crum-Cianflone, 2008; Pui et al., 2011).

Salmonella spp. belong to the family Enterobacteriaceae. Taxonomically, *Salmonella* is divided into 2 species; *S. bongori* and *S. enterica*. *S. enterica* is further divided into 6 subspecies: *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV) and *S. enterica* subsp. *indica* (VI) (Grimont and Weill, 2007). According to the WHO Collaborating Centre for Reference and Research on *Salmonella* in France, the total number of *Salmonella* serotypes recorded in 2006 was 2579 (Grimont and Weill, 2007). As the 'official' nomenclature is very long, in the literature salmonellae are commonly named using abbreviations. For instance, *Salmonella enterica* subspecies *enterica* serotype Typhimurium is abbreviated as *S.* Typhimurium (Grimont and Weill, 2007).

Salmonellosis has been documented worldwide (World Health Organization Global Salm-Surv, 2006) and most cases of salmonellosis in humans and animals are caused by serotypes of *Salmonella enterica* subspecies *enterica*. The organisms infect most species of domestic animals, especially pigs, calves and poultry (Wray and Wray, 2000). Enteric disease is the most common clinical presentation, but a wide range of forms, such as acute septicaemia, abortion, and arthritis may be seen (Wray and Wray, 2000). In addition, several host species, particularly pigs and birds, may be subclinically infected. Subclinically infected animals play an important role in *Salmonella* transmission within and between herds, and as sources of foodborne infection in humans (Hendriksen et al., 2011; Lo Fo Wong et al., 2002; Pui et al., 2011).

In calves, salmonellosis is often transmitted from subclinical infected cattle, birds or contaminated feed (Anderson et al., 2001). A wide range of *Salmonella* serotypes

have been reported in newborn calves (Anderson et al., 2001; Izzo et al., 2011; Langoni et al., 2004; Reynolds et al., 1986). Infection severity and mortality rates in young calves generally reflect the strength of immunity (Anderson et al., 2001). In the calf, immunity is enhanced by good colostrum management (Vermunt et al., 1995; Wesselink et al., 1999) and the provision of adequate nutrition, as well as optimal environment conditions that minimise pathogen load and bacterial challenge dose through contaminated milk and equipment (Mohler et al., 2009).

Salmonella spp. are identified as Gram-negative, rod shaped, highly motile organisms (with the exception of *S.* Gallinarum and *S.* Pullorum which are non-motile). Biochemically**,** *Salmonellae* are catalase-positive, oxidase-negative, non-lactose fermenting, indole -negative, urea- negative, methyl red and Simmons citrate - positive and H_2S producing (Russell and Gould, 2003). *Salmonella* has a complex life cycle in the infected host, and multiple virulence genes which enable pathogenic *Salmonella* to invade the host's intestinal epithelium and macrophages have been identified (Marcus et al., 2000). An important step in the pathogenesis of *Salmonella* infection is the bacterial interaction with the reticuloendothelial system of the host, which promotes inflammation (Chopra et al., 1999; Francis et al., 1993; Galán, 1996). Further, the capability of some *Salmonella* spp. to survive and replicate inside macrophages promotes apoptosis, and this allows evasion from the host's immune response (Aranda et al., 1992; Chopra et al., 1999; Rathman et al., 1996). *Salmonella* virulence genes are often clustered in pathogenicity islands, which are usually absent in non-pathogenic strains (Blanc-Potard et al., 1999). According to Marcus et al., (2000), these pathogenicity islands are classified into five subgroups labelled as SPI-1 through SPI-5, from which virulence genes SPI-1 and SPI-2 have higher virulence.

2.3.2.4.1 Pathogenesis and clinical signs of calf salmonellosis

Although the majority of *Salmonella* infections in young calves are acquired by the oral route, other modes of infection such as the respiratory and conjunctival routes have been reported (Mohler et al., 2009; Nielsen, 2013). After ingestion, *Salmonella* crosses the fore stomachs and abomasum and reaches the intestinal mucosa. Once in the intestine, it penetrates the ileum and upper large intestine (Hunt, 1985). *Salmonella* may then spread to the adjacent lymph nodes and invade the blood stream causing bacteraemia (Chopra et al., 1999; Nielsen, 2013). *Salmonella enterica*

serotypes that are capable of invading the intestinal epithelium can cause diarrhoea, either by inducing inflammation and/or necrosis, with increased secretion of fluid or reduced intestinal absorption. The bacteria invade the villi on the microvillus border of the intestinal epithelium, destroying cells until bacteria reach the lamina propria (Hunt, 1985).

In neonatal calves, clinical salmonellosis is more frequent after the first week of age, and is often observed between 2 to 6 weeks of age (Wray and Wray, 2000). The clinical presentation varies, with the enteric form predominating. Typically, clinical cases are characterised by fever, dullness, loss of appetite and diarrhoea which may contain blood or mucus and have an offensive odour (Millemann, 2009; Mohler et al., 2009). Affected young calves may rapidly become dehydrated and lose condition, becoming weak and emaciated (Mohler et al., 2009; Warnick et al., 2001). Some cases are mild and pass unnoticed, and others are severe, with calves suffering from septicaemia and fever (40.5–42°C), with high mortality rate within 24–48 hours, without evident diarrhoea (Mohler et al., 2009; Wray and Wray, 2000). In such cases, *Salmonella* are commonly isolated from the internal organs.

2.3.2.4.2. Epidemiology of *Salmonella* infection in neonatal calves

Salmonella spp. are important human foodborne pathogens. At risk groups for salmonellosis are newborns, immunodeficient and elderly patients (Hohmann, 2001). In the US, around 40,000 cases of human salmonellosis are recorded every year (Crum-Cianflone, 2008). Approximately 45% of *Salmonella* serotypes isolated from humans and animals in the US were serotype *S.* Typhimurium followed by *S.* Enteritidis and *S.* Heidelberg (Centers for Disease Control and Prevention, 2006). In the UK, there was a rise of human salmonellosis cases caused by *S.* Enteritidis in the 1980s, which decreased in numbers during the late 1990s. Most cases of human salmonellosis caused by *S.* Enteritidis were foodborne, associated with the consumption of poultry products, in particular eggs, and the decline was due to the application of vaccination programs for egg-laying chickens (Cogan and Humphrey, 2003). A similar trend was seen in Belgium, with a significant decrease of foodborne outbreaks due to S. Enteritidis in humans in 2005, following extensive vaccination programme in layer flocks (Collard et al., 2008).

In New Zealand, salmonellosis is considered the third most commonly notified enteric disease (after campylobacteriosis and giardiasis) (French et al., 2011). Approximately 16,500 cases have been reported each year (2001 to 2012), with an average of 150 hospitalisations. The most common risk factors for human salmonellosis in New Zealand are consumption of food from retail premises and direct contact with farm animals (ESR Annual surveillance report, 2013). The majority of *Salmonella* strains isolated in humans in New Zealand are *S.* Typhimurium Definitive type (DT) 160, *S.* Typhimurium DT1, *S.* Typhimurium DT135, *S.* Brandenburg, *S.* Typhimurium DT156, *S.* Infantis, *S.* Typhimurium DT101, *S.* Enteritidis phage type 9a, *S.* Typhimurium DT42 and *S.* Saint Paul (French et al., 2011).

As well as infecting humans, Salmonellosis is also an important infection in domesticated ruminants in New Zealand, with repeated reports in bovine clinical specimens (ESR Annual surveillance report, 2013). The most common *Salmonella* serovar isolated from New Zealand cattle is *S.* Typhimurium. Alongside this serovar, *S.* Brandenburg has also emerged as a prevalent strain, mainly in the South Island, causing enteric infections in both calves and adult cattle (Clark et al., 2004). *Salmonella* Brandenburg was reported in sheep (Baker et al., 2007), whereas *S.* Typhimurium DT 160 was reported in sheep and wild birds (Callaghan, 2001; Alley et al., 2002; French et al., 2011). *Salmonella* Agona has been reported in humans, foals, cattle, pigs and poultry (Patterson-Kane et al., 2001; Adlam et al., 2010). *Salmonella* Mbandaka is commonly reported as a human pathogen in Australia (Scheil et al., 1998) and New Zealand (Adlam et al., 2010), and the serotype has also been reported in young calves in USA (Wray et al., 1991). Although in New Zealand dairy farms salmonellosis is usually reported as sporadic cases, outbreaks continued to occur in several dairying regions. In 2012, salmonellosis outbreaks characterised by high morbidity and low mortality were reported in Taranaki. Affected cows showed a remarkable drop in milk production, diarrhoea, weight loss and death (Benschop, 2012). Besides the animal health concerns, these infections represented a zoonotic risk, as affected cows shed the bacteria in their faeces for extended periods, even without showing clinical signs. Zoonotic transmission among dairy farming families and workers can occur through direct and indirect contact with cattle faeces. Free-range poultry and birds could also carry infection between farms or to humans

while foraging over cows' paddocks areas. Drinking unpasteurised milk is also a potential hazard, as this practice is common among New Zealand dairy farmers (Benschop, 2012; Stevenson, 2012).

Finally, *S.* Dublin is an important pathogen of calves, worldwide (Lance et al., 1992; Osborne et al., 1977; Wray and Wray, 2000). In New Zealand, the organism has been reported in humans and sheep (Clark et al., 2002; Clark, 1999). In the UK, *S.* Dublin causes about 500 outbreaks every year, and the rates of infection are similar in adult cattle and young calves (Wray and Wray, 2000). In Denmark, the Netherlands and Sweden, infection in cattle with *S.* Dublin is common, compared to *S.* Typhimurium, whilst the opposite is the case in France (Visser, 1991; Wray and Wray, 2000). In Australia, *S.* Dublin infection was more common in cattle than *S.* Typhimurium until the 1990s, but subsequently, *S.* Typhimurium became more common (Wray and Wray, 2000). In the US, *S.* Dublin was isolated between 1980 and 1995, predominantly from California, New York and Pennsylvania counties (McDonough et al., 1999; Wray and Wray, 2000).

Overall, the prevalence of *Salmonella* intestinal carriage in newborn calves in New Zealand appears to be low (Grinberg et al., 2005). In comparison, *Salmonella* spp. were isolated in 23.8% of diarrheic calves in Australia (Izzo et al., 2011), 6.1% in Brazil (Langoni et al., 2004) and 12% in the UK (Reynolds et al., 1986).

2.3.2.4.3. Diagnosis and control of *Salmonella* infection in calves

The gold standard for *Salmonella* infection diagnosis in calves is the laboratory isolation of the organism from faeces or organs. Isolation of *Salmonella* can be carried out using a number of techniques that involve initial culture of the faeces in one or two of selective enrichment media (containing substances that inhibit other faecal organisms), followed by subculture onto selective-differential media, which allows differentiation of *Salmonella* from other enteropathogens (Millemann, 2009; Nielsen et al., 2011; Wray and Wray, 2000). Several biochemical tests can then be applied for the presumptive identification of the isolated strains to genus level (Bang et al., 2012; Mohler et al., 2009; Nielsen, 2013; Persson et al., 2012). Finally, anti-*Salmonella* poly-O antisera can be used to agglutinate the isolate, providing a robust identification scheme. Laboratories may then send the isolates to a reference laboratory, where the diagnosis can be confirmed and the complete identification to

serotype level established. Subtyping by phage-typing or genetic fingerprinting methods may be pursued if more detailed epidemiological information is required. In New Zealand, the final identification and subtyping of *Salmonella* isolates is performed by the enteric reference laboratory, Environmental Science and Research Ltd., Porirua (ESR).

Veterinary interventions in the course of salmonellosis outbreaks in calves are necessary in order to reduce animal suffering and the economic loss resulting from morbidity and mortality. Furthermore, as *Salmonella* spp. are of public health significance, proper management of sick animals is also essential to reduce environmental contamination and zoonotic transmission. As electrolyte imbalances, dehydration and bacteremia are common features of *Salmonella* infection in young calves (Mohler et al., 2009), treatment of infected calves is usually directed towards fluid and electrolyte replacement and inflammation control. Bacteraemia is addressed by the prudent use of antimicrobial treatments. Numerous antimicrobial drugs, such as amoxicillin, trimethoprim-sulfadiazine, chloramphenicol and ampicillin, have been evaluated for treatment of salmonellosis in calves (Constable, 2004; Fecteau et al., 2003).

Although the occurrence of antimicrobial resistance in virulent *Salmonella* serotypes is well documented (Fecteau et al., 2003), when tested clinically, several therapeutic antimicrobial agents efficacy, showed reduction in infection' severity as well as faecal shedding of the organism (Fecteau et al., 2003; Groothuis and van Miert, 1987). Vaccination attempts have been made to protect young calves and cows from *Salmonella* infection. Typically, an immunisation against *Salmonella* should prevent colonisation in the intestinal epithelium and prevent shedding of *Salmonella* and development of clinical and subclinical infection (Foster and Smith, 2009; Wray and Wray, 2000). Furthermore, an important criterion in the implementation of monitoring programmes is that antibodies produced against the strain in the vaccine should be easily distinguishable from antibodies produced in natural infections. There are three common *Salmonella* vaccines: killed *Salmonella* vaccines (bacterin), bacterial subunit, and modified-live *Salmonella* vaccines, the most commercially available being bacterins. Reports show significant variation in the efficacy of vaccine bacterin *Salmonella* preparations (Curtiss et al., 1993; House et al., 2001; Wray and Wray, 2000). According to a trial conducted by Aitken et al. (1982), the

administration of inactivated *S.* Dublin vaccine to cattle was effective when the cows were challenged by the intravenous route. Another study reported by Evans (1996) showed that the use of *S.* Typhimurium vaccine resulted in rapid reduction of *Salmonella* excretion in dairy herds infected with *S.* Typhimurium, compared to prolonged excretion detected in non-vaccinated herds. This vaccine is also capable of eliciting antibodies in pregnant cows, which can be transferred to neonatal calves in colostrum (Jones et al., 1988). From the other side, *S.* Typhimurium coated with alkali-hydrolysed lipopolysaccharide (LPS) was ineffective against challenge with this serotype (Anderson et al., 1991). Furthermore, direct vaccination of calves against *Salmonella* infection has been repeatedly reported (Segall and Lindberg, 1993; Smith et al., 1993; Smith et al., 1984). However, since calves could be infected with *Salmonella* within the first days of age, passive protection by dam immunisation is usually the preferred approach (Wray and Wray, 2000).

2.3.2.5. Other potential agents of infectious NCD

Other enteric pathogens have been described as potential causes of diarrhoea in young calves, but their aetiologic role and epidemiological importance remain poorly understood. *Clostridium perfringens* (*Cl. perfringens*) is considered pathogenic for humans and animals. The virulence of *Cl. perfringens* is determined by its ability to produce toxins, and 5 strains (A, B, C, D and E) have been documented according to the production of 5 lethal toxins named alpha, beta, epsilon, iota and enterotoxin (Rood, 1998). In young calves, haemorrhagic enteritis has been associated with type A strains of *Cl. perfringens* (Baums et al., 2004). Although *Campylobacter* spp. is reported to be more prevalent as a potential cause of enteritis in older calves, these bacteria have been also identified in the faeces of healthy, as well as diarrhoeic, newborn calves (Grinberg et al., 2005; Snodgrass et al., 1986). Finally, *Bacteroides fragilis,* which is an obligate anaerobe bacterium colonising the intestinal tract of normal animals and humans, has been reported in the faeces of diarrhoeic young calves (Border et al., 1985).

Among viruses, Haschek et al. (2006) described the occurrence of Bovine Torovirus (BoTV) in the faeces of diarrhoeic young calves in Austria, and Calicivirus-like agents (*Norovirus* and *Sapovirus*) were detected in 25% of diarrhoea outbreaks in young calves in southern England (Reynolds et al., 1986). Also, bovine viral

diarrhoea (BVD) virus, which is commonly linked with disease in older animals, has occasionally been described as a cause of endemic neonatal calf diarrhoea (Campbell, 2004). Other viruses, such as Astrovirus, Parvovirus and enteroviruses have also been detected from cases of diarrhoeic young calves, but are generally assumed to cause mild infection under normal conditions (Durham et al., 1989). Of particular interest in recent years was the isolation of Schmallenberg virus (SBV). This virus emerged in Europe in 2011 as a novel, economically significant virus in ruminants (Doceul et al., 2013). SBV belongs to the *Orthobunyavirus* genus of family Bunyaviridae. It was first identified in Germany, then in the Netherlands, Belgium, the United Kingdom and France (Doceul et al., 2013). Clinical signs are more obvious in adult livestock, and include severe diarrhoea, loss of appetite and hyperthermia. Furthermore, newborn calves may be affected with severe neurological illnesses that may lead to death (Conraths et al., 2013; Garigliany et al., 2012).

2.4. Prevention, treatment and control of NCD

The activities of NCD control aim at infection prevention and/or reduction of pathogen loads in the farm environment (Radostits, 1975), and focus on implementing appropriate levels of herd biosecurity. This includes measures for the prevention of infection such as, hand washing after handling each animal, boot disinfection and the use of quarantine pens for temporary isolation of new animals. Calf barns should be designed for easy cleaning and disinfecting with sufficient drainage systems and pens should be kept clean, dry, well ventilated and with adequate bedding, in order to reduce pathogen loads. The grouping of calves of different ages in the same pen, and overcrowding of pens should be avoided (Morgan, 1990; Perez et al., 1990). Another line of defence is to enhance calf immunity through optimal colostrum management and vaccination of mothers (Lorenz et al., 2011b).

In spite of the availability of specific interventions, for example a range of chemotherapeutic compounds for the treatment and prevention of cryptosporidiosis such as paromomycin, HL, and nitazoxanide (Fayer and Xiao, 2008; Grinberg et al., 2002; Jarvie et al., 2005), the pharmacological management of this infection remains difficult (see Chapter 3). Farm management practices for the control of *Cryptosporidium* infection have principally focused on sound colostrum feeding,

cleanliness and disinfection and early detection of diarrhoea and initiation of fluid therapy (Harp and Goff, 1998). Sometimes, broad spectrum antibiotics like oxytetracycline and neomycin are used for the treatment of calf diarrhoea if K99 or *Salmonella* spp. are involved in the diarrhoea (Vermunt, 1994). However, there is debate about the usefulness of antimicrobials for the treatment of NCD of bacterial cause in livestock, due to controversial issues around the development of antimicrobial resistance. Bacteremia is a well-known feature of *Salmonella* infection in young calves and it has been recommended that affected calves are treated with antimicrobials as soon as possible upon detection of infection (Constable, 2004).

Although there is a wide range of antimicrobials which may appear appropriate for salmonellosis, several, such as penicillin, erythromycin, and tylosin, are not recommended for use in calves because of the occurrence of resistance (Mohler et al., 2009). The occurrence of antimicrobial resistance in virulent *Salmonella* serotypes is common (Fecteau et al., 2003). Therefore, it is recommended that antimicrobial use is based on the *in vitro* susceptibility of the clinical isolate. However, broad-spectrum antimicrobials are usually used until the antibiogram is available. *Salmonella* spp. displayed varied responses with antimicrobials such as neomycin, florfenicol, ampicillin, amoxicillin, ceftiofur, amoxi-clavulonic acid, tetracycline, sulphonamides, and trimethoprim-sulfa (Noguerado et al., 1995; Groothuis et al., 1987). In a comparative experimental study, Groothuis et al. (1987) evaluated the efficacy of amoxycillin, chloramphenicol and trimethoprim on *Salmonella* infected calves at doses based on minimal inhibitory concentration, showing equal efficacy of the three drugs. Another study conducted by Fecteau et al. (2003) in which calves were orally challenged with *S.* Typhimurium followed with ceftiofur treatment (5 mg/kg, IM) showed a substantial reduction in rectal temperature, diarrhoea and faecal shedding of *Salmonella* in treated, in comparison with non-medicated calves. On the other hand, a limited effect following antimicrobial treatment was reported in other studies (Noguerado et al., 1995; Osborne et al., 1978). Overall, judicious use of antimicrobials with the narrowest spectrum of antibiotic is recommended on early infection diagnosis, based on susceptibility testing of *Salmonella* serotypes (Mohler et al., 2009; Raymond et al., 2006).

A significant factor in achieving immunity and resistance against specific infectious agents is the vaccination of pregnant cows against the major infectious agents in

order to boost specific colostral antibody titres (Bellinzoni et al., 1989; Gonzalez et al., 2010; Nagy, 1980; Saif and Smith, 1985; Snodgrass et al., 1982; Theil and McCloskey, 1995). Polyvalent vaccines for BRV, BCV and K99 given between 2 and 3 weeks before calving are available (Rotavec- Corona, http://www.msd-animal-health.co.nz/products/ rotaveccorona_ /010_ productview. Aspx; ScourGuard, http:/ / scourguard.co. nz/ farmers.html). For *Salmonella* infections, it has been reported that passive protection of young calves can be achieved by feeding colostrum from vaccinated dams. However, presently available vaccines give limited protection (Mohler et al., 2009). As previously stated, there is also no vaccination available for *Cryptosporidium* infection.

2.5. Concluding remarks and the status of NCD in New Zealand

NCD is one of the most important causes of calf morbidity and mortality worldwide. In order to increase the productivity per livestock unit, it is important to identify the etiological and risk factors involved in NCD and to develop intervention programs that reduce exposure to such factors. Although New Zealand is a major dairy producer, national data on the prevalence of enteropathogens of calves are scarce. As indicated in the sections above, epidemiological investigations have been conducted in some regions. With the exception of these small scale studies (Learmonth et al., 2003; Grinberg et al., 2005), most published studies analysed faecal specimens submitted to diagnostic laboratories (Osborne and Ensor, 1954; Burgess and Simpson, 1976; Schroeder et al., 1983; Belton, 1995; Howe et al., 2008). Extrapolating prevalence data from one country to another is problematic, due to the different environments and calf rearing systems. For instance, in New Zealand, most farms manage short and concentrated winter calving seasons, usually between July and October, which might influence the distribution of the infectious agents compared to countries managing longer calving seasons in which high numbers of susceptible calves are present year-round and the pathogens can build up in the farm environment for long periods of time. Chapter 4 reports a nation-wide prevalence study of enteropathogens that fills the gap in knowledge in this area.

Conventional wisdom assumes that the severity of NCD on farms is determined by complex interactions between enteropathogens and the environmental and husbandry factors. Thus, risk factors for NCD should ideally be analysed using multivariable

approaches. Results of NCD risk factor studies are difficult to generalise across countries, due to the significant differences, which exist among environments, hosts, and pathogen populations, and to the author's knowledge, no risk factor studies of NCD have been published in New Zealand. Chapter 5 reports a nation-wide multivariable epidemiological study of NCD performed on 97 dairy farms. Molecular epidemiological studies of *Cryptosporidium* parasites cycling in cattle have been performed in several countries (Brook et al., 2009; Budu-Amoako et al., 2012; Chen and Huang, 2012). Small-scale studies have also been performed in New Zealand (Abeywardena et al., 2012; Grinberg et al., 2008b; Learmonth et al., 2001; Learmonth et al., 2003). However, to the author's knowledge no large-scale studies characterising these parasites at a national level and comparing them to human isolates have been published. Chapter 6 describes a molecular analysis of *Cryptosporidium* parasites isolated in dairy farms located in seven New Zealand regions.

This PhD project provides a substantial amount of new data on the epidemiology of NCD in New Zealand, some of which is also directly applicable overseas.

Chapter 3

3. UTILITY OF HALOFUGINONE LACTATE FOR THE PREVENTION OF NATURAL CRYPTOSPORIDIOSIS OF CALVES, IN THE PRESENCE OF CO-INFECTION WITH ROTAVIRUS AND *SALMONELLA* TYPHIMURIUM

3.1. Abstract

Halofuginone lactate is registered in several countries for the prevention of calf cryptosporidiosis, but the compound's utility in the presence of co-infection with other enteropathogens is not well understood. We performed a randomised controlled field trial of the efficacy of HL for the prevention of natural calf cryptosporidiosis, in the presence of co-infection with BRV and *S.* Typhimurium. Newborn calves on one farm were sequentially enrolled and allocated to a full dose group (n = 15), a half dose group (n = 15), or a placebo control group (n = 15), using a randomised block design. The *Cryptosporidium* oocysts in faecal specimens collected on Days 6, 8, 10, 14 and 20 were counted and the severity of the diarrhoea was assessed using faecal consistency scores (solid, semisolid, or liquid). The oocyst numbers and faecal consistency scores were statistically compared between the groups. Ninety-one percent of the calves shed *C. parvum* oocysts during the trial. The full dose group had a longer prepatent period than the control group, but no statistical difference in the number of oocysts was identified between the groups after controlling for the effects of sex and breed. The faecal consistency scores and mortality rates did not differ between the groups. These results indicated that the anti-*Cryptosporidium* activity and clinical benefit of HL were limited. It is concluded that in order to maximise the clinical efficacy of HL in the field, diagnostic efforts should aim to rule out the presence of other enteropathogens.

3.2. Introduction

The cosmopolitan protozoan parasite *C. parvum* infects humans and a range of other hosts, and is a common agent of calf diarrhoea during the first four weeks of life (Gulliksen et al., 2009a; Uhde et al., 2008; Millemann, 2009; Trotz-Williams et al., 2005). The spectrum of severity of cryptosporidiosis in calves varies from subclinical

infection to severe diarrhoea and dehydration (Fayer et al., 1998; Klein et al., 2008; O'Handley et al., 1999). Typically, natural and experimental infections with *C. parvum* in calves have a pre-patent period of 3–6 days, followed by a patent period characterised by a bell-shaped oocyst excretion curve, with the number of faecal oocysts peaking and then rapidly decreasing to undetectable levels in a matter of 7–14 days (Fayer et al., 1998; Grinberg et al., 2002). Oocyst numbers as high as 10^7oocysts per gram of faeces have been reported at the peak of excretion (Fayer et al., 1998; Grinberg et al., 2002). Whereas infection rates as high as 100% have been reported in calves in the first month of life, a lower infection prevalence has been consistently observed in weaned animals (Bartels et al., 2010; Brook et al., 2008; Fayer et al., 2000b; Fayer and Xiao, 2008; Garber et al., 1994).

Newborn calves are therefore considered major amplifiers of potentially zoonotic *C. parvum* in nature, and the prevention of calf cryptosporidiosis is relevant from both animal and human health perspectives (Grinberg et al., 2008b; Hunter and Thompson, 2005; Kiang et al., 2006; Smith et al., 2007; Xiao and Feng, 2008; Zhou et al., 2008). Several features of the *C. parvum* life cycle make the control of cryptosporidiosis on-farms extremely difficult to achieve by means of hygienic measures alone. Calves can excrete hundreds of millions of oocysts (Grinberg et al., 2002; Naciri et al., 1999), and considering that there is a high probability of infection with a dose as low as 50 oocysts (Moore et al., 2003), an infected calf could produce enough oocysts to infect thousands of new animals. Attempts to interrupt the transmission of *C. parvum* should therefore include the immediate isolation of every infected animal, but this is rarely feasible on commercial farms. In addition, the *C. parvum* oocysts are insensitive to the action of numerous disinfectants (Chen et al., 2002; Quilez et al., 2005) and are excreted sporulated and fully infectious (Smith et al., 2005), requiring daily cleaning for their removal. Therefore, in the absence of effective immunising agents, pharmacological control strategies remain central to the prevention of cryptosporidiosis in calves.

A number of compounds, such as HL (De Waele et al., 2010; Jarvie et al., 2005; Klein, 2008; Lefay et al., 2001; Trotz-Williams et al., 2011), paromomycin sulphate (Fayer and Ellis, 1993; Grinberg et al., 2002), nitazoxanide (Ollivett et al., 2009; Schnyder et al., 2009) and decoquinate (Lallemond et al., 2006; Moore et al., 2003) have been tested for the prevention of cryptosporidiosis in calves, with variable

results. HL is a synthetic derivative of a quinazolinone alkaloid with cryptosporidiostatic activity, but it has a mode of action that is poorly characterised. In naturally and experimentally infected calves, the oral administration of 60 μg/kg HL for seven consecutive days from the first day of life delays the onset of oocyst shedding, reduces the number of oocysts excreted, and lowers the severity of diarrhoea (Jarvie et al., 2005; Joachim et al., 2003; Klein, 2008; Lefay et al., 2001; Trotz-Williams et al., 2011; Villacorta et al., 1991).

A formulation containing HL (Halocur, Intervet Ltd., Republic of Ireland) is currently the only prescription drug registered for the prevention of cryptosporidiosis of calves in several countries. The use of HL has a number of limitations, including a substantial market price and a narrow therapeutic index, with toxicity observable at approximately twice the recommended dose (Villacorta et al., 1991, Naciri et al., 1993 and Trotz-Williams et al., 2005; http://www.msd-animal-health. co.uk/ products_ public/ halocur/ 090_product_ datasheet.aspx, accessed 15 July 2012). Remarkably, notwithstanding the frequent occurrence of co-infections with *Cryptosporidium* and other enteropathogens in the field (De la Fuente et al., 1999; Naciri et al., 1999; Tzipori et al., 1980), the utility of HL in the presence of such co-infections is not well understood. Indeed, with some exceptions (Klein, 2008 and Lefay et al., 2001), most anti-*Cryptosporidium* efficacy studies of HL did not analyse or take into account the presence of co-infections. Co-infections might modify the anti-*Cryptosporidium* effect of HL in different ways. The increased fluid content and intestinal motility determined by the presence of co-infecting pathogens may reduce the activity of HL by dilution, or by reducing the transit time of the drug in the intestinal tract. Furthermore, enteric infection with rotavirus or other agents may cause exfoliation of infected cells, altering intestinal cellular function (Ramig, 2004), and potentially increasing the toxicity of HL via systemic absorption.

Motivated by the need for more data on the utility of HL in the presence of co-infections with other pathogens, we performed a randomised controlled field trial of the anti-*Cryptosporidium* preventive efficacy of the compound in calves on a New Zealand farm enzootically infected with *C. parvum*, bovine rotavirus and *Salmonella* Typhimurium.

3.3. Materials and methods

3.3.1. The farm

The study was performed between July and October 2010, on a farm situated in the Taranaki District, New Zealand. Consent for this study was obtained from the farmer and the use of animals was approved by the Animal Ethics Committee, Massey University. The farm managed split-calving herd (mainly spring calving with approximately 40% calvings in autumn) of approximately 400 milking cows and a smaller herd of beef cattle. It was recruited following a post-mortem investigation performed in March 2010 on two calves, which indicated the presence of *Cryptosporidium* oocysts in the faeces of two calves. *Salmonella* was ruled out by culture, and BRV and BCV by antigen ELISA. Sequence analysis of the *Cryptosporidium* 18S rRNA gene subsequently confirmed *C. parvum* in both animals. Notwithstanding the microbiological results performed in March, bovine rotavirus and *Salmonella* spp. were subsequently identified in multiple enrolled calves during the study (see below).

3.3.2. Study design and sample size calculation

The study was a randomised controlled field trial (see randomisation procedure below). A commercial product (Halocur, Intervet Ltd., Republic of Ireland), registered in New Zealand for the prevention and treatment of cryptosporidiosis of calves, was used to test the efficacy of HL. The product's recommended preventive dose is 4 mg (8 ml) for calves weighing 35–45 kg, and 6 mg (12 ml) for calves weighing 45–60 kg, for seven consecutive days, from birth. The initial aim was to assess the preventive efficacy of half the recommended dose of HL as compared with the full dose and with no treatment, and the following treatment groups were established: Group 1 (full dose regime; $n = 15$), calves treated orally with 8 ml Halocur; Group 2 (half dose regime; $n = 15$), calves treated with 4 ml Halocur; Group 3 (placebo control group; $n = 15$), calves treated with 4 ml water delivered using the product's dispenser. The group sizes were estimated using power analysis for the detection of a difference between means, using PASS software (NCSS, Kaysville, UT). Assumptions for the calculations were a $\log_{10}$-transformed mean number of oocysts per gram of faeces of 4.3 for the full dose and 6.2 for the untreated-control group at the peak of shedding, and a common standard deviation of 1.2. These means

were estimated from a previously published study of efficacy of paromomycin sulphate (Grinberg et al., 2002). Group sizes of eight calves per group achieved 84% power to reject the null hypothesis that both group means are 6.2. Thus, assuming a conservative infection rate of ~50%, 15 calves per group were required.

3.3.3. Animal husbandry

Management of the enrolled calves followed the same routine procedures used on the farm. Briefly, newborn calves were left on the calving paddock for 10–24 hours after birth, as commonly done in New Zealand pasture-based farms to allow for colostrum intake directly from the dam. Then the newborns were transferred to a large shed and allocated to a pen for newborn calves, with a capacity of about 10 calves. Calves were left in this pen for 2–3 days and then transferred to a new pen, in order to create space for new calves in the first pen. Subsequently, the calves progressed to new pens (often adjacent) containing 5–10 animals of the same age group every 2–3 days, until weaning. The allocation of calves to pens was done by caretakers who were not aware of the nature of the treatments given to each calf, effectively creating a commingled pen design.

Adjacent pens, with sawdust as bedding material, were separated by slatted wooden fences. The feeding regimen included the administration of complete commercial milk replacement for the first two weeks using a tank with multiple nipples, followed by feeding *ad libitum* using automatic feeders until weaning. Roughage was available from the second week of life. No routine vaccinations, preventive treatments or supplementations were administered to the calves during the study. However, inappetent calves could be tube-fed and severely diarrheic calves treated with oral electrolytes and/or other treatments by the caretakers. Severely sick calves were transferred to an isolation shed. The study protocol stipulated that the tube -fed calves could continue in the study, but animals treated by the farmer by other means or transferred to the isolation pen, were removed.

3.3.4. Field workflow, randomisation and sampling

Newborn calves were sequentially enrolled in the mornings, at arrival from the calving area at the rearing shed. Any calf born on the farm was eligible for enrolment, provided no congenital or pathological conditions were identified. At the day of enrolment (Day 0), calves were identified by the ear or neck tag number, given

a sequential number and allocated to one of the three treatment groups. Allocation of calves to treatment groups was done using a randomised block design, with blocks of three (one calf per group), using a random list prepared in advance. The fourth calf presented to the investigator after each block of three was systematically left unenrolled and continued its normal life cycle on the farm. Treatments were administered in the morning using the commercial product (see above). Faecal specimens were collected at the time of treatment from the rectum of each calf using disposable gloves, on Days 6, 8, 10, 14 and 20. At the time of collection, each specimen was scored according to its consistency as 1, solid (specimen conserved its original shape); 2, semi-solid (specimen spread across the bottom of the container but was not liquid); or 3, liquid specimen. Specimens were transported on ice to Massey University and kept in refrigeration and analysed between December 2010 and April 2011, as described below.

3.3.5. Laboratory analysis for *Cryptosporidium*

The faecal specimens were analysed by a quantitative method that estimated the number of *Cryptosporidium* oocysts present. Briefly, after mixing the specimen with a spatula, 1 g of faeces was suspended in 10 ml tap water and strained through a tea sieve. The filtrate was centrifuged at 900 × g for 10 minutes and the sediment re-suspended in 4 ml of normal saline. A 10 µl aliquot of this suspension was deposited as a drop on a slide using a micropipette, air-dried and fixed in methanol. Fixed drops were stained using a commercial immunofluoresecent anti-*Giardia* and *Cryptosporidium* monoclonal antibody (Aqua-Glo G/C Direct Comprehensive Kit, Waterborne Inc., New Orleans, USA), according to the manufacturer's instructions. The apple-green fluorescent oocysts present on the entire area of the stained drop were counted using a fluorescent microscope using an excitation wavelength of 490 nm and a 200× magnification lens. This total number of oocysts (TON) on the slide was used for statistical analysis (see below). Samples containing >1000 oocysts were difficult to count and were re-processed by a further dilution of the faecal suspension at 10^{-1} in water, followed by the staining of a 10 µl drop as above. The TON present on each re-diluted specimen was estimated by multiplying the result by 10. When the available faecal material was insufficient for counting, a direct faecal smear was stained and a qualitative result (presence/absence of oocysts) was obtained. These qualitative results could not be used for the statistical

comparison of the number of oocysts between the treatment groups, but were used for any comparison between proportions of *Cryptosporidium*-positive and negative specimens.

In March 2012, DNA from three *Cryptosporidium*-positive specimens from each treatment group was extracted from the stored faeces using a DNA extraction kit (QIAamp, DNA Stool Mini Kit, Qiagen, Hilden, GmbH), and *Cryptosporidium* parasites were identified by means of PCR-sequencing a ~ 825 bp of the 18S rRNA gene. Primers were 5-GTT AAA CTG CGA ATG GCT CA-3 (forward) and 5-CCA TTT CCT TCG AAA CAG GA-3 (reverse) (Learmonth et al., 2004). Amplification was performed in 20 µl containing 2 µl 10 x PCR buffer, 1 µl dNTP (2 mM), 1 µl $MgCl_2$ (50 mM), 2 µl non-acetylated bovine serum albumin (2 mg/ml) (New England Biolabs, USA), 4 picomoles of each primer, and 0.5 µl of Platinum® Taq DNA Polymerase (2 mg/mL) (Invitrogen Corporation, Carlsbad CA, USA). The PCR amplification was carried out in a thermocycler (SensoQuest, Goettingen, Germany) with initial denaturation at 96 °C for 2 min, followed by 40 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. PCR products were purified using an in–house ethanol purification protocol, and bidirectional sequencing of an internal segment of the amplicon was performed using primers 5-CTCGACTTTATGGAAGGGTTG-3 (forward) and 5-CCT CCAATCTCTAGTTG GCATA-3 (reverse). Forward and reverse sequences were aligned and edited manually using Geneious software version 5.6.5 (Biomatters Ltd., http://www. geneious. com/). Distal and proximal segments that could not be verified were trimmed and the resulting edited sequences aligned with sequences deposited in Genbank using the alignment algorithm BLAST (http://blast.ncbi. nlm.nih.gov.ezproxy. massey.ac.nz/Blast.cgi, accessed on April 2012).

In addition, 23 specimens were blindly collected from the samples' box by haphazard sampling (without systematic randomization) and analysed for BRV, BCV, K99 and *Salmonella* spp (Table 3.2). The analysis for BRV, BCV and K99 was performed by a commercial diagnostic laboratory using antigen-ELISA (Institut Pourquier, Montpellier, France). The analysis for *Salmonella* included parallel inoculation of faecal material into tetrathionate and Rappaport-Vassiliadis soy peptone (RVS) broths, and incubation for 24 hours at 37 °C and 42 °C, respectively. This was followed by subculture onto xylose lactose deoxycholate agar plates incubated as

above. Colonies consistent with *Salmonella* were sub-cultured into triple-sugar iron agar slopes (TSI) and L-lysine decarboxylase broth. Lysine-positive bacteria exhibiting TSI patterns consistent with *Salmonella* were subjected to *Salmonella* poly-O slide agglutination using a commercial antiserum (Institut für Immunpräparate und Nährmedien GmbH Berlin, Berlin, Germany), and agglutinating isolates were sent to the *Salmonella* Reference Laboratory (Institute of Environmental Science and Research, Porirua, New Zealand) for serotyping.

3.3.6. Statistical analysis of data

The parasitological and clinical effects of the three treatments were statistically compared. The parasitological effects were analysed by comparing the TONs between the groups using an analysis of variance for repeated measurements (rmANOVA) implemented by the PROC MIXED procedure of SAS (Statistical Analysis System, 2001, SAS Institute, Cary, NC, USA). TONs were log-transformed as $\log_{10}$ (TON + 1) and analysed using mixed models. The first model (Model 1) included the fixed effects of treatment (variable 'treatment Group'), the day of sampling as repeated factor (variable 'sampling Day'), and the interaction of treatment Group and sampling Day. Model 2 included the fixed effects as in Model 1 plus the fixed effect of sex of the calves, and Model 3 included the same factors as in Model 2 plus the effect of breed of the calves as a co-variable (variable 'Breed'; Friesian calf = 1; non-Friesian calf = 0). Fixed effects for variable 'Breed' were not included as a class due to unbalanced designs deriving from the uneven distribution of breeds across treatments. Finally, Model 4 was similar to Model 2, but did not include the data obtained from non-Freisian calves, effectively removing any variability due to the breed. All the models included the random effect of calf, to account for the within-calf variability. Using the Akaike's information criterion (Muller et al., 2013), an unstructured error structure was determined as the most appropriate residual covariance structure for repeated measures over time, within animals. Finally, the parasitological efficacy was also assessed by comparing the decline of the proportion of parasitologically negative calves as a function of time between the three groups using the non-parametric Kaplan–Meier method (KM) (Kaplan and Meier, 1958). Also in this case, the analysis was repeated after omitting all the data from the non-Friesian calves. The KM analysis was implemented using a

code available in “R” (Terry Therneau, 2012; A Package for Survival Analysis in R package version 2.36-14).

The clinical effects of the treatments were assessed by comparing the faecal consistency scores (considered ordinal data) between the groups on each sampling day, using the non-parametric Kruskal–Wallis test. These tests were performed using the codes available in “R” (Kruskal–Wallis: http://stat.ethz.ch/R-manual/R-patched/library/ stats/html/kruskal.test.html; Wilcoxon: http://stat. ethz.ch/R-manual/R-patched/ library/stats/html/wilcoxon.test.html). Comparisons between proportions of interest were performed using two-tailed Fisher's exact tests, which were interpreted using Bonferroni-adjusted critical P-values for multiple testing. Finally, the existence of association between the faecal consistency and the number of oocysts shed was tested by means of logistic regression, using the $\log_{10}$ (TON + 1) as independent variable and the presence/absence of a liquid specimen (faecal score 3) as outcome variable. This analysis was performed using a code available in “R” (http://www.ats. ucla.edu/ stat/r/ dae/logit.htm; R package version 2.36–14).

3.4. Results

3.4.1. Animals’ characteristics and adherence to the protocol

All the calves were enrolled within 24 hours from birth, over a 26-day period, between 16 July and 06 August 2010. A total of 24/45 (53.3%) calves were females and there were no statistical differences between the proportion of males and females among the treatment groups. There were 27 (60%) Friesian, 11 (25%) Angus, 6 (11%) Hereford calves and one (2%) Jersey × Angus crossbred calf (Table 3.1). Six calves haphazardly selected at enrolment and weighed using electronic scales had body weights between 35 and 40 kg (mean = 36.5, standard deviation = 1), indicating that under/over-dosing of calves was unlikely to have occurred. One calf that died on the day of enrolment (calf number 1; Table 3.1) was substituted by the subsequent calf presented to the investigator (calf number 46; Table 3.1). All the enrolled calves remained in their pens during the study and no calf was withdrawn due to concomitant treatment. Thirty- eight calves (84.5%) were followed up for the entire observation period, and seven (15.5%) died at different stages during the study. The causes of death were defined by the farmer as following: two calves from Group 1 died on Days 6 and 7, two from Group 3 on Days 7 and 9, and one from Group 2 on

Day 13, from severe diarrhoea. Two calves (Group 1 and Group 3) died from improper tube-feeding (possibly milk inhalation) on Day 7 and Day 13. There were 3 deaths in Group 1, three in Group 3 and one in Group 2, and these rates were not statistically different (Table 3.1). Only 6/225 (2.6%) faecal specimens could not be retrieved and were not analysed (Table 3.1).

3.4.2. Laboratory results

A total of 41/45 (91%) calves were *Cryptosporidium*-positive by immunofluorescence at some stage during the study. Two calves belonging to Group 2, which were able to be followed up for the entire observation period, remained parasitologically negative throughout the study (Table 3.1). Sequence analysis of the 18SrRNA gene of the 9 faecal specimens analysed by PCR-sequencing indicated the presence of *C. parvum* in all cases (matched the reference sequence in GenBank accession number: HQ009805.1) (Appendix X). Out of 23 specimens analysed for other enteropathogens, 21 (91%) were positive for BRV detected in the three treatment groups and 3 (13%) specimens were positive for *S.* Typhimurium (detected in Group 1 and 2). No BCV and K99-positive specimens were identified (Table 3.2). Although analysis for *Giardia* spp. was not the subject of this study (as the parasite is not widely considered pathogenic for calves), we note for completeness that *Giardia* cysts were observed in 20 calves by immunofluorescence.

3.4.3. Statistical results

3.4.3.1. Parasitological efficacy

The calves in Group 3 (control Group) showed an oocyst- shedding curve which was typical for natural cryptosporidiosis, with the majority of the animals becoming parasitologically positive by Day 8 and again negative by Day 20 (Table 3.1 and Table 3.3). Group 3 started shedding earlier than the other two groups. In this group, the highest mean $\log_{10}$ (TON + 1) was observed on Day 10, and on Day 20, oocysts were observed only in two calves (Table 3.1). In contrast, in Group 1 the peak mean $\log_{10}$ (TON + 1) was lower and occurred later (Day 14) than in Group 3, and on Day 20, the calves in this group were shedding more oocysts than the other two groups. However, when only Friesian calves were considered, Groups 1 and 3 showed a very similar oocyst- shedding curve, which peaked in both groups on Day

10. Comparisons between the crude mean $\log_{10}$ (TON + 1) of the three treatment groups at the various sampling days are reported in Figure 3.1.

Model 1 (which included the effects of treatment Group, sampling Day and their interaction term), indicated a significant difference between the three treatments (P = 0.04). Post hoc comparisons identified a significant difference between Group 1 (full dose) and Group 3 (control) (Group 1 < $\log_{10}$ (TON + 1) than Group 3; P = 0.01), and no significant difference between Group 2 and the other two groups. In Model 2, which included also the fixed effect of sex, the significance between the treatment Groups was preserved (P = 0.02), but there was no significant effect of sex on the outcome (P = 0.17). When variable 'Breed' was introduced as a covariate in Model 3, the P-value of the effect of treatment increased to P = 0.098, and the covariable 'Breed' was also significant (Friesian < $\log_{10}$ (TON + 1) than non-Friesian; P = 0.02). This increase in the P-value was supported by Model 4 (Friesian only), which showed P = 0.73 for the effect of treatment. All these models (Appendix I) produced similar P-values when the interaction of treatment × day was removed. In order to cross-validate these results we compared the mean $\log_{10}$ (TON + 1) between the treatment groups using bivariate ANOVA for each sampling day separately (not shown), with consistent results, whereas the inclusion of all the animals resulted in statistically significant differences between treatment Groups 1 and 3 on Days 8, 10 and 20 (P < 0.05), all the P-values were > 0.05 when only Friesian calves were analysed.

The results of the Kaplan–Meier test indicated a significantly longer prepatent period in Group 1 as compared with Group 3. In fact, 13 calves (93%) in Group 3 were parasitologically positive on Day 8, whereas only three calves from Group 1 (25%) were shedding oocysts on the same Day (Table 3.1 and Table 3.3). The duration of the prepatent period in Group 2 was intermediate, although not statistically different from the other groups. Similar results were observed when only data from Friesian calves were analysed (Figure 3.2). The results of the Fisher's exact tests showed that on Days 8 and 10, the proportion of *Cryptosporidium*-positive calves was significantly greater in Group 3 than in Group 1 (two-tailed Fisher's exact test P < Bonferroni-adjusted critical value of 0.003). On the other hand, most calves (58%) in Group 1 and only two calves in Group 2 and two in Group 3 were shedding oocysts on Day 20 (Table 3.3).

3.4.3.2. Clinical efficacy

Twenty three calves (51%) passed at least one liquid specimen in the course of the study, and the proportion of liquid specimens on Day 6 was relatively high, and very similar in the three treatment groups (Table 3.4). The results of Kruskal–Wallis and Wilcoxon tests test did not indicate any significant difference ($P > 0.05$) between the faecal consistency scores of the three groups on any sampling day (not shown). Except one significant difference between Group 2 and Group 3 on Day 8, the proportion of liquid specimens did not differ between the groups (Table 3.4), and these result persisted when only Friesian calves were included in the analysis (not shown). There was no association between the $\log_{10}$ (TON + 1) and the presence of liquid specimen by logistic regression ($P > 0.05$).

3.5. Discussion

HL is registered for the prevention of calf cryptosporidiosis in several countries, but the compound has a relatively narrow therapeutic index and a substantial market price. This study was initially designed to assess the efficacy of a reduced dosage regime of HL on a farm infected with *C. parvum*, with no evidence for the presence of co-infection. However, the identification of rotavirus and *S.* Typhimurium in multiple calves provided an opportunity to collect much needed data of the utility of the compound in the presence of such common co-infections.

When the study was designed, we predicted a longer prepatent period and a decreased number of oocysts and faecal consistency scores in the full dose group, as reported for HL in the absence of documented co-infections. The co-infections with rotavirus and *S.* Typhimurium did not affect the ability of HL (full dose) to delay the onset of shedding. Whereas most calves (5/12) in Group 1 were still shedding oocysts on Day 20, the majority of the calves in the control group were already parasitologically negative by that day (Table 3.3). The prolongation of the prepatent period was not coupled with statistically significant differences between the mean $\log_{10}$ (TON + 1) of the treatment Groups after controlling for the repeated measurements, the sex, and the breed of the calves (Models 3 and 4). We hypothesise that HL suppressed the parasite's life cycle during the first days of treatment, when the diarrhoea caused by the other pathogens was not yet overt, effectively prolonging the prepatent period in Group 1. Conversely, about 30% of the calves in Group 1 were passing liquid faeces

on Day 6, and this early diarrhoea (probably caused by the co-infecting pathogens) could have shortened the intestinal transit time of HL in the last days of treatment, effectively abolishing its anti-*Cryptosporidium* effect.

Some authors suggest that co-infections with multiple agents could cause a more severe diarrhoea than mono-infections (De la Fuente et al., 1999; García et al., 2000). Consequently, it could be hypothesised that suppression of one organism could reduce the severity of the diarrhoea. In this study, there was no significant difference between the faecal consistency scores of the treated and untreated groups, and no association between the TON and the presence of liquid faeces was found by logistic regression. Furthermore, in agreement with the results of a meta-analysis of the literature (Silverlås et al., 2009), the mortality rates did not differ between the groups. Therefore, the results do not allow conclusions to be drawn on a clinical benefit of HL in the presence of co-infections.

Infected calves are considered major amplifiers of *C. parvum* in nature. *Cryptosporidium* oocysts are very resistant and remain infective for months in cold weather and wet conditions (Smith et al., 2005). Therefore, any attempts to reduce oocysts shedding and to interrupt the transmission of *C. parvum* by pharmacological means are important also from a public health standpoint. The delay in oocyst shedding after the treatment with HL in the present study is thus very important.

Concerning prevention and control, *C. parvum* sporulated oocysts are insensitive to the action of numerous disinfectants (Chen et al., 2002; Quilez et al., 2005). Furthermore, currently no drugs, except HL are available for the pharmacological management of calf'cryptosporidiosis. Other drugs, such as paromomycin sulphate (Grinberg et al., 2002) and nitazoxanide (Ollivett et al., 2009) have been tested, with variable results, but are not registerd. It would be interesting from a clinical point of view to compare these drugs with HL in a single study.

As will be seen in Chapter 4, co-infection with two or more agents is present in a high percentage of dairy farms in New Zealand. In the current study, the anti-*Cryptosporidium* activity of HL was not fully preserved and the use of the drug was not associated with a clinical benefit in the presence of co-infection with BRV and *S.* Typhimurium in calves. Therefore, in New Zealand, diagnostic efforts should

aim to rule out the presence of other common enteropathogens in order to maximise the clinical efficacy of HL in the field.

The effect of breed on the intensity of oocyst shedding observed in this study showed that Friesian calves shed fewer oocysts than non-Friesian. This result was intriguing and requires further investigation. The author is not aware of any study of an anti-*Cryptosporidial* drug that showed different responses among breeds. However, epidemiological studies have previously reported lower *Cryptosporidium* infection prevalence in beef calves than in dairy calves (Geurden et al., 2006; Kváč et al., 2006), and in New Zealand the rate of infection was lower in Jersey cattle than in non-Jersey, although not statistically significant (Grinberg et al., 2005). Thus, cattle genetics may play a role in the susceptibility to *C. parvum* infection. As will be seen in the following chaters, however, this could not be corroborated in the prevalence study reported in Chapter 4.

3.6. Conclusions

The anti-*Cryptosporidium* activity of HL was not fully preserved and the use of the drug was not associated with a clinical benefit in the presence of enzootic co-infection with rotavirus and *S.* Typhimurium in calves. Diagnostic efforts should therefore aim to rule out the presence of other common enteropathogens in order to maximize the clinical efficacy of HL in the field.

Table 3. 1. Raw data from 46 calves enrolled in this study. F, female; M, male; D, dead calf – specimen not available; +I, sample positive for *Cryptosporidium* oocysts but faecal material insufficient for counting; MS, missing specimen (not analysed)

Enrolment sequence	Treat. Group	Gender/breed	Oocyst count (faecal consistency score)				
			Day 6	Day 8	Day 10	Day 14	Day 20
1	1	F/Friesian	D	D	D	D	D
2	2	F/Friesian	1(2)	22260(1)	41940(2)	12850(3)	0(1)
3	3	F/Hereford	0(3)	585(3)	44850(1)	256(1)	0(1)
4	1	M/Friesian	0(1)	0(1)	7(1)	146(1)	0(1)
5	3	M/Friesian	0(1)	652(1)	0(1)	635(1)	0(1)
6	2	M/Friesian	0(2)	I(1)	7260(2)	6870(2)	0(1)
7	2	M/Angus	0(1)	0(1)	0(1)	0(1)	0(1)
8	3	F/Hereford	0(1)	452(1)	D	D	D
9	1	F/Crossbreed	0(2)	0(2)	0(2)	856(1)	156(1)
10	2	M/Friesian	I(1)	0(1)	0(1)	573(2)	70(1)
11	1	M/Friesian	0(3)	I(1)	0(1)	224(1)	9(1)
12	3	F/Friesian	0(1)	487(3)	9660(3)	459(2)	0(1)
13	3	M/Friesian	MS(1)	610(2)	998(1)	29610(2)	154(2)
14	1	M/Angus	0(1)	0(2)	0(1)	0(1)	986(2)
15	2	M/Friesian	0(1)	19(2)	55(1)	488(3)	0(1)
16	3	F/Angus	0(1)	0(1)	16000(1)	I(1)	0(1)
17	2	M/Friesian	0(1)	0(1)	0(2)	0(1)	0(1)
18	1	F/Friesian	MS(1)	1(1)	0(1)	752(1)	4550(1)
19	1	M/Angus	0(1)	0(1)	0(2)	365(1)	9050(1)
20	2	M/Angus	0(1)	0(1)	12180(3)	D	D
21	3	F/Friesian	0(3)	478(3)	12850(2)	365(1)	I(1)
22	1	M/Angus	0(3)	0(1)	0(2)	9450(1)	879(2)
23	3	F/Friesian	0(1)	399(1)	41510(1)	24010(3)	0(1)
24	2	M/Hereford	I(1)	935(1)	44450(1)	37000(1)	0(1)
25	2	F/Angus	0(3)	0(2)	38950(1)	11070(3)	0(1)
26	3	M/Friesian	0(1)	303(3)	986(3)	D	D
27	1	F/Friesian	0(3)	0(2)	368(3)	8460(3)	0(3)
28	2	F/Angus	0(1)	0(1)	0(1)	7(3)	0(1)
29	1	F/Hereford	I	D	D	D	D
30	3	M/Friesian	MS	D	D	D	D
31	3	F/Friesian	0(3)	569(2)	997(3)	478(1)	0(1)
32	1	M/Angus	0(1)	0(1)	0(1)	154(2)	2(1)
33	2	M/Hereford	0(3)	0(2)	7670(2)	15820(2)	0(2)
34	3	F/Friesian	0(1)	390(1)	33560(1)	11920(1)	0(1)
35	2	M/Angus	0(3)	0(2)	8680(1)	I(1)	0(1)
36	1	F/Friesian	0(3)	D	D	D	D
37	1	F/Friesian	0(1)	125(3)	568(3)	I(3)	0(1)
38	2	F/Friesian	0(3)	0(2)	234(1)	18650(2)	0(2)
39	3	F/Friesian	0(1)	253(1)	10010(1)	478(1)	0(1)
40	1	F/Friesian	I(1)	D	D	D	D
41	3	F/Friesian	510(1)	7(3)	MS(3)	0(2)	0(2)
42	2	F/Friesian	26(1)	5810(3)	I(2)	11330(1)	0(1)
43	2	M/Friesian	MS(1)	6080(1)	I(1)	I(1)	1(1)
44	3	M/Friesian	245(3)	5(3)	989(2)	314(1)	0(1)
45	1	F/Angus	0(3)	5(2)	I(3)	MS(3)	0(2)
46	1	F/Hereford	0(1)	0(2)	115(1)	97(1)	0(2)

Table 3.2. Positives (+) and negatives (-) faecal specimens of calves from different groups for the presence of other enteropathogens.

Serial	Enrolment Sequence	Treatment group	Sampling day	Laboratory results			
				BRV	BCV	K99	*Salmonella* spp.
1.	7	2	6	+	-	-	-
2.	4	1	8	+	-	-	-
3.	10	2	10	+	-	-	+
4.	13	3	20	+	-	-	-
5.	9	1	14	+	-	-	-
6.	14	1	20	+	-	-	-
7.	31	3	10	+	-	-	-
8.	18	1	6	+	-	-	+
9.	3	3	6	+	-	-	-
10.	33	2	8	+	-	-	-
11.	27	1	8	+	-	-	-
12.	16	3	6	+	-	-	-
13.	38	2	20	+	-	-	-
14.	28	2	6	-	-	-	-
15.	23	3	14	+	-	-	-
16.	30	3	8	+	-	-	-
17.	43	2	14	-	-	-	-
18.	32	1	6	+	-	-	-
19.	37	1	14	+	-	-	+
20.	41	3	6	+	-	-	-
21.	24	2	10	+	-	-	-
22.	26	3	20	+	-	-	-
23.	45	1	10	+	-	-	-

Table 3. 3. The number of *Cryptosporidium*- negative and *Cryptosporidium*- positive calves in the three treatment groups of all breeds (left table) and of Friesian calves only (right table), on five sampling days (corresponding proportions of positive calves are in brackets). On the same day, comparisons between proportions of *Cryptosporidium*-positive calves of different treatment groups resulting in a two-tailed Fisher's exact test of $P < 0.01$ (critical probability adjusted for multiple testing), are indicated with similar superscripts.

	All calves			Friesian calves		
Sampling day	**Group 1 (full dose)**	**Group 2 (half dose)**	**Group 3 (placebo-control)**	**Group 1 (full dose)**	**Group 2 (half dose)**	**Group 3 (placebo-control)**
Day 6	13/0(0)	13/2(13)	12/2(14)	7/0(0)	6/2(25)	10/2(17)
Day 8	9/3(25)*	10/5(33)$^{+}$	1/13(93)*$^{+}$	5/2(29)	4/4(50)*	0/12(100)*
Day 10	7/5(42)*	4/11(73)	1/11(92)*	4/3(43)	2/6(75)	3/9(75)
Day 14	1/11(92)	2/12(86)	1/11(92)	2/5(71)	1/7(88)	3/9(75)
Day 20	5/7(58)*	12/2(14)*	10/2 (17)	5/2(29)	6/2(25)	10/2 (17)

Table 3.4. The number of liquid faeces (faecal score 3)/total specimens assessed; stratified by sampling days, treatment groups (the corresponding proportions are in brackets). On the same sampling day, an asterisk indicates a two-tailed Fisher's exact test $P < 0.01$ (Bonferroni-adjusted critical probability)

Sampling day	**Group 1 (full dose)**	**Group 2 (half dose)**	**Group 3 (placebo-control)**
Day 6	5/14 (0.36)	4/15 (0.27)	4/14 (0.28)
Day 8	1/12 (0.08)	1/15 (0.07)*	6/14 (0.43)*
Day 10	3/12 (0.25)	1/15 (0.07)	4/13 (0.31)
Day 14	3/12 (0.25)	4/14 (0.28)	1/12 (0.08)
Day 20	1/12 (0.08)	0/14 (0)	0/12 (0)

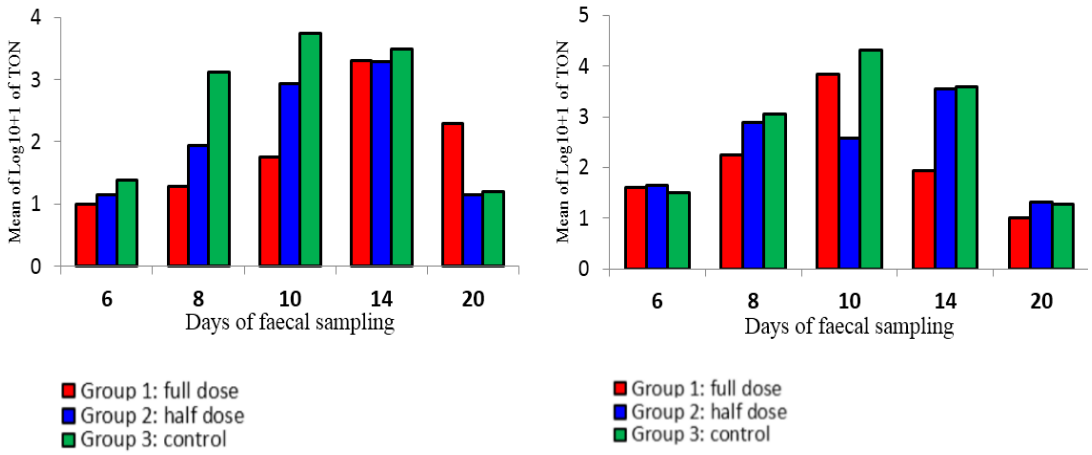

Figure 3.1. Mean Log10 of the total number of oocysts (TON+1) present on slides, stratified by treatment groups and sampling days in calves of all breeds (left graph) and of Friesian calves only (right).

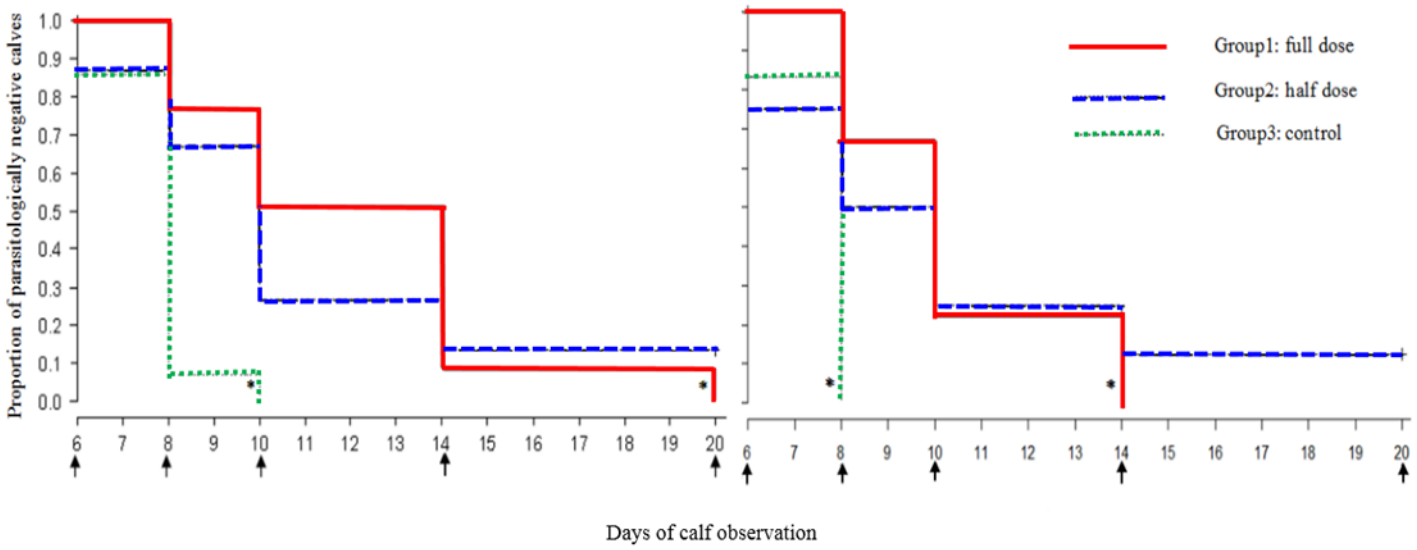

Figure 3.2. Reduction of the proportion of parasitologically negative calves (Y axis) as a function of time (X axis), in calves of all breeds (left) and in Friesian calves (right). The sampling days are indicated by arrows. Asterisks indicate a Kaplan Meier $P < 0.05$, to observe such a difference between the groups by chance alone.

MASSEY UNIVERSITY
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STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Julanda Al Mawly

Name/Title of Principal Supervisor: Dr. Alex Grinberg

Name of Published Research Output and full reference:

UTILITY OF HALOFUGINONE LACTATE FOR THE PREVENTION OF NATURAL CRYPTOSPORIDIOSIS OF CALVES, IN THE PRESENCE OF CO-INFECTION WITH ROTAVIRUS AND SALMONELLA TYPHIMURIUM.

Veterinary Parasitology 197, 59-67; 2013

In which Chapter is the Published Work: Chapter 3

Please indicate either:

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  The candidate was in the first year of the PhD program when this study was performed. He participated in study design, implemented the study (including all field and laboratory work and data analysis) and wrote the first draft of the paper. After considering the supervisors' suggestions, the candidate produced the subsequent versions and the final published paper. These contributions qualified him as first author of this publication.

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Chapter 4

4. PREVALENCE OF ENDEMIC ENTEROPATHOGENS OF CALVES IN NEW ZEALAND DAIRY FARMS

4.1. Preamble

The previous study was performed on a farm selected due to the presence of cryptosporidiosis and no laboratory evidence for the presence of other enteropathogens. However, when the study was conducted about four months later, a number of faecal specimens tested positive for *Cryptosporidium*, BRV, *Salmonella* spp. and *Giardia* spp. This prompted a reassessment of the broad objectives of the PhD project, to include studies of cryptosporidiosis and the other endemic enteropathogens of calves in New Zealand. To the author's knowledge, the study presented in this chapter represents the first national prevalence study of BRV, BCV, *C. parvum*, *Salmonella* spp. and K99 in New Zealand dairy farms. In many aspects (e.g. for *C. parvum*), this study is among the most complete in its field, worldwide.

4.2. Abstract

NCD is an economically-important condition affecting dairy farms worldwide. BRV, BCV, *Salmonella* spp., K99 and *C. parvum* are the most common infectious agents isolated from cases of NCD. The prevalences of these agents in New Zealand dairy farms are unknown. A total of 1283 faecal specimens collected during one calving season from 1-5 day-old calves and 9-21 day-old ones on a random sample of 97 dairy farms were analysed for the presence of these agents. Crude and standardised national farm-level prevalences were calculated and relationships between demographic variables and the presence of the enteropathogens were examined using multivariable logistic regression models. A total of 93/97 farms had at least one infected specimen. Among the 9-21 day-old calves, the standardised national prevalences of BRV, BCV and *C. parvum* were 57%, 30.5% and 52%, respectively. Among the 1-5 day-old calves, the farm-level prevalences for BRV, BCV, *C. parvum* and K99 were 46%, 13.7%, 17.5% and 11%. No associations between explanatory variables and the presence of enteropathogens were identified at the farm-level. At the calf level, the odds of *C. parvum* shedding, and of co-infection with any combination of pathogens were greater in 9-21 day-old, than in 1-5 day-old calves.

This study provides epidemiologically robust estimates of the national prevalence of calves' enteropathogens in dairy farms in New Zealand.

4.3. Introduction

Good calf-rearing practice forms an integral part of dairy farming due to the continuous demand for replacement animals for the milking herd. NCD, defined as diarrhoea occurring during the first month of life, is arguably the most common disease of newborn calves, worldwide. The condition has a negative impact on animal welfare and remains the main cause of mortality in many cattle-rearing countries (Virtala et al., 1996b; Svensson et al., 2003; Lorenz et al., 2011a; Pardon et al., 2012). The economic impact of NCD is due to the mortality and poor growth of the calves and the costs associated with its diagnosis and control (Uhde et al., 2008; Bartels et al., 2010; Izzo et al., 2011; Uetake, 2013).

BRV, BCV (Bartels et al., 2010; Hansa et al., 2012; Suresh et al., 2012), K99 (García et al., 2000; Uhde et al., 2008; Izzo et al., 2011), the protozoan *C. parvum* (Quilez et al., 2008; Bartels et al., 2010; Khan et al., 2010; Silverlås et al., 2010a), and *Salmonella enterica* subsp. *enterica* serovars (Wray et al., 1987; Bellinzoni et al., 1990; Tsolis et al., 1999; Vermunt, 2000) are the most commonly-reported endemic infectious agents of NCD in most cattle rearing countries. These pathogens can be found alone or in co-infections in both diarrhoeic and non-diarrhoeic calves, and the severity of the condition is widely assumed to be dependent on the interactions between the pathogens, the environmental conditions, and host-associated factors (Bazeley, 2003; Bartels et al., 2010; Smith, 2012). The pathogens induce similar clinical signs and, usually, the aetiological diagnosis of NCD can be achieved only by the laboratory identification of the agents in the faeces (Tzipori, 1981; Luginbühl et al., 2005; Millemann, 2009; Smith, 2012).

Assessment of the prevalence of the infectious agents of NCD is necessary for understanding the multifactorial aetiology of NCD and optimising the use of vaccines, passive immunoprophylatics and chemotherapeutics. Moreover, *Salmonella* spp. and *C. parvum* have zoonotic potential (Hunter and Thompson, 2005; Kiang et al., 2006; Xiao and Feng, 2008; Zhou et al., 2008), and assessment of these organisms' prevalence is important also from a public health perspective. Studies investigating the occurrence of enteropathogens of newborn calves, carried out using

different sampling strategies, revealed highly variable results. Large-scale prevalence studies analysing a random sample of farms, including diarrhoeic and non-diarrhoeic calves, for a range of enteropathogens, are lacking. De la Fuente et al. (1998) estimated the prevalence of BRV, BCV, K99 and *C. parvum* among diarrhoeic dairy calves in Spain to be 42.7%, 7.3%, 11.9% and 52% respectively. In Switzerland, the equivalent prevalence estimates were 59%, 8%, 6% and 55% in diarrheic calves (Uhde et al., 2008), while in Sweden, they were estimated to be 24%, 3%, 0%, and 11% respectively (Björkman et al., 2003). In a recent study in diarrheic calves in USA, the estimated prevalence of these agents in calves were 27%, 31.7%, 4% and 33.7% (Cho et al., 2013). Conversely, in Australia, Izzo and co-workers (2011) identified BRV as the most common enteropathogen in diarrheic calves (79.9%), followed by *C. parvum* (58.5%), *Salmonella* spp. (23.8%), BCV (21.6%) and K99 (17.4%).

New Zealand is a major dairy producer, but there is little available data on the national prevalence of enteropathogens of calves. In fact, with the exception of small scale studies (Grinberg et al., 2005), most published studies analysed faecal specimens submitted to diagnostic laboratories (Schroeder et al., 1983; Belton, 1995; Howe et al., 2008). Extrapolating prevalence data from one country to another is problematic, due to the different environments and farming systems. In particular, most New Zealand farms manage short and concentrated winter-spring calving seasons, usually between July and October. This might influence the distribution of the infectious agents compared to countries in which high numbers of susceptible calves are present year-round, with pathogens building up in the farm environment. In addition, comparison between studies is difficult, because of the different sampling strategies and testing protocols used. Therefore, we conducted a nation-wide prevalence study of BRV, BCV, *C. parvum*, *Salmonella* spp. and K99 in New Zealand dairy farms. The study, presented here, was conducted during the winter calving season of 2011 and involved sampling and laboratory analysis of 1283 faecal specimens collected from 97 dairy farms located in seven geographical regions.

4.4. Materials and Methods

4.4.1. Selection of farms and calves

This study used faecal specimens collected from newborn calves on 97 dairy farms. Sample collection was performed during the second half of the 2011 winter calving

season, in order to allow for the build-up of pathogens on farms during the first half. The target population was that of calves on farms milking more than 150 cows. This minimum farm-size was targeted to allow the sampling of multiple calves of a suitable age-range (see below), on all participating farms. The sampling frame was represented by all the farms milking more than 150 cows, registered in the 2010 version of a commercial database which included approximately 12,000 farms (AgriBase™; AsureQuality, New Zealand). There were approximately 10,600 eligible farms in the database, corresponding to 88% of the total number of farms in the country (Anonymous, 2011). Five North Island (Waikato, Wellington, Northland, Taranaki and Manawatu-Wanganui) and two South Island regions (Canterbury and Southland) were selected for the sampling based on the high density of dairy cattle reported (Figure 4.1). Collectively, these regions included 75% of the eligible farms in the database. A sample size of 120 farms was determined based on the maximum number of farms that could be reached during the limited period of time available for sampling. Assuming a perfect aggregate testing regime and a farm prevalence of 40% for *Cryptosporidium* (Grinberg et al., 2005), a sample of 120 farms from a population of 12,000 provided 95% confidence that the estimated farm-level prevalence would be between 37% and 43% (precision of 8.4%; http://epitools.ausvet.com.au/; accessed August 2011). No prevalence data were found in the literature for the other analysed agents.

The co-ordinates of all the eligible farms were plotted on a political map and the proportion of farms contributed by each region calculated. A total of 240 farms were selected using random numbers with a regionally proportional sampling scheme. Farmers were contacted by phone and the first 50% willing to participate from each region, were recruited. A total of 120 farmers were contacted by phone in order to confirm participation, and were asked to facilitate the sampling of all the 1-5 day-old and 9-21 day-old calves present on the farm. The first age group was targeted in order to assess the prevalence of K99, which is seldom pathogenic in older calves (Sherwood et al., 1983; Acres, 1985; Bazeley, 2003; Foster and Smith, 2009). Faecal specimens from these calves were also tested for the presence of BRV, BCV, *Salmonella* and *Cryptosporidium* spp. The second age group was targeted in order to maximise analytical sensitivity for *Cryptosporidium* and BRV, as these animals were predicted to be at the peak of shedding of these pathogens (Grinberg et al., 2002;

Almawly et al., 2013). The specimens collected from the older calves were tested for BRV, BCV, *Cryptosporidium* and *Salmonella* spp., but not for K99. Considering a 60-day calving season and accounting for natural mortality and culled bobby calves, we predicted that a farm milking 150 cows would have presented approximately five 1-5 day-old, and ten 9-21 day-old calves.

4.4.2. Faecal sampling

Sampling was performed by a team of samplers between August 11 and October 7, 2011. The samplers were instructed to collect faecal specimens from the rectum of calves into plastic containers, making sure that they changed disposable gloves between animals. Each specimen was scored as 1 (specimen maintained its original shape), 2 (specimen spread across the bottom of the container but was not liquid), or 3 (a liquid specimen). The breed, sex and age group of each calf were recorded, together with farm-level information obtained from the farmer. Specimens were kept in refrigeration (2-4°C) until delivery to Massey University (MU) by courier delivery.

4.4.3. Laboratory analysis of faecal specimens

Within one week of arriving at MU, the specimens were analysed for K99, BRV, BCV, *Salmonella* and *Cryptosporidium* spp. The analysis for K99, BRV and BCV was performed using a commercial enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (ELISA Calves Diarrhoea, Institut Pourquier, Montpellier, France). The optical absorbance was read with an automated ELISA plate reader using a 450 nm wave length, and absorbance values were transformed, to calculate the sample-to-positive ratio (S/P). Samples with an S/P ratio ≥ 7% were considered positive, in accordance with the manufacturer's recommendation. Additional analyses were performed in 2012 in order to cross-validate the ELISA results, in which 30 refrigerated BCV ELISA-positive specimens were subjected to a coronavirus reverse transcriptase PCR followed by semi-nested PCR (Takiuchi et al., 2006) (Appendix III). Twenty-one amplicons showed bands on gels consistent with the expected size, and four were sequenced and yielded BCV sequences. The ELISA results for BRV were cross-validated by an analysis of 25 ELISA-positive and five ELISA-negative specimens using a commercial latex agglutination kit (Rotascreen; Microgen Bioproducts Ltd., UK), with perfect

concordance between the two tests. For the K99, 14 ELISA-positive specimens were subjected to PCR (Cho et al., 2010), and 12/14 (86%) were PCR positive.

The analysis for *Salmonella* included parallel inoculation of faeces into tetrathionate and Rappaport-Vassiliadis soy peptone (RVS) enrichment broths, incubated for 24 hours at 37°C and 42°C, respectively. This was followed by subculture of the broths onto xylose lactose deoxycholate agar plates, incubated overnight at 37°C. Colonies consistent with *Salmonella* were sub-cultured into triple-sugar iron agar (TSI) slopes and L-lysine decarboxylase broth. Lysine-positive bacteria displaying growth consistent with *Salmonella* in TSI were subjected to slide agglutination with a commercial poly-O antiserum (Institut für Immunpräparate und Nährmedien GmbH Berlin, Berlin, Germany). Agglutinating isolates were sent for serotyping and phage typing at the *Salmonella* Reference Laboratory, Institute of Environmental Science and Research (ESR, Porirua, New Zealand).

Direct immunofluorescence (IF) followed by PCR-sequencing was applied for the diagnosis of *C. parvum* in faeces. A commercial anti-*Cryptosporidium* and *Giardia* FITC-conjugate was used (Aqua-Glo G/C Direct Comprehensive Kit, Waterborne Inc., New Orleans, USA). For the preparation of the slides, approximately 100 µg of faecal material was thoroughly mixed in 100 µl normal saline in the well of a ceramic plate, and a 2 µl aliquot was deposited as a drop on a slide, air-dried and fixed with methanol until evaporation. The fixed drop was covered with 5 µl of antibody, incubated for 30 minutes in a humidified chamber and washed, and the entire area of the drop was examined for the presence of apple-green fluorescent *Cryptosporidium* oocysts, using UV microscopy. The same FITC-conjugate also allowed an analysis for *Giardia* cysts on the slides. The performance of this method had been previously compared with the use of larger faecal smears and 45 µl of FITC-conjugated antibody, with perfect agreement between the two methods (Appendix IV). One hundred *Cryptosporidium* IF-positive specimens (at least one specimen per IF-positive farm) were selected in a blind fashion and subjected to an identification of species by PCR-sequencing of the 18S ribosomal RNA (18S rDNA) and the 70 kDA heat-shock protein (HSP70) genes as previously described (Almawly et al., 2013).

4.4.4. Analysis of data

For each pathogen, the crude farm-level prevalence was computed as the number of farms showing a positive faecal specimen divided by the number of analysed farms. At the calf-level, the prevalence was computed as the number of positive calves divided by the number of analysed calves. The standardised national farm-level prevalence was calculated for each pathogen using the following formula available in the R package epiR (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria):

$$\text{SNP} = \sum_{i} \frac{dw}{y}$$

Where SNP is the standardised national prevalence, *d* is the number of agent-positive farms/calves in the i_{th} region, *y* is the number of sampled farms in the i_{th} region, and *w* is a weighting factor for the i_{th} region, calculated as the number of farms milking >150 cows reported for that region in the database, divided by the sum of farms milking >150 cows in all the seven regions (we note that this SNP considers only the seven sampled regions). The 95% confidence interval for the SNP and crude prevalence was calculated by the same program, using the gamma distribution.

The relationships between demographic variables of interest and presence/absence of each enteropathogen were assessed at both farm- and calf-levels using multivariable logistic regression, as implemented by the Statistical Analysis package in R version 2.5.0 (R Development Core Team, R foundation for Statistical Computing, Vienna, Austria). The binary variable of presence or absence of an agent on the farm or the specimen was used as a binary outcome. For the farm-level analysis, a farm was considered positive for an agent if there was at least one positive specimen. Explanatory variables were: 'Herd size' (fixed effect; 1:151-450 cows; 2: 451-650 cows; 3: >650 cows); 'Farm location' (fixed effect; 1: South Island; 2: North Island); 'Breed' of calf (fixed effects; 1: Friesian; 2: Jersey; 3: other breeds). The calf-level analysis included the explanatory variables: 'Sex' of calf (fixed effect); 'Age group' (fixed effect; 1: 1-5 day-old; 2: 9-21 day-old); 'Breed'(fixed effect); 'Farm location' (fixed effect), and the variable 'Farm' (a unique identifier for each farm) modelled as random effect. In the case of *C. parvum*, the categories of *C. parvum* present or absent were determined according to the result of the PCR-sequencing. Accordingly,

all IF-positive calves on farms where *C. parvum* was identified by PCR-sequencing were coded as *C. parvum* present, and, if other *Cryptsporidium* species were identified, as *C. parvum* absent. Calves on farms for which the parasite's species could not be defined were eliminated from the multivariable analyses. Lastly, any difference between proportions of interest was assessed using two-tailed Fisher's exact tests. The 95% confidence intervals (CI) for proportions were calculated using this formula:

$$95\%\ \text{CI: } p \pm z\sqrt{\frac{p(1-p)}{n}}$$

where z=1.96, p is the proportion of interest and n is the sample size.

4.5. Results

Due to field constraints, 97/120 farms (80.8% of target) were sampled between 11 August and 07 October 2011. A total of 1,283 calves were sampled, including 429 calves that were 1–5 days-old and 797 that were 9–21 days-old, with the number of calves per farm ranging between 10 and 15. Fifty-seven (4.4%) samples were submitted without age specification, leaving 1,226 samples for the calf-level analyses. Of these calves, 643 (52.4%) were Friesian, 375 (30.6%) Friesian-Jersey, 172 (14%) Jersey, 23 (1.9%) Hereford, and 13 (1%) Ayrshire. There were 262/429 (61.1%) and 693/797 (86.9 %) female calves in the 1-5 and 9-21 day-old group, respectively. The excess of females in the 9-21 day-old group was probably due to culling of more male ('bobby') calves before the age of 9 days. A total of 116/1226 (9.5%) specimens were liquid (faecal score 3), and 51/97 (52.6 %) farms had at least one liquid specimen. The proportion of liquid specimens was significantly greater in the 1-5 day-old group (61/429, 14.2%), than in the 9-21 day-old group (55/797, 6.9%) ($p<0.01$).

4.5.1. Farm-level prevalence of enteropathogens

Tables 4.1 to 4.4 report the farm- and calf-level prevalence of the analysed enteropathogens. The five pathogens were present in most regions (Table 4.1), whereas the prevalence of BRV and BCV was similar in both age groups, *Cryptosporidium* oocysts were mainly found in the older group. *Salmonella* spp.were detected in only 4 farms, in both ages. K99 was found in 1-5 day-old calves in 11 farms. The farm-level SNP of pathogens is reported in Table 4.2. A total of 93/97 (96%) farms had specimens infected with at least one enteropathogen, and 66/97

(68%) were co-infections with two or more agents. BCV and *Cryptosporidium* co-infections were recorded in 18/97 (19%) farms, and BRV and BCV in 22/97 (23 %) farms. There was an almost significantly greater rate of co-infections on farms in the South Island than in the North Island (OR: 4.17; 95% CI: 1.1-22; p=0.06; Table 4.5). A total of 84 *Cryptosporidium*-positive faecal specimens from 55 farms were successfully identified to species level by PCR-sequencing, and DNA originating from 8 farms did not amplify at any locus. PCR-sequencing revealed only the presence of *C. parvum* and *C. bovis* (Appendix X), with only one species being identified on each IF-positive farm. *C. parvum* was the most common species, accounting for 77/84 (91.7%) of the specimens from 49/55 (89%) farms. Conversely, *C. bovis* accounted for 7/84 (8.3%) samples originating from 6/55 (11%) farms. Although assessment of the prevalence of *Giardia* spp. was not an objective of this study (as there is no consensus regarding its pathogenicity in cattle), we report for completeness that *Giardia* cysts were seen by IF in 36/429 (8.4%) 1-5 day-old calves on 23/97 (24%) farms and 314/797 (39.4%) 9-21 day-old calves on 83/97 (86%) farms ($p< 0.01$).

4.5.2. Prevalence of enteropathogens among 1-5 day-old calves

In this age group, 133/429 (31%) calves were positive for at least one agent (*Giardia* excluded). BRV was the most common pathogen, found in 86/429 (20%) specimens on 68/97 farms. The second most common agent was *Cryptosporidium*, seen by IF in 25/429 (6%) specimens. The pathogenic and potentially zoonotic species *C. parvum* was the predominant species in this age group, accounting for 14 out of 16 genotyped specimens (87.5%). Conversely, *C. bovis* was identified in 2/16 (12.5%) specimens, both from one farm in Canterbury. Twenty-three (5.4%) specimens from 17 farms were positive for BCV. K99 was found in 14/429 (3.3%) specimens from 11 farms and *Salmonella* spp. were isolated in only three specimens from 3 farms. Further analysis identified *Salmonella enterica*, serovar Typhimurium (one specimen); serovar Ruiru (one specimen); and serovar Agona (one specimen). Whereas K99 and *Salmonella* spp. infections were limited to only one or two calves on each positive farm, BRV was consistently identified in multiple calves. Co-infections K99 + BRV were detected in 9 (2%) specimens from eight farms, and K99 + *Cryptosporidium* occurred in two specimens from two farms (Table 4.3). A total of 30/133 (22.6%) specimens positive for at least one enteropathogen had a faecal score of 3, as opposed

to only 31/296 (10.5%) enteropathogen-negative specimens ($p<0.05$). A greater rate of liquid faeces was observed in the 1-5 day-old group (14.2%) compared with the older group (6.9%), as reported elsewhere (Curtis et al., 1988a; Busato et al., 1997; Wells et al., 1997).

4.5.3. Prevalence of enteropathogens among 9-21 day-old calves

A total of 311 (39%) calves of this age group were positive for at least one enteropathogen (*Giardia* excluded). Similarly to the younger group, BRV was the most common pathogen, identified in 158/797 (19.8%) specimens from 68/97 (70%) farms. There were 49 (6%) BCV-positive specimens from 37 (38%) farms. A total of 178 specimens (22%) from 57 (59%) farms were positive for *Cryptosporidium* by IF. Also in this age group, *C. parvum* was the predominant species, identified in 63/67 (94%) genotyped specimens, on 46 farms, and *C. bovis* was present in 4/67 (5.9%) specimens, on 4 farms. In all farms, only one species was identified, but parasites from 8 farms did not amplify at any locus, suggesting the possible presence of other minor *Cryptosporidium* species that display primer-site polymorphism with C. *parvum* and *C. bovis*. Only 4 (0.5%) specimens from 3 (3.1%) farms were positive for *Salmonella* spp. Further analysis identified *Salmonella enterica* serovars Typhimurium (phage types 154 and 160), Ruiru and Mbandaka. Interestingly, serovars Ruiru and Mbandaka were identified in one farm. Thirty-four out of 311 (11%) enteropathogen-positive specimens were liquid, compared with 21/486 (4.3%) enteropathogen-negative ($p< 0.01$).

4.5.4. Multivariable modeling results

Tables 4.5 and 4.6 summarise the results of the multivariate analyses. No significant associations (using an α-level of significance <0.05) between explanatory variables and the presence of enteropathogens were identified at the farm level. Consistent with the presence of a pre-patent period, the calf-level models indicated increased odds of the presence of *C. parvum* in 9-21 day-old calves (OR = 2.9; 95% CI: 1.8 - 4.6; $p<0.01$). Also, the likelihood of finding co-infections was greater in the older calves (OR=2.9; 95% CI: 1.9-4.5; $p<0.01$). Other explanatory variables showed no significant association with the presence of enteropathogens at the calf level.

4.6. Discussion

To our knowledge, this study represents the first national point-prevalence assessment of BRV, BCV, K99, *Cryptosporidium* and *Salmonella* spp. among newborn calves on dairy farms in New Zealand. In order to obtain the maximum value of prevalence, the study was carried out in the second half of a single calving season using two age groups. In fact, K99 generally causes watery diarrhoea in 1-5 day-old calves (Acres, 1985; Bendali et al., 1999a; Bazeley, 2003; Foster and Smith, 2009), whereas the other analysed agents are more commonly found in the faeces of 1-2 week-old animals (Holland, 1990; Grinberg et al., 2002; Bazeley, 2003; Almawly et al., 2013). As a measure of occurrence, prevalence values are often used in economic analyses and risk assessments. As will be seen in this discussion, comparison between studies is difficult due to the different sampling strategies and testing protocols used.

As a whole, the results indicated a wide geographical distribution of K99, BRV, BCV and *C. parvum*, low prevalence of *Salmonella* spp. and high farm-level prevalence of co-infection with two or more agents. Some authors have suggested that co-infections cause more severe disease in calves than mono-infections (Belton, 1995; García et al., 2000; Hoet et al., 2003). In this study, the rate of co-infection with two or more enteropathogens was significantly higher in 9-21 day-old calves (34.4%) than in the young group (25.3%) ($p<0.01$). This co-infection rate is intermediate between the rates reported in Australia (71%) (Izzo et al., 2011), and Europe (17-19%) (Reynolds et al., 1986; Uhde et al., 2008) in diarrhoeic calves. Limited studies sampling both diarrhoeic and non-diarrhoeic calves have been found in the literature (Waltner-Toews et al., 1986d; Bartels et al., 2010), however, and most previous studies reported analysis of samples from diarrheic calves only (Tzipori, 1981; De Rycke et al., 1986; Reynolds et al., 1986; Snodgrass et al., 1986; Bellinzoni et al., 1990; Bendali et al., 1999a; De la Fuente et al., 1999; García et al., 2000; Uhde et al., 2008; Izzo et al., 2011). In the present study, we sampled calves from a random sample of farms from the regions with the highest number of dairy farms, without collecting information about the presence of diarrhoea. It is important to note that the prevalences may be lower in other regions where dairy farm density is lower.

4.6.1. Enteropathogen prevalence among 1-5 day-old calves

At the calf level, BRV was the most prevalent agent in this age group (20%), followed by *C. parvum* (6%), BCV (5.4%), and K99 (3.3%). Conversely, *Salmonella* spp. were observed only sporadically (0.7%). In this age group, the prevalence of BRV and BCV was higher than that reported in randomly selected dairy calves in the Netherlands, but the K99 and *C. parvum* prevalence was lower (Bartels et al., 2010). As expected, the prevalence of most of these enteropathogens was lower than that reported in in diarrhoeic calves in a recent study in Australia (Izzo et al., 2011) and in some European studies (Reynolds et al., 1986). Some of these studies sampled older calves (Reynolds et al., 1986).

Although the number of K99-positive calves was relatively small, this organism was found in a significant number of farms (SNP 11%, 95% CI: 5-25). It is likely that other calves on infected farms would have been positive if repeatedly sampled, highlighting the limitations of cross-sectional studies. This farm-level prevalence was considerably lower than the prevalence in random calves in Canada (41%; Waltner-Toews et al., 1986d), similar to the prevalence in diarrhoeic calves in some European countries (11.4%; Sherwood et al., 1983) and Australia (10%; Izzo et al., 2011), but higher compared to the prevalence in diarrhoeic calves in Switzerland (2.1%; Uhde 2008). The results also showed a greater prevalence of K99 infection in calves passing liquid faeces compared with non-liquid faeces. In this context, however, causation should be assessed using multivariable analyses that control for confounding and account for the effects of the other agents.

4.6.2. Enteropathogen prevalence among 9-21 day-old calves

Some data indicate BRV as the most common agent of NCD in 1 to 2 week-old calves, worldwide (Busato et al., 1998; García et al., 2000; Uhde et al., 2008; Izzo et al., 2011). Also in this study, BRV- either alone or in co-infection - was the most common agent, found in 19.8% of the specimens, in 38% liquid and 18% non-liquid faeces ($p<0.01$), with a farm-level SNP of 57%. Conversely, the farm-level SNP of BCV was 30.5%. This farm-level prevalence was lower than that reported in Europe (40%; Snodgrass et al., 1986) and Australia (41%; Izzo et al., 2011), when diarrhoeic calves were sampled. The calf-level prevalence (6.1%) was lower than that reported in diarrheic calves in Australia (21.6; Izzo et al., 2011), North America (24.6; Marsolais, 1978), Europe (12-21.8%; Snodgrass et al., 1986; Reynolds et al., 1986;

Bendali et al., 1999a) and Africa (38.9; Abraham et al.,1992). BCV is considered an important cause of neonatal calf diarrhoea overseas (Björkman et al., 2003; Gumusova et al., 2007; Uhde et al., 2008; Izzo et al., 2011). However, the prevalence and impact of BCV in New Zealand have traditionally been considered low (Durham et al., 1979; Vermunt, 2000), and informal data indicate that the virus is found in only ~2% of the specimens submitted for analysis to diagnostic laboratories in New Zealand (Isobel and David, personal communication, 2012). We considered that the relatively high farm-level BCV prevalence found in this study could have been due to ELISA false-positive results, but RT PCR confirmed the presence of BCV in the majority of the analysed specimens (even though this analysis was performed one year after their collection), indicating the virus circulates in a high proportion of farms. BCV was not associated with the presence of liquid faeces in this study (p=0.5), but as said above, the impact of intestinal BCV on health needs to be established using multivariable analysis.

In a previous study conducted in New Zealand using microscopy and a convenience sample of 24 dairy farms, *Cryptosporidium* oocysts were identified in 40% of farms (Grinberg et al., 2005). The present study used a larger sample size and applied genotyping, which allowed the differentiation between the morphologically-similar species of *C. parvum* and *C. bovis*. In the present study, *C. bovis* was identified in six (11%) farms, whilst the majority of the IF-positive farms were infected with *C. parvum*. The predominance of *C. parvum* in the sample is consistent with previous small-scale molecular studies (Learmonth et al., 2001; Grinberg et al., 2008a) and with results from other countries which identified *C. parvum* as the predominant species in unweaned calves (Thompson et al., 2007; Quilez et al., 2008), and *C. bovis* in the post-weaning period (Starkey et al., 2006b; Fayer et al., 2007). *C. bovis* is a seemingly non-pathogenic species described for the first time in the USA in 2005 (Fayer et al., 2005). The low farm-level prevalence of *C. bovis* and possibly the other non-parvum species reinforces the diagnostic value of the phenotypic tests for *C. parvum* offered as part of the 'calf diarrhoea' panel by New Zealand diagnostic laboratories.

After removing the farms infected with *C. bovis* and non-amplifying farms, the SNP of *C. parvum* among 9-21 day-old calves was 52% (95% CI: 36-75). This prevalence is similar to the results obtained in diarrhoeic calves in Australia (58%; Izzo et al.,

2011), Mexico (54.5%; Maldonado-Camargo et al., 1998), and Switzerland (41.7%; Uhde 2008), but lower than the results obtained in Canada in random calves (80%; Olson et al., 1997) and Scotland in diarrhoeic calves (75%; Snodgrass, 1986). The calf-level prevalence of *C. parvum* (17%) was lower than that reported in diarrhoeic calves in other countries, with rates ranging from 23 to 58.5% (Garber et al., 1994; Castro-Hermida et al., 2002a; Uhde et al., 2008; Izzo et al., 2011). The *C. parvum* calf-level prevalence value is of very limited value due to the short patent period, but is reported here for completeness. In accordance with previous results (Tzipori et al., 1980; Grinberg et al., 2002; Moore et al., 2003; Sevinc et al., 2003), *C. parvum* was associated with the presence of liquid specimens at the bivariate level ($p<0.02$). Interestingly, in the 9-21 day-old group 33 *C. parvum*-positive calves had concurrent infections with BRV. Although, *C. parvum* is a frank pathogen of calves (Tzipori, 1981; Naciri et al., 1999), concurrent infections with other enteropathogens are known to occur (García et al., 2000; Uhde et al., 2008), and the attributable impact of each agent on health is difficult to assess without the application of multivariable analyses.

Only four specimens from the 9-21 day-old calves were culture-positive for *Salmonella* spp. Four *Salmonella enterica* serotypes were identified in this study, namely, *S.* Typhimurium phage type 154 and 160, serovars Ruiru, Mbandaka and Agona. All these have been reported previously in bovine faeces in New Zealand (ESR. Annual reports; http://www.esr.cri.nz/. Accessed October 2013). *S.* Typhimurium phage type 160 was responsible for a 10-year epidemic in humans and was also reported in sheep and wild birds (Callaghan, 2001; Alley et al., 2002; French et al., 2011). *Salmonella* Agona has been reported in multiple human food-borne outbreaks of gastroenteritis in Europe and USA (Killalea et al., 1996; Hendriksen et al., 2011; Nicolay et al., 2011). In New Zealand it has been reported in humans, foals, cattle, pigs and poultry (Patterson-Kane et al., 2001; Adlam et al., 2010). *S.* Mbandaka is commonly reported in humans in Australia (Scheil et al., 1998) and New Zealand (Adlam et al., 2010), and in young calves in the USA (Wray et al., 1991). The low rate of *Salmonella* isolation is consistent with a previous study, which did not find any *Salmonella* in a sample of 185 faecal specimens collected from calves on 24 New Zealand dairy farms (Grinberg et al., 2005). In comparison, *Salmonella* spp. were isolated in 23.8% of diarrhoeic calves in Australia (Izzo et al.,

2011), 6.1% in Brazil (Langoni et al., 2004) and 12% in the UK (Reynolds et al., 1986).

As mentioned previously, compared to cattle-rearing countries with year-round calving in which there is a continuous presence of young calves on farms, New Zealand's calving pattern might influence the epidemiology of NCD. A sharp increase in the numbers of immunologically naive calves during the calving season, and their reduction between the seasons might influence the environmental load of the infectious agents.

4.7. Conclusions

This report provides new epidemiologically-robust information on the occurrence of the major endemic enteropathogens of newborn calves in New Zealand. Collecting these data was necessary given the importance of the dairy sector for the New Zealand economy and the difficulty in extrapolating prevalence data from countries where the conditions and the duration of the calving season differ substantially from New Zealand. BRV and *C. parvum* circulate on the majority of the farms. BCV was found in a high proportion of farms, with a SNP of 30.5%. Although the virus is widespread, its pathogenic potential remains uncertain. *C. parvum* was the dominant species identified in newborn calves, reinforcing the diagnostic value of the phenotypic tests for *C. parvum* offered by New Zealand diagnostic laboratories. K99 was identified in 1 - 5 day-old calves in approximately 10% of the farms. In accordance with previous reports, the low prevalence of *Salmonella* spp. indicates newborn calves are probably not significant reservoirs of these organisms in New Zealand, reinforcing the high diagnostic value of a positive bacterial isolation result in the course of disease outbreak investigations. Finally, the high prevalence of *Giardia* spp. warrants further investigation. Multivariable studies are warranted to assess the relative contribution of the various pathogens to NCD in New Zealand.

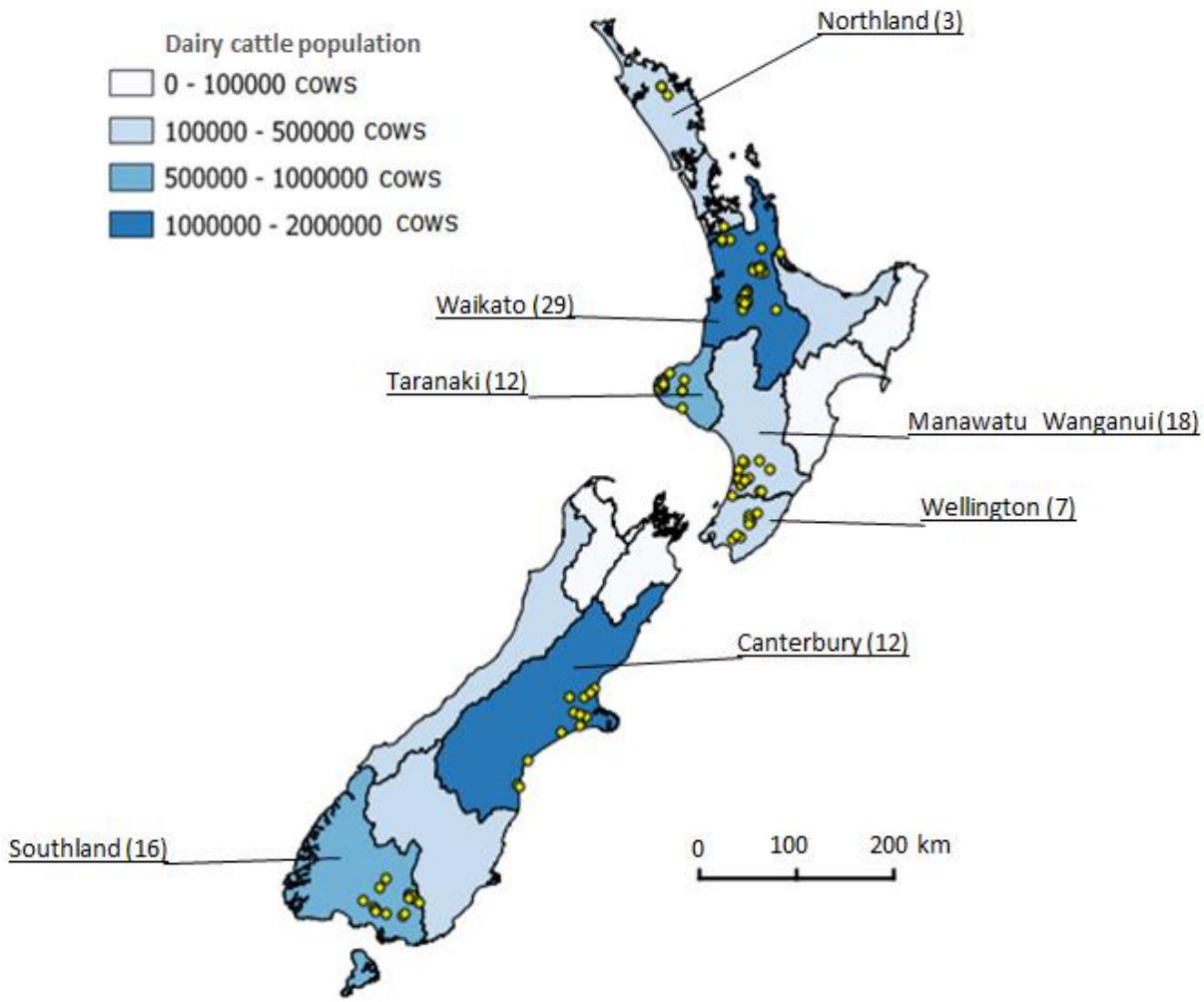

Figure 4.1. Spatial distribution of the sampled farms (dots) in the South and North Island of New Zealand. For each region, the number of sampled farms is indicated in brackets and the dairy cattle density in gray scale.

Table 4.1. The number of farms with at least one positive specimen for each enteropathogen in the different regions, according to the age groups sampled.

	Region	No. of sampled farms	BRV		BCV		*C. parvum*		K99	*Salmonella* spp.	
			9-21 d-old calves	1-5 d-old calves	9-21 d-old calves	1-5 d-old calves	9-21 d-old calves	1-5 d-old calves	1-5 d-old calves	9-21 d-old calves	1-5 d-old calves
South Island	Canterbury	12	11	9	4	4	1	1	1	0	0
	Southland	16	10	9	4	1	11	1	1	1	0
North Island	Manawatu Wanganui	18	14	9	14	6	9	0	2	1	1
	Northland	3	2	1	0	0	1	2	0	0	0
	Taranaki	12	8	7	4	1	7	3	2	0	0
	Waikato	29	12	10	8	4	17	2	3	1	2
	Wellington	7	2	3	3	1	0	0	2	0	0
	Total	**97**	**59**	**48**	**37**	**17**	**46**	**9**	**11**	**3**	**3**

Table 4.2. National standardised farm-level enteropathogens prevalence (95% confidence intervals are in brackets).

	1-5 day-old calves	9-21 day-old calves
BRV	46% (31-67)	57% (41-80)
BCV	13.7% (7-28)	30.5% (20-48)
C. parvum	17.5% (8-35)	52% (36-75)
Salmonella spp.	3% (1-15)	4% (1-16)
K99 *E. coli*	11% (5-25)	Not tested
Giardia spp.	23% (13-41)	88% (67-116)

Table 4.3. Farms positive for enteropathogen (overall, alone and in co-infection) on dairy farms in New Zealand (*n=97*), (in brackets, % followed by 95% confidence interval)

Enteropathogen	Number of positive farms
BRV (overall)	68 (70; 60-78)
BRV (alone)	34 (35; 29-39)
BRV + BCV	22 (23; 17-29)
BRV + BCV + *C.parvum*	9 (9; 2-15)
BRV + BCV + *C.parvum* + K99	2 (2; 0.06-6)
BRV + *Salmonella* spp. + BCV +K99	1 (1;0.08-5)
BCV (overall)	46 (47;38-58)
BCV (alone)	5 (5; 1-9)
BCV + *C.parvum*	18 (19; 13-25)
BCV + BRV	22 (23; 18-31)
BCV + BRV + *Salmonella* spp. + K99	1 (1;0.08-5)
C. parvum (overall)	49 (55;44-65)
C. parvum (alone)	28 (29; 21-37)
C. parvum + BRV	22 (23; 15-32)
C. parvum + BCV	18 (19; 13-25)
C.parvum + K99 + BRV + BCV	2 (2; 0.06-6)
C.parvum + *Salmonella* spp. + BRV + BCV	1 (1;0.08-5)
Salmonella spp. (overall)	4 (4;1-9)
Salmonella spp. (alone)	1 (1;0.08-5)
Salmonella spp. + BRV + BCV + *C.parvum*	1 (1;0.08-5)
Salmonella spp. + BRV + BCV +K99	1 (1;0.08-5)
Salmonella spp. + BCV	1 (1;0.08-5)
K99 (overall)	11(11;6-19)
K99 (alone)	1
K99 + BRV + BCV	7 (7; 2-15)
K99 + *C.parvum*	2 (2; 0.06-6)
K99 + *Salmonella* spp. + BRV + BCV	1 (1;0.08-5)

Table 4.4. Number of calves positive for enteropathogens (mono-infection and co-infection) on dairy farms in New Zealand (in brackets, % followed by 95% confidence interval)

Enteropathogen	Calves positive for enteropathogens/total calves	
	9-21 day-old calves ***(n=797)***	**1-5 day-old calves** ***(n=429)***
BRV	158 (19.8; 17-23)	86 (20; 16-24)
BCV	49 (6; 4-8)	23 (5.4; 3-8)
C. parvum	126 (17; 14-20)	25 (6; 4-9)
Salmonella spp.	4 (0.5;0.1-0.9)	3 (0.7;0.10-1.2)
K99	Not tested	14 (3;1-5)
BRV + BCV	26 (2.3; 1-4)	10 (2.3; 1-4)
BRV + *C. parvum*	33 (41; 32-49)	9 (2.3; 1-4)
C. parvum + BCV	20 (2.5; 0.1-5)	2 (0.50; 0.1-0.8)
K99 + BRV	Not applicable	9 (2.1; 1-4)
BRV + BCV + *C. parvum*	13 (1.6; 0.2-4)	1 (0.2; 0.08-1.3)
K99 + *C. parvum*	Not applicable	2 (0.2; 0.07-1.2)
K99 + BCV	Not applicable	2 (0.5; 0.1-0.8)
K99 + *C. parvum* + BRV	Not applicable	1 (0.2; 0.08-1.3)
BRV + *C. parvum*+ *Salmonella*	1 (0.1; 0.03-0.7)	1 (0.2; 0.07-1.3)
BRV + BCV +*Salmonella*	1 (0.1; 0.02-0.7)	1 (0.2;0.03-0.9)
BRV + BCV + K99	Not applicable	2 (0.5; 0.1-0.8)
BRV + *Salmonella*	1 (0.1; 0.02-0.7)	1 (0.2; 0.08-1.3)

Table 4.5. Farm-level multivariable logistic regression model results. In the variables for Island, Herd size and Breed, the categories "North Island", "Herd size of 151-450 cows" and "Friesian breed" were used as reference values, and are not shown. Borderline significant P-values and odds ratios are in bold

Outcome variable	Explanatory variable	P-value	Odds Ratio (95% CI)
Presence/absence of BRV	Location (South Island)	0.08	3.51 (0.92-17.6)
	Herd (451-650 cows)	0.59	0.71 (0.2-2.6)
	Herd (>650 cows)	0.40	0.61 (0.3-2.7)
	Breed (Jersey)	0.15	3.20 (0.75-22)
	Breed (other breeds)	0.96	0.97 (0.32-3)
Presence/absence of BCV	Location (South Island)	0.80	0.80 (0.27-2.7)
	Herd (451-650 cows)	0.59	1.30 (0.46-4.0)
	Herd (>650 cows)	0.30	1.10 (0.3-5)
	Breed (Jersey)	0.08	0.34 (0.09-1.1)
	Breed (other breeds)	0.04	0.36 (0.12-0.9)
Presence/absence of *C. parvum*	Location (South Island)	0.90	1.04 (0.34-3.39)
	Herd (451-650 cows)	0.60	0.75 (0.26-2.2)
	Herd (>650 cows)	0.40	0.60 (0.1-3.3)
	Breed (Jersey)	0.40	0.66 (0.2-2.15)
	Breed (other breeds)	0.80	1.10 (0.41-3.2)
Presence/absence of K99	Location (South Island)	0.90	0.90 (0.11-4.7)
	Herd (451-650 cows)	0.40	0.40 (0.02-2.63)
	Herd (>650 cows)	0.30	0.20 (0.01-2.53)
	Breed (Jersey)	0.90	0.97 (0.13-4.7)
	Breed (other breeds)	0.50	0.60 (0.08-2.84)
Presence/absence of co-infection (any combination)	Location (South Island)	0.06	4.17 (1.1-22)
	Herd (451-650 cows)	0.56	0.69 (0.19-2.5)
	Herd (>650 cows)	0.31	0.3 (0.2-2.6)
	Breed (Jersey)	0.65	1.38 (0.35-6.91)
	Breed (other breeds)	0.38	0.6 (0.19-1.86)

Table 4.6. Calf-level multivariable logistic regression model results. North Island, Friesian breed, female and 1-5day-old group were used as reference in the variables for Island, Breed, Sex and Age group, and are not shown. P-values <0.05 and odds ratios >1 are in bold. Note that K99 was only tested in the younger age group.

Outcome variable	Explanatory variable	P-value	OR (95% CI)
Presence/absence of BRV	Location (South Island)	0.16	1.61 (0.81-3.19)
	Breed (Jersey)	0.71	0.89 (0.44-1.8)
	Breed (other breeds)	0.84	0.94 (0.56-1.6)
	Sex (male)	0.81	1.06 (0.64-1.73)
	Age group (9-21 d-old)	0.66	1.08 (0.74-1.57)
Presence/absence of BCV	Location (South Island)	0.24	0.64 (0.3-1.35)
	Breed (Jersey)	0.14	0.43 (0.14-1.32)
	Breed (other breeds)	0.57	0.82 (0.41-1.46)
	Sex (male)	0.90	0.95 (0.44-2.04)
	Age group (9-21 d-old)	0.72	1.11 (0.61-2.03)
Presence/absence of *C. parvum*	Location (South Island)	0.66	1.18 (0.55-2.5)
	Breed (Jersey)	0.27	1.48 (0.73-3.01)
	Breed (other breeds)	0.31	0.71 (0.37-1.37)
	Sex (male)	0.13	0.57 (0.27-1.18)
	Age group (9-21 d-old)	**<0.01**	**2.86** (1.77-4.62)
Presence/absence of K99	Location (South Island)	0.87	0.63 (0.01-37)
	Breed (Jersey)	0.39	4.42 (0.01-33)
	Breed (other breeds)	0.21	0.13 (0.02-6.4)
	Sex (male)	0.71	2.81 (0.05-14)
Presence/absence of co-infection (any combination)	Location (South Island)	0.45	0.81 (0.47-1.39)
	Breed (Jersey)	0.42	1.27 (0.7-2.29)
	Breed (other breeds)	0.87	0.95 (0.58-1.56)
	Sex (male)	0.29	0.73 (0.4-1.31)
	Age group (9-21 d-old)	**<0.01**	**2.89** (1.87-4.48)

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STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Julanda Al Mawly

Name/Title of Principal Supervisor: Alex Grinberg

Name of Published Research Output and full reference:

Prevalence of endemic enteropathogens of calves in New Zealand dairy farms

In which Chapter is the Published Work: Four

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  The candidate has had a a major input in study design and trained the sampling team, received the samples and questionnaires from the field and performed all the laboratory analyses. He also collated and analysed the data and wrote the first draft of the Chapter. The candidate produced the definitive Chapter after several rounds of consultation with the supervisory team.

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Principal Supervisor's signature — 24/8/2014 — Date

Chapter 5

5. RISK FACTORS ANALYSIS FOR NEONATAL CALF DIARRHOEA AND ENTEROPATHOGEN SHEDDING IN NEW ZEALAND DAIRY FARMS

5.1. Preamble

The previous chapter reported a national prevalence study of BRV, BCV, *C. parvum*, *Salmonella* spp. and K99 in neonatal calves in New Zealand dairy farms. The microbiological data obtained, combined with farm-level information elicited by a questionnaire delivered to the same farmers, were used to perform the risk-factor analysis for NCD and enteropathogen shedding described in this chapter.

5.2. Abstract

The multifactorial etiology of NCD should be ascertained using representative samples of farms, comprehensive laboratory investigations and multivariable analyses, but results from studies matching these criteria are scant. To investigate age-specific risk factors for NCD, a country-wide cross-sectional study was conducted in 2011 on 97 randomly-selected New Zealand dairy farms. Faecal specimens from 1283 calves were scored as liquid, semi-solid or solid, and analysed for BRV and BCV, K99, *Salmonella* spp. and *C. parvum*. Calf- and farm-level data were collected by means of a questionnaire and the odds for the presence of liquid faeces analysed using mixed logistic regression models.

C. parvum (OR = 2.6; 95% CI: 1.3-5.6; p=0.02), BRV (OR=2.7; 95% CI: 1.3-5.9; p=0.01), and co-infection compared with mono-infection (OR=2.5; 95% CI:1.3-4.8; p=0.01) were independently associated with increased odds of liquid faeces. Conversely, vaccinating cows against calf enteropathogens (OR=0.2; 95% CI: 0.1-0.9; p=0.03), administering waste milk (OR=0.4; 95% CI: 0.1-0.8; p=0.01), and being a female calf (OR=0.2, 95% CI: 0.07-0.7; p<0.01) decreased the odds of liquid faeces. Infrastructure and management variables associated with liquid faeces were: using open barns (OR=2.1; 95% CI: 1.1-12.2; p=0.03) or partially-open barns (OR=3.5; 95% CI: 1.1-10.5; p=0.04) compared with closed barns; use of straw

bedding decreased the odds of liquid faeces (OR=0.2; 95% CI: 0.03-0.9; p=0.03) compared with sawdust.

In 1-5 day-old calves, only K99 was associated with liquid faeces (OR=4.6; 95% CI: 1.2-16.1; p=0.02). These odds were lower on farms employing female caretakers compared with farms where only males were present (OR=0.4; 95% CI: 0.01-0.9; p=0.04). The results of this study point out a number of managerial and infrastructural risk factors which might be addressed by farmers and laboratory diagnosticians, in order to reduce the burden of NCD and enhance laboratory diagnosis of this economically-important condition.

5.3. Introduction

NCD is a common health problem on dairy farms worldwide (Busato et al., 1998; De la Fuente et al., 1999; Castro-Hermida et al., 2002a; Bazeley, 2003). Incidence estimates for NCD vary between 15% and 21% among cattle herds (Waltner-Toews et al., 1986b; Wells et al., 1997; Bendali et al., 1999a). NCD has a significant economic impact due to the costs of treatment, veterinary services, decreased weight gain and diagnostic testing (Frank and Kaneene, 1993; Walker et al., 1998; De Graaf et al., 1999). The costs associated with NCD have been estimated at US$30 per calf per year in Switzerland (Huetink et al., 2001), US$70 in France (Bendali et al., 1999a) and US$71 per breeding cow per year in Australia (Gunn, 2003). In the USA the cost associated with prevention of mortality from gastrointestinal disease in young calves was estimated at US$250 million per year in 1993 (Frank and Kaneene, 1993). In addition, NCD represents an animal welfare problem that needs to be addressed by farmers (Fraser, 1989; Moran, 2002).

Knowledge of risk factors for NCD can assist the formulation of effective disease mitigation strategies. Potential risk factors for NCD can be categorised into farm-level factors associated with animal husbandry, farm management and infrastructure, and calf-level factors, such as the status of the calf's immune system, its sex and breed, and infection with specific enteropathogens. The most commonly-reported endemic enteropathogens of calves are K99 and *Salmonella* bacteria, BRV and BCV, and the protozoan parasite *C. parvum* (García et al., 2000; Uhde et al., 2008; Bartels et al., 2010; Izzo et al., 2011). Infections with *C. parvum*, BRV, BCV, K99 and *Salmonella* spp., inadequate colostrum intake and lack of dam vaccination against

calf enteropathogens have been considered significant risk factors for NCD (Bazeley, 2003; Trotz-Williams et al., 2007a; Trotz-Williams et al., 2007b; Gulliksen et al., 2009a). Other risk factors considered include the type of floors and housing; the importation of calves from other farms; winter birth; high percentage of heifers in the herd; poor cleaning of feeding tools; and the provision of inadequate shelter for calves (Waltner-Toews et al., 1986a; Quigley et al., 1995; Gulliksen et al., 2009a). However, it is difficult to generalise risk factor studies for NCD due to the differences in study design, and in environments, host, and pathogen populations. Conventional wisdom assumes that the severity of NCD is determined by complex interactions between enteropathogens and the environmental and husbandry factors. Thus, risk factors for NCD should ideally be analysed using multivariable approaches. Several NCD multivariable risk factor studies, each with its strengths and weaknesses, have been published (see Discussion). We report a New Zealand nation-wide multivariable risk factor study of NCD. The study used farm- and calf-level data collected from 97 farms by means of questionnaires and laboratory analysis (including genotyping), bearing in mind the gaps in knowledge left by previous studies. The data were analysed to assess the contribution of each agent and of environmental and host-related factors, to age-specific calf-morbidity.

5.4. Materials and methods

5.4.1. Study design and faecal sampling

This study used faecal specimens from 97 randomly-selected dairy farms. Sampling and data collection were performed in 2011, during the second half of the winter-spring calving season. Details on farms and faecal sampling have been provided in Chapter 4.

5.4.2. Laboratory analysis for enteropathogens

The analysis for BRV, BCV and K99 was performed using a commercial ELISA kit (ELISA Calves Diarrhoea, Institut Pourquier, Montpellier, France). The analysis for *Salmonella* spp. was performed by standard culture, which included in-parallel enrichment in two selective broths followed by subculture onto differential solid media (Chapter 4). The analysis for *Cryptosporidium* was performed using immunofluorescence (IF) (Aqua-Glo G/C Direct Comprehensive Kit, Waterborne Inc., New Orleans, USA) followed by species identification by sequencing of the

18S rRNA gene, preformed on at least one immunofluorescence-positive specimen from each farm. If *C. parvum* was identified by sequencing, all the IF-positve specimens from that farm were considered to be *C. parvum* Although the pathogenicity of *Giardia* spp. in calves is still debated, the presence of *Giardia* cysts was assessed on the same occasion using the same bivalent kit. Details of the laboratory methods and method validations have been provided in Chapter 4.

5.4.3. Collection of farm-level data

A questionnaire was delivered to the farmers by the samplers on the same day of sampling, to elicit information about farm demographics (breed and herd-size), infrastructure (eg. type of barns, floors, feeders, bedding, etc.), and husbandry (eg. Colostrum, milk feeding practices, disinfection methods and cows' vaccination against enteropathogens). The questionnaire was written in three phases. Initially, a draft was evaluated by 15 MU animal science post-graduate students and academic staff, with the aim of drawing the investigators' attention to potential problems of comprehension (understanding the question), information retrieval/recall (access to the information requested); judgment (willingness to provide the answer); and response (whether or not all response options were available) (Scottish government social research group social science methods series guide7; http: //www. scotland.gov.uk/ Topics/ Research/About/Social-Research/ Methods-Guides/Guide-7, accessed 15 June 2011) (Appendix V and VI). The outcome of this evaluation was used to modify some of the questions and produce a second draft which was re-assessed by three dairy farmers not enrolled in the study for further comments before the definitive version was finalised (Appendix VII).

5.4.4. Analysis of data

Several studies reported positive associations between the presence of BRV and BCV, *C. parvum*, K99 and *Salmonella* spp. in the faeces, and diarrhoea (García et al., 2000; Uhde et al., 2008; Bartels et al., 2010; Izzo et al., 2011). However, whereas K99 affects calves during the first five days of life (Bazeley, 2003; Foster and Smith, 2009; Gulliksen et al., 2009b), BRV, BCV and *C. parvum* have longer incubation periods and typically cause a diarrhoea between the second and fourth week of life (Grinberg et al., 2002; Grinberg et al., 2005; Izzo et al., 2011; Almawly et al., 2013). Therefore, in this study two age groups were sampled and analysed separately.

Laboratory and questionnaire data were coded into variables using standardised definitions. Analysis involved a descriptive phase followed by multivariable logistic regression analyses. As 55 missing data occurred in the variable 'calf's sex', the multiple imputation function in the 'Mice' package in R (Van Buuren and Groothuis-Oudshoorn, 2011) was used to assign missing values before the multivariable analyses. The programme imputed plausible values drawn randomly from a probability distribution in each cell containing a missing value (Van Buuren and Groothuis-Oudshoorn, 2011). This process allowed the inclusion of most calves in the models, maximising analytical power. There were also some missing data in the variable 'calf's age', but calves with missing age were eliminated from the multivariable analysis, as the two age-groups were treated as separate populations and analyzed separately. Farms with missing values were eliminated from the multivariable analyses as imputation of the same value for all the calves of the farms was not possible. As imputation should produce plausible values that could have been obtained if the data had been complete, imputed data were evaluated in graphical displays showing the distributions of original and imputed data.

The following research questions were addressed in multivariable analyses using the observations from all the calves:

Q1– Analysis of potential risk factors for NCD: Which variables are independently associated with the presence of a liquid specimen (faecal score 3)?

Q2– Analysis of potential risk factors for enteropathogen shedding: Which variables are independently associated with the presence of the enteropathogens in the specimens?

Mixed logistic regressions models were used for these analyses. Outcome binary variables were the presence/absence of faecal score 3 (for Q1), and presence/absence of each analysed enteropathogen (for Q2). The models included as fixed effects the explanatory variables of interest, with between-farm variability modelled as a random effect (the participating farms were a random sample of the population). The following models were fitted:

$$Y_i = \beta_0 + \beta_1 X_{1i} + \ldots + \beta_k X_{ki} + \mu_{farm(i)} + \varepsilon_i$$

Where:

$$Y = \text{outcome variable}$$

β = regression coefficient (β_0 = intercept; $\beta_{1\ldots k}$ = variable coefficients)

$X_{1i}, \ldots, X_{ki}$ = explanatory variables

$\mu_{farm(i)}$ = farm random effect for the farm of the *ith* calf

$$\mu_{farm(i)} \sim Normal(0, v^2)$$

$$\varepsilon_i \sim Normal(0, \sigma^2) \text{ (Residuals)}$$

σ^2 = Variance of residuals

v^2 = Variance of random effect of farm

The analyses were performed using the 'lme4' and 'mice' packages available on R (De Boeck et al., 2011; Van Buuren and Groothuis-Oudshoorn, 2011). Model building included an initial screening of the relationships between each explanatory variable of interest (reported in Table 5.1) and the outcome variable, with the farm identifier modelled as a random effect. This screening was done before the imputations. The variables that included at least one comparison with $p<0.2$ were selected for multivariable logistic regression (LR) analysis. LR was automatically performed by the software by the backward elimination method. Before the elimination of each variable, each model was repeated 50 times using 50 imputed datasets. A LR model was fitted separately for each imputed dataset, and the 50 estimates pooled in a final estimate (Van Buuren and Groothuis-Oudshoorn, 2011). The process progressed by backward elimination of the variable showing the highest p-value followed by re-introduction of the previously eliminated variable and selection of the model with the smallest Akaike Information Criterion score between the two (Muller et al., 2013). The analysis was completed when all the remaining variables showed a $p<0.05$. The biologically important variables of "dam vaccination against calf scours" and "calf's sex" were included in all the models, including the final one. Separate analyses were conducted for each age-group. Odds ratios (OR) and 95% confidence interval (CI) not overlapping the null value and a $p<0.05$ were considered statistically significant. Finally, the random farm effects in the final models were visually assessed using caterpillar plots, to assess the degree of variability which could be attributed to unseen variables.

5.5. Results

5.5.1. Descriptive data analysis

In total, 57/1283 (4%) calves had no age recorded, leaving 1226 calves for the descriptive analysis. In addition, one farm with a missing questionnaire and two farms with farm-level missing data were eliminated leaving 94 (97%) farms and 1183 calves for the multivariable analysis. All descriptive analysis results described (Chapter 4).

5.5.2. Multivariable analysis results: risk factors for liquid faeces and enteropathogen shedding in 9-21 day-old calves

For Q1, bivariate screening identified 16 variables with a $p< 0.2$ in this age group (Table 5.1). The variables remaining significant ($p<0.05$) after backward elimination were: presence/absence of *C. parvum*; presence/absence of BRV; calf housing type; type of milk used to feed calves; type of colostrum used to fed calves; timing of feeding colostrum; calf'gender; type of bedding; and cow vaccination status against calf enteropathogens (Table 5.2). The presence of *C. parvum* (OR=2.6; 95% CI: 1.3-5.6; P=0.02) and BRV (OR=2.7; 95% CI: 1.3-5.9, p=0.01) were independently associated with increased odds of passing liquid feces. Conversely, housing calves in closed barns reduced the odds of a liquid specimen as compared with open barns (OR=2.1; 95% CI: 1.1-12.2; p=0.03), and partially-open barns (OR=3.5; 95% CI: 1.1-10.5; p=0.04). Administering colostrum within two hours of life decreased the odds of liquid faeces compared with feeding after 6 h (OR=0.4, 95% CI: 0.02-0.8; p=0.03). Feeding stored (OR=4.8, 95% CI: 1.1-12; p=0.04) or mixed (OR=3.3, 95% CI: 1.3-8.8; p=0.03) colostrum increased the odds of diarrhoea compared with feeding the first milked colostrum to calves. Interestingly, feeding waste milk to calves was associated with decreased odds of observing a liquid specimen (OR=0.4, 95% CI: 0.1-0.8; p=0.01). The odds of liquid faeces were significantly lower in female calves compared with males (OR=0.2, 95% CI: 0.07-0.7; $p<0.01$), and on farms that used straw as bedding compared with the use of sawdust (OR=0.2, 95% CI: 0.03-0.9; p=0.03). Vaccination of cows against calf enteropathogens (combined vaccines against BRV, BCV and K99 are used in New Zealand) decreased the odds of liquid faeces (OR=0.2; 95% CI: 0.1-0.9; p=0.03) in this age group. Caterpillar plots did not indicate significant variation between the random effects of the farms,

suggesting most contributing factors were captured by the final model (appendix VIII).

Finally, in order to compare the odds of liquid faeces between co- and mono-infection, the variables presence/absence of *C. parvum*, BRV, BCV, and *Salmonella* were withdrawn from the final model and a new categorical variable including the following values was fit: 1, presence of any mono-infection; 2, presence of any co-infection; 3, absence of infection. In this new model, the odds of liquid faeces was significantly greater in co-infection compared with mono-infection (OR=2.5; 1.3-4.8; p=0.01) (the other variables retained their original effect).

For Q2, the factors that remained significantly associated with the faecal shedding of enteropathogens in 9-21 day-old calves in the final model are reported in Table 5.3. Faecal shedding of *C. parvum* was associated with the shedding of *Giardia* cysts (OR=2.2; 95% CI: 1.4-3.4; p<0.01). A similar pattern was observed for BRV and BCV co-shedding (OR=3.5; 95% CI: 1.6-7.3, p<0.01). Interestingly, dams' vaccination against calf enteropathogens was not associated with decreased odds of shedding of any enteropathogens (p>0.05).

5.5.3. Multivariable analysis results: risk factors for diarrhoea and enteropathogen shedding in 1-5 day-old calves

Analysis for Q1 indicated that K99 infection was associated with increased odds of observing a liquid specimen (OR=4.6; 95% CI: 1.2-16.1; p=0.02). Interestingly, the odds of passing liquid faeces were lower in calves on farms where only females (OR=0.4; 95% CI: 0.1-0.9; p=0.04), or females and males (OR=0.2; 95% CI: 0.01-0.8; p=0.02) were in charge of the calves, compared with farms where only male caretakers were employed (Table 5.2). Analysis for Q2 indicated no variables significantly associated with the presence of enteropathogens in this age group (p>0.05).

5.6. Discussion

With a large population of cows and short calving seasons resulting in the synchronous presence of numerous newborn calves on farms, New Zealand offered a 'natural experiment' setting for a randomised risk factor study of NCD. As this economically important condition might be determined by complex interactions

between infectious agents, the host and environmental factors, the study of its aetiology requires multivariable analytical approaches. Previous studies have provided valuable information about the risk factors of NCD. Some studies used data from questionnaires, others focused only on microbiological results without emphasising other factors, or analysed for the presence of the infectious agents at the farm, rather than the calf level (Bartels et al., 2010). Furthermore, the different ways in which variables are coded, and the statistical methods used, hinder direct comparison between studies. We present the results of a cross-sectional study performed in New Zealand, which has a number of strengths and limitations. The short calving season and the cross-sectional design allowed the sampling of a large number of calves, country-wide. Cross-sectional studies may not provide cause-effect information when the temporal relationships between the variables are not known (for instance, negative laboratory results could have indicated pre- or post-patent periods, rather than uninfected calves). In our study, this potential limitation was in part compensated by the analysis of two discrete calves' age groups, which allowed a longitudinal dimension of enquiry. Another point of strength was the *Cryptosporidium* genotyping, which allowed differentiation between *C. parvum* and the other phenotypically indistinguishable but apparently non-pathogenic species cycling in calves (Chapter 4). Due to the limitations of the questionnaire, not all the potential risk factors for NCD could be analysed. Attempts to control for potential confounding was done by design, either by using two age groups of calves or analytically, by the multivariable analysis. Confounding was also addressed by including biologically relevant variables, such as vaccination of dams against calf enteropathogens and calf sex, in all models, regardless of their initial P-value. Nevertheless, potential for uncontrolled confounding may still exist. Lastly, variability attributable to farm effects was assessed in R by visualisation of caterpillar plots, which did not indicate the existence of significant variation between the random effects of the farms, suggesting that most factors contributing to the diarrhoea were captured by the final models.

5.6.1. Risk factors for faecal score 3 in 9-21 day-old calves

In our study, the detection of BRV and *C. parvum,* but not of BCV, was independently associated with increased odds of a liquid specimen in 9-21 day-old calves. Although BCV has been considered an important causative agent of NCD in

some countries (Björkman et al., 2003; Gumusova et al., 2007; Uhde et al., 2008; Izzo et al., 2011), the prevalence and impact of this agent on calf health in New Zealand has traditionally been considered low (Durham, 1979; Vermunt, 2000). Furthermore, the virus has been identified in only ~2% of the specimens analysed by diagnostic laboratories (Isobel and David, personal communication, 2012). We later corroborated this result in a more parsimonious multivariable model which included the binary explanatory variables of presence/absence of BCV, BRV and *C. parvum*, and the binary variable of faecal score 3 (yes/no) as outcome variable (not shown). The lack of association between BCV and diarrhoea is consistent at least with one large previous study performed overseas (Bartels et al., 2010). Some authors have suggested that co-infections with multiple agents could cause a more severe diarrhoea than mono-infections (Belton, 1995; De la Fuente et al., 1999; García et al., 2000; Hoet et al., 2003), and it could be hypothesised that control of one organism could reduce the severity of diarrhoea. The results of the last LR model conducted in this study, indicated increased odds of liquid faeces in co-infection compared with mono-infection, providing statistical support to this hypothesis. Unfortunately, the complexity of the question did not allow statistical ascertainment of the differences between various possible co-infection combinations.

The results of this study suggest a number of host, environmental and husbandry factors influencing the odds of diarrhoea. The odds of passing liquid faeces was smaller on farms that housed calves in closed barns, compared to farms with open or partially-open barns. Open or partially-open barns could expose newborn calves to wind and rain, predisposing them to diarrhoeal disease. Cold or windy weather may cause calves to come together in a small confined area of the barn in order to warm up. This may increase their exposure to the enteropathogens. In this study, however, the association of the type of barn with liquid faeces was independent from the presence of the agents. The odds of liquid faeces was directly correlated with the duration of the diarrhoea. In other words, calves passing liquid faeces for longer periods of time were more likely to pass liquid faeces on the day of sampling. It is possible that exposure to the weather elements in open barns could have prolonged the recovery time beyond the patent period.

The provision of protection against adverse weather conditions seems a cost-effective way to reduce the incidence of diarrhoea and improve calf health in New Zealand, and this could be further tested in intervention studies that control for other potential confounding factors.

A number of colostrum- and milk-feeding methods associated with decreased odds of diarrhoea in the present study find support from results obtained overseas. In our study, calves on farms administering colostrum within two hours of birth showed decreased odds of diarrhoea (OR=0.4, 95% CI: 0.02-0.8; p=0.02) compared with the other groups, reinforcing the importance of feeding colostrum as soon as possible after birth. It is worth noting that there has probably been some confusion with this survey question and the timing of first colostrum feeding was probably not within two hours of birth for all the calves, as it is unlilely that farmers would collect calves so frequently. Colostrum deficiency is a known risk factor for calf scours (Bellinzoni et al., 1989; Saif and Fernandez, 1996; Fayer et al., 2000a; Bazeley, 2003). Delaying the intake of colostrum decreases the intestinal absorption of immunoglobulin and fat-soluble vitamins, which may affect calf immunity. Compared with calves that receive colostrum within the first few hours of life, calves receiving colostrum 12 to 24 hours after birth tend to have lower plasma concentrations of alpha-tocopherol, beta-carotene and retinol for almost a month after birth (Durham, 1979). These vitamins play a significant role in calf immunity and their deficiency can predispose neonates to enteric infections. Bacterial contamination may reduce the availability and the quality of the antibodies and immunoglobulins in colostrum (Godden, 2008), and colostrum which is not fed within 2 hours of collection should be kept refrigerated to reduce microbial growth, but this is not always feasible on farms. Pasteurisation is also an effective method of controling bacterial contamination of colostrum (Elizondo-Salazar et al., 2010), but it can result in immunoglobulin degradation (McGuirk and Collins, 2004). Administering first colostrum was also associated with reduced odds of a liquid specimen compared with stored (OR=4.8, 95% CI: 1.1-12; p=0.04) and mixed colostrum (OR=3.3, 95% CI: 1.3-8.8; p=0.03). In addition to having a reduced immunoglobulin content compared with first colostrum, mixing colostrum from several cows could increase the likelihood of disease transmission to multiple calves from a single infected donor cow. It is possible that some farms dilute the first colostrum with colostrum from successive milkings,

losing the benefits associated with the administration of the first colostrum before gut closure. However, this variable is difficult to interpret as in the New Zealand pastoral system calves have some time to suckle naturally and may ingest first colostrum before they are removed from the paddock to the calf barn. It should be noted that the Code of Welfare for Dairy Cattle (1999) (Anonymous, 2010a) addresses the necessity for every newborn calf to obtain colostrum from its dam or any newly calved cow as soon as possible after birth. However, it has been reported that ~50% of the calves in New Zealand might not receive sufficient colostrum from their dams, even when kept together for up to 24 hours (Vermunt et al., 1995; Wesselink et al., 1999; Irshad et al., 2012). Therefore, it is very important that calves are fed colostrum individually, to be sure that they receive an adequate amount of immunoglobulins.

Interestingly, in this study calves raised on farms that used waste milk had lower odds of diarrhoea than calves on farms not using this type of milk (OR=0.4, 95% CI: 0.1-0.8; p=0.01). Comparable results have been reported previously (Chardavoyne, 1979). Most waste milk originates from mastitic cows or from cows treated with antibiotics. It could hypothesised that the potential benefits of feeding waste milk to calves might have derived from some sort of regulation of the intestinal flora or the suppression of bacterial overgrowth in milk during storage. Although waste milk could be considered a valuable nutritional resource, its administration to calves might promote development of antimicrobial resistance in commensal intestinal bacteria. On the other hand, waste milk also represents a problematic biomass which, if unconsumed, could promote development of resistance in environmental bacteria. Although this result contributes to the discussion on the risks and benefits associated with the administration of waste milk to calves, a thorough discussion of this matter was beyond the scope of this study.

In this age group, female calves had lower odds of diarrhoea than males (OR=0.2, 95% CI: 0.07-0.7, p<0.01). Male calves in New Zealand dairy herds are of little economic value, and the long-term health of female calves is considered important, as the females are used as replacements. This result could indicate a less stringent neonatal care provided to male, compared to female calves in New Zealand farms. This problem has been examined in a study of commercial U.S. veal farms, which found that only 22% of male calves had received adequate colostral immunoglobulins and the majority had an inadequate immune system and were more susceptible to

infectious pathogens (McDonough et al., 1994). Furthermore, in some systems the rate of dystocia appears to be greater in male calves (Bellows et al., 1982; Berger, 1994; Johanson and Berger, 2003). Dystocia could decrease the effectiveness of passive immunoglobulin transfer due to a combination of reduced calf vigor and delayed ingestion of colostrum. Newborn calves which require assistance during parturition might be recumbent for longer periods after birth, and might be more exposed to faecal pathogens.

The use of straw as bedding material was associated with decreased odds of liquid faeces compared to sawdust. This finding is consistent with previous reports suggesting straw as an optimal bedding material (Panivivat et al., 2004; Hill et al., 2011). Straw provides good insulation and seems to keep calves clean, warm, dry and comfortable (Brenner et al., 2005; Andrieu, 2007; Stull and Reynolds, 2008; Mohler et al., 2009). Fecteau et al. (2009) stated that straw provides good insulation and is likely to be dry on the surface, as liquids tend to accumulate in the bottom layers. Also, when sawdust is used as bedding, calves tend to ingest it, which could disturb gastrointestinal function and act as a vehicle for the entry of enteric pathogens into the digestive tract (Andrieu, 2007).

Dam vaccination against calf enteropathogens is commonly implemented during pregnancy to increase colostral transfer of specific antibodies to the offspring. In this study, dam vaccination was associated with decreased odds of liquid faeces. This result accords with experimental evidence of the efficacy of dam vaccines (Saif and Smith, 1985; Bellinzoni et al., 1989; Theil and McCloskey, 1995; Gonzalez et al., 2010). In some studies, however, calves born on farms vaccinating dams were more likely to have diarrhoea than calves born on farms that did not apply such vaccinations (Waltner-Toews et al., 1986a; Frank and Kaneene, 1993; Bendali, 1999b). An explanation of this apparent contradiction could be that in those studies, farmers dealing with severe forms of diarrhoea tried vaccines more often than farmers experiencing mild disease, highlighting the weakness of cross-sectional studies for cause-effect relationship inference. The lack of association between BCV and diarrhoea found in this study suggests that the BCV component of the dam vaccines available in New Zealand may not contribute to the overall efficacy of these products in the field. Finally, the caterpillar plots generated from the final model for

this age group did not show variation worthy of note between the random effects of the farms.

5.6.2. Risk factors for faecal score 3 in 1-5 day-old calves

In accordance with previous reports (Snodgrass et al., 1986; Holland, 1990), there was a positive association between the presence of K99 and liquid faeces in 1-5 day-old calves (OR=4.6; 95% CI: 1.2-16.1; p=0.02). Infections with K99 are characterised by profuse diarrhoea, and in acute cases calves become dehydrated, losing around 12% of their body-weight within the first six hours of infection (Tzipori et al., 1981). Conversely, the presence of the other agents was not associated with increased odds of liquid specimens in this age group. This is consistent with a longer prepatent and incubation period of BRV and *C. parvum,* which tend to cause diarrhoea during the second and third week of life (Runnels et al., 1980; Tzipori et al., 1981; Foster and Smith, 2009).

Another interesting association in this age group was the protective effect of the presence of female caretakers on farms. This result is consistent with reports of lower neonatal calf mortality rates on farms where females were responsible for the care of the calves (Hartman et al., 1974; Losinger and Heinrichs, 1997). There is no clear explanation for this association, but it could be that female caretakers take better care of calves than males.

5.6.3. Risk factors for enteropathogens shedding

A significant association ($p<0.05$) between *C. parvum* and *Giardia* shedding was recorded in this study. A number of studies have showed concurrent *Giardia* and *C. parvum* infections in dairy calves (Bednarska et al., 1998; Björkman et al., 2003; Hamnes et al., 2006; Mark-Carew et al., 2010) and the two parasites may have similar transmission routes (Bednarska et al., 1998; Björkman et al., 2003; Graczyk et al., 2003). There is no clear explanation for this association. However, it is possible that the environment favouring *Cryptosporidium* infection could also promote the proliferation of *Giardia* spp. A similar hypothesis could be made for the association between BRV and BCV. Concurrent BRV and BCV infections have been repeatedly described (Durham et al., 1979; García et al., 2000; Uhde et al., 2008; Izzo et al., 2011). Finally, despite the decreased odds of liquid faeces in calves on farms using dam vaccination against calf enteropathogens, no association between vaccination

and enteropathogen shedding was found in this study. Perhaps, increasing specific passive immunoglobuling transfer through the use of vaccine is not suffient to eliminate the agents, which continue to cycle at levels detectable by diagnostic ELISAs but not sufficient to cause severe disease. It must be said, however, that reducing shedding is not the aim of the vaccination, which is to reduce diarrhoea burden.

5.6.4. Additional putative risk factors

The odds of passing liquid faeces and faecal shedding of enteropathogens were similar in both islands. The impact of *Salmonella* could not be assessed due to the small number of isolations and the possible presence of unexamined infectious agents, such as *Campylobacter jejuni* and *Clostridium* spp., cannot be ruled out (Cornaglia et al., 1992), although the causative role of these agents is not understood (De Rycke et al., 1986; Holland, 1990; Foster and Smith, 2009). Other factors found to be associated with NCD overseas, such as herd size, were not found to be significant in this study. It is not necessarily possible to generalise risk factors reported in one country. Furthermore, some risk factors published overseas were obtained from studies performed on beef herds (Schumann et al., 1990; Wittum et al., 1994; Bendali et al., 1999b), and/or using different study designs and analytical techniques.

Finally, there are a number of potential non-infectious risk factors of NCD, such as overfeeding/irregular feeding and milk replacer quality (Woodford et al., 1987; Holmes, 2002; Drackley, 2008) that were not analyzed. However, the caterpillar plots suggested that most variation which could be attributed to risk factors was captured by the models.

5.7. Conclusions

The present study provides data on the risk factors for calf diarrhoea in New Zealnd dairy farms, most of which manage short calving seasons. The results indicate a positive relationship, most likely causative, between infections with *C. parvum* and BRV in 9-21 day-old calves, and with K99 in 1-5 day-old calves, and liquid faeces. Conversely, the presence of BCV was not associated with increased odds of diarrhoea. This study identified a number of modifiable risk factors pertaining to

colostrum management, infrastructure and even human resource management, which could be further analysed in controlled studies and/or addressed by farmers in order to mitigate the burden of NCD on New Zealand farms. Different housing systems and the large numbers of calves present over a short period of time during the calving season in New Zealand may have an influence on the epidemiology of the enteropathogens and the risk factors for NCD compared to other countries where calves are present year round and are reared under different conditions. Furthermore, in most dairy farms in New Zealand, calvings occur on paddocks and the calves are usually allowed to suckle colostrum from their mothers, in contrast with countries where calves are separated from the mothers soon after birth and are bottle-fed. The validity of these results, therefore, do not require corroboration in other countries.

Table 5.1. Bivariate screening of the variables elicited by the questionnaire against a binary outcome variable of faecal score 3/Other faecal score, with the farm identifier used as random effect. * These variables were not included in multivariable because they dependent on the other variables.

Variable	Categories	P-value; Odds Ratio (95% Confidence Interval)	
		1-5 day-old calves	9-21 day-old calves
Calf level variables			
Cryptosporidium parvum shedding	Yes/No	0.06, 2.6 (0.9-7.3)	0.01, 2.5 (1.1-5.5)
Giardia spp. shedding	Yes/No	0.7, 1.2 (0.3-4.4)	0.04, 0.4 (0.1- 0.9)
BRV shedding	Yes/No	0.2, 1.5 (0.7-3.3)	<0.01, 3.1 (1.4-6.5)
BCV shedding	Yes/No	0.8, 1.2 (0.3-5.1)	0.5, 1.4 (0.45-5.1)
E. coli K99 shedding (1-5 day-old calves only)	Yes/No	0.02, 4.9 (1.2-19.2)	
Salmonella spp. shedding	Yes/No	0.4, 2.8 (0.2-16)	0.85, 1.2 (0.11- 13.70)
Co-infection (any combination of agents)*	Yes/No	0.9, 0.9 (0.3-3.4)	0.12, 2.2 (0.8-5.8)
Calf gender	Female/male	0.2, 0.6 (0.3-1.2)	0.12, 0.4 (0.15-1.2)
Farm-level variables			
Dam's vaccination	Yes/No	0.7, 1.1 (0.4-2.7)	0.08, 0.5 (0.08-1.00)
Feeders cleaned between pens	Yes/No	0.1, 2.5 (0.8-7.7)	0.58, 0.8 (0.42-1.60)
Using same feeders for multiple pens	Yes/No	0.5, 0.7 (0.2-2.1)	0.59, 1.2 (0.54-2.86)
Use of water blaster	Yes/No	0.09, 2.5 (0.8-7.5)	0.11, 2.1 (0.83-5.27)
Calves of different age groups in the same pens	Yes/No	0.9, 1.1 (0.4-2.5)	0.53, 1.2 (0.62-2.47)
Pens with solid partitions	Yes/No	0.8, 1.1 (0.5-2.6)	0.53, 0.7 (0.38-1.64)
Bobby calves with replacement in the same pen	Yes/No	0.4, 1.4 (0.5-3.7)	0.52, 0.7 (0.36-1.66)
Sick pen available	Yes/No	0.2, 0.6 (0.2-1.4)	0.46, 0.7 (0.39-1.52)
Water troughs are regularly cleaned	Yes/No	0.5, 1.1 (0.4-2.5)	0.93, 0.9 (0.48-1.94)
Drinking water ad libitum	Yes/No	0.5, 0.7 (0.2-2.1)	0.41, 0.7 (0.30-1.62)
Feeding calves with waste milk	Yes/No	0.2, 0.5 (0.2-1.2)	0.01, 0.5 (0.39-0.90)
Milk temperature at feeding	warm/cold	0.3, 0.7 (0.1-1.4)	0.44, 0.7 (0.40-1.49)
Milk always available for calves	Yes/No	0.6, 0.8 (0.4-1.9)	0.22, 0.6 (0.33-1.29)
Colostrum always available to calves	Yes/No	0.2, 0.2 (0.01-1.2)	0.41, 0.7 (0.37-1.49)
Importation of cows from other farms	Yes/No	0.1, 0.4 (0.1-1.3)	0.19, 0.5 (0.25-1.31)
Importation of calves from other farms	Yes/No	0.6, 0.7 (0.2-2.6)	0.35, 1.5 (0.60-3.95)
Minerals are added to drinking water	Yes/No	0.3, 0.4 (0.07-2.3)	0.61, 0.7 (0.221-2.44)
Numbers of days calves are kept housed from birth	1-30	Reference category	
	31-60	0.1, 0.3 (0.2-1.1)	0.8,0.7 (0.1-3.2)
	>60	0.2, 0.7 (0.4-2.2)	0.9, 0.3 (0.09-1.5)
Number of feeds per day	1-3	Reference category	
	>3	0.8, 0.5 (0.1-1.7)	0.2, 0.7 (0.1-1.4)
Quantity of colostrum administered daily in litres	1-3	Reference category	
	>3	0.9, 1.1 (0.3-3.7)	0.4, 0.8 (0.2-1.6)
Bedding cleaning method	Topped up	Reference category	
	Topped up+ spray disinfection	0.5, 0.7 (0.2-2.1)	0.07, 0.4 (0.1-1.1)
	Complete replacement	0.1, 0.4 (0.09-1.5)	0.09, 0.3 (0.1-1.2)
	Complete replacement and disinfection	0.1, 0.4 (0.1-1.4)	0.01, 0.2 (0.1-0.7)
Type of hard floor in pens	Concrete	Reference category	
	Gravel stone	0.7, 1.2 (0.2-5.1)	0.4, 0.5 (0.1-2.4)
	Earth	0.7, 1.3 (0.3-5.4)	0.6, 1.4 (0.3-6.1)
	More than one type	0.8, 1.2 (0.2-7.1)	0.8, 0.9 (0.1-5.4)
Bedding type	Straw	Reference category	
	Sawdust	0.6, 1.3 (0.3-4.9)	0.13, 3.5 (0.7-1.8)
	Woodchips	0.9, 1.0 (0.2-3.7)	0.50, 1.8 (0.3-10.8)
	More than one type	0.5, 1.6 (0.3-6.7)	0.87, 1.2 (0.2-9.1)
Type of barn	Closed barn	Reference category	
	Partially open	0.2, 0.5 (0.1-1.5)	0.03, 3.3 (1.1-9.9)
	Open barn	0.02, 9.4 (1.4-60.2)	0.37, 3.2 (0.2-14)
	More than one type	0.8, 0.9 (0.3-2.2)	0.31, 1.8 (0.6-5.6)
Type of sick pen	Each pen has a sick pen	Reference category	
	One sick pen for the whole farm	0.5, 0.3 (0.1-3.5)	0.98, 0.989 (0.388-2.52)

Table 5.1. (continue) Bivariate screening of the variables elicited by the questionnaire against a binary outcome variable of faecal score 3/Other faecal score, with the farm identifier used as random effect. * These variables were not included in multivariable because they dependent on the other variables.

Variable	Categories	P-value; Odds Ratio (95% Confidence Interval)	
		1-5 day-old calves	1-5 day-old calves
Type of milk fed to calves	Fresh milk	Reference category	
	Powdered	0.7, 1.3 (0.1-11)	0.73, 0.6 (0.1-11.6)
	Fresh and powdered milk	0.1, 0.3 (0.06-1.4)	0.11, 0.2 (0.02-1.5)
Time of first colostrum feeding	Within 2hrs	Reference category	
	Within 2 to 6hrs	0.2, 3.5 (0.4-24)	0.38, 2.7 (0.3-24)
	After 6 hrs	0.7, 1.3 (0.1-10)	0.32, 2.9 (0.3-25)
	More than one system	0.1, 4.2 (0.4-38)	0.19, 5.5 (0.5-28)
Type of colostrum offered to calves	First colostrum	Reference category	
	Stored colostrum	0.5, 2 (0.1-22)	0.33, 2.9 (0.2-31)
	Mixed colostrum	0.5, 1.4 (0.4-4.4)	0.02, 4.1 (1.1-15)
	More than one type	0.6, 1.2 (0.4-3.4)	0.21, 1.9 (0.6-5.9)
Vaccinate all cows or only a subset*	Not vaccinated	Reference category	
	Vaccinate all cows	0.8, 1.1 (0.5-1.9)	0.09, 0.6(0.24-1.1)
	Vaccinate only a subset of cows	0.3, 1.5 (0.8-3.6)	0.22, 0.3(0.05-1.96)
Gender of caretakers	Females	Reference category	
	Males only	0.04, 1.5 (1.00- 1.92)	0.5, 1.1 (0.6-1.6)
	Males and females	0.10, 1.3 (.20- 1.8)	0.9, 1.6 (0.2-1.8)
Calving season	Spring calving	Reference category	
	Spring and autumn calving	0.6, 1.6 (0.6-2.1)	0.21, 2.4 (0.20-1.44)
Source of drinking water	Town supply	Reference category	
	Bore hole	0.5, 0.1 (0.06-2.9)	0.34, 0.5 (0.1-2.6)
	Rain water	0.6, 2.5 (0.8-23)	0.77, 1.5 (0.2-30)
	Stream	0.1, 1.6 (0.7-9.6)	0.99, 1.0 (0.1-7.6)
	More than one source	0.4, 2.1 (0.5-12.2)	0.74, 1.9 (0.03-17.1)
Herd size	(151-450) cows	Reference category	
	(451-650) cows	0.4, 0.5 (0.05-1.6)	0.31, 0.2 (0.5-1.8)
	(>650) cows	0.7, 1.3 (0.4-2.0)	0.58, 0.9 (0.9-1.0)
Island	North Island	Reference category	
	South Island	0.8, 1.6 (0.8-3.2)	0.91, 1.1 (0.5-1.6)

Table 5.2. Variables associated with liquid faeces in the final multivariable model with random farm effect

Variable description	Outcomes	P-value	Odds Ratio (95% CI)
9-21 day-old calves			
C. parvum infection	No (reference)		
	Yes	0.02	2.6 (1.3-5.6)
BRV infection	No (reference)		
	Yes	0.01	2.7 (1.3-5.9)
Feeding calves with waste milk	No (reference)		
	Yes	0.01	0.4 (0.1-0.8)
Dam's vaccination	No (reference)		
	Yes	0.03	0.2 (0.1-0.9)
Calf gender	Male (reference)		
	Female	0.00	0.2 (0.07-0.7)
Type of colostrum offered to calves	First colostrum (reference)	0.01 (Overall p-value)	
	Stored colostrum	0.04	4.8 (1.1-12)
	Mixed colostrum	0.03	3.3 (1.3-8.8)
	More than one type	0.18	1.9 (0.7-5.1)
Timing of first colostrum feeding after birth	6 hr from birth (reference)	0.02 (Overall p-value)	
	Within the first 2 h	0.02	0.4 (0.02-0. 8)
	Within 2 to 6 h	0.08	0.3 (0.01-1.2)
	More than one system	0.10	0.6 (0.3-1.7)
Type of barn	Closed barn (reference)	< 0.01 (Overall p-value)	
	Open barn	0.03	2.1 (1.1-12.2)
	Partially open barn	0.04	3.5 (1.1-10.5)
	More than one type of barn	0.35	1.5 (0.1-4.6)
Bedding type	Sawdust (reference)	0.04 (Overall p-value)	
	Straw	0.03	0.2 (0.03-0.9)
	Woodchips	0.15	0.4 (0.1-1.5)
	More than one type	0.65	0.4 (0.1-1.2)
1-5 day-old calves			
E. coli K99 shedding	No (reference)		
	Yes	0.02	4.6 (1.2- 16.1)
Caretakers' gender	Males (reference)		
	Females	0.04	0.4 (0.1- 0.9)
	Males and females	0.02	0.2 (0.01- 0.8)

Table 5.3. Variables associated with faecal shedding of enteropathogens in the final model with random farm effects

Enteropathogen	Variable	Outcomes	P-value	Odds Ratio (95% CI)
C. parvum	Shedding of *Giardia* spp.	No		
		Yes	0.01	2.1 (1.31-3.39)
BRV	BCV infection	No		
		Yes	0.01	4.1 (1.9-8.5)
BCV	BRV infection	No		
		Yes	0.01	3.1 (1.6-6.1)

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STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Julanda Al Mawly

Name/Title of Principal Supervisor: Alex Grinberg

Name of Published Research Output and full reference:

Risk factors analysis for neonatal calf diarrhoea and enteropathogens shedding in New Zealand dairy farms

In which Chapter is the Published Work: Five

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  The candidate has had a major input in the analysis and wrote the first draft of the Chapter. In addition, he has led numerous discussions with the supervisory team, considered the supervisors' suggestions and produced the subsequent versions and the final Chapter. This effort qualified him as first author of a paper submitted for publication.

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Principal Supervisor's signature — 25/8/2014 — Date

Chapter 6

6. GENETIC DIVERSITY AND BIOGEOGRAPHY OF *CRYPTOSPORIDIUM* PARASITES ISOLATED FROM NEWBORN CALVES ON NEW ZEALAND DAIRY FARMS

6.1. Preamble

Among the enteropathogens analysed in previous chapters, *Salmonella* spp. and *C. parvum* are of significant public health impact. Whereas *Salmonella* spp. were found at low prevalence (indicating that newborn calves are probably not significant reservoirs of this organism in New Zealand), *C. parvum* was found in a high proportion of the farms. This chapter describes a molecular analysis of *Cryptosporidium* parasites isolated in the previous prevalence study, and a genetic comparison with human *C. parvum* clinical isolates collected from the same regions by diagnostic laboratories. Information provided in this study could provide the basis for future human cryptosporidiosis source attribution studies.

6.2. Abstract

Eighty-four *Cryptosporidium* parasites collected from newborn calves on 55 randomly selected New Zealand dairy farms were analysed by sequence analysis of the 18s rRNA, HSP70, and gp60 genes. Only *C. parvum* and *C. bovis* were identified. *C. parvum* accounted for 77/84 (91.7%) of the specimens, and was present on 49/55 (89%) farms. Conversely, the non-pathogenic species *C. bovis,* reported here for the first time in New Zealand, was found in 6/55 (11%) of the farms. Seven gp60 alleles belonging to the subtype families IIa and IId were found among the 77 *C. parvum.* Three of these (the IIaA16G3R1, IIaA18G3R1, and IIaA19G4R1) had been previously reported in bovine *C. parvum* in New Zealand. Comparison between the gp60 alleles of the *C. parvum* isolates with the alleles of 319 previously-genotyped human isolates from New Zealand denoted significant similarities between the parasites originating from both host species. Whereas the two most abundant gp60 alleles were geographically widespread in both the North and South Island, a number of alleles were geographically restricted, reflecting local microepidemic transmission within and between the two host populations.

6.3. Introduction

Protozoa belonging to the genus *Cryptosporidium*, in particular the intestinal species *C. hominis* and *C. parvum,* are cosmopolitan parasites of humans and livestock (Abd El Kader et al., 2012; Alves et al., 2006; Alves et al., 2003; Arslan and Ekinci, 2012; Díaz et al., 2010; Grinberg et al., 2003; Imre et al., 2013; Quilez et al., 2008). Whereas *C. hominis* is predominantly found in humans, *C. parvum* cycles extensively in animals and is considered potentially zoonotic (Budu-Amoako et al., 2012; Chalmers et al., 2011; Fayer, 2010a; Xiao, 2010; Xiao and Feng, 2008).

Currently, more than 20 species which may differ in host range and tissue tropism are recorded within the genus *Cryptosporidium* (Fayer and Santín, 2009; Plutzer and Karanis, 2009; Xiao, 2010). Of these, *C. parvum, C. bovis, C. andersoni* (Fayer et al., 2007; Santín et al., 2008), *C. ryanae* (formerly the 'deer-like' genotype) (Fayer et al., 2008), *C. ubiquitum* (formerly the 'cervine genotype') (Fayer et al., 2010b), and *C. hominis* (Abeywardena et al., 2012; Chen and Huang, 2012; Kang'ethe et al., 2012) have been isolated from cattle. Whereas the gastric species *C. andersoni* produces oval oocysts and is predominantly found in juvenile or adult cattle, *C. parvum, C. bovis, C. ryanae and C. hominis* produce round, phenotypically identical oocysts, and are mostly isolated from pre-weaned and weaned calves (Fayer and Xiao, 2008; Rzezutka and Kaupke, 2013; Silverlås et al., 2010b). In addition, *C. suis* and *C. scrofarum* (syn. Pig genotype II) have also been identified in cattle (Ng et al., 2011; Ryan and Power, 2012). The clinical significance in calves, and the zoonotic potential of *C. bovis, C. ryanae, C. ubiquitum* and *C. hominis* are not well understood. Conversely, *C. parvum* is considered worldwide a frank pathogen, and among the most important causative agents of calf diarrhoea during the first month of life (De Graaf et al., 1999; Fayer and Xiao, 2008; Foster and Smith, 2009; O'Handley et al., 1999; O'Handley, 2007; Wyatt et al., 2010; Xiao, 2010). Furthermore, *C. parvum* is also one of the most common species found in humans, and newborn calves are considered amplifiers of this potentially zoonotic species in nature (Fayer et al., 2010a; Fayer and Xiao, 2008; Santín et al., 2008; Wielinga et al., 2008; Xiao, 2010).

Humans can contract cryptosporidiosis through several transmission routes, including the human-to-human (Abd El Kader et al., 2012; Fayer, 2004; Leitch and He, 2011),

and zoonotic routes (Budu-Amoako et al., 2012; Cacciò et al., 2005; Chako et al., 2010; Xiao, 2010; Grinberg et al., 2013), or through the ingestion of contaminated food or water (Fayer et al., 2004; Mac Kenzie et al., 1994; Monge and Chinchilla, 1996; Ortega et al., 1997). The relative contribution of these transmission routes to the epidemiology of cryptosporidiosis is not completely clear due to the absence of tools for phenotypic differentiation of *Cryptosporidium* taxa. Several molecular markers developed specifically to distinguish between *Cryptosporidium* species and subtypes (Cacciò et al., 2005; Sulaiman et al., 2005; Xiao, 2010; Xiao et al., 2004), have been applied extensively in retrospective studies, enhancing the understanding of the epidemiology of cryptosporidiosis.

Molecular epidemiological studies of *Cryptosporidium* parasites cycling in cattle have been performed in several countries (Brook et al., 2009; Budu-Amoako et al., 2012; Chen and Huang, 2012; Fayer et al., 2010a; Feltus et al., 2008; Geurden et al., 2007; Imre et al., 2011; Khan et al., 2010; Wielinga et al., 2008; Xiao, 2010). Small-scale studies have also been performed in New Zealand (Abeywardena et al., 2012; Grinberg et al., 2008b; Learmonth et al., 2001; Learmonth et al., 2003).

In New Zealand, human infections with *C. parvum* follow a seasonal pattern, with the number of notifications peaking every year in spring and early summer (soon after the calving season). Learmonth et al. (2004) showed that this peak is accompanied by a substitution of the anthroponotic *C. hominis* (which is seen year round) with the potentially zoonotic *C. parvum*, suggesting a role of calves in the epidemiology of cryptosporidiosis in humans in this country. However, to our knowledge, no large-scale studies characterising these parasites at a national level and comparing them to human isolates have been published, and until this is done, this idea remains speculative.

This paper describes a molecular analysis of *Cryptosporidium* parasites isolated in 2011 from 55 randomly selected dairy farms located in seven New Zealand regions characterised by intensive dairy farming. The parasites were identified to species level by sequence analysis of two loci, and subtyped at the gp60 locus. Finally, the parasites were genetically compared to human *Cryptosporidium* collected from the same regions by diagnostic laboratories between 2003 and 2010.

6.4. Materials and Methods

6.4.1. Selection of farms

Cryptosporidium parasites were collected during a nation-wide study aimed at assessing the prevalence of the major enteropathogens of calves and the risk factors for NCD in New Zealand. Briefly, faecal specimens were collected from 1283 newborn calves on 97 randomly selected dairy farms during a single calving season. In New Zealand, most dairy farms manage short calving seasons usually starting in late winter and ending in the spring, and the sampling for this study was performed between August and October 2011. Farms in five North Island (Waikato, Wellington, Northland, Taranaki and Manawatu-Wanganui) and two South Island regions (Canterbury and Southland) were sampled (Figure 4.1). The sampling frame and randomisation procedures have been described elsewhere (Chapter 4).

6.4.2. Sampling of calves

Two subpopulations of calves were sampled in order to provide adequate diagnostic coverage for all the enteropathogens analysed. The first subpopulation was of calves aged between 1 and 5 days, and was targeted to assess the prevalence of K99 (Acres, 1985; Bazeley, 2003; Bendali et al., 1999b; Foster and Smith, 2009). In addition, the faecal specimens were also tested for *Salmonella* spp., BRV, BCV and *Cryptosporidium* spp. The second subpopulation was that of 9 to 21 days old calves, which corresponds to the age interval at the peak of shedding of *C. parvum*, BRV and BCV (Almawly et al., 2013; Bazeley, 2003; Bendali et al., 1999b; Grinberg et al., 2002; Holland, 1990). Faeces collected from these calves were also tested for *Salmonella* spp., but not for K99. Faecal specimens were collected from the calves' rectum, with handlers changing disposable gloves between animals. Specimens were transported to Massey University (MU) by overnight delivery and stored in refrigeration (4 ± 2°C) until analysed.

6.4.3. Analysis for *Cryptosporidium* and genetic characterisation of the parasites

The analysis for the presence of *Cryptosporidium* oocysts was performed by means of immunofluorescence (IFA) using a commercial kit (Almawly et al., submitted). A total of 198/1283 (15.4%) of the specimens from 57/97 (59%) farms were IFA-positive. One hundred IFA-positive faecal specimens (at least one from each positive

farm) were selected at random and genetically characterised. Genomic DNA was extracted from the faeces using a DNA extraction kit (QIAamp, DNA Stool Mini Kit, Qiagen, Hilden, GmbH) and *Cryptosporidium* parasites were identified and subtyped by PCR-sequencing of the taxonomically informative region of the 18s ribosomal RNA (18S rDNA) gene and the 70 kDA heat-shock protein (HSP70) and gp60 genes.

Primers for amplification of the 18s rRNA were 5-GTTAAACTGCGAATGGCTCA-3 (forward) and 5-CCATTTCCTTC GAAA CA GGA-3 (reverse) (Learmonth et al., 2004). The forward primer annealed with *C. parvum*, *C. bovis*, and *C. hominis* (the positive strand of this region of the gene is not reported in Genbank for *C. ryanae*, *C. andersoni* and C. *ubiquitum*). The reverse primer annealed with *C. parvum*, *C. bovis*, *C. hominis* and *C ryanane,* and differed by one nucleotide from *C. andersoni* (for *C. ubiquitum,* the positive strand of this region of the gene not being reported in Genbank). PCR amplification targeting a ~825 bp fragment of the 18S rDNA was performed in a volume of 20 µl containing 2 µl 10 x PCR buffer, 1 µl dNTP (2mM), 1 µl $MgCl_2$ (50mM), 2 µl non-acetylated bovine serum albumin (2mg/ml) (New England Biolabs, USA), 4 picomoles of each primer and 0.5 µl of Taq polymerase (2 mg/mL) (Platinum® Taq DNA Polymerase, Invitrogen Corporation, Carlsbad CA, USA). The amplification was performed in a thermocycler (SensoQuest, Goettingen, Germany) with initial denaturation at 96^{o}C for 2 min, followed by 40 cycles at 94^{o}C for 30 sec, 55^{o}C for 30 sec and 72^{o}C for 30 sec. PCR products were purified using an in house-developed ethanol purification protocol and the quality and presence of amplicon of the expected size verified by 1% agarose gel electrophoresis. In brief, equal volumes of polyethylene glycol (PEG)/NaCl were added to each micro-tube of PCR product, which was incubated at 37°C for 15 min and then spun in a micro-centrifuge at 13000 rpm for 30 min. The liquid phase was discarded and 100 µl 80% ethanol added. The micro-tubes were centrifuged again at 13000 rpm for 10 min, the liquid phase discarded and DNA dried at 37°C for 30 min. DNA was re-suspended in deionised water and its concentration measured using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Wilmington, DE, USA). Bidirectional Sanger-sequencing of an internal segment of the amplicon was performed using in house developed primers 5- CTCGACTTTATGGAAGGGTTG-3 (forward) and 5-CCTCCAATCTCTA GTTG G CATA-3 (reverse) by a commercial Sanger sequencing provider (Massey University Genome Sequencing service). The forward

sequence annealed to *C. hominis* and *C. parvum*, and differed by 3 nucleotides from *C. bovis* (again, the positive strand of this region of the gene is not reported in Genbank for *C. ryanae*, *C. andersoni* and C. *ubiquitum*). The reverse sequence aligned to *C. parvum*, *C. bovis*, *C. hominis* and *C. ryanae*, and differed by one nucleotide from *C. andersoni* (the positive strand of this region of the *C. ubiquitum* gene is not reported in Genbank). Forward and reverse sequences were aligned and edited manually using Geneious software version 5.6.5 (Biomatters Ltd., http: //www.geneious. com/). Distal and proximal sequence segments that could not be correctly verified were trimmed and the edited sequences aligned with sequences in the Genbank, using the alignment algorithm BLAST (http:// blast. ncbi. nlm.nih.gov/Blast. cgi, accessed in November 2012).

The analysed region of the HSP70 gene comprised a ~ 450 base-pair-long segment conserved within but polymorphic between *C. parvum* and *C. hominis,* and a 12-bp imperfect repeat region displaying length polymorphism within the species (Grinberg et al., 2013) (very little is known about the variability of this locus within the other species). Primers (in-house design) were 5-CACCATCCAAGAACCAAAGG-3 (forward) and 5-GCCTAAAGGTAGAGTGTGCTTTTC-3 (reverse) (Grinberg et al., 2008a). These primers aligned with *C. parvum* and *C. hominis* but the corresponding regions of the gene in the other species are not reported in Genbank. PCR reactions were performed in 20 µl containing 2 µl 10 x PCR buffer, 1 µl dNTP (2mM), 0.6 µl $MgCl_2$ (50mM), 2 µl non-acetylated bovine serum albumin, 4 picomoles of each primer, and 0.5 µl of Taq Polymerase (2 mg/mL). The amplification was performed with initial denaturation at 96^{o}C for 2 min, followed by 40 cycles at 94^{o}C for 20 sec, 57^{o}C for 20 sec, and 72^{o}C for 20 sec. PCR products were purified and the presence of amplicon examined as described above. Bidirectional Sanger-sequencing of the amplicons was performed using the same primers by the sequencing provider and the sequences aligned and edited as described above.

Finally, the *Cryptosporidium* parasites were subtyped by PCR-sequencing a ~850 bp fragment of the 60 kDa glycoprotein gene (gp60) (Strong et al., 2000). This locus is hypervariable within *C. parvum* and *C. hominis* and includes an imperfect TCA/TCG repeat encoding a homoserine, which displays extensive length polymorphism (very little is known about the variability of this locus within the other species). Isolates can contain multiple gp60 alleles, and the sequencing defined the most abundant

allele in each isolate (Grinberg et al., 2013). Primers were 5-ATTGTTCCGC TGTA TTC-3 (forward) and 5- GGAAGGAAC GATGTATCT-3 (reverse) (Peng et al., 2001). The PCR reactions were performed in 20 µl containing 2 µl 10 x PCR buffer, 1 µl dNTP (2mM), 1 µl MgCl2 (50mM), 2 µl non-acetylated bovine serum albumin (2mg/ml) (New England Biolabs, USA), 4 picomoles of each primer, and 0.5 µl of Taq polymerase (Platinum® Taq DNA Polymerase (2 mg/mL); Invitrogen Corporation, Carlsbad CA, USA). The amplification was carried out in a thermocycler (SensoQuest, Goettingen, Germany), with initial denaturation at 96^{o}C for 2 min, followed by 40 cycles at 94^{o}C for 20 sec, 57^{o}C for 20 sec, and 72^{o}C for 30 sec. The PCR products were purified and assessed using the protocol described above. Bidirectional Sanger-sequencing of an internal segment of the amplicon was performed using primers 5-TCCGCTGTATTCTCAGCC-3 (forward) and 5-GCAG AGGAACC AGCATC-3 (reverse) by the same sequencing provider. Forward and reverse sequences were aligned and edited as for the other loci. In this study, we employed the nomenclature proposed by Sulaiman et al. (2005) for the definition of gp60 alleles, according to which, *C. parvum* 'subtypes' are nominated with the prefix II, and *C. hominis* with the prefix I. This is followed by an alphabetical letter indicating the gp60 allelic family. Within each family, the gp60 alleles are defined according to the number and types of trinucleotide repeats. Alleles are defined by a letter A followed by the number of TCA repeats, then the letter G followed by the number of TCG repeats, and the letter R followed by the number of ACATCA units at the end of the repeat. For instance, the IIaA18G3R1 allele belongs to the *C. parvum* subtype family a, containing 18 TCA and three TCG repeats, and one ACATCA terminal sequence.

6.4.4. Analysis of data

The *Cryptosporidium* species and gp60 subtypes were tabulated according to the region of origin. The gp60 diversity of bovine *Cryptosporidium* was compared to the diversity of human isolates from New Zealand. Sequences of the human isolates were obtained from the New Zealand *Cryptosporidium* sequence database (NZDB), which is maintained by the Protozoa Research Unit (PRU), Massey University. At the time of this analysis, the NZDB contained sequence data for 319 human clinical isolates submitted by diagnostic laboratories for genotyping between 2003 and 2010. The gp60 allelic richness of human and bovine *C. parvum* was compared using analytical

rarefaction. This statistical technique is commonly used to compare taxonomic richness among samples taken from different collections, and has been previously used for the analysis of *Cryptosporidium* populations (Grinberg et al., 2008a). Analytical rarefaction was applied using a code available in "R" package "vegan" (http://www.jennajacobs. org/R/ rarefaction.html, accessed 15 January 2013). In order to avoid biased results due to the micro-epidemic structure of the bovine *C. parvum* sample, only one isolate per farm, for every gp60 allele, was included in the analysis. No demographic data was available for the human isolates, so micro-epidemic structures could not be removed for the human sample.

6.5. Results

6.5.1. *Cryptosporidium* species and genotypes

In total, 84/100 IFA-positive specimens from 55 farms were successfully identified to taxon level by PCR-sequencing (Table 6.1). DNA from specimens originating from eight farms did not amplify at any locus. A retrospective analysis indicated that all these specimens contained low oocysts numbers on slides.

Sequencing of the 18S rRNA revealed the presence of *C. parvum* (matched the reference sequence in GenBank accession number: AB513881.1) and *C. bovis* (matched the reference sequence in GenBank accession number KJ531689.1). *C. parvum* was the most common species identified, accounting for 77/84 (91.7%) of the specimens from 49/55 (89%) farms. Conversely, *C. bovis* accounted for 7/84 (8.3%) samples originating from 6/55 (11%) farms. To our knowledge, this is the first report of the presence of *C. bovis* in New Zealand, although a sequence 99% similar to the *C. bovis* 18S rRNA gene has previously been reported in a microscopically-negative specimen (Grinberg et al., 2013). All the specimens displaying the *C. parvum* 18s rRNA sequence showed unambiguous *C. parvum* HSP70 and gp60 sequences. The specimens showing the *C. bovis* 18s rRNA sequence were PCR-negative at both the HSP70 and gp60 loci (failure to amplify these loci could have been due to sequence divergence in the primer regions of *C. bovis* and *C. parvum,* but this could not be confirmed). There were three HSP70 alleles (matched the reference sequence in GenBank accession numbers: U69698.2, U71181.1 and U11761.1) among the 77 *C. parvum,* and all these alleles have been previously reported for human isolates in the NZDB. A total of six C. *parvum* gp60

allele types were identified. These were (with the GenBank accession number, between brackets): IIaA18G3R1 (JQ362494.1), IIaA19G4R1 (JF727803.1), IIaA20G3R1 (JQ362497.1), IIaA16G3R1 (JQ362492.1), IIaA15G2R1 (JF727755.1), and IIdA24G1 (JF727809.1) (Appendix XI). Only three of these alleles (IIaA16G3R1, IIaA18G3R1, and IIaA19G4R1) had been previously reported for bovine *C. parvum* in the NZDB. The most common gp60 allele was the IIaA18G3R1, and was observed in 48/77 (62%) isolates. The IIaA18G3R1was also the most widely distributed allele, and was present in 28 farms, in most regions (Table 6.2). The IIaA18G3R1 was also the predominant human *C. parvum* allele in the NZDB (Figure 6.1). In most farms, multiple *C. parvum* isolates had the same gp60 allele, but three farms had isolates displaying different alleles. In all cases, *C. parvum* isolates originating from the same farm and carrying the same gp60 allele, carried also the same HSP70 allele.

6.5.2. Gp60 diversity of bovine and human *C. parvum* and biogeographical features

The NZDB contained 13 human *C. parvum* gp60 allele types. With the exception of two allele types (the IIaA15G2R1 and IIaA20G3R1), four alleles identified in bovine *C. parvum* in this study were previously identified in human *C. parvum* and present in the NZDB. Conversely, eight low frequency gp60 allele types previously observed in humans were not observed in cattle in this study (Table 6.2; Figure 6.1).

A total of 24 bovine duplicates were eliminated before the rarefaction analysis, leaving 53 bovine *C. parvum* isolates. The rarefaction curve of bovine *C. parvum* approached the asymptote, indicating that these parasites were thoroughly sampled and that the number of gp60 allele types would not have significantly increased with further sampling of cattle (Figure 6.2). However, it was not possible to reject with 95% confidence the null hypothesis of non-difference between the gp60 richness of the human and bovine samples. This was because the lower boundary of the 95% confidence interval of human *C. parvum*, rarefied to the bovine sample size (53), was <7 (the observed bovine gp60 richness) (Figure 6.2).

In this study, the species of *C. parvum* and *C. bovis* were identified in both islands. The IIaA18G3R1 and IIaA19G4R1 were the two most common *C. parvum* gp60

subtypes, and were present in multiple regions (Table 6.2). Conversely, some subtypes showed marked biogeographical patterns. In fact, the IIdA24G1 was present in four farms in the Manawatu–Wanganui and two farms in Southland, and the IIaA15G2R1 was only present in two farms in Taranaki (Table 6.2).

6.6. Discussion

In a previous study conducted in New Zealand in 2002 using a convenience sample of 24 dairy farms, *Cryptosporidium* oocysts were identified microscopically in 40% of the farms (Grinberg et al., 2005). When that study was performed, *C. parvum* was considered the only species cycling in calves and no genetic analysis was performed. In recent years, new species morphologically-similar to *C. parvum* but of uncertain pathogenetic and zoonotic potential, such as *C. bovis* and *C. ryanae* (Fayer et al., 2010a; Feltus et al., 2008; Ng et al., 2011) were described, forcing the re-assessment of previous estimates of *C. parvum* prevalence using genotyping. In the present study, *Cryptosporidium* parasites from 55 randomly selected farms in 7 regions of New Zealand were identified to species level and subtyped, and genetically compared with human *C. parvum*. The primers used for the amplification and sequencing of the 18s rRNA gene annealed perfectly to *C. hominis*, *C. parvum*, *C. ryanae* and *C. bovis*, differed by one mismatch from *C. andersoni*, and by an unknown number of mismatches from *C. ubiquitum.* Therefore, the presence of these species could not be ruled out. However, the main objective of this study was to re-assess previous prevalence estimates of *C. parvum*, the only pathogenic and zoonotic species widely present in calves. As the vast majority of the isolates were identified as *C. parvum* and *C. bovis*, it can be concluded that other species, if present, circulate at a low prevalence. Another possibility could be the presence of mixed infections. It should be noted that mixed infections cannot be ruled out using Sanger sequencing, as this technology usually identifies only the most abundant taxon present in the specimen (Grinberg et al. 2013).

The results of this study contribute to the understanding of the epidemiology of cryptosporidiosis in New Zealand in a number of ways. The predominance of *C. parvum* in the sample confirms this parasite as the most common species in newborn dairy calves. This result is consistent with previous results of small-scale studies (Grinberg et al., 2008a; Learmonth et al., 2001; Learmonth et al., 2003), and with

results obtained abroad (Quilez et al., 2008; Thompson et al., 2007). Our results differ from those obtained in a recent small-scale study, which identified the unusual presence of *C. hominis* gp60 sequences in five farms, and *C. parvum* in only two farms (Abeywardena et al., 2012) in Canterbury. In general, *C. hominis* is considered anthroponotic, and this species is found only sporadically in cattle and was not found in our study. *C. bovis*, reported here for the first time in New Zealand, was identified in only 6 (11%) farms. This species was first described in the USA in 2005 (Fayer et al., 2005), and so far has not been associated with diarrhoea or zoonotic transfer. Studies indicate that the prevalence of *C. bovis* seems to increase in weaned calves compared with newborn animals (Fayer et al., 2007; Starkey et al., 2006b), but only unweaned calves were targeted in the present study.

The predominance of the zoonotic and pathogenic *C. parvum* in the sample reinforces the usefulness of the diagnostic tests for diarrhoeagenic agents offered by veterinary laboratories in New Zealand for the diagnosis of cryptosporidiosis of calves (based on light microscopy or ELISA). The differentiation between *C. parvum* and other species could be occasionally required for the investigation of severe diarrhoea outbreaks or for source tracking of human disease. In such cases, molecular analysis could easily be performed on request at dedicated laboratories.

The lack of amplification of *C. bovis* gp60 and HSP70 alleles was not surprising. Gp60 primers specific for *C. bovis* were not designed, as the polymorphic gp60 gene sequence of *C. bovis* was still unavailable when this study was performed. The results indicate a number of similarities between human and bovine *C. parvum* in New Zealand. The gp60 alleles IIaA18G3R1 and IIaA19G4R1 are the two most common alleles in both host species, collectively accounting for 79% (humans) and 77% (bovine) *C. parvum* isolates. Parasites carrying these alleles are also ubiquitous (Table 6.2). Several studies recorded the IIaA18G3R1 and IIaA19G4R1 in humans (Ng et al., 2010; Zintl et al., 2011) and cattle (Thompson et al., 2007; Xiao, 2010). The IIaA18G3R1 has previously been reported in *C. parvum* from humans, calves and horses in New Zealand (Grinberg et al., 2008a), in cattle in Australia, Canada, and Northern Ireland (O'Brien et al., 2008; Thompson et al., 2007; Trotz-Williams et al., 2006), and in humans in Australia (Jex et al., 2007; Ng et al., 2012; Waldron and Power, 2011) and Europe (O'Brien et al., 2008; Zintl et al., 2009).

Other alleles identified in this study were the IIaA20G3R1, IIaA15G2R1, IIaA16G3R1 and IIdA24G1. The IIaA20G3R1 has been reported in human *C. parvum* in the UK, in Australia (Chalmers et al., 2005), and in Jordan (Hijjawi et al., 2010). Subtype IIaA15G2R1 has been described as a very common *C. parvum* IIa subtype (O'Brien et al., 2008) in calves and humans, in countries such as Portugal (Alves et al., 2006), the United States (Xiao et al., 2007), England (Brook et al., 2009), Brazil (Meireles et al., 2011),Turkey (Arslan and Ekinci, 2012), and Romania (Imre et al., 2011). In general, parasites carrying the IIa subtype family are considered potentially zoonotic and transmissible from livestock (Alves et al., 2006; Chalmers et al., 2005; Jex et al., 2008; Trotz-Williams et al., 2006). Finally, the subtype IIdA24G1 has previously been reported in goat kids in Spain (Quilez et al., 2008) and in humans in Sweden (Gherasim et al., 2012).

In this study, the gp60 allelic richness of bovine and human *C. parvum* were compared using rarefaction analysis, a technique used to compare the taxonomic richness of samples of different sizes. Previous studies using this technique, had found a lower multilocus genotype richness in cattle, as compared with human *C. parvum* in Scotland (Grinberg et al., 2008b). In the present study, the bovine *C. parvum* gp60 allelic richness (of 7) was within the 95% confidence interval of the richness of human *C. parvum* rarefied to the bovine sample size of 53 (Figure 6.2). Thus, the null hypothesis that the gp60 richness of bovine and human *C. parvum* do not differ could not be rejected at an alpha level of 0.05. It is important to highlight that our rarefaction analysis had limitations, as human and bovine *C. parvum* were sampled over different time frames and the human isolates were not random samples, hindering an unbiased comparison between the two samples. Indeed, the fact that eight gp60 allele types (mostly present at low-frequency in the database) were present in the NZDB but were not identified in cattle in this study would suggest that some human subtypes are not endemic in New Zealand, but may have been imported through international travel, although these may be circulating in other host species.

Lastly, the results showed the existence of marked biogeographical features, with clustering of specific gp60 subtypes, such as the restriction of the IIdA24G1 to the regions of Manawatu–Wanganui and Southland, and the IIaA15G2R1 to Taranaki. This clustering could be a consequence of intense regional animal movement between farms and local cattle-human transmission events.

6.7. Conclusions

In summary, this study represents the first geographically comprehensive genetic survey of *Cryptosporidium* parasites of calves in New Zealand. The results indicate that *C. parvum* is the predominant species cycling in newborn calves in this country. The non-pathogenic species *C. bovis* is reported here for the first time. The species circulates in a small but non-negligible proportion of farms. In New Zealand, a positive phenotypic test result submitted by diagnostic laboratories should therefore provide sufficient confidence of the presence of *C. parvum* on farms in most cases. Finally, the significant genetic similarities between the human and bovine *C. parvum* observed in this study support the model considering young calves as amplifiers of potentially zoonotic *C. parvum* in New Zealand.

Table 6.1. Species, subtypes and region of origin of *Cryptosporidium* parasites analysed in this study. A - indicates negative PCR

Farm ID	Region	Cryptosporidium species	HSP70 variant	GP60 subtype
3	Wellington	-	-	-
4	Wellington	-	-	-
9	Manawatu_Wanganui	-	-	-
9	Manawatu_Wanganui	-	-	-
9	Manawatu_Wanganui	-	-	-
10	Manawatu_Wanganui	-	-	-
10	Manawatu_Wanganui	-	-	-
10	Manawatu_Wanganui	-	-	-
11	Manawatu_Wanganui	C. parvum	3	IIdA24G1
12	Waikato	C. parvum	3	IIaA18G3R1
12	Waikato	C. parvum	3	IIaA18G3R1
13	Taranaki	C. parvum	3	IIaA18G3R1
15	Manawatu_Wanganui	-	-	-
15	Manawatu_Wanganui	-	-	-
15	Manawatu_Wanganui	-	-	-
16	Manawatu_Wanganui	-	-	-
16	Manawatu_Wanganui	-	-	-
17	Manawatu_Wanganui	C. parvum	3	IIdA24G1
20	Waikato	C. parvum	3	IIaA18G3R1
20	Waikato	C. parvum	3	IIaA18G3R1
20	Waikato	C. parvum	3	IIaA18G3R1
20	Waikato	C. parvum	3	IIaA18G3R1
20	Waikato	C. parvum	3	IIaA18G3R1
20	Waikato	C. parvum	3	IIaA18G3R1
21	Waikato	C. parvum	3	IIaA18G3R1
23	Taranaki	C. parvum	3	IIaA19G4R1
24	Waikato	C. parvum	3	IIaA18G3R1
24	Waikato	C. parvum	3	IIaA18G3R1
26	Waikato	C. parvum	3	IIaA18G3R1
27	Waikato	C. bovis	-	-
29	Waikato	C. bovis	-	-
31	Waikato	C. parvum	3	IIaA18G3R1
31	Waikato	C. parvum	3	IIaA18G3R1
32	Waikato	C. parvum	2	IIaA18G3R1
33	Manawatu_Wanganui	C. parvum	3	IIdA24G1
34	Manawatu_Wanganui	C. parvum	2	IIdA24G1
37	Canterbury	C. parvum	3	IIaA18G3R1

Table 6.1. (continue) Species, subtypes and region of origin of *Cryptosporidium* parasites analysed in this study. A - indicates negative PCR

Farm ID	Region	Cryptosporidium species	HSP70 variant	GP60 subtype
38	Canterbury	C. parvum	2	IIaA18G3R1
40	Southland	C. parvum	2	IIaA18G3R1
40	Southland	C. parvum	2	IIaA18G3R1
41	Southland	C. parvum	3	IIaA18G3R1
41	Southland	C. parvum	3	IIaA18G3R1
42	Canterbury	-	-	-
42	Canterbury	-	-	-
44	Waikato	C. parvum	3	IIaA18G3R1
45	Waikato	C. parvum	2	IIaA19G4R1
46	Waikato	C. parvum	3	IIaA19G4R1
47	Waikato	C. parvum	2	IIaA18G3R1
50	Waikato	C. parvum	3	IIaA18G3R1
52	Waikato	C. parvum	3	IIaA18G3R1
54	Manawatu_Wanganui	C. parvum	2	IIaA18G3R1
54	Manawatu_Wanganui	C. parvum	2	IIaA18G3R1
56	Southland	C. parvum	2	IIaA20G3R1
57	Southland	C. parvum	2	IIaA20G3R1
59	Waikato	C. parvum	3	IIaA16G3R1
60	Northland	C. parvum	2	IIaA18G3R1
60	Northland	C. parvum	2	IIaA20G3R1
60	Northland	C. parvum	2	IIaA18G3R1
61	Northland	C. parvum	3	IIaA20G3R1
63	Southland	C. bovis	-	-
64	Southland	C. parvum	3	IIaA20G3R1
64	Southland	C. parvum	3	IIaA20G3R1
66	Manawatu_Wanganui	C. parvum	3	IIaA18G3R1
66	Manawatu_Wanganui	C. parvum	3	IIaA19G4R1
67	Waikato	C. parvum	3	IIaA18G3R1
68	Taranaki	C. parvum	3	IIaA19G4R1
69	Taranaki	C. parvum	1	IIaA18G3R1
69	Taranaki	C. parvum	1	IIaA18G3R1
69	Taranaki	C. parvum	1	IIaA18G3R1
69	Taranaki	C. parvum	-	IIaA18G3R1
69	Taranaki	C. parvum	-	IIaA18G3R1
70	Waikato	C. parvum	3	IIaA18G3R1
71	Waikato	C. parvum	3	IIaA18G3R1
73	Canterbury	C. bovis	-	-

Table 6.1. (continue) Species, subtypes and region of origin of *Cryptosporidium* parasites analysed in this study. A - indicates negative PCR.

Farm ID	Region	Cryptosporidium species	HSP70 variant	GP60 subtype
74	Southland	C. parvum	3	IIaA18G3R1
74	Southland	C. parvum	3	IIaA18G3R1
74	Southland	C. parvum	3	IIaA18G3R1
76	Southland	C. parvum	3	IIaA16G3R1
76	Southland	C. parvum	3	IIaA20G3R1
78	Southland	C. parvum	3	IIaA16G3R1
79	Southland	C. parvum	3	IIdA24G1
80	Southland	C. parvum	3	IIdA24G1
82	Taranaki	-	-	-
84	Taranaki	C. parvum	3	IIaA15G2R1
85	Taranaki	C. parvum	3	IIaA15G2R1
85	Taranaki	C. parvum	3	IIaA19G4R1
85	Taranaki	C. parvum	3	IIaA19G4R1
87	Taranaki	C. parvum	1	IIaA18G3R1
87	Taranaki	C. parvum	1	IIaA18G3R1
87	Taranaki	C. parvum	1	IIaA18G3R1
89	Canterbury	C. bovis	-	-
89	Canterbury	C. bovis	-	-
91	Southland	C. parvum	3	IIaA18G3R1
93	Manawatu_Wanganui	C. bovis	-	-
94	Manawatu_Wanganui	C. parvum	2	IIaA19G4R1
94	Manawatu_Wanganui	C. parvum	2	IIaA19G4R1
94	Manawatu_Wanganui	C. parvum	2	IIaA19G4R1
95	Manawatu_Wanganui	C. parvum	3	IIaA18G3R1
96	Southland	C. parvum	3	IIaA18G3R1
97	Manawatu_Wanganui	C. parvum	2	IIaA19G4R1

Table 6.2. *Cryptosporidium parvum* gp60 allelic types identified in calves (number of farms in brackets), and humans (data from the New Zealand *Cryptosporidium* sequence database), according to the geographical regions

	South Island				North Island										Other regions	Total
	Canterbury		Southland		Northland		Manawatu–Wanganui		Taranaki		Waikato		Wellington			
	Bovine	Human	Bovine	Human	Bovine	Human	Bovine	Human	Bovine	Human	Bovine	Human	Bovine	Human	Human	
IIaA15G2R1	-	-	-	-	-	-	-	-	2 (2)	-	-	-	-	-	-	2
IIaA16G3R1	-	-	2 (2)	1	-	-	-	1	-	-	1 (1)	-	-	8	-	13
IIaA18G3R1	2 (2)	5	9 (5)	45	2 (1)	-	4 (3)	22	9 (3)	-	22 (14)	63	-	9	33	225
IIaA19G4R1	-	1	-	33	-	-	5 (3)	19	4 (3)	10	2 (2)	6	-	4	8	92
IIaA20G3R1	-	-	5 (4)		2 (1)	-	-	-	-	-	-	-	-	-	-	7
IIaA20G4R1	-	-	-	7	-	-	-	-	-	-	-	-	-	-	-	8
IIdA24G1	-	-	2 (2)	-	-	-	4 (4)	-	-	-	-	-	-	-	2	8
IIaA14G1R1	-	-	-	-	-	-	-	2	-	-	-	-	-	-	1	3
IIaA17G1	-	1	-	-	-	-	-	-	-	-	-	-	-	1	-	2
IIaA18G2R1	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	2
IIaA18G4R1	-	-	-		-	-	-	-	-	1	-	-	-	-	-	1
IIaA19G3R1	-	1	-	4	-	-	-	1	-	-	-	1	-	-	-	7
IIaA20G5R1	-	-	-	7	-	-	-	-	-	-	-	-	-	1	6	14
IIaA21G4R1	-	-	-	3	-	-	-	2	-	-	-	-	-	-	-	5
IIdA23G1	-	-	-	1	-	-	-	1	-	-	-	1	-	1	4	8
Total	2	9	18	103	4	-	13	49	15	11	25	71	-	24	52	395

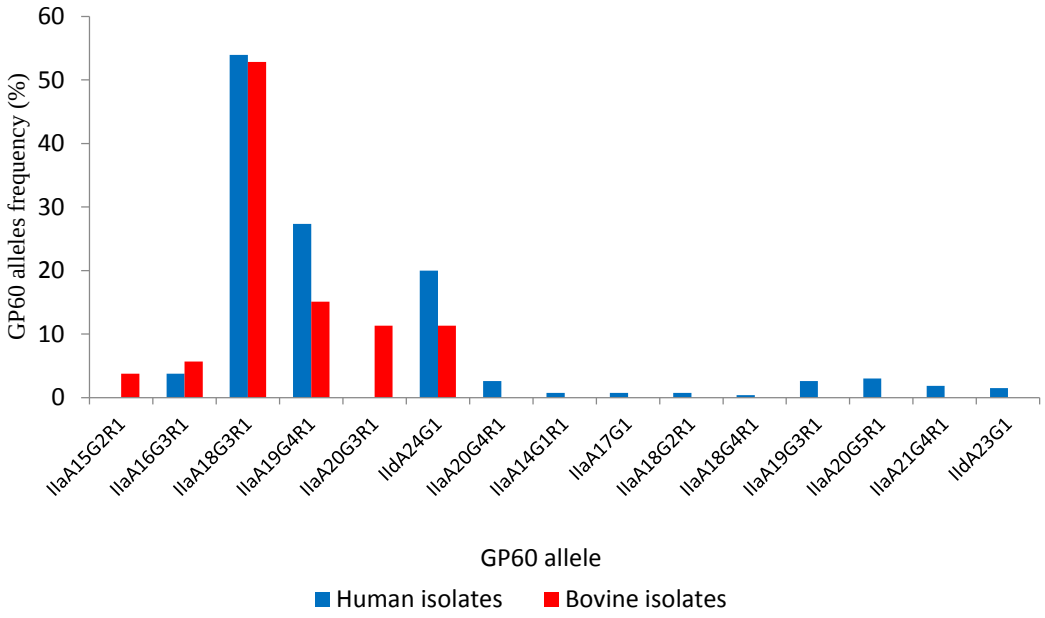

Figure 6.1. The frequency (in %) of each *C. parvum* gp60 allele type in human isolates registered in the NZDB (*n*=319), and the bovine samples (*n*=53) analysed by rarefaction analysis in this study.

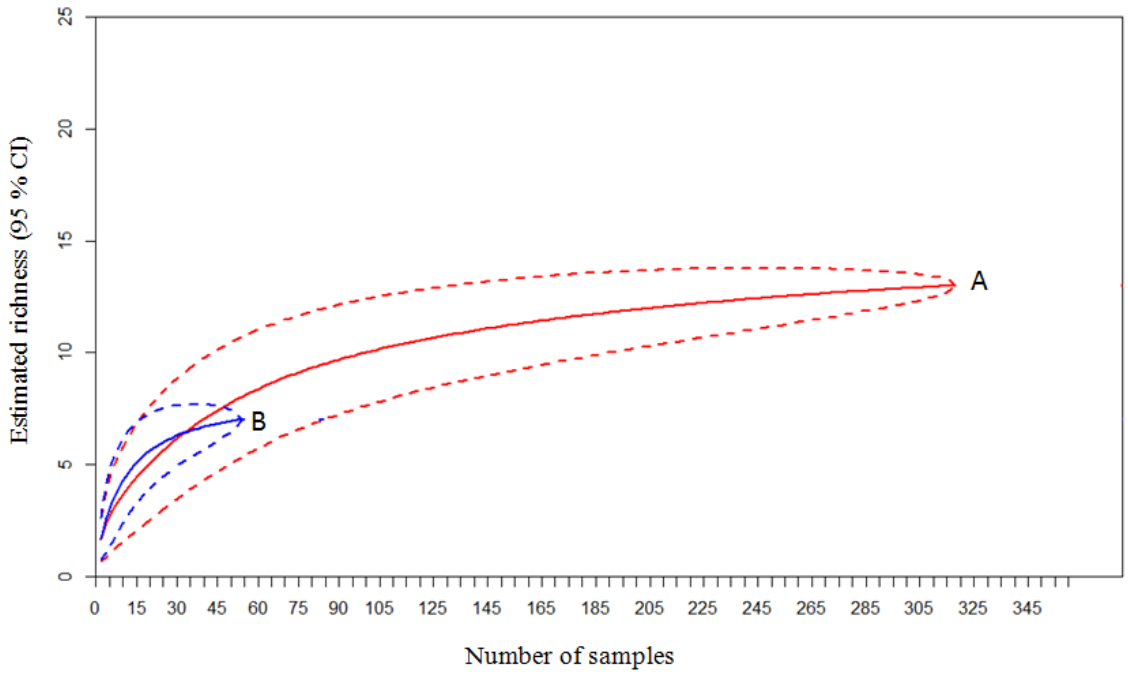

Figure 6.2. Rarefaction curves of the gp60 allelic types of human (A) and bovine (B) *C. parvum.* The sample sizes on the horizontal axis and the estimated gp60 allelic-type richness on the vertical axis. The calculated rarefied richness is reported with 95% confidence intervals (dotted lines).

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Julanda Al Mawly

Name/Title of Principal Supervisor: Alex Grinberg

Name of Published Research Output and full reference:

Genetic diversity and biogeography of cryptosporidium parasites isolated from newborn calves on New Zealand dairy farms

In which Chapter is the Published Work: Six

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  The author has had a major input in this Chapter, including performing all the laboratory analyses, sequence editing and sequence searches. He wrote the first draft of the Chapter and after considering his supervisors' feedback produced subsequent versions and the final Chapter.

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GRS Version 3– 16 September 2011

Chapter 7

7. GENERAL DISCUSSION

The dairy industry is a significant contributor to the New Zealand economy (Anonymous, 2010b; Schilling et al., 2010). Morbidity and mortality in cattle significantly reduce the income of cattle operations. Diarrhoeal disease, in particular NCD, is one of the major causes of morbidity and mortality in cattle. The work presented in this thesis investigated epidemiological aspects of NCD and the role of the main endemic enteropathogens of calves (BCV and BRV, *Cryptosporidium*, *Salmonella* spp. and K99) in dairy farms in New Zealand. The project filled several gaps in knowledge, as limited information on the epidemiology of these agents in New Zealand dairy cattle was available. Except for the first study, the focus of the project was on an analysis at a national level. This approach required the collection of data from a representative sample of farms. Procurement of samples and data from a large number of farms from both the North Island and the South Island during the short calving season was challenging due to the short calving season. As a comparison, in a previous study performed during the calving season in 2002 (Grinberg et al., 2005), the authors reached only 24 farms in one geographical region. In this project, the presence of sponsor's personnel in the territory allowed 97 farms nation-wide to be reached during a two-month period.

The objectives of this PhD project were:

- To study the utility of halofuginone lactate for the prevention of cryptosporidiosis in newborn calves in the presence of co-infection.
- To assess the farm-level prevalence of BRV, BCV, K99, *Salmonella* spp. and *Cryptosporidium parvum* in dairy farms in New Zealand.
- To identify potential risk factors for NCD in New Zealand dairy farms.
- To genetically characterise *Cryptosporidium* parasites infecting calves and compare them with human isolates.

This PhD project contributes to the understanding of the epidemiology of NCD in New Zealand and internationally in several areas. The first study (Chapter 3) provides new data about the efficacy of halofuginone lactate (HL) in the prophylaxis of calf cryptosporidiosis, in the presence of co-infection with BRV and *S.* Typhimurium. One of the most important findings from this work was that the anti-*Cryptosporidium* activity of HL (mainly the delayed onset of shedding) was partially conserved, but there was no evidence of a significant clinical benefit of the drug when used in the presence of BRV and *S.* Typhimurium co-infection. In fact, there was no significant difference between the faecal consistency scores of treated and untreated calf groups. Given the relatively high rate of *C. parvum*-BRV co-infections observed in the prevalence study presented in Chapter 4, this limitation should be taken into account by veterinarians in New Zealand when prescribing HL. Studies in multiple co-infected farms are needed in order to assess the generalisability of this result to the most common form of co-infection found in New Zealand, namely BRV and *C. parvum* (in the absence of *Salmonella*).

A greater understanding of how infectious agents and stressors contribute to NCD could help in designing interventions aimed at reducing disease burden. Before the completion of this project, there was very little data on the epidemiology of the major enteropathogens of calves in New Zealand. The author found a single research-based paper about the prevalence of *Cryptosporidium* parasites and *Salmonella* performed using a limited number of dairy farms in one geographical region (Grinberg et al., 2005). Prevalence studies for the BRV, BCV, K99 were not found in the literature. To the author's knowledge, the study described in Chapter 4 is the first nation-wide prevalence survey of these enteropathogens in New Zealand. The study used a random sample of farms located in the most important dairy regions. Initially, 240 eligible farms were selected from the seven regions using a stratified random sampling scheme based on regionally-proportional sampling. Budgetary and logistical limitations prevented the study from including all the New Zealand regions as well as multiple visits to farms. It would have been interesting to sample farms on multiple occasions and collect repeated samples from each calf, as some agents, like *C. parvum*, have short patent periods. Repeated sampling of a large number of farms, some of which were situated more than 500 km from Massey University was not possible due to budget and time constraints. When the sampling was performed, the

samples from some regions were not properly regionally proportional, as this was dependent on the availability of samplers and field logistics. Therefore, standardised national farm-level prevalence (SNP) was calculated for each pathogen in Chapter 4. The results indicate that BRV and *C. parvum* circulate on the majority of the farms. BCV was found in 47% of the farms, with a SNP of 30.5% and 13.7% in 9-21 and 1-5 day-old calves, respectively. In spite of the presence of BCV in a large proportion of farms, the presence of this virus was not associated with increased odds of liquid faeces in the study presented in Chapter 5. Thus, in New Zealand BCV is a high prevalence virus, but its pathogenic potential in neonatal calf diarrhoea remains uncertain.

C. parvum was the dominant *Cryptosporidium* species identified in newborn calves, with the non-pathogenic species *C. bovis* present in a small proportion of farms, reinforcing the diagnostic value of the phenotypic tests for *C. parvum* offered by most New Zealand diagnostic laboratories. The differentiation between *C. parvum* and other species could occasionally be required for the investigation of severe diarrhoea outbreaks, or for source-tracking of human disease. K99 was identified in 1-5 day-old calves in approximately 10% of the farms. Besides its impact on animal health, *Salmonella* infections represent a zoonotic risk as the infected animals may shed this bacterium with the faeces for extended periods of time even without showing clinical signs, increasing the potential for transmission to farmers, families and workers. In accordance with previous reports, the low prevalence of *Salmonella* spp. indicates that newborn calves are probably not significant reservoirs of *Salmonella enterica* in New Zealand, reinforcing the diagnostic value of the isolation of this bacterium in the course of diarrhoeal disease outbreaks in calves.

One of the most interesting and challenging studies in this PhD project was the risk factors analysis for NCD presented in Chapter 5, which was a natural continuation of the prevalence study presented in Chapter 4. In the risk factor study the faecal score 3 was a proxy of diarrhoea duration. In spite of the inherent limitation of the cross sectional design, the relatively large sample size allowed detection of a number of significant variables. There was a positive relationship, most likely causative, between infections with *C. parvum* and BRV in 9-21 day-old calves, and with K99 in 1-5 day-old calves, and the presence of diarrhoea. However, the presence of BCV was not associated with increased odds of liquid faeces. This study also identified a

number of modifiable factors pertaining to colostrum management, infrastructure and even human resources management, which could be further analysed and/or addressed by farmers in order to mitigate the burden of NCD, underlying the importance of appropriate housing and management programs to maintain calf health and wellbeing. Interestingly, mothers' late pregnancy vaccination against BRV, BCV and K99 was associated with reduced odds of liquid faeces in 9-21 day- old calves. However, the vaccine did not reduce the odds of shedding of pathogens. The use of vaccine is, therefore, not suffient to eliminate the agents, which continue to cycle at levels detectable by diagnostic ELISA.

Although the questionnaire used to elicit the variables for Chapter 5 was subject to significant scrutiny and modification before its delivery, some difficulties in determining the parameters of the variables using the responses obtained were found. In future studies, I recommend the introduction of an additional step in the cognitive assessment of questionnaires used to build variables. This step would involve the coding of the values into variables using a small number of preliminary responses, in order to reveal further areas of improvement before its delivery. I also recommend introducing standardised definitions for disease categories, and refining some of the questions about herd management and housing. For instance, some questions relating to colostrum management could be improved (as mentioned in Chapter 5). It is important to note that, although the questionnaire delivered to the farmers provided detailed information about management and husbandry, the impact of many non-infectious causes of diarrhoea, such as overfeeding, irregular feeding, or poor quality milk replacer (Woodford et al., 1987; Holmes, 2002; Drackley, 2008) could not be assessed. In addition, the possible presence of infectious agents which were not analysed, such as *Campylobacter jejuni* and *Clostridium* spp. should not be ignored (Cornaglia et al., 1992). These agents could have also contributed to the diarrhoea, as 6.6% (52/782) calves passed liquid faeces and were negative for enteropathogens, although their aetiologic role is not well understood (De Rycke et al., 1986; Holland, 1990; Foster et al., 2009). However, analysis of caterpillar plots of the random farm effects (Appendix VIII) indicated that these effects were evenly distributed among the farms, suggesting that most epidemiologically significant risk factors were captured in the final multivariable model.

The risk factor study had a number of limitations, mainly associated with the cross-sectional design and the potential for some selection bias during the recruitment of the farms by phone. Whereas cross-sectional studies allow a sampling of large numbers of farms, such design might not provide cause-effect information when temporal relationships between the variables are unknown. As always, larger sample sizes will also increase statistical power. Another limitation was the potential for confounding by variables not included in the final models (although some biologically relevant variables were included in all models; see Chapter 5). Confounding is always a possibility, so readers are invited to consider these results critically. Specifically designed follow-up studies could be required in order to assess the presence of confounding in the future, in particular in the case of waste milk. Administration of waste milk could increase the risk of development of antimicrobial resistance in the gut flora. Thus, the author recommends an assessment of the effects of waste milk in specifically designed studies, before any recommendation about the use of this potentially useful resource is made.

So what could dairy farmers do to control calf diarrhoea? According to the results of this project it could be theoretically possible to control diarrhoea by controlling a limited number of simple risk factors. For example, designing closed barns for calves, which provide shelter from adverse climatic conditions, could be a cost-effective way to reduce one factor. However, this might require some educational work, as some farmers in New Zealand might think that providing access to pasture is beneficial for calves' health. Employing females as caretakers for the very young calves could also be useful, as well as vaccinating mothers against NCD and ensuring optimal colostrum management. It is well documented that ~50% of newborn calves in New Zealand might not receive sufficient colostrum from their dams, even when kept together for up to 24 hours, as highlighted in Chapter 5. Therefore, it is very important to feed individual calves the best possible colostrum by hand as soon as possible after birth, to be sure that they receive an adequate amount of immunoglobulins. Furthermore, providing good bedding to keep the calves clean, dry and comfortable is also very important. An adequate layer of clean straw will not only absorb moisture and urine, but will also help insulate the calf from the cold ground underneath, providing a comfortable place to lay down. In cold weather, straw also allows the newborn calf to nestle down on the straw to stay warmer.

Despite all these interventions, diarrhoea may persist, especially due to the presence of *C. parvum*, which is difficult to control. The use of HL could be beneficial in farms infected with pure cryptosporidiosis, as emphasised in Chapter 3.

Research to understand molecular epidemiology of zoonotic *Cryptosporidium* parasites is ongoing. Chapter 6 presents the first geographically comprehensive national genetic survey of *Cryptosporidium* parasites of calves in New Zealand. The results indicated that *C. parvum* is the predominant species cycling in newborn calves. The significant genetic similarities between the human and bovine *C. parvum* observed support the model considering young calves as amplifiers of potentially zoonotic *Cryptosporidium* in New Zealand. Thus, farmers and veterinarians in New Zealand should be aware of the potential zoonotic risk of cryptosporidiosis through direct contact with newborn calves, and apply caution.

A number of areas for future studies have been identified. According to Chapter 3, although there were no statistically significant differences between Group 2 (half dose of HL) and the other groups of calves, Group 2 showed intermediate results in the two parasitological outcomes measured, which warrants further investigation of half dose regimes in more powerful multi-site studies. *C. parvum* oocysts are extremely resistant in the environment. As public health is a major concern of agricultural policy, future studies should assess the usefulness of differerent treatments, as applied to the inactivation of *C. parvum* oocysts in biomasses originating from calf-rearing activities (mainly bedding material), in order to prevent the release of viable, potentially zoonotic oocysts in the environment.

As there is no consensus regarding the pathogenicity of *Giardia* in calves, the assessment of the prevalence of *Giardia* spp. was not an objective of this project. However, the high prevalence of *Giardia* spp. found in this study warrants further investigation using molecular tools, also to assess the zoonotic potential of these parasites in New Zealand. Indeed, there is increasing evidence from overseas that some *Giardia* assemblages (A and B) infecting domestic animals can also infect humans, although transmission patterns are not clear (Thompson, 2004; Traub et al., 2004).

Because of the cross-sectional nature of the studies presented in Chapters 4 and 5, future investigations could complement, or even contradict, some of the findings. Some of the findings, such as the administration of waste milk, should be analysed in more detail, preferably in well-designed, prospective, randomised controlled studies.

Finally, molecular tools and technology continue to develop, and it is becoming reasonably inexpensive to determine the whole genome sequence of infectious agents. Genotypic techniques offer an extraordinary ability to distinguish between single isolates, and typing and sub-typing are now techniques for studying the epidemiology of pathogens, allowing researchers to strengthen surveillance programs and diagnostic procedures. Considering the findings of Chapter 6, future use of Next Generation Sequencing is likely to provide a clearer picture of all possible subtypes present in the single isolates (Grinberg et al., 2013). Although the majority of human cryptosporidiosis in New Zealand are caused by two species, *C. parvum* and *C. hominis* (see Chapter 6), other species, including *C. felis, C. meleagridis, C. canis, C. suis, C. muris,* are also able to infect humans, especially children and immunocompromised individuals. Therefore, studies evaluating the possible zoonotic impact of *Cryptosporidium* parasites cycling in other domestic and wild animals in New Zealand, such as sheep, goats, deer, possums and birds, are warranted.

To conclude, this PhD project provides a significant amount of new data in support of interventions aimed at controlling NCD in New Zealand. On methodological grounds, the thesis also presents a range of approaches for data collection and analysis that could stimulate research in this, and other areas.

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APPENDICES

APPENDIX I: MODELS OF ANALYSIS OF VARIANCE FOR REPEATED MEASUREMENTS (RMANOVA) IMPLEMENTED BY THE PROC MIXED PROCEDURE OF SAS PRESENTED IN CHAPTER3

Model-1

Effect	Treatment	Day	Estimate	SE	DF	P-value
treat	1		0.9772	0.2092	37	<.0001
treat	2		1.4095	0.1904	37	<.0001
treat	3		1.7279	0.1969	37	<.0001
day		6	0.2754	0.1365	37	0.0507
day		8	1.3014	0.2183	37	<.0001
day		10	2.8025	0.2613	37	<.0001
day		14	2.3462	0.2758	37	<.0001
day		20	0.1322	0.07842	37	0.1001
treat*day	1	6	0.2892	0.247	37	0.249
treat*day	1	8	0.7485	0.4096	37	0.0755
treat*day	1	10	2.4129	0.4806	37	<.0001
treat*day	1	14	1.4356	0.4842	37	0.0052
treat*day	1	20	1.0005	0.1365	37	1
treat*day	2	6	0.2394	0.2343	37	0.3132
treat*day	2	8	1.1488	0.3595	37	0.0028
treat*day	2	10	2.6121	0.4292	37	<.0001
treat*day	2	14	2.907	0.4655	37	<.0001
treat*day	2	20	0.1401	0.1255	37	0.2714
treat*day	3	6	0.2976	0.2275	37	0.1987
treat*day	3	8	2.007	0.3634	37	<.0001
treat*day	3	10	3.3825	0.4463	37	<.0001
treat*day	3	14	2.6959	0.4831	37	<.0001
treat*day	3	20	0.2564	0.1447	37	0.0844

Model-2

Effect	Sex	Treatment	Day	Estimate	SE	DF	P-value
treat		1		0.9774	0.2148	37	<.0001
treat		2		1.4047	0.1958	37	<.0001
treat		3		1.7553	0.204	37	<.0001
day			6	0.2992	0.1356	37	0.0336
day			8	1.3038	0.2209	37	<.0001
day			10	2.8051	0.2647	37	<.0001
day			14	2.3516	0.2767	37	<.0001
day			20	0.1359	0.07745	37	0.0877
sex	f			1.3313	0.1293	37	<.0001
sex	m			1.4269	0.133	37	<.0001
treat*day		1	6	0.2939	0.2443	37	0.2366
treat*day		1	8	0.7485	0.4142	37	0.0789
treat*day		1	10	2.4089	0.4871	37	<.0001
treat*day		1	14	1.4356	0.4857	37	0.0054
treat*day		1	20	7.63017	0.134	37	1
treat*day		2	6	0.2591	0.2321	37	0.2715
treat*day		2	8	1.1355	0.3641	37	0.0035
treat*day		2	10	2.6048	0.4352	37	<.0001
treat*day		2	14	2.8971	0.4671	37	<.0001
treat*day		2	20	0.1271	0.1244	37	0.3135
treat*day		3	6	0.3445	0.2275	37	0.1383
treat*day		3	8	2.0275	0.3686	37	<.0001
treat*day		3	10	3.4016	0.4524	37	<.0001
treat*day		3	14	2.7221	0.4856	37	<.0001
treat*day		3	20	0.2805	0.1474	37	0.0648

Model-3

Effect	Treatment	Day	Estimate	SE	DF	P-value
treat	1		1.0256	0.2116	37	<.0001
treat	2		1.4207	0.192	37	<.0001
treat	3		1.6722	0.1998	37	<.0001
day		6	0.2714	0.1348	37	0.0514
day		8	1.2867	0.2134	37	<.0001
day		10	2.8328	0.2666	37	<.0001
day		14	2.343	0.2796	37	<.0001
day		20	0.1301	0.07749	37	0.1016
treat*day	1	6	0.3627	0.2455	37	0.148
treat*day	1	8	0.7682	0.4005	37	0.0629
treat*day	1	10	2.4782	0.4907	37	<.0001
treat*day	1	14	1.4772	0.4917	37	0.0048
treat*day	1	20	0.0416	0.1363	37	0.7618
treat*day	2	6	0.2207	0.2309	37	0.3452
treat*day	2	8	1.1343	0.3515	37	0.0026
treat*day	2	10	2.6708	0.4381	37	<.0001
treat*day	2	14	2.9284	0.4715	37	<.0001
treat*day	2	20	0.1491	0.1241	37	0.2375
treat*day	3	6	0.2308	0.2255	37	0.3128
treat*day	3	8	1.9577	0.3558	37	<.0001
treat*day	3	10	3.3495	0.4558	37	<.0001
treat*day	3	14	2.6233	0.4905	37	<.0001
treat*day	3	20	0.1996	0.1444	37	0.1751

Model-4

Effect	Sex	Treatment	Day	Estimate	SE	DF	P-value
treat		1		1.1741	0.3533	20	0.0034
treat		2		1.4772	0.2847	20	<.0001
treat		3		1.7078	0.2407	20	<.0001
day			6	0.4812	0.2558	20	0.0746
day			8	1.683	0.3114	20	<.0001
day			10	2.8323	0.335	20	<.0001
day			14	2.0676	0.3663	20	<.0001
day			20	0.2011	0.1368	20	0.1572
sex	f			1.4243	0.1847	20	<.0001
sex	m			1.4818	0.1962	20	<.0001
treat*day		1	6	0.6331	0.5297	20	0.246
treat*day		1	8	1.2506	0.6461	20	0.0672
treat*day		1	10	3.0437	0.6945	20	0.0003
treat*day		1	14	0.9374	0.7464	20	0.2236
treat*day		1	20	0.005754	0.2781	20	0.9837
treat*day		2	6	0.4483	0.4309	20	0.3106
treat*day		2	8	1.7292	0.516	20	0.0032
treat*day		2	10	2.2578	0.5574	20	0.0006
treat*day		2	14	2.6891	0.6237	20	0.0003
treat*day		2	20	0.2618	0.2205	20	0.2489
treat*day		3	6	0.3623	0.3421	20	0.3022
treat*day		3	8	2.0691	0.4361	20	0.0001
treat*day		3	10	3.1956	0.4665	20	<.0001
treat*day		3	14	2.5764	0.5123	20	<.0001
treat*day		3	20	0.3356	0.2086	20	0.1233

APPENDIX II: SAMPLING FORM USED IN CHAPTER 4

Region:
Staff name:

Study ID number of the farm: **Date:**

s	Eartage	Faecal score	Age	Sex	Breed
1		□ Liquid □ Semi- liquid* □ Solid (normal)	□ 1-5 days old □ 9-20 days old	□Male □Female	□Friesian □Jersey □ Friesian - Jersey Crossbreed □Other, please state……
2		□ Liquid □ Semi- liquid* □ Solid (normal)	□ 1-5 days old □ 9-20 days old	□Male □Female	□Friesian □Jersey □ Friesian - Jersey Crossbreed □Other, please state……
3		□ Liquid □ Semi- liquid* □ Solid (normal)	□ 1-5 days old □ 9-20 days old	□Male □Female	□Friesian □Jersey □ Friesian - Jersey Crossbreed □Other, please state……
4		□ Liquid □ Semi- liquid* □ Solid (normal)	□ 1-5 days old □ 9-20 days old	□Male □Female	□Friesian □Jersey □ Friesian - Jersey Crossbreed □Other, please state……
5		□ Liquid □ Semi- liquid* □ Solid (normal)	□ 1-5 days old □ 9-20 days old	□Male □Female	□Friesian □Jersey □ Friesian - Jersey Crossbreed □Other, please state……
6		□ Liquid □ Semi- liquid* □ Solid (normal)	□ 1-5 days old □ 9-20 days old	□Male □Female	□Friesian □Jersey □ Friesian - Jersey Crossbreed □Other, please state……
7		□ Liquid □ Semi- liquid* □ Solid (normal)	□ 1-5 days old □ 9-20 days old	□Male □Female	□Friesian □Jersey □ Friesian - Jersey Crossbreed □Other, please state……
8		□ Liquid □ Semi- liquid* □ Solid (normal)	□ 1-5 days old □ 9-20 days old	□Male □Female	□Friesian □Jersey □ Friesian - Jersey Crossbreed □Other, please state……
9		□ Liquid □ Semi- liquid* □ Solid (normal)	□ 1-5 days old □ 9-20 days old	□Male □Female	□Friesian □Jersey □ Friesian - Jersey Crossbreed □Other, please state……
10		□ Liquid □ Semi- liquid* □ Solid (normal)	□ 1-5 days old □ 9-20 days old	□Male □Female	□Friesian □Jersey □ Friesian - Jersey Crossbreed □Other, please state……
11		□ Liquid □ Semi- liquid* □ Solid (normal)	□ 1-5 days old □ 9-20 days old	□Male □Female	□Friesian □Jersey □ Friesian - Jersey Crossbreed □Other, please state……
12		□ Liquid □ Semi- liquid* □ Solid (normal)	□ 1-5 days old □ 9-20 days old	□Male □Female	□Friesian □Jersey □ Friesian - Jersey Crossbreed □Other, please state……
13		□ Liquid □ Semi- liquid* □ Solid (normal)	□ 1-5 days old □ 9-20 days old	□Male □Female	□Friesian □Jersey □ Friesian - Jersey Crossbreed □Other, please state……
14		□ Liquid □ Semi- liquid* □ Solid (normal)	□ 1-5 days old □ 9-20 days old	□Male □Female	□Friesian □Jersey □ Friesian - Jersey Crossbreed □Other, please state……
15		□ Liquid □ Semi- liquid* □ Solid (normal)	□ 1-5 days old □ 9-20 days old	□Male □Female	□Friesian □Jersey □ Friesian - Jersey Crossbreed □Other, please state……

*** Faecal score** is <u>semi-liquid</u> when faecal sample spreads across the bottom of the container but it is not liquid.

<u>Please complete these questions:</u>

- What is the No of milking cows in the farm? .....
- Mothers have been vaccinated with: □ Rotavec Corona □ Scourguard □ None (please tick the appropriate)
- Percentage of cows that calved so far: □10-20% □21-40% □41-50% □> 50% (please tick the appropriate)

APPENDIX III: ASSESSMENT OF THE PERFORMANCE OF THE DIAGNOSTIC TESTS FOR ENTEROPATHOGENS USED IN CHAPTERS 4 AND 5

Introduction

Diagnostic laboratories must evaluate the performance of the diagnostic tests used. Not only must these tests be acceptable in terms of specificity and sensitivity, but they must also be clinically applicable, cost-effective, and they must provide rapid results, particularly when it is necessary to screen large numbers of samples. A variety of diagnostic techniques, each with its own advantages and limitations, was available for the organisms analysed in the studies presented in this thesis (Garcia et al., 1997; Bialek et al., 2002; Weitzel et al., 2006; Van Zijll et al., 2010). Furthermore, enzyme immunoassays (ELISA) for detection of BCV, BRV, K99 and *Cryptosporidium* are commercially available and widely used in diagnostic laboratories in New Zealand and overseas. In general, some of these tests were shown to be highly sensitive and specific (Garcia, et al., 1997; Bialek, et al., 2002; Van Zijll et al., 2010). In this Appendix, the methods used for the evaluation of the diagnostic tests used in the project before or after study completions, are described.

Materials and Methods

Assessment of the performance of the ELISA for BCV, BRV and K99

There were no cost-effective alternatives to the commercial ELISA kits for the diagnosis of BRV, BCV and K99. When this project started, the generally held view in New Zealand was that BCV is found only sporadically in calves. However, in this project BCV was identified in a relatively large proportion of farms, raising questions about the specificity of the ELISA kit used. The aim of the study described in this Appendix was to evaluate the performance of the BCV, BRV and K99 ELISA. This evaluation was performed retrospectively in view of the relatively high rate of positive results for BCV observed in the study. The evaluation was not designed as a proper validation, due to its retrospective nature and to the lack of budget.

In this project, faecal samples were screened for BCV, BRV and K99 using a commercial combined ELISA test kit (ELISA Calves Diarrhoea, Institut Pourquier, Montpellier, France). Prior to the test, 0.2 g of each faecal sample was suspended in

0.8 dilution buffer. The suspension was thoroughly mixed and homogenised by means of vortexing. The ELISA kit was used according to the manufacturer's instructions. In brief, 100 µl of diluted samples were placed into a well of a micro-plate coated with the appropriate antisera. The plate was held at room temperature (~25°C) for 30 minutes and then washed three times using a plate-washing machine with the washing solution supplied. A unique conjugate (specific for the tested pathogen) was added to the appropriate wells, and the plate was held at room temperature for a further 30 minutes. Following a final wash, chromogen substrate was added to all wells and held at room temperature for 10 minutes. A stop solution (0.5 mol/L H_2SO_4) was added to stop the reaction and the absorbance was read with an automated ELISA plate reader at 450 nm wave length. The absorbance was transformed according to the manufacturer's instructions, to calculate the sample to positive ratio (S/P). Samples with an S/P ratio $\geq$ 7% were considered to be positive (according to the manufacturer's instructions). Reported sensitivity and specificity of the ELISA kit used for testing *E. coli* were 91.3 % and 100% (compared to PCR), for rotavirus were 98.5 % and 100% (relative to dsRNA electrophoresis), and for coronavirus of 95,7 % and 89,7% (relative to electron microscopy).

Assessment of the performance of BRV ELISA using latex agglutination test

In May 2012, 30 faecal specimens taken randomly from the collection and kept in refrigeration since 2011 (25 positives and 5 negatives) were re-tested by latex agglutination (Rotascreen; Microgen Bioprod- ucts Ltd., UK). The test was performed according to the manufacturer's instructions. Briefly, 10% faecal suspension was prepared in dilution buffer by transferring 100 mg of faeces into 1ml dilution buffer. The sample was allowed to stand for two minutes and then centrifuged at 1000 g for 10 minutes. Two drops of supernatant were transferred onto a test slide and mixed with one drop of the latex reagent coated with anti-rotavirus antibody, and examined for the development of agglutination within two minutes. Negative control latex was added to a second drop of faecal suspension. A positive control which showed agglutination with the test latex reagent was provided, and was included in all tests.

Assessment of the performance of BCV ELISA using reverse transcriptase (RT) PCR

In May 2012, in order to confirm the presence of BCV in some of the specimens, reverse-transcription PCR (RT-PCR) was performed on 30 ELISA- positive faeces collected in this project. The reactions described here were kindly developed by Dr. Indira Rasiah and Laryssa Howe from IVABS, to whom I am very grateful. RNA was extracted from the faeces using the QIAamp viral RNA Mini Kit according to manufacturer's instructions (QIAamp viral RNA mini kit [Qiagen, Cat. no. 52904]). Prior to extraction, 1 gram of faeces was suspended in 5 mL 0.89% NaCl. The solution was clarified by centrifugation at 4000xg for 20 minutes. The supernatant was filtered through a 0.22 µM filter, and 140µl was used as the starting material for RNA extraction. RNA samples were stored at -80^{o}C until analysis.

The protocols for the RT PCR and the following semi-nested PCR were adapted from Takiuchi et al., (2006). The RT reaction was performed using the Invitrogen Superscript III RT/Platinum Taq kit according to the manufacturer's instructions. The primers BCoV1 sense (5'CGATGAGGCTATTCCGAC3') and BCoV2 antisense (5'TGTGGGTG CGAGTTCTGC3') were used in the RT reaction. Briefly, 5 µl of each RNA sample were added to 1 µl BCoV2 and incubated at 97^{o}C for four minutes. The reaction was held at 4^{o}C and 44 µl of a mixture containing BCoV1 (1µl), RT Taq (1 µl), 2x supermix (25µl) and water (17µl) was added to each tube. The first round of amplification reaction was performed as follows: one cycle of 42^{o}C/30 min and 1 cycle of 95^{o}C/5 min followed by 40 cycles of 94^{o}C/1 min, 55^{o}C/1 min, 72^{o}C/1 min and a final extension of 72^{o}C/ 7min. For the second round (semi-nested PCR) of amplification, 3 µl of product from the first round were added to 47µl of semi-nested reaction mixture containing 10x PCR buffer (5 µl), 2 mM $MgCl_2$ (2 µl), 0.2mM dNTPs (1 µl), BCoV3 sense (1µl) (5'TTGCTAGTCTTGTTCTG GC3') and BCoV2 antisense (1 µl) (5'TGTGGGTGCGAGTTCTGC3') and 2.5 U Platinum Taq DNA polymerase (0.5µl) (Invitrogen, Carlsbad, CA, USA). Cycling conditions were as follows: 1 cycle of 94^{o}C/4 min, 30 cycles of 94^{o}C/1 minute, 55^{o}C/1 minute, 72^{o}C/1 minute and a final extension of 72^{o}C/ 7minute. The PCR amplifications were carried out on an automated thermocycler system (Goettingen, Germany).

Assessment of the performance of the K99 ELISA using PCR

The presence of *E. coli* (K99) in ELISA positive faeces was confirmed by PCR chain reaction. Briefly, genomic DNA from faecal specimens was extracted using a commercial DNA extraction kit (QIAamp, DNA Stool Mini Kit, Qiagen, Hilden, GmbH). The protocol for PCR was adapted from Cho et al. (2010). PCR-sequencing a ~ 80 bp of the K99 gene was performed using forward primer (5'GCGA CTACCAATGCTT CTGCG AATAC3') and reverse primer (5'GAACCAG ACCA GTCAATACGAGCA3'). PCR reactions were performed in a volume of 50 µl containing 2 µl DNA, 5 µl 10 x PCR buffer, 1 µl dNTP (2mM), 1.5 µl $MgCl_2$ (50mM), 1 µl of each primer and 0.2 of Platinum® Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad CA, USA). The thermal cycling conditions were of initial denaturation at 95 ^oC for 15 minutes, followed by 40 cycles at 94 ^oC for 30 sec, 55^oC for 66 sec, 72 ^oC for 60 sec and 72^oC for 7 minutes. The PCR products of BCV and *E. coli* (K99) were analysed by electrophoresis in a 2% agarose gel in 0.5% TBE buffer pH 8.4, stained with 0.5ug/mL ethidium bromide and visualised under UV light. A number of amplicons were verified by sequencing. Three and four amplicons were verified by sequencing for K99 (matching the sequence deposited in the GenBank under accession number: GU951525.1, and BCV (matching the sequence deposited in the GenBank under accession number: DQ811784.2, respectively (Appendix X).

Results

For BRV, BCV and K99, the results are descriptive. For BCV, a total of 21/30 (70%) specimens showed bands on gels consistent with the expected amplicon size. Four of the amplicons were further analysed by sequencing and yielded BCV sequences (not shown). The ELISA results of BRV were in perfect agreement with the latex agglutination test. Of the 14 K99 ELISA-positive specimens undergoing PCR, 12 (86%) were PCR-positive and three amplicons were further verified by sequencing.

Conclusions

The latex agglutination test for BRV and the PCR for *E. coli* K99 confirmed the accuracy of the ELISA results. In the case of BCV, whereas the RT PCR confirmed the presence of viral RNA in the majority of the specimens tested, only 70% of the ELISA positive results were positive by RT PCR. The results argue against the generally held view that BRV is rare in New Zealand. However, it should be

remembered that BCV is an RNA virus and that the RT PCR was performed on faeces stored for one year in refrigeration. The negative PCR results could thus be explained by RNA degradation during storage. Therefore, overall, the accuracy of this assessment cannot be ascertained.

APPENDIX IV: ASSESSMENT OF THE PERFORMANCE OF *CRYPTOSPORIDIUM* ELISA AND A MODIFIED IFA

Introduction

A variety of diagnostic techniques, each with its own advantages and limitations, were available for the organisms analysed in the studies presented in this thesis (Garcia et al., 1997; Bialek et al., 2002; Weitzel et al., 2006; Van Zijll et al., 2010). Enzyme immunoassays (ELISA) and direct immunofluorescence (IFA) for the detection of *Cryptosporidium*, are commercially available and widely used in diagnostic laboratories in New Zealand and overseas. In general, some of these tests were shown to be highly sensitive and specific (Garcia et al., 1997; Bialek et al., 2002; Van Zijll et al., 2010) and the IFA test for *Cryptosporidium* is in a way considered the ‘‘gold standard’’ (Van Zijll et al., 2010; Chalmers et al., 2011), as it detects entire oocysts rather than soluble antigens or naked DNA, indicating the presence of a cycling parasite. However, the high costs of the IFA reactive make it impractical for non-commercial research projects requiring a large number of tests. An inexpensive alternative to the IFA is direct microscopy of acid-fast stained smears for the visualisation of the acid-fast *Cryptosporidium* oocysts, but this method was deemed inadequate by the author’s research supervisors (Grinberg, personal communication, 2012). Modified IFA methods have been devised by diluting the conjugate to increase the number of tests that can be performed with a single kit. For the present project, in order to reduce the costs of testing for *Cryptosporidium* by IFA, I have developed a method which uses small volumes of IFA conjugate.

The aim of the work described here was to assess the sensitivity and specificity of the modified IFA and the ELISA test for *Cryptosporidium*, compared with a gold standard based on IFA. This evaluation was performed before the commencement of the studies.

Materials and methods

In order to develop a cost- effective method of screening for *Cryptosporidium* oocysts in faecal specimens , an ELISA and a modified IFA method were evaluated against the IFA method recommended by the manufacturer using a set of solid and liquid faeces from 23 calves aged between 5 and 20 days. Positive (of low, medium and high oocysts count), and negative samples were collected at random from the sample collected previously in the study presented in Chapter 3 and were held

refrigerated (4-6°C) until tested. This assessment was performed in April 2011 using the following diagnostic techniques:

Standard immunofluorescent assay (IFA)

The 23 faecal specimens were re-tested with the direct immunofluorescent assay (Aqua-Glo- G/C Direct Comprehensive Kit) method used in Chapter 3. According to this method, a 10 µl aliquot of the specimens was deposited as a drop on a slide using a micropipette, air-dried and fixed in methanol until evaporation. The slides were stained using a commercial immunofluoresecent anti-*Cryptosporidium* monoclonal antibody following the manufacturer's instructions (Merifluor C/G, Meridian Bioscience, Cincinnati, Ohio, USA). Briefly, 45 µl of fluorescein isothiocyanate conjugated anti-*Cryptosporidium* monoclonal antibody added, then the slide incubated for 30 minutes in a humidified chamber. The slide was then washed with deionised distal water, and was held until partially to completely dry, then a mounting medium was added and the slide was covered with a coverslip. The entire area of the smear was examined using a fluorescence microscope with a 200 x magnification lens, containing a filter with wavelength of 490 nm. The sample was considered positive when at least one brilliant apple green coloured oocyst similar in morphology and size to the positive controls of *Cryptosporidium* oocysts was seen using a fluorescence microscope, and it was considered negative when no such oocysts were found.

Modified immunofluorescent assay

The 23 faecal specimens were tested using a modified IFA assay. Basically, the modification involved the homogeneisation of the faeces in saline, the deposit of a small drop of homogenate on a slide and staining using only 5 µl of IFA conjugate. In a special ceramic plate containing 12 wells, 100 µg of faecal material were mixed with 100 µl normal saline. A 2 µl aliquot was deposited as a drop on a glass slide, air-dried and fixed with methanol for one minute. Five µl of the anti-*Cryptosporidium* fluorescein isothiocyanate-conjugated monoclonal antibody were added to cover the drop and the slide incubated for 30 minutes in a humidified chamber. The slide was then washed with deionised water, a mounting medium and coverslip added and the entire area of the smear was examined using a fluorescence microscope as in the standard IFA method mentioned above.

ELISA

The faecal samples were screened for *Cryptosporidium* spp. using a commercial ELISA (Bio-X Diagnostics, Jemelle, Belgium). The testing was performed according to the manufacturer's instructions. Briefly, 100 µl of supplied dilution buffer and 100 µl of faeces were deposited in an appropriate well of a 96 microplate coated with anti-*Cryptosporidium* antibodies. The plate was left at room temperature (15-20°C) for one hour, and then washed three times with the supplied washing solution using a plate-washing machine. A unique conjugate for *Cryptosporidium* was then added and the plate was held at room temperature for one hour. Following a final wash, chromogen substrate solution was added to all the wells and held at room temperature for 10 minutes. A stop solution was added to stop the reaction and the optical absorbance read at 450 nm wavelength using an ELISA plate reader. Each test was considered positive or negative for *Cryptosporidium* according to the manufacturer's recommendations, by determining sample to positive (S/P) ratios. Samples with an S/P ratio > 6 % were deemed *Cryptosporidium*-positive.

Results

Twenty- one samples (91%) were ELISA-positive and, with IFA and modified IFA, the number of positives was 17 (74.8%) (Table IVa). Although the sensitivity of the ELISA and modified IFA were equal, comparing with the IFA as gold standard, the specificity of the ELISA was low (33.33%), and that of the modified IFA was 100%. The agreement of ELISA with IFA was 'moderate', with a Cohen's kappa coefficient of 0.43 (95% CI: 0.09 - 0.75). There was 'perfect agreement' between the modified IFA and IFA test results (Cohen's kappa coefficient=1; 95% CI: 0.59 - 1.4). The sensitivities, specificities, positive predictive values and negative predictive values for ELISA and modified IFA are shown in Table IVb.

The intensity of fluorescence and the morphology of the *Cryptosporidium* oocysts were similar in the IFA and modified IFA and very similar to the intensity in the positive control. The ELISA procedure required about three hours for a batch of 96 samples, whereas the IFA and modified IFA required only 40-60 minutes for 50 samples. Interpretation of the immunofluorescent smears was straightforward and required less than one minute per specimen using a fluorescence microscope. Although the ELISA was considered to be more adapted to batch testing because of the possibility of performing the test simultaneously with the testing of the other

agents, its performance compared to the IFA test was not encouraging. Both IFA assays were easy to perform, with the Modified IFA significantly less expensive. Reagent cost (including positive control) for standard IFA assay was NZ$ 9.00 per sample, whereas the cost of Modified IFA assay was only NZ$ 1.00 per test.

Conclusions

For *Cryptosporidium*, we compared a modified IFA assay and the ELISA with the IFA, which is the most widely used test in research worldwide. The sensitivity of the two tests was 100%, which is in consistent with previous studies (Garcia, et al., 1997; Bialek, et al., 2002). However, the ELISA was considerably less specific. This could be due to the presence of cross- reactive antigens or soluble *Cryptosporidium* antigens ingested from the environment, or it could be that the ELISA has a higher analytical sensitivity than the IFA itself in detecting cycling parasites. The results obtained in this test are in agreement with the results reported by Van Zijll (2010), who tested 95 faecal specimens with different diagnostic methods including IFA. The IFA assays detect only intact *Cryptosporidium* oocysts, and the ELISA detects *Cryptosporidium* antigens, which may be ingested from the environment or persist after the animal stops shedding parasites. Therefore, the results may not be false-positives or represent recently cured cases. There have been reports of ELISA kits *Cryptosporidium* of low sensitivity and specificity, resulting in considerable numbers of false-positive and false-negative results (Doing et al., 1999; Hanson et al., 2001).

The results of this study demonstrated that, the Enzyme immunoassay was less specific than the immunofluorescent assays for the detection of *Cryptosporidium*. Such tests with a low specificity should not be used as screening tests or as the sole method of diagnosing cryptosporidiosis, in particular in low-prevalence populations. The standard immunofluorescent assay and Modified immunofluorescent assay were very easy to read as the brilliant apple-green-fluorescent organisms with typical morphology were visible at 200 x magnification lens, and could be easily identified at 400 x magnification, thus requiring much less technologist time. The test does require a fluorescent microscope, but this is becoming a standard portion of equipment in many microbiology laboratories worldwide. The results of this test suggested that the modified IFA had excellent specificity and sensitivity compared with the routine test. The cost of the modified IFA was five-fold lower than the IFA, so it was decided to use the modified method for the testing in this project.

Table IVa. Performance of a commercial antigen ELISA, direct immunofluorescence and Modified direct immunofluorescence in the detection of *Cryptosporidium* oocysts in 23 faecal specimens

Specimen	ELISA	Direct immunofluorescence (oocysts count)*	Modified direct immunofluorescence
1.	Positive	Negative	Negative
2.	Positive	Positive (low)	Positive
3.	Positive	Positive (medium)	Positive
4.	Positive	Positive (high)	Positive
5.	Positive	Negative	Negative
6.	Negative	Positive (low)	Positive
7.	Positive	Positive (medium)	Positive
8.	Positive	Positive (high)	Positive
9.	Positive	Negative	Negative
10.	Positive	Positive (low)	Positive
11.	Positive	Positive (medium)	Positive
12.	Positive	Negative	Negative
13.	Positive	Positive (low)	Positive
14.	Positive	Positive (medium)	Positive
15.	Positive	Positive (high)	Positive
16.	Positive	Negative	Negative
17.	Positive	Positive (low)	Positive
18.	Positive	Positive (medium)	Positive
19.	Positive	Positive (high)	Positive
20.	Positive	Negative	Negative
21.	Positive	Positive (low)	Positive
22.	Positive	Positive (medium)	Positive
23.	Negative	Positive (high)	Positive

* low count:1-10 oocycts; medium count:11-1000 oocycts; high count: >1000 oocycts

Table IVb. Sensitivity and specificity of ELISA and modified immunofluorescence for the detection of *Cryptosporidium* oocysts in 23 faecal specimens, compared with direct immunofluorescence (considered 'gold standard')

	Positive specimens	Negative specimens	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Cohen's kappa (CI)
ELISA	21	2	100	33.33	80.95	100	0.425 (0.09 - 0.75)
Modified immunofluorescence	17	6	100	100	100	100	1 (0.5913 - 1.409)

APPENDIX V: COGNITIVE ASSESSMENT OF THE QUESTIONNAIRE USED IN CHAPTERS 5

The questionnaire used in Chapter 5 included questions about herd demographics, farm infrastructure and husbandry practices, which elicited farm-level data for the NCD risk factor analysis. In order to maximise the accuracy and completeness of the data, a draft of the questionnaire was initially circulated among 15 MU Animal Science post-graduate students and academic staff, for a cognitive evaluation (Appendix VI). This assessment drew the investigators' attention to potential problems of comprehension (understanding the question), information retrieval/recall (possibility of retrieval or recall of the information requested); judgment (willingness to provide the answer); and response (whether or not all response options are available) (Scottish Government social research group social science methods series guide7; http: //www. scotland.gov.uk/ Topics/ Research/About/ Social-Research/ Methods-Guides/Guide-7, accessed 15 June 2011). The results of this evaluation were used to modify some questions, a new draft was delivered to three dairy farmers not enrolled in the study for further comments, and the final version of the questionnaire was developed (Appendix VII). Table V summarises the cognitive concepts evaluated in the questionnaire.

Table V. Cognitive issues evaluated in cognitive testing of the questionnaire

	Definition	**Response Errors/Question Problems**
Comprehension	Understanding and interpretation of questions	Unknown terms, Ambiguous concepts, Long and overly complex questions.
Retrieval/Recall	Respondent searches memory for relevant information to answer questions	Recall difficulty, perceived irrelevance of topic.
Judgement	Respondent evaluates question and/or estimates response in deciding on an answer	Estimation difficult
Response	Respondent provides information in response to the question	Incomplete response options

APPENDIX VI: QUESTIONNAIRE VERSION USED FOR COGNITIVE TESTING

Farm study ID: Address:

Date of sampling (if known): Phone Number of the farm:

Role of the person who is completing the questionnaire about the farm:

□ Farm Owner □ Farm manager □Farm worker □ other, please state:…….

1. Farm details

1.1 What is the effective area of the dairy farm?	ha
1.2 What is the main breed of cows in milking herd?	
1.3 What is the number of cows in the milking herd?	
1.4 Does this farm receive calves from other farms?	□Yes □No □I don't know
1.5 Does this farm receive cows from other farms?	□Yes □No □I don't know
1.6 What is the type of dairy set-up? (please tick one box only)	
□ Totally spring calving herd	Start date of calving is ……..
□ Totally autumn calving herd	Start date of calving is ……..
□ Run a mix of spring calving and autumn calving herds	Start dates of calving is ……

2. Drinking Water

2.1 What is the main source of drinking water for calves? (please tick one box only)
☐ Town supply/mains
☐ Bore-hole
☐ Rain water-collected into header tank
☐ Stream/pond
☐ Other, please state:

2.2 Have you added minerals to the calves' water?	□Yes □No □I don't know
If yes, what is the name of the products (minerals) added to the water?	

3. History of calf scours

3.1 How would you rate the following conditions as a problem in calves up to one month of age on your farm? Please, use a ranking of 0 (Less important) to 4 (More important).

	Less important	→			More important
➢ Weak-born calves	0	1	2	3	4
➢ Inadequate colostrum intake	0	1	2	3	4
➢ Scours or diarrhoea	0	1	2	3	4
➢ Respiratory problems	0	1	2	3	4
➢ Bovine Viral Diarrhoea (BVD)	0	1	2	3	4
➢ Joint or navel problems	0	1	2	3	4
➢ Trauma	0	1	2	3	4
➢ Other (please state which condition)	0	1	2	3	4

Com	Ret	Jud	Res	Non

3.2 At what age do your calves usually start scouring in current calving season?

□ 0-5 days of age □ 6-21 days of age □ 3-6 weeks of age □ 7-16 weeks of age

3.3 Usually, are calves still housed when they start scouring? □Yes □No □I don't know

Com	Ret	Jud	Res	Non

3.4 To the best of your knowledge, how many calves had scours and how many of them died in the following calving seasons? (If you don't calve in autumn, please put NA)

	Had scours	Died	Number of calves born during the season	
Spring 2011	_____	_____	_____	□ Don't recall
Autumn 2011	_____		_____	□ Don't recall
Spring 2010	_____		_____	□ Don't recall

Com	Ret	Jud	Res	Non

3.5 If the causes of scours were diagnosed, how was the diagnosis in your **previous** calving season determined? (**not** in the current spring 2011 season)
(Please, tick all applicable options)

Com	Ret	Jud	Res	Non

□ Vet diagnosis (after vet visit)	□ You diagnosed it yourself, because you diagnosed it previously
□ Vet diagnosis (after talking to a vet)	□ You diagnosed it yourself after talking to other farmers with similar problems

Com	Ret	Jud	Res	Non

3.6 If calf scours occurred in other years, please indicate when (please tick):

□ 2005	□ 2006	□ 2007	□ 2008	□ 2009	□Don't recall

3.7 Have scouring calves ever been sampled for laboratory diagnosis? □Yes □No □I don't know

Com	Ret	Jud	Res	Non

If yes, which bugs have been found last years?

□ Rotavirus □ Coronavirus □ Cryptosporidium □ Giardia □ E. coli (K99)
□ Coccidiosis □ Salmonella □ Other, please state,……..
□ No bugs were isolated.

4. Interventions

4.1Do you vaccinate your cows for prevention of calf scours prior to calving? □Yes □No □I don't know

Com	Ret	Jud	Res	Non

4.2 If **yes** do you vaccinate **all** your cows prior to calving or just a **subset**?	☐All ☐ Subset
4.3 If you do vaccinate just a **subset** of cows which cows are chosen for vaccination?	Com | Ret | Jud | Res | Non
If you do vaccinate prior to calving, which product was used this year?	☐Rotavec Corona ☐Scourguard ☐ Other, please state: …… Com | Ret | Jud | Res | Non ☐Don't recall
4.4 Do you vaccinate early and late calving cows at different times?	☐Yes ☐No ☐I don't know Com | Ret | Jud | Res | Non
4.5 What mineral/vitamin/tonics are routinely given to calves up to a month of age? (if none , please write none)	Com | Ret | Jud | Res | Non
4.6 Are there any treatments routinely given to scouring calves?	☐Yes ☐ No ☐ I don't know If yes, what is the name of the product(s) used? …… Com | Ret | Jud | Res | Non
4.7 Do you carry out other interventions on young calves up to one month of age? (e.g. castration, disbudding)	☐Yes ☐ No If yes, what intervention is done? Com | Ret | Jud | Res | Non …… At what age? ……..

5. Cost

5.1 On average how much time do you spend treating a scouring calf each day? Com | Ret | Jud | Res | Non	
☐1- 9 minutes per scouring calf, per day ☐ 20-40 minutes per scouring calf, per day ☐ Other, please state	☐10-20 minutes per scouring calf, per day ☐ 40-60 minutes per scouring calf, per day
5.2 Have you estimated the cost of calf scours to your farm? ☐Yes ☐No ☐ I don't know	
If yes what is the cost per sick calf? $NZ_____ What is the cost to your farm per year? $NZ_____ Com | Ret | Jud | Res | Non	

6. Feeding of calves

Colostrum	(To the best of your knowledge, please tick appropriate box)
6.1 What is the type of colostrum?	☐ First colostrum ☐ Mixed colostrum ☐ Stored colostrum ☐ I don't know ☐ Other, please state:……..
6.2 When calves are fed colostrum after birth? Com | Ret | Jud | Res | Non	☐Within the first 2 hrs ☐Within 2 to 6 hrs ☐After 6 hrs ☐ Other, please state:……
6.3 What is the amount administered per calf per day (in	Com | Ret | Jud | Res | Non

litres)?	
6.4 What is the number of feeds per day?	Com Ret Jud Res Non
6.5 Is colostrum always available for all calves?	☐Yes ☐ No ☐ I don't know Com Ret Jud Res Non
Milk	
6.6 What type of milk is administered to the calves?	☐Fresh milk ☐ Powdered milk ☐ Others, please state:…… Com Ret Jud Res Non
6.7 What is the amount of milk administered to each newborn calf per day (in litres)	• Upto 2 weeks of age …… • At third and fourth weeks of age …….. Com Ret Jud Res Non
6.8 What is the number of feeds per day	
6.9 At what temperature is milk administered to calves?	☐ Warm ☐ Cold Com Ret Jud Res Non
6.10 Is milk always available for calves?	☐Yes ☐ No ☐ I don't know Com Ret Jud Res Non
Water	
6.11 Is water always available?	☐Yes ☐ No ☐ I don't know Com Ret Jud Res Non
6.12 Are water troughs regularly cleaned?	☐Yes ☐ No ☐ I don't know Com Ret Jud Res Non
6.13 Is waste milk (milk from mastitis cows etc) fed to calves?	☐Yes ☐ No ☐ I don't know Com Ret Jud Res Non If yes, is it milk ☐ Antibiotic milk ☐Mastitis

7. Housing and rearing system

7.1 How many people are responsible for rearing calves?	Com Ret Jud Res Non …… Male(s) ……..Female(s)
7.2 For how many days are calves kept housed from birth?	Com Ret Jud Res Non days
7.3 What type of housing is used for calves? (Please, if more than one option are applicable, tick them)	☐ Closed barn, calves totally inside ☐ Patio barn, calves have a restricted area open to outside ☐ Open barn, calves have free access to outside ☐ Others, please state:…… Com Ret Jud Res Non
7.4 Is there a sick pen on the farm?	☐Yes ☐No ☐ I don't know
If Yes, how is the sick pen designed? (Please tick) Com Ret Jud Res Non	☐ One sick pen for the whole farm ☐ Each calf house has a sick pen ☐ Other, please describe:
7.5 Are the bobby calves housed in the same shed with heifer calves?	☐Yes ☐No Com Ret Jud Res Non
If yes, are they kept in the same pen with the heifer calves?	☐Yes ☐No Com Ret Jud Res Non
7.6 Do you keep calves of different age in the same pens?	☐Yes ☐No Com Ret Jud Res Non
If yes, at what age do you join calves of different ages?	……weeks

7.7 How many sheds for calves are on the farm?	Com Ret Jud Res Non
7.8 How many pens for calves?	……
7.9. What are the approximate sizes of the pens? (for example: 15 X 20 meters; or 20 X 30 meters)	
7.10.What is the maximum number of calves housed in one pen?	……
7.11 Do pens have solid partitions?	□Yes □No
7.12 What type of litter is used in pens?	Com Ret Jud Res Non

☐ Straw	☐ Sawdust	☐ Woodchips	☐ Wooden slats	☐ Other, please state

7.13 What type of hard floor is used in the pens?	Com Ret Jud Res Non

☐ Concrete	☐ Slag	☐ Gravel/stone	☐ Earth	☐ Unknown	☐ Other, please state

7.14 How are pens cleaned? Com Ret Jud Res Non	☐ Complete replacement of litter ☐ Litter topped up ☐ Litter not changed during the season ☐ Litter sprayed with disinfectant ☐Other, please state:……
7.15 What is the frequency of cleaning?	☐ At beginning of calving season ☐ Every month ☐ Other, please state:…… Com Ret Jud Res Non
7.16 What is the calf feeding system?	☐Buckets ☐Calf feeders ☐Other, please state:…… Com Ret Jud Res Non
7.17 What is the number of teats per feeder?	• Upto 2 weeks of age …… • At third and fourth weeks of age ……..
7.18 Are the same feeders used for multiple pens (depends on size of pens in the farm)?	☐Yes ☐No ☐ I don't know
If yes, are the feeders cleaned between pens?	☐Yes ☐No ☐ I don't know
7.19 What are the chemicals used to disinfect the feeders/teats/buckets?	……. Com Ret Jud Res Non

7.20 Do you use a water blaster for cleaning calf pens? Com Ret Jud Res Non	☐Yes ☐ No ☐ I don't know	If yes, how frequency it is used? ☐ Once a week ☐ Once a month ☐ Other, please state ……
7.21 What chemicals are used to disinfect calf shed? Com Ret Jud Res Non		(If any, please state) ……..
7.22 Any other comments about calf scours? Com Ret Jud Res Non		

APPENDIX VII: QUESTIONNAIRE USED IN CHAPTER 5

Institute of Veterinary, Animal and Biomedical Sciences

A study of neonatal calf diarrhoea (Calf scours) In New Zealand

We are conducting a study investigating the causes of calf scours on dairy farms in New Zealand and identifying the risk factors associated with the condition. This will help us to design appropriate measures that could assist New Zealand farmers in the prevention and management of this disease on dairy farms. We would like to invite you to participate in the study, which will involve filling in a short questionnaire about how calves are managed on your farm, and having a representative take faecal samples from some of your calves. The study will directly benefit your farm, as you will receive the laboratory test results from us free of charge. We will be testing the samples for a number of pathogens that are common causes of calf scours in New Zealand.

The results of the study will benefit both your farm and the whole industry, and we would very much appreciate your involvement.
Participation in the study is anonymous and the enrolled farms will not be identified by any means during the dissemination of the results.

Participating staff:

Alex Grinberg (IVABS, Massey University)
Kevin Lawrence (Farm Services, VTH, Massey University)
John Moffat (**Intervet**/Schering-Plough)
Julanda Al Mawly (Hopkirk Research Institute, Massey University)

Note: Our representative who is taking samples from your calves would be happy to assist you in completing this questionnaire. For farmers who would prefer to complete the questionnaire themselves, please post it to the following address using the attached, pre-paid envelope:

Alex Grinberg
Private Bag 11222, IVABS
Massey University
Palmerston North 4442

Farm study ID:	**Address:**
Date of sampling:	**Phone Number of the farm:**
Role of the person who is completing the questionnaire about the farm: □ Farm Owner □ Farm manager □Farm worker □ other, please state:…….	

8. Farm details

1.1 What is the total area available for newborn calves?	Indoor area: ……m^2 Outdoor area: ……. Ha
1.2 What is the main breed of cows in the milking herd?	
1.3 What is the number of cows in the milking herd?	
1.4 Does this farm receive calves from other farms?	□Yes □No □I don't know
1.5 Does this farm receive cows from other farms?	□Yes □No □I don't know

1.6 What is the type of dairy set-up? (please tick one box only)	
□ Totally spring calving herd	Start date of calving is ……..
□ Totally autumn calving herd	Start date of calving is ……..
□ Run a mix of spring calving and autumn calving herds	Start dates of calving are ……

9. Drinking Water

2.1 Please indicate the percentage of drinking water for calves that comes from each of the following sources:	
□ Town supply/mains	……%
□ Bore-hole	……%
□ Rain water-collected into header tank	……%
□ Stream/pond	……%
□ Other, please state:	
2.2 Have you added minerals to the calves' water?	□Yes □No □I don't know
If yes, what is the name of the products (minerals) added to the water?	

10. History of calf scours

3.1 How would you rate the following conditions as a problem in calves up to one month of age on your farm? Please, use a ranking of 0 (Less important) to 4 (More important).

	Less important	→			More important
➢ Weak-born calves	0	1	2	3	4
➢ Inadequate colostrum intake	0	1	2	3	4
➢ Scours or diarrhoea	0	1	2	3	4
➢ Respiratory problems	0	1	2	3	4
➢ Bovine Viral Diarrhoea (BVD)	0	1	2	3	4

➢ Joint or navel problems	0	1	2	3	4
➢ Trauma	0	1	2	3	4
➢ Other (please state which condition)	0	1	2	3	4

3.2 At what age do your calves usually start scouring in the current calving season?

□ 0-5 days of age □ 6-21 days of age □ 3-6 weeks of age □ 7-16 weeks of age

3.3 Usually, are calves still housed when they start scouring?	□Yes □No □I don't know

3.4 To the best of your knowledge, how many calves had scours and how many died in each calving seasons? (If you don't calve in autumn, please put NA)

	Had scours	Died	Number of calves born during the season	
Spring 2011 (to date)	_____	_____	_____	□ Don't recall
Autumn 2011	_____	_____	_____	□ Don't recall
Spring 2010	_____	_____	_____	□ Don't recall

3.5 If the causes of scours were diagnosed, how was the diagnosis in your **previous** calving season determined? (**not** in the current spring 2011 season)
(Please, tick all applicable options)

□ Vet diagnosis (after vet visit)	□ You diagnosed it yourself, because you diagnosed it previously
□ Vet diagnosis (after talking to a vet)	□ You diagnosed it yourself after talking to other farmers with similar problems

3.6 If calf scours occurred in previous years, please indicate when (please tick):

□ 2005	□ 2006	□ 2007	□ 2008	□ 2009	□Don't recall

3.7 Have scouring calves ever been sampled for laboratory diagnosis?	□Yes □No □I don't know

If yes, which bugs have been found last years?

□ Rotavirus □ Coronavirus □ Cryptosporidium □ Giardia □ E. coli (K99)
□ Coccidiosis □ Salmonella □ Other, please state,……..
□ No bugs were isolated.

11. Interventions

4.1Do you vaccinate cows for prevention of calf scours prior to calving?	□Yes □No □I don't know
4.2 If **yes** do you vaccinate **all** your cows prior to calving or just a **subset**?	□All □ Subset
4.3 If you do vaccinate just a **subset** of cows which cows are chosen for vaccination?	
If you do vaccinate prior to calving, which product was used this year?	□ Rotavec Corona □ Scourguard □ Other, please state: ……. □ Don't recall
4.4 Do you vaccinate early and late calving cows at different times?	□Yes □No □I don't know
4.5 What mineral/vitamin/tonics are routinely given to calves up to a	

month of age? (if none , please write none)	
4.6 Are there any treatments routinely given to scouring calves?	☐Yes ☐ No ☐ I don't know If yes, what is the name of the product(s) used? …….
4.7 Do you carry out other interventions on young calves up to one month of age? (e.g. castration, disbudding)	☐Yes ☐ No If yes, what interventions? At what age? interventions / Age …… …… ……

12. Cost

5.1 On average how much time do you spend treating a scouring calf each day?	
☐1- 9 minutes per scouring calf, per day ☐ 20-40 minutes per scouring calf, per day ☐ Other, please state	☐10-20 minutes per scouring calf, per day ☐ 40-60 minutes per scouring calf, per day
5.2 Have you estimated the cost of calf scours to your farm? ☐Yes ☐No ☐ I don't know	
If yes what is the cost per sick calf? $NZ_____ What is the cost to your farm per year? $NZ_____	

13. Feeding of calves

Colostrum	(To the best of your knowledge, please tick appropriate box)
6.1 What is the type of colostrum?	☐ First colostrum ☐ Mixed colostrum ☐ Stored colostrum ☐ I don't know ☐ Other, please state:……..
6.2 When calves are fed colostrum after birth?	☐Within the first 2 hrs ☐Within 2 to 6 hrs ☐After 6 hrs
6.3 What is the amount administered per calf per day (in litres)?	
6.4 What is the number of feeds per day?	
6.5 Is colostrum always available for all calves?	☐Yes ☐ No ☐ I don't know
Milk	
6.6 What type of milk is administered to the calves?	☐Fresh milk ☐ Powdered milk ☐ Other, please state:……
6.7 What is the amount of milk administered to each newborn calf per day (in litres)?	• Up to 2 weeks of age …… • At third and fourth weeks of age ……..
6.8 What is the number of feeds per	

day?	
6.9 At what temperature is milk administered to calves?	☐ Warm ☐ Cold
6.10 Is milk always available for calves?	☐Yes ☐ No ☐ I don't know
6.11 Is waste milk (milk from mastitis cows etc) fed to calves?	☐Yes ☐ No ☐ I don't know If yes, is it ☐ Antibiotic milk ☐Mastitis milk
Water	
6.12 Is water always available?	☐Yes ☐ No ☐ I don't know
6.13 Are water troughs regularly cleaned?	☐Yes ☐ No ☐ I don't know

14. Housing and rearing system

7.1 How many people are responsible for rearing calves?	……. Male(s) ……..Female(s)
7.2 For how many days are calves kept housed from birth?	…….. days
7.3 What type of housing is used for calves? (Please, tick all that apply)	☐ Closed barn, calves totally inside ☐ Patio barn, calves have a restricted area open to outside ☐ Open barn, calves have free access to outside ☐ Other, please state:……
7.4 Is there a sick pen on the farm?	☐Yes ☐No ☐ I don't know
If Yes, how is the sick pen designed?	☐ One sick pen for the whole farm ☐ Each calf house has a sick pen ☐ Other, please describe:
7.5 How many sheds for calves are on the farm?	……
7.6 How many pens for calves?	……
7.7 What are the approximate sizes of the pens? (for example: 15 X 20 meters; or 20 X 30 meters)	
7.8 Are the bobby calves housed in the same shed with heifer calves?	☐Yes ☐No
If yes, are they kept in the same pen with the heifer calves?	☐Yes ☐No
7.9 Do you keep calves of different age in the same pens?	☐Yes ☐No
If yes, at what age do you join calves of different ages?	……weeks
7.11.What is the maximum number of calves housed in one pen?	……
7.11 Do pens have solid partitions?	☐Yes ☐No

7.12 What type of litter is used in pens?				
☐ Straw	☐ Sawdust	☐ Woodchips	☐ Wooden slats	☐ Other, please state …….. ……..

7.13 What type of hard floor is used in the pens?					
☐ Concrete	☐ Slag	☐ Gravel/stone	☐ Earth	☐ Unknown	☐ Other, please state

7.14 How are pens cleaned?	☐ Complete replacement of litter ☐ Litter topped up ☐ Litter not changed during the season ☐ Litter sprayed with disinfectant ☐Other, please state:……
7.15 What is the frequency of cleaning?	☐ At beginning of calving season ☐ Every month ☐ Other, please state:……

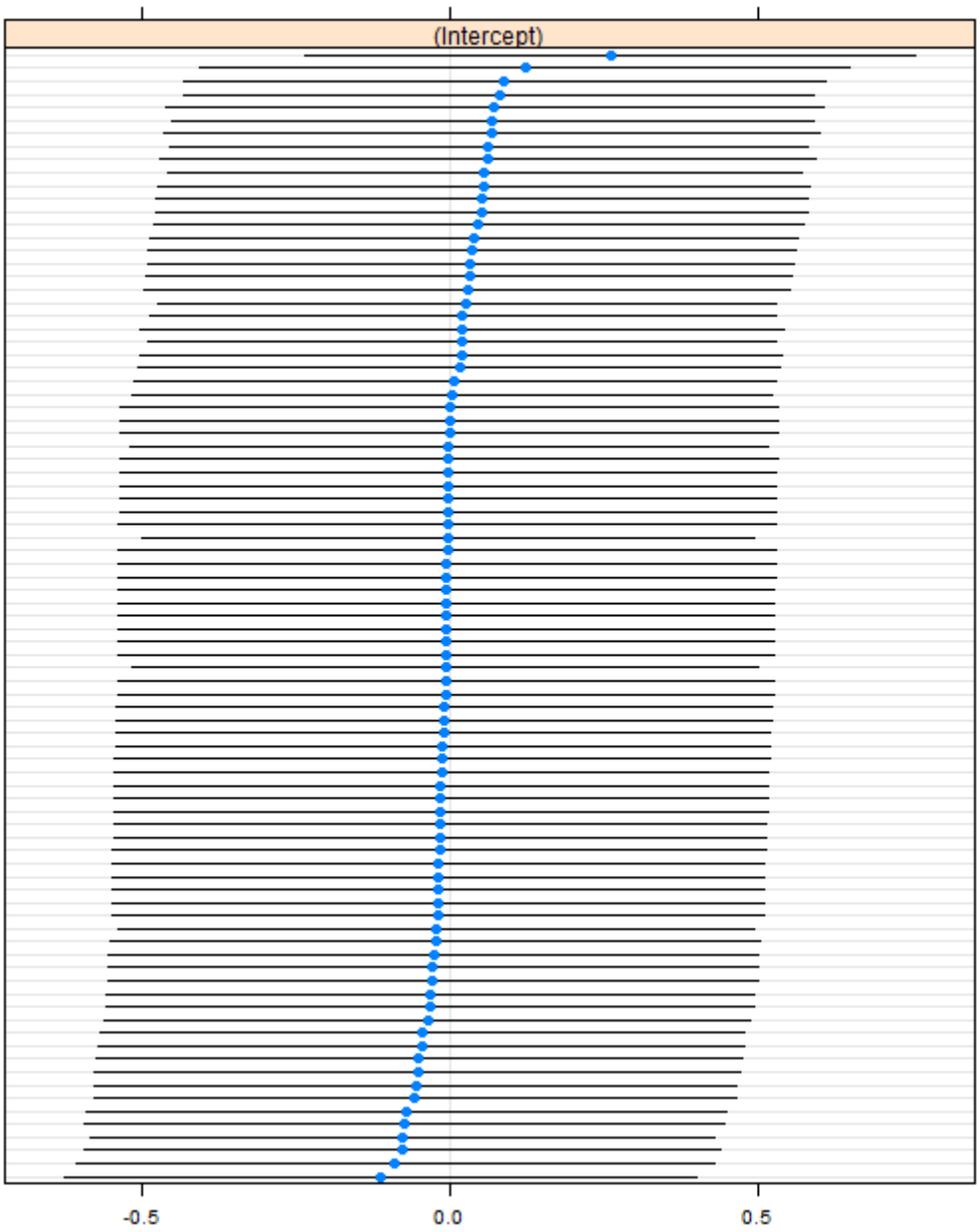
7.16 Do you use a water blaster for cleaning calf pens?	☐Yes ☐ No ☐ I don't know	If yes, how frequency it is used? ☐ Once a week ☐ Once a month ☐ Other, please state ……
7.17 What chemicals are used to disinfect calf shed?	(If any, please state) ……..	
7.18 What is the calf feeding system?	☐Buckets ☐Calf feeders ☐Other, please state:……	
7.19 What is the number of teats per feeder?	• Up to 2 weeks of age …… • At third and fourth weeks of age ……..	
7.20 Are the same feeders used for multiple pens?	☐Yes ☐No ☐ I don't know	
If yes, are the feeders cleaned between pens?	☐Yes ☐No ☐ I don't know	
7.21 What are the chemicals used to disinfect the feeders/teats/buckets?	……	
7.22 Any other comments about calf scours?		

Thank you very much for participating.

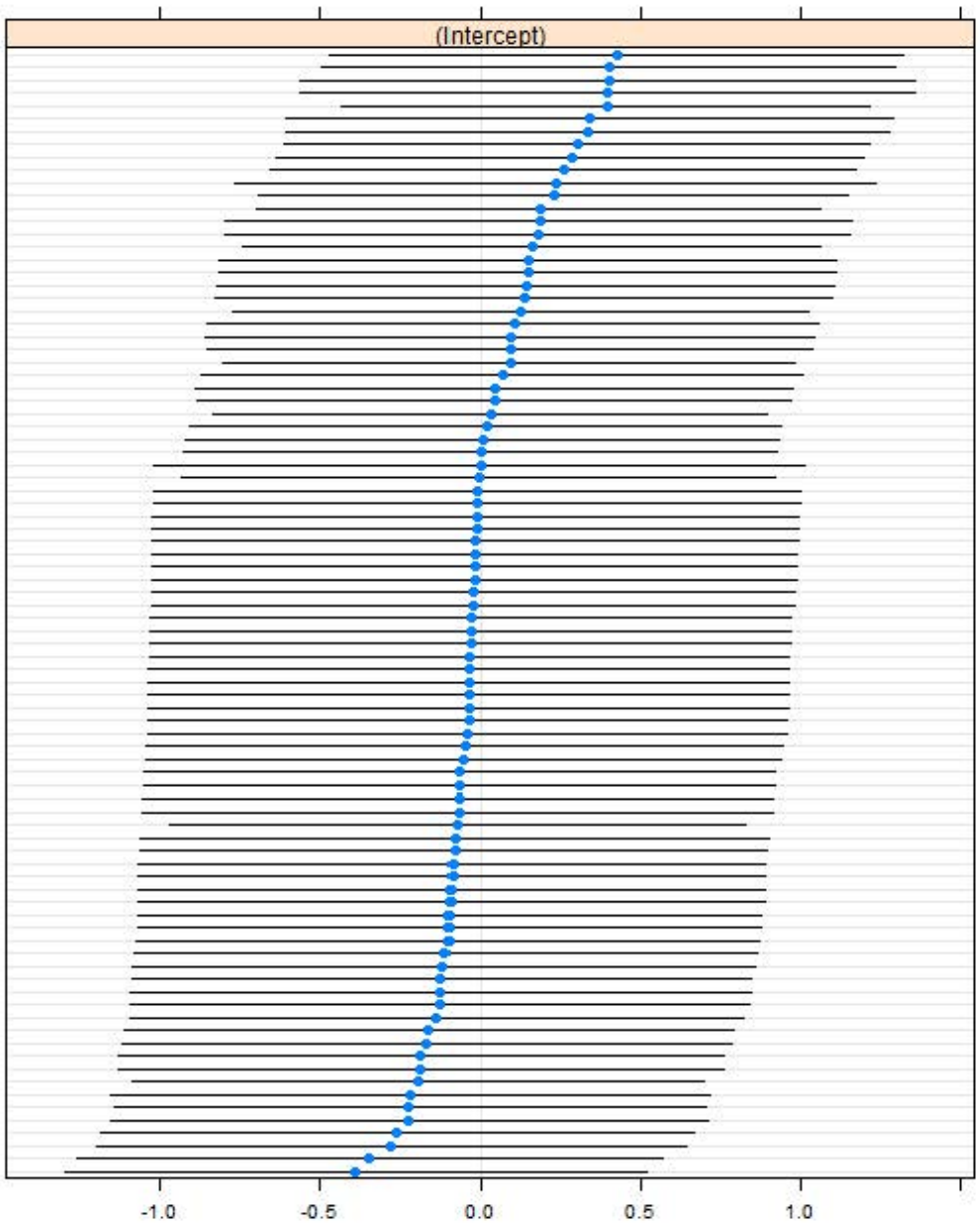
Staff name: ……

APPENDIX VIII:

A: Caterpillar plot of random effect farms of final model of 9-21 day-old calves used in Chapter 5



B: Caterpillar plot of random effect farms of final model of 1-5 day-old calves used in Chapter 5



APPENDIX IX: R PACKAGES AND FUNCTIONS USED IN THE PROJECT

R Packages and functions used in chapter 3

```
library(survival)
d2<-read.csv(file.choose(),na.string="NA",header=TRUE)
head(d2)
names(d2)
attach(d2)

d2.surv<-Surv(d2$tim,d2$stat)
surv.est<-survfit(d2.surv~1,conf.int=FALSE)
surv.est<-survfit(d2.surv~d2$treat,conf.int=FALSE)
summary(surv.est)
## to create plot and legend with 3 colours
plot(surv.est)
plot(surv.est,col=c("red","blue","green"),lty=c(1),lwd=4,xlab="Days of calf
observation",xlim=c(6,21),
xaxt="n",yaxt="n",ylab="Proportion of parasitologically negative calves", main="",bty="n")
legend("topright", legend=c("Group1:full dose","Group2:half dose","Group3:untreated control"),
cex=0.8, col=c("red","blue","green"),
      lty=c(1), lwd=3, bty="n")

axis(1, at = seq(6, 21, 1), las = 1)
axis(2, at = seq(0.0, 1.0, 0.1), las = 1)
```

R functions used in chapter 4

```
d1<-read.csv(file.choose(),na.string="NA",header=TRUE)
names(d1.2)
d1.1 <- subset(d1, age_group == 1)

d1.2 <- subset(d1, age_group == 2)
library(lme4)
library (mice)
library (raster)
summary(d1$herd)
d1$area<-relevel(d1$area,ref="Wellington")
d1$Island<-relevel(d1$Island,ref="North")
str(d1)
d1$breed2<-relevel(d1$breed2,ref="j")

## area , breed  , sex  , X1_3_herd_population  ## rota  corona  crypto.parvum crypto_yes_no  k99
salm  giardia ## age_group
## Canterbury  Manawatu_Wanganui  Northland  Southland  Taranaki  Waikato  Wellington  ##
##diar.yes.no  rota.yes.no  corona.yes.no  k99.yes.no  giard.yes.no  sal.yes.no
X1_3_herd_population  ##
##  crypto.yes.no  mother.vaccin.yes.no  Area ##
## Canterbury Manawatu-Wanganui Northland  Southland  Taranaki  Waikato Wellington #



class(d1$Area)
d1$ age_group <-factor(d1$ age_group)

d1$X1_3_herd_population<-as.numeric(d1$ X1_3_herd_population)
d1$X1_3_herd_population <-cut( d1$X1_3_herd_population , 3,label=c("150-450","451-
650",">650"))
d1$herd <-cut( d1$herd , 3,label=c("150-450","451-750",">750"))
```

```
d1$herd <-cut( d1$herd , 3,label=c("150-450","451-650",">650"))
d1$herd <-cut( d1$herd , 3,label=c("150-300","301-550",">550"))
d1$herd <-cut( d1$herd , 2,label=c("<501",">500"))
d1$herd <-factor(d1$ herd)
d1$ herd<- as.numeric (d1$ herd)
##########
##random effect logistic regression
gm1 <- glmer(calf_scours_yes_no ~ IIaA18G3R1.yesno+ (1 | farm_serial),family = binomial, data =
d1.2)
log1<- glm(multi_infection  ~ Island+herd+breed2, family=binomial(link="logit"), data=d1)
summary(log1)
summary(gm1)
exp(fixef(gm1))
lower <- coef(summary(gm1))[,1] + qnorm(.025)*coef(summary(gm1))[,2]
upper <- coef(summary(gm1))[,1] + qnorm(.975)*coef(summary(gm1))[,2]
cbind(coef(summary(gm1)), lower, upper)
exp(lower)
exp(upper)
cbind( exp(lower), exp(upper))
```

R Packages and functions used in chapter 5

```
d1<-read.csv(file.choose(),na.string="NA",skip=1,header=TRUE)
names(d1)
d1.1 <- subset(d1, age_group == 1)
d1.2 <- subset(d1, age_group == 2)
library(lme4)
library (mice)
library (raster)

## data frams
s1<- d1.1[,c("calf_scours_yes_no" ,
"crypto_parvum","rota","k99","males","females","corona","giardia","sex",
        "X7_14a_litter_topped_up","X7_2_days_calves_kept_housed","males","closed_barn",
"X6_11_waste_milk_feeding",
        "X6.3colostrum_adminster_daily_liters","X7_4_sick_pen_avail",
"mother_vaccin_yes_no","X1_3_herd_population","X1_4_.calves_from_other_farms",
"X7_16_water.blaster",
        "Rotavec_Corona","Scourguard","X7_13a_concrete", "X7_13c_gravel_stone","open_barn",
"mastitis_milk","antibiotic_milk","X7_8_bobby.calves_with_heifer_calves",
        "X7_9_different_age_same.pens","X7_12a_straw",
"rota_yes_no","corona_yes_no","k99_yes_no","crypto_yes_no","giard_yes_no",
        "source_drinking_water","colost_type","time_fed_colost_after_birth",
"milk_type","people_rearing_calves",
        "barn_type", "litter_type", "type_hard_floor","How_pens_cleaned",
"X6_9_milk_temp","col_fed_within_first_2hrs","X6_1c_mixed_colost","farm_serial")]


s2<- d1.2[,c("calf_scours_yes_no" , "crypto_parvum","rota","corona","giardia","sex",
        "X7_14a_litter_topped_up","X7_2_days_calves_kept_housed","males","closed_barn",
"X6_11_waste_milk_feeding",
       "X6.3colostrum_adminster_daily_liters","X7_4_sick_pen_avail",
"mother_vaccin_yes_no","X1_3_herd_population","X1_4_.calves_from_other_farms",
"X7_16_water.blaster",
       "Rotavec_Corona","Scourguard","X7_13a_concrete", "X7_13c_gravel_stone","open_barn",
"mastitis_milk","antibiotic_milk","X7_8_bobby.calves_with_heifer_calves",
       "X7_9_different_age_same.pens","X7_12a_straw",
"rota_yes_no","corona_yes_no","k99_yes_no","crypto_yes_no","giard_yes_no",
        "source_drinking_water","colost_type","time_fed_colost_after_birth",
"milk_type","people_rearing_calves",
```

```
          "barn_type", "litter_type", "type_hard_floor","How_pens_cleaned",
"X6_9_milk_temp","col_fed_within_first_2hrs","X6_1c_mixed_colost","farm_serial")]


## imputation
imp50s1 <- mice(s1, m = 50, seed = 10)
imp50 <- mice(s2, m = 50, seed = 10)

## multivariable model with imputation

fit1 <- with(imp50s1, glmer (calf_scours_yes_no ~   + k99+males  +  (1 | farm_serial),family =
binomial))

fit2 <- with(imp50, glmer (calf_scours_yes_no ~ crypto_parvum + rota +X6_11_waste_milk_feeding
+mother_vaccin_yes_no+
                    sex+colost_type  + time_fed_colost_after_birth +milk_type+ barn_type +
litter_type+
                     +  (1 | farm_serial),family = binomial))

fit4 <- with(imp50, glmer (corona ~  rota + litter_type +   (1 | farm_serial),family = binomial))

d1.2$coinfection_yesno <-factor(d1.2$ coinfection_yesno)
d1.2$coinfection_yesno<-relevel(d1.2$coinfection_yesno,ref="1")
table(d1.2$coinfection_yesno,d1.2$calf_scours_yes_no)

summary(gm1)
exp(fixef(gm1))
lower <- coef(summary(gm1))[,1] + qnorm(.025)*coef(summary(gm1))[,2]
upper <- coef(summary(gm1))[,1] + qnorm(.975)*coef(summary(gm1))[,2]
cbind(coef(summary(gm1)), lower, upper)
exp(lower)
exp(upper)
cbind( exp(lower), exp(upper))
```

fisher's exact test

```
d2 <- matrix(c(64,444,52,782), 2, 2, byrow=TRUE)
fisher.test(d2,alternative="two.sided")
```

National standardized prevalence

```
library(epiR)
### Numbar of positive farms in regions##
  obs <- matrix(data = c(10,17,3,14,12,24,3), nrow = 1, byrow = TRUE,
            dimnames = list("", c("ca","ma","no","so","ta","wa","we")))
obs
##population of farms in regions
pop <- matrix(data = c(12,18,3,16,12,29,7), nrow = 1, byrow = TRUE,
              dimnames = list("", c("ca","ma","no","so","ta","wa","we")))
##standard farms population in regions

nsp  <- matrix(data = c(612,584,739,538,1748,3547,167), nrow = 1, byrow = TRUE,
           dimnames = list("", c("ca","ma","no","so","ta","wa","we")))
nsp
### nsp and crude prevalence
epi.directadj(obs, pop, std, units = 1, conf.level = 0.95)
```

R functions used in chapter 6

```
library(vegan)
x<-read.csv(file.choose(),na.string="NA",header=FALSE)
x <- as.matrix(x)
```

```
y1<-apply(x, 1, sum)
rare.data<-x
select<-unique(sort(c((apply(x, 1, sum)), (seq(0,(max(y1)), by=subsample)), recursive=TRUE)))
storesummary.e<-matrix(data=NA, ncol=length(rare.data[,1]),nrow=length(select))
rownames(storesummary.e)<-c(select)
colnames(storesummary.e)<-rownames(x)
storesummary.se<-matrix(data=NA, ncol=length(rare.data[,1]),nrow=length(select))
rownames(storesummary.se)<-c(select)
colnames(storesummary.se)<-rownames(x)
for(i in 1:length(select))                    #the for loop
{
 select.c<-select[i]                  #assigns the 'i'th element of select to select.c
 foo<-rarefy(x,select.c, se=T)          #use whatever vegan fn you want
  storesummary.e[i,]<-foo[1,]
 storesummary.se[i,]<-foo[2,]
 }
storesummary.e<-as.data.frame(storesummary.e)
richness.error<<-storesummary.se
for (i in 1:(length(storesummary.e)))
{
 storesummary.e[,i]<-ifelse(select>sum(x[i,]), NA, storesummary.e[,i])
}
################plot result#################################
if (plot==TRUE)
{
 if(color==TRUE){
  plot(select,storesummary.e[,1], xlab="Individuals in Subsample",
       xlim=c(0,max(select)), ylim=c(0, 5+(max(storesummary.e[,1:(length(storesummary.e))],
na.rm=TRUE))),
       ylab="Mean Species Richness", pch =16, col=2, type="n")
      for (j in 1:(length(storesummary.e))){
    points(select, storesummary.e[,j], pch=16, col=j+1, type="b", lty=1)}
      if(error==TRUE){
    for (m in 1:(length(storesummary.e))){
     segments(select, storesummary.e[,m]+storesummary.se[,m],select, storesummary.e[,m]-
storesummary.se[,m])
    }
  }
  if (legend==TRUE){
    legend("bottomright", colnames(storesummary.e), inset=0.05, lty=1,
col=1:length(storesummary.e)+1, lwd=2)
  }
 }
 else
 {
  plot(select,storesummary.e[,1], xlab="Individuals in Subsample",
       xlim=c(0,max(select)), ylim=c(0, 5+(max(storesummary.e[,1:(length(storesummary.e))],
na.rm=TRUE))),
       ylab="Mean Species Richness", pch =16, col=2, type="n")
      for (j in 1:(length(storesummary.e))){
    points(select, storesummary.e[,j], type="l", lty=1)}
      for (k in 1:(length(storesummary.e))){
    symbol<-ifelse(symbol<length(storesummary.e),rep(symbol,2),symbol)
    points(as.numeric(rownames(subset(storesummary.e,
storesummary.e[,k]==max(storesummary.e[,k],na.rm=TRUE)))),
max(storesummary.e[,k],na.rm=TRUE), pch=symbol[k], cex=1.5)}
      if(error==TRUE){
    for (m in 1:(length(storesummary.e))){
      points(select, storesummary.e[,m]+storesummary.se[,m], type="l", lty=2)
      points(select, storesummary.e[,m]-storesummary.se[,m], type="l", lty=2)}}
      k<-1:(length(storesummary.e))
```

```
    if (legend==TRUE){
      legend("bottomright", colnames(storesummary.e), pch=symbol[k], inset=0.05, cex=1.3)
    }
  }
}
print("rarefaction by J. Jacobs, last update April 17, 2009")
if(error==TRUE)(print("errors around lines are the se of the iterations, not true se of the means")  )
list("richness"= storesummary.e, "SE"=richness.error, "subsample"=select)
}
```

APPENDIX X: SEQUENCES OF BCV, K99 AND *CRYPTOSPORIDIUM* SPECIES DESCRIBED IN CHAPTER 4

> BCV (99% matching the sequence deposited in the GenBank under accession number: DQ811784.2)

TTGTGGGTGCGAGTTCTGCAAGAATGGGGAACTGTGGATCACTAGTTCCAAGTTTTAACA
TTTCTCCACCACCAAAATTCTGATTGGGGCCTCTCTTCCCAAAACACTGCTGAACAGTGCA
TTGTTTATTGGGGCTCCTCTTCTGACGGGGCTTATTCAAAATTTTCTGTCTAATTTCTTTGG
CAGTCTGCTTAGTTACTTGCTGTGGCTTAGTGGCATCCTTGCCAAGTTTTGCCAGAACAAG
ACTAGCAAAAAACTTGGCAGGAGCCTAAGCCAAGCCGTACTAACAGACTGCCAAGAAAT
TAGACAAAAATTTGAATAACCCCTTCGAGAGAC

> K99 (100% matching the sequence deposited in the GenBank under accession number: GU951525.1)

AACCAGACCAGTCAATACGAGCATTTGTTTTCGCTAGGCAGTCATTACTGCCGGGCGCTG
GTTTTAGTTTAAAATCCACTACAGTGCCATGACCACTAATAGCAGCCTGCCCAAGATCTA
TAGTTGATGTACGATTACCATTGACCTCAGGCTCAATTGTACAAGTAGCACTCGTTATTTT
GCCATTGAAGTTAATAGTACCTGTATTCGCAGAAGCATTGGTAGTCG

> *Cryptosporidium parvum* (100% matching the sequence deposited in the GenBank under accession number: HQ009805.1)

ATAAAGAACCAATATAATTGGTGACTCATAATAACTTTACGGATCACATTAAATGTGACA
TATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGCCTACCGTGGCAAT
GACGGGTAACGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCA
CATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTAATACAGGGAGGTAGTGAC
AAGAAATAACAATACAGGACTTTTTGGTTTTGTAATTGGAATGAGTTAAGTATAAACCCC
TTTACAAGTATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCA
ATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTCTGTTAATAATTT
ATATAAAATATTTTGATGAATATTTATATAATATTAACATAATTCATATTACTATATATTT
TAGTATATGAAATTTTACTTTGAGAAAATTAGAGTGCTTAAAGCAGGCATATGCCTTGAA
TACTCCAGCATGGAATAATATTAAAGATTTTTATCTTTCTTATTGGTTCTAAGATAAGAAT
AATGATTAATAGGGACAGTTGGGGGCATTTGTATTTAACAGTCAGAGGTGAAATTCTTAG
ATTTGTTAAAGACAAACTAATGCGAAAGCATTTGCCAAGGATGTTTTCATTAATCAAGAA
CGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACTATGCC
AACTA

> *Cryptosporidium bovis* (100% matching the sequence deposited in the GenBank under accession number: KJ531689.1)

TCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGCCTACCGTGGCTATGA
CGGGTAACGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACA
TCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTAATACAGGGAGGTAGTGACAA
GAAATAACAATACAGAACCTTACGGTTTTGTAATTGGAATGAGTTAAGTATAAACCCCTT
AACAAGTATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAAT
AGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTAATCTTCTGTTAATTTTTATA
TATAATATCACGATATTTATATAATATTAACATAATTCATATTACTTTTTAGTATATGAAA
CTTTACTTTGAGAAAATTAGAGTGCTTAAAGCAGGCTATTGCCTTGAATACTCCAGCATG
GAATAATATTAAGGATTTTTATTCTTCTTATTGGTTCTAGAATAAAAATGATGATTAATAG
GGACAGTT

APPENDIX XI: SEQUENCES OF *C. PARVUM* gp60 SUBTYPES DESCRIBED IN CHAPTER 6

> IIaA18G3R1 (100% matching the sequence deposited in the GenBank under accession number: JQ362494.1)

CCTGTTGAGGGCTCATCATCGTCATCGTCATCGTCATCATCATCATCATCATCATCAT
CATCATCATCATCAACATCAACCGTCGCACCAGCAAATAAGGCAAGAACTGGAGAAGAC
GCAGAAGGCAGTCAAGATTCTAGTGGTACTGAAGCTTCTGGTAGCCAGGGTTCTGAAGAG
GAAGGTAGTGAAGACGATGGCCAAACTAGTGCTGCTTCCCAACCCACTACTCCAGCTCAA
AGTGAAGGCGCAACTACCGAAACCATAGAAGCTACTCCAAAAGAAGAATGCGGCACTTC
ATTTGTAATGTGGTTCGGAGAAGGTACCCCAGCTGCGACATTGAAGTGTGGTGCCTACAC
TATCGTCTATGCACCTATAAAAGACCAAACAGATCCCGCACCAAGATATATCTCTGGTGA
AGTTACATCTGTAACCTTTGAAAAGAGTGATAATACAGTTAAAATCAAGGTTAACGGTCA
GGATTTCAGCACTCTCTCTGCTAATTCAAGTAGTCCAACTGAAAATGGCGGATCTGCGGG
TCAGGCTTCATCAAGATCAAGAAGATCACTCTCAGAGGAAACCAGTGAAGCTGCTGCAA
CCGTCGATTTGTTTGCCTTTACCCTTGATGGTGGTAAAAGAATTGAAGTGGCTGTACCAAA
CGTCGAAGATGCATCTAAAAGAGACAAGTACAGTTTGGTTGCAGACGATAAACCTTTCTA
TACCGGCGCAAACAGCGGCACTACCAATGGTGTCTACAGGTTGAATGAGAACGGAGACT

> IIaA19G4R1 (100% matching the sequence deposited in the GenBank under accession number: JF727803.1)

ATAATGTTTTCCTCTGTATTATCAGCCCCACCCGTTCCACTCAGAGGAACTTTAAAGGATG
TTCCTGTTGAGGGCTCATCATCGTCATCGTCATCGTCATCGTCATCATCATCATCATCATC
ATCATCATCATCATCATCATCAACATCAACCGTCGCACCAGCAAATAAGGCAAGAACTGG
AGAAGACGCAGAAGGCAGTCAAGATTCTAGTGGTACTGAAGCTTCTGGTAGCCAGGGTT
CTGAAGAGGAAGGTAGTGAAGACGATGGCCAAACTAGTGCTGCTTCCCAACCCACTACTC
CAGCTCAAAGTGAAGGCGCAACTACCGAAACCATAGAAGCTACTCCAAAAGAAGAATGC
GGCACTTCATTTGTAATGTGGTTCGGAGAAGGTACCCCAGCTGCGACATTGAAGTGTGGT
GCCTACACTATCGTCTATGCACCTATAAAAGACCAAACAGATCCCGCACCAAGATATATC
TCTGGTGAAGTTACATCTGTAACCTTTGAAAAGAGTGATAATACAGTTAAAATCAAGGTT
AACGGTCAGGATTTCAGCACTCTCTCTGCTAATTCAAGTAGTCCAACTGAAAATGGCGGA
TCTGCGGGTCAGGCTTCATCAAGATCAAGAAGATCACTCTCAGAGGAAACCAGTGAAGCT
GCTGCAACCGTCGATTTGTTTGCCTTTACCCTTGATGGTGGTAAAAGAATTGAAGTGGCTG
TACCAAACGTCGAAGATGCATCTAAAAGAGACAAGTACAGTTTGGTTGCAGACGATAAA
CCTTTCTATACCGGCGCAAACAGCGGCACTACCAATGGTGTCTACAGGTTGAATGAGAAC
GGAGACTTGGTTGATAAGGACAACACAGTTCTTTGAAGGATGCTGGTTCCTCTGCTTTTG
GACTCAGATACATCCTTTCCTTCC

> IIaA20G3R1 (100% matching the sequence deposited in the GenBank under accession number: JQ362497.1)

CCTGTTGAGGGCTCATCGTCATCGTCATCGTCATCATCATCATCATCATCATCATCAT
CATCATCATCATCATCAACATCAACCGTCGCACCAGCAAATAAGGCAAGAACTGGAGAA
GACGCAGAAGGCAGTCAAGATTCTAGTGGTACTGAAGCTTCTGGTAGCCAGGGTTCTGAA
GAGGAAGGTAGTGAAGACGATGGCCAAACTAGTGCTGCTTCCCAACCCACTACTCCAGCT
CAAAGTGAAGGCGCAACTACCGAAACCATAGAAGCTACTCCAAAAGAAGAATGCGGCAC
TTCATTTGTAATGTGGTTCGGAGAAGGTACCCCAGCTGCGACATTGAAGTGTGGTGCCTA
CACTATCGTCTATGCACCTATAAAAGACCAAACAGATCCCGCACCAAGATATATCTCTGG
TGAAGTTACATCTGTAACCTTTGAAAAGAGTGATAATACAGTTAAAATCAAGGTTAACGG
TCAGGATTTCAGCACTCTCTCTGCTAATTCAAGTAGTCCAACTGAAAATGGCGGATCTGC
GGGTCAGGCTTCATCAAGATCAAGAAGATCACTCTCAGAGGAAACCAGTGAAGCTGCTG
CAACCGTCGATTTGTTTGCCTTTACCCTTGATGGTGGTAAAAGAATTGAAGTGGCTGTACC
AAACGTCGAAGATGCATCTAAAAGAGACAAGTACAGTTTGGTTGCAGACGATAAACCTTT
CTATACCGGCGCAAACAGCGGCACTACCAATGGTGTCTACAGGTTGAATGAGA

> IIaA16G3R1 (100% matching the sequence deposited in the GenBank under accession number: JQ362492.1)

CCTGTTGAGGGCTCATCATCGTCATCGTCATCGTCATCATCATCATCATCAtCATCATCATC
ATCATCAACATCAACCGTCGCACCAgcAAATAAGGcAAGAaCTGGAGAAGACGCAGAAGG
CAGTCAAGATTCTaGTGGTACTGAAGCTTCTGGTAGCCAGGGTTCTGAAGAGGAAGGTAG
TGAAGACGATGGCCAAACTAGTGCTGCTTCCCAACCCACTACTCCaGCTCAAAGTGaAGGC
GCAACTACCGAAACCATAGAAGCTACTCCAAAAGAAGAATGCGGCaCTTCATTTGTaATGt
GGTTCGGAGAAGGTACCCCaGCTGCGACATTGAAGtGtGGTGCCTACACTATCGTCTATGCa
CCTATAAAAGACCAAACAGATCCCGCACCAAGATATATCTCTGGTGAAGTTACATCTGTa
ACCTTTGAAAAGAGTGATAATACAGTTAAAATCaAGGtTAACGGTCAGGATTTCAGCACTC
TCTCTGCTAATTCAAGTAGTCCAACTGAAaATGGCGGATCTGCGGGTcAgGcTTCATCAAG
ATCAAGAAGATCACTCTCAGAGGAAACCAGTGAAGCTGCTGCaACCGTCGATTTGtTTGCC
TTTACCCTTGaTGGTGGTAAAAGAAtTGAAGTGGcTGTACCAaACGTCGAAGATGCATCTAA
AAGAGACAAGTACAGttTGGTTGCAGACGATAAAcCTTTCTATACCGGCGCAAACAGCGGC
ACTACCaATGGTGTCTACAgGTTGAATGAGAACGGAGACTTG

> IIaA15G2R1 (100% matching the sequence deposited in the GenBank under accession number: JF727755.1)

CCTGTTGAGGGCTCATCATCGTCATCGTCATCATCATCATCATCATCATCATCATCAT
CAACATCAACCGTCGCACCAGCAAATAAGGCAAGAACTGGAGAAGACGCAGAAGGCAGT
CAAGATTCTAGTGGTACTGAAGCTTCTGGTAGCCAGGGTTCTGAAGAGGAAGGTAGTGAA
GACGATGGCCAAACTAGTGCTGCTTCCCAACCCACTACTCCAGCTCAAAGTGAAGGCGCA
ACTACCGAAACCATAGAAGCTACTCCAAAAGAAGAATGCGGCACTTCATTTGTAATGTGG
TTCGGAGAAGGTACCCCAGCTGCGACATTGAAGTGTGGTGCCTACACTATCGTCTATGCA
CCTATAAAAGACCAAACAGATCCCGCACCAAGATATATCTCTGGTGAAGTTACATCTGTA
ACCTTTGAAAAGAGTGATAATACAGTTAAAATCAAGGTTAACGGTCAGGATTTCAGCACT
CTCTCTGCTAATTCAAGTAGTCCAACTGAAAATGGCGGATCTGCGGGTCAGGCTTCATCA
AGATCAAGAAGATCACTCTCAGAGGAAACCAGTGAAGCTGCTGCAACCGTCGATTTGTTT
GCCTTTACCCTTGATGGTGGTAAAAGAATTGAAGTGGCTGTACCAAACGTCGAAGATGCA
TCTAAAAGAGACAAGTACAGTTTGGTTGCAGACGATAAACCTTTCTATACCGGCGCAAAC
AGCGGCACTACCAATGGTGTCTACAGGTTGAATGAGAACGGAGACTTGG

> IIdA24G1 (99% matching the sequence deposited in the GenBank under accession number: JF727809.1)

CCTGTTGAGTCATCATCATCATCATCATCATCATCATCATCATCATCATCATCATCGT
CATCATCATCATCATCAACATCGACTGTAGCACCAACTCCAAAGAAAGAAAGAACTGGA
GAGGAAGTAGGTAATCCAGGTTCTGAAGGTCAGGACGGTAAAGGAGACAATGAAGAAAC
AGAAGACAATCAGACCGAGAGTACTGTTTCTCAAAATACTTCAGCTCAAACTGAAGGCAC
AACTACCGAAACCACAGAAGCTGCTCCAAAGAAAGAGTGCGGTACTTCATTTGTTATGTG
GTTCGGAGAGGGTGTTCCAGTTGCATCTTTGAAGTGTGGCGACTATACTATGGTCTATGC
ACCAGAAAAGGACAAAACAGATCCCGCACCAAGATATATCTCTGGTGAAGTTACATCTGT
AACCTTTGAAAAACAAGAGAGCACAGTTACAATCAAGGTTAATAATGTAGAGTTCAGCA
CTCTTTCTACTAGCTCAAGTAGTCCAACTGAAAATAGCGGATCTGCAGGTCAGGTTCCAT
CAAGATCAAGAAGATCACTCTCAGAGGAGGCTAGTGAAACTGCAACCGTCGATTTGTTTG
CCTTCACCCTTGATGGTGGTAAAAGAATTGAAGTTGCTGTACCAAGCGACGAAGATGCAT
CTAAAAGAAACCAGTACAGTTTGGTTGCAGACGATAAACCTTTCTATACCGGCTCAAATA
GCGG