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Investigations into critical aspects of the health and welfare of bobby calves and dairy cows in Victorian dairy systems

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I have not failed. I have just found 10,000 ways that won't work.

- *Thomas A. Edison*

Abstract

Animal welfare is becoming critical to the general public, farmers and dairy industry. In Australian dairy farming systems, maintenance of good welfare of large numbers of surplus non-replacement young male dairy calves (bobby calves) that go for slaughter at an early age of 5 to 10 days old is essential. Another major area of concern for the sustainable dairy industry in Australia is the active control measures against infectious pathogens that may cause severe diseases and production loss such as enteric pathogens in neonatal calves and *Mycoplasma bovis (M. bovis)* infection in cattle. This thesis examines the health and welfare conditions of bobby calves after transportation for slaughter and the potential of bobby calf blood samples to detect herd level *M. bovis* infection in dairy cattle in Victoria, Australia.

Blood samples from bobby calves were collected at a commercial abattoir in Victoria after transportation and lairage to assess their welfare by examining plasma biochemical profile. A quarter of the calves had a failure of passive transfer with a variation of passive immune status depending on which region they came from suggesting colostrum management practices are not similar in all the farms. Very few calves experienced severe hypoglycaemia and dehydration before slaughter. However, most of the calves had higher plasma creatine kinase and lactate indicative of muscular fatigue. It is not clear from this study whether increased creatine kinase was also due to the muscular bruising in calves. Distance or duration of journey and lairage time had no significant effect on energy metabolites, hydration state or muscular fatigue in bobby calves before slaughter. Most of the calves at the time of slaughter showed no evidence of substantial physiological compromise, and there was no significant association either between the transport distance and plasma analytes nor between the total duration of transport and lairage in relation with plasma analytes. These results highlight that bobby calves can be well managed from the property of origin to

abattoir under existing management system in Victoria without unduly compromising their welfare.

The enteric pathogen *E. coli* K99 was the most common pathogen (37.4%) followed by bovine rotavirus (8.1%), *Salmonella* spp. (5.1%) and bovine coronavirus (2.6%) in the faeces of bobby calves after their transportation. Infected calves with the higher acquisition of passive immunity had lower amounts of bovine rotavirus (BRV), bovine coronavirus (BCV) and *Salmonella* spp. in faeces. Hypoglycaemia was associated with increased amounts of shedding of *E. coli* K99 and BRV in the faeces of infected calves. Increased distance of transportation was associated with a higher excretion of BRV only. Breed and sex had no influence on pathogen prevalence in the faeces. This study highlights that the prevalence of major enteric pathogens in bobby calves is minimal except *E. coli* K99 compared to previously reported prevalence of enteric pathogens in Australian dairy calves with diarrhoea, and higher acquisition of passive immunity may play an important role in lowering pathogen load in faeces of infected calves.

The potential for bobby calf blood samples to be used to detect maternal antibody against *M. bovis* for the estimation of herd-level *M. bovis* prevalence in dairy cattle in Victoria was also assessed. Antibodies were detected using antibody capture ELISA. Sera were evaluated for adequate transfer of passive immunity before screening for *M. bovis* specific maternal antibody. All the *M. bovis* positive samples were detected from the sera with adequate passive immunity which was consistent with *M. bovis* specific antibody being transferred from cows to bobby calves. A proportion of 33.3% and 32.9% positive herds against *M. bovis* were detected in the northern and south-eastern dairy region respectively. These results indicate that *M. bovis* is a common pathogen in the major dairy regions in Victoria. This study also suggests that the collection of blood from bobby calves at the abattoir is

convenient and could be used as a source of samples for *M. bovis* prevalence and surveillance study in Victoria, Australia.

Declaration

I at this moment declare that the work carried out in this thesis is my own and that this thesis has not been presented for award or degree at any other university except where indicated in the preface.

Due acknowledgement has been made where necessary.

The thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Monoar Sayeed Pallab

11 September 2019

Publications

Work from this thesis has been presented as follows:

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Preface

All the research work presented here in this thesis was conducted in the laboratories of the Melbourne Veterinary School, and samples were collected from one abattoir in Victoria, Australia. All the samples were collected by the author with active assistance from the primary supervisor, Peter Mansell and two fellow PhD students. Subsequent sample processing, storage and laboratory testing were done by the author with some technical laboratory assistance. All the data analysis and transcripts were drafted by the author, with some modification after reviewing from the supervisors.

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Lastly, I am grateful to my family. My beautiful wife, who stayed with me during my entire PhD study quitting her study in Bangladesh and encourage me to complete the project despite

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Table of Contents

List of Figures

List of Tables

Commonly used abbreviations

AHA	Animal Health Australia
BCV	Bovine Coronavirus
BHB	Beta-hydroxy Butyrate
BRV	Bovine Rotavirus
cDNA	Complementary DNA
CI	Confidence Interval
CK	Creatine Kinase
Ct	Cycle threshold
DEDJTR	Department of Economic Development, Jobs, Transport and Resources
DNA	Deoxyribonucleic Acid
E. coli	*Escherichia coli*
ELISA	Enzyme-linked Immunosorbent Assay
FFA	Free Fatty Acid
GGT	Gamma-glutamyltransferase
HPA	Hypothalamic Pituitary Adrenal cortex
IgG	Immunoglobulin G

M. bovis	*Mycoplasma bovis*
MCOP	Model Code of Practice
MilA	Mycoplasma Immunogenic Lipase A
NCD	Neonatal Calf Diarrhoea
NEFA	Non-Esterified Fatty Acid
NLIS	National Livestock Identification System
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
PIC	Property Identification Code
qPCR	Quantitative PCR
RFID	Radio-frequency Identification
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
RSPCA	Royal Society for the Prevention of Cruelty to Animals
TOF	Time Off Feed
TPP	Total Plasma Protein

1 General Introduction

Over the last fifty years maintaining good welfare of farm animals has been a significant area of concern, both for the general public and animal scientists. Due to technological advancement and improved genetic make-up, there has been an increased intensification of farm animal management which has brought new challenges to maintaining good welfare. One such problem in Australian dairy farming systems is the maintenance of well-being of large numbers of surplus non-replacement young calves that go for slaughter at an early age of 5 to 10 days old.

In addition to animal welfare, another major area of concern for the sustainable dairy industry in Australia is the active control measures against infectious pathogens that may cause severe diseases and production loss such as enteric pathogens in neonatal calves and *Mycoplasma bovis* infection in dairy cattle.

In Australia, each year, approximately 770,000 non-replacement young calves (bobby calves) are transported from farms to abattoirs for slaughter. Welfare issues may arise in these young calves due to transport at a very young age, poor on-farm management practices, prolonged periods of food and water deprivation, long duration of transportation and lairage, and other management practices associated with the supply chain before these animals are slaughtered. Some regulations have been developed based on science-backed data from experimental studies to minimise undue stress in bobby calves. However, assessment of the welfare outcomes of these calves under field condition is lacking.

Therefore, it is appropriate to examine the performance of the bobby calf supply chain with regulatory standards in place and to investigate mechanisms for ongoing monitoring of calf health and welfare for the industry to demonstrate its operating welfare performance. If the

standard is to be sustainable, it is necessary to show that there are acceptable animal welfare outcomes in practice.

Bobby calves, due to their young age, may get infected with enteric pathogens at farms. Also, farm management practices and other stressful events such as transportation might increase the enteric pathogen shedding before being slaughtered. Enteric pathogen infection at the neonatal stage of calves is an important issue regarding the health and welfare of calves. Enteric pathogen infection is also related to food safety for human consumers of veal and associated products from bobby calves as some of the enteric pathogens are foodborne such as *Salmonella* spp. Existing literature on the prevalence of enteric pathogens and their associated risk factors in bobby calves are limited.

Mycoplasma bovis infection in dairy cattle is also a significant concern for dairy farmers due to its high morbidity and subsequent production loss. Controlling this pathogen in dairy herds is difficult due to a lack of effective vaccines and drug treatments. A suitable control strategy is to maintain a continuous monitoring and surveillance programme which allows improved understanding of the epidemiology of *M. bovis* in existing dairy farming systems and prevent the introduction of this pathogen into a naïve herd. One of the challenges under Australian dairy farming systems, where dairy farms are distributed widely, is the lack of readily available appropriate samples for the detection of *M. bovis* infection at the herd level for continuous monitoring and surveillance. Serological testing of bobby calves at abattoirs may represent a reliable and straightforward way to monitor the infection status of their herds of origin.

Therefore, the overall aims of this thesis were to investigate bobby calf health and welfare issues under commercial management practice and to assess the utility of these calves for the detection of herd-level *M. bovis* infection in dairy cattle herds.

In Chapter 2, a review of literature is presented highlighting what is known and what are the gaps in current knowledge relating to bobby calf health and welfare and the use of these animals in the detection of *M. bovis* in dairy herds.

In Chapter 3, blood samples from bobby calves from an abattoir in Victoria were collected to assess the physiological variables indicative of welfare status in these calves. Associated management data were also collected to test for associations between physiological status and key transport and management factors, including journey distance and duration.

In Chapter 4, the occurrence of major bacterial and viral enteric pathogens in bobby calves and their associated risk factors were assessed after testing and analysing faecal samples in association with associated management practices and plasma analytes.

In Chapter 5, blood samples from bobby calves were used as a proxy to detect *Mycoplasma bovis* infection in dairy cattle and herds in Victoria to assess the potential of blood samples from bobby calves from abattoir as a sample of choice for future *M. bovis* surveillance study.

In Chapter 6, an overall discussion, limitations of the study, conclusion and future recommendations have been presented for the improvement of the health and welfare of bobby calves and detection of *M. bovis* in herd level under existing dairy farming systems in Victoria, Australia.

2 Literature Review

2.1 The welfare of bobby calves

2.1.1 Overview of the dairy industry in Australia

In Australia, one of the most critical rural industries is dairying. In terms of farmgate and export sales value, dairying ranks third after the beef and wheat industries. In the international dairy market, Australian dairy products represent 6% of the trade and hold the fourth position after New Zealand, The European Union and the USA. Currently, 80% of Australian exports market is in Asia; and China, Japan, Singapore, Indonesia and Malaysia are the top five export countries. In 2016/2017 financial year, this industry generated more than 3 billion Australian dollars.

In 2017, there were 5,789 registered dairy farms in Australia having 1.51 million dairy cows which produced around 9 billion litres of milk, of which 37% was exported. The distribution of dairy farms in different regions in Australia is shown in Figure 2.1. About 42,100 Australians are directly employed on dairy farms, and a significant number of the employee are associated with related service industries (Dairy Australia 2017).

Australian dairying is predominantly pasture-based, and most of the dairy farms are in the coastal areas due to rainfall and available pasture. All the states have dairy farms; however, 67% of Australian dairy farms are in Victoria due to its favourable climate and natural resources for dairying. The main breeds kept are Holstein which accounts for approximately 65% of all dairy cattle.

The other breeds are Jersey, the Holstein and Jersey cross, Brown Swiss, Ayrshire and some local breeds such as the Australian Red and Illawarra.

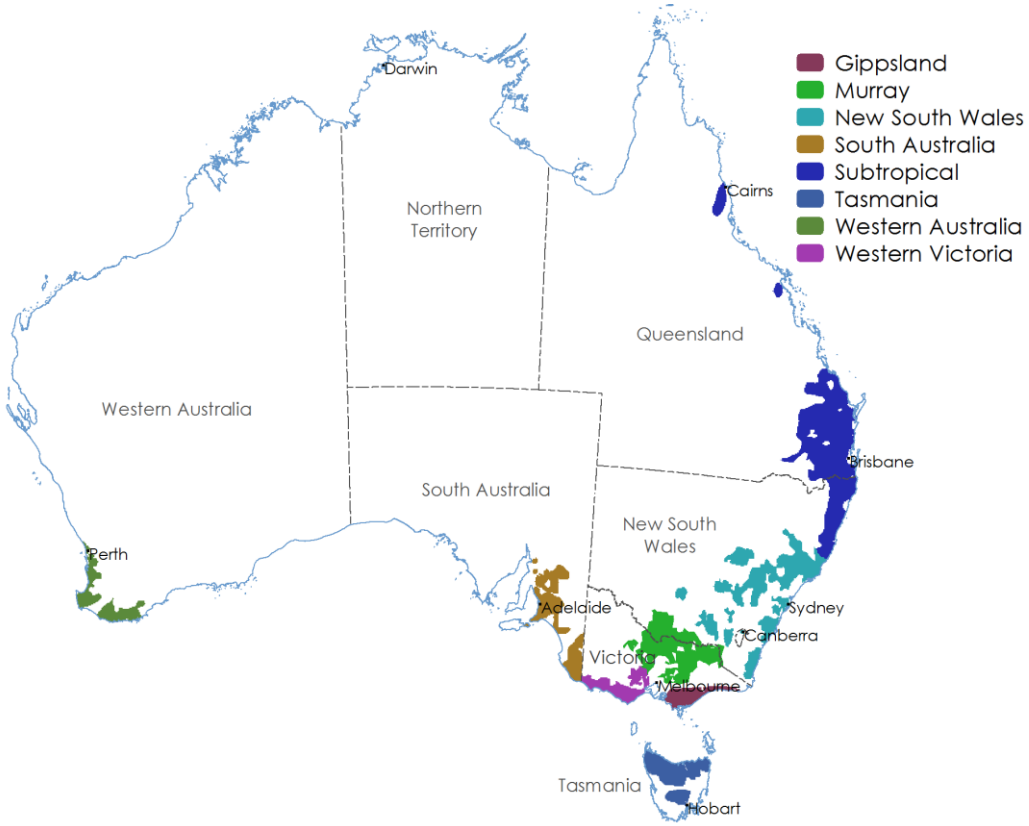

Figure 2.1: The major dairy regions and distribution of dairy properties in Australia. The map is adapted from the Australian Dairy Industry In Focus 2017 (Dairy Australia 2017).

From 1979/80 to 2016/2017, some dramatic changes have occurred in the Australian dairying industry, specifically a reduction of farm numbers and the number of dairy cows in the national herd, and an increase of the average herd size and milk production per cow. In 1979/80, there were 21,994 registered dairy farms, and now it is 5,789; this is a three-quarters reduction over 37 years. A decline in many cows has also occurred, it was 1.88 million cows in 1979/80, compared to 1.51 million now. Average herd size has increased, almost tripling from 93 cows to 262 cows, and milk production has nearly doubled from 5.43 billion litres to 9 billion litres. Average milk production per cow has almost doubled, increasing from 2,848 litres to 5,819 litres over the last 37 years. This increased milk production with a smaller

number of cows is due to the improvement of genetic merit and better nutritional management (Dairy Australia 2017).

To maximise milk production in the Australian pasture-based production system, it is vital that cows calve approximately every year. This synchronises the peak of herd nutritional demand associated with peak lactation to the season when pasture, as the cheapest source of nutrition, is most readily available. Subsequently, most Australian dairy cattle calve during the Spring (August-October), especially in the south-eastern states (Dairy Australia 2017). Accordingly, a large proportion of dairy calves are born within these months.

2.1.2 Bobby calves

In Australia, a non-replacement or bobby calf means a calf not accompanied by its dam, less than 30 days old and weighing less than 80 kg live weight (Agriculture Victoria 2018). In Australia, dairy cows give birth on average every 12-14 months. Most female calves are usually kept as potential herd replacements. Male calves and heifers that are not suitable for herd replacement are mostly sent to slaughter at 5-10 days old as bobby calves. Annually, around 770,000 such non-replacement calves are born in Australia, of which 63% are from Victoria (Table 2.1). Approximately 700,000 bobby calves are slaughtered at commercial abattoirs. The remainder is humanely destroyed on the farms or reared as a beef cattle although the dairy beef market in Australia is limited.

Veal and by-products from the bobby calves are mostly exported to Japan and in the US market. Annually, the Australian bobby calf trade generates approximately 200 million dollars including farmgate value, associated industry services such as transport, processing of veal and export income (Meat & Livestock Australia 2011). Therefore, the trade is of significant economic importance to the industry.

Table 2.1: Showing approximate numbers of bobby calves in Australia by state and their end destination (Animal Welfare Standards 2011).

State	No of dairy cows	No of bobby calves	End destination
Victoria	1,020,000	492,000	Most are slaughtered at the age of 5-7 days; small numbers go for beef rearing, and undersized or weak calves are killed on the farm.
Tasmania	132,000	63,000	Most are slaughtered at the age of 5-7 days; small numbers go for beef rearing, and undersized or weak calves are killed on the farm.
South Australia	100,000	48,000	Most are slaughtered at the age of 5-7 days. In some regions, bobby calves are taken to a collection point, fed and transported for slaughter in Victoria. In other areas, limited numbers are used for beef rearing or humane destruction on the farm.
New South Wales (NSW)	185,000	89,000	Most calves from Southern NSW are sent for slaughter in Victoria. Calves from other areas are either humanely destroyed on a farm or used for beef rearing.
Queensland	110,000	53,000	Mostly humane destruction on the farm. Limited numbers used for commercial slaughter and beef rearing.
Western Australia	53,000	25,000	Mostly humane destruction on the farms. Limited numbers used for commercial slaughter and beef rearing.
Total	1,600,000	770,000	

2.1.3 Management of bobby calves

2.1.3.1 Calving, housing and feeding

In Victoria, most herds are either seasonal calving in spring and autumn or split calving, and most cows are calved in the paddock (Dairy Australia 2010a; Phipps *et al.* 2018b). Shortly after birth calves are separated from cows and are reared in groups. Typically, bobby calves are separated from heifer replacement calves and identified as sales calves. Most of the sale calves go for commercial slaughter at the abattoirs at 5 to 10 days old (Dairy Australia 2016; Phipps *et al.* 2018a).

Published articles regarding on-farm management of bobby calves are limited. Bobby calves only spend a very brief period in farms after birth during which they are housed together and fed colostrum or milk or milk replacer before being sold (Dairy Australia 2010b). In Victoria, mostly calves are separated from their mothers shortly after birth and are fed 1-4 L of colostrum within 24 hours (Vogels *et al.* 2013; Phipps *et al.* 2018b).

A recent survey of calf rearing practices at northern Victorian dairy farms indicates that 65% of farms provide additional roughage and concentrate in addition to milk or milk replacer at less than one week of age (Phipps *et al.* 2018a). Calves should get free access to fresh water after birth; however, in the northern Victorian dairy farms indicate that the mean age of access to water is 4.7 days with a range of minimum 1 day to 48 days (Phipps *et al.* 2018a). Another survey conducted by Dairy Australia in 2010 reported that 99% of farms have the provision of fresh water to replacement heifer calves while 76% in case of sale calves (Dairy Australia 2010b). During transport, 96% of cases bobby calves are given their last feeding within 6 hours before transport where the average time is 2.6 hours (Dairy Australia 2016).

2.1.3.2 Transportation, lairage and slaughter

In approximately 35% of cases travelling calf buyers purchase the bobby calves directly from the farms. The rest of the calves are sent to local calf scales or saleyards. Transportation of

bobby calves from the farms to local saleyards are usually done using small trucks or trailers. Calves are gathered in the saleyards by morning and then transferred to the larger vehicles for transport directly to the abattoirs by afternoon or evening in the same day (Cave *et al.* 2005; Animal Welfare Standards 2011).

After unloaded, calves are rested in lairage pens at the abattoirs for the whole night and are slaughtered at high priority early in the morning. During the lairage time, bobby calves have access to water; however, they have no access to feed and bedding (Animal Welfare Standards 2011). The electric stunning method is commonly used in bobby calves before being slaughtered at the commercial abattoirs.

2.1.3.3 Bobby calves rearing for beef or humane destruction

Approximately 91% of bobby calves are commercially slaughtered, and the remaining are either go for beef rearing or humanely destroyed at the farms. For humane destruction, current practice is on-farm slaughter or use of gunshot or captive bolt or blunt trauma in the forehead to kill the calves. As they are male calves of dairy origin, their growth is not optimal compared to beef breed. Hence, farmers are less interested in rearing bobby calves as beef, and the dairy beef market is also limited (Animal Welfare Standards 2011; Dairy Australia 2016).

The inevitable process is that each year majority of the bobby calves go for slaughter at the very early stage of life and may, therefore, arise some welfare issues. Before going to further discussion on the potential welfare risks in bobby calves, it is crucial to understand what is meant by ‘animal welfare’ and how the welfare of animals can be assessed, particularly during and after transport.

2.1.4 What does 'animal welfare' mean?

Scientists, government bodies and legislators use the term 'animal welfare' from a scientific point of view to measure the quality of living animals. Animal welfare emerged as a new fundamental scientific concept during the 1960s after debates on intensive farming (Woods 2012). A key event was the publication of the book 'Animal Machines' by Ruth Harrison in 1964 where she indicated that animals are often considered as inanimate machines rather as living beings by the animal production industries (Harrison 1964). In 1965, in response to this book, the British government took the initiative and formed a committee to report on the matter. This report considered the biology of the animals to be important to explain their needs and introduced the concept of 'the five freedoms' being *Freedom from hunger and thirst, Freedom from discomfort, Freedom from pain, injury and disease, Freedom to express normal behaviour and Freedom from fear and distress* (Brambell 1965). In the 1970s and early 1980s, although the term 'animal welfare' was used, many people involved in science did not consider animal welfare as a science and the term was not defined clearly. However, from the late 1980s, the concept of animal welfare become more apparent to the biologists and veterinarians (Broom 2011). As the term welfare was frequently being used in science, in-laws and in different discussions to explain the effect of different treatment or factors in animal and measurable consequence of that treatment; a precise scientific definition was necessary.

Animal welfare lacks a complete universal definition (Hewson 2003). To date, the definition of animal welfare has been developed highlighting three approaches- coping, feeling and naturalistic approaches those emphasise biological function and the relation between body and mind (Hewson 2003; Marina and Corrado 2010).

The coping approach highlights the biological functioning of the organism which includes health, reproduction, growth as well as behaviour. An example of the definition about this

approach was given by Broom who stated that "The welfare of an individual is its state as regards its attempts to cope with its environment" (Broom 1986). The word 'coping' was meant as total control of mental and bodily stability. Animal welfare can vary from very good to very poor depend upon how the animal responds to a treatment or a factor. If they are unable to cope with the stimuli or factor, the welfare will be reduced. Animal welfare can be measured through their behavioural, physiological, immunological response or reflections of other components that are coordinated from the brain (Broom 1998; Broom 2001, 2011).

The second approach highlights the animal's mental state and feelings (Duncan 1996, 2005; Marina and Corrado 2010). This approach emphasises the psychological aspects of welfare, where feelings and emotions are the key components to determine the quality of the life of an animal. Feelings are associated with the sensory system in the brain and make the animal conscious of its surrounding environment. Negative subjective emotional states reflect the poor welfare and positive subjective emotional states represent the good welfare (Duncan 2002).

The third approach highlights the concept of naturalness where animals are allowed to live naturally and express their full range of natural behaviour (Kiley-Worthington 1989; von Keyserlingk *et al.* 2009; Marina and Corrado 2010). In this approach, however, the animal may suffer physically such as feeling cold during extreme weather or mentally due to fear response in the event of being preyed (Hewson 2003).

None of the above approaches explains the meaning of animal welfare entirely. Hence, three conceptions of animal welfare such as primary health and functioning, affective states and natural living were compiled in a single statement and defined as "Animal welfare comprises the state of the animal's body and mind, and the extent to which its nature is satisfied" (Duncan and Fraser 1997; Fraser 2008a).

An official definition by the World Organisation for Animal Health (OIE) also includes these three domains and states that "Animal welfare means how an animal is coping with the conditions in which it lives. An animal is in a good state of welfare if it is healthy, comfortable, well-nourished, safe, able to express innate behaviour, and if it is not suffering from unpleasant states such as pain, fear, and distress" (OIE World Organization for Animal Health 2005). Australia also accepts this definition under the Australian Animal Welfare Strategy (Department of Agriculture and Water Resources 2006).

2.1.5 Animal welfare assessment methods

From the early 1990s, animal scientists agreed that animal welfare is a scientific concept because the welfare outcome is measurable (Fraser 2008b). The science-based measurement is indicative of how good or how poor welfare is. Various indicators are useful to monitor the welfare of an animal, such as assessment of physiology, behaviour, growth rate, health status and mortality, and reproductive success (Broom 1988; Broom 2014). Different types of feelings such as pain, fear and pleasure are components of the mechanisms for attempting to cope and should be evaluated during welfare assessment (Broom 1998; Broom 2001; Broom 2006). It should keep in mind that rational integration of the outcome measures of behavioural, physiological and pathological conditions of an animal is the key for the valid interpretation of the welfare status of that animal.

2.1.5.1 Physiological measures

Some physiological values are indicative of poor welfare status of animals such as increased heart rate and adrenal activity or reduced immunological response against pathogens (Broom and Fraser 2015). Due to an increased level of stress, hypothalamic-pituitary-adrenal (HPA) cortex activity may elevate, which leads to a higher level of cortisol or corticosterone production. However, interpretation of physiological measurements should be considered cautiously as normal activities such as courtship and mating can lead to increased HPA

activity (Broom 2014; Broom and Fraser 2015). Age is also a factor regarding HPA activity as it responds poorly in young calves of less than one-month-old due to less development of the HPA axis (Mormede *et al.* 1982; Van Reenen *et al.* 2005). Several studies reported no increase of either plasma cortisol or heart rate in response to transport stress in young calves less than one month of age (Mormede *et al.* 1982; Knowles 1995; Knowles *et al.* 1997). However, several other physiological values are useful to assess the welfare status of animals during transport or short-term treatments. Prolonged deprivation from food and water and prolong transport may lead to increased metabolic activity, dehydration, bruising which can be reflected by changes in blood biochemistry. For example, increased free fatty acids (FFA), beta-hydroxybutyrate (BHB) and urea and decreased plasma glucose is the indication of food deprivation. The interrelation among these metabolic pathways is highly correlated and gives an accurate reflection of individual's energy status and well-established indices to measure energy level in ruminants (Leng 1970; Bell 1979; Sykes and Russel 1979; Clarenburg 1992; Todd *et al.* 2000; Stafford *et al.* 2001). Plasma biochemical values that are convenient and straightforward measures of dehydration are plasma osmolality, total plasma protein and PCV. In general, PCV and total plasma protein increase when there is a loss of fluid from the body. However, total plasma protein can be changed due to dietary effect or due to certain diseases, and reserve red blood cells can be released from the spleen during excitement and stressor, and thus can change the PCV percentage (Knowles and Warriss 2007). Creatine kinase is an enzyme that is found in skeletal muscle, cardiac muscle and brain (Baird *et al.* 2012), and released during trauma and intense muscular exercise (Wrogemann and Pena 1976; Warren *et al.* 2002). On the other hand, lactate is produced in anaerobic conditions during glycolysis from pyruvate (Andersen *et al.* 2013). An elevated concentration of serum or plasma lactate is positively correlated with heavy exercise (Cerretelli and Samaja 2003; Siegel *et al.* 2008), muscle damage (Knowles and Warriss 2007) and trauma (Andersen *et al.*

2013). Increased creatine kinase (CK) activity and lactate concentrations in blood are indications of physical exertion, muscular bruising and fatigue (Knowles *et al.* 2014; Broom and Fraser 2015).

2.1.5.2 Behavioural measures

Behavioural measures also have value for assessing animal welfare. When an animal actively avoids an object or a situation due to bad feelings, this may alter their behaviour and may indicate poor well-being (Broom and Fraser 2015). For example, during rough handling in the event of transportation, mature cattle feel discomfort and attempt to escape, vocalise, kick or struggle (Grandin 1997). Knowles *et al.* (1999) reported the more extended duration of standing at the initial stage of transport and then progressive declination after 10 hours of transportation in 1-2 week old calves when they were transported up to 19 hours. Overall, they found the longer duration of standing behaviour in calves during the journey compared to when those calves were in the farm pen before transportation. Fisher *et al.* (2014) reported similar findings when they transported 5 to 9-day old male dairy calves for 12 hours. They found calves spent 22-32% of time lying down during transport compared to 50% in control calves. Kent and Ewbank reported that calves of less than 4 weeks of age spent 33 to 36% of the time lying during transport in contrast to 6-month-old calves which spent only 5.7% lying when transported (Kent and Ewbank 1983, 1986a). They also reported that 6 month old calves defecated and urinated profusely during the transport and indicated these abnormal behaviours are due to compromised welfare (Kent and Ewbank 1983). Tarrant (1990) reported a similar increased frequency of urination during the transportation of cattle and interpreted this behaviour possibly due to fear. Adult cattle spend most of the time standing during transport and show increased lying behaviour after transport due to transport fatigue (Kenny and Tarrant 1987a, 1987b; Tarrant *et al.* 1992).

2.1.5.3 Pathological measures

Good health is the key to good welfare, and poor health reflects compromised welfare (Broom 1991; von Keyserlingk *et al.* 2009). Hence, the outcome of the measurements of morbidity and mortality are indicative of animal welfare status. The key measurable variables are the presence of diseases or disease conditions, injury, reduced productivity and death (Broom 1988, 1991; von Keyserlingk *et al.* 2009). The prepathological state of the animal such as suppressed immunity (hence vulnerability to diseases), reduced growth and reproduction ability are suggestive of animal suffering and are indicators of the poor welfare state (Broom 1991; Hemsworth *et al.* 1995; Moberg 1998; Broom 2014; Staley *et al.* 2018). Cattle transport is a complex operation which causes direct injury, tissue damage or muscle bruising and sometimes death of the animal. Animals are stressed due to transport which may suppress immunity which enhances susceptibility to infection and diseases (Tarrant 1990; Trunkfield and Broom 1990; Moberg 1998; Swanson and Morrow-Tesch 2001; Broom 2005; Knowles *et al.* 2014; Yun *et al.* 2014). Therefore, measuring the extent of the tissue damage and injury, clinical diseases, the immune function of the animal and mortality rate are useful indicators of the welfare status of the animal during and after transport.

2.1.6 Welfare risk factors in bobby calves

2.1.6.1 Feeding management

Soon after birth, feeding sufficient and good quality colostrum is essential for both heifer replacement calves and sale calves. A calf should be fed an amount of colostrum which is equal to 10% of its bodyweight just after birth but not later than 12 hours and the same amount within the next 12 hours. As a rule of thumb, this volume is 2 litres in the first 12 hours and another 2 litres in the next 12 hours. After 24 hours, whole milk (colostrum or milk), milk replacer or a combination of milk and milk replacer should be fed equally to 10% of its body weight. A small amount of grain or grain-based concentrate from day 1 and good quality fibre from day 3 ensure healthy rumen function in calves (Khan *et al.* 2007; Dairy

Australia 2011). From the day after birth, access to clean, freshwater provides normal body function, increase feed intake and rumen development of the calves (Kertz *et al.* 1984; Dairy Australia 2011). Published literature on the management of feeding in bobby calves in Victoria, as well as in Australia, is limited. Bobby calves, due to their lower economic value, might not get adequate attention and feeding compared to the replacement heifer calves. Vogles *et al.* (2013) investigated colostrum management practice in south-west Victorian dairy farms. In this study, a total of 825 heifer calves and 193 bull calves from 97 dairy herds from south-west Victorian dairy farms were included. Out of 97 dairy herds, they found different management practices between heifer replacement calves and bull calves in 36 herds. They then sampled 33 bull calves from 12 of the 36 dairy herds and found either minimal or no hand-fed extra colostrum at all in bull calves compared to heifer replacement calves. They reported a higher prevalence of failure of passive immunity in male dairy calves (44%) compared to heifer replacement calves (36.6%). A survey conducted by Dairy Australia in 2014 reported that 62% of farmers always give extra colostrum to heifer replacement calves compared to 52% in sale calves (Dairy Australia 2014). A similar survey in 2016 reported a higher percentage of farmers (81%) provide additional colostrum to most calves; although they did not clarify whether sale calves were included in this category (Dairy Australia 2016).

2.1.6.2 Time off feed

Bobby calves are either picked up from the farms and go directly to abattoirs for slaughter or are initially sent to local saleyards from where they are then transported to the slaughterhouses. Bobby calves are usually collected from the farms in the morning and are delivered to the slaughterhouses during the afternoon or evening of the same day. These calves are then rested overnight in the abattoirs before being slaughtered next day at early

morning. From the time of collection from the farm of origin they generally remain without liquid or solid feed but are given access to water during the lairage time at the abattoirs.

An experimental study of 1 to 3-week old calves showed that 18 hours off feed did not have a significant effect on plasma biochemical values compared to 6 hours of deprivation except decreasing level of plasma glucose after 6 hours of fasting (Kent and Ewbank 1986a). Another study conducted by Knowles *et al.* (1997) reported weight loss, dehydration and increased utilisation of reserved body energy in one-month-old calves when transported for 24 hours without feed compared to normally fed calves without transportation.

In New Zealand and Australia, two experimental studies have been conducted in bobby calves to assess the effect of deprivation of feed and transportation time (Todd *et al.* 2000; Fisher *et al.* 2014). In the New Zealand study, Todd *et al.* (2000) found that glucose concentration in 5 to 10-day old bobby calves started to decline after 10 to 13 hours of starvation and continued this trend up to 24 hours and then remained constant until 31 hours. From 19 hours up to 31 hours plasma glucose level in starved bobby calves were significantly lower than the control adequately fed calves. They observed an increased level of β-hydroxybutyrate, indicative of lipid metabolism to supplement energy, and little increase of plasma urea concentration, indicative of some utilisation of amino acid to provide energy, in the starved bobby calves compared to controlled adequately fed calves. They concluded that bobby calves experienced hypoglycaemia after 30 hours of starvation, but they were not extremely hypoglycaemic as they maintained normothermia and catalysed minimal amino acid for supplementing energy. An Australian experimental study conducted in bobby calves by Fisher *et al.* (2014) reported that blood glucose concentration declined in calves after 18-24 hours of off feed; and β- hydroxybutyrate, which is the indication of lipolysis, increased after that time. Fisher *et al.* (2014) also stated that 12% of calves had glucose concentrations

at 30 hours below 2.8 mmol/L, which was below the relevant reference value and an indication of hypoglycaemia. However, Todd *et al.* (2000) reported that bobby calves experienced hypoglycaemia after 30 hours of off feed.

2.1.6.3 Age at transport

A few studies have looked at the effect of age on blood chemistry and behaviour of calves during and after transportation and reported that 1 to 3-week old calves were affected less compared to 3 to 6 months old calves (Kent and Ewbank 1983, 1986a, 1986b). For example, calves of less than 4 weeks of age spent 33 to 36% of the time lying during transport in contrast to 6-month-old calves which spent only 5.7% lying when transported (Kent and Ewbank 1983, 1986a). Jongman and Butler (2014) examined the effect of age with transportation in bobby calves. The authors reported no significant variation in metabolic profiles after 12 hours transport among 3, 5 and 10 day old bobby calves. However, they found 3 day old calves had frequent lying behaviour than other groups. In another study, they reported that 5 to 11-day old calves are easier to handle compared to 3-day old calves (Jongman and Butler 2013).

2.1.6.4 Transportation

Several other factors may affect the welfare of calves due to transportation such as ways of loading and unloading, space and bedding materials provided in the trucks, distance and duration of transport, attitude towards bobby calves by the people who are involved in their transportation, the climatic and other transport conditions.

2.1.6.5 Handling

Handling during loading and unloading is thought to be one of the most stressful aspects of transportation in both calves and older cattle (Agnes *et al.* 1990; Trunkfield and Broom 1990; Maria *et al.* 2004). Duration of loading, the design of ramp and animal handling during loading and unloading have a potential effect on animal welfare during transport (Nielsen *et*

al. 2011). In commercial beef cattle transport, it was found that cattle with longer loading and unloading time had significantly increased plasma CK and lactate compared to cattle which required less time (Maria *et al.* 2004). Studies highlighting the effect of duration of loading, loading technique and handling during commercial bobby calf transport would be interesting to see whether those factors have any potential welfare risk in bobby calves under existing management practice.

2.1.6.6 Distance and duration

The long duration of transport is more likely to have an adverse effect on animal welfare compared to short duration transport (Nielsen *et al.* 2011). However, it is essential to recognise that other factors such as feed and water deprivation, extreme environmental temperature, loading and unloading methods and other transport conditions strongly influence the impact of journey duration on animal welfare. Hence, healthy and physically fit animals provided with all other optimal conditions can tolerate travel longer without compromising welfare compared to unfit animals (Nielsen *et al.* 2011). In very young calves of 7 to 21 days of age, Kent and Ewbank (1986a) did not find a significant difference between the metabolic profile and behavioural response when compared 6 hours versus 18 hours of transport. Knowles *et al.* (1997) found an increased level of NEFA and BHB after 24 hours of transport in calves less than one-month-old probably due to the mobilisation of reserve energy. In addition to the increased level of free fatty acids, Grigor *et al.* (2001) reported an increased level of plasma CK due to physical exertion when young calves were transported for 9 hours of transport with 1 to 12 hours of lairage and again 9 hours of transport compared to non-transported calves. A similar result of increased plasma CK was observed in bobby calves when transported experimentally for 12 hours compared to pre-transport and calves those stayed at farms (Fisher *et al.* 2014).

2.1.6.7 Stocking density

Appropriate space allowance in the truck during road transport is vital for calves. A generous space allowance (low stocking density) is problematic for calves to maintain their balance as they cannot use surrounding animals to support each other for maintaining stability. Small space allowance (high stocking density) may make it difficult for calves to orient themselves to the direction of the vehicles during transport and they are also more likely to be squashed (Tarrant *et al.* 1992; Knowles 1999). One experimental study was conducted to assess the effect of stocking density and type of suitable bedding material during road transport in bobby calves in Australia (Jongman and Butler 2014). The authors reported that 0.3 m^2 space allowance per calf in the truck is beneficial compared to 0.2 m^2 space. Regarding bedding materials, they found straw bedding to be more comfortable for calves compared with no bedding during transportation but acknowledged that straw might not be practical under commercial transport because of cost and biosecurity issues. The authors also indicated that perforated rubber matting could be a suitable alternative to straw bedding; however, this needs to be investigated under experimental and commercial conditions (Jongman and Butler 2014).

2.1.6.8 Other factors

Climatic conditions such as temperature and humidity and the introduction of the cattle into a new environment may also lead to welfare risks (Kenny and Tarrant 1987b; Tarrant and Grandin 2000; Grigor *et al.* 2001; Nielsen *et al.* 2011). The attitude of the people involved with transport and their attitude towards animals is also critical. Careless management can cause injuries or undue stress throughout the process of transportation. The effect of these risk factors during the transport of bobby calves has not been examined before.

2.1.6.9 Effects of transport

Research indicates that animals can be severely stressed due to transportation (Trunkfield and Broom 1990) and significant transport stress can cause the death of an animal (Vecerek *et al.*

2006). Thus, increased mortality due to transport could be an indicator of poor welfare. The stress of calves increases during transportation and the level of stress could be influenced by regrouping, handling during loading and unloading, space allowance, driving performance, transport and lairage time (Vecerek *et al.* 2006; Jongman and Butler 2014). In Australia, the majority of the bobby calves are transported from dairy farms to regional abattoirs (Cave *et al.* 2005; Animal Welfare Standards 2011). As bobby calves are young and vulnerable, they need special attention during transportation. Cave *et al.* (2005) investigated the effect distance of transport and mortality in bobby calves by analysing mortality data from 1998 to 2000 from an abattoir in Northern Victoria. They reported 0.64% mortality in bobby calves during and after transport and a positive correlation between the distance of bobby calf transport and increased mortality. They also observed that increased mortalities occurred during transportation or just after arrival compared to death overnight at the abattoir. However, limited information has been found as to why long distance increases the mortality. They also found that the highest mortality was in October followed by September and the lowest in August. They hypothesised that induction of calving in the later part of the calving season (September-October) might be one of the reasons for higher mortalities in October and September compared to August. It would be expected that induced calves are weaker than calves those are not induced. Thomas and Jordaan (2013) reported a 0.7% pre-slaughter mortality risk in bobby calves in New Zealand, which has a similar industry to Victoria, Australia. They did gross post-mortem analysis of dead bobby calves to determine the reasons for pre-slaughter mortality and reported that disorders in the digestive tract, omphalitis and septicaemia were the most common causes of death. Studies have not previously been carried out in Australia into causes of mortality in bobby calves. Both gross and microscopic post-mortem analysis could help to determine the possible causes of bobby calf mortality that might inform management actions aimed at reducing further calf mortality.

An extended period of transport without food and water causes loss of body weight in cattle. Warriss (1990) reported 3 to 11% of losses of initial body weight during the first 24 hours of transport. Knowles *et al.* (1997) reported decreasing trends of body weight losses during the 24 hours of transport in very young calves of less than one month of age. Fisher *et al.* (2014) found a similar result of 6.7% losses of initial body weight after 30 hours of food withdrawal and 12 hours of transport in bobby calves in an experimental study. Todd *et al.* (2000) also reported 0.63 to 1.79 kg loss of initial weight after 30 hours of food deprivation in bobby calves during the experimental study. Although initial weight loss is due to the loss of gut fill, long-term deprivation of food and water can cause carcass weight loss due to dehydration and depletion of energy reserves from the body (Knowles *et al.* 2014). In the commercial bobby calf supply chain, weight loss before and after the transport has not been assessed to see how this value influences the economics of veal production.

2.1.6.10 Lairage

Few studies have been conducted to see the effect of lairage duration and its impact on calf welfare after transportation. In an experimental study, minimal physiological and behavioural responses were observed between 1 h vs 12 h lairage duration in young calves (Grigor *et al.* 2001). In contrast, a field study indicates that longer lairage duration is likely to have adverse welfare effects on bobby calves (Stafford *et al.* 2001). An observational study conducted during commercial transportation of bobby calves in New Zealand showed that after 12-15 h lairage blood glucose concentrations declined and BHB concentrations increased indicating a shortage of carbohydrate in the body and increased lipid metabolism (Stafford *et al.* 2001). They also found a significantly higher level of CK after long lairage time. Data regarding the duration of lairage after commercial transport and its effect on the welfare of bobby calves before slaughter in Australia are lacking.

2.1.7 Public Concern

Animal welfare concerns are becoming increasingly crucial to the industry, consumers, and the public, both in Australia and internationally. There is considerable evidence that public interest in animal welfare has been increasing. The public is now more concerned regarding farm animal welfare in several countries including United States, United Kingdom, Spain, Sweden, Finland, Denmark and Australia (Maria and María 2006; Coleman *et al.* 2015; Coleman 2018; Coleman *et al.* 2018; Malek *et al.* 2018; McKendree *et al.* 2018; Sinclair *et al.* 2018). As bobby calves are very young and vulnerable to transport; some sectors of the Australian community are concerned about their ethical management, transportation, and handling (RSPCA Australia 2018).

2.1.8 Regulations for the management of bobby calves

Over the years, community values and expectations to farm animals have changed dramatically, and international trading partners also want to ensure livestock welfare throughout the supply chain. For the last 35 years, the Australian government and industry in consultation with the community and relevant stakeholders have developed a series of Model Codes of Practice (MCOP) to ensure the welfare of animals in Australia. After a review in 2005, the MCOP was developed into Australian Animal Welfare Standards and Guidelines. In 2009, the Primary Industries Ministerial Council endorsed the Australian Animal Welfare Standards and Guidelines-Land Transport of Livestock. During the development process for this document, it was highlighted that the transport and management of bobby calves remain a contentious area. One of the most critical welfare concerns was the maximum permissible duration of time off feed (TOF). Animal Health Australia (AHA) was then appointed for the Regulation Impact Analysis to recommend a science-based standard for the maximum allowable TOF for bobby calves. Considering cost-benefit and the welfare of bobby calves supported by relevant scientific studies, AHA recommended a 30 hours TOF as a maximum

enforceable limit (Animal Welfare Standards 2011). This standard is supported by the research conducted under experimental condition in bobby calves in Australia and New Zealand (Todd *et al.* 2000; Fisher *et al.* 2014). This industry standard accompanies the other requirements required under the Australian Animal Welfare Standards and Guidelines for bobby calf transport. According to the criteria for the transport of bobby calves for slaughter in Australia; bobby calves must be at least 5 days old, healthy and fit for transport, be fed within 6 h before loading, be transported for no more than 12 h (Animal Welfare Standards 2011). Although regulations exist, there is a lack of data regarding the impact of these regulations on the welfare outcomes for bobby calves in commercial practice.

2.1.9 Conclusion

Ensuring the welfare of bobby calves is a high priority for the dairy industry in Australia. There are some standards and regulations have been developed to maintain good welfare outcomes across the bobby calf supply chain based on relevant scientific studies. However, current literature indicates that there are still some areas of research gap worthy of further studies in commercial bobby calf supply chain.

- The first task is to establish the measurable criteria for the welfare determination in bobby calves in commercial practice.
- Another gap is the lack of available field data which may establish the health and welfare performance of bobby calves after commercial transport and lairage. It is also essential to explore the data in commercial practice whether existing commercial transport of bobby calves are following the standard of 30 hours TOF before being slaughtered.
- There is also limited data on other risk factors during transportation of bobby calves which may affect their welfare such as the handling, loading and unloading, attitude

of people involved with bobby calf transport, climatic conditions, distance and duration of bobby calf transport, and lairage duration.

- Another potential area of further study is to understand the on-farm management of bobby calves, specifically the on-farm feeding management to see how feeding management affect their health and welfare before slaughter.

2.2 Enteric pathogens in bobby calves

2.2.1 Bacterial and viral enteric pathogens in young calves

Calf diarrhoea or calf scours is a multifactorial disease of neonatal calves and responsible for the significant economic loss to farmers globally (Wittum *et al.* 1993; Busato *et al.* 1997; Barrington *et al.* 2002; Osteras *et al.* 2007; Lorenz *et al.* 2011; Cho and Yoon 2014). Although both infectious and non-infectious factors are responsible for neonatal calf diarrhoea, the major causes are the bacterial, viral and parasitic agents (Acres *et al.* 1977; Reynolds *et al.* 1986; Snodgrass *et al.* 1986; Clement *et al.* 1995; Bendali *et al.* 1999b; Barrington *et al.* 2002; Trotz-Williams *et al.* 2007; Gulliksen *et al.* 2009; Bartels *et al.* 2010; Izzo *et al.* 2011; Cho and Yoon 2014).

Among the infectious agents, the major bacterial and viral enteric pathogens in neonatal calves are bovine rotavirus (BRV), bovine coronavirus (BCV), *Escherichia coli* K99/F5, and *Salmonella spp*. (Torres-Medina *et al.* 1985; Reynolds *et al.* 1986; Snodgrass *et al.* 1986; Holland 1990; Trotz-Williams *et al.* 2007; Gulliksen *et al.* 2009; Bartels *et al.* 2010; Izzo *et al.* 2011). Less common pathogens are enteropathogenic *E. coli*, enterohemorrhagic *E. coli*, Shiga toxin-producing *E. coli*, *Clostridium difficile*, *Clostridium perfringens*, and bovine torovirus (Haschek *et al.* 2006; Rodriguez-Palacios *et al.* 2006; Kirisawa *et al.* 2007; Rodriguez-Palacios *et al.* 2007; Wieler *et al.* 2007; Ferrarezi *et al.* 2008; Bartels *et al.* 2010).

2.2.1.1 Bovine rotavirus (BRV)

Mebus *et al.* (1969) first demonstrated a virus that caused neonatal calf diarrhoea and later the virus was named as rotavirus (Mebus 1969; Flewett *et al.* 1974; Derbyshire and Woode 1978). Bovine rotavirus, which is a non-enveloped virus having 11 double-stranded RNA segments, belongs to the genus rotavirus under the Reoviridae family (Derbyshire and Woode 1978; Kalica 1978; Parashar *et al.* 1998). Based on the antigenic and genetic similarities of the intermediate capsid protein (VP6), rotavirus is classified into seven serogroups designated

A through G (Pedley *et al.* 1983; Parashar *et al.* 1998). The most common rotaviruses associated with infection and diarrhoea in young farm animals are group A rotaviruses (Holland 1990). Based on genetic and antigenic similarities of protease-sensitive protein (VP4) and glycoprotein (VP7), group A rotaviruses can be further classified as P or G types (Estes and Cohen 1989; Parashar *et al.* 1998; Desselberger 2014). Globally, the most prevalent rotaviruses in young calves are the G6, G10, P[5] and P[11] genotypes (Hussein *et al.* 1995; Garaicoechea *et al.* 2006; Howe *et al.* 2008; Swiatek *et al.* 2010; Papp *et al.* 2013; Pourasgari *et al.* 2016). Bovine rotaviruses are widespread and usually affect young calves at 1 to 2 weeks of age (Acres *et al.* 1977; Holland 1990; Bendali *et al.* 1999a; Garcia *et al.* 2000; Cho and Yoon 2014), although older calves can be affected (Bridger 1994).

2.2.1.2 Bovine coronavirus (BCV)

Mebus *et al.* (1973) first reported coronaviruses as a cause of calf diarrhoea back in 1973 and now these viruses are widely distributed in the world and are associated with three clinical syndromes in cattle: calf diarrhoea, winter dysentery in adult cattle and respiratory infection in cattle of different ages (Clark 1993; Saif 2010; Maclachlan and Dubovi 2017). Bovine coronaviruses (BCV) belong to the antigenic subgroup 2a under the Coronaviridae family in the Nidovirales order (Clark 1993; Saif 2010). Calf dialarhoea due to BCV infection commonly occurs in calves of less than three weeks of age, although older calves up to 3 months of age can be affected (Reynolds *et al.* 1986; Maclachlan and Dubovi 2017).

2.2.1.3 Enterotoxigenic *Escherichia coli*

Intestinal *E. coli* those can produce diseases are classified into six categories: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) (Nataro and Kaper 1998). Among these bacteria, the most common pathotype that causes neonatal diarrhoea in calves is ETEC strains that has K99 (F5) adhesion antigen,

commonly called as *E. coil* K99, and produce the heat-stable enterotoxin (ST) (Myers and Guinee 1976; Sivaswamy and Gyles 1976; Burgess *et al.* 1978; Guinée 1979; Sherwood *et al.* 1983; Acres 1985; Holland 1990; Bazeley 2003). ETEC commonly infects and causes diarrhoea in the first four days of life in both dairy and beef calves, although rarely causes diarrhoea to older calves and adult cattle (Myers and Guinee 1976; Acres *et al.* 1977; Sherwood *et al.* 1983; Acres 1985).

2.2.1.4 Salmonella *spp.*

Salmonella is a genus under Enterobacteriaceae family. There are two species under *Salmonella*, *Salmonella enterica* and *Salmonella bongori* (Brenner *et al.* 2000). Under *Salmonella enterica,* serovars *S. Typhimurium* and *S. Dublin* mainly responsible for diarrhoea in dairy and beef calves. Neonatal disease outbreaks in calves due to *Salmonella* spp. are mostly observed between 2 to 6 weeks of age (Sojka *et al.* 1977; Barrington *et al.* 2002; Mohler *et al.* 2009; Barrow and Methner 2013).

2.2.2 Passive immunity and enteric pathogens in young calves

The syndesmochorial placenta of cows act as a barrier to pass immunoglobulins from dam to bovine foetus in utero; as a result, calves are born agammaglobulinemic (Weaver *et al.* 2000). After birth, it requires at least 2 to 4 weeks or more time to be functional of the active immunity of calves. Therefore, calves rely on maternal immunologic assistance through colostrum during the first month of their life (Barrington and Parish 2001; Reber *et al.* 2006; Chase *et al.* 2008). The significant components of colostrum, which establish passive immunity in the early life of calves, are antibodies, cytokines and immune cells such as leukocytes (Clover and Zarkower 1980; Lee *et al.* 1980; Norcross 1982; Hagiwara *et al.* 2000). Failure of adequate passive immunity in neonatal calves predisposes to gastrointestinal and systemic infection. Colostrum thus provides the first line of defence in newborn calves (Norcross 1982; Holland 1990; Wells *et al.* 1996; Donovan *et al.* 1998; Chase *et al.* 2008).

Several studies indicate that passive immunity can prevent infection to enteric pathogens causing diarrhoea provided that ingested colostrum contains specific antibody against the specific enteric pathogens (Logan 1974; Saif *et al.* 1983; Besser *et al.* 1988; Bok *et al.* 2018; Lora *et al.* 2018). To ensure a high level of antibody in the colostrum, one of the strategies is the maternal vaccination against major enteric pathogens which results in a high level of antibody in colostrum and protect calves against enteric pathogens if calves were fed adequate colostrum at the right time (Acres *et al.* 1979; Saif *et al.* 1983; Acres 1985; Saif and Fernandez 1996; Maclachlan and Dubovi 2017). Hence, maternal vaccination and colostrum management are important factors for adequate protection against enteric pathogens in the neonatal period of calves. Farm-level data regarding the colostrum management of bobby calves in Australia is limited. It is hypothesised that as they are sale calves, they might not get the adequate colostrum and farmers might give the colostrum to heifer replacement calves first which might have delayed colostrum feeding to the bobby calves. Therefore, passive immunity in bobby calves and how they affect the shedding of enteric pathogens in bobby calves under Victorian dairy systems is an important area of further research.

2.2.3 Road transport and enteric pathogen shedding in neonatal calves

Physical and psychological stimuli during transportation cause significant transport stress to cattle. Factors that are positively correlated to transport stress but not limited to are pre-transport management, noise, vibration, novelty, duration of transport, mixing with unfamiliar animals, stocking density, climatic factors, restraints, loading and unloading, age, and feed and water derivation (Trunkfield and Broom 1990; Grandin 1997; Swanson and Morrow-Tesch 2001; Fike and Spire 2006). Transport stress is immuno-suppressive in calves (Kent and Ewbank 1986a, 1986b; Mackenzie *et al.* 1997; Fike and Spire 2006), which in turns may increase the chance of disease susceptibility and increased pathogen shedding (Swanson and Morrow-Tesch 2001). The relationship between the influence of stress and

increased pathogen shedding in the faeces of ruminants has been well documented (Freestone and Lyte 2010). Barham *et al.* (2002) reported an increased incidence of *Salmonella* spp. in the faeces of beef cattle after transportation. Bach *et al.* (2004) also reported a similar trend of increased faecal incidence of *E. coli* and *E. coli* O157: H7 in calves after long-haul transport. However, few authors have reported no significant effect of transport and lairage on the faecal prevalence of *E. coli* O157 in cattle (Minihan *et al.* 2003; Fegan *et al.* 2009). In Australia, a research was conducted in sheep to see the effect of 12 or 24 h transport in combination with 12 and 24 h food and water derivation before transport on the levels of *E. coli* and *Salmonella* spp. shedding in the faeces. No significant effect of transport time and food and water deprivation on the rates of pathogen shedding in the faeces of sheep had been observed (Meat & Livestock Australia 2007).

Research has not been conducted yet to see the effect of transport on enteric pathogen shedding in bobby calves which is important in relation to health and welfare of bobby calves and food safety perspective as the veal from bobby calves predominantly export to Japan and the USA.

One such primary foodborne pathogen is *Salmonella* spp. and common causes of bacterial gastroenteritis in human. Meat and meat products from the cattle are the important sources of Salmonella infection in human (McEvoy *et al.* 2003). During the slaughterhouse operation, hair and skin of cattle, gut content and faeces, and the external portion of the urogenital tract are the primary sources of meat contamination (Nørrung and Buncic 2008). Hence, excretion of *Salmonella* spp. in cattle at slaughter and subsequent cross-contamination of meat and meat product is a significant food safety issue. Slaughtering pathogen-free animal is one of the approaches to improve meat hygiene (Swanenburg *et al.* 2001).

2.2.4 Prevalence of enteric pathogen in neonatal calves in Australia

Neonatal calf diarrhoea (NCD) or calf scours in dairy and beef calves is a significant problem for the producers. Neonatal calf diarrhoea has a negative impact on both farm economics and animal welfare (Bazeley 2003; Mellor and Stafford 2004; Uetake 2013; Meganck *et al.* 2015; Abuelo 2016). A survey conducted back in 1966 reported that 80% of beef properties in Victoria had calf scour problem (Dickson 1966). Despite being one of the top dairy issues, investigation on etiologic agents and risk factors associated with NCD in calves in Australian dairy farming systems is limited. So far, few small scales and one large-scale studies reported on etiologic agents of NCD and their prevalence in Australia (Jerrett and Snodgrass 1981; Huang *et al.* 1992; Becher *et al.* 2004; Swiatek *et al.* 2010; Izzo *et al.* 2011). In 1988, during the outbreak of calf diarrhoea, 78 calf faecal samples from 3 dairy farms in Victoria were tested for rotaviruses; of those 38 samples were positive for group A rotavirus (Huang *et al.* 1992). Another study conducted during 2004-2005, where 72 faecal samples from diarrhoeic calves and 28 samples from calves without diarrhoea from the Gippsland region in Victoria were tested for rotaviruses. Group A rotavirus was found in 26% samples, 22 faecal samples were positive from diarrhoeic calves and four from calves without diarrhoea (Swiatek *et al.* 2010). *Cryptosporidia* spp. was detected and reported in calves from 5 of 9 outbreaks of calf diarrhoea on farms in the Gippsland and central Victoria in the year of 1980 (Jerrett and Snodgrass 1981). In Western Australia, 54 faecal samples from Holstein dairy calves from 2 dairy properties were tested for *Cryptosporidium parvum*, and 48% of samples were positive (Becher *et al.* 2004). A large-scale study conducted between May 2007 and September 2008, and samples were collected from outbreaks of diarrhoea in calves less than six weeks of age. A total of 597 samples tested from 84 Australian dairy and dairy beef properties from all states of Australia, of those 95% samples was positive for enteric pathogens. They reported that rotavirus was the most common pathogen detected (79.9%) followed by

Cryptosporidium parvum (58.5%), *Salmonella* spp. (23.8%), coronavirus (21.6%) and *E. coli* K99 (17.4%) (Izzo *et al.* 2011).

Although these studies highlight the presence and extent of infectious agents during NCD in neonatal calves while somewhat limited to predict the situation in bobby calves. There is a possibility of less attention and care in bobby calves due to their low market value. Also, a stressful journey from farms to abattoirs might influence to get them infected with enteric pathogens. At present, data on the prevalence of major enteric pathogens and associated risk factors in bobby calves in Australia are limited.

2.2.5 Conclusion

- Above available literature highlights the fact that prevalence and risk factors associated with enteric pathogens in bobby calves have not been explored before in Australia.
- Stressful transportation and other farm management may influence increased shedding of enteric pathogens in bobby calves which is a potential area of further research.

2.3 *Mycoplasma bovis* in dairy cattle

2.3.1 Mycoplasma

At the initial phase of discovery, mycoplasmas were considered as a virus because they could pass through the filters which prevent other bacteria from passing through (Reimann 1938; Saraya 2016). Later, mycoplasmas were thought as L-forms of bacteria due to some similarities as both have a lack of cell wall and form fried egg shape colony. This confusion came to an end after genomic data analysis by DNA hybridisation method in the late 1960s (Razin 1969). However, current research findings based on rRNA sequencing data support the degenerative evolution of mycoplasmas from gram-positive bacteria (Woese *et al.* 1980). They are now considered as a group of eubacteria without any cell walls and the smallest prokaryotes capable of self-replication (Razin and Hayflick 2010). Phenotypically they are different from bacteria by their minute size, the diameter of 0.1 to 0.3 µm and length of up to 98 µm, and complete lack of cell wall (Razin 1987; Razin *et al.* 1998; Razin and Hayflick 2010). Instead of a cell wall, they have a plastic cell membrane and mostly spherically shaped (Razin *et al.* 1998).

2.3.2 Taxonomic Classification of *Mycoplasma bovis*

Mycoplasma genus belongs to the family *Mycoplasmataceae* under the order *Mycoplasmatales* of the class *Mollicutes* (Tully et al. 1993). This class comprises eight different genera: *Mycoplasma, Ureaplasma, Entomoplasma, Mesoplasma, Spiroplasma, Acholeplasma, Anaeroplasma and Asteroplasma* based on genome size, requirement of cholesterol for growth, G-C content, shape, NADH oxidase lactation, urease reaction, habitat and other growth requirements (Tully *et al.* 1993; Razin *et al.* 1998). The genus *Mycoplasma* has 126 recognised species, including *Mycoplasma bovis* (Razin *et al.* 1998).

2.3.3 History of *Mycoplasma bovis*

Mycoplasma bovis first isolated from the milk of a mastitic cow in the USA back in 1961. After discovery, this pathogen was named as *Mycoplasma agalactiae* var. *bovis* because of much similarities of the disease called contagious agalactia in goat and sheep caused by *Mycoplasma agalactiae* (Hale *et al.* 1962). Later, it was termed as *Mycoplasma bovimastidis* as a separate species (Jain *et al.* 1967), but Freundt and Edward (1971) provided the evidence that it had many similarities with *M. agalactiae* based on serological growth-inhibitory antibody and gel-diffusion precipitation methods and proposed the name *M. agalactiae* subsp. *bovis* . Finally, it was established as separate species by Askaa and Erno (1976) based on nucleic acid hybridisation technique.

2.3.4 Epidemiology of *M. bovis* in dairy cattle

2.3.4.1 Host specificity

Mycoplasma bovis has been isolated from goats, chickens, pigs and human other than bovine animals but it mainly causes pathogenesis in cattle (Pfutzner and Sachse 1996; Bashiruddin *et al.* 2005; Pitcher and Nicholas 2005; Ongor *et al.* 2008; Spergser *et al.* 2013).

2.3.4.2 Transmission and risk factors

Mycoplasma bovis can harbour in the upper respiratory tract of healthy calves and young cattle and shed the pathogen via nasal discharge for months to years (Pfutzner and Sachse 1996; Nicholas *et al.* 2002; Maunsell *et al.* 2011). The other vital sites where it harbours are mammary gland (Bennett and Jasper 1977a; Gonzalez *et al.* 1992), joints (Stalheim and Page 1975), lungs (Thomas *et al.* 2002), urogenital tracts (Winter *et al.* 2006; Aurich *et al.* 2008; Bruegmann *et al.* 2012; Hazelton *et al.* 2018) and middle ear (Francoz *et al.* 2004) in cattle. Among these predilection sites, upper respiratory tract and the mammary gland are the preferred sites for colonisation and shedding (Bennett and Jasper 1977a; Pfutzner and Sachse 1996; Punyapornwithaya *et al.* 2010). Bacteraemia has been reported after *M. bovis* infection

(Bennett and Jasper 1977a; Nicholas *et al.* 2002) and consequently infection in the joint, ear canal and occasional in the mammary gland (Fox *et al.* 2005; Fox 2012). In the tissue, *M. bovis* mainly colonises in the mucosal surfaces and persist with or without clinical diseases (Punyapornwithaya *et al.* 2010; Maunsell *et al.* 2011). The subclinical nature of infection makes the animal as a silent carrier and play a vital role to transmit *M. bovis* into the naïve population (Gonzalez *et al.* 1992; Nicholas *et al.* 2008; Maunsell *et al.* 2011). A different stressful condition such as transportation, stress from cold, during feedlot entry and comingling initiate increased nasal shedding and facilitate infection into uninfected animals (Boothby *et al.* 1983; Woldehiwet *et al.* 1990). In dairy herds, the primary route of transmission is from udder to udder (Jasper *et al.* 1974; Gonzalez *et al.* 1992; Gonzalez and Wilson 2003). From udder to calves is another important route of transmission. Calves get an infection after ingestion of contaminated or mastitic milk from infected cows (Bennett and Jasper 1977b). The report also suggests that even without feeding of contaminated milk calves may get an infection by sharing the same calving premises with infected cows and occasionally via the congenital route (Pfutzner and Sachse 1996; Maunsell *et al.* 2011). Aerosols created from nasal secretion, nasal contact from infected to uninfected animals, contaminated feed, water, fomites or semen are thought to be the other sources of transmission and infection (Jasper *et al.* 1974; Eaglesome and Garcia 1990; Gonzalez *et al.* 1992; Gonzalez and Wilson 2003; Haapala *et al.* 2018). Experimentally, *M. bovis* infection in calves by inhalation has been established (Nicholas *et al.* 2002; Wawegama *et al.* 2014) which support that inhalation as a mode of transmission in calves when they live in infected premise or during commingling. *M. bovis* may persist in the dairy shed and surrounding environment in variable duration depending upon the environmental condition. Cold and humid condition help to survive *M. bovis* in the environment whereas increased temperature and sunlight reduce the survivability (Ruffo *et al.* 1969; Pfutzner 1984). The environment

thus might have a potential source of infection; however, further studies are required to confirm this is the case.

2.3.4.3 Distribution and Prevalence

M. bovis has a worldwide distribution. In Asia, *M. bovis* has been reported in Israel (Lysnyansky *et al.* 2016), China (Menghwar *et al.* 2017) and Japan (Higuchi *et al.* 2011) and in many countries in Europe including, but not limited to, Great Britain (McAuliffe *et al.* 2004), France (Arcangioli *et al.* 2011), Germany (Kirchhoff and Binder 1986), Denmark (Kusiluka *et al.* 2000), Switzerland (Aebi *et al.* 2015), Ireland (Byrne *et al.* 1998), and Netherland (ter Laak *et al.* 1992b). Several reports have been published from the USA (Gonzalez *et al.* 1992; Kirk *et al.* 1997; Fox *et al.* 2003) and Canada (Olde Riekerink *et al.* 2006) highlighting the presence of *M. bovis* in cattle from different regions. In Australia, the presence of *M. bovis* has been reported in 1970 (Cottew 1970). New Zealand was free from *M. bovis* before 2017. Even after a strong biosecurity management recent report of the occurrence of the *M. bovis* in New Zealand dairy cattle highlights its possible worldwide distribution. Level of the prevalence of *M. bovis* varies from country to country and type of cattle industry. In the USA, 7-20% *M. bovis* prevalence has been reported in dairy herds based on bulk milk samples testing (Fox *et al.* 2003; Wilson *et al.* 2009). In European countries, 1.5 to 5.9% herd prevalence of *M. bovis* has been reported (Filioussis *et al.* 2007; Passchyn *et al.* 2012; Arede *et al.* 2016). However, the higher prevalence of *M. bovis* has been reported in feedlot cattle. In a US study, Hanzlicek *et al.* (2011) reported a positive *M. bovis* specific antibody in 27% beef calves on the first date of feedlot entry. In France, 28-90% herd prevalence was estimated and reported depending upon the location of the beef herds in a different county (Le *et al.* 2002). In a study in beef cattle in northern Italy, 76% animal was tested seropositive against *M. bovis* (Radaelli *et al.* 2008). Sero-conversion rate is also higher in feedlot cattle where 60-100% seroconversion of *M. bovis* has been reported by

various authors around the world (Arcangioli *et al.* 2008; Hanzlicek *et al.* 2011; Wawegama *et al.* 2016). On the other hand, the nasal prevalence of *M. bovis* in young calves in infected herds in the endemic area has been reported up to 100% (Bennett and Jasper 1977b; Springer *et al.* 1982; ter Laak *et al.* 1992a; ter Laak *et al.* 1992b).

2.3.5 Diseases caused by *M. bovis* in dairy cattle

In adult dairy cattle, *M. bovis* mainly responsible for mastitis either in the subclinical or severe clinical form (Gonzalez and Wilson 2003). *M. bovis* can affect any stage of lactation in cows, including a dry period (Bicknell *et al.* 1978; Otter *et al.* 2015) and prepubertal stage (Fox *et al.* 2008). *M. bovis* associated mastitis can occur alone or in combination with arthritis or respiratory disease or both (Pfutzner and Sachse 1996; Gonzalez and Wilson 2003; Wilson *et al.* 2007).

M. bovis associated pneumonia occurs mostly in young dairy and beef calves although any age of cattle is susceptible (Pfutzner and Sachse 1996; Caswell and Archambault 2007; Maunsell and Donovan 2009; Maunsell *et al.* 2011). Other form of *M. bovis* associated diseases in young calves are otitis media, arthritis (Stalheim and Page 1975; Stipkovits *et al.* 1993; Walz *et al.* 1997; Francoz *et al.* 2004; Maunsell and Donovan 2009), and less commonly tenosynovitis, decubital abscess and meningitis (Kinde *et al.* 1993; Stipkovits *et al.* 1993; Adegboye *et al.* 1996).

Other less commonly occurring *M. bovis* associated diseases are keratoconjunctivitis (Alberti *et al.* 2006), myocarditis (Haines *et al.* 2004), and genital disorders such as genital infection and abortion in cows (Pfutzner and Sachse 1996).

2.3.6 Diagnosis of *M. bovis* infection in dairy cattle

2.3.6.1 Clinical signs

Clinical signs are not very specific to detect *M. bovis* infection; however, clinical manifestation indicates *M. bovis* infection which needs to be confirmed by an appropriate laboratory test.

M. bovis associated mastitis generally affect more than one quarter. General systemic illness is mild; however, a drastic reduction in milk production is evident. The mammary gland may be swollen without intense pain sensation having with gritty or purulent secretion and inadequate response to antibiotics (Pfutzner and Sachse 1996; Gonzalez and Wilson 2003). Fever, tachypnoea, dyspnoea, loss of appetite with or without nasal discharge and coughing are associated with pneumonia caused by *M. bovis*. These signs are nonspecific as pneumonia caused by other organisms have many similar signs (Pfutzner and Sachse 1996; Stipkovits *et al.* 2000; Caswell and Archambault 2007). Eardrop, ptosis, head shaking, rubbing the ears, nystagmus, circling and falling are some of the signs due to a middle ear infection by *M. bovis.* In an advanced case, a middle ear infection can develop leading to meningitis (Francoz *et al.* 2004; Lamm *et al.* 2004). Arthritis in calves and dairy cows are manifested like septic arthritis with inadequate response to treatment. Clinical signs include lameness with a swollen joint, pain with fever and anorexia. *M. bovis* arthritis and concurrent infection leading to pneumonia or mastitis is a common feature of *M. bovis* infection (Adegboye *et al.* 1996; Wilson *et al.* 2007).

2.3.6.2 Necropsy and Histopathology

Mild to severe multifocal fibrinosuppurative, caseonecrotic, or granulomatous lesions are seen in mammary gland due to *M. bovis* associated mastitis (Bennett and Jasper 1977a; Radaelli *et al.* 2011). Pathological lesions due to *M. bovis* associated pneumonia are similar to typical respiratory tract lesions manifested with the consolidation of lungs cranio-ventrally

and multiple necrotic foci having yellowish to whitish caseous content (Rodriguez *et al.* 1996; Caswell and Archambault 2007). *M. bovis* associated pneumonia is generally subacute to chronic bronchopneumonia associated with suppuration and necrotising lesions (Caswell and Archambault 2007). *M. bovis* antigen is usually seen in the peripheral lesions by immunohistochemistry (Gagea *et al.* 2006). Microscopically, a large number of lymphoid cells aggregate around the bronchi, bronchioles and vasculature, and alveolar septa appear thick due to infiltration of lymphoid cells and formation of lymphoid follicles (Howard *et al.* 1987; Sarradell *et al.* 2003). Suppurative or caseous exudate in tympanic bullae and osteolysis are seen in mycoplasma associated otitis media (Lamm *et al.* 2004; Van Biervliet *et al.* 2004). In arthritis, odourless fibrinous to caseous exudate is seen in the joint and fibrosis occurs due to the chronic nature of infection (Adegboye *et al.* 1996; Gagea *et al.* 2006). Infection commonly spread in the surrounding areas of the joint including tendons, synovial sheath, muscle and connective tissue. Histologically, multifocal caseous necrosis with extensive fibrosis is seen in the affected periarticular tissues (Adegboye *et al.* 1996; Gagea *et al.* 2006).

Clinical signs in combination with necropsy and histopathology help to reach a tentative diagnosis but isolation and identification of *M. bovis* by culture and molecular technique is still needed for confirmation.

2.3.6.3 Culture

Detection of *M. bovis* by culture is suitable for an individual animal (Sachse *et al.* 1993) and still considered as the ‘gold standard’ method to isolate mycoplasma from clinical samples (Sachse *et al.* 1993; Pitcher and Nicholas 2005). However, the technique is somewhat limited in a situation where rapid screening is necessary to limit the spread of the pathogen due to the slow-growing nature of *M. bovis*. Minimum 3-5 days are required to see the visible colony (Jasper *et al.* 1979) and up to 10 days waiting period is recommended to declare the sample is

negative (Sachse *et al.* 1993; Maunsell *et al.* 2011). Another major limitation is that detection by culture is possible when the animal is in the clinical state; an animal with chronic stage or intermittent shedder are likely to be undetected by this method (Jasper 1981). In a report by Gonzalez and Wilson (2003) found a 56 days long period without shedding of *M. bovis* from cows with chronic mastitis. As a result, three consecutive bulk milk tank (BTM) samples with 3-4 days interval is recommended to collect and culture. Even though the probability is 70% that herd is negative (Gonzalez and Wilson 2003).

2.3.6.4 PCR-based detection

Compared to culture, *M. bovis* detection by PCR method has higher efficiency, specificity and sensitivity. The other benefit is that PCR requires less time compared to culture and no need for the viable organism (Sachse *et al.* 1993).

Successful amplification of 16s rRNA gene-specific to *M. bovis* and *M. agalactiae* by conventional PCR method was first described in the 1990s (Chavez Gonzalez *et al.* 1995). The disadvantage of this PCR is that it cannot differentiate between *M. bovis* and *M. agalactiae* due to much similarities of nucleotide sequences in the 16s gene in both species. Later, a modification of this PCR was done by Johansson and others to make this PCR species-specific (Ayling *et al.* 1997). PCR targeting the uvrC gene reported as a better target as it prevents cross-amplification between *M. bovis* and *M. agalactiae* (Bashiruddin *et al.* 2005). Targeting oppD/F genes to detect *M. bovis* from milk and nasal swab have been described by several authors (Hotzel *et al.* 1996; Sachse *et al.* 2010). For rapid turnaround and quantification of *M. bovis* has also been successful using quantitative PCR targeting uvrC and oppD/F genes (Clothier *et al.* 2010; Rossetti *et al.* 2010; Sachse *et al.* 2010). Several commercial PCR kits are available to detect mycoplasma species such as PathoProof assays (Thermo Fisher Scientific; Scoresby, Victoria, Australia), Mastit4 assays (DNA Diagnostic A/S; Risskov, Denmark), the bactotype Mastitis HP3 PCR kit (QIAGEN Pty Ltd, Chadstone

Centre, Victoria, Australia), and Bovicheck *M. bovis* PCR kit (Biovet Inc., Quebec, Canada). However, more field-based data regarding their diagnostic sensitivity and specificity are needed for field application.

The major limitation of either conventional or real-time PCR method is that like culture method this technique is also not reliable to identify chronic infection or silent carrier animal. As a result, the PCR-based technique is not a useful tool for extensive screening and prevalence studies on herd-level.

2.3.6.5 Immune-based detection

Several immunological methods such as indirect hemagglutination, agar gel diffusion, ring precipitation, complement fixation test, western blot, immunohistochemistry and ELISA to detect *M. bovis* have been described previously (Cho *et al.* 1976; Andrew and Carter 1977; Boothby *et al.* 1981; Rosendal and Martin 1986; Uhaa *et al.* 1990; Sachse *et al.* 1992; Nicholas *et al.* 2000; Haines *et al.* 2001; Haines *et al.* 2004). However, the most widely used technique is the indirect ELISA due to its easy application, quicker to get results and cost-effective particularly in screening and prevalence study (Le *et al.* 2002; Nicholas and Ayling 2003). Boothby *et al.* (1981) first described in-house ELISA to detect *M. bovis* specific IgG in bovine serum; however, cross-reaction occurs with other mycoplasmas. Subsequently there are other in-house ELISA methods were characterized by various authors where they either use the whole cell as antigen (Thomas *et al.* 1987; Boughton and Liberal 1992; Ghadersohi *et al.* 2005) or recombinant proteins (Pfutzner *et al.* 1993; Brank *et al.* 1999; Robino *et al.* 2005; Fu *et al.* 2014; Sun *et al.* 2014). However, sensitivity and specificity remained as an issue in some of these ELISAs due to the selection of inappropriate recombinant protein for the development of ELISA or cross-reaction occurred with other mycoplasmas when whole-cell lysates were used.

The recent development of an antibody capture ELISA, using a membrane protein from the *M. bovis* strain 3683 which is called mycoplasma immunogenic lipase A (MilA), has been shown promise with high sensitivity and specificity (Wawegama *et al.* 2014). There are few commercial ELISA kits available for the diagnosis of *M. bovis* such as BIO K302 and BIO K260 (BioX Diagnostics, Belgium). However, in a recent study, it has been shown that MilA ELISA outperformed compared to the above mentioned commercially available ELISA kits (Wawegama *et al.* 2016). In the field condition, this MilA ELISA was evaluated by detecting *M. bovis* infection in the sera from feedlot cattle in Australia and reported a higher diagnostic sensitivity of 94.3% and specificity of 94.4% (Wawegama *et al.* 2016).

An indirect ELISA to assess the immune response of cattle after *M. bovis* infection is a better serological assay, especially for the prevalence study provided that the test is highly sensitive and specific. An indirect ELISA does not require active shedding of the pathogen or presence of a pathogen in the samples; thus, it can detect past exposure and the subclinical infection where PCR and culture only detect active infection. After infection, the *M. bovis* specific antibody remains in the blood for several months in dairy cattle (Byrne *et al.* 2000; Nicholas and Ayling 2003). Hence, indirect ELISA is an excellent serological assay for the active screening and surveillance study for the biosecurity management, prevention and control of *M. bovis* in dairy herds.

2.3.7 Control of *M. bovis* infection in dairy cattle

A vaccine is not useful against *M. bovis* associated disease in cattle (Boothby *et al.* 1987; Maunsell *et al.* 2009). Few reports suggest that vaccination can generate enough protective immunity against *M. bovis* in experimental settings; however, they are ineffective in the field trial (Howard *et al.* 1980; Nicholas *et al.* 2002; Nicholas *et al.* 2006).

β-lactam group of antibiotics are not as useful as *M. bovis* has no cell wall. Sulphonamides also do not work as this bug does not synthesise folic acid. Antibiotics that interfere with protein synthesis such as tetracycline, macrolides or prevent DNA synthesis (fluoroquinolones) were promising in *vitro* studies although erythromycin was resistant (Francoz *et al.* 2005; Rosenbusch *et al.* 2005). Tulathromycin and florfenicol are two antimicrobials approved in the USA for the treatment of bovine respiratory disease (BRD) associated with *M. bovis* and gamithromycin in Canada (Maunsell *et al.* 2011). In the experimental setup, tulathromycin was highly effective in *M. bovis* associated BRD in calves (Godinho *et al.* 2005). Although some antimicrobials are effective against *M. bovis* associated BRD after experimental and natural infection in cattle, information regarding the efficacy of antimicrobials against other naturally occurring *M. bovis* associated diseases in cattle is limited (Maunsell *et al.* 2011). Treatment responses to *M. bovis* associated arthritis is poor (Stipkovits *et al.* 1993; Adegboye *et al.* 1996) and same in case of *M. bovis* associated mastitis (Maunsell *et al.* 2011).

Therefore, active biosecurity management has paramount importance to control *M. bovis* infection in dairy cattle. Maintaining a closed herd is the best option to prevent *M. bovis* introduction, and if not possible then screening and quarantine before introduction into a naïve herd is a must (Gonzalez *et al.* 1992; Step 2001). During the purchasing of cattle, *M. bovis* history of the herd of origin should be considered. This can be done by knowing the records of bulk milk tank culture or antibodies in the milk or PCR results or serology of the herd of origin or if not available then 3 consecutive samples 3-4 days interval should be tested (Bicknell *et al.* 1978; Byrne *et al.* 2000; Gonzalez and Wilson 2003). In the case of *M. bovis* associated mastitis, monthly monitoring is vital in both mycoplasma free and infected herds by testing the bulk milk test. Record of response to treatment against *M. bovis* associated mastitis also has a value. Active surveillance and immediate segregation and

culling infected cows is an excellent approach to minimise the spread of infection and reduction of mastitis in a dairy herd (Brown *et al.* 1990; Byrne *et al.* 1998; Fox *et al.* 2003; Gonzalez and Wilson 2003). The strict hygienic practice of milking is advisable at all times to prevent udder to udder transmission (Bicknell *et al.* 1978; Gonzalez and Wilson 2003). Calves should not be fed with milk or colostrum from infected dams. An approach of not pooling the colostrum, and pasteurisation of the colostrum or milk before feeding can prevent the infection from cows to calves (Butler *et al.* 2000; Stabel *et al.* 2004). Respiratory aerosol from infected calves to healthy calves could be minimised by avoiding direct contact with sick calves, and segregation and culling of infected calves. Although *M. bovis* can survive in the environment for a certain extent, this pathogen is highly susceptible to commonly used disinfectant such as cholerine, chlorhexidine, acid or iodine-based compounds. Accordingly, maintaining proper sanitation of housing and equipment used in the dairy operation is strictly advisable. Existing epidemiological studies suggest that the principle of control of *M. bovis* infection in dairy herds is the quick identification and elimination of the infected animals. However, the development of an effective vaccine or antimicrobial therapy and an in-depth understanding of transmission and survival of *M. bovis* within herds are needed for effective control in future.

2.3.8 Economic and welfare impact of *M. bovis* in the dairy industry

In the USA, yearly estimated loss in the beef industry associated with *M. bovis* infection is about US$32 million due to reduced weight gain and carcass value, and roughly US$108 million in the dairy industry because of *M. bovis* mastitis (Rosengarten and Citti 1999). In the UK, the estimated cost is £153 million due to respiratory disease and death of calves. In Europe, this could be as much as 576 million euros (Nicholas *et al.* 2000). As *M. bovis* is an important pathogen causing respiratory diseases, the probability is that one-fourth to one-third of this cost is due to *M. bovis* infection (Nicholas *et al.* 2000; Nicholas and Ayling

2003). In Australia, the direct cost of *M. bovis* associated diseases in the dairy industry is unknown. The costs per case due to *M. bovis* infections in Australian dairy could be substantial as *M. bovis* associated diseases are chronic (Wilson *et al.* 1997; Fulton *et al.* 2009). In addition to that, production loss, drugs and labour cost, death and culling, diagnosis and implementation of control measures incurred an enormous cost irrespective of the country (Maunsell *et al.* 2011).

Other than economic cost, the animal-welfare impact should not be underestimated as *M. bovis* related diseases are chronic and response to therapy is minimal (Nicholas and Ayling 2003; Maunsell *et al.* 2011).

2.3.9 *M. bovis* in cattle in Australia

Simmons and Johnston (1963) and Cottew (1970) were first to describe the presence of *Mycoplasma* spp. in both sick and healthy cattle in Australia. They isolated different strains of mycoplasma from joints of cattle with arthritis, from milk in mastitis cases and also from lungs and lymph node. Simmons and Johnston (1963) isolated mycoplasma from calves in Queensland; however, Cottew (1970) did not mention the state information. Later, Shiel *et al.* (1982) reported group 7 mycoplasma in a calf with polyarthritis from a farm in northeastern Victoria which is the first case report of mycoplasma in cattle in Victoria. In 1983, an outbreak of mastitis in cows and polyarthritis and pneumonia in calves associated with *Mycoplasma* spp. bovine group 7 in a farm in Australia was reported (Alexander *et al.* 1985). In this report, the authors did not mention state information. In total 21 cattle were affected, of them 11 cows with mastitis, nine calves with arthritis and one calf having both arthritis and pneumonia. After a long silence, in 1998 another outbreak of mastitis and arthritis in cows and calves associated with *Mycoplasma* spp. bovine group 7 was reported in 3 large dairy farms in New South Wales (Hum *et al.* 2000). This time about 30% of calves developed arthritis due to mycoplasma, and out of 10 mastitic quarters, four samples were positive for

mycoplasma (Hum *et al.* 2000). During the similar time a broader scale of study conducted to see the prevalence of *M. bovis* in the dairy cattle herds in Victoria and North Queensland by testing bulk milk samples from 186 herds in Victoria and 167 herds from North Queensland using *M. bovis* specific PCR and reported a higher prevalence of 43% and 62% of herds positive for *M. bovis* respectively (Ghadersohi *et al.* 1999). However, prevalence results were questionable due to the lack of the diagnostic sensitivity and specificity of this *M. bovis* specific PCR and they also did not mention how they calculated the required sample size. In 2014, another report published regarding *M. bovis* prevalence in dairy cattle herds in Australia where authors reported a low herd-level prevalence of 0.9% (95% probability interval 0.1-3.7%) (Morton *et al.* 2014). They tested bulk milk samples, using commercial *M. bovis* specific PCR (Pathoproof ^{TM}, Dairy Technical Services Ltd, VIC, Australia), from 238 dairy herds and only one herd was positive. The diagnostic sensitivity of the commercial PCR was estimated as 76% (95% probability interval 34-98%) (Morton *et al.* 2014). One of the limitations in this study is that PCR assay is not a suitable diagnostic assay for the herd level screening of *M. bovis* as it failed to detect the subclinical infection or past exposure. To date, limited information is available regarding herd-level seroprevalence of *M. bovis* in dairy cattle in Australia. A recent serological study conducted in feedlot cattle in Australia highlighted some 13.1% seropositive cattle against *M. bovis* during entry into feedlots and a 73.5% seropositive after six weeks follow-up (Wawegama *et al.* 2016). A study conducted in Israel where they tested *M. bovis* antibody in 295 calves imported from Australia and reported a 94% seropositive calf against *M. bovis* (Lysnyansky 2017). Although this is a higher proportion of calves they detected against *M. bovis* in calves imported from Australia, it should be in mind that these calves were shipped from Australia to Israel and it might be that due to stress and closer contact higher proportion of calves got infected. While *M. bovis* has been circulating in the Australian cattle population for a long time, a recent study

revealed a minimal genetic variation among different isolates of *M. bovis* in Australia. From 2006 to 2015, a total 94 isolates from various anatomical sites from healthy and sick cattle from different geographical locations in Australia were selected, and of them, after quality control, 82 *M. bovis* isolates sent for whole-genome sequencing (WGS). Later, an SNP analysis was performed which highlighted that a single *M. bovis* strain is circulating throughout Australia with many genomic similarities (Parker *et al.* 2016).

One of the challenges that limit the screening of *M. bovis* in the dairy herds is the appropriate diagnostic test having high sensitivity and specificity. Another limitation is the readily available appropriate sample to detect *M. bovis* under Australian dairy farming systems where dairy farms are distributed widely. A large number of bobby calves are slaughtered each year at a very young stage. Hence, their blood samples can be used to detect maternal *M. bovis* antibody of a herd of origin. MilA ELISA has been shown promise to detect *M. bovis* antibody in the laboratory, and in-field condition in feedlot cattle sera in Australia. However, this ELISA has not been tested to explore its potential to detect *M. bovis* antibody of maternal origin in the sera from very young calves to detect herd level *M. bovis* infection in Australian dairy herds.

2.3.10 Conclusion

- Based on the literature review it appears that despite *M. bovis* is a significant pathogen of cattle population it's epidemiology in Australian dairy cattle herds was not explored adequately.
- Wide-scale prevalence study with continuous monitoring and understanding of the herd-level risk factors in dairy cattle would be one of the significant areas of further research to control this pathogen for the sustainable dairy industry in Australia.
- For the large-scale herd-level study, it would be a potential area of research to assess the possibility of detecting maternal antibody against *M. bovis* in bobby calves' sera

by MilA ELISA for its possible future use in surveillance study of *M. bovis* in Australia.

3 Assessment of plasma analytes of bobby calves after transportation for slaughter

3.1 Abstract

This study evaluated the plasma biochemical profile of bobby calves to assess calf health and welfare before their slaughter. Blood samples were collected at slaughter from bobby calves (n=482) from a commercial abattoir in Victoria, Australia for the measurement of passive immunity (gamma-glutamyl transferase activity), metabolic profile (plasma glucose, beta-hydroxybutyrate, non-esterified fatty acids and urea), dehydration state (packed cell volume), and muscular exhaustion and bruising (plasma creatine kinase and lactate). Plasma analytes were measured by the multi-channel biochemical analyser. A quarter of the calves had a failure of passive transfer with a variation of GGT level depending on which region they came from suggesting colostrum management practices are not similar in all the farms. Very few calves experienced severe hypoglycaemia and dehydration before slaughter. However, most of the calves had higher than reference value limits of plasma creatine kinase and lactate, indicative of muscular fatigue. Distance or duration of journey and time spent in lairage had no significant effect on energy metabolites, hydration state or muscular fatigue in bobby calves before slaughter ($P > 0.05$). Breed or sex was not correlated with plasma metabolites, except for Jersey calves which had higher total plasma protein and gamma-glutamyl transferase activity ($P < 0.05$) than Holstein-Friesian calves. In conclusion, most calves at the time of slaughter showed no evidence of physiological compromise, apart from muscular fatigue, and there did not appear to be a significant association between plasma analytes and the transport distance or with a duration of transport and lairage time in the calves in this study. These results suggest that bobby calves can be well managed from the property of origin to the abattoir under existing management systems in Victoria without unduly compromising their welfare.

3.2 Introduction

In Australia, most of the male dairy calves and heifers that are not intended for use as herd replacements are sent to regional abattoirs for slaughter at 5-10 days old as bobby calves for veal. Annually, about 770,000 non-replacement calves are commercially slaughtered in abattoirs in Australia of which 63% of calves are from Victoria. Calves generally arrive at slaughter plants in the afternoon and are killed early the following morning after a period of lairage of approximately 9-18 hours. During this time, calves typically will not have access to liquid feed and are without bedding (Animal Welfare Standards 2011).

The recent development process for the Australian Standards and Guidelines for the Welfare of Animals: Land Transport of Livestock highlighted that the transport and management of bobby calves remains a contentious area. According to welfare standards for transport for slaughter in Australia, bobby calves must be at least 5 days old, healthy and fit for transport, be fed no more than 6 h before loading, be transported for no more than 12 h and must be slaughtered within 30 h from their last feed (Animal Welfare Standards 2011). This standard is supported by research conducted using bobby calves under experimental conditions in New Zealand and Australia (Todd *et al.* 2000; Fisher *et al.* 2014). A New Zealand study conducted under experimental conditions by Todd *et al.* (2000) indicated that bobby calves could cope well with 12 h of transport and total time off feed up to 30 h. A controlled study was conducted in Australia by Fisher *et al.* (2014) to provide additional scientific evidence for an Australian standard. The conclusions of the research aligned with the New Zealand data and supported 24 h off-feed duration as a good standard with a 30 h maximum time off feed as an outer limit. A field study conducted by Stafford *et al.* (2001) in New Zealand indicated that welfare problems could arise in bobby calves arriving at abattoirs. This study reported that a proportion of calves (0.4%) arrived at slaughterhouses was in the poor physical condition and that this category of calves typically was hypoglycaemic, had evidence of the failure of

passive transfer of immunity, muscular bruising and dehydration. Their results also showed that in bobby calves after a long 12-15 h lairage period, blood glucose concentrations declined and beta-hydroxybutyrate (BHB) concentrations increased, indicating a shortage of carbohydrate in the body and increased lipid metabolism. In the Australian study conducted by Fisher *et al.* (2014) using bobby calves managed under experimental conditions, blood glucose concentration declined in calves after 18-24 h without feed and BHB increased after this time while remaining within reference values. They also found that 12% of calves had glucose concentrations at 30 h below 2.8 mmol/L, which was below the relevant reference value and an indication of hypoglycaemia. Accordingly, Fisher *et al.* (2014) recommended that 30 h off feed as an outer limit for bobby calves and that best practice should be no more than 24 hours.

It is often challenging to translate the outcomes of research conducted in experimental conditions to conditions in commercial practice. Data regarding bobby calf health and welfare before slaughter in Victoria under commercial conditions is limited. Therefore, it is appropriate to examine the welfare of bobby calves at the end of the commercial supply chain. Various indicators such as assessment of mortality, health status, growth, reproductive success, physiology and behaviour are useful to monitor the welfare of an animal (Broom 1988). Physiological change, behavioural response, injury or mortality are most commonly evaluated to measure short-term effects during and after transportation of animals (Broom 2005). Therefore, the objective of this study was to evaluate the welfare status of bobby calves at an abattoir in Victoria by assessing their physiological status by measuring blood chemistry indicative of passive immunity, metabolic and energy status, dehydration, and muscular bruising and fatigue.

3.3 Materials and methods

3.3.1 Animals

Data for 482 bobby calf samples were collected from a commercial abattoir in Victoria during spring in 2015 (n =113) and during autumn in 2016 (n = 369). The abattoir processes calves originating from dairy herds in Victoria, South Australia, and New South Wales. During spring in 2015, we visited the abattoir on three occasions to collect samples, with an average of 40 samples per day. In 2016 during autumn, we visited on five occasions and collected on average 74 samples per day. Breed and sex of the calves were determined based on visual assessment.

3.3.2 Sample collection

During our sample collection, calves were presented for slaughter randomly from the lairage yard. We sampled calves without reference to their breed, size or gender and as frequently as the speed of the killing chain allowed. Based on the speed of the killing chain, blood samples were randomly collected from approximately every tenth calf from the killing line at the slaughter plant. Blood was collected into a sterile container by free catch from the severed jugular veins immediately after stunning and sticking and then immediately transferred to a 10-ml vacutainer containing lithium heparin (BD Vacutainer®) for further processing. Samples were kept cool in an icebox for the period between collection and processing (a maximum of 6 h).

3.3.3 Identification of the bobby calves

In Australia, radio-frequency identification ear tags (RFID) and the national livestock identification system (NLIS) are used for identifying and tracking livestock. For the identification of each calf, a portable reader (Allflex® RS 420, Allflex® Livestock Intelligence, Australia) was used to scan RFID at the time of blood collection. At the same time, external tag NLIS identification numbers were also noted and recorded. The electronic data was downloaded each day from the RFID reader to a spreadsheet compilation and data

screening. Subsequently, RFID data was sent to the Victorian Department of Economic Development, Jobs, Transport and Resources (DEDJTR), whose staff provided calf data relating to origin and transport. Information regarding state, shire and parish of origin of each calf, excluding the specific farm-level data, and time of collection of bobby calves from farms and slaughter time of each calf were provided by DEDJTR. Each NLIS unique identification consists of 16 characters of letters and numbers. The first 8 characters of the NLIS ID is the property identification code (PIC) which is unique for each property and allows locating the individual property. Thus, calves that came from the same herd were identified based on having the same PIC.

3.3.4 Estimation of travel distance

Based on the Google Map website, the approximate distance of travel by each calf from the property of origin to the abattoir was calculated (Google Maps). From the PIC of each calf based on the NLIS database, we identified the parish of origin of each calf and its postcode. Subsequently, we estimated travel route from the centre of the parish of origin to the abattoir using Google Maps and calculated the distance. The point locations of the source of the calves from Victoria state, a map (Figure 3.1) was generated using QGIS (QGIS Development Team, 2018) and R (R Core Team 2017).

3.3.5 Determination of total transport and lairage duration

The NLIS database available from DEDJTR provided the time at which calves were loaded onto the truck at the property of origin. The time of slaughter was recorded during the blood collection process by scanning the RFID ear tag. The difference between these two time points was the total duration of transport and lairage for each calf. The time of arrival and unloading at the abattoir was not available. Consequently, the duration of transport and lairage time could not be separated.

3.3.6 Estimation of total off feed duration

We added 6 hours to the calculated duration of transport and lairage to estimate the maximum time off-feed (TOF) for each calf. This additional 6 hours was added assuming that the last feed to the calves was no more than 6 hours before leaving the property, as per the state regulations.

3.3.7 Haematological and biochemical measurements

The packed cell volume (PCV) of each blood sample was measured within 6 h of blood collection using the microhaematocrit technique as described previously (Jain 1986). Blood was drawn in the capillary tube. The end of the capillary tube was then inserted into plasticine to seal one end. The tubes were then placed in the haematocrit centrifuge and centrifuged at 2000 x *g* for 10 minutes. The PCV was then determined by examining the tube using a microhematocrit reader.

The remainder of the blood sample was centrifuged at 2000 x *g* for 10 minutes and the separated plasma stored at -20º C until analysis. The plasma was analysed to determine concentrations of glucose, beta-hydroxybutyrate (BHB), urea, non-esterified fatty acids (NEFA), total plasma protein (TPP), gamma-glutamyl transferase (GGT), creatine kinase (CK) and lactate using a multi-channel biochemical analyser (COBAS INTEGRA® 400 plus, Roche Diagnostics International Ltd., Switzerland) following the manufacturer's instructions. In brief, 200 µl plasma in Eppendorf cup per sample was loaded into the sample rack. Cassettes containing all the reagents for the tests was also loaded. Based on the absorbance photometry and turbidometry plasma analytes were then measured and recorded. A detailed description of the principles of each test and reagents used to determine each biochemical variable is provided in Appendix 3.

3.3.8 Statistical analyses

Data were analysed with the Minitab® statistical software version 18.1 (Minitab Inc., 2018). The Anderson-Darling test was performed to check data normality. The Student *t*-test was conducted to analyse the difference of the means of glucose, lactate, TPP and PCV between two groups. Mann-Whitney test was performed to see the difference between two groups for BHB, urea, NEFA, CK and GGT. One-way analysis of variance (ANOVA) was performed to know the difference among the groups for glucose, lactate, TPP and PCV. Kruskal-Wallis test was performed to see the difference among the groups for BHB, urea, NEFA, CK and GGT. Pearson's correlation and regression were performed to determine the association between each of the plasma analytes and the variables of transport distance and the total duration of transport and lairage. A value of $P < 0.05$ was considered statistically significant.

3.4 Results

3.4.1 Characteristics of the study samples

Data from a total of 482 bobby calves were included in the study. We could not scan RFID in 94 calves and unable to record NLIS ID in 23 calves out of 482 calves during sample collection at the abattoir. Hence, we could not locate the property of origin for all of the calves. Department of Economic Development, Jobs, Transport and Resources (DEDJTR) provided the information of pick up time of 271 calves only. Accordingly, we estimated the transport duration and lairage time and total off feed time for 271 calves. Information regarding missing data has been cited appropriately where necessary. Most of the bobby calves (92.4 %) were male. The predominant breeds were Holstein-Friesian (60.0%), Jersey (11.3%) and cross-bred calves (28.7%). Most of the calves in this study originated from Victorian herds (96.7%), with 2.1% and 1.2% of calves coming from South Australia and New South Wales, respectively. In Victoria, most of the calves came from northern and south-eastern dairy regions (Figure 3.1).

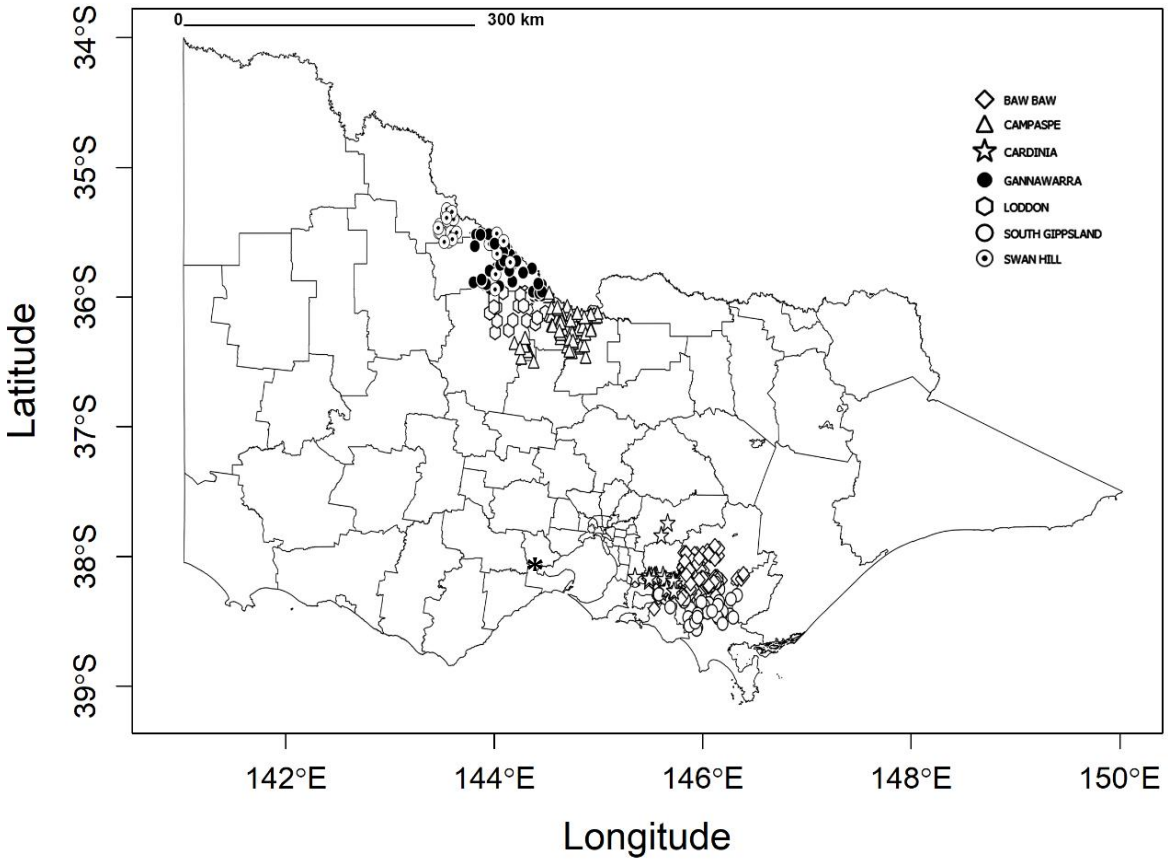

Figure 3.1: Map showing Victoria state and spatial distribution of origin of bobby calves from different shires of northern and south-eastern dairy regions in Victoria. Each symbol represents one calf and their respective shire location. (*Abattoir location).

The mean of the estimated distance of transport before slaughter was approximately 220 km with a range of 112 to 358 km (Figure 3.2). The minimum, maximum, and mean duration of total transport and lairage times were 12.4 h, 23.3 h and 18.7 h respectively (Figure 3.3).

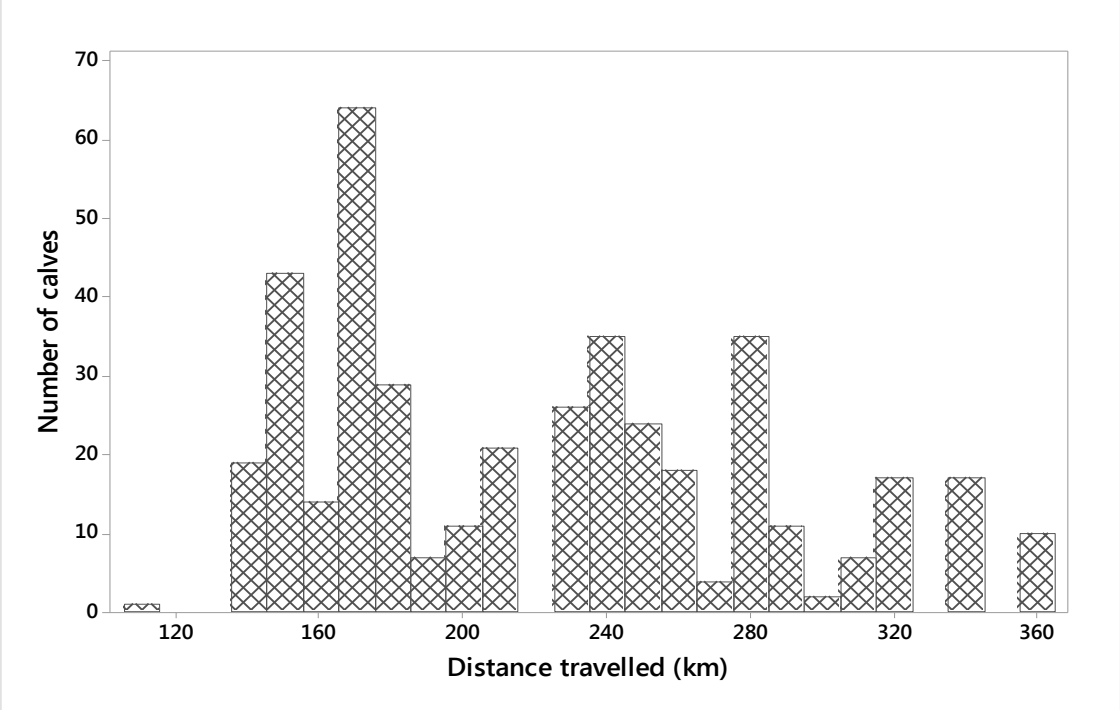

Figure 3.2: Histogram showing estimated distance of transport of bobby calves before slaughter based on Google Maps from approximate property location to abattoir (n = 415).

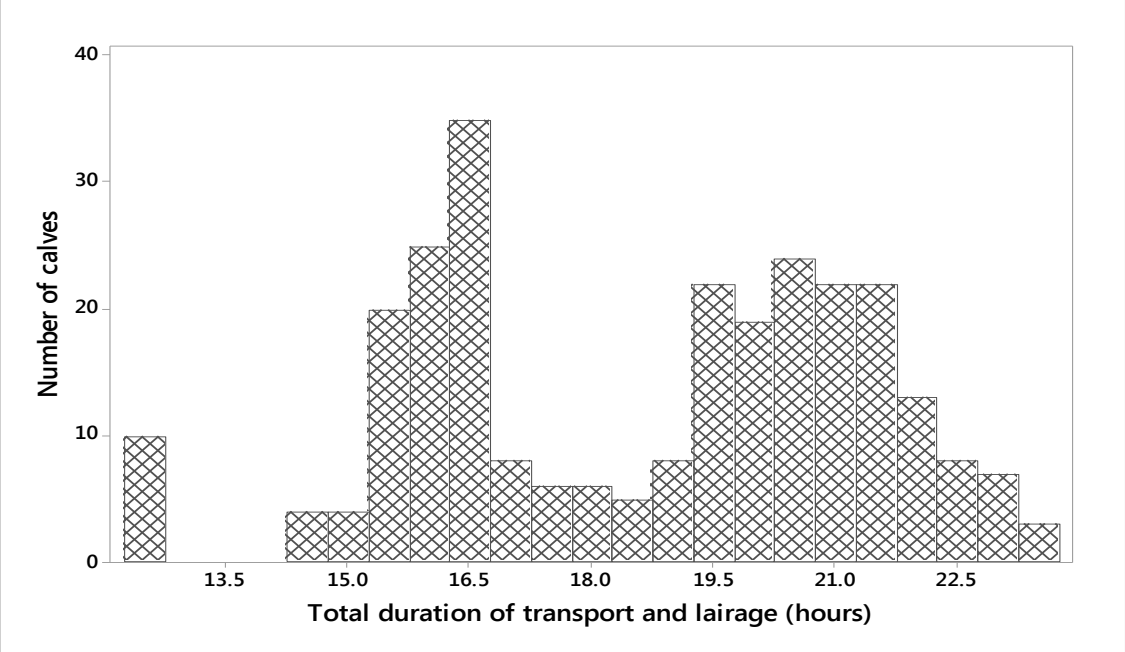

Figure 3.3: Histogram showing total duration of transport and lairage time of bobby calves before slaughter (n = 271).

3.4.2 Passive immunity

The mean plasma value of GGT, an enzyme which indicates the transfer of passive immunity via colostrum, was high (388.7 IU/L) with a wide range of values (9-4220 IU/L) and the distribution was highly right-skewed (Figure 3.4).

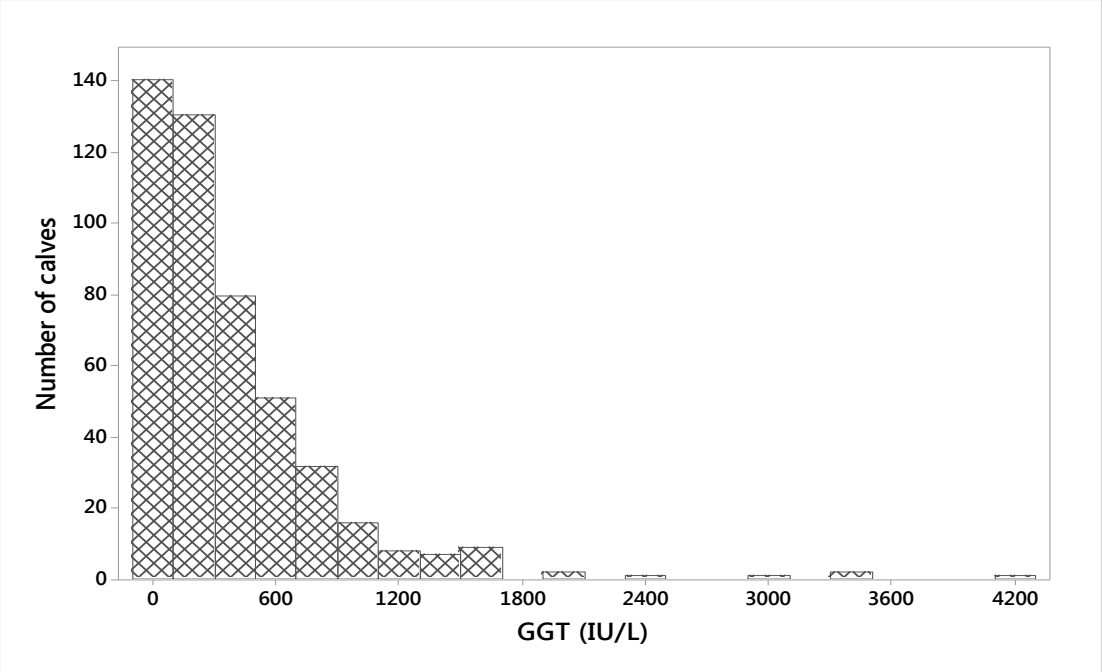

Figure 3.4: Histogram showing distribution of plasma GGT in bobby calves just after slaughter. Normal reference range of GGT in calves is ≥ 75 IU/L (Parish *et al.* 1997) (n = 482).

Failure of passive transfer (GGT < 75 IU/L) was evident in 23.03% of calves (Table 3.1). A positive correlation was found between GGT and total plasma protein (Figure 3.5). Calves from the south-eastern dairy region had both higher plasma protein and GGT than calves from the northern dairy region (Table 3.3). Similarly, calves during autumn had both higher plasma protein and GGT than in spring (Table 3.4). Sex did not influence plasma protein and GGT. However, calves identified as being of the Jersey breed had significantly higher plasma protein and GGT than the other two breeds (Table 3.5 & Figure 3.6).

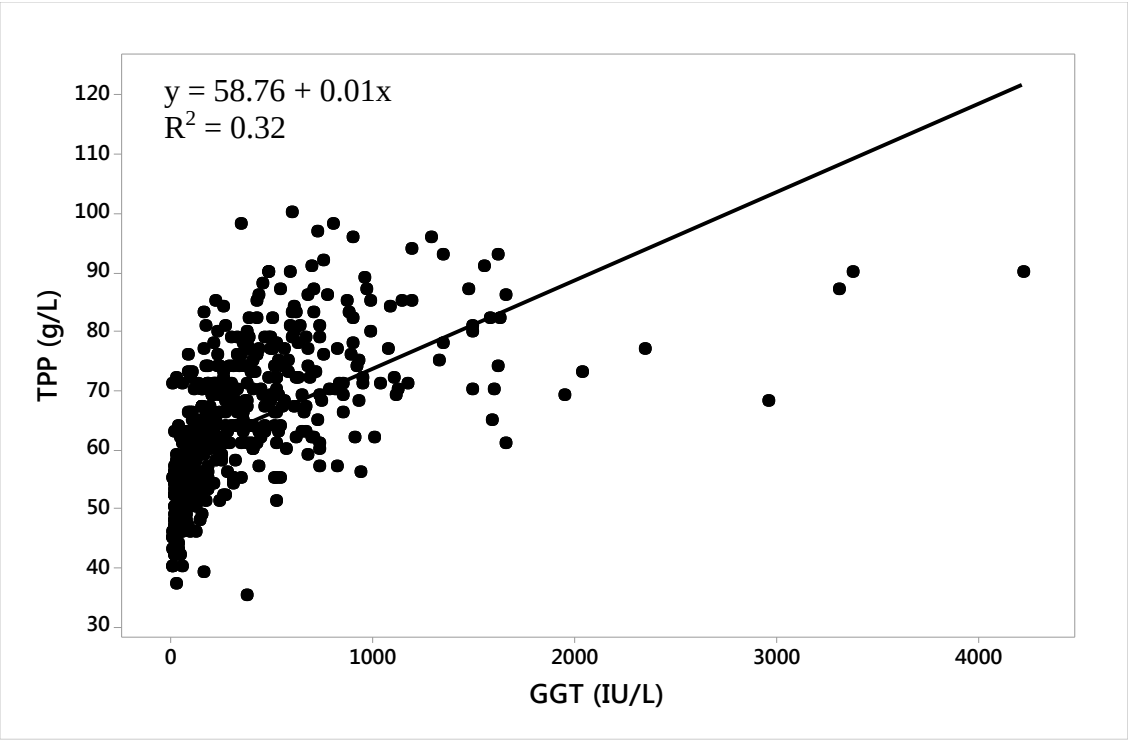

Figure 3.5: Relationship between plasma TPP and GGT in bobby calves just after slaughter.

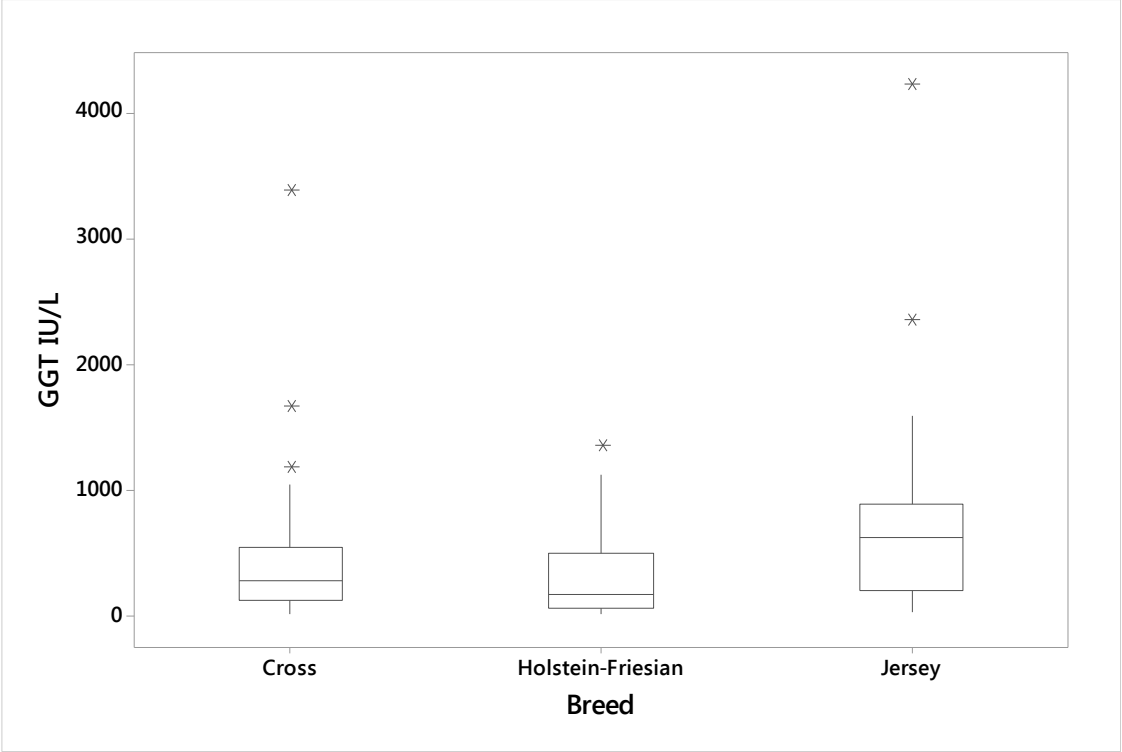

Figure 3.6: Box plot showing plasma GGT levels in different breeds of bobby calves after slaughter.

3.4.3 Time off feed duration and energy level

The minimum, maximum and mean estimated duration of time off feed were 18.4 h, 29.3 h and 24.7 h respectively (Figure 3.7, left).

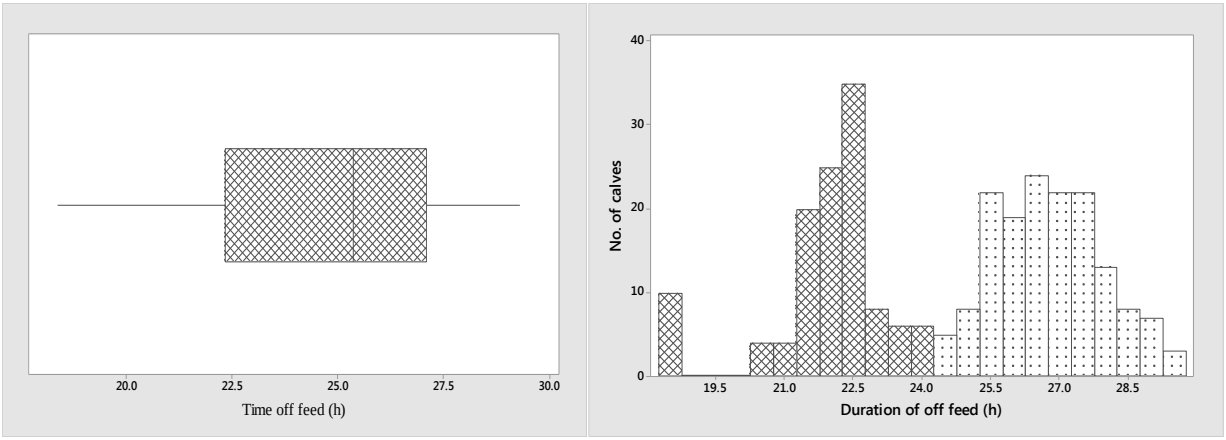

Figure 3.7: Box plot (left) showing estimated total duration of off feed and histogram (right) showing a bimodal distribution of TOF (n = 271).

A bimodal distribution pattern of estimated TOF was observed, whereby a cohort of calves was without feed for ≤ 24 h and another cohort of calves was without feed for > 24 h (Figure 3.7, right). However, no significant difference was found between these two groups for plasma analytes except for higher BHB concentrations in calves having more than 24 h TOF (Table 3.2). For the determination of energy status, key biochemical variables indicative of energy status such as glucose, BHB, NEFA and urea were measured (Table 3.1). The plasma glucose concentrations in bobby calves were normally distributed, and the mean glucose value in most of the bobby calves just after the slaughter was within the published physiological ranges, except for 2.5% calves having blood glucose <2.8 mmol/L (Table 3.1 and Figure 3.8). The first and third quartile and mean plasma glucose values were 4.0 mmol/L, 5.2 mmol/L and 4.6 mmol/L, respectively. About one half (55.17%) of the calves exceeded the physiological range of BHB (Table 3.1). In addition to this, the mean values of other biochemical parameters indicative of energy status such as NEFA and urea were within the reference ranges; however, 18.92% and 1.87% calves had higher NEFA and urea level

respectively compared to maximum normal reference values (Table 3.1). A significant ($P <$ 0.000) inverse correlation was observed between plasma BHB and plasma glucose (Figure 3.9). No significant association ($P > 0.05$) was found either between plasma glucose and transport distance nor between plasma glucose and the total duration of transport and lairage (Figures 3.10 & 3.11). Plasma glucose concentrations in bobby calves did not differ significantly depending on which shire they came from except Loddon (Table 3.3). Blood samples collected during spring had higher plasma glucose concentrations compared with autumn (Table 3.4). No differences were found in plasma analytes indicative of energy status in relation to calf breed and sex (Table 3.5).

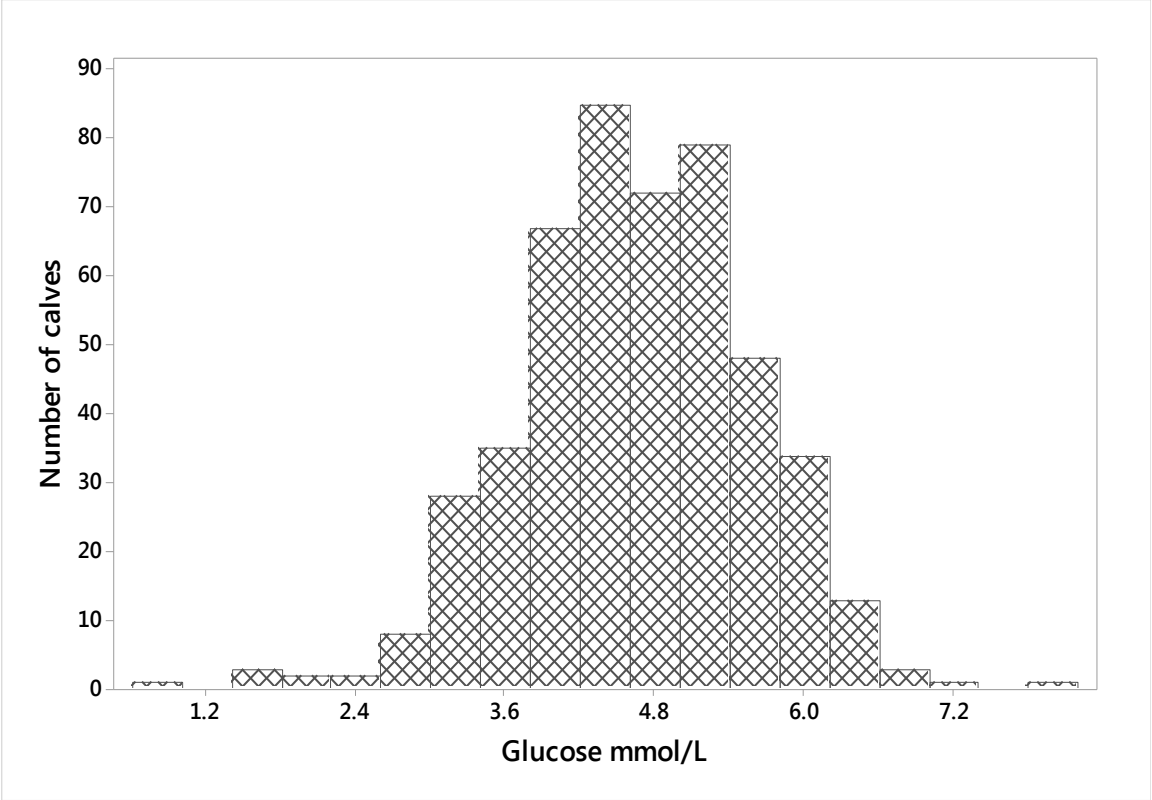

Figure 3.8: Distribution of plasma glucose in bobby calves immediately after slaughter. Normal reference range of glucose in calves is 2.8-7.5 mmol/L (Lumsden *et al.* 1980) (n = 482).

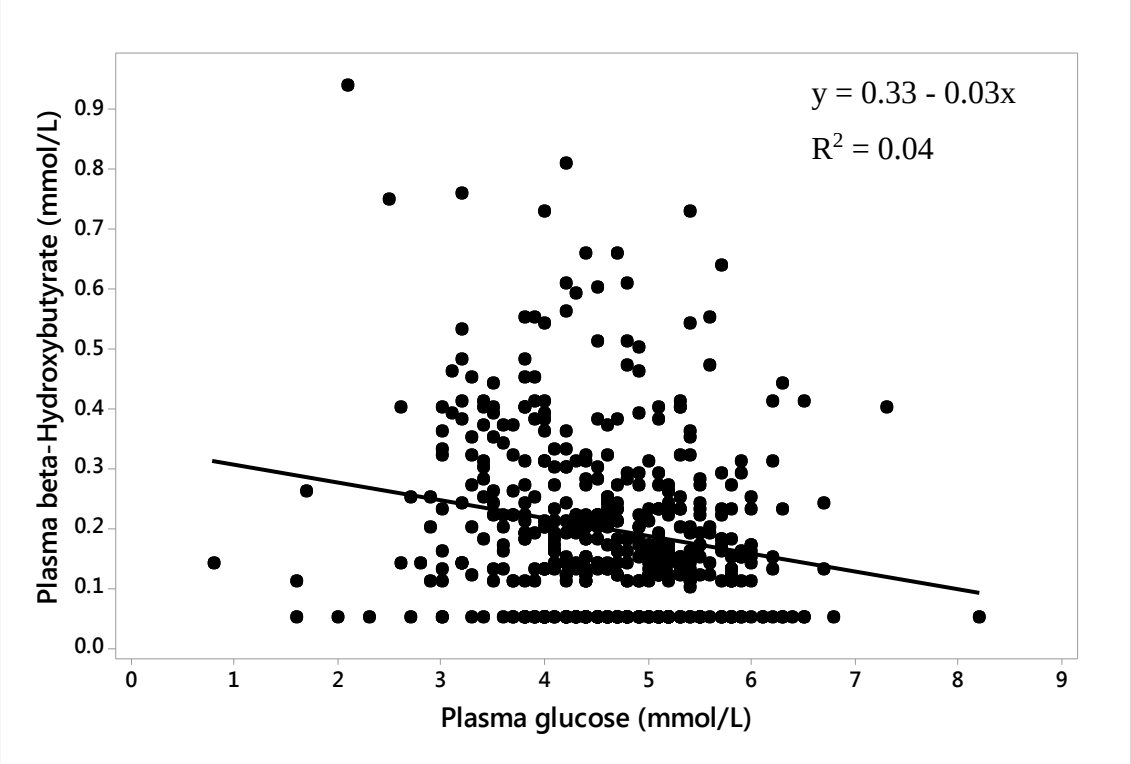

Figure 3.9: Scatter plot showing inverse relationship between plasma BHB and plasma glucose in bobby calves just after slaughter (n = 482).

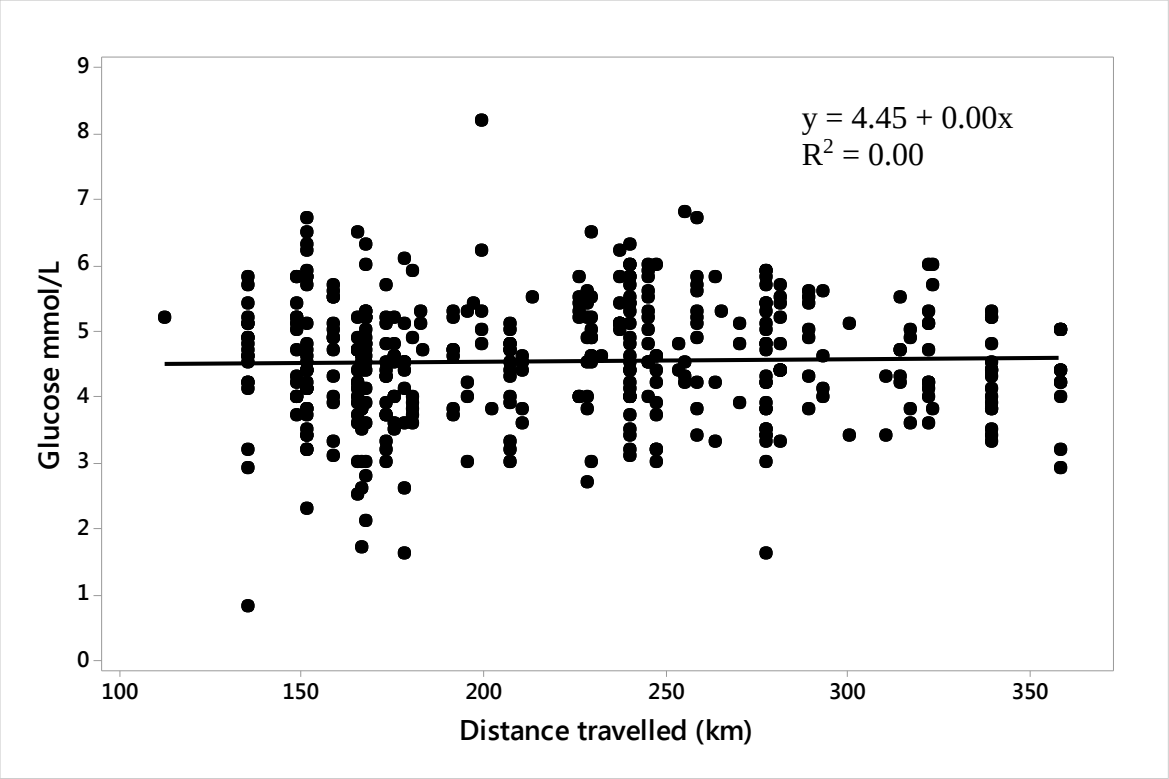

Figure 3.10: Association between plasma glucose at slaughter in bobby calves and distance of transport to the abattoir (n = 415).

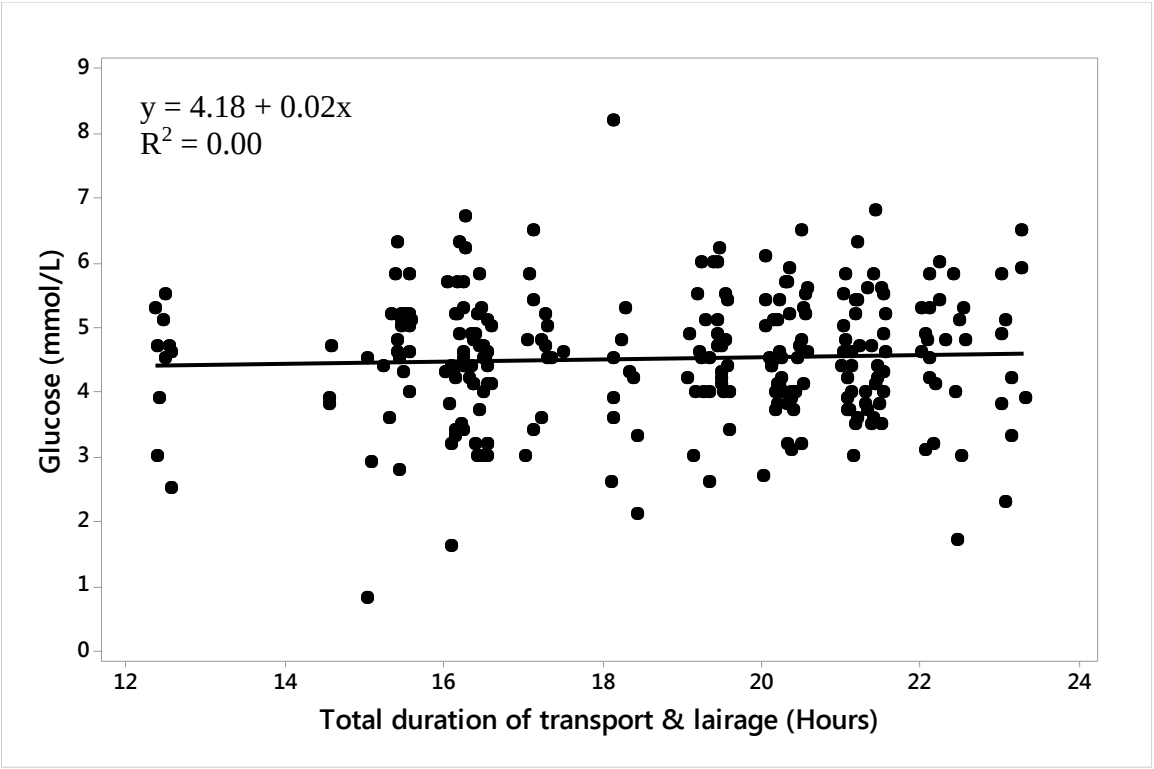

Figure 3.11: Association between plasma glucose at slaughter in bobby calves and total duration of transport and lairage (n = 271).

3.4.4 Dehydration

A normal distribution of PCV was found (Figure 3.12), and only 1.2% of calves had higher PCV (> 47%) than the reference range (Table 3.1). No significant association ($P > 0.05$) was observed between PCV and transport distance, or between PCV and the total duration of transport and lairage (Figures 3.13 & 3.14).

Table 3.1: Mean (± SE) concentration of plasma analytes and PCV in bobby calves (n = 482) at slaughter following transport to the abattoir and lairage.

Parameters	Glucose, mmol/L	BHB, mmol/L	Urea, mmol/L	NEFA, mmol/L	PCV, %	TPP, g/L	CK, IU/L	Lactate, mmol/L	GGT, IU/L
Mean (±SE)	4.6 ± 0.04	0.19 ± 0.01	4.96 ± 0.17	0.31 ± 0.01	33.34 ± 0.31	64.55 ± 0.57	387.30 ± 12.20	5.22 ± 0.07	388.70 ± 21.70
Range (Min -Max)	0.80 - 8.20	0.05 - 0.94	1.10 - 50.50	0.03 - 1.24	13 - 56	35 - 100	2.50 - 2578	0.11 - 13.04	9 - 4220
Reference range	2.8 - 7.5[a]	0.10 - 0.14[c]	3.6 - 15.7[a]	0.26 - 0.42[c]	17 – 47[a]	39 - 70[a]	11 - 125[a]	1.11 - 1.89[d]	≥ 75[b]
% Calves outside reference range	2.49	55.17	1.87	18.92	1.22	31.33	97.51	99.17	23.03

Standard error (SE)
Reference range from [a] (Lumsden *et al.* 1980), [b] (Parish *et al.* 1997), [c] (McCoard *et al.* 2014) and [d] (Lindt and Blum 1994).

Table 3.2: Mean (± SE) concentrations of plasma analytes at slaughter in bobby calves, categorised by time off feed (TOF) before slaughter.

Descriptive variable		No. of calves	Glucose, mmol/L ± SE	BHB, mmol/L ± SE	Urea, mmol/L ± SE	NEFA, mmol/L ± SE	PCV, % ± SE	Total Protein, g/L ± SE	CK, IU/L ± SE
TOF	≤ 24h (18.35-23.48h)	105	4.48 ± 0.09	0.23 ± 0.01	5.08 ± 0.39	0.34 ± 0.01	34.01 ± 0.65	66.17 ± 1.27	365.1 ± 20.6
	> 24h (24.09-29.30h)	158	4.54 ± 0.07	0.27 ± 0.01	4.98 ± 0.37	0.33 ± 0.01	33.16 ± 0.48	64.77 ± 0.99	385.9 ± 20.3
	Estimate for difference (with 95% CI)		0.00 (0.2, - 0.2)	0.03 (- 0.00, 0.06)	0.1 (- 0.3, 0.5)	0.01 (- 0.03, 0.05)	0.85 (- 0.76, 2.46)	1.40 (- 1.77, 4.57)	4 (- 34, 39)
	***P* value**		**0.955**	**0.064**	**0.558**	**0.553**	**0.299**	**0.385**	**0.849**

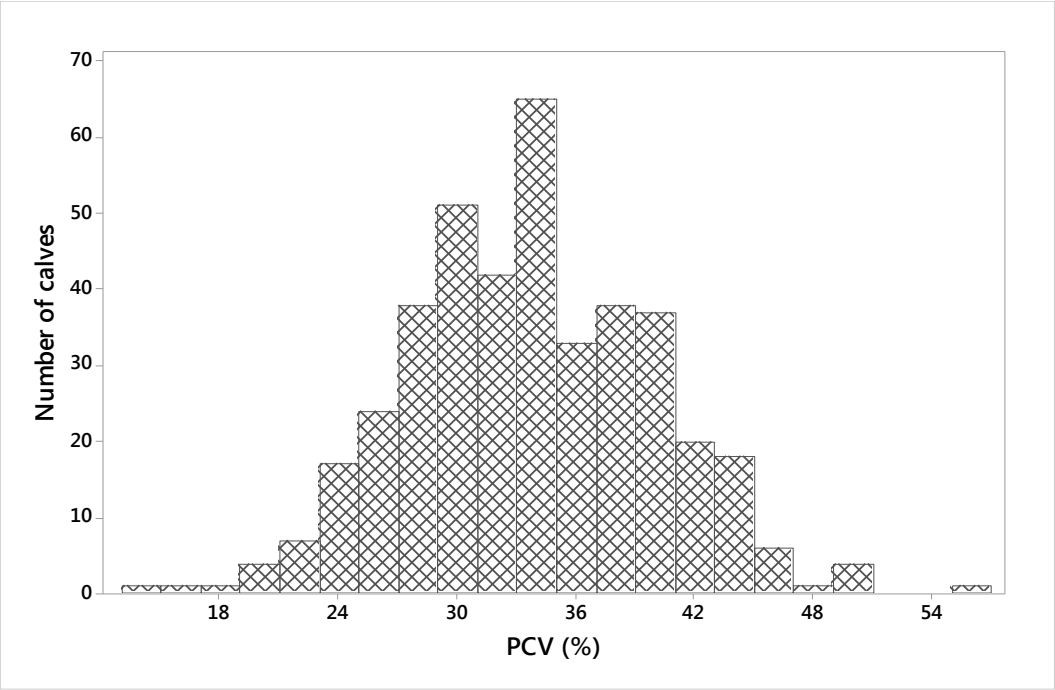

Figure 3.12: Histogram showing distribution of packed cell volume (PCV) in bobby calves immediately after slaughter. Normal reference range of PCV in calves is 17 - 47% (Lumsden *et al.* 1980) (n = 409).

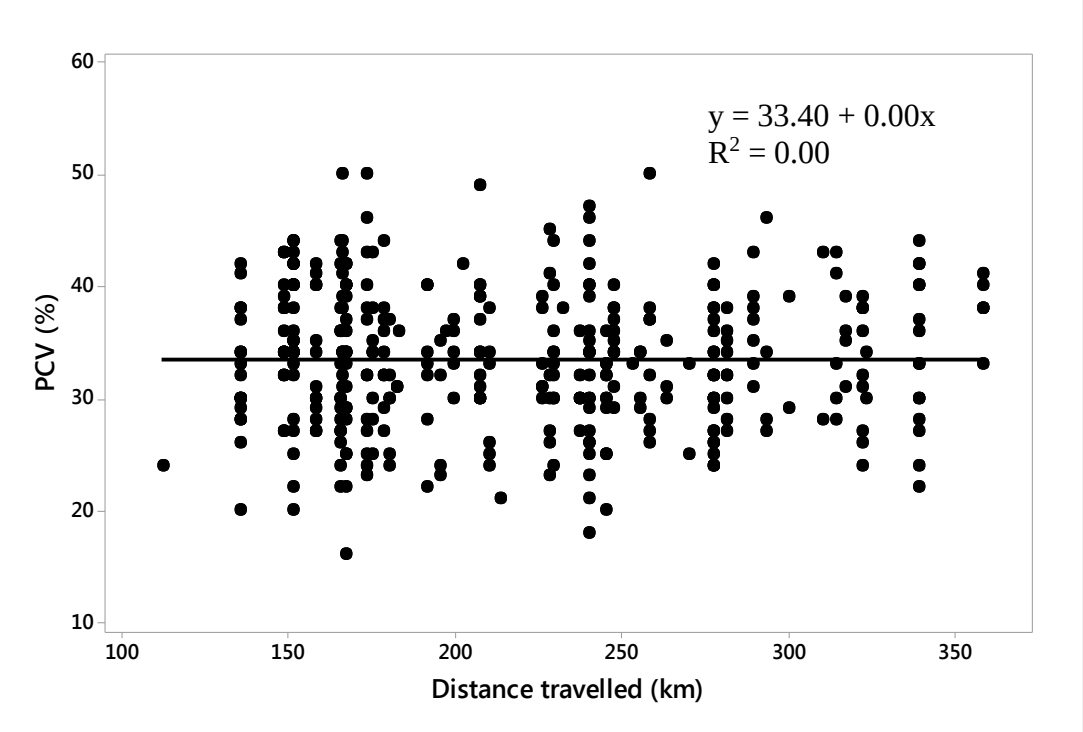

Figure 3.13: Association between PCV at slaughter in bobby calves and distance of transport to the abattoir (n = 409).

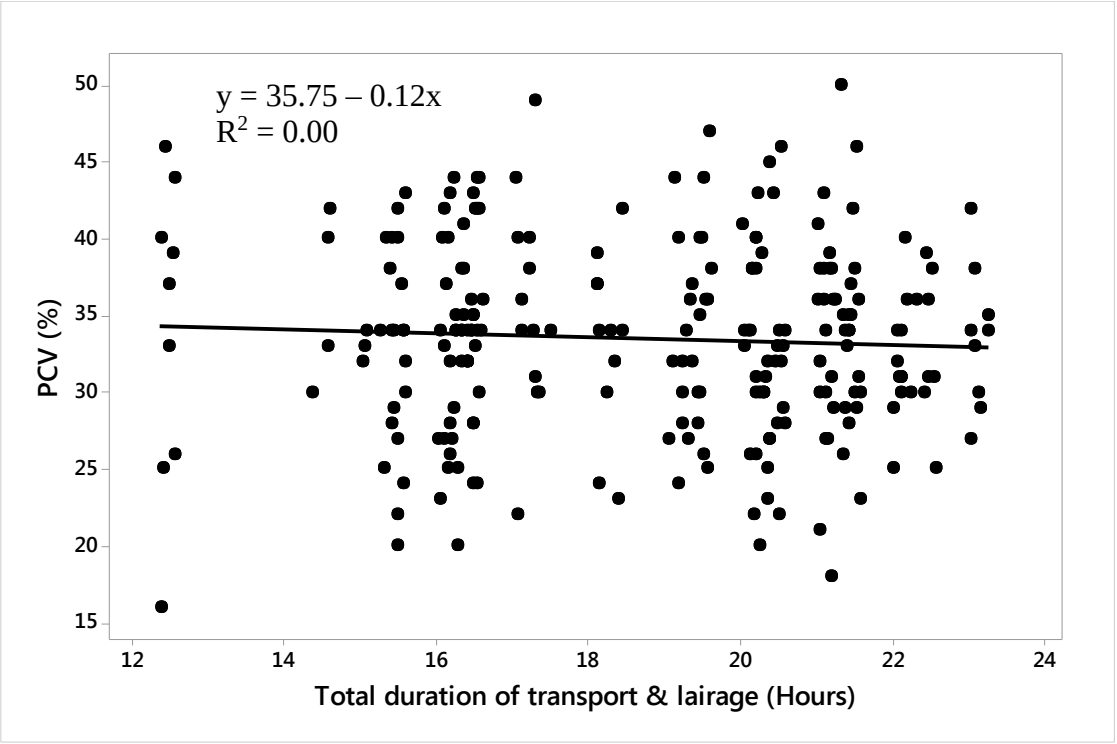

Figure 3.14: Association between PCV at slaughter in bobby calves and total duration of transport to the abattoir and lairage (n = 271).

3.4.5 Muscular bruising or fatigue

A right-skewed distribution was observed for plasma CK (Figure 3.15). More than 95% of calves had higher CK (>125 IU/L) and lactate (>1.89 mmol/L) values at slaughter compared with the relevant reference ranges, and the mean values were also significantly higher than the reference ranges (Table 3.1). There were no correlations between plasma CK and transport distance or with transport duration and lairage time (Figures 3.16 & 3.17). Within Victoria, calves from the northern dairy region had higher plasma CK than calves from the south-eastern region (Table 3.3). Samples collected during autumn had higher plasma CK than spring (Table 3.4). Breed and gender did not affect plasma CK (Table 3.5).

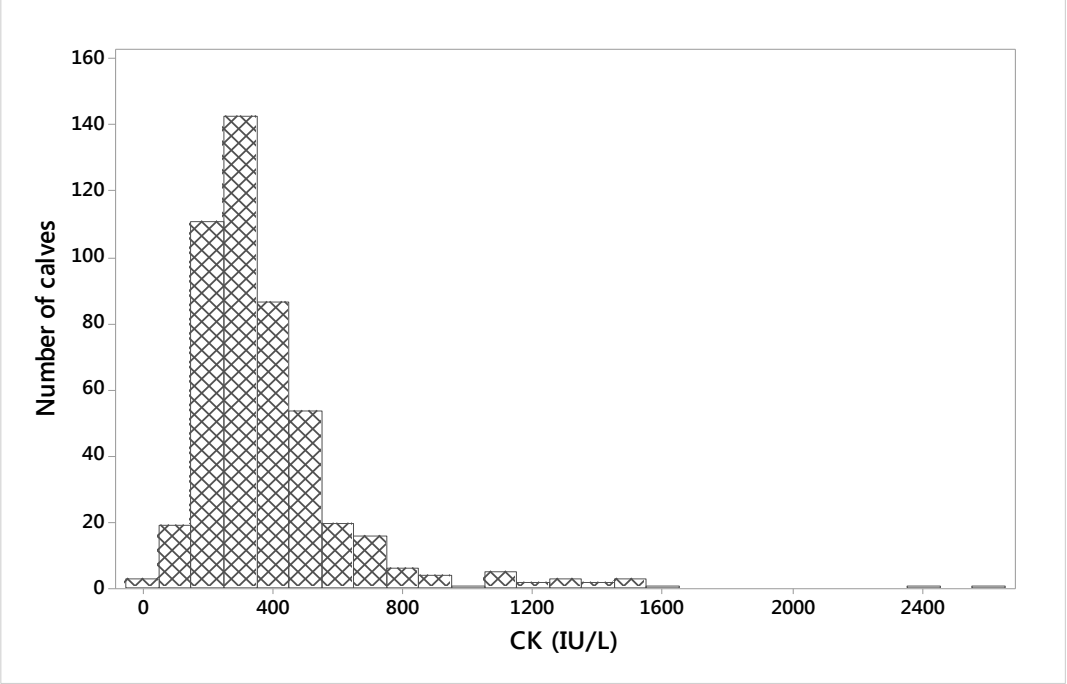

Figure 3.15: Histogram showing distribution of plasma CK in bobby calves at slaughter. Normal reference range of CK in calves is 11-125 IU/L (Lumsden *et al.* 1980) (n = 482).

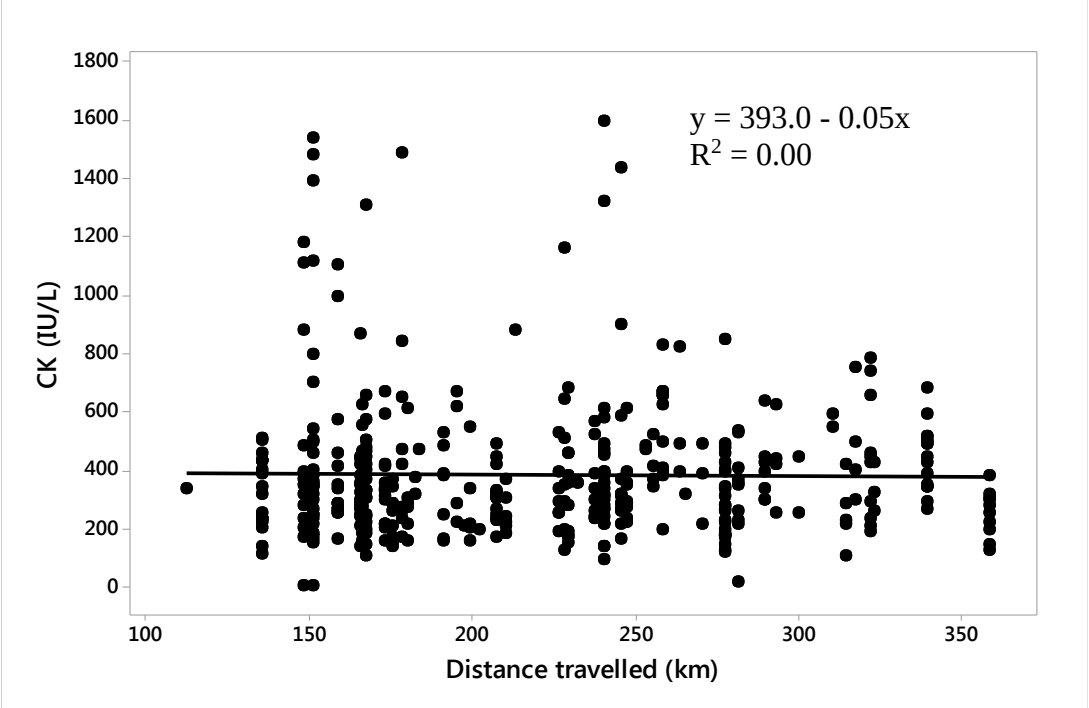

Figure 3.16: Association between plasma CK at slaughter in bobby calves and distance of transport to the abattoir (n = 415).

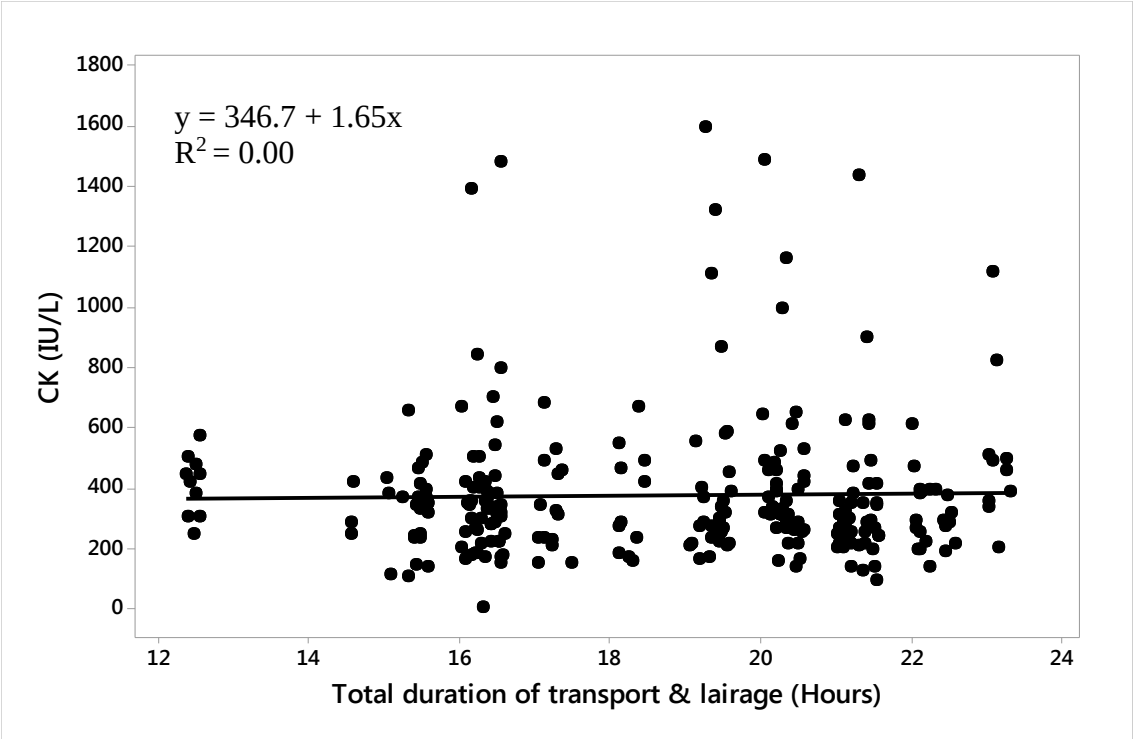

Figure 3.17: Association between plasma CK at slaughter in bobby calves and the total duration of transport to the abattoir and lairage time (n = 271).

Table 3.3: Mean (± SE) concentrations of plasma analytes of bobby calves at slaughter from different shires in northern and south-eastern dairy regions in Victoria.

Dairy regions	Shire	No. of herds	No. of calves	Glucose, mmol/L ± SE	PCV, % ± SE	Total protein, g/L ± SE	GGT, IU/L ± SE	CK, IU/L ± SE
Northern dairy	Campaspe	45	80	4.72 ± 0.10	32.61 ± 0.70	63.39 ± 1.36	371.40 ± 44.10	400.40 ± 30.40
	Gannawarra	30	61	4.69 ± 0.13	33.59 ± 0.86	63.48 ± 1.48	365.0 ± 67.8	387.0 ± 41.0
	Loddon	10	22	5.26 ± 0.16	32.86 ± 1.71	60.55 ± 2.54	245.7 ± 49.5	443.3 ± 38.8
	Swan Hill	05	28	4.34 ± 0.14	33.29 ± 1.31	62.57 ± 2.78	326.30 ± 74.70	447.00 ± 29.80
***P* value**				**0.006**	**0.847**	**0.784**	**0.794**	**0.026**
Total value for northern dairy		90	191	4.70 ± 0.06	33.03 ± 0.48	62.59 ± 0.86	339.40 ± 29.00	395.60 ± 18.10
South-eastern dairy	Baw Baw	72	143	4.50 ± 0.08	33.35 ± 0.55	66.61 ± 1.06	466.70 ± 45.30	379.50 ± 22.20
	South-Gippsland	18	61	4.49 ± 0.10	33.43 ± 0.85	65.33 ± 1.45	373.90 ± 71.90	326.80 ± 18.70
	Cardinia	18	38	4.60 ± 0.15	33.73 ± 0.98	68.21 ± 2.13	358.40 ± 51.60	387.70 ± 40.40
***P* value**				**0.425**	**0.948**	**0.531**	**0.487**	**0.501**
Total value for south-eastern dairy		108	242	4.50 ± 0.06	33.44 ± 0.42	66.54 ± 0.80	426.30 ± 33.40	367.50 ± 15.40
Estimate for difference between northern and south-eastern dairy (with 95% CI)				0.20 (0.00, 0.4)	- 0.41 (- 1.60, 0.78)	- 3.95 (- 6.22, -1.68)	- 46 (- 97, -4)	43 (14, 70)
***P* value**				**0.018**	**0.499**	**0.001**	**0.029**	**0.003**

Table 3.4: Mean (± SE) concentrations of plasma analytes of bobby calves in different seasons

Descriptive variable		No. of herds	No. of calves	Glucose, mmol/L ± SE	PCV, % ± SE	Total protein, g/L ± SE	GGT, IU/L ± SE	CK, IU/L ± SE
Season	Spring	61	113	4.87 ± 0.09	34.05 ± 0.86	61.50 ± 1.04	328.1 ± 41.2	347.4 ± 33.3
	Autumn	161	369	4.52 ± 0.05	33.25 ± 0.33	65.57 ± 0.67	408 ± 25.5	395.6 ± 11.8
	Estimate for difference (with 95% CI)			0.35 (0.16, 0.55)	0.80 (- 0.66, 2.26)	- 4.07 (- 6.66, - 1.48)	- 47 (- 99, - 5)	- 72 (- 102, - 44)
	***P* value**			**0.000**	**0.281**	**0.002**	**0.026**	**0.000**

Table 3.5: Mean (± SE) concentrations of plasma analytes of bobby calves at slaughter in relation to calf breed and sex

Descriptive variable		No. of herds	No. of calves	Glucose, mmol/L ± SE	PCV, % ± SE	Total protein, g/L ± SE	GGT, IU/L ± SE	CK, IU/L ± SE
Calf Breed	Holstein-Friesian	84	144	4.56 ± 0.07	33.33 ± 0.52	63.79 ± 1.09	303.50 ± 24.7	414.90 ± 18.0
	Jersey	19	27	4.40 ± 0.19	31.76 ± 1.11	73.73 ± 2.59	750 ± 172.0	402.30 ± 34.5
	Cross	44	67	4.47 ± 0.09	33.21 ± 0.97	67.89 ± 1.44	421.6 ± 62.0	397.90 ± 27.4
	***P* value**			**0.572**	**0.455**	**0.001**	**0.015**	**0.855**
Sex	Male	161	341	4.54 ± 0.05	33.13 ± 0.34	65.32 ± 0.69	407.70 ± 27.0	394.90 ± 12.5
	Female	21	28	4.26 ± 0.17	34.80 ± 1.04	68.61 ± 2.56	411.60 ± 72.0	404.40 ± 34.3
	Estimate for difference (with 95% CI)			0.28 (- 0.08, 0.64)	- 1.67 (- 3.79, 0.45)	- 4 (- 9, 2)	- 21 (- 145, 71)	- 28 (- 80, 28)
	***P* value**			**0.129**	**0.118**	**0.175**	**0.631**	**0.317**

3.5 Discussion

The results of this study indicated that the proportion of failure of passive transfer (FPT) in bobby calves was similar to findings reported in northern America where the prevalence of FPT in dairy calves varied from 19% to 37% (Trotz-Williams *et al.* 2008; Beam *et al.* 2009). Vogels *et al.* (2013) reported a higher proportion (38%) of FPT in calves in southwest Victorian dairy herds compared with our finding (23.03%). This difference might be because of regional variation of colostrum management, as Vogels *et al.* (2013) conducted their study in the south-west dairy region in Victoria whereas our study focused on the north and south-eastern dairy regions of Victoria. Another reason may be that the farmers are now more aware of bobby calf welfare and pay more attention to feed colostrum to bobby calves after successive educative campaigns by Dairy Australia. The animal husbandry survey in 2016 conducted by Dairy Australia indicated that most of the dairy farms provide additional colostrum to most of the calves (81%) and large farms always provide extra colostrum to nearly all calves (Dairy Australia 2016).

Variations of plasma TPP and GGT level in bobby calves between northern and south-eastern dairy regions might be due to different colostrum management practices in various properties. The reason behind higher plasma GGT and protein in Jersey calves in our study may be due to their higher capacity to absorb immunoglobulin compared to Holstein-Friesian calf. In one study, Jones *et al.* (2004) found that after 24 hours later of colostrum ingestion Jersey calves had higher IgG concentrations than Holstein calves (16.47 ± 0.71 g/L and 11.12 ± 0.60 g/L respectively). They also found that Jersey maintained higher plasma IgG concentrations compared to Holstein calves from day 1 through day 15.

Although the mean glucose concentration was within the normal reference ranges of glucose in bobby calves, a quarter of the calves had glucose levels towards the lower end of the

quoted reference range. In relation to the relevant reference ranges, our results showed a higher mean BHB in more than half of the calf plasma samples and a higher concentration of NEFA in one-fifth of the calves, indicating lipolysis to supplement their energy. These findings are similar to the study conducted in bobby calves at abattoirs in New Zealand (Stafford *et al.* 2001) and those reported in a study conducted under experimental conditions in Australia (Fisher *et al.* 2014). In the New Zealand study, Stafford *et al.* (2001) reported higher mean BHB level (0.22 mmol/L) in a proportion of bobby calves when mean glucose level was 4.3 mmol/L. Fisher *et al.* (2014) reported 4.14 mmol/L mean glucose with 0.18 mmol/L of mean BHB level after 24 hours off feed and 3.43 mmol/L mean glucose with 0.24 mmol/L mean BHB level after 30 hours off feed in bobby calves. In our study, we found similar trends of glucose and BHB level in a moderate proportion of calves which is not surprising as the tentative mean off feed period in bobby calves in commercial practice was 24.67 h. An inverse correlation between BHB and glucose concentrations in our data set indicates that a reasonable proportion of calves had commenced lipolysis due to depletion of glucose.

However, the bobby calves after transportation to the abattoir in Victoria did not suffer severe negative energy balance as reflected in their low plasma urea concentration, a parameter indicative of amino acid catabolism during severe shortfall of energy and very few calves exceeded the physiological ranges. We did not find any association either between transport distance and plasma glucose concentration nor between total transport duration and lairage with plasma glucose. The reason may be that in our study, calves were transported relatively short distances and duration before being slaughtered. Fisher *et al.* (2014) reported that plasma glucose concentrations in bobby calves remained stable for about 18 hours after feeding when they experimentally transported calves from a farm. They also found that

glucose concentrations started declining after 18 hours and found the lowest plasma glucose at 30 hours of time off feed, the maximum time point of their experiment. In our field study, the mean duration of transport and lairage was around 18 hours although some calves experienced a longer duration up to 23.3 hours. It would appear that plasma glucose remained relatively stable for bobby calves in our study, with no association of plasma glucose with the duration of transport and lairage time. Another contributing influence may be variation in the time of final feeding on-farm. Some calves might be fed immediately before pick up, and some might be fed well before pick up. Hence, the correlation between transport duration and lairage with plasma glucose may be variable due to the variation of the final feeding time point. This effect may also have contributed to the two TOF groups having no difference in energy metabolites between ≤ 24 h and > 24 h TOF. We added 6 h to the total duration of transport and lairage to estimate TOF, assuming that calves were fed within 6 h according to industry compliance. In reality, calves might be fed within 2 h before loading or at some other time, possibly more than 6 h beforehand. Although we observed higher BHB concentrations in calves with > 24 h TOF, indicating lipolysis, we are not confident based on our existing data set that higher TOF had an impact on the energy level in bobby calves after transport as there was no difference for other energy metabolites- i.e. glucose, NEFA and urea. The glucose concentrations did not vary significantly across the shires, except in one shire which might be due to the differences in management between farms of origin. Plasma glucose in bobby calves differed between spring and autumn. Although this difference was statically significant, biologically it might not be substantial as the difference in plasma glucose between the two seasons was minimal.

Based on the PCV results, very few calves experienced dehydration. Previous reports indicate that total plasma protein is positively correlated with colostrum intake (Selim *et al.* 1995;

Tyler *et al.* 1996). We found a positive correlation between GGT and total plasma protein. The proportion of calves found with elevated plasma protein in our study is probably because of colostrum intake rather than due to dehydration. Accordingly, we interpreted the dehydration level based on the PCV only. However, it is important to note that PCV can be affected by sympathetically-mediated splenic contraction following acute stress in calves (Constable *et al.* 1998) and thus assessing hydration level in calves based on PCV should interpret cautiously. We analysed the degree of a correlation either with the journey distance and PCV or with the journey duration and lairage time in relation with PCV, and neither yielded an apparent relationship. These findings are similar to the results of Todd *et al*. (2000) and Fisher *et al*. (2014), where they reported very little change in hydration status after bobby calf transport. Our field study and previous experimental studies suggest that the overall impact of transport on the hydration state in bobby calves is minimal.

The significant main effect of commercial bobby calf transport on the calves was seen in blood CK and lactate concentrations, which is similar to the findings from the field study in New Zealand where Stafford *et al.* (2001) reported a high percentage of bobby calves processed at slaughter plants with elevated CK. The probable reasons behind the higher levels of CK and lactate, compared to the standard reference range, was either calves had minor or significant muscular trauma or bruises or muscular fatigue, or that an anaerobic metabolism of glucose occurred due to strenuous muscular activity during truck movements and handling during loading and unloading. During our study, we did not physically examine the calves after slaughter, nor had access to the bruising or injury record of the calves from the abattoir. Hence, we could not determine the direct link between the CK level in relation to muscular injury in this study. However, there are some reasons which might explain the relationship between elevated CK in bobby calves with muscular injury or fatigue in this case. Bobby

calves are transported at a very young age and may be loaded and unloaded several times before they reach slaughter plants (Jongman and Butler 2013). The muscle at this stage is very tender and may be traumatised due to improper loading and unloading. Trauma could also occur during parturition, yarding at the slaughter plants and during transport with high stocking densities (McCausland *et al.* 1977; Tarrant *et al.* 1992; Jongman and Butler 2014). In a field study in New Zealand where they assessed 16,400 bobby calves at commercial slaughter plants, the researchers found bruises in the stifle area in 50% of calves (McCausland *et al.* 1977). Increased CK concentrations were also observed when bobby calves were experimentally transported without bedded flooring and at a small space allowance of 0.2 m^2 compared to straw bedding and 0.5 m^2 of space allowance (Jongman and Butler 2014). Currently, in commercial practice, bobby calves are transported to abattoirs without bedding in trucks in Victoria. Apart from trauma during loading and unloading; lack of bedding, stocking density during commercial transport and high stocking densities during lairage may have contributed to the elevated concentrations of CK in calves in our study. Further study involving a physical examination of calves in association with blood CK would help us to interpret CK values and its relationship with a muscular injury.

In experimental bobby calf studies, Todd *et al*. (2000) and Fisher *et al.* (2014) reported higher CK concentrations when calves were transported for longer durations. McNally and Warriss (1996) also reported a similar trend in cattle transported to abattoirs. However, we did not find an association between transport duration and lairage with plasma CK. The probable reason might be due to different management practices for a different cohort of calves. Some may be loaded and unloaded gently, and some might have a different experience. Apart from that, we could not separate the transport duration from the lairage time. As a result, some calves may have been transported for a longer duration and some not. Due to the lack of

consistent data for each calf, our correlation result of total transport duration and lairage time with plasma CK need to be interpreted cautiously. The lack of management data on loading and unloading of calves also limit to an explanation for reasons of variation of CK in different regions and different seasons. It is also possible that the statistically significant difference of plasma CK between two different dairy regions and seasons might not be biologically significant, given the relative differences involved.

3.6 Conclusions

The key findings of our study were that in commercial practice, bobby calf welfare at an abattoir after transportation was not significantly compromised apart from some indicators of muscular fatigue. Very few calves experienced a severe shortfall of energy, and most of them were well hydrated. Around a quarter of the calves had a lack of passive immunity; however, this is a general problem in young dairy calves. Transport distance and the total duration of transport and lairage had no significant effect on plasma analytes in bobby calves measured at slaughter. Overall, our field study indicates that bobby calves can be well managed from the property of origin to abattoir under existing management systems in Victoria without unduly compromising their welfare.

4 Prevalence of major enteric pathogens in the faeces of transported bobby calves and risk factors associated with shedding

4.1 Abstract

Faecal samples (n = 273) from bobby calves collected after transportation to an abattoir in Victoria were screened for *E. coli* K99, *Salmonella* spp., bovine rotavirus (BRV) and bovine coronavirus (BCV) using qPCR to estimate the prevalence and faecal load of major enteric pathogens. Risk factors associated with the prevalence and faecal load of the different pathogens were also investigated. *E. coli* K99 was the most prevalent pathogen (37.4%), followed by BRV (8.1%), *Salmonella* spp. (5.1%) and BCV (2.6%). Infection with more than one pathogen was detected in 10.3% of the samples. Calves with a higher plasma gamma-glutamyl transferase (GGT) concentration, reflective of better acquisition of passive immunity, had lower concentrations of BRV, BCV and *Salmonella* spp. in faeces of infected calves. Low plasma concentrations of glucose and high plasma urea, which are indicative of hypoglycaemia, were associated with increased amounts of shedding of *E. coli* K99 and BRV in infected calves. There was no association between plasma creatine kinase or lactate concentrations, which are indicative of muscular fatigue, and pathogen shedding. Increased distance of transportation was only associated with an increased amount of shedding of BRV in infected calves. There was regional variation in the prevalence of excretion of the different pathogens, with BCV only detected in calves from the south-eastern dairying region. Calves from the south-eastern dairy region were also more likely to be co-infected with the different pathogens than those from the northern dairying region. Breed and sex were not associated with any differences in the presence or absence of pathogens in the faeces of the bobby calves. These findings suggest that the main factor influencing amounts of excretion of enteric pathogens in infected bobby calves is the efficacy of transfer of passive immunity, although

hypoglycaemia and transportation distance can have some effect on amounts of excretion with specific pathogens.

4.2 Introduction

Although the Australian dairy industry has made significant improvements in herd management, animal care, nutrition and health management, neonatal calf diarrhoea is still a significant concern, and its management is complicated by the multifactorial nature of the disease (Izzo *et al.* 2011). The causal pathogens of calf diarrhoea are well known, but the prevalence of the different pathogens varies between countries and production systems (Izzo *et al.* 2011). Many prevalence studies of the major enteric pathogens causing diarrhoea in dairy calves have been reported internationally (Reynolds *et al.* 1986; Garcia *et al.* 2000; Uhde *et al.* 2008; Gulliksen *et al.* 2009), but only a few small-scale prevalence studies of individual pathogens (Jerrett and Snodgrass 1981; Huang *et al.* 1992; Becher *et al.* 2004; Swiatek *et al.* 2010) and one large-scale study of the major pathogens (rotavirus, coronavirus, *Cryptosporidium parvum, Escherichia coli* K99, and *Salmonella* spp.) associated with diarrhoea in dairy calves in Australia have been reported (Izzo *et al.* 2011).

Numerous factors can influence the apparent prevalence of enteric pathogens in dairy calves, including management, age, season and the diagnostic techniques used to detect the pathogens (Acres 1985). Previous studies have shown that transportation can result in increased susceptibility to pathogens in adult cattle and calves, and can also influence the prevalence of shedding of these pathogens (Frank and Smith 1983; Barham *et al.* 2002; Bach *et al.* 2004; Arthur *et al.* 2007; Dewell *et al.* 2008; Aperce *et al.* 2014). Animals can be severely stressed by transportation (Trunkfield and Broom 1990). In calves, stress increases during transport, and the level of stress can be influenced by regrouping, handling during loading and unloading, space allowances, the performance of the driver, and the transport and lairage time

(Vecerek *et al.* 2006; Jongman and Butler 2014). In addition to these factors, pre-transport management, noise, vibration, the novelty of the experience, restraint and deprivation from feed and water also contribute to transport stress (Swanson and Morrow-Tesch 2001). Transport stress can result in immune suppression and thus may lead to increased susceptibility to disease and shedding of pathogens in cattle (Swanson and Morrow-Tesch 2001).

Infectious enteritis is a significant health and welfare issue in neonatal calves. Moreover, some of the enteric pathogens of calves can cause foodborne disease in human. For example, infection with *Salmonella* spp. causes acute diarrhoea and systemic illness in calves and can pose a significant threat to public health (Mead *et al.* 1999; Cho and Yoon 2014). Meat and other products derived from bobby calves are mainly exported to the USA and Japan (Meat & Livestock Australia 2011). Therefore, regular monitoring of the excretion of foodborne pathogens by these calves is critical to preserving public health and export markets. Bobby calves, because of their low value compared to heifer replacement calves, may not be given sufficient colostrum or an optimal standard of housing, feeding, cleanliness, care and attention. This is possible that transportation and current management practices in the commercial bobby calf supply chain may increase the prevalence and enteric pathogen load in bobby calves. Therefore, understanding the prevalence of excretion of enteric pathogens and the risk factors associated with a higher prevalence of excretion in bobby calves is worthy of greater investigation.

To date, published reports about the prevalence of enteric pathogens and their level of shedding in bobby calves and dairy heifer replacement calves in animals that are not suffering from diarrhoea are limited. Therefore, this study firstly aimed to estimate the prevalence and load of the major enteric pathogens (*Salmonella* spp., *E. coli* K99, rotavirus and coronavirus)

in the faeces of transported bobby calves to understand how common these pathogens are in bobby calves after arrival at the abattoir and, to some extent, in dairy calves in Victoria in general. Secondly, the study aimed to identify potential risk factors associated with the prevalence and load of enteric pathogens in the faeces of bobby calves presented for commercial slaughter.

4.3 Materials and methods

4.3.1 Animals and collection of samples and transport data

In March and April 2016, a total of 273 faecal samples were collected from bobby calves on the killing line at an abattoir in Victoria. Faecal samples were collected directly from the rectum with sterile gloves and stored in 50 ml sterile containers for approximately 2 hours in a box containing ice before being shifted to 1 ml Eppendorf tubes and stored at -80º C for further processing. The procedure of blood collection and identification of bobby calves have been described previously in section 3.3.2 and 3.3.3. Methods of calculation of transport management data such as distance and duration of transport and lairage have been described previously in section 3.3.4 and 3.3.5.

4.3.2 Haematological and biochemical measurements

The procedure of haematological and biochemical measurements has been described previously in section 3.3.7.

4.3.3 Extraction of nucleic acids from faeces

The DNeasy® PowerSoil® kit (Qiagen) was used to extract the nucleic acids from each sample of calf faeces using the manufacturer's protocol. Depending upon the sample volume, 0.1 to 0.2 g of faeces was added to the Power Bead Tube, and the mixture vortexed gently. After vortexing, 60 µl of solution C1, which contains SDS, was added and the mixture vortexed briefly to achieve cell lysis. To ensure complete homogenization and cell lysis, an additional 30 seconds of vortexing was performed in a tissue homogeniser. The tube was then

centrifuged at 10,000 x *g* for 30 seconds. A 500 µl volume of the supernatant was then transferred to a clean 2 ml collection tube and 250 µl of solution C2, which contains a reagent to remove potential inhibitors, was added. The tube was then incubated at 2-8°C for 5 minutes. After incubation, the tube was centrifuged at 10,000 x *g* for 1 minute. A 600 µl volume of the supernatant was then transferred to a clean 2 ml collection tube, and 200 µl of solution C3 was added and the solution vortexed briefly. The tube was then incubated at 2-8°C for 5 minutes. Solution C3 also contains a reagent to remove further potential inhibitors to ensure purity of the nucleic acids for downstream analysis. After incubation, the tube was then centrifuged at 10,000 x *g* for 1 minute. Up to 750 µl of the supernatant was transferred to a clean 2 ml collection tube and 1200 µl of solution C4 added and the solution was vortexed for 5 seconds. Solution C4 contains a high concentration of salt, which ensures binding of the nucleic acids to the spin filters. A total of 675 µl of the solution was then loaded onto an MB Spin Column and the column was centrifuged at 10,000 x *g* for 1 minute. The flow-through was discarded. This step was repeated twice until all the solution had been processed. A 500 µl volume of solution C5, which is an ethanol-based wash solution used to clean the nucleic acids further, was added to the spin column and the column was centrifuged at 10,000 x *g* for 30 seconds. The flow-through was discarded. The tube was centrifuged further at 10,000 x *g* for 1 minute. The MB Spin Column then placed into a clean 2 ml collection tube and 100 µl Solution C6, which is a sterile elution buffer, was added to the centre of the white filter membrane. After 10 minutes of incubation at room temperature, the tube was then centrifuged at 10,000 x *g* for 30 seconds to elute the nucleic acids. Extracted nucleic acids were then measured for quality and quantity using a NanoDrop™ Spectrophotometer ND1000 (ThermoFisher Scientific Inc., USA). An A260: A280 ratio value of approximately 2.0 was

regarded optimal purity of the nucleic acids. The eluted nucleic acids were then stored at -80°C for further analysis.

4.3.4 Reverse Transcriptase Polymerase Chain Reactions (RT-PCR)

For detection of rotavirus and coronavirus RNA, complementary DNA (cDNA) was generated from the extracted nucleic acids using Superscript III Reverse Transcriptase (Invitrogen) and random hexamer primers. To reduce secondary structure, 5 µl of nucleic acids and 1 µl of 10 µM random hexamers were incubated at 95°C for 5 minutes before being placed on ice. Reverse transcription was then performed at 55°C for 50 minutes in a reaction mixture containing 4 µl of 5 X first strand buffer (Invitrogen), 1 µl of 0.1 M dithiothreitol (Invitrogen), 1 µl of 10 mM deoxynucleotide triphosphates (dNTPs), 1 µl of 40 U of RNaseOUT (Invitrogen) and 1 µl of 200 U of Superscript III RT, with sterile water added to obtain a final reaction volume of 20 µl. After the reaction was finished, the reverse transcriptase was inactivated by incubation at 70°C for 15 minutes.

4.3.5 Real-time PCR assay

The quantitative PCR (qPCR) assays to detect *E. coli* K99, *Salmonella* spp., rotavirus and coronavirus, and the internal control 16S rRNA, were performed in reactions containing 1 µl of DNA or cDNA template in 1 X GoTaq Flexi Buffer containing 1.5 mM $MgCl_2$ (Promega Corporation, USA), 0.2 mM dNTPs, 0.4 µM of each of the forward and reverse primers, 8 µM of SYTO 9 (Invitrogen™, USA) and 0.05 U/µl of GoTaq DNA polymerase, with the volume of the reaction made up to 20 µl with sterile water.

The real-time PCR amplification of the target gene was performed on a Rotorgene-600, (Qiagen®, Germany). Previously validated primers were used (Table 4.1). The cycling conditions for each target pathogen gene were as follows: for the K99 gene, an initial incubation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 15 seconds; for the *invA*

of *Salmonella* spp., an initial incubation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 15 seconds; for the NSP3 gene of bovine rotavirus, an initial incubation at 94°C for 1 minute, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 68°C for 30 seconds; for the N gene of bovine coronavirus, an initial incubation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 15 seconds; for the 16S rRNA gene, an initial incubation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 15 seconds. After 40 cycles, a melt curve was generated for each PCR product using SYTO 9 based green fluorescence, with a ramp speed of 0.03°C/sec between 70°C and 95°C.

4.3.6 Cloning of PCR products in pGEM-T

The target gene sequences were amplified by PCR using the same primers and cycling conditions used for qPCR, except for an additional final extension cycle for 5 minutes. After amplification of the target sequence, the PCR products were purified and ligated into the pGEM-T Vector. The vector was then introduced into electrocompetent DH5α cells by electroporation and transformants were cultured on LB agar containing ampicillin at 50 µg/ml. The identities of cloned fragments were confirmed by colony PCR followed by sequencing.

Table 4.1: Nucleotide sequences of primers used for PCR and qPCR

Pathogen (target gene)	Amplicon (bp)	Primer name	Sequence (5'-3')	Reference
Salmonella spp. (*invA*)	132	*invA*-F	CATTTCTATGTTCGTCATTCCATTACC	Pusterla *et al.* 2010
		invA-R	AGGAAACGTTGAAAAACTGAGGATTCT	
E. coli K99 (K99 gene)	230	K99-F	GCGACTACCAATGCTTCTGCGAATAC	Cho *et al.* 2010
		K99-R	GAACCAGACCAGTCAATACGAGCA	
Rotavirus (NSP3 gene)	87	NSP3-F	ACCATCTACACATGACCCTC	Pang *et al.* 2004
		NSP3-R	GGTCACATAACGCCCC	
Coronavirus (N gene)	87	BCoV-F	CTAGTAACCAGGCTGATGTCAATACC	Cho *et al.* 2010
		BCoV-R	GGCGGAAACCTAGTCGGAATA	
Bacterial 16S Ribosomal RNA genes (16S rRNA gene)	60	16S-F	GGGTTGCGCTCGTTGC	Muziasari *et al.* 2016
		16S-R	ATGGYTGTCGTCAGCTCGTG	

4.3.7 PCR product clean-up

PCR products were purified from the reactions using the UltraClean® PCR clean-up kit (MO BIO Laboratories, Inc.) according to the manufacturer's instructions. The Spinbind solution was shaken and 100 µl added to the 20 µl PCR reaction. This suspension was then mixed well by pipetting. The suspension was then transferred into a spin filter unit. The unit was centrifuged for 30 seconds at 10,000 x *g*. After centrifugation, the spin filter basket was removed, and the liquid flow-through was discarded from the tube. The spin filter basket was then placed back into the same tube. A 300 µl volume of SpinClean buffer was then added to

the spin filter and the filter was centrifuged at 10,000 x *g*. The spin filter was then transferred to a clean collection tube. A 50 µl volume of elution buffer (10 mM Tris) was added to the centre of the spin filter membrane. The tube was then centrifuged for 60 seconds at 10,000 x *g*. Finally, the spin filter was discarded, and the purified DNA in the eluate was stored at -80°C.

4.3.8 Ligation of the PCR product into pGEM-T

The PCR products were ligated into pGEM-T (Promega) according to the manufacturer's instructions. The pGEM-T vector and Control Insert DNA tubes were centrifuged briefly to collect the contents at the bottom of the tube. The ligation reactions were then set up in 0.5 ml tubes as described in Table 4.2.

Table 4.2: Reagents and their amount used for the ligation during cloning

Reagents	**Standard reaction**	**Positive control**
2 X Rapid Ligation buffer	5 µl	5 µl
pGEM-T vector (50 ng)	1 µl	1 µl
PCR product	* µl	-
Control insert DNA	-	2 µl
T4 DNA Ligase	1 µl	1 µl
Nuclease-free water to a final volume of	10 µl	10 µl

The reactions were then mixed by pipetting and incubated overnight at 4°C.

* The molar ratio of PCR product (insert) and the vector was 3:1. The amount of insert was calculated using the following formula.

$$\frac{\text{ng/ vector} \times \text{kb inserts}}{\text{size of vector}} \times \frac{3}{1}$$

4.3.9 Bacterial electrotransformation (electroporation)

A 2 µl ligation mixture was added into 40 µl of electro-competent DH5α cells, mixed by flicking with a finger and the mixture incubated on ice for 30 min. A 0.2 cm gap Gene Pulser cuvette (Bio-Rad Laboratories) was placed on ice for 10 minutes to chill. After incubation, the cell suspension was transferred to the chilled cuvette by pipetting and tapped on the benchtop to exclude any air bubbles. The cuvette was then placed into a holder and positioned in the chamber of a Bio-Rad Gene Pulser. The machine was set with a voltage of 2.5 kV, 25 µF capacitance and 200 Ω resistance. A single pulse was delivered, with a time constant between 4 and 5 milliseconds. Immediately after electroporation, 1 ml of room temperature LB broth was added to the cuvette. The solution was then transferred to a 1.5 ml tube and incubated at 37°C for 30 minutes in a shaking incubator at 200 rpm.

4.3.10 Screening transformants for inserts

The transformed electro-competent DH5α cells were plated on LB agar containing ampicillin (50 µg/ml), 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG) and 2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and incubated at 37°C for overnight.

Successful cloning interrupts the coding sequence of β-galactosidase in the pGEM-T vector. This interruption results in colour change in the colonies grown in the presence of IPTG and X-gal. Usually, a clone containing insert produces white colonies, while those containing a plasmid without an insert produce blue colonies.

White colonies were picked and tested to confirm successful cloning by colony PCR and sequencing. For colony PCR, a small amount of a white colony was picked up using a sterile pipette tip and mixed with 20 µl of nuclease-free water in an Eppendorf tube. The tube was then incubated at 95°C for 5 minutes in a heating block and the supernatant collected as a template for the subsequent PCR. For PCR, 2 µl of the supernatant was added to a reaction mixture containing 1X GoTaq Flexi Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of M13 forward and reverse primers, and 0.5 U GoTaq DNA polymerase in a final volume of 20 µl. The reaction was cycled through an initial activation at 95°C for 2 minutes, then 35 cycles of denaturation at 94°C for 20 seconds, annealing at 55°C for 20 seconds, and extension at 72°C for 30 seconds. A final extension at 72°C for 5 minutes for 1 cycle was also included.

The M13 primer sequences were:

M13F: CCCAGTCACGACGTTGTAAAACG

M13R: AGCGGATAACAATTTCACACAGG

The M13 reverse sequencing primer binding site in the pGEM-T vector sequence is at bases 174-196, and the M13 forward sequencing primer binding site is at bases 2946-2968. The M13 forward and reverse primers amplify 250 base pairs (bp). Hence, 250 bp was added to the insert size to calculate the expected PCR product size. For example, the coronavirus N gene insert size was 87 bp, and additional sequences amplified using the M13 forward and reverse primers were 250 bp, so the final expected product size was 337 bp.

4.3.11 Storage of transformants containing cloned PCR products

Samples of colonies suspected to contain pGEM-T were picked up using a sterile pipette tip and inoculated into 5 ml LB broth containing ampicillin (100 µg/ml). The broth culture was

incubated at 37°C overnight at 200 rpm in a shaking incubator. A sample of the culture was used for DNA extraction, and the rest was mixed with glycerol (1:1 ratio) and stored at -80°C.

4.3.12 Extraction of plasmid DNA

Plasmid DNA was extracted from cultured transformants using the Wizard Plus SV Minipreps DNA purification system (Promega) following the manufacturer's instructions. A 1.5 ml sample of an overnight culture was centrifuged at 10,000 x *g* for 5 minutes. The pellet was then resuspended in 250 µl of Cell Resuspension Solution, 250 µl of Cell Lysis Solution was added and the suspension mixed by inversion of the tube. A 10 µl volume of Alkaline Protease Solution was added, the suspension mixed by inversion of the tube and then incubated for 5 minutes at room temperature. After incubation, 350 µl of Neutralisation Solution was added and the tube inverted 4 times to mix the contents. The solution was then centrifuged at 14,000 x *g* for 10 minutes at room temperature to clear the lysate. A spin column was inserted into a collection tube. The cleared lysate was then decanted into the spin column and the column centrifuged at 14,000 x *g* for 1 minute at room temperature. The flowthrough was discarded, and the column was reinserted into the collection tube. For washing, 750 µl of Wash Solution was added and the column centrifuged at 14,000 x *g* for 1 minute. This washing step was repeated using 250 µl of Wash Solution, with the column centrifuged at 14,000 x *g* for 2 minutes at room temperature. The spin-column was then transferred to a sterile 1.5 ml microcentrifuge tube and 100 µl nuclease-free water added to the centre of the spin column and the column centrifuged at 14,000 x *g* for 1 minute at room temperature. Finally, the column was discarded, and the eluted DNA was stored at -80°C for later use as a template for generating the standard curve for the qPCR assay. DNA was also sent to Australian Genome Research Facility (AGRF) for sequencing to confirm that the insert had the expected sequence.

4.3.13 Generation of standard curves for qPCR assays

A standard curve for quantifying the amount of *Salmonella* spp. DNA in a sample was generated using genomic DNA from *Salmonella enterica* subsp. *enterica* Typhimurium strain (TW-Stm6). The strain used was previously isolated from pig faeces, and its complete genome had been sequenced (Dyall-Smith *et al.* 2017). In brief, a single *Salmonella* colony was picked from LB agar and used to inoculate 5 ml LB broth. The inoculum was incubated overnight at 37°C with shaking at 200 rpm. Genomic DNA was then extracted using the Wizard Genomic DNA extraction kit (Promega) according to the manufacturer's instructions. DNA quality and quantity were assessed using a NanoDrop ND1000 spectrophotometer (ThermoFisher Scientific). To generate the standard curve, a concentration of 10^7 copies/µl was prepared based on the genome size and the DNA concentration in the stock solution using the ThermoFisher DNA copy number and dilution calculator (ThermoFisher Scientific 2018). For *E. coli* K99, rotavirus and coronavirus, targets were amplified by PCR from a commercial calf vaccine (Bovilis Rotavec Corona Calf Scours Vaccine, Coopers Animal Health Intervet). After extraction of the nucleic acids from the vaccine, PCR products were amplified and cloned into pGEM-T (Promega). Using the ThermoFisher DNA copy number and dilution calculator, a solution containing 10^8 copies/µl was prepared from the plasmid DNA containing the cloned PCR product and stored in aliquots for subsequent assays. A tenfold dilution series, ranging from 10^8 copies/µl to 10^1 copies/µl were prepared and assayed by qPCR to generate a standard curve. Serial dilutions were prepared using a QIAgility robot (Qiagen) according to the manufacturer's instructions. The amplification efficiency (E) of the qPCR assay was estimated from the slope of the standard curve, where $E = -1 + 10^{(-1/\text{slope})}$ (Nybo 2011). A PCR efficiency of 100% corresponds to a slope of -3.32.

4.3.14 Measurement of the efficiency and sensitivity of the qPCR assays and identification of inhibition of any qPCR assays

The efficiency and sensitivity of the qPCR assays were calculated using the software provided with the Rotorgene-600 qPCR system (Qiagen). Each standard curve for each target was run at least 3 times to obtain a reliable estimate of the efficiency of the assays (Figure 4.1). After optimisation of the standard curve, unknown samples were run along with the standard curve and results were only accepted when the standard curve had an efficiency between 90% and 105% with an $R^2 > 0.98$. Based on the standard curve, the detection limit for each target and the cut-off Ct were determined. Samples from which PCR products were amplified beyond the cut-off Ct for the target gene were considered negative. A melting curve analysis was also performed to confirm that the expected PCR product had been amplified in each positive sample (Figure 4.2). Any unknown samples yielding a product with a similar melting temperature and melting curve to those of the standard were considered to be positive. To detect inhibition of the qPCR assay, an assay amplifying the 16S rRNA gene was performed as a positive control. If this assay did not yield a product the assays for the different pathogens were assumed to be inhibited. All samples were analysed in parallel with the standard curve for the assay, and positive and negative controls to check for PCR inhibition and contamination.

4.3.15 Estimation of pathogen load in faeces

Template copy numbers were converted to numbers of organisms, with target genes assumed to occur as single copies on the genome, and the bacterial and viral genomes assumed to be haploid. To calculate this, the copy number/µl template was multiplied by the total volume in which the nucleic acid was eluted, and this was then corrected to account for the mass of the faecal sample from which the nucleic acid had been extracted to yield the copy number/g of faeces. The results were reported as organisms/g (copy/g) of faeces.

4.3.16 Statistical analysis

Data were analysed with the Minitab® version 18.1 (Minitab). The Anderson-Darling test was performed to assess the normality of the data. To compare differences of plasma analytes in relation to presence and absence of pathogens, *t*-test and Mann-Whitney tests were performed. Linear regression analysis was performed when outcomes and explanatory variables were continuous; otherwise binary logistic regression analysis was applied to examine associations between categorical outcomes and explanatory variables. Fisher's exact test was performed when the binary logistic regression model failed due to cells with expected counts less than 5. P <0.05 was considered significant.

4.4 Results

4.4.1 PCR efficiency and sensitivity

Around 95% of samples were positive in the 16S rRNA gene qPCR assay, with an average Ct of 17.24 and a standard deviation of 2.6. The mean concentration of 16S rRNA was 3.3 x 10^8 /µl of DNA.

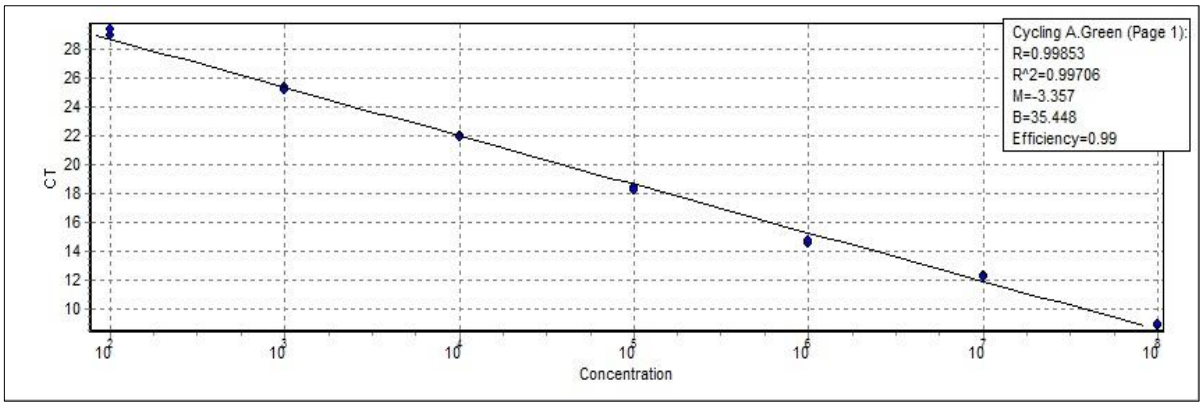

Figure 4.1: Standard curve for the coronavirus N gene qPCR assay showing detection concentration of N gene between 10^8 and 10^2 copies/µl. The assay had an efficiency of 99% and the correlation coefficient (R^2) was 0.997.

Based on the standard curves for each assay, the limit of detection of the target genes for BRV, BCV, *Salmonella* spp. and *E. coli* K99 was 100 copies/ µl (Figure 4.1 & 4.2), and the cut-off Cts were 29, 34, 33 and 29, respectively.

4.4.2 Prevalence and concentration of enteric pathogens in bobby calves

The overall estimated prevalence of *E. coli* K99, *Salmonella* spp., BRV and BCV in faecal samples from bobby calves from a single abattoir in Victoria were 37.4%, 5.1%, 8.1% and 2.6%, respectively. Overall, any single infection was detected in 38.1% of faecal samples, and co-infection with two or more pathogens was detected in 10.3% of samples (Table 4.3). The estimated prevalence and the loads of enteric pathogens in different dairy regions and shires in Victoria are given in Table 4.4. The pathogen with the highest prevalence was *E. coli* K99. Bovine coronavirus only detected in the south-eastern dairy region and had the lowest prevalence of detection among the four enteric pathogens.

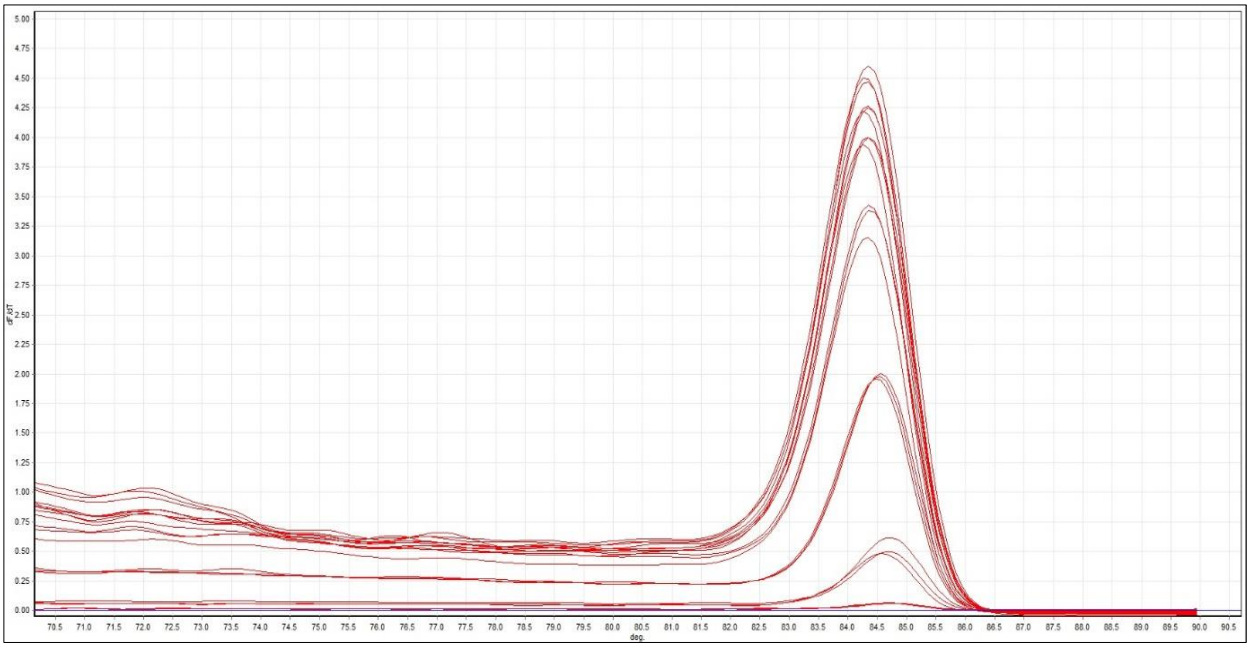

Figure 4.2: Melting curve of the products amplified in coronavirus N gene standard curve, generated after serial dilutions from 10^8 to 10^1 copies/µl, demonstrating that lowest concentration detected was 10^2 copies/µl (the low peak) and highest concentration detected was 10^8 copies/µl (high peak). There was no evidence of primer-dimer formation, with a single melting point (T^m) indicating amplification of a single target. Each assay was performed in triplicate.

4.4.3 Factors associated with enteric pathogen shedding in the faeces of bobby calves

The plasma analytes measured in bobby calves just after slaughter are shown in Table 4.5, with the relationships between these measurements and excretion of enteric pathogens shown in Tables 4.6 to 4.13, and Figure 4.3 and 4.4. Presence or absence of pathogens had no significant effect on plasma analytes except the lower level of plasma glucose in the positive rotavirus samples compared to negative rotavirus samples (Table 4.6 to 4.9). A significant ($P < 0.05$) linear relationship was detected between the log loads of *E. coli* K99 in faeces and log plasma urea concentrations in those calves that were found to be shedding *E. coli* K99. Similarly, the log loads of BRV shed in faeces were significantly associated with log plasma glucose concentrations and the distance the infected calves had been transported. Shedding amounts of BRV, *Salmonella* spp. and BCV, but not *E. coli* K99 was inversely correlated

with log plasma GGT and log total plasma protein concentrations in infected calves (Table 4.10 and in Figure 4.3 and 4.4). However, GGT had no significant effect on the presence or absence of enteric pathogens in the faeces (Table 4.11). Breed and sex were not correlated with pathogen shedding in faeces. Calves from the south-eastern dairy region were more likely to have two or more pathogens detectable in their faeces than calves from the northern dairy region (Table 4.12 and Table 4.13).

Table 4.3: Estimated prevalence of enteric pathogens in faecal samples (n = 273) from bobby calves in Victoria.

Pathogen	Percent positive (95% CI)
E. coli K99	37.36 (31.61, 43.40)
Salmonella spp.	05.13 (2.83, 8.45)
BRV	08.06 (05.11, 11.95)
BCV	02.56 (01.04, 05.21)
E. coli K99 + BRV	03.67 (01.77, 06.63)
E. coli K99 + BCV	01.10 (00.23, 03.18)
Salmonella spp. + BRV	00.73 (00.09, 02.62)
Salmonella spp. + BCV	00.73 (00.09, 02.62)
E. coli K99 + *Salmonella* spp.	02.56 (01.04, 05.21)
BRV + BCV	00.37 (00.01, 02.02)
BRV + BCV + *E. coli* K99	00.37 (00.01, 02.02)
BRV + BCV + *Salmonella* spp.	Not detected
E. coli K99 + *Salmonella* spp. + BRV	00.37 (00.01, 02.02)
E. coli K99 + *Salmonella* spp. + BCV	00.37 (00.01, 02.02)
E. coli K99 + *Salmonella* spp. + BRV + BCV	Not detected
Any single infection	**38.10 (32.31, 44.14)**
Any co-infection	**10.26 (06.92, 14.48)**

Table 4.4: Estimated prevalence of shedding of enteric pathogens by, and faecal loads of pathogens in positive samples from, bobby calves from different dairy regions and shires in Victoria at slaughter.

Dairy region	Shire	No. calves tested	*E. coli* K99		*Salmonella* spp.		BRV		BCV	
			Prevalence (%, 95% CI)	Median organisms/gm (range)	Prevalence (%, 95% CI)	Median organisms/gm (range)	Prevalence (%, 95% CI)	Median organisms/gm (range)	Prevalence (%, 95% CI)	Median organisms/gm (range)
Northern	Campaspe	34	32.4 (17.4 - 50.5)	4.1×10^6 (5.6×10^5 - 5.9×10^8)	ND	ND	2.9 (0.1 - 15.3)	2.2×10^9 (2.2×10^9 - 2.2×10^9)	ND	ND
	Gannawarra	23	30.4 (13.2 - 52.9)	2.7×10^6 (6.8×10^5 - 2.2×10^8)	4.4 (0.1 – 22.0)	2.0×10^7 (2.0×10^7- 2.0×10^7)	17.4 5.0 - 38.8)	1.4×10^8 (1.8×10^7 - 1.5×10^{11})	ND	ND
	Loddon	08	37.5 (8.5 - 75.5)	7.5×10^6 (4.6×10^6- 9.2×10^7)	ND	ND	ND	ND	ND	ND
	Swan Hill	20	25.0 (8.7 - 49.1)	1.8×10^6 (5.4×10^5- 5.1×10^7)	ND	ND	5.0 (0.1 – 24.9)	2.6×10^8 (2.6×10^8 - 2.6×10^8)	ND	ND
South-eastern	Baw Baw	115	39.13 (30.2 - 48.7)	3.8×10^6 (1.0×10^5- 1.4×10^{10})	9.6 (4.9 – 16.5)	1.0×10^6 (9.3×10^4- 4.3×10^9)	9.6 (4.9 – 16.5)	2.1×10^6 (4.0×10^5 - 1.3×10^{10})	5.2 (1.9 – 11.0)	3.6×10^5 (1.5×10^5 - 7.8×10^5)
	South Gippsland	41	51.22 (35.1 - 67.1)	3.6×10^6 (2.5×10^5- 2.2×10^{10})	4.9 (0.6 – 16.5)	4.7×10^6 (4.5×10^5- 8.9×10^6)	12.2 (4.1 – 26.2)	1.9×10^6 (1.0×10^6 - 7.2×10^8)	ND	ND
	Cardinia	25	28.00 (12.1 – 49.4)	1.1×10^6 (1.7×10^5- 1.0×10^7)	ND	ND	ND	ND	4.0 (0.1 – 20.4)	2.2×10^5 (2.2×10^5 - 2.2×10^5)

ND, not detected

Table 4.5: Concentrations of plasma analytes in bobby calves at slaughter in Victoria.

	Glucose (mmol/L)	BHB (mmol/L)	Urea (mmol/L)	NEFA (mmol/L)	PCV (%)	TPP (g/L)	CK (IU/L)	Lactate (mmol/L)	GGT (IU/L)
Mean (±SE)	4.54 ± 0.06	0.20 ± 0.01	4.81 ± 0.17	0.33 ± 0.01	33.01 ± 0.39	65.54 ± 0.78	392.10 ± 14.0	5.39 ± 0.09	417.20 ± 28.6
Range (Min -Max)	0.80 - 8.20	0.05 - 0.94	1.10 - 35.0	0.09 - 0.85	13 - 50	40 - 100	2.50 - 1485	0.11 - 13.04	9 - 3380

Table 4.6: Difference of plasma analytes in relation with presence or absence of *E. coli* in the faeces of bobby calves.

Descriptive variables	No. of calves	Glucose mmol/L ± SE	BHB mmol/L ± SE	Urea mmol/L ± SE	NEFA mmol/L ± SE	PCV % ± SE	GGT IU/L ± SE	TPP g/L ± SE	CK IU/L ± SE	Lactate mmol/L ± SE
Presence of *E. coli*	102	4.6 ± 0.1	0.18 ± 0.01	5.2 ± 0.4	0.32 ± 0.01	33.1 ± 0.7	444.3 ± 51.3	65.2 ± 1.3	379.8 ± 22.5	5.5 ± 0.2
Absence of *E. coli*	171	4.5 ± 0.1	0.22 ± 0.01	4.6 ± 0.2	0.33 ± 0.01	32.9 ± 0.5	401 ± 33.9	65.7 ± 0.9	399.4 ± 17.9	5.3 ± 0.11
Estimate for difference (95% CI)		0.1 (-0.1, 0.3)	-0.02 (-0.1, 0.0)	0.0 (-0.4,0.4)	0.0 (-0.04,0.03)	0.1 (-1.4, 1.6)	14 (-43, 77)	-0.5 (-3.7, 2.7)	-16 (-53, 21)	0.18 (-0.1, 0.5)
***P*-value**		**0.55**	**0.13**	**0.92**	**0.79**	**0.91**	**0.62**	**0.76**	**0.38**	**0.25**

Table 4.7: Difference of plasma analytes in relation to the presence or absence of *Salmonella* spp. in the faeces of bobby calves.

Descriptive variables	No. of calves	Glucose mmol/L ± SE	BHB mmol/L ± SE	Urea mmol/L ± SE	NEFA mmol/L ± SE	PCV % ± SE	GGT IU/L ± SE	TPP g/L ± SE	CK IU/L ± SE	Lactate mmol/L ± SE
Presence of *Salmonella* spp.	14	4.7 ± 0.3	0.23 ± 0.1	5.2 ± 0.8	0.30 ± 0.04	35.5 ± 1.4	380.9 ± 75.8	65.2 ± 3.5	359.5 ± 51.8	5.8 ± 0.6
Absence of *Salmonella* spp.	259	4.5 ± 0.1	0.20 ± 0.0	4.8 ± 0.2	0.32 ± 0.0	32.9 ± 0.4	419.1 ± 29.8	65.6 ± 0.8	393.8 ± 14.5	5.4 ± 0.1
Estimate for difference (95% CI)		0.2 (-0.5, 0.9)	-0.01 (-0.1, 0.0)	0.1 (-0.9, 1.4)	-0.02 (-0.1,0.01)	2.6 (-0.4, 5.7)	23 (-128, 205)	-0.4 (-8.0, 7.3)	-7 (-97, 76)	0.7 (-0.04, 1.4)
***P*-value**		**0.63**	**0.44**	**0.79**	**0.54**	**0.08**	**0.80**	**0.92**	**0.87**	**0.06**

Table 4.8: Difference of plasma analytes in relation to the presence or absence of BRV in the faeces of bobby calves.

Descriptive variables	No. of calves	Glucose mmol/L ± SE	BHB mmol/L ± SE	Urea mmol/L ± SE	NEFA mmol/L ± SE	PCV % ± SE	GGT IU/L ± SE	TPP g/L ± SE	CK IU/L ± SE	Lactate mmol/L ± SE
Presence of BRV	22	4.2 ± 0.2	0.15 ± 0.02	5.2 ± 0.4	0.28 ± 0.03	34.6 ± 0.9	282 ± 58.3	65.1 ± 2.5	315 ± 22	5.36 ± 0.3
Absence of BRV	251	4.6 ± 0.1	0.21 ± 0.01	4.8 ± 0.2	0.33 ± 0.01	32.9 ± 0.4	429 ± 30.6	65.6 ± 0.8	398.8 ± 15	5.39 ± 0.1
Estimate for difference (95% CI)		0.4 (-0.8, -0.1)	-0.04 (-0.1, -0.0)	0.7 (-0.0, 1.5)	-0.05 (-0.1,0.0)	1.8 (-0.3, 3.8)	-60 (-200, 27)	-0.4 (-5.9, 4.9)	-41 (-103,16)	-0.01(-0.6,0.6)
***P*-value**		**0.03**	**0.07**	**0.05**	**0.05**	**0.09**	**0.21**	**0.86**	**0.16**	**0.97**

Table 4.9: Difference of plasma analytes in relation to the presence or absence of BCV in the faeces of bobby calves.

Descriptive variables	No. of calves	Glucose mmol/L ± SE	BHB mmol/L ± SE	Urea mmol/L ± SE	NEFA mmol/L ± SE	PCV % ± SE	GGT IU/L ± SE	TPP g/L ± SE	CK IU/L ± SE	Lactate mmol/L ± SE
Presence of BCV	07	4.6 ± 0.3	0.22±0.1	4.9 ± 0.7	0.45 ± 0.1	34.3 ± 0.8	535 ± 128	70.3 ± 5.4	445 ± 190	5.1 ± 0.9
Absence of BCV	266	4.5 ± 0.1	0.20 ± 0.01	4.8 ± 0.9	0.32 ± 0.01	32.9 ± 0.4	414.1 ± 29.1	65.4 ± 0.8	390.7 ± 13.6	5.4 ± 0.1
Estimate for difference (95% CI)		0.1 (-0.7, 0.9)	-0.0 (-0.1, 0.1)	0.4 (-0.9,1.8)	0.2 (0.03,0.3)	1 (-3,5)	210.5 (-90,412)	7 (-5,17)	-64 (-225,178)	0.4 (-1.1, 1.5)
***P*-value**		**0.76**	**0.87**	**0.54**	**0.02**	**0.51**	**0.18**	**0.22**	**0.50**	**0.47**

Table 4.10: Linear regression analysis of factors associated with enteric pathogen load in positive samples.

Outcome variable	Explanatory variable	Co-efficient	95% CI	SE	*P*-Value
E. coli K99	Plasma urea	1.16	0.14, 2.18	0.51	0.03
Salmonella spp.	Plasma GGT	-1.49	-2.46, -0.53	0.44	0.01
	TPP	-10.41	-16.56, -4.27	2.82	0.01
BRV	Plasma GGT	-1.78	-3.07, -0.49	0.61	0.01
	TPP	-9.35	-17.86, -0.85	4.08	0.03
	Transport distance	7.11	1.11, 13.12	2.88	0.02
	Plasma glucose	-9.29	-17.19, -1.40	3.79	0.02
BCV	Plasma GGT	-0.34	-0.67, -0.01	0.12	0.04

Only significant explanatory variables for the loads different enteric pathogens are shown. All data were log-transformed.

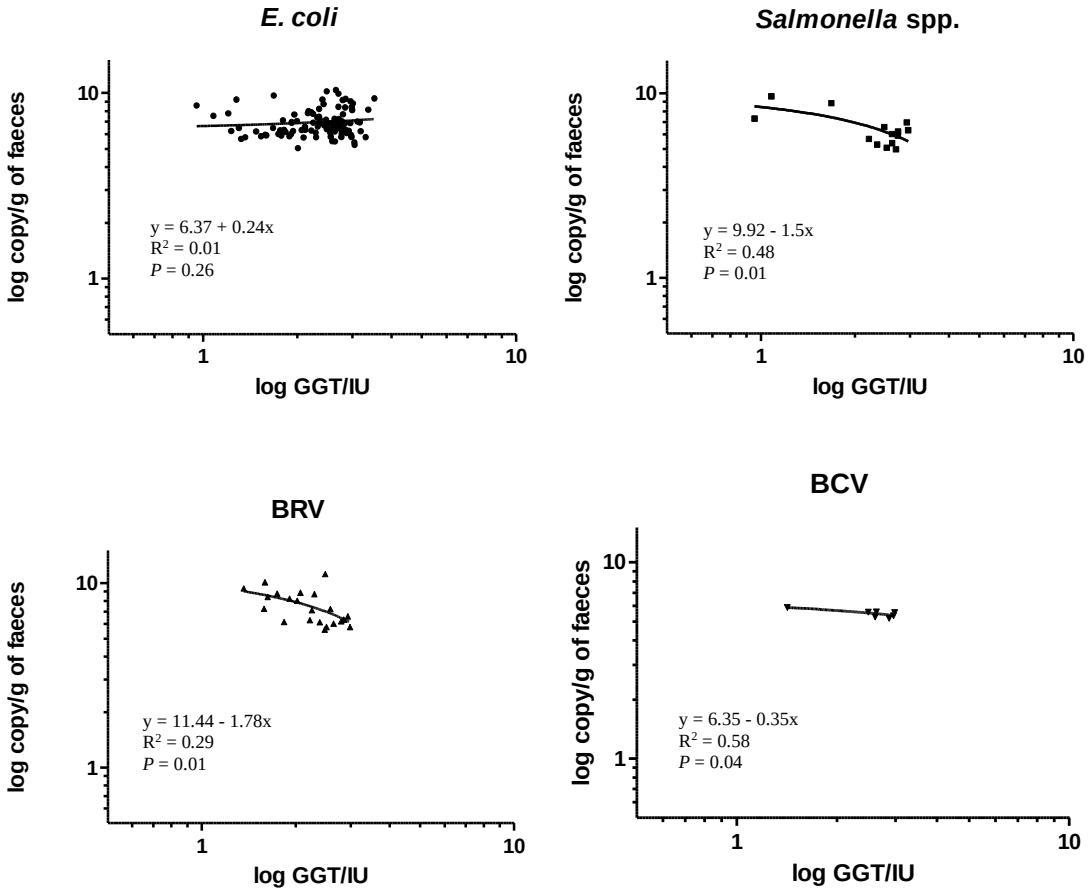

Figure 4.3: Scatter plots showing the negative correlations between log plasma GGT concentrations and log copy number of BRV, BCV and *Salmonella* spp. in positive samples. There was no correlation between log plasma GGT and log copy numbers of *E. coli*.

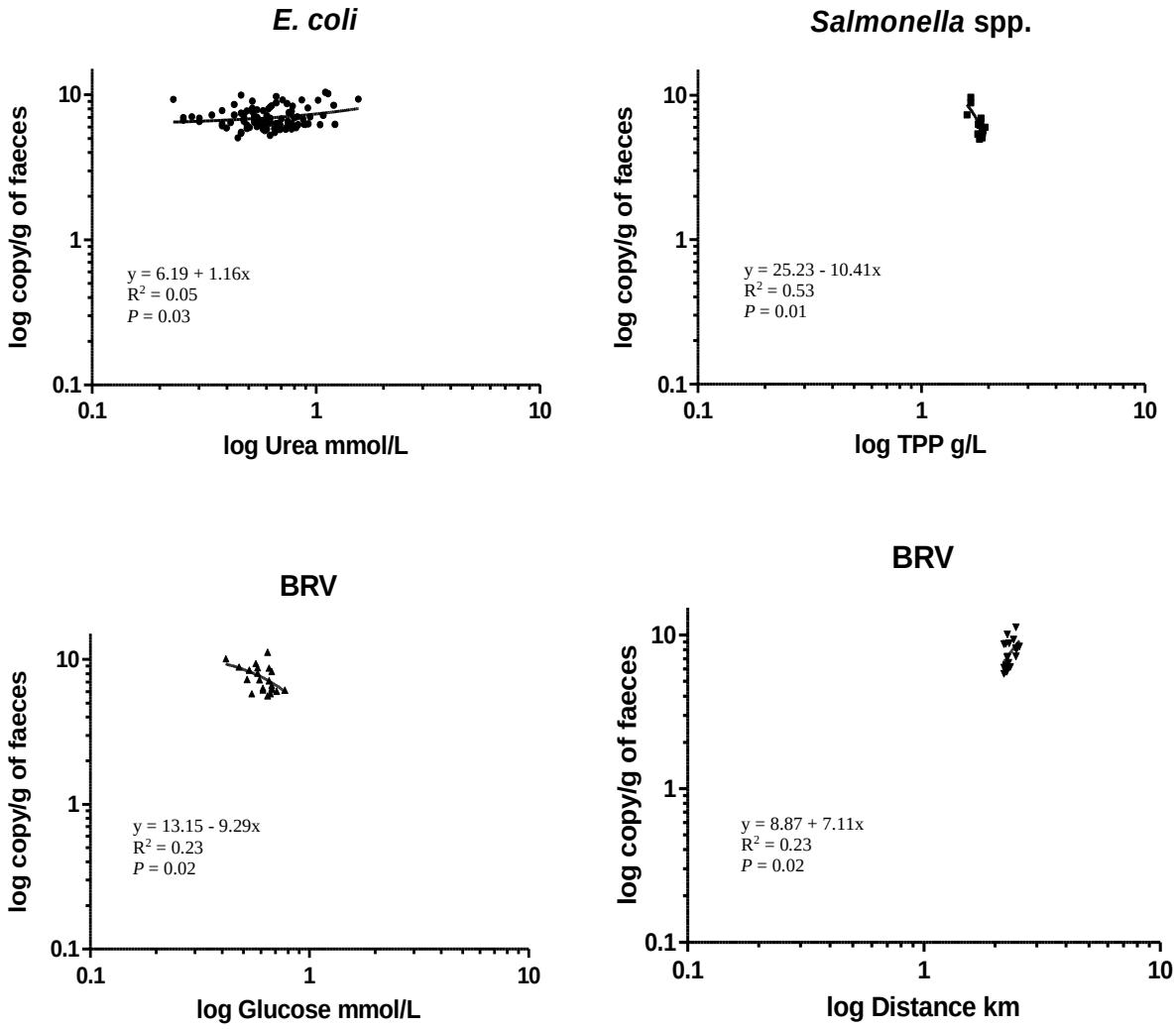

Figure 4.4: Scatter plots showing positive correlation between log plasma urea and log copy number of *E. coli*, and between log distance of transport and log copy number of BRV. In *Salmonella* spp., it shows inverse correlation between log TPP and log copy number, and similar trend between log glucose and log copy number of BRV.

Table 4.11: Results of binary logistic regression analysis to identify the association of transfer of passive immunity and enteric pathogens in the faeces of bobby calves.

Outcome variables	No. of calves	Explanatory variables (Transfer of passive immunity)	OR (95% CI)	*P*-value
Presence of *E. coli* K99	218	Adequate passive transfer (GGT ≥75 IU/L[*]) (Reference group)		
	55	Failure of passive transfer (GGT <75IU/L[*])	0.95 (0.51, 1.75)	0.86
Presence of *Salmonella* spp.	218	Adequate passive transfer (Reference group)		
	55	Failure of passive transfer	1.09 (0.29, 4.03)	0.90
Presence of BRV	218	Adequate passive transfer (Reference group)		
	55	Failure of passive transfer	1.55 (0.58, 4.16)	0.40
Presence of BCV	218	Adequate passive transfer (Reference group)		
	55	Failure of passive transfer	0.65 (0.07, 5.56)	0.68

***** Parish *et al.* 1997

Table 4.12: Binary logistic regression analysis to identify potential categorical variables associated with faecal shedding of enteric pathogens by bobby calves at slaughter

Outcome variable	No. of Calves	Explanatory variable	OR (95% CI)	*P*-value
Presence of		Breed		
E. coli K99	91	Holstein-Friesian (Reference group)		
	21	Jersey	0.78 (0.26, 2.35)	0.69
	51	Cross	1.25 (0.60, 2.62)	0.69
		Sex		
	20	Female (Reference group)		
	253	Male	1.12 (0.43, 2.90)	0.82
		Dairy region		
	85	Northern dairy (Reference group)		
	181	South-eastern dairy	1.53 (0.87, 2.66)	0.12
Presence of		Breed		
Salmonella spp.	91	Holstein-Friesian (Reference group)		
	21	Jersey	*	*
	51	Cross	1.08 (0.24, 4.69)	0.92
		Sex		
	20	Female (Reference group)		
	253	Male	0.26 (0.07, 1.01)	0.08
		Dairy region		
	85	Northern dairy (Reference group)		
	181	South-eastern dairy	6.50 (0.84, 50.53)	0.02
Presence of		Breed		
BRV	91	Holstein-Friesian (Reference group)		
	21	Jersey	1.81 (0.33, 10.04)	0.66
	51	Cross	0.70 (0.13, 3.76)	0.66
		Sex		
	20	Female (Reference group)		
	253	Male	0.77 (0.17, 3.57)	0.74
		Dairy region		
	85	Northern dairy (Reference group)		
	181	South-eastern dairy	1.28 (0.48, 3.39)	0.62
Presence of		Breed		
co-infection (any combination)	91	Holstein-Friesian (Reference group)		
	21	Jersey		
	51	Cross	1.35 (0.29, 6.32)	0.92
		Sex	1.08 (0.12, 10.26)	0.92
	20	Female (Reference group)		
	253	Male	0.41 (0.11, 1.53)	0.22
		Dairy region		
	85	Northern dairy (Reference group)		
	181	South-eastern dairy	**4.58 (1.03, 20.22)**	**0.01**

* Quasi-complete separation of data points due to minimal cell counts, when cells with expected counts less than 5, where it was not possible to observe the events with low probabilities by the binary logistic regression model. For example, in Jersey calves none of them was positive against *Salmonella* spp. which yielded 0 cell count. Similar results were found in BCV. Hence, Fisher's exact test was performed in those analyses (Table 4.13).

Table 4.13: Results of Fisher's exact test analysis to identify potential categorical variable associated with faecal shedding of the enteric pathogens.

Outcome variable	No. of Calves	Explanatory variable	*P*-value
Presence of *Salmonella* spp.		Breed	
	91	Holstein-Friesian (Reference group)	
	21	Jersey	0.58
Presence of BCV			
	91	Holstein-Friesian (Reference group)	
	21	Jersey	1.00
	51	Cross	1.00
		Sex	
	20	Female (Reference group)	
	253	Male	1.00
		Dairy region	
	85	Northern dairy (Reference group)	
	181	South-eastern dairy	0.10

4.5 Discussion

The 16S rRNA gene was amplified as a universal internal control to check the PCR inhibition due to impurities that were not entirely removed by the extraction process from complex faecal materials. The 16S rRNA genes were amplified from a high proportion of samples with low Ct values, indicating that the qPCR assays on most samples were not likely to be inhibited by the faecal extracts and the detection limit of the assays was also low at 100 copies/µl.

The prevalence of excretion of *E. coli* K99 in this study was higher than in a previous study of diarrhoeic calves (Izzo *et al.* 2011). This higher prevalence can be attributed to the age specificity of *E. coli* K99 infection in calves. *E. coli* K99 can only infect calves in the first week after birth (Smith 1965), with several epidemiological studies finding that over 80% of calves with diarrhoea associated with infection with *E. coli* K99 occur in calves less than 4 days of age (Acres *et al.* 1977; Sherwood *et al.* 1983; Acres 1985). Age-related resistance develops rapidly because the intestinal epithelial cells bearing receptors for K99 fimbriae are replaced in the week after birth by cells that lack the

receptor (Runnels *et al.* 1980; Acres 1985). A survey conducted in 2016 by Dairy Australia reported that 77% of bobby calves were transported for slaughter at the minimum allowable age of 5 days (Dairy Australia 2016). A recent study of dairy calf rearing practices on northern Victoria dairy farms found that the mean age of bobby calves at the sale was 5.1 ± 1.2 days (Phipps *et al.* 2018a). Although the exact age of the calves in the study described here could not be determined, these earlier surveys suggest that most of the calves sampled were likely to have been under one week old, and thus would have been susceptible to infection with *E. coli* K99, partially explaining the high prevalence of infection that was detected.

The prevalence of excretion of *Salmonella* spp. detected in the study described here was lower than in a previous study of diarrhoeic calves (Izzo *et al.* 2011). This might be expected, given that the animals sampled in the study described here were apparently healthy and predominantly less than one week of age. Several studies have found that excretion of *Salmonella* spp. in faeces is minimal in healthy calves (Lance *et al.* 1992; Busato *et al.* 1999; Naciri *et al.* 1999). In addition, previous studies have found that infection with *Salmonella* spp. is more common in calves between 10 days and 3 months of age (Fossler *et al.* 2005). However, it is not uncommon for infection to occur in calves as young as four days of age during an outbreak (Anderson *et al.* 2001). The rate of excretion of *Salmonella* spp., although relatively low, could still represent a reducible public health risk if the factors that contribute to a higher likelihood of shedding are able to be better controlled. It was notable that higher levels of passive transfer of immunity reduced the faecal loads of *Salmonella* spp. in infected calves, suggesting that optimising the administration of colostrum to bobby calves on a farm may have an impact on amounts of shedding of this important zoonotic pathogen at slaughter.

The prevalence of excretion of both BRV and BCV detected in the study described here was also lower than in a previous study of diarrhoeic calves (Izzo *et al.* 2011). As discussed above, the apparently healthy status of the calves sampled in the study report here is likely to partially explain the lower rate of detection of viral pathogens. A previous case-control study found a higher prevalence of both BRV and BCV in the faeces of diarrhoeic calves than in healthy calves (Cho *et al.* 2013).

The prevalence of excretion of the four enteric pathogens varied geographically. However, the prevalence of excretion of *E. coli* K99 was high across all regions. The likelihood of infection with more than one pathogen was higher in calves from the south-eastern region than in those from the northern region; however, it is not possible to distinguish whether this resulted from infection on the farm of origin or as a result of commingling with other calves during transportation and lairage. A more controlled study aiming to sample of calves before loading, after loading and before slaughter would help in better understanding the relative contribution of these different potential sources of exposure to infection of bobby calves with enteric pathogens, and thus measures that might be introduced to reduce the risk of infection.

Plasma analytes of bobby calves were measured to detect any associations with excretion of the different pathogens to gain a better understanding of metabolic and immunological factors that may be associated with shedding of enteric pathogens by bobby calves after transport. Plasma urea and glucose were associated with increased levels of shedding of *E. coli* K99 and BRV in calves that were infected. Plasma urea increases during severe shortfalls in energy and decreased plasma glucose is indicative of hypoglycaemia. Both of these parameters could be indicative of a longer period of time off-feed and thus higher levels of stress.

This study has shown that adequate passive transfer of immunity is associated with lower loads of some enteric pathogens in infected bobby calves, but this association was not seen with *E. coli* K99. While colostral antibody against *E. coli* K99 can be protective, it needs to be present in the intestine to bind to the K99 fimbriae, blocking their capacity to bind to their receptors on the intestinal mucosa, thus preventing colonisation (Morris *et al.* 1980; Acres *et al.* 1982). Thus, circulating passively transferred antibody may not reflect ongoing feeding of colostrum or milk containing anti-K99 antibody. In an experimental study, calves fed colostrum 2 h before the challenge were protected against *E. coli* K99 and did not develop diarrhoea, but calves fed 2 to 6 h after challenge did develop diarrhoea (Logan *et al.* 1977). Thus, the ongoing feeding of colostrum is likely to be important in protecting against *E. coli* K99. Other experiments had shown that even early feeding of colostrum was not protective when calves were challenged with large doses of enterotoxigenic *E. coli* (Myers *et al.* 1973; Acres *et al.* 1979; Bellamy and Acres 1979; Nagy 1980; Acres *et al.* 1982). Passively acquired systemic antibody is protective against generalised colisepticaemia, but to offer little to no protection against enterotoxigenic *E. coli* (Logan *et al.* 1974; Logan *et al.* 1977; Smith and Huggins 1979). As bobby calves are destined for slaughter, they may be less likely that to be fed colostrum than replacement heifer calves, but at this stage, there is little information about any differences in the management of sale calves and replacement heifer calves in Victoria.

Only the faecal loads of BRV in infected calves were associated with transport distance. A transported animal is exposed to multiple stressors, which can have adverse effects on immunity and may thus result in increased pathogen shedding. However, this depends on the nature of the pathogens and the intensity of the stress experienced during transportation (Swanson and Morrow-Tesch 2001; Appleby 2008). Studies on

associations between the shedding of enteric pathogens and the distance of transportation are limited. Some studies have found increased rates of shedding of *E. coli* O157 and *Salmonella* spp. after long-distance transport of cattle and pigs transport while others have not (Rajkowski *et al.* 1998; Swanson and Morrow-Tesch 2001; Barham *et al.* 2002; Minihan *et al.* 2003; Arthur *et al.* 2007; Dewell *et al.* 2008; Fegan *et al.* 2009; Aperce *et al.* 2014). In our study calves were transported reasonably short distances and this may have reduced the impact on pathogen shedding.

Although presence or absence of enteric pathogens had no significant influence on plasma analytes; some of the metabolic, immunological and transport parameters were correlated with amounts of pathogen excretion in infected calves suggested that proper management can lower the amounts of pathogen excretion at the end of bobby calve supply chain; however, may not affect on rates of pathogen shedding.

4.6 Conclusions

This study indicates that the prevalence of major enteric pathogens in the faeces of transported bobby calves is low except *E. coli* K99 compared to previously reported prevalence of enteric pathogens in Australian dairy calves. The results also suggest that the main factor influencing amounts of excretion of enteric pathogens in infected bobby calves is the efficacy of transfer of passive immunity, although hypoglycaemia and transport distance can have some effect on amounts of excretion with specific pathogens.

5 Detection of *Mycoplasma bovis* specific antibody by indirect ELISA in the serum of bobby calves from two major dairy regions in Victoria, Australia

5.1 Abstract

A survey was conducted to detect *Mycoplasma bovis* specific antibodies in the sera of bobby calves from two major dairy regions in Victoria. Antibodies were detected using antibody capture ELISA. Sera were assessed for adequate transfer of passive immunity before screening for *M. bovis* specific maternal antibody based on serum GGT level. Sera with failure of passive transfer were also screened as a negative control. All the *M. bovis* positive samples were detected from the sera with adequate passive immunity which indicate *M. bovis* specific antibody was transferred from cows to bobby calves. All the shires of the two dairy regions had *M. bovis* positive dairy herds in varying proportion ranging from 22.2% to 100%. True proportion of positive herds between two dairy regions did not differ much. With adjustment of test sensitivity and specificity, a proportion of 33.3% and 32.9% positive herds against *M. bovis* were detected in the northern and south-eastern dairy region respectively. These results indicate that *M. bovis* is a common pathogen in the major dairy regions in Victoria. This study also suggests that the collection of blood from bobby calves at the abattoir is convenient and could be used as a source of samples for *M. bovis* prevalence and surveillance study in Victoria, Australia.

5.2 Introduction

Mycoplasma bovis (*M. bovis*) belongs to the genus *Mycoplasma* within the class *Mollicute*. This organism plays a vital role in the respiratory diseases, otitis media, and arthritis in young dairy calves and mastitis in adult dairy cattle (Walz *et al.* 1997; Stipkovits *et al.* 2000; Giovannini *et al.* 2013). *M. bovis* has a worldwide distribution and is recognised as the most frequently occurring pathogenic bovine mycoplasma in Europe and the USA (Nicholas *et al.* 2008). *M. bovis* prevalence in dairy herds have been studied by many authors from various countries (Stipkovits *et al.* 2000; Fox *et al.* 2003; Filioussis *et al.* 2007; Radaelli *et al.* 2008; Wilson *et al.* 2009; Fox 2012; Passchyn *et al.* 2012; Soehnlen *et al.* 2012; Giovannini *et al.* 2013; Arede *et al.* 2016); however, widescale studies highlighting the prevalence, risk factors and impact of *M. bovis* in dairy herds in Victoria as well as in Australia is limited although *M. bovis* first isolated back in 1970 from dairy cattle in Australia (Cottew 1970).

Animals sub-clinically infected with *M. bovis* can act as reservoirs and shed the organism sporadically for many months to years (Pitcher and Nicholas 2005). This organism is highly contagious and after infection it can spread via respiratory secretion (Nicholas *et al.* 2002; Maunsell *et al.* 2011), from udder to udder (Jasper *et al.* 1974; Gonzalez *et al.* 1992; Gonzalez and Wilson 2003), in young calves by ingesting infected milk (Bennett and Jasper 1977b; Butler *et al.* 2000) or indirectly from feed, housing or other fomites (Jasper *et al.* 1974; Gonzalez and Wilson 2003). *M. bovis* can also spread by semen in dairy herds (Haapala *et al.* 2018). The cold and humid condition may lead to longer survival of *M. bovis* in the environment (Pfutzner 1984), and the presence of *M. bovis* even reported for months in recycled sandbags (Justice-Allen *et al.* 2010). The most preferred site for colonisation of *M. bovis* in cattle is the upper respiratory tract (Nicholas *et al.* 2002) and mammary gland (Bennett and Jasper 1977a). In those

locations, *M. bovis* persist and shed with or without any clinical sign (Bennett and Jasper 1977a; Punyapornwithaya *et al.* 2010), and shedding of the pathogen can occur for few months to years (Bennett and Jasper 1977b; Biddle *et al.* 2003; Punyapornwithaya *et al.* 2010). Once established where there are cattle of multiple ages, *M. bovis* is difficult to eradicate (Nicholas *et al.* 2008), indicating the importance of knowing the epidemiology of *M. bovis* for effective planning and future control programs.

There are limited data available on the economic impact of *M. bovis* alone or *M. bovis* associated disease (*Mb*AD) in dairy calves and dairy herds in Australia. However, the reports from different countries suggest that on the individual farm level *Mb*AD could result in significant losses due to treatment costs, death and culling, and economically devastating outbreaks with very high morbidity rates and death losses up to 30% (Walz *et al.* 1997; Butler *et al.* 2000; Stipkovits *et al.* 2000). The infection rate of *M. bovis* leading to mastitis is up to 70% of a herd in the USA, which causes a considerable economic loss (Brown *et al.* 1990). For example, in the Iowa state of USA, bulk tank positive herds experienced culling rates of 30-70% cows (Rosengarten and Citti 1999). In Canada, 52% of dairy herds are affected with mastitis caused by *M. bovis* which resulting in roughly 33% culling rate and losses of 6.4 litres milk per cow every day (Rosengarten and Citti 1999). The expense of diagnosis, control measures and premature culling may also contribute a considerable additional economic cost (Maunsell *et al.* 2011). In addition to the financial consequences, *M. bovis* must be considered important from a welfare perspective. *Mycoplasma bovis* associated disease is often chronic. It responds poorly to antibiotic therapy (Nicholas and Ayling 2003). A substantial proportion of calves, heifers and adult cattle in the herd may be infected, and

those affected may experience permanent health issues (Stipkovits *et al.* 2000; Maunsell and Donovan 2009).

Disease due to *M. bovis* can be diagnosed using culture and identification of the organism (Sachse *et al.* 1993), PCR (Ayling *et al.* 1997; Murai *et al.* 2014) or serology (Andrew and Carter 1977; Boothby *et al.* 1981; Rosendal and Martin 1986; Uhaa *et al.* 1990). Of these methods, culture is considered to be the gold standard (Sachse *et al.* 1993). Due to the slow-growing nature of *M. bovis* (Jasper *et al.* 1979; Razin *et al.* 1998) and possibilities of the contamination and interference during culture to isolate (Gonzalez and Wilson 2003) make this method not suitable for the rapid diagnosis. PCR is highly sensitive, specific and fast, but limitation applies to detect subclinical infection due to the absence of *M. bovis* in the samples. Hence, PCR is not preferable in the herd level screening due to false-negative results. On the contrary, serological assay such as antibody capture ELISA can detect subclinical carrier animals due to the presence of *M. bovis* specific antibody in the blood for several months after *M. bovis* infection and this method is also rapid (Nicholas and Ayling 2003). However, limited sensitivity to detect *M. bovis* specific antibody in serum and milk (Wawegama *et al.* 2016) limit their effectiveness in the field condition for herd-level screening and surveillance (Nielsen *et al.* 2015). The recent development of antibody capture ELISA based on a recombinant fragment of the Mycoplasma immunogenic lipase A protein (MilA ELISA) has shown promising results both in experimental and field condition compared to other commercial ELISAs. In a laboratory setting, the sensitivity and specificity of this MilA ELISA was tested comparing positive and negative animals after experimental infection with *M. bovis*. MilA ELISA detected *M. bovis* specific IgG with a sensitivity and specificity of 92.86% and 98.7% respectively (Wawegama *et al.* 2014). The diagnostic sensitivity of this ELISA was also compared with two commercial ELISAs where it

found that the MilA ELISA had good diagnostic sensitivity of 87% compared to BIO K302 ELISA (37%) and BIO K260 ELISA (13%). In the same paper authors also reported a 94.3% diagnostic sensitivity with a 94.4% diagnostic specificity of this ELISA using Bayesian latent class modelling during the screening of *M. bovis* in feedlot cattle (Wawegama *et al.* 2016).

Bulk milk tank testing is an established method to detect *M. bovis* to estimate herd-level prevalence in dairy cattle (Fox *et al.* 2003; Wilson *et al.* 2009; Fox 2012). However, this technique may be somewhat disadvantageous as bulk milk only come from healthy cows and may lead to underestimating the actual prevalence due to not testing the samples from sick animals. Mastitis milk and milk from cows being treated with antibiotics for any disease are withheld from the vat in Australia. So bulk tank vat milk is less likely to include milk from sick cows, and they are the ones most likely to be carrying infections like *M. bovis*. Another limitation is the detection of a lower level of antibody by ELISA using bulk milk samples. Due to the dilution effect in bulk milk samples, *M. bovis* specific antibody might be detected at a low level by ELISA which may lead to a false-negative result. Therefore, an alternate sample to detect *M. bovis* antibody by ELISA needs to be evaluated to screen herd level *M. bovis* prevalence in Australian dairy systems. Blood samples from bobby calves those go for slaughter at an early stage of age might be a sample of choice for detecting maternal *M. bovis* antibody for herd screening.

Two published articles indicate the very high to the very low level of *M. bovis* prevalence in dairy herds in Australia. Ghadersohi *et al.* (1999) estimated *M. bovis* exposure and subsequent mastitis among dairy cattle populations in Victoria and northern Queensland, and reported that *M. bovis* was present in 43% and 62% of

Victorian and North Queensland dairy herds respectively. On the contrary, Morton *et al.* (2014) reported the very low level of 0.09% herd-level prevalence of *M. bovis* in dairy herds in Australia. PCR was used to detect *M. bovis* in both studies which has its limitation for herd-level *M. bovis* prevalence study. Therefore, serological study to detect *M. bovis* antibody by MilA ELISA to assess how common *M. bovis* infection in Victorian dairy herds is an important area of further research.

Collection of blood directly from dairy cattle in farms to screen *M. bovis* for estimating prevalence and surveillance study is challenging in Australian dairy farming systems. Challenges in terms of logistics supports and practicalities as large number of dairy herds and their wide distribution. However, it could be easier to collect an adequate number of blood samples from bobby calves to indirectly measure the *M. bovis* specific maternal antibody for the estimation of herd-level prevalence and routine surveillance study under existing bobby calves' supply chain.

As bobby calves are mostly slaughtered at the age of 5 to 10 days old, it is likely that *M. bovis* antibody will appear in the serum of bobby calves due to ingestion of colostrum from *M. bovis* infected dam. Thus, the presence of *M. bovis* antibody in the sera of bobby calves is indicative of maternal infection.

In Australia, the National Livestock Identification System (NLIS) requires that cattle be identified with an NLIS accredited device. The NLIS allows the traceability of each cattle from the property of birth to slaughter. Each livestock property is allocated for unique Property Identification Code (PIC) by state or Territory governments form which is the part of the NLISID number. Hence, bobby calves are fitted with a white RFID ear tag device before they leave the property of birth. The NLISID number is printed on the outside of the RFID ear tag. The NLISID number is read visually while

the RFID number is read electronically by an electronic reader such as Allflex® RS 420 (Allflex® Livestock Intelligence, Australia). All the NLIS or RFID number are linked to the central database stored by the Department of Economic Development, Jobs, Transport and Resources (DEDJTR). Therefore, retrospective identification of herd origin of a calf is possible under existing NLIS. Annually about 770,000 bobby calves are commercially slaughtered in abattoirs in Australia, of which 63% calves are from Victoria State (Animal Welfare Standards 2011). Bobby calves are mostly sent to few slaughterhouses for slaughter and processing because all the abattoirs are not equipped with infrastructure that supports slaughter facilities of bobby calves. As such, collection of blood samples from bobby calves from a single abattoir may be convenient and economical which represent samples from different herds across Australia.

Accordingly, the first aim of this study was to detect the *M. bovis* specific maternal antibody by MilA ELISA in the sera from bobby calves to assess the potential of MilA ELISA as a diagnostic method and sera of bobby calves as a sample of choice for future herd-level *M. bovis* prevalence study in Australia. Secondly, to assess how common *M. bovis* infection across the dairy herds in Victoria, Australia.

5.3 Materials and methods

5.3.1 Animals and sample collection

Information on animals and the procedure of collection of blood samples from bobby calves from the abattoir have been described previously in section 3.3.1 and 3.3.2.

5.3.2 Identification of the bobby calves

The procedure of identification of bobby calves and obtaining information regarding shire and parish of origin of each calf have been described previously in section 3.3.3.

5.3.3 Measurement of transfer of passive immunity

Serum was analysed for the concentrations of γ-glutamyl transferase (GGT) using a multi-channel biochemical analyser (COBAS INTEGRA® 400 plus, Roche Diagnostics International Ltd., Switzerland) following manufacturer instructions. In brief, 200 µl serum in Eppendorf cup per sample was loaded into the sample rack. Cassettes containing all the required reagents(TRIS 100 mmol/L, Glycylglycine 100 mmol/L, Acetate 1.63 mmol/L, L-γ-glutamyl-3-carboxy-4-nitroanilide 3.7 mmol/L) for the test was also loaded. Based on the absorbance photometry (absorbance at 409 nm), serum GGT was then measured and recorded. Plasma GGT < 75 IU/L was considered a failure of passive transfer based on the previous literature (Parish *et al.* 1997). Calves with adequate passive immunity and without passive immunity were then selected for subsequent ELISA.

5.3.4 Measurement of *M. bovis* specific maternal antibody

Serum samples from calves were tested with the indirect ELISA for detecting *Mycoplasma bovis* specific maternal antibody. Serum samples were run in duplicate for ensuring repeatability and omitting intra-assay variation by in house ELISA previously developed by Wawegama *et al.* (2014). Antigen GST-MilA, a recombinant fusion proteins of glutathione S-transferase and mycoplasma immunogenic lipase A from *M. bovis*, was diluted to 12 µg/ml in a coating buffer and added 100 µl well to well of Nunc Maxisorp plate. The plate was then covered and incubated overnight at 4ºC. The antigen from the plate was then removed and washed with 2X phosphate-buffered saline containing 0.05% [vol/vol] Tween 20 (PBST). The unoccupied sites of the plate wells were then blocked by 150 µl of block buffer per well, covered and incubated for 2h at 37 ºC. During the incubation period, 300 µl test serum was prepared with a diluent buffer in a ratio of 1:300 (serum: diluent). For positive control, 250 µl primary antibody

dilution/well was prepared with *M. bovis* specific calf serum in dilution buffer with a 2-fold dilution series from 1/75 to 1/9600. A 300 µl of 1:300 dilution of the negative calf serum, sera from the experimental calves those were not exposed to *M. bovis* during the MilA ELISA development, was also prepared. The blocking agents from the plate were removed after incubation by washing the plates with 2X PBST. Positive, test and negative sera of 100 µl/well in duplicates were then added. A duplicate blank well was also included in the ELISA plate. The plate then covered and placed on a shaker for 2h at room temperature. Afterwards, the sera were removed, and the plate was washed for 4X with PBST and plate was dried by banging in a clean towel. As a conjugate, HRP-conjugated anti-bovine sheep IgG-heavy and light-chain antibodies (Bethyl Laboratories, Inc.) were diluted at 1:2000 in dilution buffer and added 100 µl/well. The plate was then covered and placed on a shaker for 45 min. After incubation the conjugate then removed, and the plate was washed 4X with PBST. After complete wash, 100 µl/well peroxidase substrate, 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), was added to each well. The plate was then incubated for 7 minutes at room temperature. After incubation, 100 µl/well of 1% SDS was added to stop the reaction. The absorbance of each well was then read using a Hybrid multimode microplate reader (Bio Tek) at a wavelength of 450 nm. The antibody titres for each sample was obtained by comparing the average optical density (OD) of each duplicate pair of samples with the optical density (OD) values of the standard curve generated by positive control samples using DeltaSoft 3 (Bio-metallic, Inc.) software. Antibody concentration of >105 antibody unit (AU) was considered *M. bovis* positive based on the previous literature (Wawegama *et al.* 2016).

5.3.5 Statistical analysis

Animal and herd-level apparent prevalence were calculated by dividing the positive animal or herd by the total number of animal or herd tested respectively. A herd was considered positive when ≥1 animal within the herd was positive. Frequentist approach applied to calculate animal and herd level true prevalence using the formula from Rogan and Gladen, 1978 (Rogan and Gladen 1978) using EpiTools (Sergeant 2018). To calculate herd-level prevalence, herd sensitivity (*HSe*) and herd specificity (*HSp*) were first calculated using the following formulas from Dohoo *et al.* (2009).

$$HSe = 1- (1-AP_{pos})^n$$

$$HSp = Sp^n$$

Where AP_{pos} is the apparent prevalence representing the proportion of disease within infected herds, *Sp* is the diagnostic specificity of the indirect ELISA we used in this study and n is the average number of animals sampled per herd. Wilson's and Blaker's method were used to estimating the confidence intervals for apparent and true prevalence respectively (Sergeant 2018). Pearson's correlation was performed to check the association between the serum GGT and *M. bovis* antibody concentration. $P < 0.05$ was considered statistically significant.

A sample size calculation for animal-level and herd-level prevalence was done by using EpiTools under the section of sample size to estimate true prevalence with an imperfect test (Sergeant 2018) to compare with sample size in this study. During calculation, we accounted field level *Se* and *Sp* of the indirect ELISA which has recently been reported based on *M. bovis* serology in feedlot cattle in Australia using Bayesian latent class modelling (Wawegama *et al.* 2016). Sample size calculation for animal-level prevalence was based on an expected prevalence of 13%, a desired absolute precision of 5% at the

level of 95% confidence interval. For herd-level, the expected prevalence of 1% with 5% precision and 95% confidence interval were considered. Due to lack of animal-level prevalence data on *M. bovis* infection in dairy cattle, expected animal-level prevalence estimate for dairy cattle was obtained from the recent study conducted by Wawegama *et al*. (2016) in feedlot cattle where they reported 13.1% of cattle were seropositive for infection with *M. bovis* on entry into feedlots in Australia. For herd-level, the previous report by Morton *et al*. (2014) was considered where they reported 0.09% herd-level *M. bovis* prevalence in Australia.

5.4 Results

5.4.1 Description of the samples tested

A total of 269 calves with adequate passive immunity were screened for *M. bovis*. They represent 106 calves from 63 dairy farms in the northern dairy region and 163 calves from 82 dairy farms in the south-eastern dairy region, overall 145 dairy herds, in Victoria. As a negative control, 69 calves having a failure of passive immunity were also screened. The number of bobby calves sampled by shires is presented in Figure 5.1. The number of calves tested per herd varies from minimum 1 calf to a maximum of 7 calves. Relative distribution of bobby calves by herds are shown in Figure 5.2.

5.4.2 Required sample size

The required sample size to detect animal-level and herd-level prevalence were 286 and 428 respectively with the absolute precision of 5% at the level of 95% confidence interval.

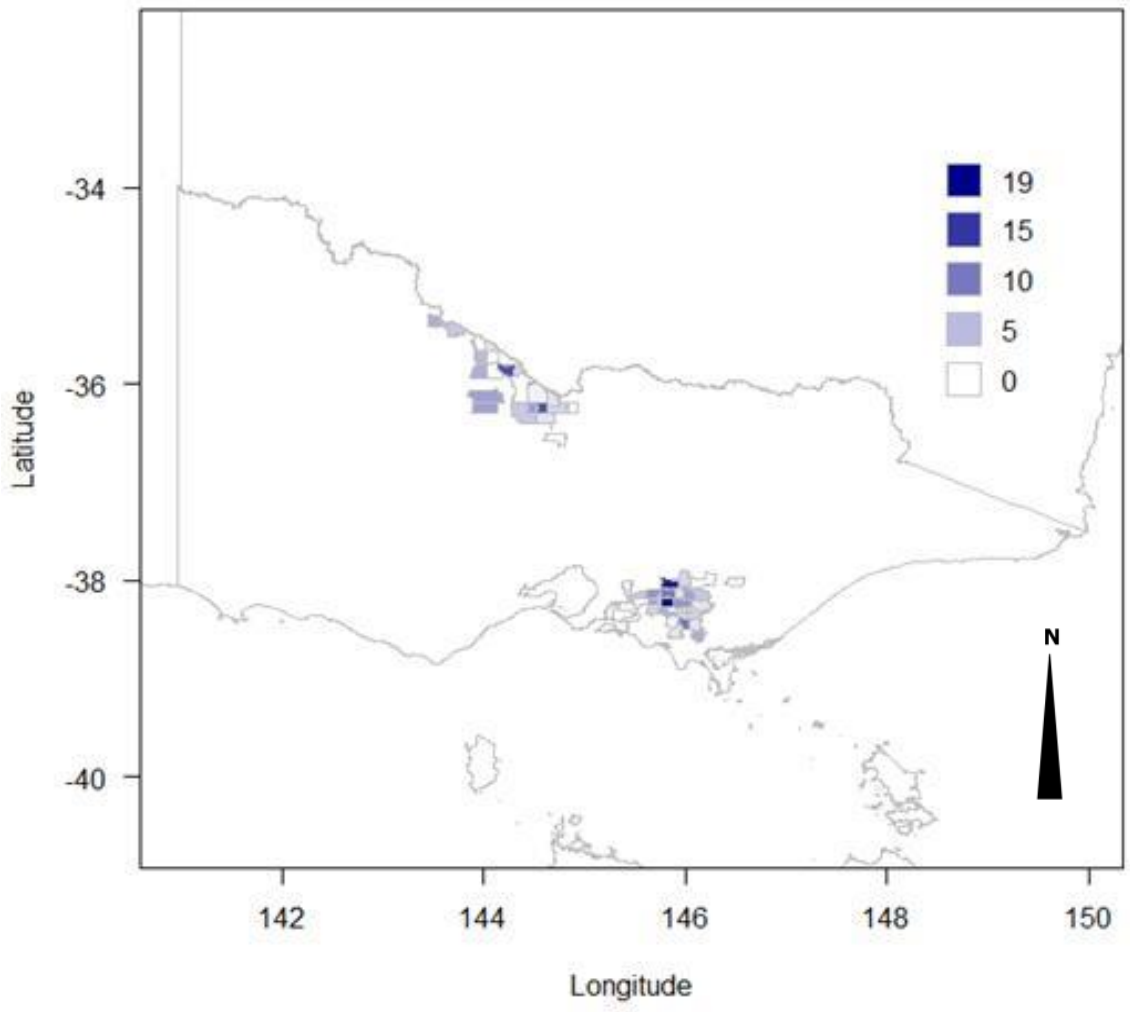

Figure 5.1: The Victorian map showing distribution of sample of bobby calves by shires in north and south eastern dairy regions. The intensity of blue colour is proportionate to the number of calves sampled.

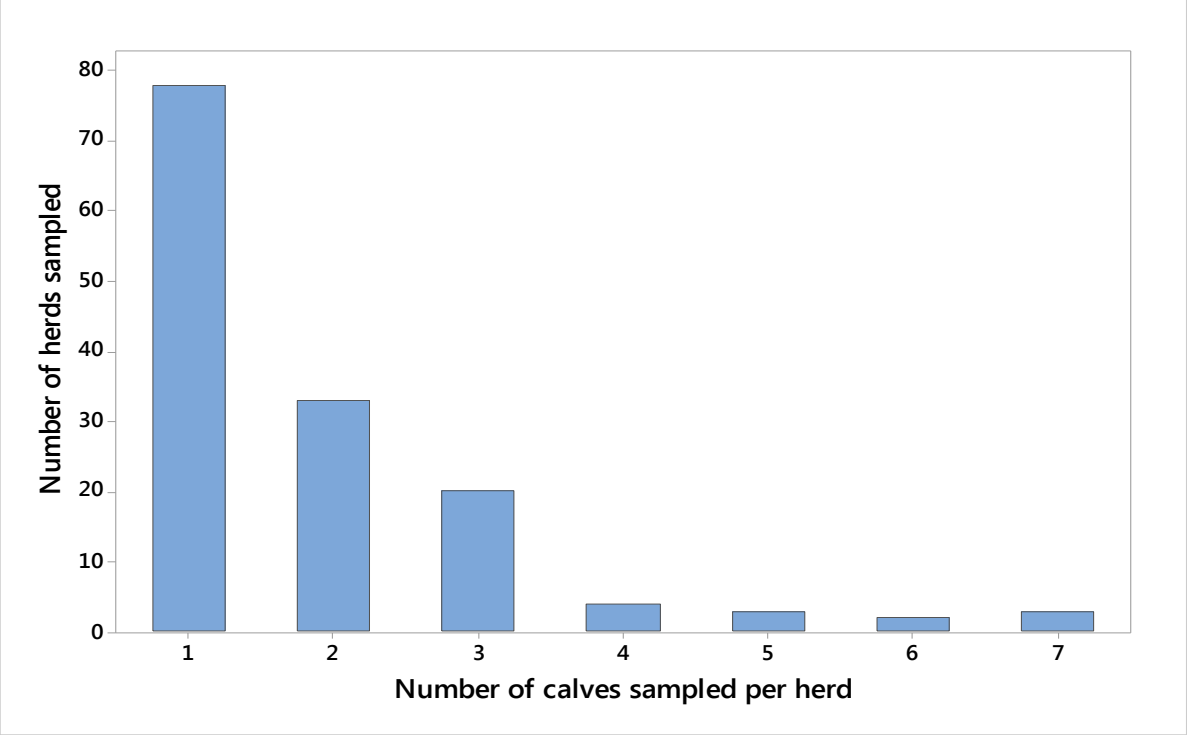

Figure 5.2: Histogram showing number of bobby calves and corresponding number of herds sampled for *M. bovis* antibody detection in bobby calves.

5.4.3 Maternal antibody against *M. bovis* in bobby calf serum

None of the serum samples was positive against *M. bovis* from the calves having a failure of passive transfer. The *M. bovis* antibody concentration in this group of calves was also low compared to calves with adequate passive transfer (Figure 5.3). Positive sera of *M. bovis* specific antibody was only detected from the calves with adequate passive transfer (Figure 5.3). A positive (r = 0.32) and statistically significant (*P* = 0.000) correlation were observed between the serum GGT and *M. bovis* antibody concentration (Figure 5.4).

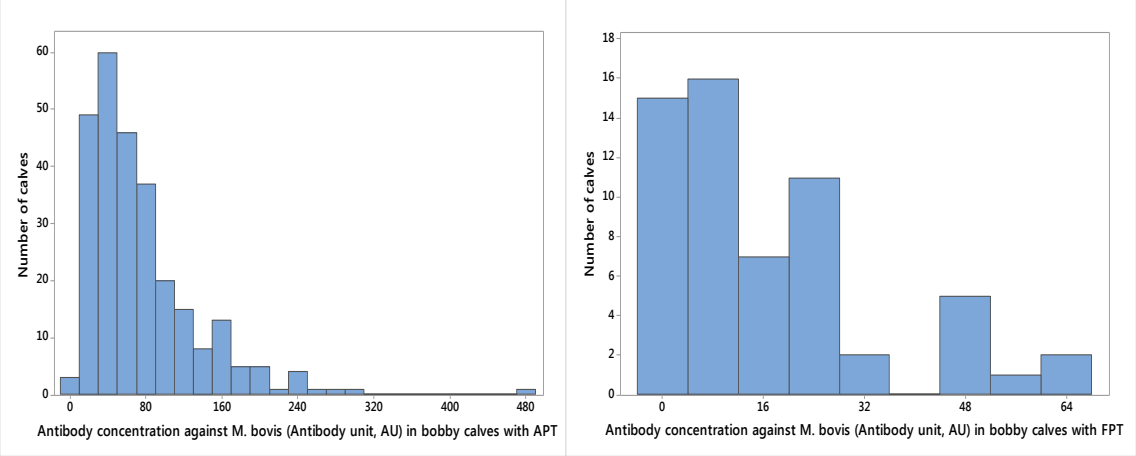

Figure 5.3: Histogram showing *M. bovis* antibody titre in the sera of bobby calves with adequate passive transfer (APT) of immunity (left) and in calves with failure of passive transfer (FPT) of immunity (right). Antibody concentration of >105 antibody unit (AU) was considered *M. bovis* positive.

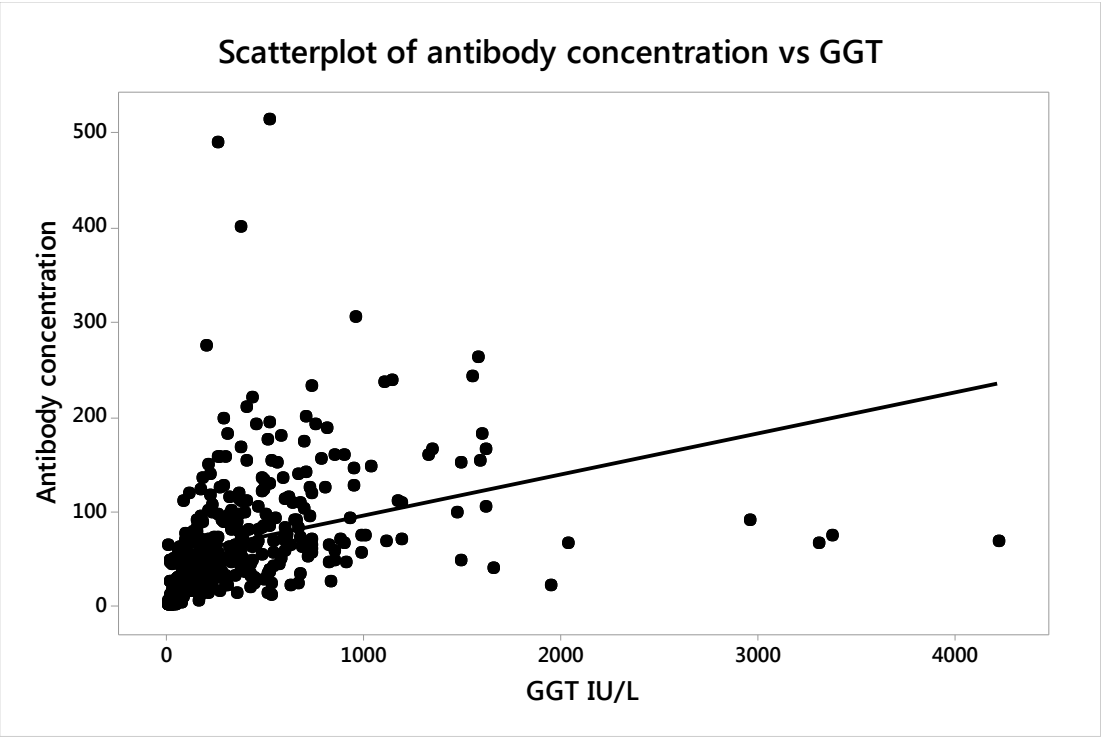

Figure 5.4: Scatter plot showing the positive correlation between serum GGT and *M. bovis* antibody concentration in the sera from bobby calves.

5.4.4 The proportion of *M. bovis* positive dairy cattle and herds in the two dairy regions

All the shires of the two dairy regions had *M. bovis* positive dairy herd in varying proportion ranging from 22.2% to 100% (Table 5.1). Percentage of positive herd between two dairy regions did not differ much. Without adjustment of test sensitivity

and specificity, a proportion of 33.3% and 32.9% positive herd against *M. bovis* were detected in the northern and south-eastern dairy region respectively. A lower percentage of positive *M. bovis* animal was observed compared to herd-level in both overall and region-specific in Victoria, 24.5% in northern dairy and 19.6% in south-eastern dairy without adjustment of the test sensitivity and specificity. This trend has not been changed much after correction of the test characteristics (Table 5.2 & 5.3). Regarding animal-level positive samples, both overall and region-specific animal-level true positive proportion was lower than the apparent proportion of positive *M. bovis*. However, both overall and region-specific herd-level true positive proportion has not been changed much compared to apparent positive proportion after adjusting herd-level sensitivity and specificity (Table 5.2 & 5.3).

Table 5.1: Proportion of *M. bovis* positive herd in different shires of two dairy regions in Victoria, 2015-2016.

Region	Shire/Province	Total number of herds tested	Total number of herds tested positive	% Positive herd (95% CI[a])
Northern dairy	Campaspe	31	07	22.58 (11.40-39.81)
	Gannawarra	20	09	45.00 (25.82-65.79)
	Swan Hill	03	03	100.00 (43.85-100.00)
	Loddon	09	02	22.22 (6.32-54.74)
South-eastern dairy	Baw Baw	52	15	28.85 (18.33-42.27)
	Cardinia	13	06	46.15 (23.21-70.86)
	South-Gippsland	17	06	35.29 (17.31-58.70)

[a] Confidence interval calculated using Wilson's CI

Table 5.2: Results of the apparent proportion of seropositive samples of *M. bovis* by frequentist approach at the herd-level and animal-level in Victoria, 2015-2016.

Level (n)	Apparent proportion	(95% Confidence Interval[a])
Herd-level, overall (145)	0.331	(0.259, 0.411)
Northern dairy (63)	0.333	(0.229, 0.456)
South-eastern dairy (82)	0.329	(0.237, 0.436)
Animal-level, overall (269)	0.215	(0.170, 0.268)
Northern dairy (106)	0.245	(0.173, 0.335)
South-eastern dairy (163)	0.196	(0.142, 0.264)

[a] Confidence interval calculated using Wilson's CI

Table 5.3: Results of true proportion of seropositive samples of *M. bovis* by frequentist approach at the herd-level and animal-level in Victoria, 2015-2016.

Level (n)	True proportion	(95% Confidence Interval[b])
Herd-level, overall (145)	0.335	(0.221, 0.462)
Northern dairy (63)	0.318	(0.163, 0.502)
South-eastern dairy (82)	0.343	(0.192, 0.519)
Animal-level, overall (269)	0.176	(0.125, 0.237)
Northern dairy (106)	0.210	(0.128, 0.312)
South-eastern dairy (163)	0.154	(0.093, 0.231)

[b] Confidence interval calculated using Blaker's CI

5.5 Discussion

The critical findings of this study were the detection of maternal antibody specific to *M. bovis* in the sera of bobby calves by MilA ELISA. Hence, the possibility of using bobby calves as a source of samples for *M. bovis* screening in dairy herds in Victoria. Another finding was that *M. bovis* is a more common pathogen in Victorian dairy herds than previously anticipated.

We tested 59 calf sera from bobby calves having a failure of passive transfer as a negative control, and none of those samples tested positive and overall antibody unit was low. However, calves with adequate passive immunity had higher antibody unit, and all the positive sera were detected from this group of calves. These results indicate that *M. bovis* specific antibody was not generated in bobby calves after infection; rather it passed from dam to calf via colostrum. Gamma-glutamyl transferase is an enzyme

which presents in the colostrum and absorbed through intestine along with immunoglobulin (Parish *et al.* 1997; Wilson *et al.* 1999). We found a statistically significant positive correlation between GGT and *M. bovis* specific antibody in the sera of bobby calves which support that this antibody was absorbed from colostrum. A survey report by Dairy Australia indicates that 77% of bobby calves are slaughtered in less than 1 week old (Dairy Australia 2016). Phipps *et al.* (2018a) also reported in the recent survey of dairy calf management in northern Victoria that the mean age of selling bull calves or excess heifer is 5.1 (SD 1.15) days. Within this limited age, it is not possible to generate *M. bovis* specific antibody after infection in calves. Wawegamma *et al.* (2014) in her experimental infection study showed that it requires about 3 weeks in calves to generate high titre of *M. bovis* specific antibody to detect by indirect ELISA. These all data support that *M. bovis* specific antibody we detected in bobby claves' sera was from cows not from bobby calves after they infected with *M. bovis*. Also, our experience suggests that the collection of blood from bobby calves from killing line is easy and straight forward. Scanning digital RFID from each calf make it easier to identify the property of origin retrospectively which could potentially facilitate to explore herd-level risk factors by case-control study and to limit the spread of the pathogen after detection of the positive herd.

This study confirms that *M. bovis* is circulating in the dairy cattle of the two-dairy regions, north and south-eastern dairy, in Victoria. These results support the previous findings of the *M. bovis* prevalence in Australia with some disagreement about the proportion of *M. bovis* present in dairy herds in Australia with our current results. Ghadersohi *et al.* (1999) reported a prevalence of 43% and 62% in Victoria and northern Queensland dairy herds respectively. However, the accuracy of the results was uncertain due to the lack of information regarding diagnostic sensitivity and specificity

of their PCR assay and the limitation of the amplification of the *M. bovis* specific DNA. The PCR assay they used can amplify the other DNA fragments in addition to *M. bovis* specific DNA (Mansell and Browning 1999). On the contrary, Morton *et al.* (2014) reported the very low herd-level prevalence of 0.9% (95% probability interval 0.1-3.7%) by PCR testing of bulk milk samples of 238 randomly selected dairy herds in Australia. It is not clear the way they calculated the required sample size for the precise estimation of the herd-level prevalence of *M. bovis*. The PCR assay they used had the sensitivity of 76% (95% probability interval 34-98%) (Morton *et al.* 2014). Considering small sample size, PCR sensitivity of 76% and sub-clinical nature of *M. bovis*, which limit *M. bovis* detection by PCR, might have been contributed to low detection in their study. Worldwide *M. bovis* prevalence varies widely in dairy herds. In the USA, 7 to 20 % dairy herds were positive based on bulk tank milk sample testing reported in different periods in different regions by various authors (Fox *et al.* 2003; Wilson *et al.* 2009; Fox 2012). In Europe, the estimated prevalence has been reported between 1.5% and 5.4% (Filioussis *et al.* 2007; Passchyn *et al.* 2012; Arede *et al.* 2016). Before considering these comparisons with our current findings; it is important to note that different study populations, design of the studies and assay to detect *M. bovis* with its test characteristics have an ultimate effect on the final estimate.

An increased proportion of *M. bovis* positive samples detected in the herd-level compared to animal-level in our study was probably because of the fewer samples size screened in the herd-level compared to animal-level. The other reason might be that we declared a herd is positive even if a single calf within a herd was positive which might have increased the herd-level positive percentage. Within the dairy region across all the shires had positive herd, and the proportion of positive samples detected in herd-level and animal-level between two dairy regions were close. These indicate that *M. bovis* in

dairy cattle is widespread in these two-dairy regions instead clustered in a specific area. This wide distribution of *M. bovis* across the shires in two dairy regions in Victoria is not surprising because of the infection dynamics and transmission epidemiology of these infectious agents. The previous study highlighted that cattle could be sub-clinically infected and act as a reservoir which facilitates the shedding of the pathogen sporadically from many months to years (Pitcher and Nicholas 2005). Chronic infection without any symptoms makes it easier for *M. bovis* to persist within the herd and introduction into a naïve population. Once established in a herd, *M. bovis* can easily transmit from infected to uninfected cattle and very difficult to eradicate due to subclinical nature of infection and presence of different age category of cattle in the same herd (Nicholas *et al.* 2008; Maunsell and Donovan 2009; Maunsell *et al.* 2011). The indirect ELISA we used had been validated in the laboratory and filed condition with good sensitivity and specificity compared to other commercial ELISA kits (Wawegama *et al.* 2014; Wawegama *et al.* 2016). The excellent test characteristics of MilA ELISA ensure the good accuracy of our results and valid estimation of the proportion of positive samples we found.

A major limitation of this study was the limited sample size within the herd and number of herds screened which limited the precise and accurate estimation of the herd-level prevalence in the north and south-eastern dairy regions in Victoria. However, information about the existing colostrum management practice in the dairy herds in Victoria might have been advantageous in our case. In a report of colostrum management in south-west Victorian dairy herds, around 64% pooled colostrum was given to calves (Vogels *et al.* 2013). In another report in northern dairy around one-third of the herds pooled raw colostrum from multiple cows (Phipps *et al.* 2018b). Accordingly, some of the calves sampled in this study might have been fed pooled

colostrum which means that testing a calf's serum represents many dairy cows. However, this is our observation based on the current colostrum management system in Victoria where we did not assess what colostrum management was considered for the bobby calves during this study.

We also went for convenience random sampling rather than systematic multistage sampling due to the limitation of the allocated resources for this survey. Due to the lack of required sample size, this would be over ambitious to infer in the population-level prevalence of *M. bovis* based on our preliminary results from this single sero-survey. However, within this limited samples, this study confirms the presence of *M. bovis* with a range of percentages of positive *M. bovis* samples in animal and herd level having uncertainty with exact prevalence in the two dairy regions in Victoria. These findings highlight the widespread distribution of the circulating *M. bovis* in dairy cattle and herds in Victoria and warrant further investigation of the accurate estimation of the prevalence of *M. bovis* in Victoria as well as other states in Australia and identification of the probable risk factors to limit the spread of this pathogen.

5.6 Conclusion

This study concludes that sera from bobby calves could be a potential source of samples to estimate the herd-level *M. bovis* prevalence and surveillance study by MilA ELISA to control this infectious agent in dairy cattle herds in Victoria. The results also suggest that *M. bovis* in the dairy cattle of the northern and south-eastern dairy regions is common and widespread.

6 General Discussion

This PhD project was entirely focused on to assess the health and welfare of bobby calves in commercial dairy practice in Victoria, Australia and assessment of blood samples from bobby calves as a sample of choice for the identification of *M. bovis* in dairy herds in Victoria for future large-scale prevalence and surveillance study.

Good management practices and maintaining adequate welfare in bobby calves across the bobby calf supply chain have been recognised as a priority area for the sustainable dairy industry in Australia by the industry, dairy farmers and the general public. Accordingly, in the first study (Chapter 3) we looked at the physiology of bobby calves after transportation to the commercial slaughterhouse and assessed their plasma analytes indicative of welfare status. We also analysed the management factors in parallel with biochemical data to find out possible risk factors of poor welfare before their slaughter. Enteric pathogens those are responsible for enteritis in neonatal calf are undoubtedly essential factors for deteriorating health in young calves and some of them also responsible for foodborne diseases. Therefore, in the second study (Chapter 4) we looked at the prevalence and amounts of enteric pathogen shedding in bobby calves at the end of the supply chain and their associated risk factors. A considerable number of bobby calves are sent for slaughter each year in few abattoirs in Australia. Collection of blood samples from bobby calves in the slaughterhouse is convenient which tempted us to investigate whether we could use their blood samples to identify *M. bovis* antibody of maternal origin to detect herd level *M. bovis* in Victoria which is a significant area of concern for the dairy industry and farmers nowadays in Australia. We, therefore, in the third study (Chapter 5) measured *M. bovis* antibody in the serum from booby calves by indirect MilA ELISA to assess the potential of this ELISA and bobby calves as a source of samples for larger-scale herd level *M. bovis* prevalence and surveillance study in

Australia. We have presented the results and discussed accordingly in this thesis. However, some of the explicit messages that we have found from this project are worthy for further general discussion.

6.1 Poor welfare indicators observed in bobby calves in our study

Some of the physiological variables indicate that the welfare of bobby calves was compromised in the existing management systems.

6.1.1 Muscular bruising or fatigue

A higher level of plasma CK and lactate compared to previously published reference range have been observed in most of the bobby calves during our study indicate that bobby calves might have been experienced muscular fatigue before being slaughtered. However, from our data set, we could not ascertain the exact risk factors of muscular fatigue in bobby calves. Transport distance and duration were not correlated with CK. We assume strenuous muscular activity during truck movement, handling during loading and unloading, and transporting of bobby calves in the trucks without bedding materials in the floor result in muscular trauma or bruises that have been contributed increased CK level in bobby calves.

6.1.2 Failure of passive transfer

Although 77% of calves had an adequate passive transfer of immunity, failure of passive transfer of immunity in one-fourth of the calves during our study indicate that further improvement is required. Gamma-glutamyl transferase and TPP values in bobby calves varied depending on which dairy regions they came from suggesting that colostrum management might not be similar across the dairy farms and regions in Victoria.

6.2 Good welfare indicators observed in bobby calves in our study

Some of the physiological variables indicating that the welfare of bobby calves was adequate at the end of the supply chain.

6.2.1 Energy level

Most of the calves maintained an adequate energy level, and none of the calves exceeded a maximum duration of 30 hours off feed limit according to the Australian Animal Welfare Standards before slaughter. Transport management such as distance and duration did not affect plasma glucose level. Few calves that experienced hypoglycaemia was probably due to a long period of food deprivation or less amount of liquid feed provided; however, we could not determine the actual risk factors with limited data set in this study.

6.2.2 Hydration level

Majority of the bobby calves were well hydrated before slaughter. We could not find any correlation between transport distance and PCV or transport duration and lairage time with PCV. One of the reasons for optimum hydration level in bobby calves at the abattoir might be access to enough drinking water in the lairage facility.

6.2.3 The lower prevalence of major enteric pathogens in bobby calves

Infection with enteric pathogens leading to diarrhoea is one of the critical health and welfare problems at the early age of calves. We have found in our study that presence of major enteric pathogens those are responsible for enteritis are low, except *E. coli* K99, in bobby calves compared to previously published Australia wide study on the enteric pathogen in calves during a diarrhoeal outbreak. This message ensures that although bobby calves go for slaughter at an early age having a lot of stressful events, they are not shedding enteric pathogens at an alarming situation. Under the existing management

system, bobby calves are not vulnerable to get exposed and infection with enteric pathogens, and subsequent excretion. However, from our study, we have found that few factors might increase the higher amount of the enteric pathogen excretion in the faeces in the infected bobby calves and should be considered in future bobby calf management strategy to lower a load of enteric pathogens in the faeces.

6.2.4 Factors that increased the amount of enteric pathogen shedding

Lower acquisition of passive immunity is an apparent risk factors of increased amounts of enteric pathogen shedding in the faeces of infected bobby calves. Reasonable on-farm colostrum management might prevent a higher load of pathogen excretion through faeces in infected calves. From our study, we found that *Salmonella* spp., BRV and BCV had a lower copy number per gram of faeces when calves had a higher acquisition of passive immunity. This finding suggests that boosting passive immunity by on-farm proper colostrum management for bobby calves must not be forgotten by the dairy farmers although bobby calves are slaughtered mostly at one week of age.

6.2.5 Factors that did not affect enteric pathogen shedding

Transport factors such as the higher distance of transportation did not affect increased enteric pathogen shedding except bovine rotavirus. In our study, bobby calves were transported shorter distance from farm to abattoir which might not be stressful enough to shed higher enteric pathogens in faeces. This result highlights that bobby calves those come from Victorian dairy farms and are processed within the abattoirs in Victoria are not supposed to be vulnerable for increased enteric pathogen shedding due to transport stress. Also, breed and sex had no significant effect on pathogen prevalence in bobby calves.

6.3 Blood samples from bobby calves could be useful for future *M. bovis* prevalence and surveillance study in Australia

From our research, we can confidently say that *M. bovis* is not an uncommon pathogen in the dairy herds in Victoria. As we analysed a relatively small number of samples without farm-specific management information, a more comprehensive scale of prevalence study with farm-level data is required to understand the epidemiology of this pathogen under Australian dairy farming systems to control this pathogen in future. The other important messages to the dairy industry and researcher are that MilA ELISA is a useful diagnostic tool to detect maternal *M. bovis* antibody in the serum of bobby calf. Blood sample from bobby calves is easy to collect, economical and convenient. A large number of blood samples is possible to collect from the bobby calves in the killing line of the abattoir within a shorter duration of time. Due to NLIS, it is easy to trace back property location and other important animal and herd-level data of a sample. Accordingly, we recommend that blood samples from bobby calves from the abattoir is a good option for sampling for the larger scale prevalence and surveillance study of *M. bovis* in Australia.

6.4 Summary

The key conclusion of this study is:

- Under existing management systems in Victoria, bobby calves are well managed from property of origin to abattoir without unduly compromising their welfare. Possible welfare issue is muscular fatigue or bruising, which needs to be addressed in future.
- Excretion of major enteric pathogens is minimal except *E. coli* K99. Better acquisition of passive immunity may play an important role in lowering pathogen load in faeces in infected calves.

- *Mycoplasma bovis* is not uncommon in Victorian dairy herds. Bobby calf blood sample can be used to detect the maternal antibody against *M. bovis* using MilA ELISA for the prevalence and surveillance study.

6.5 Limitations and future direction

In our studies, some of the results may not reflect accurate information due to lack of data.

In chapter 3, we investigated the welfare status of bobby calves at the end of the supply chain by examining samples from bobby calves from one commercial abattoir. One of the limitations of this study was that we did not have on-farm control bobby calves to compare the plasma analytes between on-farm bobby calves with the calves those were slaughtered after transportation. We compared the plasma analytes of bobby calves to the normal reference range of young stage of calves which might not be similar to the normal reference range of bobby calves under Victorian dairy farming systems. Also, from the NLIS database, we only got the pickup time and slaughter time of each calf. As a result, we could not determine the exact duration of transport from farm to the abattoir and also the lairage duration to investigate precisely how the duration of transport or lairage time separately influenced on the physiology of bobby calves after transportation. Furthermore, in this study, we also could not determine the exact duration of time off feed in bobby calves before slaughter due to lack of data on the last feeding time of each calf. Hence, the analysis we have done based on tentative time off feed duration needs cautious interpretation. Although this study had some limitations, this is the first time we now know the baseline information of the welfare status of bobby calves after commercial transport in Victoria. In future, a progressive cohort study with following bobby calves from farm to abattoir is recommended to eliminate these limitations and to identify potential welfare risk factors accurately. Behaviour,

physical examination and mortality data need to be assessed in addition to examining physiological status of bobby calves to evaluate their welfare and possible risk factors in commercial practice. Risk factors of elevated CK and lactate during commercial bobby calf transportation need to be addressed. Specifically, the loading and unloading, stocking density and bedding material in the truck during commercial transportation and their effect on blood CK and lactate would improve our understanding of the cause of high CK and lactate in bobby calves after transport. On-farm survey to understand the potential farm management factors that might lead to poor welfare in bobby calves also needs to be addressed in future. The broader scale of the study and periodic monitoring by the industry are also recommended to ensure bobby calf welfare and demonstrate their welfare performance for the sustainable dairy industry.

In chapter 4 and 5, we only assessed limited samples to estimate the prevalence of enteric pathogens in bobby calves and *M. bovis* in dairy herds respectively. Although these studies give us some ideas on the prevalence of enteric pathogens and *M. bovis* in dairy herds in Victoria, the results do not reflect the exact prevalence status. We also did not have farm-level data to identify potential farm management factors leading to pathogen prevalence in our studies. Hence, future studies with adequate sample size having farm management data are required to determine the prevalence of enteric pathogens in bobby calves and *M. bovis* in dairy herds, and to identify potential farm-level risk factors to control these pathogens.

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Appendix 1: Solutions and reagents used in chapter 3

Glucose measurement:

Test principle:

Enzymatic reference method with hexokinase. Hexokinase (HK) catalyses the phosphorylation of glucose by ATP to form glucose-6-phosphate and ADP. A second enzyme, glucose-6-phosphate dehydrogenase (G6PDH) is used to catalyse the oxidation of glucose-6-phosphate by $NADP^+$ to form NADPH.

$$\text{D-glucose + ATP} \xrightarrow{\text{HK}} \text{D-glucose-6-phosphate + ADP}$$

$$\text{D-glucose-6-phosphate + } NADP^+ \xrightarrow{\text{G6PDH}} \text{D-6-phosphogluconate + NADPH + } H^+$$

The concentration of the NADPH formed is directly proportional to the glucose concentrations. Thus, glucose is determined by measuring the increase in absorbance at 340 nm.

Reagents and working solutions:

Components	Concentrations			
	R1	R2 = SR	Test	Unit
TRIS	100		74	mmol/L
ATP	1.7		1.3	mmol/L
Mg^{++}	4	4	3.5	mmol/L
NADP	1		0.7	mmol/L
HEPES		30	4.5	mmol/L
HK (yeast)		≥ 130	≥ 19	µKat/L
G6PDH (microbial)		≥ 250	≥ 37	µKat/L
p^H	7.8	7.0	7.8	

Pipetting parameters:

Components		Diluent (H_2O)
R1	**150 µl**	
Serum/ plasma	**2 µl**	20 µl
SR	**30 µl**	
Total volume	**202 µl**	

NEFA measurement:

Test principle:

$$\text{NEFA} + \text{ATP} + \text{CoA} \xrightarrow{\text{Acyl CoA Synthetase}} \text{Acyl CoA} + \text{AMP} + \text{PPi}$$

$$\text{Acyl CoA} + O_2 \xrightarrow{\text{Acyl CoA Oxidase}} \text{2, 3, - trans-Enoyl-CoA} + H_2O_2$$

$$2H_2O_2 + \text{TOOS} + \text{4-AAP} \xrightarrow{\text{Peroxidase}} \text{purple adduct} + 4H_2O$$

(4-AAP = 4-aminoantipyrine

TOOS = N-ethyl-N-(2hydroxy-3-sulphopropyl) m-toluidine)

The concentration of NEFA in the sample is determined by measuring the absorbance at 552 nm.

Reagents and working solutions:

Contents	Initial Concentration of Solutions	
R1a.	**Buffer**	
	Phosphate buffer	0.04 mol/L, pH 6.9
	Magnesium Chloride	3 mmol/L
	Surfactant	
R1b.	**Enzyme/ Coenzymes**	
	Acyl Coenzyme A Synthetase	≥ 0.3 U/ml
	Ascorbate oxidase	≥ 1.5 U/ml
	Coenzyme A	0.9 mmol/L
	ATP	5.0 mmol/L
	4-aminoantipyrine	1.5 mmol/L
R2a.	**Enzyme Diluent**	
	Phenoxyethanol	0.3% (w/v)
	Surfactant	
R2b.	**Maleimide**	10.6 mmol/L
R2c.	**Enzyme Reagent**	
	Acyl Coenzyme A Oxidase	≥ 10 U/ml
	Peroxidase	7.5 U/ml
	TOOS	1.2 mmol/L

Pipetting parameters:

Components		Diluent (H_2O)
R1	**100 µl**	10 µl
Serum/ plasma	**5 µl**	20 µl
SR	**80 µl**	10
Total volume	**225 µl**	

β-Hydroxybutyrate measurement:

Test principle:

D-hydroxybutyrate + NAD^+ $\xrightarrow{\text{3-hydroxybutyrate dehydrogenase}}$ Acetoacetate + H^+ + NADH

The concentration of β-hydroxybutyrate in the sample is determined by measuring the absorbance at 340 nm.

Reagents and working solutions:

Contents	Initial Concentration of Solutions	
R1a.	**Buffer**	
	Tris buffer	100 mmol/L, pH 8.5
	EDTA	2 mmol/L
	Oxalic acid	20 mmol/L
R1b.	**Enzyme/ Coenzymes**	
	NAD^+	2.5 mmol/L
	3-HBDH	0.12 U/ml

Pipetting parameters:

Components		Diluent (H_2O)
R1	**125 µl**	10 µl
Serum/ plasma	**4 µl**	20 µl
Total volume	**159 µl**	

Urea measurement:

Test principle:

Kinetic test with urease and glutamate dehydrogenase (GLDH).

Urea is hydrolysed by urease and from ammonium and carbonate. In the subsequent reaction, 2-oxoglutarate reacts with ammonium in the presence of GLDH and the coenzyme NADH to produce L-glutamate. In the second reaction, 2 moles of NADH are oxidised to NAD for each mole of urea hydrolysed. The rate of decrease in the NADH concentration is directly proportional to the urea concentration in the sample. The level of urea in the sample is then determined by measuring the absorbance at 340 nm.

$$\text{Urea} + 2\,H_2O \xrightarrow{\text{Urease}} 2NH_4^+ + CO_3^{2-}$$

$$NH_4^+ + \text{2-oxoglutarate} + \text{NADH} \xrightarrow{\text{GLDH}} \text{L-glutamate} + NAD^+ + H_2O$$

Reagents and working solutions:

Components	Concentrations		
	R	Test	Unit
TRIS	220	45	mmol/L
2-oxoglutarate	73	15	mmol/L
NADH	2.5	0.5	mmol/L
ADP	6.5	1.4	mmol/L
Urease	≥ 300	≥ 61	µKat/L
GLDH	≥ 80	≥ 16	µKat/L
p^H	8.6	8.6	

Pipetting parameters:

Components		Diluent (H_2O)
R	**50 µl**	95 µl
Serum/ plasma	**2 µl**	98 µl
Total volume	**245 µl**	

γ-Glutamyltransferase measurement:

Test principle:

Enzymatic colorimetric assay.

Gamma-glutamyltransferase transfers the γ-glutamyl group of L-γ- glutamyl-3-carboxy-4-nitroanilide to glycylglycine.

L-γ- glutamyl-3-carboxy-4-nitroanilide + glycylglycine $\xrightarrow{GGT}$ L-γ- glutamyl-glycylglycine + 5-amino-2-nitrobenzoate

The amount of 5-amino-2-nitrobenzoate released is proportional to the GGT activity in the sample. GGT thus determined by measuring the increase in absorbance at 409 nm.

Reagents and working solutions:

Components	Concentrations			
	R1	R2 = SR	Test	Unit
TRIS	492		100	mmol/L
Glycylglycine	492		100	mmol/L
Acetate		10	1.63	mmol/L
L-γ- glutamyl-3-carboxy-4-nitroanilide		22.5	3.7	mmol/L
p^H	8.25	4.5		

Pipetting parameters:

Components		Diluent (H_2O)
R1	**25 µl**	35 µl
Serum/ plasma	**3 µl**	20 µl
SR	**20 µl**	20
Total volume	**123 µl**	

Total protein measurement:

Test principle:

Colorimetric assay.

Divalent copper reacts with protein and forms the purple-coloured biuret complex.

$$\text{Protein} + Cu^{2+} \xrightarrow{\text{Alkaline } p^H} \text{Cu-protein complex}$$

Protein is determined by measuring the increase in absorbance at 552 nm.

Reagents and working solutions:

Components	Concentrations			
	R1	R2 = SR	Test	Unit
Sodium hydroxide	400	400	321	mmol/L
Sodium potassium tartrate	89	89	71	mmol/L
Potassium iodide		61	13	mmol/L
Cupric sulphate		24.3	5	mmol/L
p^H	13.4	13.2	13.3	

Pipetting parameters:

Components		Diluent (H_2O)
R1	**90 µl**	0 µl
Serum/ plasma	**2 µl**	28 µl
SR	**32 µl**	0
Total volume	**152 µl**	

Creatine Kinase measurement:

Test principle:

$$\text{Creatine phosphate + ADP} \xrightarrow{\text{CK}} \text{creatine + ATP}$$

$$\text{ATP + D-glucose} \xrightarrow{\text{HK}} \text{ADP + G6P}$$

$$\text{G6P + } NADP^{+} \xrightarrow{\text{G6PDH}} \text{D-6-phosphogluconate + NADPH + } H^{+}$$

The rate of the NADPH formation is directly proportional to the catalytic CK activity. CK thus determined by measuring the increase in absorbance at 340 nm.

Reagents and working solutions:

Components	Concentrations			
	R1	R2 = SR	Test	Unit
Imidazole	58		29	mmol/L
N-Acetylcysteine	40		20	mmol/L
EDTA	3	3	2	mmol/L
AMP	10		5	mmol/L
Diadenosine pentaphosphate	24		12	µmol/L
NADP	9.5		4.8	mmol /L
Mg^{++}	20		10	mmol /L
D-Glucose	40		20	mmol /L
Creatine phosphate		180	30	mmol /L
HK (yeast)		≥ 600	98	µkat/L
G6PDH (microbial)		≥ 600	98	µkat/L
N-Methyldiethanolamine		69	11	mmol /L
ADP		12	2	mmol /L
Sodium azide		0.09	0.015	%
p^H	6.0	9.1	6.6	

Pipetting parameters:

Components		Diluent (H_2O)
R1	**61 µl**	9 µl
Serum/ plasma	**3 µl**	19 µl
SR	**20 µl**	10
Total volume	**122 µl**	

Lactate measurement:

Test principle:

Enzymatic calorimetric method.

L-lactate is oxidised to pyruvate by the specific enzymes lactate oxidase (LOD).

Peroxidase (POD) later generates a coloured dye using the hydrogen peroxidase.

$$\text{L-Lactate} + O_2 \xrightarrow{\text{LOD}} \text{pyruvate} + H_2O_2$$

$$2\ H_2O_2 + \text{H donor} + \text{4-AAP} \xrightarrow{\text{POD}} \text{chromogen} + 2\ H_2O$$

The intensity of the colour formed is proportional to the L-lactate. Thus, lactate is determined by measuring the increase in absorbance at 552 nm.

Reagents and working solutions:

Components	Concentrations			
	R1	R2 = SR	Test	Unit
Hydrogen donor	1.75		1.1	mmol/L
Ascorbate oxidase	501		326	µkat/L
4-aminoantipyrine		5	0.65	mmol/L
Lactate oxidase		251	32.6	µkat/L
Peroxidase		401	52.2	µkat/L

Pipetting parameters:

Components		Diluent (H_2O)
R1	**125 µl**	
Plasma	**2 µl**	20 µl
SR	**25 µl**	20 µl
Total volume	**192 µl**	

Appendix 2: Solutions and reagents used in chapter 4

Agarose gels:

1-1.2% small (35 ml) and large gels (100-120 ml) were made by adding 1-1.2 g of agarose per 100 ml distilled water and microwaved until dissolved. The solution was allowed to cool, and nucleic acid staining solutions were added before gel was poured into moulds and allowed to set.

LB broth:

Tryptone/peptone 8 g

NaCl 2 g

dH2O 800 ml

Sterilise by autoclaving.

LB plate

To make plates add 12 g bacto-agar to the broth, then aseptically dispense 20 ml per Petri dishes. Allow plates to set then dry for a ½ hour before packing.

Antibiotics

Ampicillin- 50 mg/ml in dH2O, filtered to sterilise and stored at -20^{0}C.

2% X-Gal

Dissolve 40 mg X-Gal in N, N'-dimethylformamide to a final volume of 2 ml. Dispense into 500 µl aliquots, and store protected from light at -20^{0}C. The final concentration of X-Gal is 20 mg/ml. The stock solution of X-Gal should be stable for 2-4 months at this temperature.

1M IPTG

IPTG MW = 238.3 g/ mol

So, 2.383 grams dissolved in 10 ml dH2O, filtered and stored at -20^{0}C.

Appendix 3: Solutions and buffers used in chapter 5

Coating buffer (bicarbonate/ carbonate buffer):

Solution 1: 0.1M Na_2CO_3 (can be stored at 4^0C for a long time)

Solution 2: 0.1M $NaHCO_3$ (can be stored at 4^0C for a long time)

Store solutions 1 and 2 at 4^0C to prevent growth. Mix 16 ml of solution 1 and 34 ml of solution 2 and calibrate to p^H 9.6. Once mixed then can be stored for 1 week at 4^0C.

PBST:

0.05% (v/v) Tween 20 in 1xPBS (0.5 ml of Tween 20 in 1L of 1xPBS).

10xPBS:

NaCl 80g

KCl 2g

Na_2HPO_4 11.5g

KH_2PO_4 2g

Makeup to 1 L with dH2O, sterilise by autoclaving.

Blocking buffer 2% BSA

10g Bovine serum albumin (BSA)

Add 1xPBS up to 500 ml

Once fully dissolved, filter through 0.45 µm filter.

Diluent 1% BSA, 0.05% Tween

5g BSA

Add 1xPBS up to 500 ml

Add 0.25 ml Tween-20

Once fully dissolved, filter through 0.45 µm filter.

Note: Blocking and diluent buffers can be stored at -20^{0}C in 50 ml aliquots and thaw overnight at 4^{0}C before use. Freeze again once used at 4^{0}C.