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INVESTIGATION OF THE INFECTIOUS CAUSES OF DIARRHOEA IN AUSTRALIAN THOROUGHBRED FOALS

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ABSTRACT

Diarrhoea is a common disease in foals that is costly and labour intensive to manage. A large number of potential enteric pathogens have been detected in the faeces of foals, however, the role of these infectious agents in causing clinical disease is not clearly understood and their prevalence in Australia is unknown. In addition, timely methods for definitive diagnosis are not readily available for some of these agents. Therefore, this study aimed to develop rapid molecular detection assays to investigate the presence of equine rotaviruses, equine coronaviruses, *Salmonella* spp. and *Clostridium difficile* in Australian thoroughbred foals with and without diarrhoea.

A prospective case control study was conducted on five thoroughbred breeding farms in the Hunter Valley (New South Wales, Australia) during the 2010 breeding season. Faecal samples were collected from age-matched foals with diarrhoea and without diarrhoea from the same farm (age-matched pair). In addition, faeces were collected from foals with diarrhoea from an equine veterinary hospital. All faecal samples were analysed for equine rotaviruses, equine coronaviruses, *Salmonella* spp. and *Clostridium difficile* by quantitative PCR (qPCR) assay. All faecal samples were also cultured for *Salmonella* spp. using selective growth media. Samples positive by qPCR and 15 randomly selected control foal samples negative by qPCR were cultured anaerobically for *Clostridium difficile*.

Faecal samples were collected from 117 pairs of age-matched case control foals and 26 hospitalised foals with diarrhoea. In the age-matched case control foals, equine rotaviruses were the most frequently detected infectious agent (25% case foals, 5% control foals) and the only infectious agent significantly associated with the presence of diarrhoea. In hospitalised foals, *Clostridium difficile* (23%) was the most frequently detected infectious agent. In this investigation co-infections were detected in 4% of matched case foals and 4% of hospital foals, with equine rotaviruses and *Salmonella* spp. being the most frequent combination. Four different *Clostridium difficile* ribotypes were detected, including ribotype 012 and 078. Importantly, this is the first report of the detection of *C. difficile* ribotype 078 in Australian horses. As this ribotype has been associated with severe disease in humans, this finding may have public health

implications. The availability of rapid molecular screening tests for infectious causes of foal diarrhoea enhances the veterinary practitioner's ability to instigate appropriate therapy and control measures in foals with diarrhoea. However, the detection of pathogens in foals without diarrhoea highlights the need for more research into the role some of these pathogens play in clinical disease both individually and in combination.

DECLARATION

This is to certify that:

i. The thesis comprises only my original work towards the PhD except where indicated in the Preface

ii. Due acknowledgement has been made in the text to all other material used

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

PREFACE

I, Kirsten Erin Bailey, declare that I performed all the work described in this thesis, except for the following:

- Foal faecal samples were collected by myself, stud farm staff, hospital veterinary nurses or hospital veterinarians due to the logistics of sampling foals at multiple locations at the same time.
- Anaerobic bacterial culture of *Clostridium difficile* was performed with the assistance and direct supervision of Dr. Kate Mackin, at The Department of Microbiology, Monash University, Victoria, Australia
- Toxinotyping of *Clostridium difficile* isolates was performed by Dr Kate Mackin, Department of Microbiology, Monash University, Victoria, Australia.
- Ribotyping of *Clostridium difficile* isolates was performed by The Department of Pathobiology, Ontario Veterinary College, University of Guelph, Ontario, Canada.

Sample collection was performed in the Hunter Valley in New South Wales and laboratory work was conducted at the Equine Infectious Diseases Laboratory, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne. The work was carried out under the supervision of Professor James Gilkerson, Dr Carol Hartley and Dr Sally Symes. No work was carried out prior to PhD candidature enrolment or submitted for other qualifications. The author was a recipient of an Australian Postdoctoral Award scholarship.

Scientific manuscripts in preparation for publication relevant to this thesis are listed below:

K.E. Bailey, S.J. Symes, K.E. Mackin, J. Rousseau, D. Lyras, J.S. Weese, J.E. Axon, C.M. Russell, C.A. Hartley, G.F. Browning, J.R. Gilkerson. First detection of *Clostridium difficile* Ribotype 078 in Australian Thoroughbred foals. (Manuscript in preparation).

K.E. Bailey, S.J. Symes, C.A. Hartley, G.F. Browning, M. Devlin, J.R. Gilkerson. A prospective age-matched case-control study of infectious causes of diarrhoea in Australian Thoroughbred foals. (Manuscript in preparation).

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K.E. Bailey, S.J. Symes, C.A. Hartley, G.F. Browning, J.M. Devlin, J.R. Gilkerson. Case-control study of infectious foal diarrhoea. Oral presentation - Bain Fallon Memorial lectures, Gold Coast, Australia (2014).

K.E. Bailey, S.J. Symes, C.A. Hartley, G.F. Browning, J.M. Devlin, J.R. Gilkerson. How important is rotavirus as a cause of foal diarrhoea? A case control study of diarrhoea in Australian foals. Oral presentation - British Equine Veterinary Association Congress, Manchester, United Kingdom (2013).

K.E. Bailey, S.J. Symes, C.A. Hartley, G.F. Browning, J.M. Devlin, J.R. Gilkerson. The role of rotavirus in Australian foals: an age matched case control study. Oral presentation - Bain Fallon Memorial lectures, Melbourne, Australia (2013).

K.E. Bailey, S.J. Symes, C.A. Hartley, G.F. Browning, J.M. Devlin, J.R. Gilkerson. A case control study of foal diarrhoea: rotavirus detection by reverse transcription quantitative polymerase chain reaction. Oral presentation - 9th International equine infectious diseases conference, Lexington, Kentucky, USA (2012).

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In Scone, I would like to acknowledge the cooperation and assistance provided by the Scone Equine Hospital, the Hunter Valley Research Centre and the thoroughbred stud farms that participated in the case control study. I would also like to thank Dr. Catherine Chicken for her friendship, encouragement and support that made it possible to keep going during challenging times.

Other external collaborators I would like to acknowledge for providing technical guidance and assistance include: Associate Professor Carl Kirkwood (Murdoch Childrens Research Institute) and Associate Professor Barbara Coulson (The University of Melbourne) for advice on troubleshooting rotavirus RT-PCR genotyping; Professor Dena Lyras and Dr Kate Mackin (Monash University) for assistance with *Clostridium difficile* isolation and performing the *Clostridium difficile* toxinotyping; and Professor Scott Weese and Joyce Rousseau (University of Guelph) for performing the *Clostridium difficile* ribotyping.

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Last, but certainly not least, I would like to thank my parents, Jim and Gail, my sister, Lauren, my amazing husband, Andrés and our son Thomas. It has been a long journey to complete this work, thank-you all for your patience and support, I am extremely grateful and so lucky to have you all in my life.

ABBREVIATIONS

aa	amino acid
ANOVA	analysis of variance
BCoV	bovine coronavirus
bp	base pairs
C. difficile	*Clostridium difficile*
cDNA	complementary DNA
CDS	calibrated dichotomous sensitivity
CDT	binary toxin
cfu	colony forming units
CHEF	contour clamped homogeneous electric fields
CI	confidence interval
CMB	cooked meat medium broth
Ct	cycle threshold
CV	coeffieicent of variation
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphates
EDTA	ethylene diamine tetraacetic acid
EqRV	equine rotavirus
ESP	EDTA –sarcosine-proteinase buffer
F	forward
x *g*	unit of gravity
G	glycoprotein
HCl	hydrogen chloride

HIS	hypoxic ischaemic syndrome
IgG	immunoglobulin G
invA	invasion A gene
IPTG	isopropyl-β-d-thiogalactopyranoside
kb	kilo bases
LB	Luria Bertani
LBamp	Luria Bertani broth supplemented with 100 µg/ml ampicillin
M	molar
MCA	MacConkey Agar
$MgCl_2$	Magnesium chloride
min	minute
mm	millimetres
mM	millimolar
MPU	Media Preparation Unit
NSP3	non structural protein 3
NTC	no template control
NVP3	non-structural viral protein 3
ORF	open reading frame
P	protease sensitive
PaLoc	pathogenicity locus
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFGE	pulse field gel electrophoresis
qPCR	quantitative polymerase chain reaction
R	reverse
RFLP	restriction fragment length polymorphism

RNA	ribonucleic acid
RT	reverse transcription
RT-qPCR	reverse transcription quantitative polymerase chain reaction
SD	standard deviation
SET	sodium chloride EDTA Tris buffer
TAE	Tris-acetate-EDTA buffer
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA buffer
Tm	melting temperature
tpi	triose phosphate isomerase gene
µg	microgram
µl	microlitre
µM	micromolar
VP7	viral protein 7
VP4	viral protein 4
v/v	volume/volume
w/v	weight/volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside
XLD	xylose lysine desoxycholate
yo	year old

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CHAPTER 1 LITERATURE REVIEW

1.1 INTRODUCTION

Diarrhoea is one of the most common clinical complaints in foals (Cohen, 1994). While many cases may be mild and self-limiting, others can prove rapidly fatal. Some may be due to non-infectious causes such as excessive milk intake or incorrect preparation of milk replacer, mechanical irritation of the gastrointestinal tract due to the ingestion of sand, dirt and stable bedding, or hypoxic injury to the gastrointestinal tract due to peri-partum asphyxia syndrome (Magdesian, 2005; Mallicote *et al.*, 2012).

Transient diarrhoea, often named foal heat diarrhoea as it often coincides with the dam's first oestrus period post-partum, may be observed in as many as 50-80% of foals in the first few weeks of life (Kuhl *et al.*, 2011; Masri *et al.*, 1986). Affected foals remain systemically well and do not require treatment. This diarrhoea is thought to be related to changes in the bacterial flora as the foal's gastrointestinal tract matures (Kuhl *et al.*, 2011).

Studies of causes of disease and death in foals have shown the first few weeks of life to be the highest risk period (Cohen, 1994; Galvin and Corley, 2010), with diarrhoea being among the most common infectious diseases reported in neonatal foals (Cohen, 1994; Wohlfender *et al.*, 2009). Diarrhoea is labour intensive and costly to manage, therefore, distinguishing between mild and severe cases of diarrhoea and determining the cause of diarrhoea is of both clinical and economical importance to enable prompt, appropriate therapy and to minimise mortalities. In addition, with antimicrobial resistance increasing and posing a global threat to public health, appropriate use of antimicrobials is paramount.

There are numerous potential bacterial, viral and parasitic enteric pathogens in foals (Table 1-1), however, the clinical significance of many of these infectious agents in foal diarrhoea is poorly understood. Many have been reported in case studies of individual foals or investigations of foal diarrhoea outbreaks, and some are recognised as causes of diarrhoea in other animal species and have been extrapolated to be possible causes of diarrhoea in foals. For example, *E. coli* is one of the most common

causes of diarrhoea in neonatal calves (Izzo *et al.*, 2011), and while it is commonly involved in foals with bacteraemia, it is not recognised as a common primary cause of diarrhoea in foals.

Table 1-1 Potential foal diarrhoea pathogens

Pathogen type	Infectious agent	Reference
Bacterial	*Salmonella* spp.	Edwards (1934)
	Clostridium difficile	Jones *et al.* (1988)
	Clostridium perfringens	Dickie *et al.* (1978)
	Lawsonia intracellularis	Duhamel and Wheeldon (1982); Lavoie *et al.* (2000)
	Escherichia coli	Ike *et al.* (1987); Ward *et al.* (1986)
	Rhodococcus equi	Zink *et al.* (1986)
	Aeromonas hydrophila	Browning *et al.* (1991b)
	Campylobacter jejuni/coli	Atherton and Ricketts (1980)
	Bacteroides fragilis	Myers *et al.* (1987)
	Enterococcus durans	Tzipori *et al.* (1984)
	Actinobacillus spp.	Stewart *et al.* (2002)
	Yersinia enterocolitica	Browning *et al.* (1991b)
Viral	Rotavirus	Flewett *et al.* (1975)
	Coronavirus	Bass and Sharpee (1975)
	Adenovirus	Studdert and Blackney (1982)
	Parvovirus	Baker and Ames (1987)
Protozoal	*Cryptosporidium* spp.	Coleman *et al.* (1989); Fernandez *et al.* (1988)
	Eimeria leuckarti	Dwyer *et al.* (1990b); Reppas and Collins (1995)
	Giardia spp.	Frederick *et al.* (2009)
Helminth	*Strongyloides westeri*	Enigk *et al.* (1974)
	Parascaris equorum	Frederick *et al.* (2009)
	Strongylus spp.	Frederick *et al.* (2009)

There are relatively few in-depth survey studies investigating a range of pathogens in foals in an attempt to determine whether detection of these infectious agents is actually associated with diarrhoea (Browning *et al.*, 1991b; Dwyer *et al.*, 1990b; Frederick *et al.*, 2009; Holland *et al.*, 1991; Netherwood *et al.*, 1996b; Slovis *et al.*, 2014; Traub-Dargatz *et al.*, 1988). Each of these studies investigates a different range of infectious agents, with only rotaviruses and *Salmonella* being common to all. All except two of

these studies were conducted over 20 years ago and relied on traditional diagnostic methods based on direct visualisation, microbiological culture and antigen detection. The uptake of molecular methods based on detection of nucleic acids, such as polymerase chain reaction (PCR) assays, quantitative PCR (qPCR) assays and next generation sequencing in equine veterinary medicine has only occurred in the last few years. Traditional detection methods generally have a lower sensitivity and a slower turnaround time than molecular methods. Bacterial culture from faeces usually relies on enrichment media and/or selective media, and requires several days to obtain results. In a clinical situation, this makes obtaining microbiological results in a timely manner challenging. Effective isolation of some anaerobic bacteria, such as *Clostridium difficile* (*C. difficile*) and *Clostridium perfringens*, requires specific media and specialised laboratory equipment that is not available in many veterinary laboratories. These requirements have possibly limited or prevented the investigation of anaerobic bacteria previously (Holland *et al.*, 1991). Over time, there has also been modification and improvement of bacterial culture methods, which has enabled greater success when isolating these fastidious organisms from clinical samples in recent years.

Identification of viral infectious agents in foal faeces have largely been dependent on electron microscopy (EM) (Browning *et al.*, 1991b; Dwyer *et al.*, 1990b; Frederick *et al.*, 2009; Holland *et al.*, 1991; Traub-Dargatz *et al.*, 1988). This is a valuable diagnostic tool, but one requiring specialised equipment that is generally limited to research institutes. Rotaviruses have been the most common virus investigated in previous studies of foal diarrhoea, likely due to the availability of latex agglutination and enzyme linked immunosorbent assays (ELISA) based on the common VP6 protein for the detection of group A rotaviruses in humans. While one study used an ELISA based on polyclonal antibodies to bovine coronavirus to look for coronaviruses in foals (Browning *et al.*, 1991b), other studies have relied solely upon EM for detection of coronaviruses, and occasionally other viruses. The use of a variety of detection methods also means there is variation in the sensitivity and specificity of these methods.

In addition to the challenges faced in detection of infectious agents in foal diarrhoea studies, the relative prevalence of different infectious agents is not directly comparable both within and between studies. This is due to variation in some studies during the

study period in the infectious agents investigated and the detection methods used. In addition, not all samples collected in individual studies were tested for the same infectious agents. For example, a three-year study of foal diarrhoea in Central Kentucky tested all samples with a latex agglutination test for the detection of rotavirus, and used electron microscopy for detection of other viruses in the first and third year of the study, but not the second (Dwyer *et al.*, 1990b). In addition, only 16% of the samples collected in the first year and 36% of the samples collected in the third year of the study were examined by EM. Parasitologic examinations were only performed in the first two years of the study and in 59% and 20% of samples collected each year, respectively.

All these studies were performed in the northern hemisphere where climatic conditions, management practices and farm sizes differ to those on Australian thoroughbred farms. For example, in a prospective longitudinal study of disease on equine farms in Australia, foal diarrhoea frequency was greater on large farms (mare population >125) compared to small farms (Gilkerson, 2006). In a study of foals with diarrhoea conducted in Washington, USA, the farms were comparatively much smaller, with a range of 5 to 79 foals per farm, and therefore likely represent different selection pressures (Traub-Dargatz *et al.*, 1988). Additionally, mares in Australia generally foal outdoors and the mare and foal pair are then housed outdoors unless there is a medical condition requiring confinement. In the northern hemisphere, it is more common for mares to foal and then be housed in stables. These differences mean extrapolation of the prevalence and importance of potential diarrhoea pathogens in these studies to Australian foals may not be appropriate.

To evaluate whether the detection of an infectious agent in a foal with diarrhoea equated with the cause of disease, most of these studies included foals with and without diarrhoea. The selection of foals without diarrhoea varied between studies. In some studies the healthy animals were sourced from a relatively small proportion of farms in comparison to case foals (Browning *et al.*, 1991b; Slovis *et al.*, 2014). Ideally a control foal represents comparable exposure and risk factors to a case foal except for the presence of the disease of interest. If neonatal foals are at a greater risk of disease and death than older foals, comparison of control foals with case foals of different ages is less than ideal. Only one previous study matched foals by age, however faecal samples were only collected from 19 foals with diarrhoea and 10 age-related control

foals, even though these samples were part of the larger study which collected data on 297 foals, of which 144 foals developed diarrhoea (Traub-Dargatz *et al.*, 1988). This sample size was too small to draw significant conclusion from.

One recent study has used molecular detection methods to investigate infectious agents in foals with gastrointestinal disease and healthy foals (Slovis *et al.*, 2014). This study overcomes some of the limitations in earlier studies, by using the same type of detection method for all infectious agents and testing all samples with the same panel of tests, allowing direct comparison of the prevalence of agents detected within the study. However, some aspects of this study limit the ability to compare the findings to other studies. Firstly, a commercial panel of molecular tests was used, for which the reaction details and primer sequences were not reported, resulting in insufficient experimental detail to enable reproduction of this work. Secondly, the use of the term gastrointestinal disease encompassed foals with ultrasonographic evidence of excessive fluid in the small intestine and/or colon either in the presence or absence of diarrhoea. The inclusion criteria for case foals is therefore broader than other studies. Additionally, healthy foals were mainly sourced from two of 32 farms involved in the study. It is unlikely these foals were exposed to the same range of individual farm environmental and managerial factors as foals with diarrhoea, or in this case, gastrointestinal disease.

1.2 EQUINE ROTAVIRUSES

Rotaviruses are the most common infectious agent detected in foals with diarrhoea, with a frequency of detection of between 20-77% (Browning *et al.*, 1991b; Conner and Darlington, 1980; Dwyer *et al.*, 1990b; Netherwood *et al.*, 1996b). Although rotaviral diarrhoea has a high morbidity in foals, clinical disease is usually self-limiting, however, dehydration may lead to mortalities (Dwyer *et al.*, 1990b). Rotaviruses were first observed in the faeces of a foal with diarrhoea in 1975 in Great Britain (Flewett *et al.*, 1975) and are considered to be ubiquitous in horse populations. The evidence for the widespread nature of rotavirus infection includes the high prevalence of rotavirus antibodies in adult horses (Conner and Darlington, 1980; Eichhorn and Huan-Chun, 1987; Goto *et al.*, 1981; Pearson *et al.*, 1982) and the detection of rotaviruses in horse populations from many countries, including the United Kingdom (Flewett *et al.*, 1975; Strickland *et al.*, 1982), the USA (Kanitz, 1976), Japan (Imagawa *et al.*, 1984),

Australia (Studdert *et al.*, 1978; Tzipori and Walker, 1978), New Zealand (Durham *et al.*, 1979; Schroeder *et al.*, 1983), Germany (Elschner *et al.*, 2005), Italy (Monini *et al.*, 2011), Greece (Ntafis *et al.*, 2010), France (Puyalto-Moussu and Taouji, 2002), the Netherlands (van der Heide *et al.*, 2005), Venezuela (Ciarlet *et al.*, 1994), Argentina (Barrandeguy *et al.*, 1998) and India (Gulati *et al.*, 2009). An inactivated maternal vaccine has been available commercially since the mid 1990s (Powell *et al.*, 1997).

Rotaviruses belong to the family *Reoviridae*, they are non-enveloped viruses, with a genome consisting of 11 segments of double stranded RNA encoding six viral proteins and six non-structural proteins (Figure 1-1) (Newman *et al.*, 1975; Rodger *et al.*, 1975). The 6th RNA segment codes for the VP6 protein, which is used to classify rotaviruses into Groups A-H, of which only Group A rotaviruses have been identified in horses (Browning *et al.*, 1991b; Dwyer, 2007; Matthijnssens *et al.*, 2012).

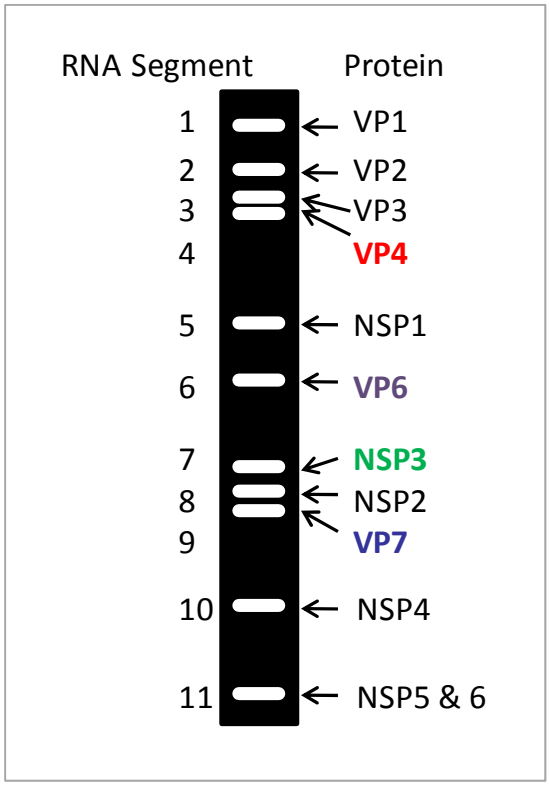

Figure 1-1 Equine rotavirus genome electrophoretic pattern - 11 double stranded RNA segments encoding six viral proteins (VP) and six non-structural proteins (NSP)

The VP7 glycoprotein in the outer capsid is the major neutralising antigen. This protein is used to classify group A rotaviruses into 27 G types (Matthijnssens *et al.*, 2011). Six

of these G types have been identified in horses, with G3 and G14 types predominating (Browning and Begg, 1996; Browning *et al.*, 1992a; Ciarlet *et al.*, 1994; Collins *et al.*, 2008; Elschner *et al.*, 2005; Garaicoechea *et al.*, 2011; Hardy *et al.*, 1991; Isa *et al.*, 1996; Monini *et al.*, 2011; Nemoto *et al.*, 2011; Tsunemitsu *et al.*, 2001). The VP4 protease sensitive protein is a minor neutralising antigen, and the basis for determining the P type, most equine rotaviruses (EqRV) are P genotype 12. There have been single isolates of a number of other G types from horses including G5, G6, G8, G10, G13, which are thought to be cases of cross infection from other species (Browning *et al.*, 1991c; Hoshino *et al.*, 1983a, b; Imagawa *et al.*, 1994; Isa *et al.*, 1996).

Numerous techniques have been used to detect EqRV in the faeces of foals. Initially EM examination of faecal samples was used, but this requires specialised equipment and expertise. Furthermore, the diagnostic sensitivity is low, with the lower threshold for detection exceeding 10^7 viral particles per ml of faeces (McIntosh, 1996).

Polyacrylamide gel electrophoresis has been used to detect rotaviral RNA in faeces, with the advantage that the electrophoretic migration pattern of the 11 segments of double stranded RNA can be used to distinguish between groups of rotaviruses, as well as different strains. In a typical equine group A rotavirus pattern (Figure 1-1), gene segments 3 and 4 migrate close together, and segments 7, 8, and 9 form a well spaced triplet (Hardy *et al.*, 1991; Ohta *et al.*, 1990; Snodgrass and Browning, 1991).

The development of ELISAs to detect rotaviruses in faeces has superseded the use of EM due to the greatly improved sensitivity and the accessibility of these techniques for routine diagnostic laboratories. Many immunoassays are based on the VP6 protein of the intermediate capsid. This protein is the most abundant rotaviral protein, is present in particles that have lost the outer capsid and is highly conserved in group A rotaviruses from a number of species. This is advantageous, as commercial assays developed for detection of rotaviruses in humans can also be used to detect EqRV. There are a number of antigen detection kits, based on immunochromatography or latex agglutination, for detection of rotavirus in humans that are rapid, simple and can be performed on the farm. Some of these kits have been evaluated for use in horses and, while the sensitivity of rapid antigen detection kits is not as high as that of ELISAs, these kits can be useful for veterinarians in the field (Dwyer *et al.*, 1988; Imagawa *et al.*, 1989; Nemoto *et al.*, 2010a). However, in a study conducted by Slovis and collaborators in 2014, 13 foal faecal samples positive for EqRV by qPCR were

negative by a rapid immunoassay with specificity for human rotaviruses (ImmunoCard STAT! Rotavirus, Meridian Bioscience, Inc., Cincinnati, Ohio, USA) (Slovis *et al.*, 2014).

Molecular methods such as reverse transcription polymerase chain reaction (RT-PCR) assays can be used to detect rotaviruses, but are more frequently used in a research setting for G and P genotyping of rotaviruses, thus providing information on the molecular epidemiology of outbreaks (Fukai *et al.*, 2006; Garaicoechea *et al.*, 2011; Gentsch *et al.*, 1992; Gouvea *et al.*, 1994; Tsunemitsu *et al.*, 2001).

A reverse transcription loop-mediated isothermal amplification assay targeting the VP4 gene has been described for sensitive and rapid detection of EqRV (Nemoto *et al.*, 2010b). A commercial RT-qPCR assay for the detection of EqRV has become available in the USA, and more recently Australia (IDEXX). However, experimental details such as primer sequences and reaction components have not been published.

1.3 EQUINE CORONAVIRUSES

Coronaviruses are associated with respiratory, gastrointestinal and neurological diseases in a large variety of mammalian and avian species. Coronaviruses belong to the family *Coronaviridae*, subfamily *Coronavirinae* and are subdivided into four genera based on serological cross reactivity and genetic analysis, known as *alpha-*, *beta-*, *gamma-* and *deltacoronaviruses*. Equine coronavirus (ECoV) belongs to the genera *Betacoronavirus*, species *Betacoronavirus*-1 which also includes bovine coronavirus (BCoV), human coronavirus (HCoV) OC43, and porcine haemagglutinating encephalomyelitis virus (PHEV) (Balasuriya, 2013).

The closely related BCoV causes winter dysentery in adult cattle and is a common cause of neonatal diarrhoea in calves, second only to rotavirus (Boileau and Kapil, 2010; Zhang *et al.*, 2007). Coronaviruses were first observed in foals with gastroenteritis by electron microscopy in 1975 (Bass and Sharpee, 1975) and have since been detected in foals with and without diarrhoea (Browning *et al.*, 1991b; Dwyer *et al.*, 1990b; Frederick *et al.*, 2009; Guy *et al.*, 2000; Slovis *et al.*, 2014). While there have been several case reports of coronaviruses in foals with severe disease (Davis *et al.*, 2000; Mair *et al.*, 1990; Solis and Foreman, 2010), other complicating

factors have also been present. Little is known about the pathogenic and etiologic role of ECoV in relation to clinical disease in foals.

Coronaviruses are enveloped, non-segmented, positive stranded RNA viruses. The ECoV genome contains eleven open reading frames (ORF) encoding two replicase polyproteins, five structural proteins and four accessory proteins (Zhang *et al.*, 2007). The structural proteins include hemagglutinin-esterase (HE), spike (S), envelope (E), membrane (M) and nucleocapsid (N) proteins.

Until relatively recently, ECoV was detected by EM, or using antibody against BCoV for antigen capture ELISA in faeces (Davis *et al.*, 2000), immunohistochemistry in intestinal sections (Bass and Sharpee, 1975; Davis *et al.*, 2000) or to measure rising antibody titres (Anzai *et al.*, 2001; Davis *et al.*, 2000; Imagawa *et al.*, 1990; Narita *et al.*, 2011). Viral isolation of the first ECoV was from a foal with diarrhoea in 2000 (Guy *et al.*, 2000) and the whole genome was subsequently sequenced in 2007 (Zhang *et al.*, 2007). Due to difficulties in isolation of ECoV, the availability of ECoV sequence for development of molecular tests has been limited until the recent detection of ECoV in a number of outbreaks of anorexia and pyrexia in adult horses with and without enteric disease (Oue *et al.*, 2011; Oue *et al.*, 2013; Pusterla *et al.*, 2013). These outbreaks have led to an increase in investigation of coronaviruses, resulting in viral isolation and whole genome sequencing of three equine coronaviruses, and subsequent development of molecular detection methods and development of an ELISA for detection of IgG antibodies against the ECoV spike glycoprotein (Kooijman *et al.*, 2016; Nemoto *et al.*, 2015c). Molecular methods developed have included RT-PCR assays (Narita *et al.*, 2011; Oue *et al.*, 2011; Oue *et al.*, 2013), a LAMP assay (Nemoto *et al.*, 2015a) and RT-qPCR assays (Miszczak *et al.*, 2014; Pusterla *et al.*, 2013) with most targeting the gene encoding the nucleocapsid (N) protein.

1.4 *SALMONELLA*

Salmonellae are Gram-negative rods belonging to the Family *Enterobacteriaceae*. There are two species in the Genus *Salmonella*, *Salmonella bongori* and *Salmonella enterica*. The majority of equine clinical cases are due to *Salmonella enterica* subspecies *enterica* of which there are more than 1450 serovars (Brenner *et al.*, 2000). Commonly these are written as the genus name italicised, followed by the serovar

name, for example *Salmonella* Newport rather than *Salmonella enterica* subspecies *enterica* serovar Newport.

Salmonella has long been recognised as a pathogen of foals and adult horses, as well as an important zoonotic disease. The source of infection in outbreaks is often not determined and may be due to oral contact with faeces from asymptomatic carriers or clinical cases, contaminated feed, water, environmental surfaces and hands of personnel. *Salmonella* can persist in the environment for periods as long as 9 months (Carter *et al.*, 1979).

Horses infected with *Salmonella* may have a spectrum of disease ranging from asymptomatic shedding or mild clinical disease involving fever, depression and anorexia; to severe diarrhoea with or without septicaemia; or septicaemia with or without diarrhoea (Smith *et al.*, 1979). Foals are thought to be at higher risk of severe disease and can also experience localised infections, such as septic arthritis, physitis and osteomyelitis, alone or in combination with systemic disease (Bryans *et al.*, 1961). Transmission is by the faeco-oral route and disease presentation may be influenced by dose. Disease has been reproduced experimentally in both foals and adult horses with an incubation period as short as 24 hrs (Bryans *et al.*, 1965; Smith *et al.*, 1979). A number of risk factors for development of clinical disease have been identified, largely in adult horses, including age, stress and overall health status. Stress may be related to transportation, general anaesthesia, surgery, antimicrobial medication, feed restriction, and change of diet (Baker, 1970; Ekiri *et al.*, 2009; Owen *et al.*, 1983; Traub-Dargatz *et al.*, 1990).

Faecal shedding can be intermittent, and the organism may be shed in low numbers which can make culture challenging (Roberts and O'Boyle, 1981). If the organism is present in large numbers, it may be detected on primary culture. However, overnight enrichment in a selective broth, followed by plating on selective media is usually required (Bryans *et al.*, 1961; Duijkeren *et al.*, 1995; Smith *et al.*, 1979). In some circumstances pre-enrichment in buffered peptone water can also be used. There are multiple selective broths available including Rappaport-Vassiliadis broth, selenite broth and tetrathionate broth and numerous selective agar plates, such as XLD agar, Brilliant green agar and Hektoen enteric agar. In contrast to *Salmonella* testing in human and food samples, there is no standardised method for isolation of *Salmonella* in veterinary microbiology laboratories. Identification of presumptive *Salmonella*

colonies requires confirmation using biochemical tests. The turnaround time for bacterial isolation and confirmation can be up to 5 days (Hyatt and Weese, 2004). To increase the likelihood of detection of *Salmonella* in horse faeces it is recommended to culture at least 5 consecutive daily faecal samples (Smith *et al.*, 1978).

Subtyping of Salmonellae can provide valuable information when investigating the epidemiology of an outbreak. There are numerous typing methods, including serotyping, phage typing, antimicrobial susceptibility testing, and pulsed-field gel electrophoresis (PFGE) (Hyatt and Weese, 2004; Wattiau *et al.*, 2011). Serotyping differentiates isolates into subtypes or serovars within a subspecies based on agglutination with antisera to the somatic (O) and flagellar (H) surface antigens. Serotyping is time consuming, requires skilled personnel and over 150 specific antisera and is therefore usually performed by enteric reference laboratories. Phage typing, based on patterns of bacterial lysis by bacteriophages, is used to further discriminate between *Salmonella* strains within subtypes. This method also requires skilled personnel and a collection of typing phages. Serotyping and phage typing results can take several weeks to obtain, limiting their usefulness in the face of an outbreak (Wang *et al.*, 2008). Antimicrobial susceptibility testing is routinely performed in veterinary microbiology laboratories and provides relatively rapid results that may enable some differentiation of *Salmonella* strains while also informing appropriate antimicrobial therapy. PFGE genotyping is based on migration patterns of DNA fragments after digestion by restriction enzymes and is highly discriminatory (Ridley *et al.*, 1998). Many other *Salmonella* molecular typing methods exist, including whole genome sequence comparison, which with time may become more affordable and accessible.

There have been several PCR assays and one qPCR assay published for the detection of *Salmonella* in horses (Amavisit *et al.*, 2001; Cohen *et al.*, 1996; Kurowski *et al.*, 2002; Pusterla *et al.*, 2010; Ward *et al.*, 2005). PCR and qPCR detection of *Salmonella* is more sensitive than culture and has the advantage of a rapid turnaround time (Amavisit *et al.*, 2001; Cohen *et al.*, 1996). Sensitivity is further improved when performed on nucleic acids extracted from an enrichment broth rather than directly from faeces (Pusterla *et al.*, 2010; Stone *et al.*, 1994). Although, this depends on the enrichment broth used, as Rappaport-Vassiliadis and tetrathionate broths have been shown to be inhibitory to PCR (Stone *et al.*, 1994).

1.5 CLOSTRIDIUM DIFFICILE

C. difficile was first isolated from faeces of healthy newborn infants in 1935 (Hall and O'Toole, 1935), however it was not thought of as a pathogen until the 1970's when antibiotic associated pseudomembranous colitis emerged in humans due to the use of new classes of broad spectrum antibiotics (Bartlett *et al.*, 1978). The organism was first reported in foals with diarrhoea in 1987 (Jones *et al.*, 1987a) and has since been associated with disease ranging from mild diarrhoea to severe haemorrhagic necrotising enteritis in horses of all ages.

Clinical signs in foals include mild abdominal discomfort and pasty faeces, colic with severe watery or haemorrhagic diarrhoea and death following acute abdominal pain within 24 hours. (Jones *et al.*, 1987a; Jones *et al.*, 1987b). Adult horses may present with fever, depression, anorexia, colic, mild diarrhoea to severe acute colitis and endotoxaemia (Gustafsson *et al.*, 1997). Foals tend to develop lesions in the small intestine, with loss of epithelium from the tips of villi, and subepithelial oedema progressing to epithelial sloughing, necrosis and haemorrhage (Jones *et al.*, 1987b). There may be mild to severe necrotizing colitis as well. Lesions in adult horses typically involve haemorrhage and necrosis of the caecum and colon (Gustafsson *et al.*, 1997; Perrin *et al.*, 1993).

The organism is a spore forming, obligate anaerobic, Gram positive rod. Both vegetative cells and spores are infectious. Toxigenic strains produce two potent toxins, enterotoxin A and cytotoxin B, encoded by the *tcdA* gene and *tcdB* gene, respectively, in the pathogenicity locus (PaLoc), a 19.2 kb region of the bacterial chromosome (Figure 1-2). PaLoc also includes three accessory genes *tcdR*, *tcdC* and *tcdE* involved in regulating toxin production and secretion. In non-toxigenic strains PaLoc has been replaced by a unique 115bp sequence (Braun *et al.*, 1996).

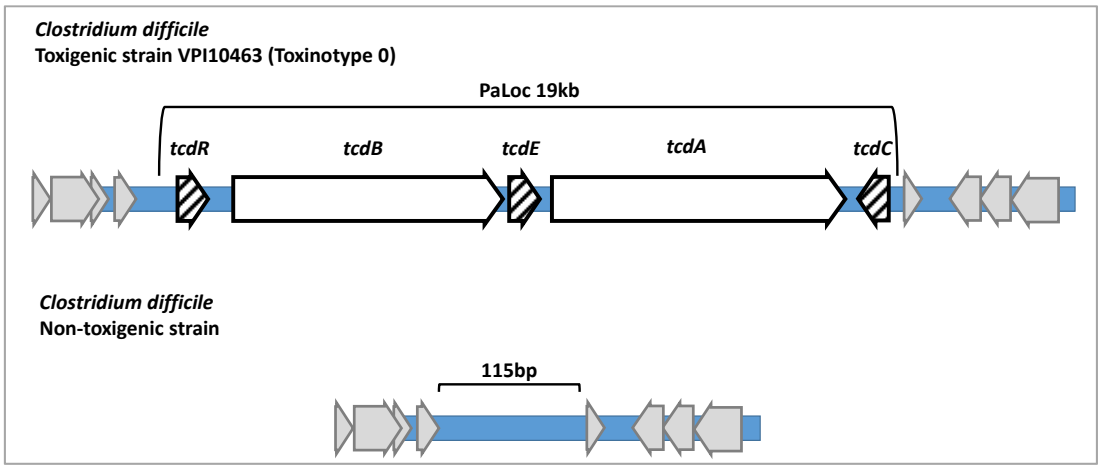

Figure 1-2 Schematic diagram of the pathogenicity locus (PaLoc) of toxigenic C. difficile strain VPI10463, which represents toxinotype 0, and a non-toxigenic strain where PaLoc has been replaced by 115 bp. Genes in the PaLoc include tcdA and tcdB which encode for toxin A and B respectively, and genes tcdR, tcdE and tcdC involved in regulation of toxin production (Adapted from Rupnik (2010))

Another virulence factor is the binary toxin (CDT), encoded by the *cdtA* gene (enzymatic component) and the *cdtB* gene (binding component), with regulation of toxin production by the *cdtR* gene. These genes lie in a different region of the chromosome to PaLoc, known as the CdtLoc (Perelle *et al.*, 1997). Strains containing significant changes in the *tcdA* and *tcdB* genes in comparison to strain VPI10463 are more likely to possess the CdtLoc. The presence of binary toxin genes correlates with toxinotype and ribotype (Stubbs *et al.*, 1999). In non-toxigenic strains CdtLoc has been replaced by a unique 68 bp sequence (Carter *et al.*, 2007).

Non-toxigenic strains and those that produce both toxin A and B, toxin B alone, and binary toxin have all been detected in horses (Arroyo *et al.*, 2007; Arroyo *et al.*, 2006; Madewell *et al.*, 1995; Magdesian *et al.*, 2006; Ossiprandi *et al.*, 2010; Thean *et al.*, 2011).

C. difficile associated disease requires disruption of the normal enteric flora, colonisation, and toxin production by toxigenic *C. difficile* strains. Risk factors for disease in humans such as prior treatment with antibiotics and nosocomial infection, have been associated with disease in horses, but are not a requirement for development of disease (Baverud *et al.*, 1998; Baverud *et al.*, 1997; Gustafsson *et al.*, 1997; Madewell *et al.*, 1995; Weese *et al.*, 2006). In adult horses, disruption of the enteric

flora can occur following hospitalisation, fasting or change in diet, surgery and/or treatment with antibiotics leading to acute and sometimes fatal, colitis (Baverud *et al.*, 1997; Chapman, 2009). An example of this is the development of colitis in the dams of foals being treated for *Rhodococcus equi* pneumonia with erythromycin and rifampicin (Baverud *et al.*, 1998). In foals, disease is less frequently associated with prior exposure to antibiotics. Disease may present as sporadic cases (Weese *et al.*, 1999) or occasionally as outbreaks on breeding farms and in veterinary hospitals (Jones *et al.*, 1987b; Madewell *et al.*, 1995; Magdesian *et al.*, 1999).

A large proportion of healthy children in the first 2 years of life are asymptomatic carriers of *C. difficile* (Stark *et al.*, 1982) Carriage of *C. difficile* has been detected in healthy foals at a low prevalence, although two studies reported a relatively high prevalence of 29-44% in healthy foals (Baverud *et al.*, 2003; Ossiprandi *et al.*, 2010). Whether there is an age association with asymptomatic colonisation in foals is not known.

Animals can be colonised with non-toxin producing strains of *C. difficile*, therefore diagnosis depends on detection of the organism and demonstration of toxin production. Vegetative *C. difficile* is extremely sensitive to oxygen, slow growing and readily overgrown by other bacteria, making culture difficult. It is for this reason that the organism was originally named *Bacillus difficilis* (Hall and O'Toole, 1935). With non-survival of vegetative cells occurring after only 15 minutes of aerobic exposure, specialised anaerobic incubation chambers, as opposed to anaerobic jars, enhance isolation of *C. difficile* from clinical samples (Buggy *et al.*, 1983; Cox *et al.*, 1997). Spores on the other hand are highly resistant to oxygen, heat, desiccation, and disinfectants, surviving in horse faeces in ambient conditions for up to 4 years (Baverud *et al.*, 2003). Selective media containing the antibiotics cycloserine and cefoxitin is used to inhibit growth of contaminating faecal flora and the addition of bile salts, such as sodium taurocholate, is used to enhance growth from *C. difficile* spores (George *et al.*, 1979). Non-culture detection methods of *C. difficile* include an ELISA for the glutamate dehydrogenase antigen and PCR assays (Lemee *et al.*, 2004b).

The gold standard for detection of Toxin B is a cytotoxicity assay (Bartlett *et al.*, 1978). This method demonstrates a cytopathic effect when filtered culture supernatant or clarified faecal sample is inoculated onto cell culture. Results are then confirmed by

neutralisation with antiserum against *C. difficile* toxin B. While this technique has the advantage of being sensitive, it is technically complex, has a turnaround time of 48 hrs and requires cell culture skills and facilities.

Enzyme-linked immunoassays based on monoclonal antibodies against either toxin A, toxin B, or both, are rapid and simple to perform. However, the sensitivity is not as high as the cytotoxicity assay, and many have not been validated for use in horses. A study in dogs found the sensitivity and specificity insufficient for detection of toxin in canine faecal samples (Chouicha and Marks, 2006). A comparison of the *C. difficile* Tox-A/B II ELISA (TECHLAB, Blacksburg, VA) to the cell cytotoxicity assay for detection of *C. difficile* toxins in horse faeces, reported a sensitivity of 84%, a specificity of 96%, and a positive predictive value of 89%, suggesting this ELISA was a useful diagnostic screening tool in horses (Medina-Torres *et al.*, 2008).

Deletions in the *tcdA* gene can render the gene product undetectable by ELISA methods based solely on detection of Toxin A. Therefore, ELISAs targeting both toxins are preferable as tests that only look for toxin A would not detect or distinguish between A-B+ strains and A-B- strains (Kader *et al.*, 1998; Kato *et al.*, 1999). The detection of toxins in faeces can also be hindered by degradation at ambient temperatures. If a delay in toxin testing is anticipated, it is best to store samples below 4°C (Weese *et al.*, 2000b).

Genes encoding *C. difficile* toxins A and B can also be detected by numerous PCR or qPCR assays. However, variant strains may have deletions in the *tcdA* and *tcdB* genes, so interpretation of the results can be problematic depending on the region the primers target and whether the deletions result in production of a functional toxin or not (Kato *et al.*, 1999).

Characterisation of *C. difficile* isolates for epidemiological analysis was initially, based on phenotypic characteristics such as antibiograms and serogroup. This was followed by molecular typing methods such as restriction endonuclease analysis (REA), PFGE, arbitrarily primed PCR (AP-PCR), restriction fragment length polymorphism (RFLP) toxin typing and PCR-ribotyping.

PFGE is highly reproducible and highly discriminatory for typing of *C. difficile*, however the procedure is time consuming, taking a minimum of 4 days. AP-PCR is

quicker to perform than PFGE but is poorly reproducible and therefore of limited use for inter-laboratory correlation of strain typing (Bidet *et al.*, 2000).

PCR-RFLP is used to classify *C. difficile* isolates into toxinotypes by amplifying fragments of the genes encoding toxin A and B and comparing restriction enzyme digestion patterns to the reference laboratory strain VPI 10463 (Rupnik *et al.*, 1997). This strain is designated as toxinotype 0 and variant strains have differences in the *tcdA* and *tcdB* genes. These differences are typically deletions in the 3' third of *tcdA* and point mutations in the 5' third of *tcdB*, although in some strains the complete *tcdA* gene is lacking (Janezic *et al.*, 2015). Currently there are 34 toxinotypes, designated I to XXXIV (Rupnik and Janezic, 2016).

PCR-ribotyping, based on DNA fragment patterns produced by amplicons of the intergenic spacer region between the 16S and 23S rRNA genes, is relatively quick, is highly reproducible and correlates well with toxinotyping (Bidet *et al.*, 1999; Rupnik *et al.*, 2001). Some ribotypes have been associated with increased epidemic and virulence potential and are referred to as hypervirulent strains. Two examples are ribotypes 027 and 078 (Goorhuis *et al.*, 2008). Ribotype 027 was initially identified in severe outbreaks in human hospitals in Quebec, Canada (Pépin *et al.*, 2005). It has since been shown to produce more toxin than other ribotypes and have a higher sporulation rate (Åkerlund *et al.*, 2008; Warny *et al.*, 2005). Ribotype 078 was initially considered an animal strain that was predominant in calves and pigs. This strain has also been identified in outbreaks of *C. difficile* associated disease in humans internationally (Goorhuis *et al.*, 2008).

There are relatively few studies reporting ribotypes of equine *C. difficile* isolates and most have been conducted in North America (Arroyo *et al.*, 2007; Arroyo *et al.*, 2006; Medina-Torres *et al.*, 2011; Niwa *et al.*, 2013; Ossiprandi *et al.*, 2010; Rodriguez *et al.*, 2015; Schoster *et al.*, 2012a; Songer *et al.*, 2009a; Thean *et al.*, 2011; Uzal *et al.*, 2012). The only previous study in Australian horses identified 6 ribotypes amongst equine isolates, including ribotype 014, the most common ribotype identified in humans in Australia (Thean *et al.*, 2011). In contrast to North America where ribotypes 078 and 027 have been detected in horses (Keel *et al.*, 2007; Medina-Torres *et al.*, 2011; Niwa *et al.*, 2013; Schoster *et al.*, 2012a; Schoster *et al.*, 2012b; Songer *et al.*, 2009a; Uzal *et al.*, 2012), these ribotypes have not been detected in Australian horses, cattle, sheep or pigs (Knight and Riley, 2013; Knight *et al.*, 2015; Knight *et al.*, 2013;

Thean *et al.*, 2011). Similarly in humans, these ribotypes have only been detected at a low prevalence in Australia compared to North America and Europe (Barbut *et al.*, 2007; Bull *et al.*, 2012; Cheng *et al.*, 2016; Mackin *et al.*, 2015; Tickler *et al.*, 2014). With the emergence of hypervirulent strains in human hospital outbreaks and the detection of these strains in some animal populations, the surveillance of the strains present in foals is of heightened importance to public health authorities.

1.6 RESEARCH AIMS

The purpose of this study was to: 1) Establish a collection of faecal samples from foals with and without diarrhoea that could be archived for future studies. 2) Develop molecular assays to enable rapid screening of foal faecal samples for the presence of rotaviruses, coronaviruses, *Salmonella* and *Clostridium difficile.* 3) Establish whether these infectious agents are present in the Australian thoroughbred foal population. 4) Determine whether there was an association between the detection of these infectious agents and the presence of diarrhoea in Australian thoroughbred foals.

CHAPTER 2 MATERIALS AND METHODS

2.1 SAMPLE COLLECTION AND STORAGE

2.1.1 Sample collection

Samples of faeces from foals with and without diarrhoea were collected for this study using a convenience sampling frame. Five farms were visited on multiple occasions each week between August 2010 and December 2010 when mares were being examined for breeding purposes, providing access to foals in a crush or small yard.

Where possible, freshly voided faeces were sampled to avoid having to handle or restrain the foal. If no voided faecal sample was available, a faecal sample was obtained per rectum by one of two methods:

1. Gently inserting a gloved, well-lubricated finger into the rectum and using gentle digital manipulation to remove some faecal material which was then placed into a 70 ml sterile specimen jar.
2. By insertion of a lubricated plastic blood collection tube into the rectum, that then filled passively by peristalsis.

2.1.2 Sample storage

Following collection and transport to the laboratory, faecal samples were divided into four aliquots:

2.1.2.1 Nucleic acid preservation: 0.5 ml or 0.5 g of foal faeces was added to 2.5 ml RNAlater ® (Sigma) and thoroughly mixed with a vortex, followed by incubation at 4°C overnight to allow the solution to thoroughly penetrate the sample. The sample was then frozen at -20°C overnight, followed by long term storage at -70°C until RNA/DNA extraction and qPCR assay.

2.1.2.2 Anaerobic bacteriological preservation: 1 ml or 1 g of foal faeces was placed into cooked meat broth (Schaedlers) with glucose (CMB) in a 20 ml McCartney bottle (STM 2028 Media Preparation Unit (MPU) The University of Melbourne) and vortexed. The McCartney bottle with the lid ajar was then reduced in an anaerobic container for 24 hrs (BD GasPak®

EZ Large Incubation Container with three BD GasPak® EZ Anaerobe with Indicator Sachets). Once reduced, the McCartney lid was screwed on tightly, sealed with plastic paraffin film and stored at room temperature until processing.

2.1.2.3 Aerobic bacteriological preservation: For aerobic bacteriological preservation 0.3 ml or 0.3 g of foal faeces were added to 3.5 ml of a glycerol storage broth (2% w/v tryptone, 20% v/v glycerol, MPU The University of Melbourne). The sample was then vortexed and allowed to equilibrate for 24 hours at 4°C, before being frozen at -20°C overnight and then stored long-term at -70°C until examination by bacteriological culture methods (Section 2.6).

2.1.2.4 Untreated stool sample: The remaining foal faecal material was placed into a sterile tube and stored overnight at -20°C, before long-term storage at -70°C.

Given the different consistencies of faeces obtained in this study, liquid and solid samples were handled and measured differently. Liquid faecal samples were vortexed to ensure an even consistency and the appropriate volume distributed into each media using a 1,000 µl pipette tip with the tip cut off. In contrast, faecal samples with a more solid consistency were weighed and distributed into each media using a wooden spatula.

If the size of the foal faecal sample was insufficient to place the above described amounts into their respective containers, either a smaller volume/weight was used or, if the sample weighed less than 1.2g, it was diluted with a known volume of phosphate buffered saline (PBS, 136 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4), vortexed and then divided into the 4 containers. The volume of PBS added and the sample volume or weight was recorded for every sample.

The majority of samples (94%) were processed within 24 hrs of collection, however, due to geographical limitations some samples were processed later than 24 hrs after collection. If this occurred, the date of collection and the date of processing were recorded. The maximum time until processing was 7 days, which occurred for 2 samples.

2.2 NUCLEIC ACID METHODS

Nucleic acids were purified for a number of reasons in this study as outlined below.

- RNA was extracted from archived foal faecal samples for use as positive controls during development and validation of RT-qPCR assays to detect rotavirus and coronavirus.
- RNA and DNA were extracted from foal faecal samples collected during this study and screened for rotavirus and coronavirus by RT-qPCR assay and *Salmonella* and *C. difficile* by qPCR assay.
- Bacterial DNA was extracted from overnight selenite enrichment broth culture of foal faeces for detection of *Salmonella* spp. by qPCR assay.
- Genomic DNA was extracted from overnight broth culture of *C. difficile* for molecular typing assays (eg. toxinotyping & ribotyping).

2.2.1 Nucleic acid extraction from archived foal faecal samples

The QIAamp Viral RNA mini kit (Qiagen) was used to extract nucleic acids from archived foal faecal samples. Prior to extraction, 200 µl of homogenised foal faeces were diluted in 800 µl of PBS and mixed with a vortex, followed by centrifugation at 2,700 x *g* for 10 minutes (mins). Viral RNA was then extracted from 140 µl of supernatant using the QIAamp Viral RNA mini kit spin protocol.

2.2.2 Nucleic acid extraction from foal faecal samples collected in this study stored in RNAlater®

Total nucleic acids were extracted from 225 µl of foal faeces stored in RNAlater® (Section 2.1.2.1) using the PowerSoil® - htp 96 Well Soil DNA Isolation Kit (MoBio) according to the manufacturer's directions (Appendix 1) with some modifications, as described below.

In step six of the manufacturer's "*experienced user combination vacuum and centrifugation protocol"* the bead plate was placed on an orbital shaker for 20 mins instead of in a MOBIO 96 well plate shaker.

The protocol from step 18 onwards was automated using a QIAxtractor (Qiagen). Briefly, at step 18 the spin plate was manually preloaded with 350 µl of sample from step 17. The partially preloaded spin plate was then placed in the QIAxtractor on top

of a 2 ml collection plate above the vacuum chamber. The 2 ml collection plate with the remaining 1,600 µl of sample was placed on the deck of the QIAxtractor. The QIAxtractor was then programmed to transfer 800 µl from the collection plate to the spin plate. A 30 kPa vacuum was then applied for 5 mins. Before turning the vacuum off, all wells were checked to ensure liquid had passed through. If any wells had liquid remaining, the spin plate membrane was pierced with an 18 gauge needle. Once the liquid had passed through, the vacuum was turned off. The remaining 800 µl of sample was then loaded and vacuumed in the same manner.

Five hundred µl of solution C5-D was then added to each well of the spin plate by the QIAxtractor and a 30 kPa vacuum applied for 3 mins. This wash step was repeated to maximise removal of residues and contaminants.

A 25 kPa vacuum was then applied for a further 3 mins to dry the sample prior to elution. The spin plate was then transferred to sit above a capture plate and 100 µl of solution C6 was added to the spin plate by the QIAxtractor and allowed to incubate for 2 mins. Nucleic acids were then eluted into the capture plate by application of a 30 kPa vacuum for 1 min. To maximise retrieval of eluted nucleic acids the spin plate was sealed with centrifuge tape and removed from the QIAxtractor with the capture plate beneath it and centrifuged for 5 mins at 2,500 x *g* (Allegra X-12R Centrifuge, Beckman Coulter). The spin plate was then discarded and the capture plate sealed and stored at -70°C. These nucleic acids were then screened for rotavirus and coronavirus by RT-qPCR and *Salmonella* spp. and *C. difficile* by qPCR.

2.2.3 Nucleic acid extraction from selenite broth

During bacterial culture of faecal samples stored in glycerol broth for *Salmonella*, a 4 ml aliquot of overnight selenite enrichment broth was collected and stored at -70°C. These aliquots were thawed and bacterial DNA was extracted from 200 µl of selenite broth in 96 well extraction plates with the DX Liquid Sample DNA extraction protocol using a QIAxtractor (Qiagen). This DNA was then screened for *Salmonella* spp. by qPCR assay.

Each 96 well extraction plate had six positive controls and six negative controls distributed across the plate. The positive extraction controls were prepared from a dilution in PBS of an overnight *Salmonella* culture in Luria-Bertani (LB) culture media

(1% w/v tryptone, 0.5% w/v yeast extract and 0.5% w/v sodium chloride). Negative extraction controls included either uninoculated selenite broth or sterile water.

2.2.4 *C. difficile* genomic DNA preparation method

Genomic DNA was prepared from *C. difficile* cultures in pre-boiled heart infusion supplement broth (heart infusion broth (Oxoid) supplemented with 0.5% w/v yeast extract, 1.5% w/v glucose, 0.1% w/v L-cysteine) as described by O'Connor *et al.* (2006) modified from Pospiech and Neumann (1995) but without RNase treatment.

Briefly, 5 ml of overnight culture was pelleted and resuspended in 495 µl SET buffer (75 mM NaCl, 25 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0, 20 mM Tris HCl pH 7.5 in distilled water) followed by addition of 50 µl of 10 mg/ml lysozyme (Sigma) and incubated at 37°C for 1 hour. Then sodium dodecyl sulphate and proteinase K were added to final concentrations of 0.1% w/v and 25 µg/ml, respectively, and the sample mixed and incubated for 1 hour at 55°C.

Two hundred µl NaCl and 700 µl chloroform:isoamyl alcohol (24:1) were added and incubated at room temperature for 30 mins with inversion every 5 mins. Then the sample was centrifuged at 20,238 x *g* for 30 mins at room temperature and the aqueous phase transferred to a new tube. The DNA was then precipitated by the addition of 700 µl of isopropanol and centrifuged at 20,238 x *g* for 10 mins. The supernatant was then carefully removed, the DNA pellet washed with 500 µl of ice cold 70% v/v ethanol and allowed to dry. The DNA was then resuspended with 100 µl of distilled water and left on ice for 10 mins to allow the DNA to resuspend. This genomic DNA was used to further characterise *C. difficile* isolates using molecular typing assays.

2.2.5 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA from PCR products or plasmid preparations. Unless otherwise stated, agarose gels from 0.8 to 2.5% (w/v) were prepared with molecular biology grade agarose (Scientifix) dissolved in SYBR safe DNA gel stain (Invitrogen) in TBE buffer (45 mM Tris-HCl, 45 mM boric acid, 1 mM EDTA pH 8.0). Electrophoresis was performed in TBE buffer. DNA was visualised and an image captured by UV transillumination using the ChemiDoc XRS+ molecular imager (BioRad).

2.2.6 Gel purification of DNA

Silica-membrane-based purification of DNA from agarose gel slices was performed using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

2.2.7 Spectrophotometric measurement of nucleic acid concentration

The ND-1000 spectrophotometer was used to quantitate nucleic acid concentration of 1µl of sample by measuring absorbance at 260 nm.

2.2.8 Synthesis of cDNA by reverse transcription

For rotavirus detection, complementary DNA (cDNA) was generated from viral RNA (Section 2.2.2) using Superscript III Reverse Transcriptase (Invitrogen) and random primers. To reduce secondary RNA structure, 5 µl of RNA sample and 100 ng of random primers (Invitrogen) were denatured at 95°C for 5 mins, before being placed on ice.

RNA was then reverse transcribed at 46°C for 50 mins in a reaction mixture containing first strand buffer (Invitrogen), 10 mM dithiothreitol (Invitrogen), 1.5 mM deoxynucleotide triphosphates (dNTPs), 20 U of RNaseOUT (Invitrogen), 100 U of Superscript III in a final volume of 20 µl with sterile water. The reverse transcriptase enzyme was then inactivated by incubation at 70°C for 15 mins.

For coronavirus detection, cDNA (Section 2.2.2) was generated from RNA using Superscript III Reverse Transcriptase (Invitrogen) and the BCoV-rev primer (Table 2-1). To reduce secondary RNA structure, 5 µl of RNA sample and 1 µl of 20 µM of BCoV-rev primer were denatured at 80°C for 5 mins, before being placed on ice.

RNA was then reverse transcribed at 50°C for 50 mins in a reaction mixture containing first strand buffer, 10 mM dithiothreitol, 0.5 mM dNTPs, 20 U of RNaseOUT (Invitrogen), 100 U of Superscript III made up to a final volume of 20 µl with water. The reverse transcriptase enzyme was then inactivated by incubation at 70°C for 15 mins.

2.3 POLYMERASE CHAIN REACTION METHODS

2.3.1 Primers and probes

The primer and probe sequences for PCR and qPCR assays were adopted from published primers and are detailed in Table 2-1. The coronavirus forward primer BCoV-fwd, designed to amplify bovine coronavirus (Cho *et al.*, 2010), was modified in the present study, to account for GenBank sequence differences in the gene encoding the nucleocapsid in equine and bovine coronaviruses, and renamed BCoV-fwd alt.

Primers were manufactured and supplied by GeneWorks Pty Ltd (Australia) and probes by Biosearch Technologies (USA).

2.3.2 Machine parameters and data acquisition

Conventional PCR assays and reverse transcription (RT) assays were performed in an iCycler (BioRad). All quantitative PCR assays were performed on the Mx3000P Real Time PCR machine (Stratagene) and analysed using the accompanying MxPro software. The common fluorescent threshold or interrun calibrator setting was used to compare cycle threshold (Ct) values across multiple plates.

In assays using SYTO® 9 green fluorescent nucleic acid stain (Invitrogen), fluorescence data was recorded with the SYBR filter at the end of each annealing step. After 40 amplification cycles a dissociation melt cycle was performed from 55°C to 95°C at the rate of 0.01°C per second. A dissociation plot of the first derivative of the fluorescence as a function of temperature was used to visualise melting peaks and to confirm the specificity of the amplicon.

For probe based assays, fluorescence data was recorded with the SYBR filter and the ROX filter.

Table 2-1 Primer and TaqMan probe sequences for qPCR assays

Target	Amplicon (bp)	Primer name	Sequence (5'-3')	Reference
Rotavirus				
NSP3	87	Rota NVP3-F	ACCATCTACACATGACCCTC	Pang *et al.* (2004)
		Rota NVP3-R	GGTCACATAACGCCCC	
Coronavirus				
N	87	BCoV-fwd alt	CTAACAAGCAGGCTGATGTTAATACC	Cho *et al.* (2010) modified
		BCoV-rev	GGCGGAAACCTAGTCGGAATA	Cho *et al.* (2010)
Salmonella				
invA	132	*invA*-156f	CATTTCTATGTTCGTCATTCCATTACC	Pusterla *et al.* (2010)
		invA-288r	AGGAAACGTTGAAAAACTGAGGATTCT	
		invA-189p	FAM-TCTGGTTGATTTCCTGATCGCACTGAATATC-TAMRA	
Clostridium difficile				
tpi	230	*tpi*-F	AAAGAAGCTACTAAGGGTACAAA	Lemee *et al.* (2004b)
		tpi-R	CATAATATTGGGTCTATTCCTAC	

2.3.3 SYTO® 9 based qPCR assays

The qPCR assays to detect rotavirus, coronavirus and *C. difficile* were performed using SYTO® 9 (Invitrogen), GoTaq Flexi DNA Polymerase, Green GoTaq Flexi buffer and $MgCl_2$ (Promega). The primer and master mix components varied between assays and are described individually below. The cycling conditions for each assay are detailed in Table 2-2.

The qPCR assay to detect rotavirus was conducted using 5 µl of cDNA (Section 2.2.8) in a 20 µl reaction mixture containing Green GoTaq Flexi Buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of primers Rota NVP3-F and Rota NVP3-R (Table 2-1), 1.6 µl of SYTO® 9 and 0.8 U of GoTaq DNA polymerase.

The qPCR assay to detect coronavirus was conducted using 5 µl of cDNA (Section 2.2.8) in a 25 µl reaction mixture containing Green GoTaq Flexi Buffer, 2 mM $MgCl_2$, 0.2mM dNTPs, 0.35 µM of primers BCoV-fwd alt and BCoV-rev (Table 2-1), 2 µl of SYTO® 9 and 1 U of GoTaq DNA.

A touchdown qPCR assay to detect *C. difficile* was conducted using 5 µl of extracted DNA (Section 2.2.2) in a 25 µl reaction mixture containing Green GoTaq Flexi Buffer, 2.0 mM $MgCl_2$, 0.2mM dNTPs, 0.4µM *tpi*-F primer and 0.5µM *tpi*-R primer, 2 µl of SYTO® 9 and 1 U of GoTaq DNA polymerase.

Table 2-2 SYTO® 9 qPCR assay cycling conditions

Assay / Target	Number of cycles	Temperature °C	Time
Rotavirus NSP3	1	94	1 min
	40	94	30 sec
		50*	30 sec
		68	30 sec
	1	dissociation 55°C to 95°C @ 0.01°C/sec	
Coronavirus N	1	95	5 mins
	40	95	30 sec
		60*	30 sec
		72	45 sec
	1	dissociation 55°C to 95°C @ 0.01°C/sec	
C. difficile tpi	1	95	5 mins
	11	95	30 sec
		65-55, decrease 1°/cycle*	30 sec
		72	30 sec
	29	95	30 sec
		55*	30 sec
		72	30 sec
	1	dissociation 55°C to 95°C @ 0.01°C/sec	

Data collected: end of each annealing step (*) and all of dissociation ramp

2.3.4 Probe based qPCR assay

The quantitative PCR assay for the detection of *Salmonella* used TaqMan probe chemistries as described below. The assay cycling conditions are detailed in Table 2-3.

For the detection of *Salmonella*, qPCR was conducted using 5 µl of extracted DNA (Section 2.2.3) in a 20 µl reaction containing Brilliant II QPCR master mix (Agilent Technologies), 0.2 µM *invA*-156f primer, *invA*-288r primer, and *invA*189p probe, and 30 nM ROX reference dye (Agilent Technologies).

Table 2-3 TaqMan Probe qPCR assay cycling conditions

Assay	Number of cycles	Temperature °C	Time
Salmonella invA	1	95	10 min
	40	95	15 sec
		60	1 min

2.3.5 Intra- and inter-assay variability

To assess the variation within a single qPCR assay run (intra-assay variation) and between runs (inter-assay variation) the coefficient of variation (CV) was calculated and expressed as a percentage of deviation from the mean Ct value.

$$CV(\%) = (SD/mean) \times 100$$

The larger the CV, the greater the variability in the assay.

2.4 PREPARATION OF QPCR STANDARDS

For rotavirus, coronavirus, *Salmonella* and *C. difficile* qPCR assays, a DNA quantification standard was prepared by cloning the qPCR amplicon from a positive control into a pGEM-T (Promega) plasmid vector. The viral positive controls were sourced from archived foal faecal samples in which rotavirus and coronavirus had previously been detected using alternative PCR assays, while bacterial isolates were used as positive controls for *Salmonella* (Section 7.4.1) and *C. difficile* assays (Section 2.6.4). Purified plasmid DNA standards were diluted to create aliquots of stock containing 10^8 copies and stored at -70°C. To minimise variation between standard dilution series used in multiple assay runs, serial 10-fold dilutions from 10^7 to 10^0 copies were prepared from thawed 10^8 stock, and used within 48hrs with storage at 4°C in between runs.

2.4.1 Cloning of DNA

PCR amplicons were excised after agarose gel electrophoresis (Section 2.2.5) and purified using the QIAquick Gel Extraction Kit (QIAGEN) (Section 2.2.6). Products were then ligated into the pGEM-T cloning plasmid (Promega) according to manufacturer's instructions with a 3:1 molar ratio of insert DNA to vector and incubated overnight at 4°C.

2.4.2 Bacterial transformation

One µl of ligation reaction (2.4.1) was added to a thawed 40 µl aliquot of electrocompetent *E. coli* DH5α or JM109 cells, mixed by pipetting and then transferred to a chilled electroporation cuvette. Electro-transformation was conducted in a Gene Pulser Apparatus (BioRad) with a single pulse at a voltage of 2.4 kV, a capacitance of 25 µFD and a resistance of 200 Ω. Following electroporation, cells were suspended in 1 ml of LB broth and incubated for 1 hour at 37°C. Following incubation, 100 µl was then spread onto LB plates containing 100 µg/ml ampicillin, 50 µg/ml isopropylthio-β-D-galactoside (IPTG) and 40 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). Positive recombinants were selected based on white colony colour, due to disruption of the β-galactosidase coding sequence, and inoculated into LB broth supplemented with 100 µg/ml ampicillin (LBamp). A PCR assay was then used to screen for insertion of the PCR product into the plasmid vector using 2 µl of a 1/50 dilution of overnight LBamp broth as template. In cases where DNA inserts were less than 100 bp, blue colonies were also selected and screened by PCR for the presence of the insert as the coding sequence of β-galactosidase may not have been sufficiently disrupted.

2.4.3 Plasmid preparation

Plasmid DNA was extracted from 3 ml of overnight LBamp broth cultures of positive recombinants using the Wizard® Plus SV Miniprep DNA Purification System (Promega) according to the manufacturer's directions.

2.5 DNA SEQUENCING

2.5.1 Sequencing reaction

Sequencing reactions were conducted using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). For PCR products sized 100 - 200 bp, 200 - 500 bp or 1,000 – 2,000 bp, each reaction contained at least 5 ng, 10 ng or 40 ng of purified nucleic acid (Section 2.2.6), respectively, in a 20 µl reaction. Cycling was performed on an iCycler (BioRad) at 94°C for 5 mins, followed by 30 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 mins.

2.5.2 Precipitating DNA sequencing reactions

To precipitate the sequencing extension products, 80 µl of 75% v/v isopropanol was added to each reaction, vortexed briefly, followed by incubation at room temperature for 15 mins. Sequencing products were then pelleted by centrifugation at 20,238 x *g* for 20 mins and the supernatant removed. To ensure removal of unincorporated dye terminators, sequencing products were washed with 250 µl of 75% v/v isopropanol followed by centrifugation and discard of the supernatant. Finally, sequencing extension products were dried at 90°C for 1 min. Dried sequencing products were then submitted to Applied Genetic Diagnostics, Department of Pathology, The University of Melbourne, for capillary electrophoresis on ABI3130xi capillary genetic analysers.

2.5.3 Analysis of sequence data

Sequence chromatogram results were analysed using Geneious® (version 9.0.5 Kearse *et al.* (2012)). The sequences obtained were compared to each other and to published sequences.

2.6 BACTERIAL METHODS

2.6.1 Bacterial culture of *Salmonella* from foal faeces

Isolation of *Salmonella* spp. was performed by bacterial culture of foal faecal samples stored in glycerol storage broth at -70°C (Section 2.1.2.3). Samples were thawed and vortexed to ensure mixing of the sample. Each sample was directly streaked onto MacConkey agar (MCA) plates (MPU The University of Melbourne) with a sterile loop and 1 ml was inoculated into selenite enrichment broth (MPU The University of Melbourne) and incubated at 37°C overnight. After overnight incubation, selenite broths were streaked onto MCA and xylose lysine desoxycholate (XLD) agar plates (MPU The University of Melbourne) with a sterile loop and incubated as above. A 4 ml aliquot of selenite broth from each sample was stored at -70°C for later DNA extraction (Section 2.2.3) and testing by qPCR assay (Section 2.3.4) for the presence of *Salmonella*. Non-lactose fermenting colonies cultured on MCA agar and acid negative, H_2S positive colonies on XLD agar were subcultured onto both MCA and XLD plates.

2.6.2 Biochemical testing for *Salmonella* identification

Colonies consistent with *Salmonella* spp. were streaked onto a urea agar slope (MPU The University of Melbourne) (Cowan and Steel, 1993) and incubated at 37°C for up to 24 hours. Urease negative colonies, based on the absence of colour change in the media, were then subjected to a latex agglutination test based on polyvalent antisera to *Salmonella* flagella antigens (Oxoid *Salmonella* Test Kit, Thermo Scientific). Presumptive isolates of *Salmonella* spp. from the latex agglutination test were then tested biochemically in a BBL Enterotube II (BD) as per manufacturer's directions. Briefly, a single colony was picked up with the end of the provided needle and the media inoculated by withdrawing the needle through all of the test compartments, followed by reinsertion of the needle and re-capping of the tube. The aerobic test compartments were pierced and then the tube incubated for 48 hours at 37°C. Reactions for glucose acid, glucose gas, lysine, ornithine, H_2S, indole, adonitol, lactose, arabinose, sorbitol, dulcitol, phenylalanine deaminase, urea and citrate were then interpreted and a 5 digit biocode derived. The identity of the organism was then determined using the 5 digit code in the Enterotube II coding manual.

2.6.3 Antimicrobial sensitivity of *Salmonella* isolates

Antibiotic susceptibility testing of *Salmonella* isolates was performed by Rhys Bushell, Laboratory of Clinical Microbiology, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, using the calibrated dichotomous sensitivity (CDS) method (Bell *et al.*, 2011). Briefly, a Sensitest agar (Oxoid) plate was inoculated with 10^7 cfu/ml of the test organism, allowed to dry and loaded with discs of the antibiotics to be tested. The plate was then incubated at 37°C overnight. The annular radius of the zone of inhibition for each antibiotic disc was then measured. If the annular radius was greater than or equal to 6 mm the organism was considered sensitive, while those with an annular radius less than 6 mm were considered resistant.

The antibiotic discs tested were ampicillin (25 μg), tetracycline (30 μg), sulfafurazole (300 μg), trimethoprim (5 μg), enrofloxacin (5 μg), gentamicin (10 μg), ceftiofur (30 μg), methicillin (5 μg) and ticarcillin (75 μg).

2.6.4 *C. difficile* bacterial isolates for use as positive controls

C. difficile bacterial isolates were used as positive controls during development and validation of the *C. difficile tpi* qPCR assay and in toxinotyping and characterisation

assays. These isolates included two equine isolates, CM09-0928 and CM09-0930, with unknown toxin A and toxin B profiles, provided by the Laboratory of Clinical Microbiology, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne. Four *C. difficile* isolates with different toxin profiles were also provided by Associate Professor Dena Lyras, Department of Microbiology, Monash University (Table 2-4).

Table 2-4 Control *C. difficile* strains used in this study

Strain	Characteristics	Toxin production	Source or reference	Assays used in
VPI10463	PaLoc +ve toxinotype 0 reference strain	A+B+CDT-	Dove 1990	*tpi* qPCR
M7404	toxinotype III / ribotype 027 human isolate	A+B+CDT+	J.Pepin Carter et al 2007	*tpi* qPCR, toxinotyping
JGS6133	toxinotype V / ribotype 078 porcine isolate	A+B+CDT+	J.G.Songer Iowa State Uni	toxinotyping
CD37	PaLoc –ve isolate	A-B-CDT-	T.D.Wilkins Anaerobe Laboratory, Virginia Tech Smith 1981	*tpi* qPCR toxinotyping

2.6.5 Bacterial culture of *C. difficile* from foal faeces

Bacterial culture of *C. difficile* was performed from foal faecal samples stored anaerobically in CMB (Section 2.1.2.2) with the assistance of Kate Mackin, Department of Microbiology, Monash University.

To aid in the sampling of spores, anaerobically stored CMB were carefully opened with minimal disturbance and 1 ml aspirated from the bottom layer of sediment. This was then added to 1 ml of ethanol and incubated at room temperature for 1 hour (alcohol shocked). Samples were then centrifuged at 2,300 x *g* in a swing-out rotor benchtop centrifuge (Sigma, type 2-5) for 5 mins to pellet spores and debris. The majority of the supernatant was then tipped off leaving approximately 400 µl behind. The remaining liquid was used to resuspend the pellet and inoculate selective medium

for *C. difficile* consisting of heart infusion broth (Oxoid) supplemented with 0.5% w/v yeast extract, 1.5% w/v glucose, 0.1% w/v L-cysteine, 0.1% w/v sodium taurocholate, 1.5% w/v agar and Oxoid CDMN supplement (32 µg/ml moxalactam, 12 µg/ml norfloxacin). Plates were incubated at 37°C for 48 hours in a Coy anaerobic chamber in an atmosphere of 10% H_2, 10% CO_2 and 80% N_2.

Colonies consistent with *C. difficile* were subcultured onto *C. difficile* selective medium. Once a pure culture with appropriate colony morphology and odour was obtained (Jones, 1989; Wren, 2010), a colony PCR for the *spo0A* gene was performed to confirm the isolation of *C. difficile*.

2.6.6 *C. difficile* colony identification using *spo0A* targeted PCR

A colony consistent with *C. difficile* was selected from an agar plate and resuspended in 10 µl of distilled water, incubated in a heat block at 90°C for 10 mins, then immediately placed on ice for 1-2 mins. The lysate was then centrifuged for 1 min.

A PCR targeting the *spo0A* gene (Mackin *et al.*, 2013) was performed using 5 µl from the supernatant fraction of the colony lysate as template in a 20 µl reaction containing FailSafe Buffer E (Epicentre Technologies), 2.5 µM of primers JRP2527 and JRP258 (Table 2-5), and 2.5 U Taq polymerase (Roche).

Table 2-5 Primer sequences for *C. difficile spo0A* PCR assay

Target	Primer name	Sequence (5'-3')	Reference
spo0A	JRP2527	AGTTCTAGAGAGTATTTGTCTAATGAAGATG	Mackin (2014)
	JRP2528	GATAATGTTGAGCTTTTAGGTGCAG	Mackin *et al.* (2013)

The assay cycling conditions involved an initial denaturation cycle at 95°C for 5 min, followed by 30 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at 50°C and extension for 1 min at 72°C, with a final additional 12 min extension period at 72°C.

PCR products were separated by electrophoresis on a 0.8% w/v agarose gel in TAE buffer (40 mM Tris, 40 mM acetate, 1 mM EDTA pH 8.0). DNA was visualised by UV transillumination after being stained with Gel Red (Biotium).

2.7 TYPING METHODS

The primer sequences used in molecular typing assays of rotavirus and *C. difficile* are described in Table 2-6. The rotavirus VP7-F deg primer sequence (Gomara *et al.*, 2001) was modified to contain degenerate bases to allow for differences in sequences from Genbank (www.ncbi.nlm.nih.gov/genbank/) of the gene encoding VP7 of equine and human rotaviruses.

2.7.1 One-step RT-PCR for rotavirus G typing

For rotavirus G typing, 4 µl of RNA was denatured at 97°C for 4 mins in the presence of 0.6 µM of primers VP7-Fdeg/VP7-Rdeg or Beg9/End9 (Table 2-6), then immediately cooled on ice. The RT-PCR was then conducted as per the protocol provided by Carl Kirkwood, WHO Rotavirus Collaborating Center, Melbourne with the Qiagen OneStep RT-PCR Kit in a 30 µl reaction mixture volume, under the following cycling conditions: 30 mins at 42°C, followed by inactivation of the reverse transcriptase and activation of the DNA polymerase at 95°C for 15 mins, 40 cycles of denaturation at 94°C for 30 seconds, annealing at 42°C for 30 seconds and extension at 72°C for 1 min, with a final extension for 7 mins at 72°C.

VP7 RT-PCR products (from primer sets VP7-Fdeg/Rdeg and Beg9End9) were excised from a 1% w/v agarose gel after electrophoresis, purified (Section 2.2.6) and then sequenced (Section 2.5). The sequences obtained were compared to each other and to representative EqRV VP7 sequences. Sequence alignments were used to generate an unrooted maximum-likelihood phylogenetic tree using Geneious® (version 9.0.5 Kearse *et al.* (2012)).

2.7.2 Pulse field gel electrophoresis

Pulse field gel electrophoresis (PFGE) was performed on isolates of *Salmonella* identified by latex agglutination and biochemical testing (Section 2.6.2).

Table 2-6 Primer sequences for typing assays

Target / Purpose	Amplicon (bp)	Primer name	Sequence (5'-3')	Reference
Rotavirus				
VP7/G typing	1,062	Beg9	GGCTTTAAAAGAGAGAATTTCCGTCTGG	Gouvea *et al.* (1990)
		End9	GGTCACATCATACAATTCTAATCTAAG	
	884	VP7-Fdeg	ATGTATGGTATTGAATRTACCRC*	Gomara *et al.* (2001) modified
		VP7-Rdeg	AACTTGCCACCATYTYTTCC**	World Health Organization (2009)
Clostridium difficile				
cdtB / detection of binary toxin	510	*cdtB*pos	CTTAATGCAAGTAAATACTGAG	Stubbs *et al.* (2000)
		*cdtB*rev	AACGGATCTCTTGCTTCAGTC	
A3 fragment of *tcdA* /toxinotyping	3,100	A3C	TATTGATAGCACCTGATTTATATACAAG	Rupnik *et al.* (1997)
		A4N	TTATCAAACATATATTTTAGCCATATATC	
B1 fragment of *tcdB* /toxinotyping	3,100	B1C	AGAAAATTTTATGAGTTTAGTTAATAGAAA	
		B2N	CAGATAATGTAGGAAGTAAGTCTATAG	
tcdC / detection of deletions	718	C1	TTAATTAATTTTCTCTACAGCTATCC	Spigaglia and Mastrantonio (2002)
		C2	TCTAATAAAAGGGAGATTGTATTATG	
PaLoc absence in non-toxigenic isolates	115	Lok3	TTTACCAGAAAAAGTAGCTTTAA	Braun *et al.* (1996)
		Lok1	AAAATATACTGCACATCTGTATAC	
16s - 23s rRNA / ribotyping	225-700	16S	GTGCGGCTGGATCACCTCCT	Bidet *et al.* (1999)
		23S	CCCTGCACCCTTAATAACTTGACC	

*R = A or G

**Y = C or T

2.7.3 Preparation of bacterial cells

To propagate *Salmonella* bacterial cells for PFGE, a single colony from an agar plate was inoculated into 5 ml of LB broth and incubated overnight at 37°C in a Ratek OM25 orbital mixer incubator.

Bacterial cells were harvested from 1 ml of broth by centrifugation at 16,000 x *g* for 1 min, and washed by resuspending in 1 ml of Tris/NaCl buffer (1 M NaCl, 10 mM Tris pH7.6). The centrifugation was repeated and the final resuspension was in 800 µl of Tris/NaCl buffer.

2.7.3.1 Preparation of agarose-embedded bacterial DNA plugs

A 2% w/v agarose solution was prepared by dissolving low melting point agarose (Seaplaque, FMC) in Tris/NaCl buffer in a 50°C incubator. An equal volume of agarose solution was added to 300 µl of bacterial cell suspension in a microcentrifuge tube and mixed gently. Volumes of 100 µl of this suspension were then dispensed into six wells of a PFGE plug mould. The mould was then placed on ice for 10 mins to allow the agarose to solidify.

Solidified agarose plugs were then expelled from the mould into individual microcentrifuge tubes containing 1 ml of cell lysis buffer (1 M NaCl, 6 mM Tris-HCl pH7.6, 100 mM EDTA pH 7.6) , 1% w/v N-lauroyl-sarcosine (Sigma) and 1 mg/ml of lysozyme (Boehringer Mannheim). Plugs were then incubated at 37°C overnight.

Following overnight incubation, cell lysis buffer and lysozyme were removed from the microcentrifuge tube and replaced with 1 ml of EDTA–sarcosine-proteinase (ESP) buffer (0.5 M EDTA pH 9.2, 1% w/v N-lauroyl-sarcosine, and 1 mg/ml proteinase K (Boehringer Mannheim)). Plugs were then incubated in ESP buffer for 4 days at 50°C.

ESP buffer was removed from each tube and plugs were washed six times for 30 mins in 1 ml TE buffer (10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0) at room temperature. Plugs were then stored in 1 ml of 0.25 M EDTA pH 8.0 at 4°C.

2.7.3.2 Restriction Enzyme digestion of plugs

One mm slices of each plug were cut and transferred to a microcentrifuge tube containing 100 µl of NEBuffer 4 (New England BioLabs) and incubated at room temperature for 30 mins. Buffer was then discarded and the slice resuspended in 100 µl of digestion solution containing NEBuffer 4, 10 µg BSA (New England BioLabs)

and 20 U of XbaI restriction endonuclease (New England BioLabs). Slices were then digested at 4°C for 1 hr followed by 37°C overnight.

2.7.3.3 Gel electrophoresis

DNA grade agarose (Progen Biosciences) was dissolved in TBE buffer and poured into a CHEF-DR III gel casting stand (Bio-Rad) to prepare a 1% w/v agarose gel. Once the gel solidified, the comb was removed and 50 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) was pipetted into each well. A lambda ladder DNA size standard 0.05 to 1 Mb (BioRad) and the digested plug slices were then carefully loaded into the wells using a spatula, taking care not to introduce air bubbles. Warm 1% agarose was then pipetted over the slices to seal the wells.

The levelled CHER-DR III electrophoresis chamber was then filled with 2 L of TBE buffer and cooled to 14°C. Once the TBE cooled to 14°C the gel was removed from the casting stand and placed into the frame in the chamber, ensuring the top of the gel was at least 2 mm below the surface of the buffer.

Once the gel was submerged in buffer, 5 µl of lambda DNA/HindIII Marker (Fermentas) was loaded into a single well to assess the effectiveness of electrophoresis.

Pulse field gel electrophoresis separation of XbaI digested fragments was performed using a contour-clamped homogenous electrophoresis field (CHEF) system with a field strength of 6 V/cm, a 120° included angle and a pulse time ramped from 2 to 50 seconds over 22 hours.

2.7.3.4 Staining and visualisation of PFGE pattern

To stain DNA for visualisation the gel was immersed in an ethidium bath containing distilled water and 0.5 µg/ml ethidium bromide for 45 mins, followed by 30 mins destaining in distilled water alone. The DNA bands were then visualised and imaged by UV transillumination using the ChemiDoc XRS+ molecular imager (BioRad).

2.7.4 Serotyping of *Salmonella* isolates

An isolate representative of each PFGE pattern was sent to the Microbiological Diagnostic Unit, Public Health Laboratory, The University of Melbourne for serotyping and phage typing.

2.7.5 Toxinotyping of *C. difficile* isolates

C. difficile isolates from this study were toxinotyped by the Department of Microbiology, Monash University, Clayton, Victoria. This involved PCR-RFLP analysis of fragments of the genes encoding toxin A (*tcdA*) and B (*tcdB*) and comparison of restriction enzyme digestion patterns as described by Rupnik *et al.* (1997). As the differences between strains are predominantly found in the 5' third of the *tcdB* (B1 fragment) and the 3' third of *tcdA* (A3 fragment) only the primer sets B1C/B2N and A3C/A4N (Table 2-6) were used.

If a product was detected, restriction digestion was performed. A3 fragments were digested with EcoRI (New England Biolabs) and B1 fragments with HincII and AccI (New England Biolabs). The digested products were separated by electrophoresis in a 1% w/v agarose gel in TAE buffer and the restriction fragment length polymorphisms compared to those of known toxinotypes (http://www.mf.uni-mb.si/tox/). *C. difficile* strains VPI10463, M7404, JGS6133 were used as positive controls for toxinotypes 0, III and V respectively (Table 2-4).

2.7.6 Ribotyping of *C. difficile* isolates

Using genomic DNA (Section 2.2.4) as template, *C. difficile* isolates were ribotyped in Dr. Weese's laboratory, The Department of Pathobiology, Ontario Veterinary College, University of Guelph, Ontario, Canada. Briefly, the PCR-ribotyping method used primer 16S and 23S (Table 2-6) to amplify the intergenic spacer regions between the 16S and the 23S rRNA genes as previously described by Bidet *et al.* (1999) except the DNA fragments were separated by electrophoresis in 1.5% agarose (UltraPure Agarose-1000, Invitrogen) in TBE and visualised under UV light after staining with Gel Red (Biotium) for 30 mins.

Ribotype banding patterns were visually compared to the laboratory's ribotype reference library of animal and human isolates to assign internationally recognised numerical nomenclature. Unique ribotype banding patterns were assigned an internal laboratory designation using the prefix KB.

2.7.7 Characterisation of *C. difficile* isolates

To further characterise the toxigenicity of the *C. difficile* isolates, three PCR assays were conducted.

2.7.7.1 PCR assay targeting *cdtB* gene

Primers *cdtB*pos and *cdtB* rev (Table 2-6) were used to detect the gene encoding the binding component of binary toxin (*cdtB*) located in a separate region of the chromosome to PaLoc known as CdtLoc. The presence of this gene correlates with toxinotype and ribotype.

2.7.7.2 PCR assay targeting *tcdC* gene

The *tcdC* gene, which is a negative regulator of *tcdA* and *tcdB* expression located in the pathogenicity locus, was detected with primers C1 and C2 (Table 2-6). Genetic variability in the *tcdC* gene has been detected in strains with high levels of cytotoxicity.

2.7.7.3 PCR assay to detect the absence of PaLoc

The absence of PaLoc in non-toxigenic isolates of *C. difficile* was confirmed with primers Lok3 and Lok1 (Table 2-6). These isolates do not generate a product in the toxinotyping assay (targets genes in PaLoc) but can be ribotyped (16s).

PCR assays were performed using between 20-100 ng of template DNA (Section 2.2.4) in individual reactions containing buffer (Roche), 0.5 μM of primers *cdtB*pos/*cdtB*rev, C1/C2 or Lok3/Lok1 (Table 2-6), 200 μM each dNTP and 1.25 U Taq polymerase (Roche) made up to a final volume of 20 μl with water.

Cycling conditions involved an initial denaturation cycle at 95°C for 5 mins followed by 30 cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 52°C (*cdtB* and Lok3/1 assay) or 50°C (*tcdC* assay), and extension for 1 min at 72°C with a final additional 10 min extension period at 72°C. PCR products were separated by electrophoresis on a 0.8% (w/v) agarose gel in TAE buffer. DNA was stained using Gel Red (Biotium) and visualised by UV transillumination

CHAPTER 3 FOAL DIARRHOEA IN THE FARM SETTING

- A MATCHED CASE CONTROL STUDY

3.1 INTRODUCTION

Diarrhoea is one of the most common infectious diseases to affect young foals (Cohen, 1994; Wohlfender *et al.*, 2009). Numerous bacterial, viral, protozoan and parasitic agents have been detected in foals with diarrhoea, however, the pathogenic importance of many of these agents is poorly understood.

Numerous case reports and studies have investigated aspects of individual diarrhoeal pathogens of foals, however, there are relatively few in-depth survey studies that investigated a comprehensive range of foal diarrhoea pathogens (Browning *et al.*, 1991b; Dwyer *et al.*, 1990b; Frederick *et al.*, 2009; Netherwood *et al.*, 1996b; Slovis *et al.*, 2014). Studies including control foals are also limited (Browning *et al.*, 1991b; Holland *et al.*, 1991; Netherwood *et al.*, 1996b; Slovis *et al.*, 2014) and, when included, the selection of these control animals has often been by convenience sampling. Therefore, with no temporal or spatial relationship between case and control animals, it has been difficult to draw robust conclusions from these studies.

In addition, there have been no previous studies investigating a range of infectious causes of diarrhoea in foals in Australia. In contrast to thoroughbred breeding conditions in the northern hemisphere where mares and foals are often housed in stables, mares due to foal on thoroughbred stud farms in Australia are typically housed in large paddocks close to the foaling unit where they can be closely monitored until they show signs of imminent foaling. At this point they are typically moved to a smaller yard on their own to foal down. The mare and foal then tend to be housed in small yards or paddocks and only stabled for conditions requiring confinement or medical treatment. These different management practices may influence the selection pressures and disease dynamics of infectious diarrhoea in Australian foals. For reference, in Australia, the thoroughbred breeding season commences on the 1st September resulting in foals being born from the 1st August generally until early December.

The purpose of the present study was to conduct a case control study to investigate the association between the detection of an infectious agent and the presence of diarrhoea. The results and conclusions from this study could be used to further elucidate pathogen epidemiology and lead to the development of improved disease prevention and control strategies.

3.2 STUDY DESIGN

A prospective 1:1 matched case control study of diarrhoea in foals was designed and conducted on five thoroughbred breeding farms in close geographical proximity. Faecal samples were collected from foals with diarrhoea and an age matched control foal on the same farm between September and December during the 2010 foaling season.

3.2.1 Case definition

A case was defined as any foal with evidence of diarrhoea on the day of sampling, irrespective of whether the foal had received treatment for diarrhoea. Evidence of diarrhoea included observation of the passage of fluid faeces, and/or wet faecal material on the tail, perineum or hind legs. Daily collection of faecal samples from case foals for the duration of the diarrhoea episode was included where possible.

3.2.2 Control definition

Each case foal was matched with one control foal based on age, farm and time of sampling. A control was defined as a foal without diarrhoea on the day of sampling, with no known history of diarrhoea in the week preceding sampling, in residence on the same farm as the case foal, and born within 7 days of the case foal. A single faecal sample was collected from each control foal within seven days of sampling the diarrhoea case. If diarrhoea was observed in a control foal in the week following sampling, the control sample was excluded from the matching analysis and if possible an alternative control foal selected and sampled. If no age-matched-control was available on a particular property, a sample was collected from the next nearest-by-age eligible control foal on that farm.

3.2.3 Farm population and sample size

This study was conducted in the Hunter Valley in NSW, as this region has a high concentration of thoroughbred breeding farms, allowing access to a large number of foals in a relatively small geographical area. Sample size calculations using a two independent

proportions power analysis, showed 203 matched case-control foal pairs needed to be sampled to detect an odds ratio of 3 or greater, at a confidence level of 95% and a power of 80%, assuming a 4% prevalence of all four infectious agents in the control group.

Five thoroughbred breeding farms (designated A-E) located within a 100 km radius of the town of Scone were selected. These five farms had a population of 944 pregnant broodmares during the 2010 breeding season (Table 3-1). The number of pregnant broodmares on each recruited farm ranged between 114 and 297. The properties ranged in size from 412 to 3,237 hectares. Of note, farm A only housed mares owned by the stud, while visiting mares foaled on a separate property and these horses were not involved in the study. The other farms had a mixed population of resident mares owned by the stud and non-resident agisted mares. Farm B routinely administered neomycin/penicillin within 24 hours of birth while farm C was the only farm that did not routinely vaccinate mares for rotavirus in late gestation.

Table 3-1 Details in 2010 of the five thoroughbred breeding farms recruited to this study

Farm	Farm area[a]	No. foaling mares	Comments
A	412	136	Only housed mares owned by stud farm
B	775	114	Foals routinely administered neomycin/penicillin antibiotics at birth
C	902	297	Mares not routinely vaccinated against Rotaviruses
D	1,010	161	Mare colostrum quality data not available
E	3,237	236	

[a]hectares

3.2.4 Farm records

Information was obtained from farm records and the Australian studbook (http://www.studbook.org.au) to assess if any factors were associated with a foal being a case or a control. This information included foal date of birth, gender, serum IgG level 12-24 hours post-partum, mare age, last date of service, and parity. Gestation length was calculated from the interval between the last date the mare was serviced by the stallion and the foal date of birth.

Data on colostrum quality were available on all farms except for farm D. Colostrum quality was measured indirectly with a refractometer, calibrated in the Brix scale, where values were

read as a percentage between 14 - 32%. Data were also recorded on whether foals were supplemented with stored freeze-thawed colostrum.

3.2.5 Statistics

Descriptive statistics reported the mean for exposure variables with a normal distribution and the distribution was compared using independent t-tests if there were two independent samples, or a One-way ANOVA if there were more than two independent samples.

The median was reported for exposure variables with a non-normal distribution and the distribution compared using non parametric tests. For two independent samples a Mann-Whitney U test was used, while a Kruskal Wallis test was used if there were more than two independent samples.

A matched pair analysis was performed to estimate if exposure to a risk factor was associated with being a case or a control foal. This analysis described each age-matched case-control pair as either concordant or discordant (Table 3-2). An age-matched pair was concordant when both the case and control foal in the pair had the same exposure status. For example, both the case and the control foal were positive, or both were negative for a particular exposure variable. An age-matched pair was discordant when the exposure status of the case was different to the exposure status of the control. In this scenario, if the case was positive for exposure, the control was negative for exposure, or if the case was negative for exposure, the control was positive for exposure. The ratio of discordant pairs was then used to calculate an odds ratio based on the Fisher's Exact test. If an observation was missing for either a case or a control, the pair was excluded from the analysis. Age, farm and time of sampling variables were not analysed as these were constant within each matched set.

Table 3-2 Exposure status of cases and controls for matched pair analysis

Matched pair analysis		Case	Control
Exposure	Concordant	+	+
		−	−
	Discordant	−	+
		+	−

For all statistical tests the level of significance was set at P ≤0.05. Statistical analysis was performed in SPSS Statistics Version 22.0 (IBM Corp., NY) and OpenEpi, Version 3.03a.

3.3 DESCRIPTIVE RESULTS

3.3.1 Samples collected

During the study period, 168 case foals were sampled. Fifty one case foals were excluded from the matched case-control analysis because no control foal was available. Therefore, there were 117 1:1 age and farm matched case-control pairs sampled in this study.

Daily faecal sample collection from case foals for the duration of each episode of diarrhoea was included where possible, with multiple faecal samples collected from 19 of the 117 matched case foals. In total, 51 samples were collected from these 19 foals (Figure 3-1). For the purposes of analysis, the foal was considered positive if a microorganism was detected in any faecal sample collected during the episode of diarrhoea.

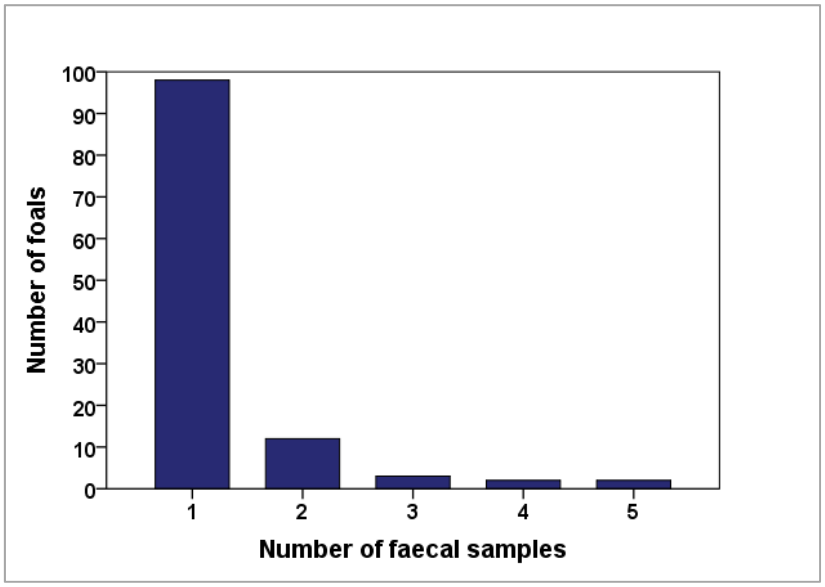

Figure 3-1 Number of foal faecal samples collected per episode of diarrhoea

While the selection criteria for a control foal were that it be born within 7 days of the case foal and sampled within 7 days of the case foal, this was not possible in seven instances and so the next nearest foal in age or time of sampling was selected. In relation to age difference, this resulted in two control foals with age differences of 8 days, one with an age difference of 9 days and another with a difference of 10 days from the corresponding case foal. In

relation to sampling time, two control foals were sampled within 8 days and one within 9 days of the respective case foal.

The gender of the matched foals sampled included 132 females and 102 males. Sixty percent (70/117) of case foals and 53% (62/117) of control foals were females (Figure 3-2).

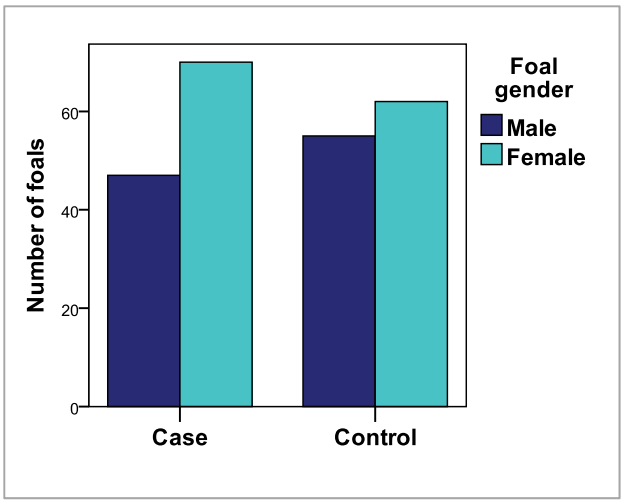

Figure 3-2 Gender distribution of case and control foals

Case foals were sampled between September and December 2010 (Table 3-3). The greatest number of samples were collected in October (n=49).

Table 3-3 Number of matched case foals sampled per month on each farm during the study period

Farm	September	October	November	December	Total cases (Total foals born on farm)
A	9	14	10	4	37 (136)
B	2	1	4	3	10 (114)
C	7	21	8	0	36 (297)
D	2	2	4	0	8 (161)
E	3	10	8	5	26 (236)
Total	23	49	33	12	117 (944)

3.3.2 Foal age

The age of case foals included in the matched analysis ranged from 1 day to 111 days of age with a median age of 22 days. The age of control foals ranged from 2 to 109 days of age with

a median age of 23 days. The majority of case foals were aged less than one month at the time of sampling (Figure 3-3).

There was some variation in the age distribution of case foals sampled on each farm (Figure 3-4). In particular, the median age at the time of sampling of case foals was 11 days on farm C and 52 days on farm B. On farms A, D and E the median age at the time of sampling of case foals was 26, 25 and 27 days respectively. A pairwise comparison of the median age of case foals by farm showed case foals sampled on farm C were significantly younger than case foals from farms A, B, D and E ($P \leq 0.001$, Kruskal Wallis test).

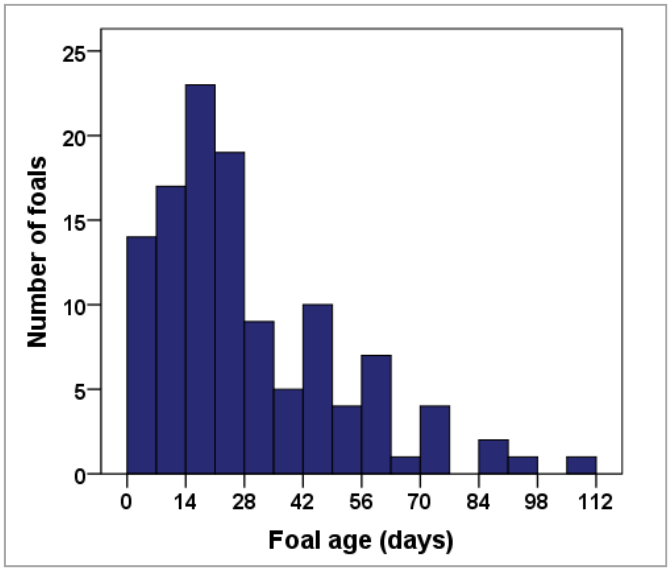

Figure 3-3 Age distribution of matched diarrhoea case foals

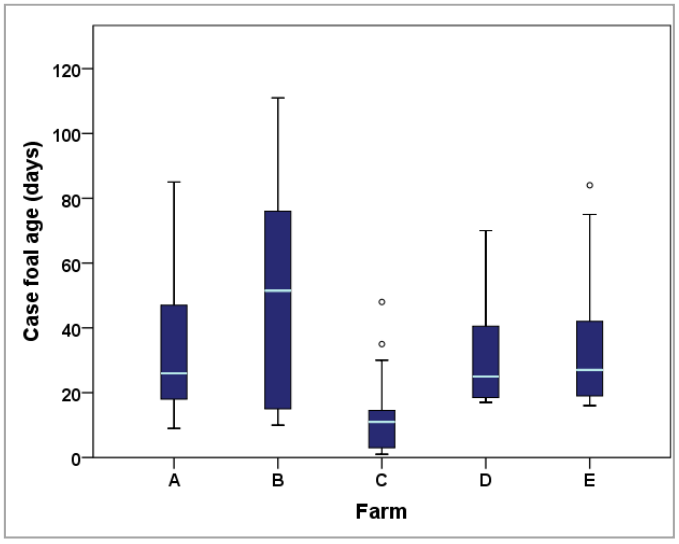

Figure 3-4 Age of diarrhoea case foals by farm. The median is represented by the horizontal light blue line, the interquartile range by the box and whiskers, and the outliers by open circles.

3.3.3 Colostrum quality and passive transfer of immunity

Foal serum IgG concentration was >8 g/L in 114/117 (97.4%) control foals and 113/117 (96.6%) case foals. Partial failure of passive transfer (IgG 4 - 8 g/L) was recorded for one control foal and three case foals, including one case foal sampled with diarrhoea on two separate occasions. The dams of two of these foals had been noted to have had clinical evidence of premature placental separation by the attending staff. The other two foals were born to mares with low quality colostrum (15% and 16%) and were supplemented with colostrum of a higher quality (29% and 32%) from a colostrum bank. One further control foal had complete failure of passive transfer (IgG <2 g/L) and was subjected to dystocia and experienced hypoxia during birth resulting in hospitalisation. All seven foals with partial and complete failure of passive transfer were supplemented with intravenous plasma. Post-partum IgG results were not available for one control foal as it was born on a farm not enrolled in the study and then subsequently moved to farm C with its dam.

Foals born to mares with lower quality colostrum were often supplemented with stored colostrum of a higher quality. There was no colostrum refractometer reading that was strictly adhered to as a cut off for supplementing foals on each farm or across the farms (Figure 3-5). Overall, foals born to mares with a colostrum refractometer reading of >26% were not supplemented with stored colostrum. Supplementation of foals born to mares with a colostrum refractometer reading of <26% varied on each farm (

Figure 3-6). Colostrum refractometer readings were not available for four mares.

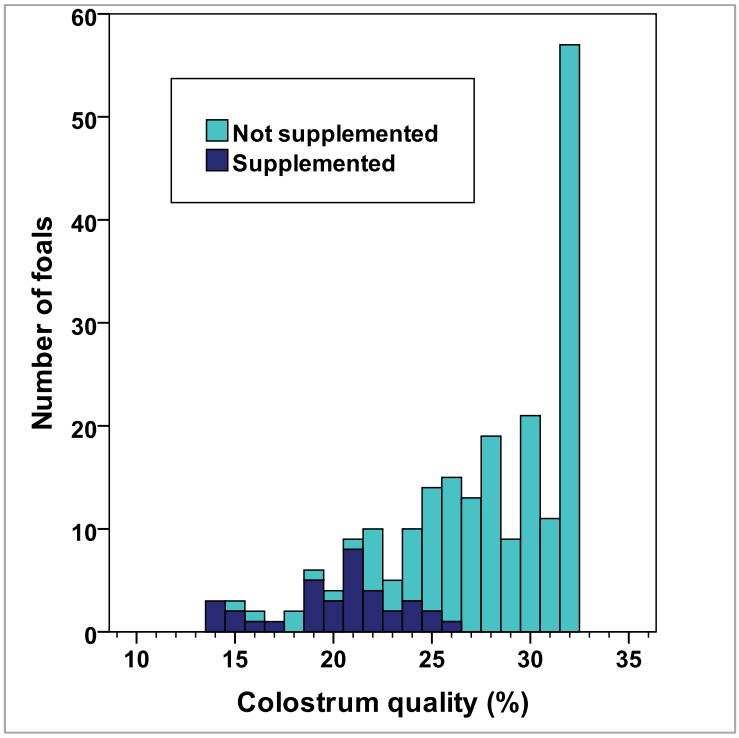

Figure 3-5 Dam colostrum quality and supplementation of foals with stored colostrum

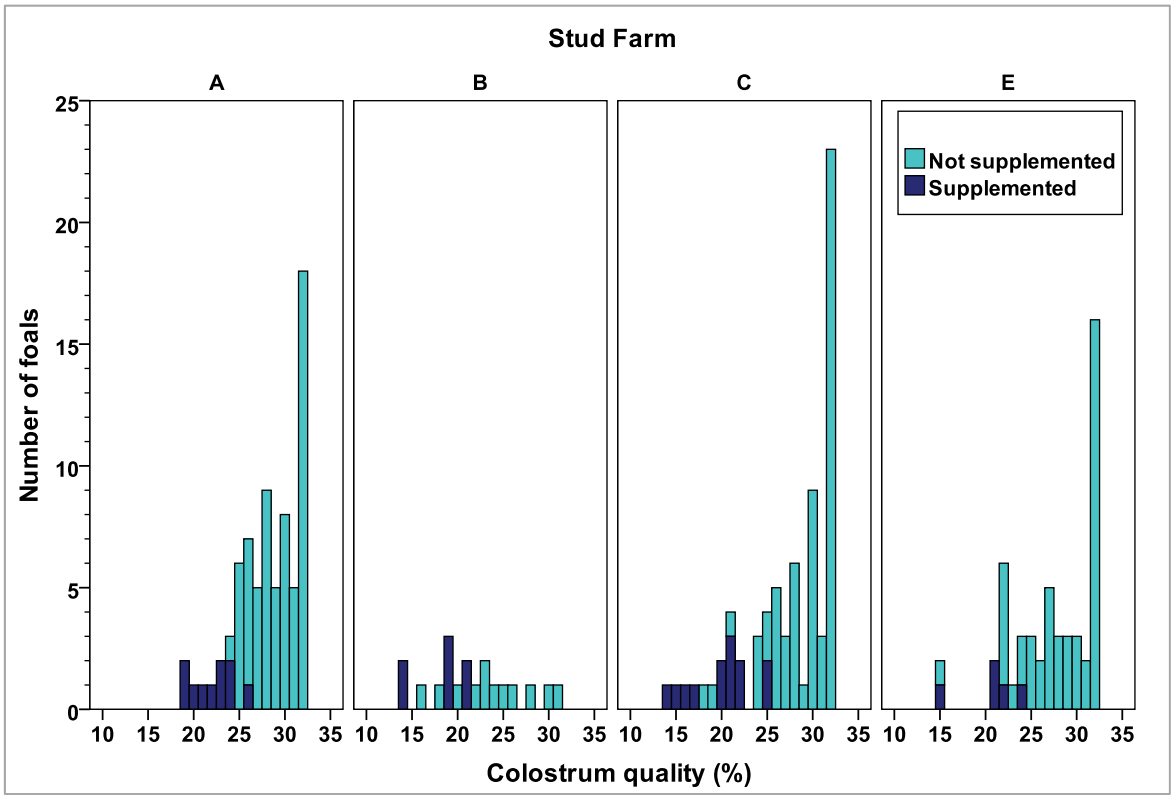

Figure 3-6 Dam colostrum quality and foal supplementation with stored colostrum by farm (Data not available for farm D)

3.3.4 Mare age

The dams of case foals ranged in age from 4 - 22 years with a median of 7 years (Figure 3-7 A). The dams of control foals ranged in age from 4 - 21 years with a median of 8 years (Figure 3-7 B). The median age of dams of case foals and dams of control foals was not significantly different (P=0.170, Mann-Whitney U test).

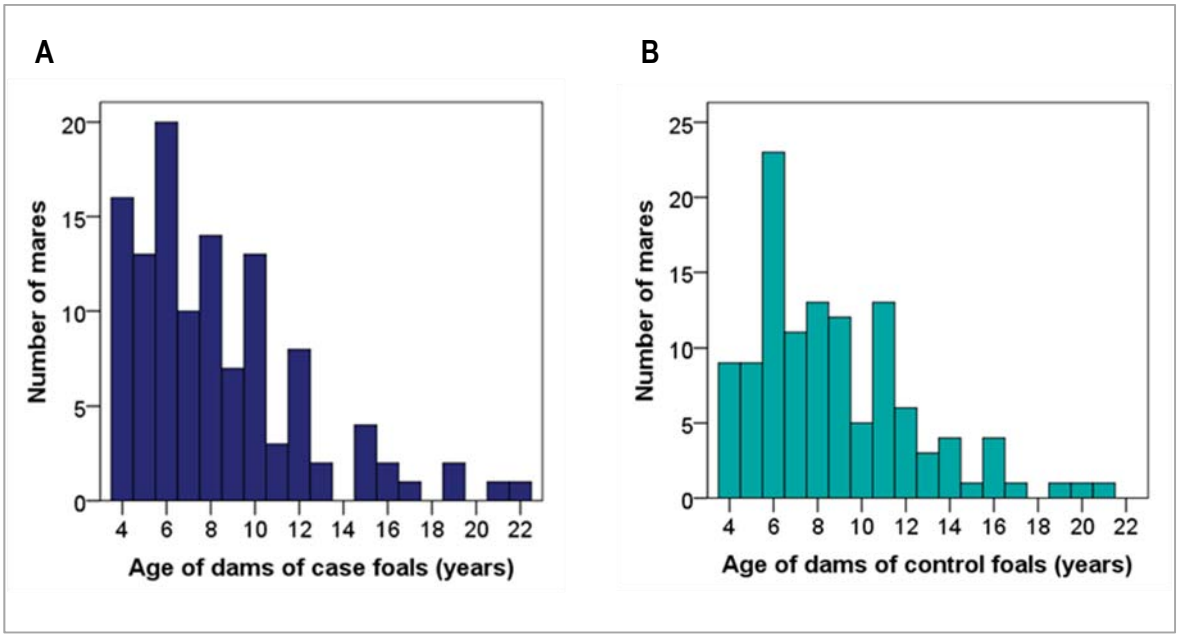

Figure 3-7 Age distribution of (A) dams of case foals and (B) dams of control foals

When the age of dams by farm was considered, the median age of dams of case foals was not significantly different between farms (Figure 3-8 A). In contrast a pair wise comparison of the median age of dams of control foals shows dams of control foals on farm B were significantly older than dams of control foals on farm C (P=0.003, Kruskal Wallis test) and farm A (P=0.012, Kruskal Wallis test), but not farm D and farm E (Figure 3-8 B).

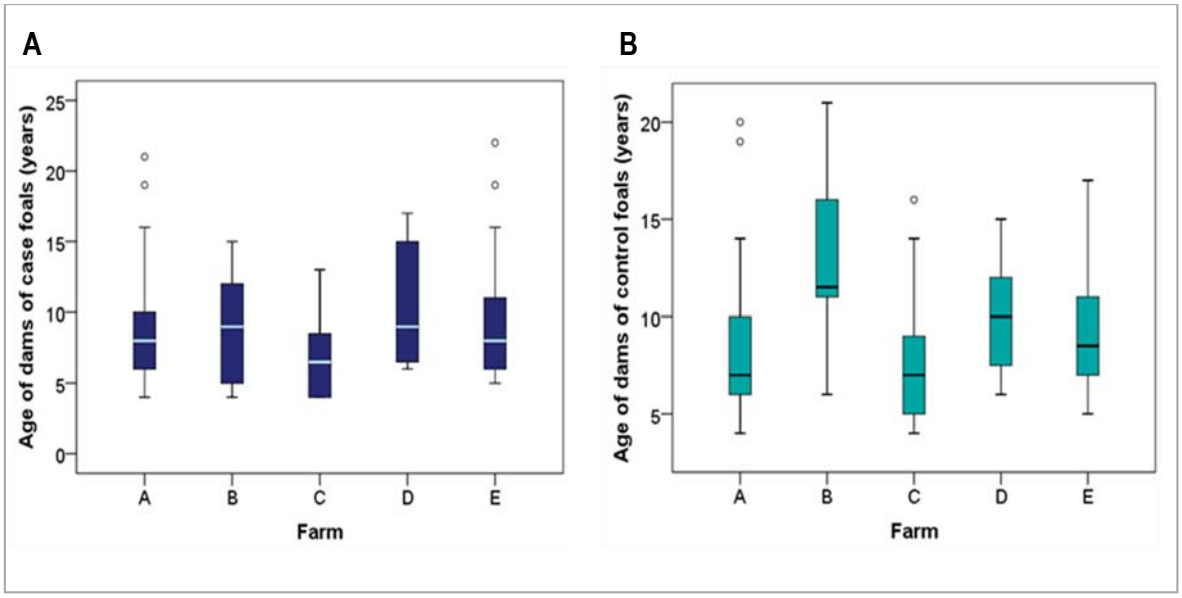

Figure 3-8 Age distribution by farm of (A) dams of case foals and (B) dams of control foals

3.3.5 Mare colostrum quality

The colostrum quality of dams of case foals and dams of control foals ranged from 14 to 32% and both groups had a median of 28% (Figure 3-9). The median colostrum quality of dams of case foals and dams of control foals was not significantly different (P=0.896, Mann-Whitney U test).

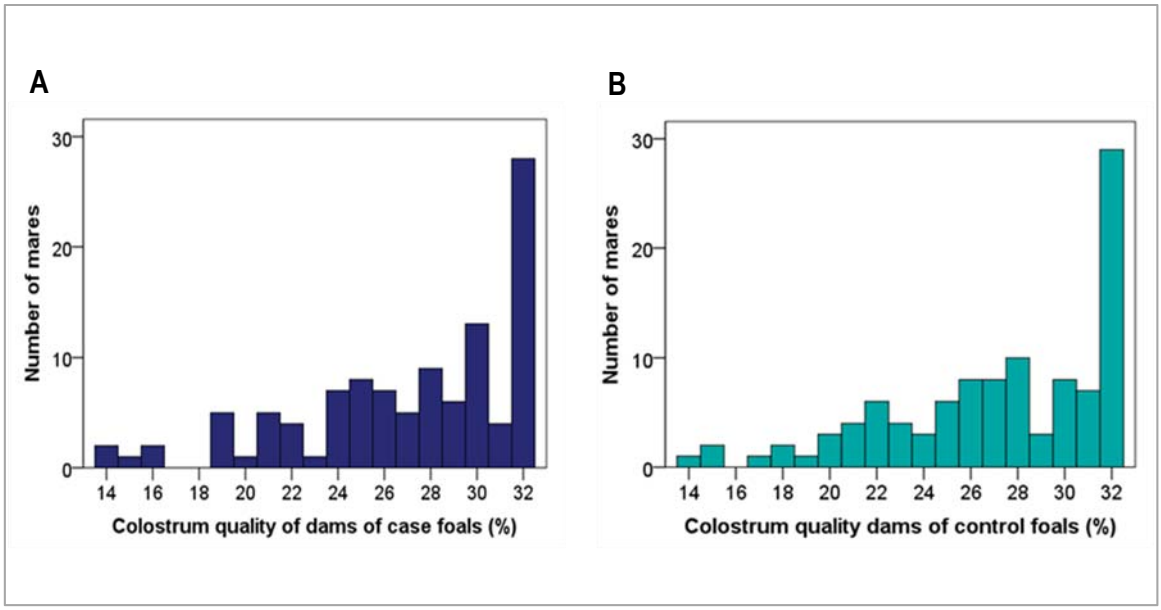

Figure 3-9 Colostrum quality distribution of (A) dams of case foals and (B) dams of control foals

When the colostrum quality of dams by farm was considered, the median colostrum quality of dams of case foals was 19% on farm B, while the median ranged between 27% and 30% on the other three farms with available data (Figure 3-10 A). A pairwise comparison of the median colostrum quality of dams of case foals on farm B was significantly lower than farm A (P=0.001), farm C (P<0.001) and farm E (P=0.028, Kruskal Wallis test).

The median colostrum quality of dams of control foals was lower on farm B (23%) than on the other three farms (27 - 31%), but was not significantly different (P=0.058, Kruskal Wallis test) (Figure 3-10 B).

When comparing the colostrum quality on farm B alone, the dams of control foals had a median colostrum quality of 23%, while dams of case foals had a median colostrum quality of 19% (Figure 3-10). This difference was significantly different (P=0.049, Mann-Whitney U test).

The range of colostrum readings in dams of case foals on farm B was 14 - 26%, while the range of colostrum readings in dams of case foals on farms A, C and E was 19 - 32%, 16 - 32% and 15 - 32%, respectively. The range of colostrum readings in dams of control foals for farms A, B, C and D was 19 - 32%, 18 - 31%, 14 - 32% and 15 - 32%, respectively.

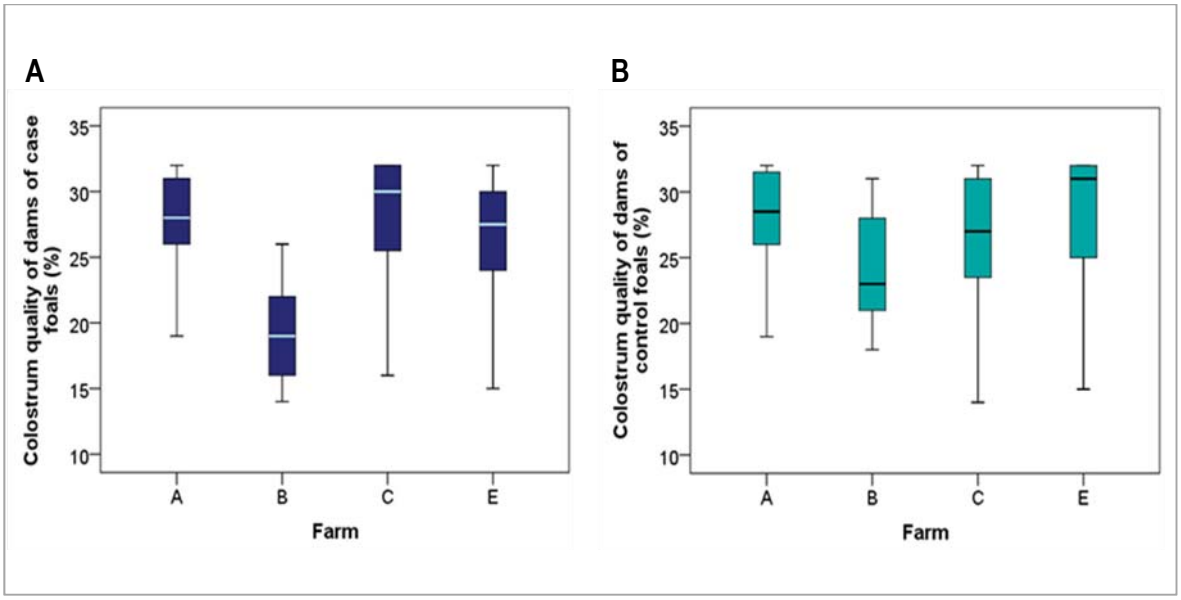

Figure 3-10 Colostrum quality distribution by farm of (A) dams of case foals and (B) dams of control foals

3.3.6 Gestation length

The gestation length of case foals ranged between 326 and 363 days (mean 343 days) while the gestation length of control foals ranged between 326 and 367 days (mean 344 days) (Figure 3-11). The mean gestation length of case foals was not significantly different from control foals (t(232) = -0.805, P=0.422, Independent t-test). Similarly, when the mean gestation length of case foals and control foals was compared by farm there was no significant difference (F(4, 112) = 0.754, P=0.558, One-way ANOVA) (Figure 3-12).

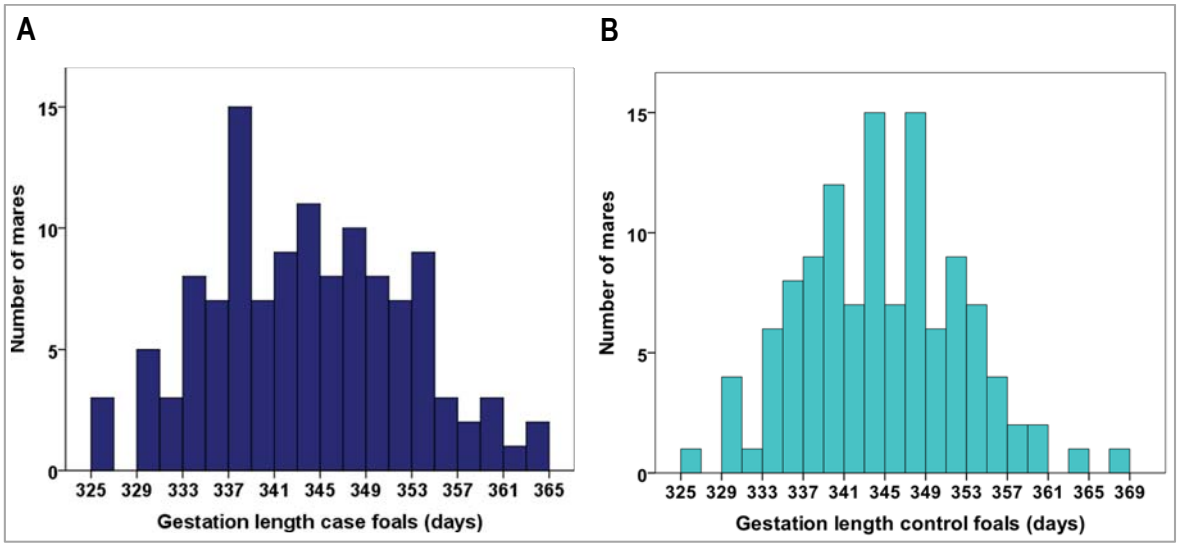

Figure 3-11 Gestation length distribution of (A) dams of case foals and (B) dams of control foals

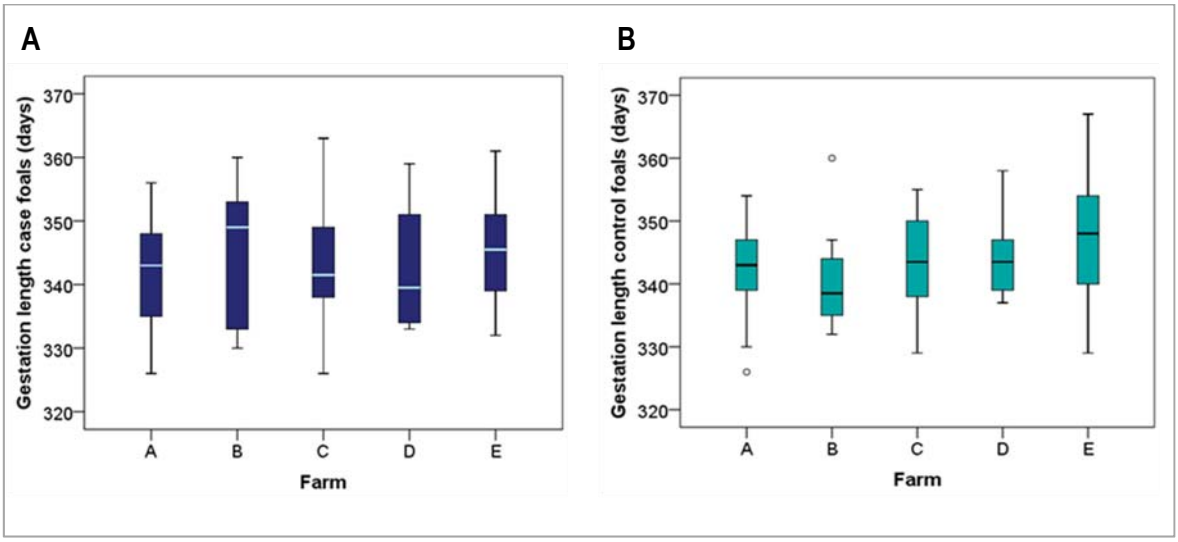

Figure 3-12 Gestation length distribution by farm of (A) dams of case foals and (B) dams of control foals

Overall, the age of the mare, the quality of colostrum and the gestational length were not significantly different between dams of foals with and without diarrhoea (Table 3-4).

Table 3-4 Summary of variables associated with dams of foals with diarrhoea and dams of foals without diarrhoea

Variable		Mean	Median	Range	P-value
Age of dam (yrs)	Cases	8	7	4 - 22	0.170[a]
	Controls	9	8	4 - 21	
Colostrum quality of dam (%)	Cases	27	28	14 - 32	0.896[a]
	Controls	27	28	14 - 32	
Gestation length (days)	Cases	343	343	326 - 363	0.422[b]
	Controls	344	344	326 - 367	

[a] Mann-Whitney U test of significance

[b] Independent t-test of significance

3.4 MATCHED ANALYSIS RESULTS

A matched pair analysis of risk factors such as foal gender, parity of the dam, age of the dam, dam colostrum quality and gestation length was conducted (Table 3-5). There was no significant association found between any of these variables and the likelihood of a foal being a case or a control ($P>0.05$).

Table 3-5 Matched analysis of risk factors for case and control foals

	In each age & farm matched pair					
Risk Factor	**Case exposed to risk factor**	**Control exposed to risk factor**		**Pair-matched OR**	**95% CI**	**P value[a]**
		yes	**no**			
Filly	yes	39	31	1.35	0.761 - 2.421	0.341
	no	23	24			
Maiden	yes	9	31	1.41	0.79 - 2.554	0.272
	no	22	55			
Mare <8 yo	yes	29	30	1.30	0.733 - 2.351	0.410
	no	23	35			
Gestation <344 d	yes	32	28	1.22	0.676 - 2.213	0.576
	no	23	34			
Colostrum <23%	yes	4	15	1.00	0.456 - 2.195	0.999
	no	15	71			

[a]P value Fisher's Exact

3.5 DISCUSSION

This study is the first prospective, matched, case-control study of this size to investigate infectious causes of diarrhoea in foals on thoroughbred breeding farms. Diarrhoea is a common cause of disease in foals and although often mild, in some instances it can be life threatening requiring veterinary intervention. In previous foal diarrhoea case reports and outbreak investigations, a large number of infectious agents have been detected; however, detection of these agents does not necessarily indicate causation.

A well designed case control study provides a method of determining if there is an association between detection of a particular infectious agent and the presence of diarrhoea. Ideally, control animals should be comparable to cases in every way except for the presence of the disease of interest. Therefore, a control subject should have the same chance of becoming classified as a case, if they had become diseased. However, this is difficult to achieve and selection of control foals is challenging. In the present study, control foals were selected to be representative of the same population, exposure factors and time at risk as the

case foals, in an attempt to determine if there was an association between the presence of an infectious agent and the presence of diarrhoea.

Currently there are a limited number of foal diarrhoea studies that include samples from control foals. Of these studies, a common limitation was that control foals were sourced from different properties or geographical regions to case foals (Browning *et al.*, 1991b; Netherwood *et al.*, 1996b; Slovis *et al.*, 2014). Only one study specifically aimed to assess the association between diarrhoea and various infectious agents by sourcing control samples from foals that were "in-contact" with case foals, as well as foals "not-in-contact" (Netherwood *et al.*, 1996b). This was to account for potential subclinical infection in the "in contact" foals. However, it is not clear in this study whether foals "not in contact" were from the same properties as the case foals. If control foals are sourced from different properties to the case foals, it is possible they were not exposed to the same infectious agents, or that conditions on that property did not favour infection. To overcome these limitations, our study matched foals by farm to reduce the variation in exposure to different environmental and managerial factors and exposure to different pathogens that may have occurred on different farms.

Another limitation of previous studies was that foals of different ages were being compared. In our study, foals were matched by age, as the susceptibility to particular pathogens may differ between neonatal foals and older foals, not only due to effects of waning maternal antibody, but also due to changes in the maturity of the foals own gastrointestinal and immune systems. Only one previous study also matched case and control foals by age, however, the sample size was too small to draw conclusions about the association between the pathogens detected and their importance in clinical disease (Traub-Dargatz *et al.*, 1988).

In addition to matching foals by farm and age, samples in this study were collected from case and control foals as close together in time as possible. This temporal matching was to control for confounding effects of time and differences in levels of exposure during the breeding season. For example, presumably a property with 30 foals resident in September has different exposure factors than the same farm with 300 foals resident in December.

As foal diarrhoea is a common disease, a disadvantage of using a matched case control study design was the difficulty recruiting eligible control foals at times, particularly around the time of foal heat. Also, as mares became pregnant again, handling of mare and foal pairs became less frequent, resulting in fewer opportunities to collect diarrhoea samples from

older foals and difficulty sourcing age matched control foals. This led to exclusion of unmatched diarrhoea samples from older foals.

Recruitment of all foals that developed diarrhoea during the study period was not possible due to different farm/staff attitudes towards what constituted a foal with diarrhoea and availability of staff with an already busy daily schedule to assist with moving mares and foals to an area safe for restraint and sample collection. Importantly, this means the prevalence of diarrhoea in the study population cannot be inferred from these data.

In regards to sample size, this study was conducted in the Hunter Valley, NSW, the epicentre of Australian thoroughbred horse breeding to maximise access to a large population of horses in an attempt to meet sample size calculations for statistical power. The collection of samples from 117 matched pairs did not meet the *a priori* sample size calculation, however, it was sufficient to detect an odds ratio of 4, with a 95% confidence interval and a power of 80% assuming an exposure level of 4% in the control foals.

Enrolment of a larger population was not possible due to the logistics and resources required to physically monitor foals and collect samples. Selection of un-matched control foals would have been easier and resulted in a larger sample set, however, the comparability of cases and controls would have been reduced and any associations between the presence of an infectious agent and diarrhoea may be confounded by other factors.

The classification of foals into those with diarrhoea and those without diarrhoea was subjective and challenging at times. Case definitions of foal diarrhoea in other studies have been broad or not provided (Browning *et al.*, 1991b; Dwyer *et al.*, 1990b; Netherwood *et al.*, 1996b; Traub-Dargatz *et al.*, 1988). One study defined foals with gastrointestinal disease as those with watery diarrhoea for >24 hours, decreased milk consumption and/or ultrasonic evidence of enterocolitis (Slovis *et al.*, 2014). In human epidemiological studies, diarrhoea may be defined as the passage of looser than normal or watery faeces three or more times in a 24 hour period, where the faecal sample takes the shape of the container in which it is collected (Gidudu *et al.*, 2011). In a large field study such as this, with 915 pregnant mares in residence during the study period, recording the number of diarrhoeic faecal samples foals passed per day was not feasible as all mares and foals could not be watched 24 hours a day. Measuring the faecal water content of samples could have been used to objectively classify them into normal or diarrhoeic faeces, however, this could not be performed stall side and would have resulted in a delay for collection of control samples.

An alternative case definition that included only sampling foals treated for diarrhoea based on the premise that foals requiring treatment for diarrhoea are more likely to be passing multiple diarrhoeic faeces per day was considered, but, rejected for two reasons. Firstly, the treatment approach varied greatly between farms, with some farms treating every foal with evidence of diarrhoea regardless of whether the foal appeared clinically ill (inappetent, depressed, febrile, dehydrated), while other farms only treated if there was clinical illness or a more prolonged duration of diarrhoea. Secondly, the opportunity to collect a faecal sample early in the disease course and detect a pathogen shed in the faeces, would be reduced if sampling was delayed until after initiation of treatment or observation of multiple diarrhoeic faeces. Additionally, treatment with antibiotics prior to collection of a faecal sample may influence the likelihood of detecting a bacterial pathogen. Therefore, we defined cases of diarrhoea as any foal with evidence of diarrhoea at the time of sampling.

To evaluate whether any factors that could influence passive transfer were having an effect on whether a foal was more or less likely to be a case foal than a control foal, data was collected on foal serum IgG levels, mare age, colostrum quality and gestation length. Foals rely on passive transfer of immunoglobulins from colostrum for protection against infectious diseases during the first few weeks and months of life, as they are essentially agammaglobulinaemic at birth (Jeffcott, 1974).

Overall, the age of dams, the quality of colostrum and the length of gestation were not significantly different between dams of foals with diarrhoea and dams of foals without diarrhoea. However, on Farm B dams of both case and control foals had lower median colostrum readings compared to the other farms. Factors previously associated with lower colostrum quality include premature lactation and increasing maternal age (Giguère and Polkes, 2005; Leblanc *et al.*, 1992). Data on premature lactation was not available in this study, while the median age of dams of control foals, but not dams of case foals, on farm B was significantly higher than the other farms, suggesting advancing maternal age could be contributing to lower quality colostrum.

Only seven foals had IgG levels consistent with failure of passive transfer and were subsequently supplemented with an intravenous source of IgG. Matched analysis showed dam colostrum quality did not contribute to the risk of a foal developing diarrhoea. This result is not surprising, as it is common practice on thoroughbred breeding farms to measure the colostrum quality of mares shortly after foaling to identify foals that may be at risk of

failure of passive transfer and allow oral supplementation with stored colostrum prior to measurement of serum IgG levels.

Similarly, the matched analysis of risk factors for case foals and control foals showed foal gender, dam parity, dam age, and gestation length were not associated with the likelihood of a foal being a case or a control. Therefore, we can compare foals with diarrhoea and foals without diarrhoea where potentially the main difference is whether or not an infectious agent is present, to determine if a particular infectious agent is truly associated with disease.

CHAPTER 4 FOAL DIARRHOEA IN AN EQUINE REFERRAL HOSPITAL

4.1 INTRODUCTION

Neonatal foals are susceptible to a number of conditions requiring hospitalisation for veterinary treatment. In a large multicentre study of hospitalised foals, diarrhoea was the most common presenting complaint (Borchers *et al.*, 2012). Treatment of hospitalised foals is often expensive and very labour intensive, while the prognosis and likelihood of survival can be difficult to predict. With the spectrum of diarrhoeal disease ranging from mild and self-limiting to severe and life threatening, determining the cause of diarrhoea helps to inform the prognosis and management.

Faecal samples from foals with diarrheoa were collected from an equine referral hospital servicing the same region. These samples were screened for the same infectious agents as the samples from the case control study (Chapter 3), however, the results were considered separately to the case-control study. This enabled comparison of the infectious agents in foals requiring hospitalisation for treatment, as these were likely those foals with more severe disease. In addition, these samples represent a foal population sourced from a wider range of stud farms in the region, providing information on what agents are circulating in the wider region.

4.1.1 Hospital case definition

Foals hospitalised at the Scone Equine Hospital for diarrhoea, or that developed diarrhoea during hospitalisation, were sampled as hospital case foals. Daily collection of faecal samples for the duration of the diarrhoea episode was included where possible. There were no control foals defined or sampled in this population. Samples were collected between September and November in 2010, the same foaling season as the collection of the farm case-control samples.

4.1.2 Hospital records

Information was obtained from hospital records and the Australian studbook on the foal's presenting complaint and treatment, date of hospital admission, date of hospital discharge, date of birth, gender, IgG result, the mare's age, last date of service, and parity. Gestation length was calculated from the interval between the mare's last date of service and the foal's date of birth.

4.2 VETERINARY HOSPITAL FOAL RESULTS

4.2.1 Samples collected

In the hospital case series faecal samples were collected from 26 foals with diarrhoea. Daily collection of faecal samples for the duration of the diarrhoea episode was not achieved for all foals. However, multiple faecal samples from an episode of diarrhoea were collected from eight hospitalised foals resulting in a total of 38 faecal samples being collected from the 26 foals (Figure 4-1). These foals came from 19 different properties, including two foals from farm A, three foals from farm C, and one foal each from farm D and farm E.

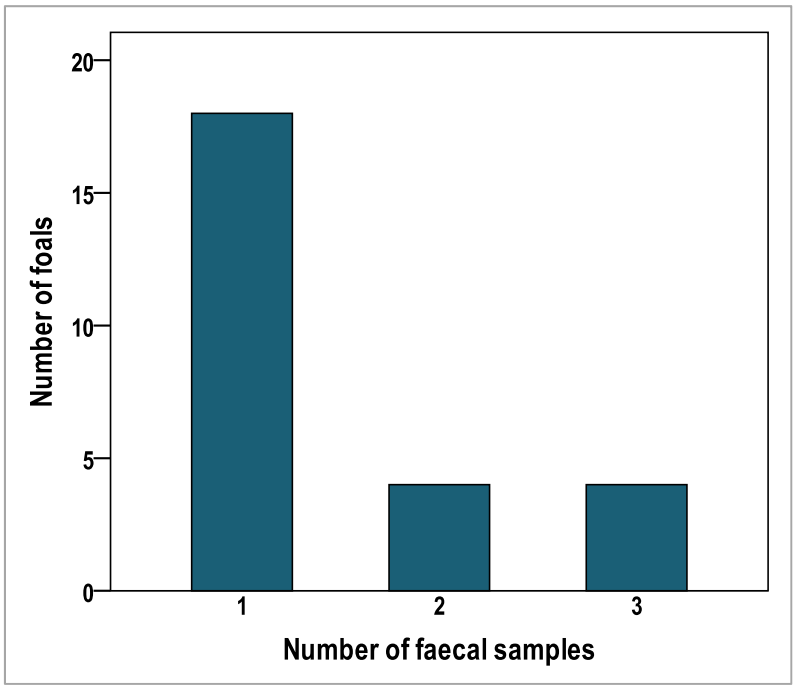

Figure 4-1 Number of foal faecal samples collected per episode of diarrhoea

In this population, there were equal numbers of fillies and colts sampled. The age of hospitalised foals sampled with diarrhoea ranged from less than 24 hrs of age up to 81 days of age, with a median age of 3.5 days. The majority (80%) of hospitalised foals sampled with diarrhoea were less than 14 days of age (Figure 4-2).

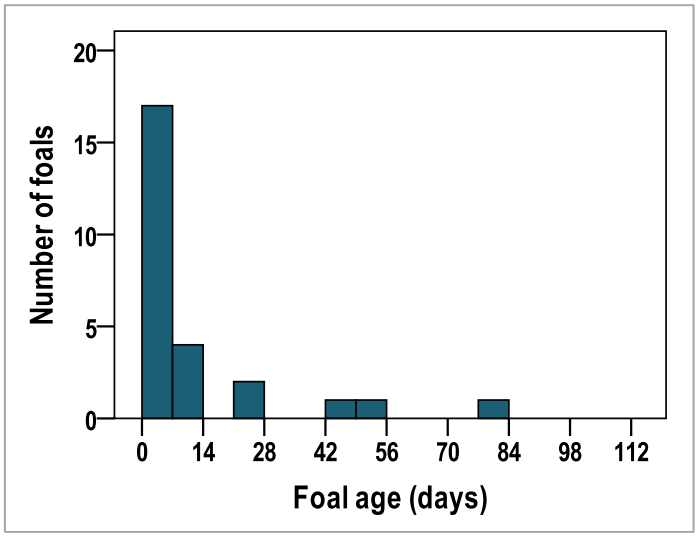

Figure 4-2 Age distribution of hospitalised foals with diarrhoea

The greatest number of faecal samples were collected from hospitalised foals in September 2010 (Figure 4-3).

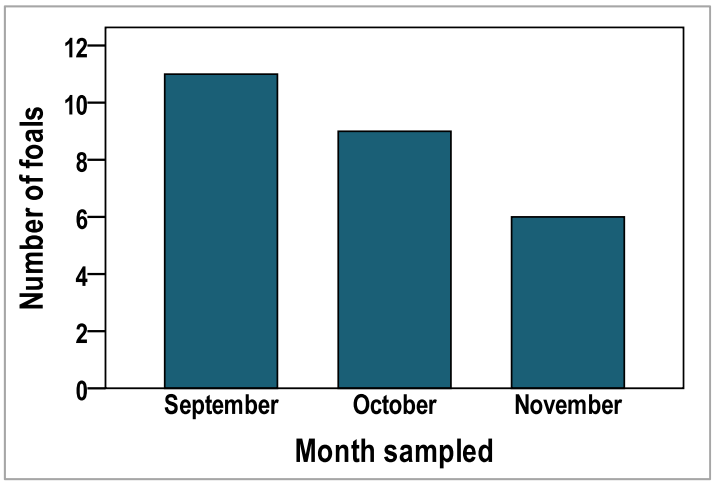

Figure 4-3 Distribution of hospitalised foals with diarrhoea by month of sample collection

Twenty three of the 26 hospitalised foals had diarrhoea at the time of admission. The presenting complaint was diarrhoea alone in 65% of foals (17/26). Six foals presented

with diarrhoea in combination with lameness, sepsis, hypoxic ischaemic syndrome (HIS), colic or ill thrift. Three foals developed diarrhoea while in hospital, one after presenting with meconium impaction, one with colic and the other after presenting with a septic joint (Table 4-1). Foal #3 had a history of diarrhoea for three days before admission to hospital at 4 days of age. This foal received antimicrobials on the day of admission, which was also the day the first faecal sample was collected, but from the history in the foal's hospital record it is unknown whether this foal received any antimicrobials on the farm prior to admission. In relation to the day of hospital admission, 83% of foals presenting with diarrhoea had the first faecal sample collected within 48 hours of hospitalisation (Figure 4-4).

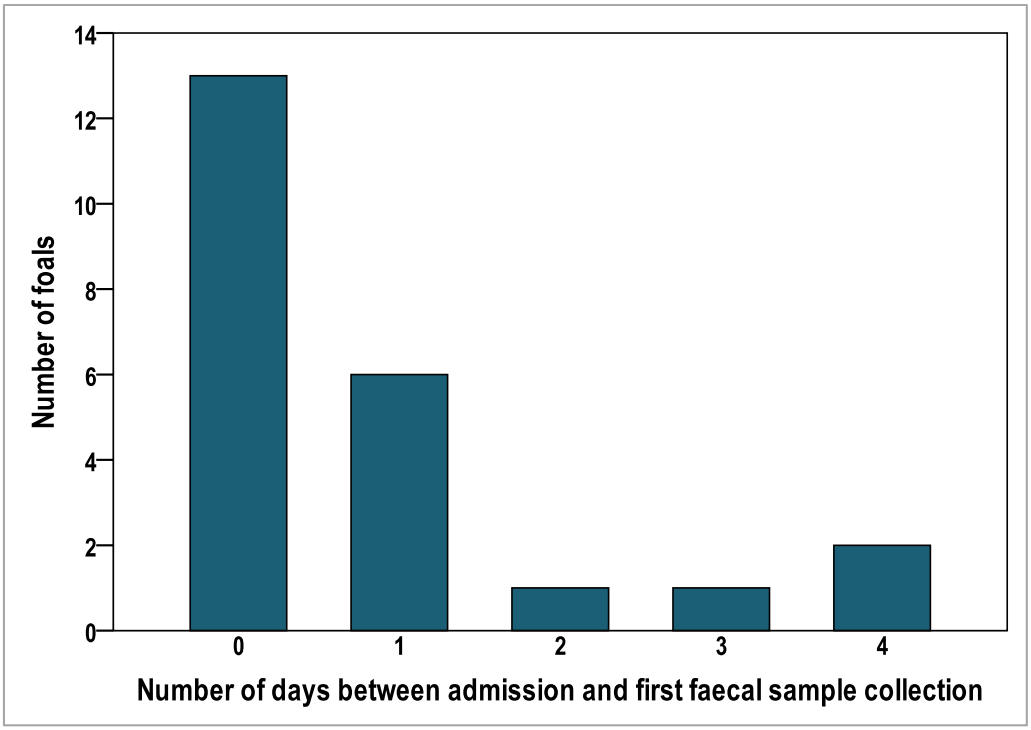

Figure 4-4 Timing in relation to the day of admission and collection of the first faecal sample in foals hospitalised with diarrhoea

Table 4-1 Presenting complaint and antibiotic history of hospitalised case foals

Foal ID#	Presenting complaint	Antibiotics administered prior to day of sampling?		
		Sample 1	Sample 2	Sample 3
1	Lame, Diarrhoea	Yes	-	-
2	Diarrhoea	Yes	-	-
3	Diarrhoea	Unknown	-	-
8	Diarrhoea	No	-	-
11	HIS, Sepsis, Diarrhoea	No	-	-
12	Diarrhoea	No	Yes	Yes
13	Diarrhoea	No	-	-
15	Diarrhoea [a]	Yes	-	-
16	Diarrhoea	Yes	-	-
17	Diarrhoea	Yes	Yes	-
62	Diarrhoea	No	No	Yes
101	Septic joint, Diarrhoea	No	Yes	Yes
142	Diarrhoea	Yes	-	-
143	Diarrhoea	Yes	-	-
166	Colic	Yes	-	-
167	Diarrhoea	Yes	-	-
168	Diarrhoea	Yes	-	-
169	Diarrhoea	Yes	-	-
190	Diarrhoea	No	-	-
191	Meconium impaction[b]	Yes	-	-
218	Diarrhoea	No	Yes	-
224	Colic, Diarrhoea	No	-	-
242	Septic joint [b]	Yes	Yes	-
247	Diarrhoea	Yes	Yes	Yes
259	Ill thrift, Diarrhoea	Yes	-	-
260	HIS, Sepsis, Diarrhoea	Yes	-	-

[a] Foal previously hospitalised and discharged for HIS. Re-admitted dull, with diarrhoea and omphalitis. Euthanased day of re-admission & sampling

[b] Diarrhoea developed during hospitalisation

-, No sample collected; HIS, Hypoxic ischaemic syndrome;

The results of infectious agents detected in foals hospitalised with diarrhoea are briefly summarised in Table 4-2 and described in more detail in the following chapters.

Table 4-2 Summary of infectious agents detected in hospitalised foals with diarrhoea

Infectious agent	qPCR positive (%)	Culture positive (%)
EqRV	3 (12%)	
ECoV	0 (0%)	
Salmonella	4 (15%)	0 (0%)
Clostridium difficile	6 (23%)	5 (19%)

4.2.2 Mare data

Dams of hospitalised diarrhoea foals ranged in age from 5-17 years, with a median age of 7 years (Figure 4-5).

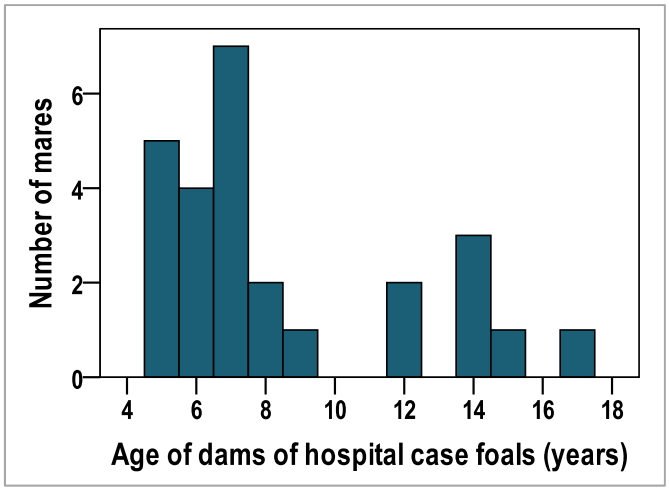

Figure 4-5 Age distribution of dams of hospitalised foals with diarrhoea

The gestation period of dams of hospitalised foals with diarrhoea ranged from 329 to 365 days, with a median of 341.5 days (Figure 4-6).

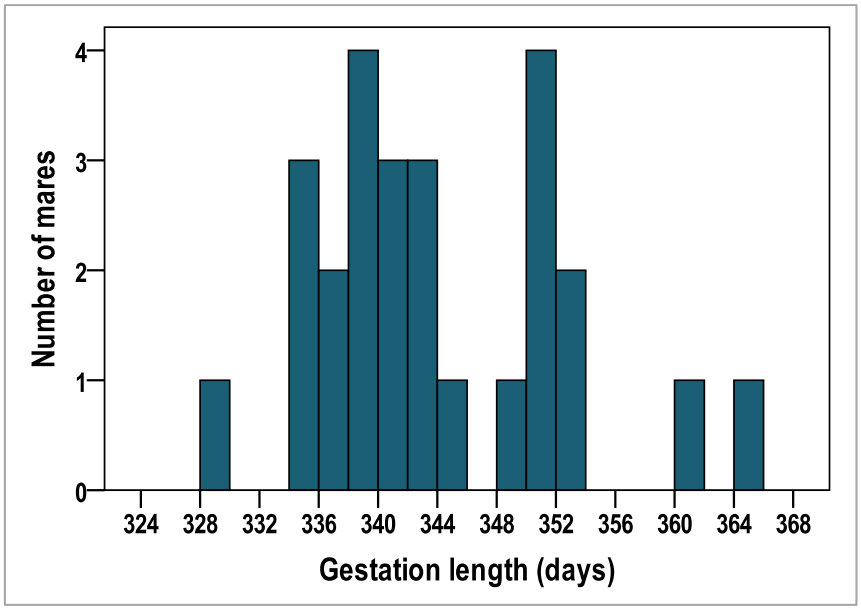

Figure 4-6 Gestation length distribution of dams of hospitalised foals with diarrhoea

4.3 DISCUSSION

The hospitalised foals sampled with diarrhoea represent a different population to the farm case-control foal population in a number of ways. Firstly, hospital foals sampled with diarrhoea were younger than the age matched farm case foals (median age 3.5 days and 22 days respectively). This was not unexpected as foals in the first week of life are at a greater risk of disease and death, with major causes of disease including septicaemia, diarrhoea and musculoskeletal issues (Cohen, 1994). In addition, while the majority of hospital foals in this study presented with diarrhoea alone, 35% of foals presented with other complicating problems and 64% of sampled hospital foals received antibiotic treatment prior to the day of faecal sample collection. Another study of bacteraemia in hospitalised neonatal foals with diarrhoea found 50% had bacteraemia at admission with no association between blood culture status and antimicrobial treatment prior to admission (Hollis *et al.*, 2008). Without faecal samples collected prior to antimicrobial administration and blood culture at admission the effect of antimicrobials on faecal flora and bacteraemia cannot be assessed. However, these results do suggest the hospitalised foal population represent foals requiring more specialised or intensive veterinary care due to more severe or complicated disease than the farm case-control population.

Another difference is hospitalised foals with diarrhoea that were sampled in this study came from 19 thoroughbred breeding properties in the catchment area of the equine referral hospital (including 4 of the 5 farms in the case-control study), therefore representing a wider geographical region than the case-control study. Foals sourced from a wider range of properties likely experience exposure to a greater variety of infectious agents, environmental and management conditions. Screening these hospital foal faecal samples for infectious agents provides information on whether the same infectious agents and particular viral and bacterial strains are circulating in the wider region, whether any are only present in more severely diseased foals and whether any appear to be nosocomial. Although, in terms of mare factors such as age and gestation length, the dams of hospitalised foals were very similar to dams of the farm case-control foals, property size and management details were not available, which together with the lack of a control population, limits the ability to draw conclusions.

Selection of hospitalised control animals is challenging, as even foals admitted for non-infectious conditions such as correction of an angular limb deformity may have complicating issues. This risks the detection of misleading associations between pathogens and severe disease. Perhaps the best criteria for selecting control foals in the hospital would be foals that are hospitalised due to their dam requiring treatment. Although this may reduce the number of control foals available for recruitment and restrict the study sample size and therefore the ability to detect any associations.

CHAPTER 5 DETECTION OF ROTAVIRUSES

5.1 INTRODUCTION

Rotaviruses are the most common cause of infectious diarrhoea in foals, with surveys implicating rotavirus in 20 - 77% of cases (Browning *et al.*, 1991b; Conner and Darlington, 1980; Dwyer *et al.*, 1990b; Netherwood *et al.*, 1996b). An inactivated maternal vaccine has been available commercially since the mid 1990s. Despite this, rotaviruses are still a major cause of diarrhoea in foals. Rotaviral diarrhoea has a high morbidity in foals and, although clinical disease is often self-limiting, dehydration may lead to mortalities.

Rotaviruses are non-enveloped viruses belonging to the family *Reoviridae*. The genome consists of 11 segments of double stranded RNA (Rodger *et al.*, 1975) encoding six virion proteins and six non-structural proteins. The VP7 glycoprotein in the outer capsid is the major neutralising antigen. This protein is used to classify group A rotaviruses into 27 G types. Initially rotaviruses were classified into G types based on cross-neutralisation assays with panels of hyperimmune sera (Hoshino *et al.*, 1984; Kalica *et al.*, 1981; Offit and Blavat, 1986). Serotyping has largely been replaced by genotyping, as the amino acid sequence in antigenic regions correlates closely with G serotype designations (Gouvea *et al.*, 1990). While there have been 6 G types identified in horses (Browning *et al.*, 1991a; Browning *et al.*, 1991c; Hoshino *et al.*, 1983a, b; Imagawa *et al.*, 1994; Isa *et al.*, 1996), the predominant EqRV G types are G3 and G14 (Begg *et al.*, 1994; Garaicoechea *et al.*, 2011; Matthijnssens *et al.*, 2015; Nemoto *et al.*, 2011). G3 EqRV have been further classified into two subtypes, G3A and G3B, based on cross neutralisation assays, which also correlate with five amino acid polymorphisms in three VP7 antigenic regions (Browning *et al.*, 1992b; Dyall-Smith *et al.*, 1986; Tsunemitsu *et al.*, 2001).

Numerous techniques are used to detect rotaviruses in the faeces of foals, including electron microscopy (EM), polyacrylamide gel electrophoresis (PAGE), and immunoassays. Reverse transcription polymerase chain reaction (RT-PCR) assays can be used to detect rotaviruses, but are more frequently used in a research setting for G and P genotyping of rotaviruses, thus providing information on the molecular

epidemiology of outbreaks (Fukai *et al.*, 2006; Garaicoechea *et al.*, 2011; Gentsch *et al.*, 1992; Gouvea *et al.*, 1994; Tsunemitsu *et al.*, 2001). The limitations of these methods for clinical diagnosis of rotavirus in foals with diarrhoea include availability, test result turn-around-time and sensitivity. A more rapid and sensitive detection method with potential for high throughput is RT-qPCR (Pang *et al.*, 2004). Rapid detection assays would assist veterinary clinicians to tailor treatment regimens so as to avoid the unnecessary use of antibiotics in cases of viral diarrhoea.

At the time this study was initiated there was no RT-qPCR assay described to detect EqRV. This chapter describes the development and validation of an RT-qPCR assay to detect EqRV in the faeces of thoroughbred foals in Australia.

5.2 APPROACH TO THE DETECTION OF ROTAVIRUSES IN FOAL FAECAL SAMPLES

To screen foal faecal samples for the presence of EqRV, primers previously described for the detection of rotavirus in children (Pang *et al.*, 2004) were adapted for use in a two-step RT-qPCR assay. Development of this assay involved the use of archived foal faecal samples positive for rotavirus to optimise assay conditions and generate a qPCR standard. To screen foal faecal samples collected in this study, nucleic acids were extracted from faeces stored in RNAlater® (Section 2.1.2.1) with the PowerSoil®-htp 96 Well Soil DNA Isolation kit (Section 2.2.2). Complementary DNA, generated using random primers (Section 2.2.8) was used as template in the qPCR assay (Section 2.3.3). Samples with amplification products matching the rotavirus profile were tested in a one-step G-typing VP7 RT-PCR assay (Section 2.7.1) to both further characterise the rotaviruses present and confirm the detection of rotaviruses in these samples.

5.3 RESULTS

5.3.1 Development and validation of an RT-qPCR to detect EqRV in foal faeces

At the time of assay development there were no published EqRV RT-qPCR assays in the literature. Two sets of primers were designed, with one set targeting conserved regions of the gene encoding the VP7 protein and the other set targeting the conserved regions of the VP4 protein. In addition, primers Rota NVP3-F and Rota NVP3-R (Table 2-1) were selected from an RT-qPCR assay developed for the detection of

rotavirus in children that targets a highly conserved region of the gene encoding non-structural viral protein 3 (NVP3) (Pang *et al.*, 2004).

The three sets of primers were tested on nine archived foal faecal samples that had previously tested positive for EqRV (Browning and Begg, 1996) by RT-PCR targeting the gene encoding VP7 (kindly provided by Professor Glenn Browning, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne). Another 2 archived foal faecal samples that had previously tested negative for rotaviruses were used as negative controls. Nucleic acids were extracted from these samples (Section 2.2.1) and cDNA generated using random primers (Section 2.2.8).

The primers targeting VP4 did not generate a specific amplification product (data not shown) and were not tested further. The primers targeting VP7 and NVP3 both generated an amplification product with a single melting temperature (Tm). To select the superior primer set, rotavirus positive archived foal faecal samples #114, #71 and #60, representing the genotypes G3A, G3B, and G14, respectively, were tested. Based on poor amplification of the genotype G14 sample (#60) by the VP7 primers (data not shown), the NVP3 primer set was selected for further optimisation of the assay conditions. Conditions tested included comparison of Ct values with forward (F) to reverse (R) primer ratios of 1:1, 1:3, 1:5, 5:1 and 3:1, $MgCl_2$ concentrations of 1.5 mM, 2.0 mM, 2.5 mM and 3.0 mM and annealing temperatures of 45°C, 50°C and 55°C (Table 5-1).

In relation to Ct value, there was no obvious advantage of a particular primer ratio, $MgCl_2$ concentration or annealing temperature, so optimal assay conditions were selected to minimise non-specific amplification. These optimal assay conditions consisted of a forward to reverse primer ratio of 1:1, an $MgCl_2$ concentration of 2.5 mM and an annealing temperature of 50°C.

Table 5-1 Comparison of Ct values with variations in assay conditions for rotavirus positive archived foal faecal samples representing EqRV serotypes G3A, G3B and G14

Assay condition variable	Mean Ct value[b] (Tm value) for each rotavirus serotype		
	G3A	G3B	G14
Primer ratio F:R[a]			
1:1	31.2 (79.5°C)	27.9 (79.5°C)	32.8 (79.0 C)
5:1	30.4 (79.5°C)	27.1 (79.7°C)	32.7 (79.4°C)
3:1	31.6 (79.5°C)	28.1 (79.5°C)	32.8 (79.0°C)
1:3	30.9 (79.5ºC)	28.7 (79.7°C)	32.9 (79.4°C)
1:5	32.0 (79.5°C)	27.9 (79.5°C)	33.2 (79.0°C)
$MgCl_2$ concentration per reaction[c]			
1.5 mM	26.3 (79.1°C)	20.9 (78.6°C)	28.0 (79.1°C)
2.0 mM	26.3 (79.7°C)	21.0 (79.2°C)	29.1 (79.1°C)
2.5 mM	27.0 (79.1°C)	20.6 (79.6°C)	26.3 (79.1°C)
3.0 mM	26.9 (79.2°C)	21.2 (79.2°C)	28.3 (79.1°C)
Annealing temperature[d]			
45°C	26.6 (79.1°C)	20.3 (79.0°C)	27.6 (79.0°C)
50°C	27.1 (79.2°C)	21.3 (79.1°C)	28.8 (79.6°C)
55°C	30.0 (79.2°C)	22.2 (79.1°C)	29.8 (79.1°C)
Mean Tm	79.3°C	79.3 °C	79.2°C

Template – cDNA from archived foal samples 114 (G3A), 71(G3B) and 60(G14)

F, forward; R, reverse;

[a] Annealing temperature 45°C, [$MgCl_2$] 2.5 mM

[b] Mean of two replicates

[c] Annealing temperature 45°C, primer ratio 1:1

[d] Primer ratio 1:1, [$MgCl_2$] 2.5 mM

Separation and visualisation of the NVP3 qPCR products by electrophoresis (Section 2.2.5) on an agarose gel demonstrated the expected 87 bp bands (not shown). These products were then purified (Section 2.2.6) and cloned into a plasmid vector for use as a DNA standard (pGEM-T.NVP3.G14) (Section 2.4). DNA sequencing (Section 2.5) of the plasmid inserts and comparison with rotavirus NSP3 sequence (GenBank Accession no. X81436) confirmed the specificity of the NVP3 qPCR assay (Figure 5-1).

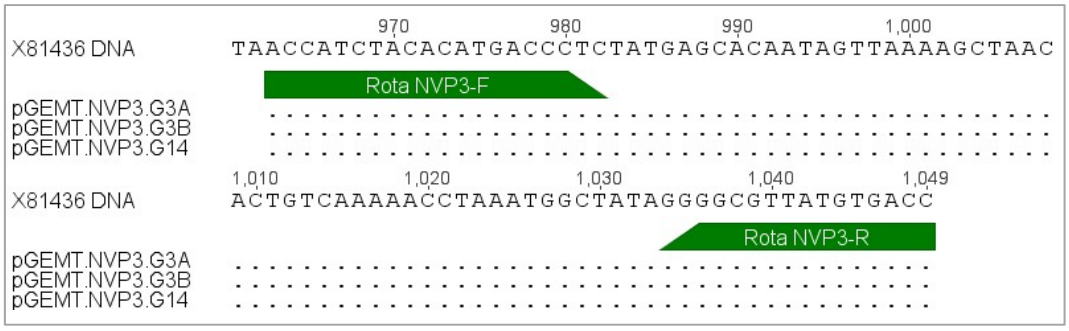

Figure 5-1 Nucleotide sequence alignment of the rotavirus NVP3 qPCR product of EqRV serotypes G3A, G3B and G14 cloned into the pGEM-T plasmid with the human rotavirus NSP3 sequence (GenBank Accession no. X81436) used by Pang *et al.* (2004) to design the primers Rota NVP3-F and Rota NVP3-R (represented by green arrows). Identical nucleotides are indicated by a dot (.).

A DNA standard curve was generated by testing 10-fold serial dilutions of plasmid DNA (pGEM-T.NVP3.G14) in triplicate using the optimised NVP3 qPCR conditions (Figure 5-2). The efficiency of DNA amplification was high (103.6%). The assay was linear over 7 orders of magnitude, from 10^1 to 10^7 DNA copies per reaction. The lower limit for quantification was defined as 10 copies per reaction.

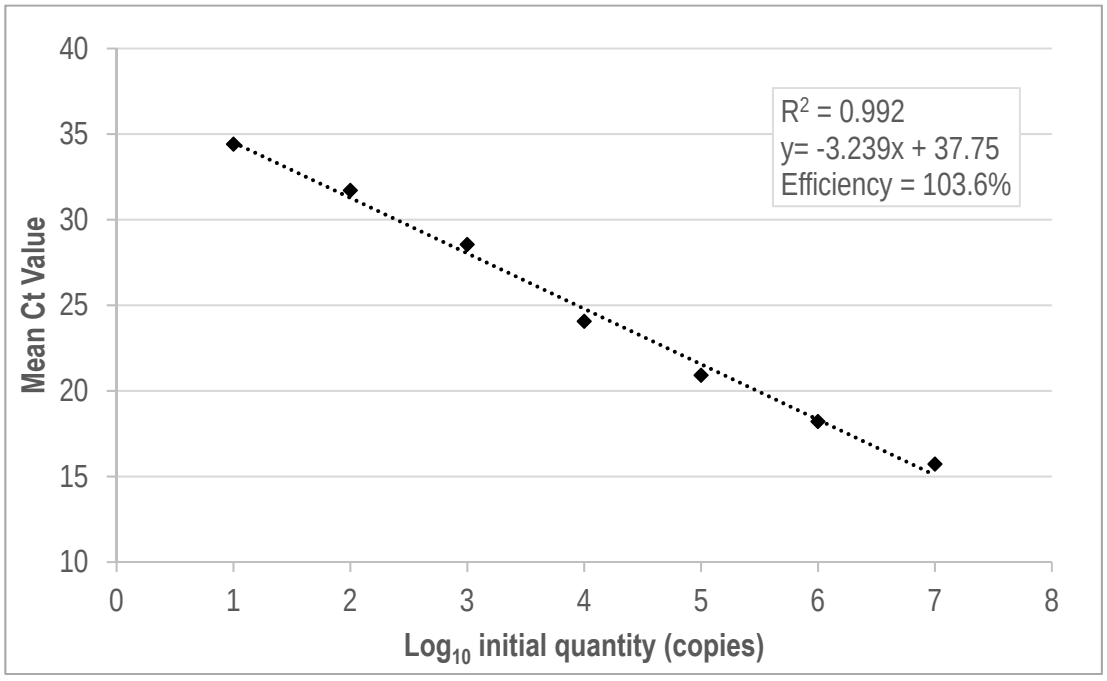

Figure 5-2 EqRV NVP3 qPCR assay standard curve produced from 10 fold serial dilutions of pGEM-T.NVP3.G14 plasmid DNA

To evaluate the reproducibility of the EqRV NVP3 qPCR assay, the within assay (intra-) and between assay (inter-) variation was determined. The intra-assay variation

was determined by calculating the CV% (Section 2.3.5) between Ct values of three replicates of a 10-fold serial standard dilution series with between 10^1 and 10^7 DNA copies/reaction (Table 5-2), while the inter-assay variation was determined by calculating the CV% between the mean Ct values of three replicates of a standard dilution series with between 10^1 and 10^7 DNA copies/reaction performed in three independent assay runs (Table 5-3). Both the intra- and inter-assay variation were low (≤2.28 CV% and ≤4.19 CV%, respectively), which indicated that the assay was precise and reproducible.

Table 5-2 Intra-assay variation of the EqRV NVP3 RT-qPCR assay

Copies[a]	Ct value			Mean	SD	CV%
	Replicate					
	1	2	3			
7	15.9	15.5	15.6	15.7	0.18	1.16
6	18.2	17.9	18.4	18.1	0.28	1.56
5	21.1	20.3	21.1	20.8	0.47	2.28
4	24.2	23.7	24.2	24.0	0.25	1.06
3	28.7	28.2	28.6	28.5	0.25	0.86
2	31.6	31.7	31.7	31.7	0.04	0.11
1	35.0	34.5	33.7	34.3	0.65	1.90

[a]$\log_{10}$ DNA copies per reaction

Table 5-3 Inter-assay variation of the EqRV NVP3 RT-qPCR assay

Copies[a]	Ct value[b]			Mean	SD	CV%
	Assay					
	1	2	3			
7	16.2	16.7	15.7	16.2	0.51	3.15
6	19.7	18.7	18.1	18.9	0.78	4.13
5	22.6	22.0	20.8	21.8	0.91	4.19
4	25.7	25.3	24.0	25.0	0.92	3.67
3	29.2	29.2	28.5	29.0	0.40	1.37
2	32.2	31.6	31.7	31.8	0.35	1.10
1	34.8	33.0	34.3	34.0	0.94	2.75

[a]$\log_{10}$ DNA copies per reaction

[b]Mean of 3 replicates

5.3.2 Detection of EqRV in foal faeces

Having optimised assay conditions and established that the NVP3 qPCR was highly reproducible and capable of detecting the three most common EqRV G types from archived foal faecal samples, the assay was used to screen foal faecal samples collected in this study. Foal faecal samples were considered potentially positive for EqRV if there was an NVP3 qPCR amplification product with an appropriate dissociation melt curve (Tm 77.5-80.5°C) and a Ct value less than 40. These criteria were met in 51 samples from 45 foals (Table 5-4).

Table 5-4 NVP3 qPCR Ct values of foal faecal samples potentially EqRV positive

Foal ID #	Sample type	NVP3 qPCR Ct[a] Sample #				
		1	2	3	4	5
203	Case	26.2	-[e]	-	-	-
175	Case	27.4	-	-	-	-
179	Control	27.8	-	-	-	-
79	Case	29.9	-	-	-	-
59	Case	No Ct	30.2	-	-	-
93	Case	30.7	-	-	-	-
1	Hospital	30.8	-	-	-	-
183	Case	31.1	-	-	-	-
264	Case	31.2	-	-	-	-
128	Case	31.3	-	-	-	-
47	Case	32.4	-	-	-	-
255[b]	Case	32.5	-	-	-	-
188[b]	Case	33.1	-	-	-	-
113	Case	No Ct	33.4	No Ct	No Ct	No Ct
251	Case	34.5	-	-	-	-
31	Case	34.7	36.1	No Ct	35.7	-
135	Case	34.7	-	-	-	-
174	Control	35.1	-	-	-	-
101	Hospital	35.3	29.8	No Ct	-	-
176	Case	35.4	-	-	-	-
311	Case	35.4	No Ct	-	-	-
49	Case	35.4	-	-	-	-

Foal ID #	Sample type	NVP3 qPCR Ct[a] Sample #				
		1	2	3	4	5
192	Case	35.5	-	-	-	-
45	Case	35.9	-	-	-	-
270	Case	36.0	-	-	-	-
58	Case	36.0	-	-	-	-
238[c]	Case	36.0	-	-	-	-
39[c]	Control	36.4	-	-	-	-
14	Case	36.1[d]	37.0	38.0	-	-
144	Case	37.3	-	-	-	-
182	Control	37.3	-	-	-	-
42	Case	37.4	-	-	-	-
72	Case	37.7	-	-	-	-
167	Hospital	37.8	-	-	-	-
271	Case	37.9	-	-	-	-
231	Case	37.9	35.3	No Ct	No Ct	-
309[b]	Case	38.0	-	-	-	-
266[c]	Control	38.1	-	-	-	-
233	Case	38.3	-	-	-	-
284	Control	38.3	-	-	-	-
161	Control	38.5	-	-	-	-
71	Control	38.8	-	-	-	-
256	Case	39.5	-	-	-	-
70	Case	39.8	36.2	-	-	-
22[c]	Case	39.9	No Ct	No Ct	No Ct	37.8 [d]

[a] Common fluorescence threshold 49.4

[b] Unmatched case foal

[c] VP7 & NVP3 sequence not obtained

[d] Tm of amplicon outside of expected range; Tm range = 77.5 – 80.5°C

[e]- Sample not collected

To confirm the presence of rotavirus while simultaneously determining the G type of EqRV present in this foal population, these 51 samples were tested with a one-step RT-PCR assay that amplified the gene segment encoding VP7 (Section 2.7.1) using

primers VP7-Fdeg and VP7-Rdeg (Table 2-6). If amplification failed with these primers, the sample was re-tested using primers Beg9 and End9 (Table 2-6). VP7 RT-PCR amplification products were sequenced in both directions, with primers VP7-Fdeg/Rdeg or Beg9 and End9, to confirm the detection of rotavirus. If VP7 sequence could not be detected in these samples, the NVP3-qPCR amplicons were separated by electrophoresis (Section 2.2.5) and excised for purification (Section 2.2.6) and sequencing (Section 2.5) to confirm the detection of rotavirus.

Using these methods, EqRV was confirmed in foals by the detection of VP7 sequence in 28 foals (31 samples) and NVP3 sequence consistent with rotavirus in an additional 13 foals (16 samples) (Figure 5-3). There were four foals from which VP7 sequence and NVP3 sequence were not obtained. For subsequent analyses, these samples were considered negative. Rotaviruses were therefore confirmed in 41 foals, including 29 age-matched case foals, 6 age-matched control foals, 3 unmatched case foals and 3 hospital foals.

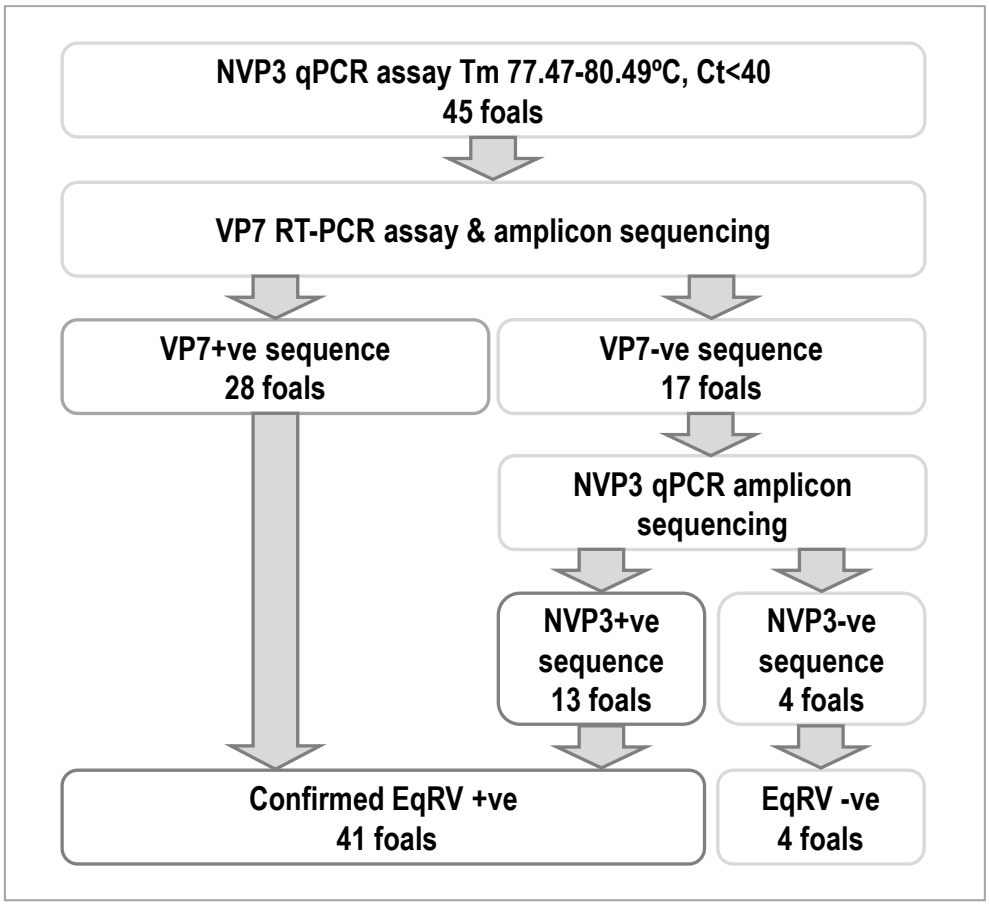

Figure 5-3 Flow chart describing the process used to verify the detection of EqRV in foal faeces by NVP3 qPCR, VP7 RT-PCR and amplicon sequencing. Abbreviations: +ve = positive, -ve = negative.

5.3.3 Comparison of EqRV NVP3 qPCR Ct value and diarrhoea status

To assess if there was any relationship between the EqRV viral load and clinical disease, the NVP3 qPCR Ct values of case and control foals were compared. The Ct values of case samples ranged from 26.2 to 39.8, with a median of 35.4, and control sample Ct values ranged from 27.8 to 38.8, with a median of 37.8 (Figure 5-4). The median Ct values of EqRV positive (EqRV+ve) case samples and EqRV+ve control samples were not significantly different (P=0.170, Mann-Whitney U test). Therefore there was no association between viral load at the time of sampling and diarrhoea status.

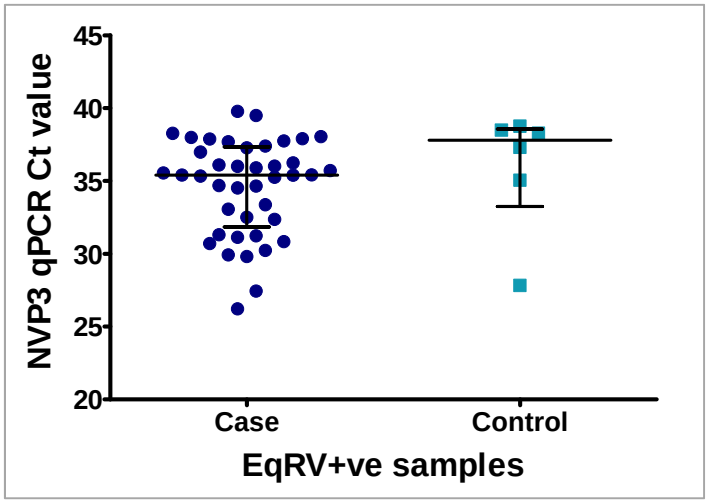

Figure 5-4 Comparison of NVP3 qPCR Ct values for EqRV+ve case and control samples. The bars represent the median and interquartile range.

To assess if there was any relationship between the NVP3 qPCR Ct value and the amplification of VP7 sequence, the Ct values from EqRV+ve samples were compared. The foal faecal samples from which VP7 sequence was amplified had NVP3 qPCR Ct values between 26.2 and 39.8, with a median of 35.4, while the foal faecal samples from which VP7 sequence could not be amplified had NVP3 qPCR Ct values between 30.8 and 39.9, with a median of 36.1 (Figure 5-5). There was no significant difference between the median Ct values in foals from which VP7 sequence was amplified and foals from which VP7 sequence was not amplified (P=0.239, Mann-Whitney U test).

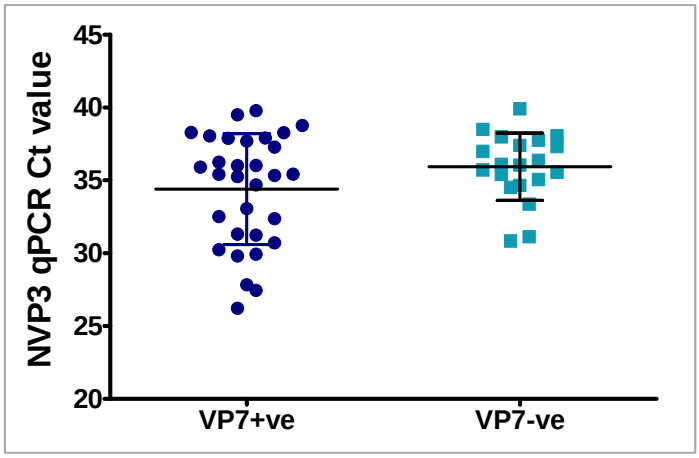

Figure 5-5 Comparison of NVP3 qPCR Ct values from VP7 +ve and VP7 -ve samples. The bars represent the median and interquartile range.

5.3.4 Analysis of EqRV detected in age matched case control foals

In the age-matched case control faecal samples, EqRV was detected by NVP3 qPCR in 5% of control foals and 25% of case foals (Table 5-5).

Table 5-5 Rotavirus detection in age matched case and control foals

Sample	EqRV+ve	Total	% EqRV+ve
Case	29	117	25%
Control	6	117	5%

Rotavirus was detected in foals from all five farms. There were 18 case foals that had more than one faecal sample collected during an episode of diarrhoea. Four of these foals were positive for EqRV in more than one faecal sample. One matched case foal in which rotavirus was detected died the following day due to a ruptured stomach.

To establish if there was a significant association between the detection of EqRV and the presence of diarrhoea, the EqRV status of each age matched case-control pair was determined (Table 5-6). A matched analysis of the discordant pairs showed the odds of detecting rotavirus in a case were 6.75 times that of detecting rotavirus in a control, which was significant ($P<0.001$, 95% CI 2.35-26.6).

Table 5-6 Matched analysis of case-control pairs in relation to EqRV status.

Case-control pair exposure	EqRV
Case +ve Control +ve	2
Case +ve Control –ve	27
Case –ve Control +ve	4
Case −ve Control –ve	84
Total number of case-control pairs	117

The age of case foals positive for EqRV ranged from 11 to 85 days, with a median of 27 days (Figure 5-6 A). The age of control foals positive for EqRV ranged from three to 75 days, with a median of 14.5 days of age (Figure 5-6 B). The median age of case and control foals positive for EqRV was not significantly different (P=0.134).

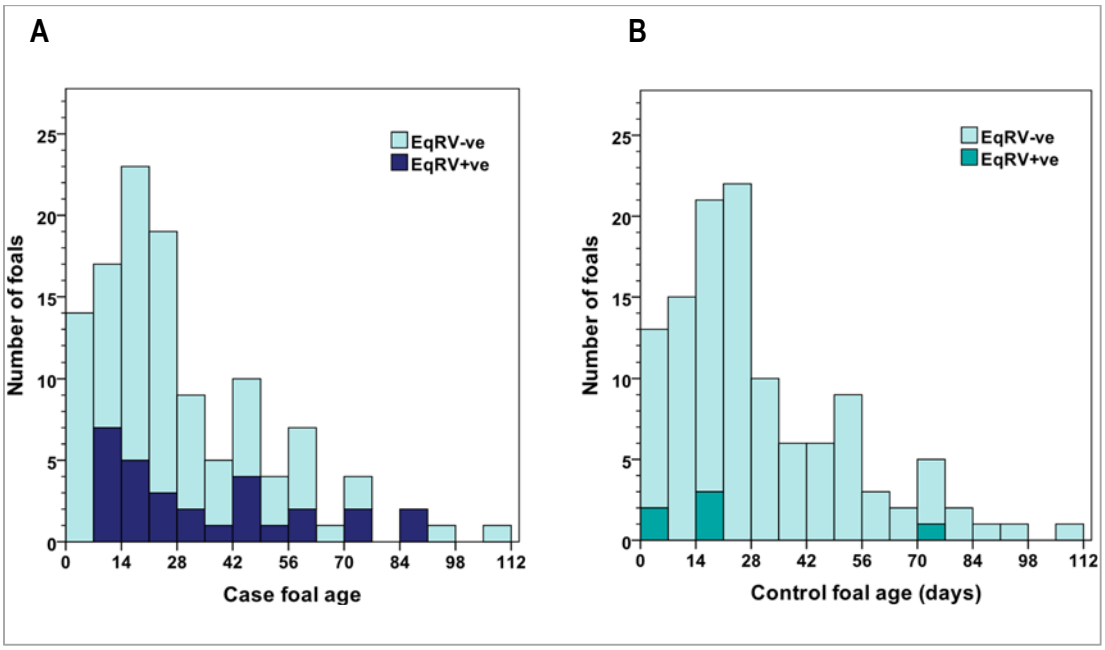

Figure 5-6 Age distribution of age-matched (A) cases and (B) controls and those EqRV positive

The month with the highest number of foals sampled and the highest number of foals positive for EqRV was October (Figure 5-7). A total of 85% (799/944) of mares on the enrolled farms had foaled by the end of October in 2010.

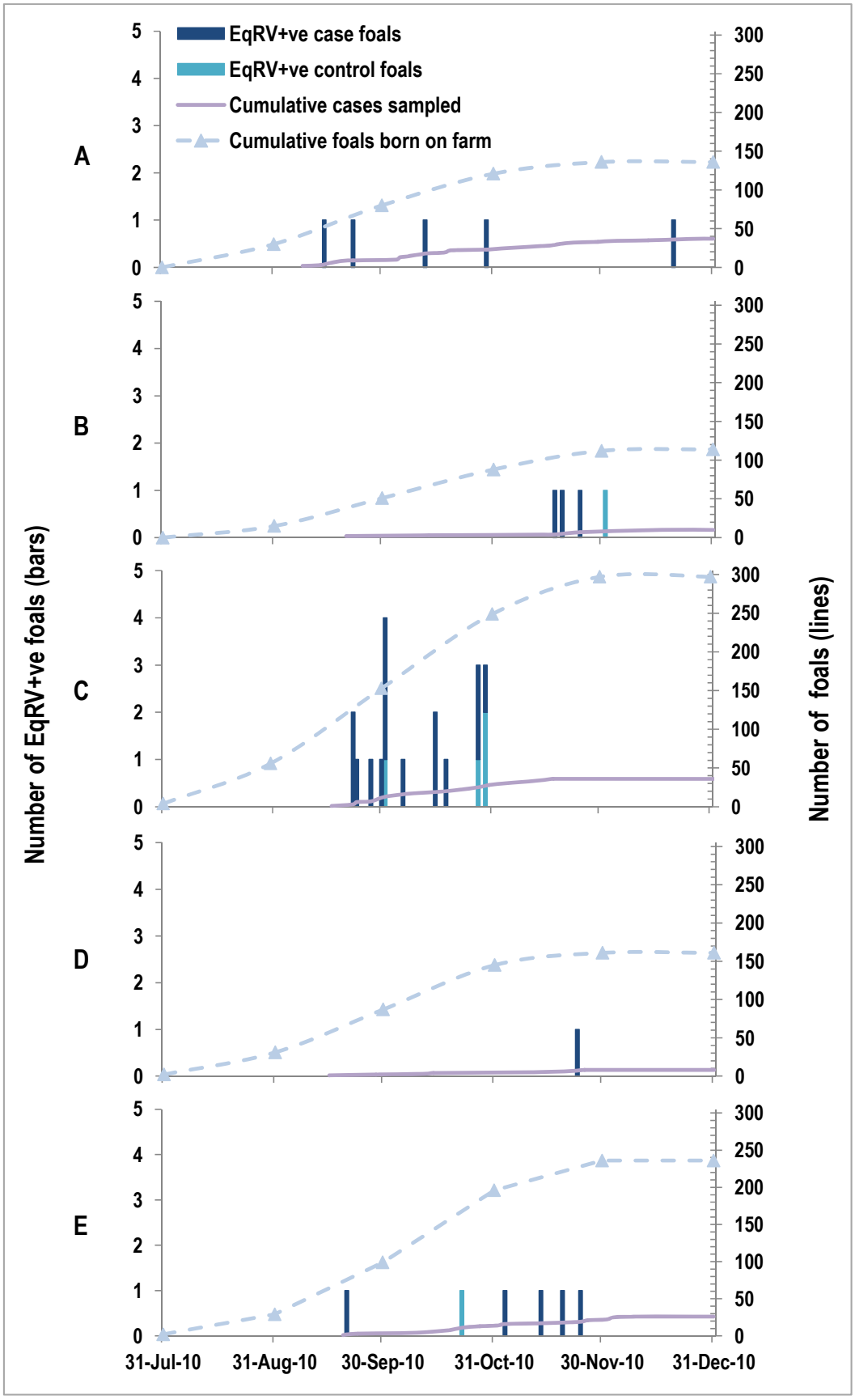

Figure 5-7 Temporal distribution of EqRV positive case and control foals in relation to the number of cases sampled and the number of foals born on each farm (A-E) in 2010

5.3.5 Analysis of EqRV detected in hospital foals with diarrhoea

In the hospital case series, 26 cases of diarrhoea were sampled, representing foals from 19 different properties. Of these, 3 foals were RT-qPCR positive for EqRV, with two of these foals being from farm C. The age of hospitalised foals positive for EqRV was 4, 13 and 21 days and samples were collected in September and October (Figure 5-8).

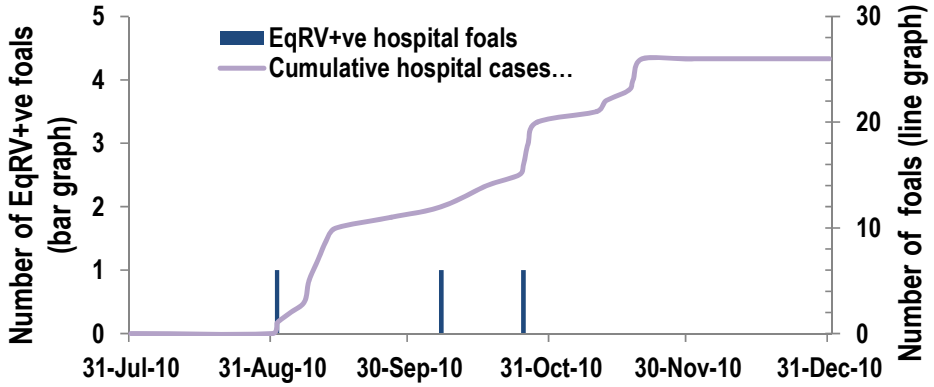

Figure 5-8 Temporal distribution of EqRV positive hospital foals in relation to the number of hospital cases sampled

There were eight hospitalised foals that had more than one diarrhoeic faecal sample collected. One of these foals was positive in more than one sample, demonstrating faecal shedding of EqRV for a period of at least four days.

5.3.6 G-typing and phylogenetic analysis of EqRV VP7 sequences

As well as confirming the presence of EqRV in foal samples, VP7 nucleotide sequence obtained from the one-step VP7 RT-PCR products was used to determine the G genotype of the EqRV present in this foal population. Nucleotide sequence consistent with VP7 was obtained from 28 foals, including 21 age-matched case foals, 3 age-matched control foals, 3 unmatched case foals and 1 hospital foal.

Nucleotide alignment of VP7 sequence products fell into two distinct genotypes/strains with 98.9% nucleotide identity, represented by samples 135 and 203 (Figure 5-9). The genotype represented by sample 135 was detected in 16 foals, all from farm C, including 13 age-matched case foals, 2 age-matched control foals and 1 hospital foal. The second genotype represented by sample 203, was detected in 12 foals from farms other than farm C. This included 8 age-matched case foals, 1 age-matched control foal and 3 unmatched case foals.

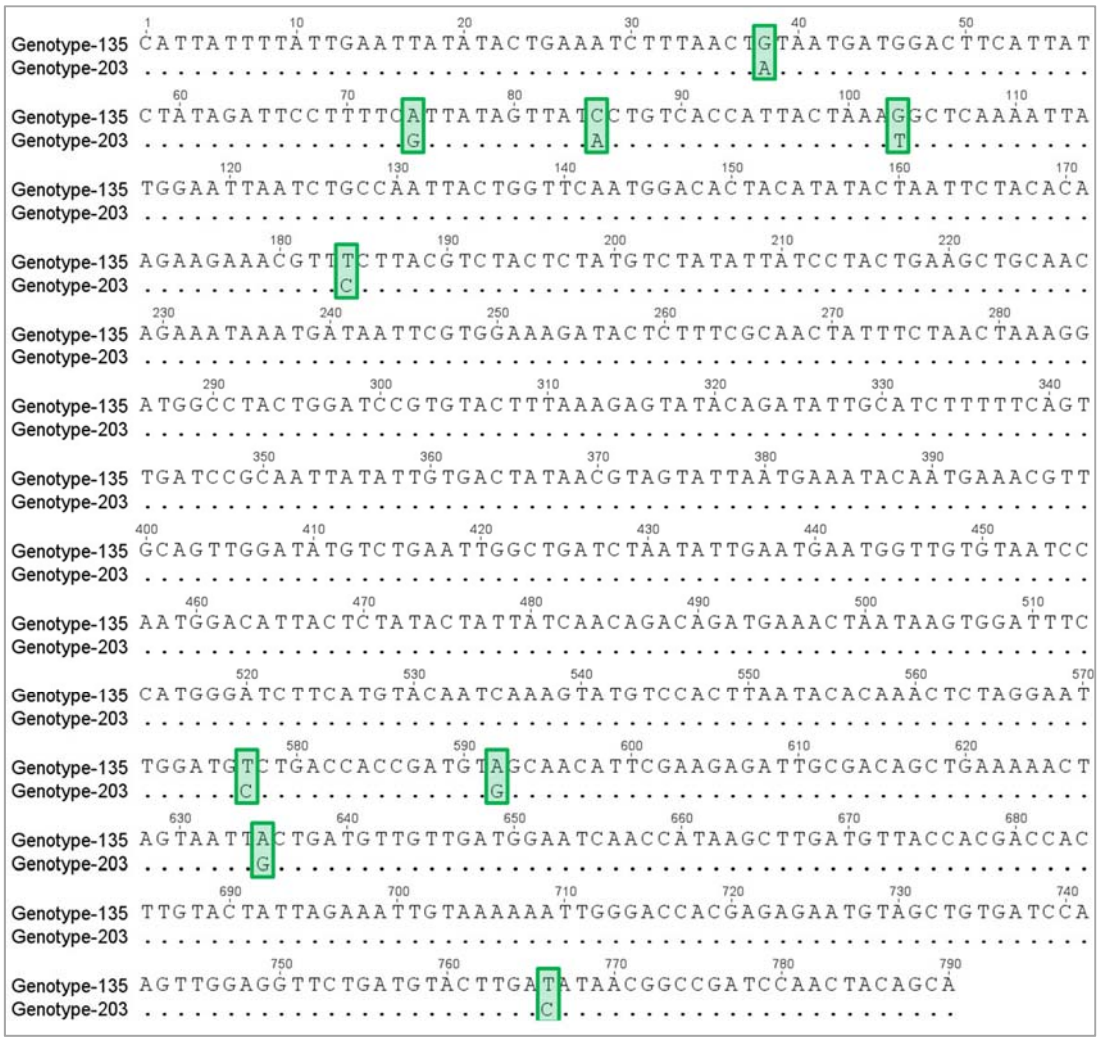

Figure 5-9 Nucleotide sequence alignment of EqRV VP7 sequences from samples 135 and 203, representing the two genotypes detected in this study. Identical nucleotides are indicated by dots (.) and non-identical nucleotides are shown shaded in green.

Based on phylogenetic comparisons of the VP7 nucleotide sequences from sample 135 and 203 with 11 other representative equine Group A rotavirus VP7 sequences, the EqRVs detected in this survey were genotype G3A (Figure 5-10). VP7 sequence from samples 135 and 203 were most similar to strain Erv316 (97.4% and 98.2% identity respectively), isolated in Australia in 1991 (GenBank accession number L49043), and strain 4698G5 (97.7% and 98.4% respectively) isolated in Germany sometime between 1993 and 2002 (GenBank accession number AY750922). The H-2 EqRV strain (GenBank accession number HM160096), originally isolated from a foal with

diarrhoea in 1976 in the UK, is the strain included in the inactivated maternal rotavirus vaccine commercially available in Australia.

The amino acid sequences were deduced from the VP7 nucleotide sequences of isolates 135 and 203 and aligned with the translated VP7 sequences of the same 11 EqRV strains discussed above. The amino acid (aa) sequence obtained in this study was shorter than the full length VP7 protein (326 aa) because of the location of the VP7-Fdeg and VP7-Rdeg primer binding sites. The deduced VP7 amino acid sequences from samples 135 and 203 had 98.9% identity with each other, and 95.4-98.5%, 91.4-92.8% and 79.8-87.8% amino acid identity with G3A, G3B and G14 strains, respectively.

Previously the amino acid sequences in three antigenic regions, A, B and C, have been correlated with serotype-specific neutralization epitopes (Dyall-Smith *et al.*, 1986; Tsunemitsu *et al.*, 2001). The two deduced amino acid sequences from this study have 100% amino acid identity with the H-2 vaccine strain in current use and the Australian Erv316 strain in regions A and B. In antigenic region C, the two strains in this study were identical to each other, but differed from the H-2 vaccine strain, which has a threonine at position 217 instead of a glutamic acid, and the ERv316 strain, which has a phenylalanine instead of an isoleucine at position 215 (Figure 5-11).

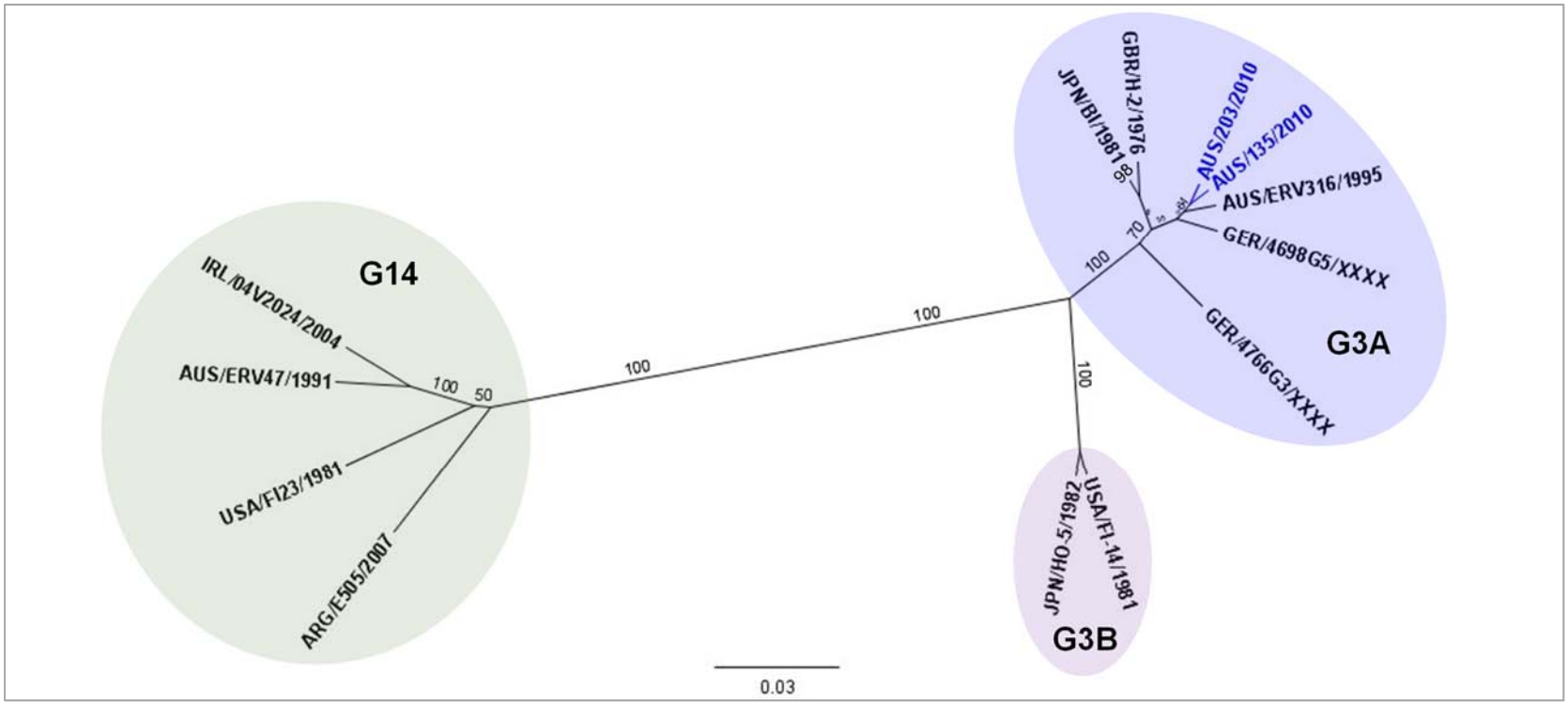

Figure 5-10 Unrooted phylogenetic tree comparing VP7 sequences representative of equine Group A rotaviruses type G14 (green), G3A (blue) and G3B (purple), including 135 and 203 from this study (blue text). ARG=Argentina, AUS= Australia, GER=Germany, GBR=Great Britain, ITA=Italy, IRL=Ireland, JPN=Japan, USA=United States of America. The tree was inferred using the maximum likelihood method and bootstrap analysis with 100 replicates in Geneious® (version 9.0.5, Kearse *et al.* (2012)). Bootstrap consensus values are indicated on the branches. Genbank accession numbers: E505 GU373936, Erv47 L49042, Erv316 L49043, 4698G5 AY750922, 4766G3 AY750924, H-2 HM160096, 04V2024 JN903517, HO-5 AB046464, BI KC815685, FI23 M61876, FI14 KM454486.

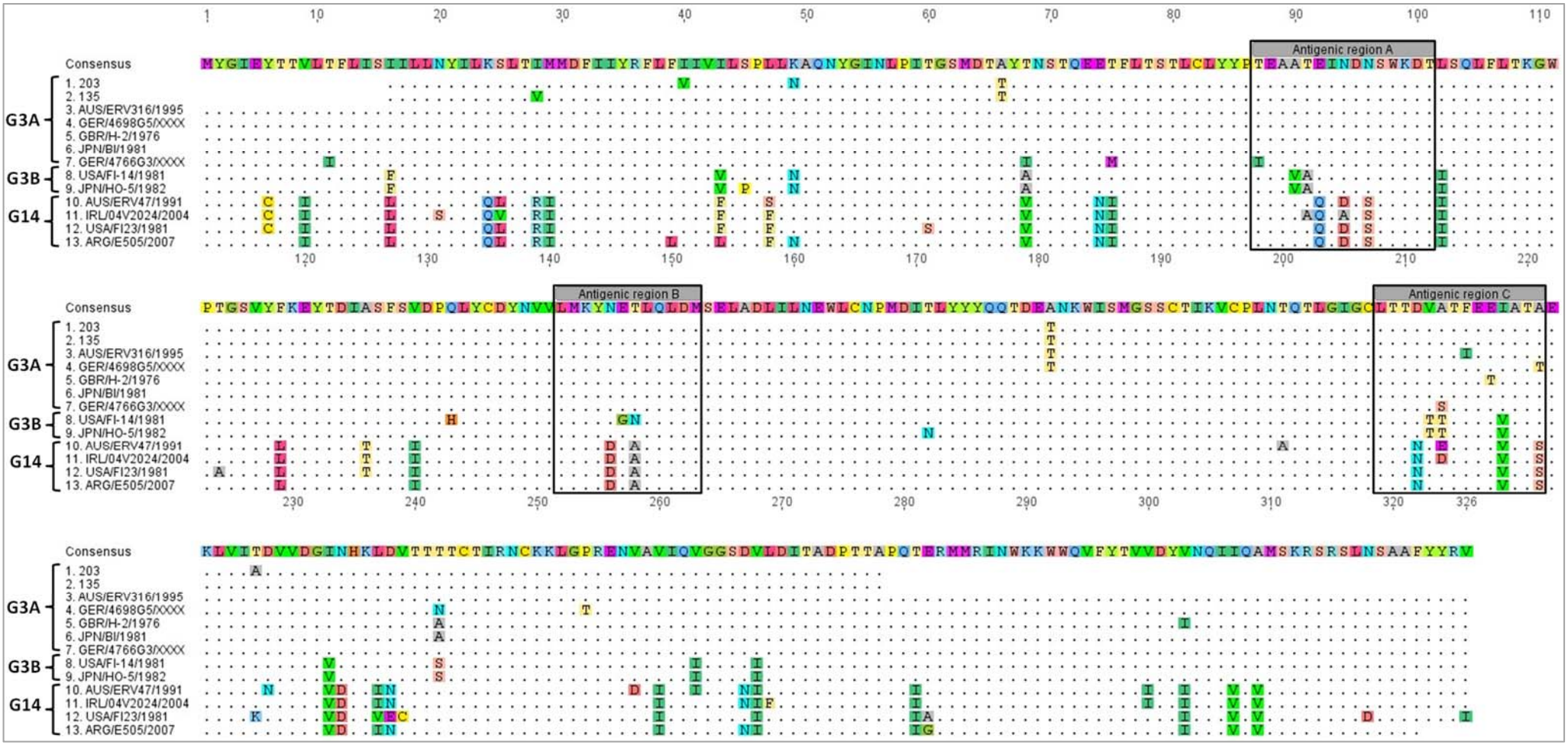

Figure 5-11 Alignment of the deduced VP7 amino acid (aa) sequences of the two EqRV strains detected in this study with 11 EqRV strains representing types G3A, G3B and G14. Antigenic regions A (aa 87-101), B (aa 141-152) and C (aa208-221) are outlined.

5.4 DISCUSSION

This is the first matched case control study to find a significant association between the detection of rotaviruses and the presence of diarrhoea in foals while accounting for variation in exposure due to temporal, spatial and age factors. Previous studies have also detected an association between rotavirus and diarrhoea in foals (Browning *et al.*, 1991b; Netherwood *et al.*, 1996b; Slovis *et al.*, 2014), but apart from one study which separated controls into those in-contact and those not-in-contact with cases (Netherwood *et al.*, 1996b), the selection of control animals in those studies was not representative of the same population, exposure factors and time at risk as the case foals.

Rotaviruses are the most common infectious agents identified in foals with diarrhoea (Dwyer *et al.*, 1990b; Holland *et al.*, 1991; Slovis *et al.*, 2014) and the detection of rotaviruses in 25% of case foals in this study is consistent with other studies. The detection of rotaviruses in 12% of hospitalised foals with diarrhoea was low in comparison to previous studies examining equine hospital populations (20%-23%) (Frederick *et al.*, 2009; Holland *et al.*, 1991).

In foals without diarrhoea, rotaviruses have been reported at frequencies of between 3-10% (Browning *et al.*, 1991b; Dwyer *et al.*, 1990b; Netherwood *et al.*, 1996a; Slovis *et al.*, 2014), which may represent subclinical infection, animals in the incubation phase, or animals in the convalescent phase of disease. Rotaviruses have previously been detected in the faeces of foals 1-4 days before the onset of diarrhoea and up to 6 days after the faeces returns to normal consistency (Dwyer *et al.*, 1990b; Higgins *et al.*, 1987). The criteria used to select control foals in the current study, by only sampling control foals with no evidence of diarrhoea in the seven days before or after sampling, were chosen to reduce the likelihood of sampling animals about to develop disease or those recovering from disease. Therefore, the 5% of control foals in which rotaviruses were detected, more likely represent infection without clinical disease.

Studies in pigs have demonstrated that clinical rotavirus infection can occur with as few as 90 viral particles (Payment and Morin, 1990), but the infectious dose for foals has not been established. In this study, it was interesting to note that although rotaviruses were associated with the presence of diarrhoea, there was no association

between the viral load (inferred by the NVP3 qPCR Ct value), and a foal being a case or a control. While there has been some investigation of the duration of viral shedding in the faeces of foals with diarrhoea (Dwyer *et al.*, 1990b; Higgins *et al.*, 1987), little is known about the dynamics of viral load over time. Future studies analysing a greater number of serial faecal samples from EqRV+ve foals both with and without diarrhoea would be beneficial as there were too few foals positive in serial faecal samples in this study to enable conclusions to be drawn on this aspect of infection. As qPCR is unable to distinguish between infectious virus and virus nucleic acid, a decrease in Ct values (increase in viral load) in serial samples may clarify whether RT-qPCR detection of rotavirus represents an active infection.

Alternatively, an epidemiological factor other than viral dose may be contributing to the presence or absence of disease. Colostral antibody and the continued presence of neutralising antibodies in milk play an important role in protecting young animals from enteric infections. Of the EqRV+ve foals without diarrhoea in this study, 5/6 were less than 19 days old, which may suggest maternal antibody was protecting these foals from developing clinical disease. However, four of these foals were from farm C, where mares were not vaccinated against EqRV, and so maternal antibody was not boosted by vaccination. There is the possibility that maternal antibody could have been boosted by natural exposure, given that this farm had the most cases of EqRV in the study. Collection of dam milk samples in future studies of this type would enable examination of the correlation between neutralising antibody in milk and the presence or absence of diarrhoea in foals in which EqRV is detected.

The peak detection of rotaviruses occurred in October, with 10/12 positive case foals being on Farm C. In addition, 4/6 EqRV positive control foals were from farm C and were sampled in October. This coincided with a rapid increase in the population density and in horse movement, as the peak months of foaling were September and October on all five farms. Farm C, in particular, had the highest population density in terms of the number of mares foaling on the property, which may have contributed to the spread and contamination of the environment with rotaviruses. In addition, this farm was the only farm in the study that did not routinely vaccinate mares against rotaviruses. Rotavirus vaccination was hastily implemented on farm C in October for mares that hadn't yet foaled, but only one or two of the three recommended intramuscular injections one month apart were administered.

This chapter also describes the development of an RT-qPCR assay to rapidly screen foal faecal samples for EqRV. This assay was adapted from primers published for the detection of rotavirus in children with diarrhoea (Pang *et al.*, 2004). Continued use of the assay for surveillance of human gastroenteritis led to the discovery by these authors that rotaviruses with the G2 genotype were either not detected or yielded higher Ct values (Pang *et al.*, 2011). When they compared NSP3 sequence of various G types of human rotavirus strains, 3-5 nucleotide mismatches were identified in the forward primer sequence, which, when modified, led to an improvement in the sensitivity of the assay across a number of genotypes.

At the time the primers from the original assay were used to screen foal faeces in our study, there was no EqRV NSP3 sequence available. However, this situation has changed and there are now EqRV NSP3 sequences available for ten G3 strains, eight G14 strains and one G13, one G5, and one G6 strain. Alignment of the NVP3-F primer with G3 and G14 equine NSP3 sequences revealed between 0 and 4 nucleotide mismatches with the forward primer and no mismatches with the NVP3-R primer. Alignment of these sequences with the modified NVP3-F2 primer published by Pang *et al.* (2011) revealed even more mismatches (3-6) than with the original primer (Figure 5-12).

With this new information, further improvements to the existing assay would include alterations to the forward primer sequence to account for EqRV NSP3 sequence mismatches and investigation of the effect this has on the sensitivity of the assay on foal faecal samples. Other future work might also include comparison of the sensitivity of this assay with an RT-qPCR assay that has become commercially available in Australia since the initiation of this study.

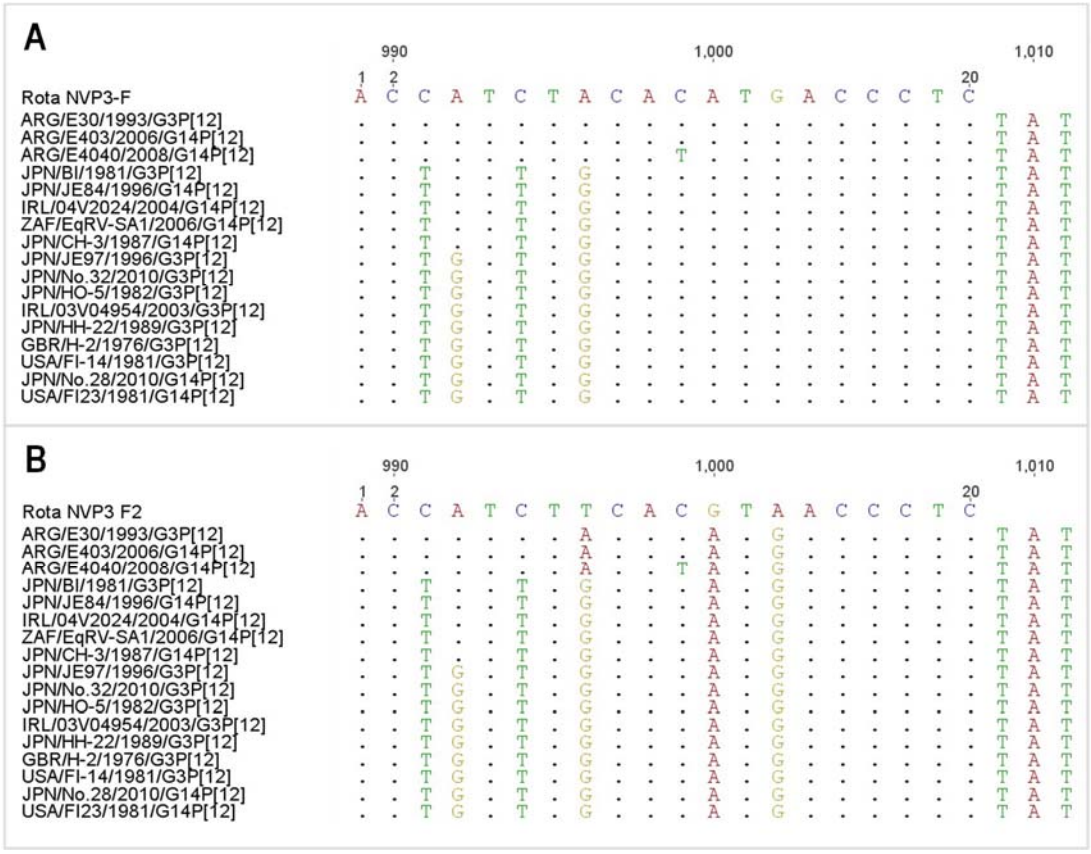

Figure 5-12 Forward primer sequence alignment with G3 and G14 EqRV sequences for the gene encoding NSP3. Alignment with the original NVP3-F primer (A) used in this study revealed between 0 and 4 nucleotide mismatches. Alignment with the modified NVP3-F2 primer (B) published by Pang *et al.* (2011) revealed between 3 and 6 nucleotide mismatches.

In this study there was no correlation between Ct value and the likelihood of being able to sequence a VP7 product. This is in contrast to Matthijnssens *et al.* (2015), where no samples with a Ct value above 36.10 could be G or P genotyped. The detection assay used in that study targeted the NSP5 gene and the G typing assay used primers Beg9/End9. In our study, optimisation of the VP7 RT-PCR assay led to better amplification with the VP7-Fdeg/VP7-Rdeg primers than the Beg9/End9 primers, although some samples that were negative with the VP7-Fdeg/VP7-Rdeg primers were positive with the Beg9/End9 primers. Greater variation in VP7 sequence and the larger amplicon size are possible reasons why the VP7 RT-PCR assay did not perform as well as the NVP3 qPCR assay. This theory is supported by the numerous different primer pairs used in human Rotavirus G typing (6 primer pairs are described in the

WHO Manual of rotavirus detection and characterization methods). Secondary structure at primer binding sites could have been another reason influencing the performance of the VP7 RT-PCR.

Only EqRV type G3 was detected in foals in this study, even though EqRV types G3 and G14 have both previously been detected in Australian foals (Browning and Begg, 1996). This could be due to geographical variation and periodic fluctuations in the prevalence of circulating G3 and G14 viruses, which have been described in Ireland, Argentina and Japan (Collins *et al.*, 2008; Garaicoechea *et al.*, 2011; Nemoto *et al.*, 2011). As the VP7-Fdeg/VP7-Rdeg primers align with the majority of G14 EqRV sequences available in Genbank, the absence of G14 sequences in this study population was unlikely to be the result of an inability of these primers to bind. In support of this, samples that didn't amplify with the VP7-Fdeg/VP7-Rdeg primers were also tested with Beg9/End9 primers, which have successfully amplified G14 EqRV in other studies (Collins *et al.*, 2008; Monini *et al.*, 2011; Nemoto *et al.*, 2011).

One EqRV sequence type detected in this study was found in foals from four of the five farms, suggesting the endemic maintenance and transmission of one virus between multiple farms, while the other EqRV sequence type was only found in foals from Farm C. Given the frequent movement of mares and foals between stud farms for breeding purposes, transmission between farms is not surprising. Biosecurity measures to prevent introduction of disease from visiting mares and foals to resident mares and foals on Australian thoroughbred breeding farms tend to focus on housing visiting mares in dedicated visiting yards in an area of the farm separate from resident horses. However, visiting mares returning to their own farm are generally not quarantined. Therefore, if a visiting mare and foal contaminate a yard with rotaviruses, and then another visiting mare and foal use the same yard, this could result in that mare and foal taking rotaviruses back to their own farm and transmitting disease. This could be an important consideration for prevention of EqRV transmission between farms.

CHAPTER 6 DETECTION OF CORONAVIRUSES

6.1 INTRODUCTION

Coronaviruses are single stranded, positive sense, enveloped RNA viruses belonging to the family *Coronaviridae*. Equine coronaviruses (ECoV) belong to the genus *Betacoronavirus* and are closely related to bovine coronavirus (BCoV), human coronavirus OC43 and porcine haemagglutinating encephalomyelitis virus (Zhang *et al.*, 2007). Coronaviruses were first detected in foals with diarrhoea in 1975 by electron microscopy (Bass and Sharpee, 1975) and first isolated in cell culture from the faeces of a foal with diarrhoea in 1999 (Guy *et al.*, 2000). More recently there have been multiple outbreaks of ECoV reported in adult horses in Japan and the USA (Oue *et al.*, 2011; Oue *et al.*, 2013; Pusterla *et al.*, 2013). Clinical signs of disease in adult horses have predominantly been inappetance, fever and diarrhoea and disease has been experimentally reproduced in adult draft horses (Nemoto *et al.*, 2014; Oue *et al.*, 2011; Oue *et al.*, 2013; Pusterla *et al.*, 2013). These outbreaks have led to further investigations of the prevalence of ECoV, largely in adult horses, and the development of RT-PCR, RT-qPCR and RT-LAMP molecular assays for the detection of ECoV in faecal samples (Nemoto *et al.*, 2015a; Pusterla *et al.*, 2013) and an ELISA for the detection of antibodies against ECoV (Kooijman *et al.*, 2017; Kooijman *et al.*, 2016).

In a survey of thoroughbred foals with and without diarrhoea a recent study in Japan found ECoV was neither prevalent nor related to diarrhoea (Nemoto *et al.*, 2015b). While in Kentucky in the USA, ECoV were detected at a prevalence of nearly 30% of samples tested and at a similar frequency in foals both with, and without gastrointestinal disease (Slovis *et al.*, 2014). Therefore, the significance of ECoV in the pathogenesis of diarrhoea in foals is unclear. Detection of ECoV in the US study used a real-time PCR assay panel that screened foal faeces for 9 infectious agents. This assay has become commercially available in both the USA and more recently Australia (IDEXX), although the protocol has not been published. At the time this research project was initiated there was no ECoV detection method readily available to horse

owners and veterinarians in Australia. This chapter describes the development of an RT-qPCR assay to screen the faeces of thoroughbred foals in Australia for ECoV.

6.2 APPROACH TO THE DETECTION OF CORONAVIRUSES IN FOAL FAECAL SAMPLES

To screen foal faecal samples for the presence of ECoV previously described primers for the detection of coronavirus in cattle (Cho *et al.*, 2010) were adapted for use in a two-step RT-qPCR assay. Assay development, optimisation and generation of a qPCR standard were performed using an archived foal faecal sample positive for coronavirus. The assay was then used to screen nucleic acids extracted from foal faeces stored in RNAlater® (Section 2.1.2) with the PowerSoil®-htp 96 well Soil DNA Isolation kit (Section 2.2.2). Complementary DNA was generated (Section 2.2.8) and used as template in the qPCR assay (Section 2.3.3).

6.3 RESULTS

6.3.1 Development and validation of a qPCR to detect ECoV in foal faeces

At the time primers were being selected for detection of ECoV there were no published ECoV RT-qPCR assays. Alignment of the sequences available for the gene encoding the nucleocapsid (N gene) protein for five ECoV in GenBank was used to design four sets of primers with Geneious® software (version 9.0.5, Kearse *et al.* (2012)). These primers were tested on an archived foal faecal sample (#219) previously found to be ECoV positive using an in house unpublished nested RT-PCR assay. Only one of the primer sets (KBECoV F2 and R2) could amplify an ECoV product, but it also resulted in amplification of non-specific products. An attempt to optimise primer concentration and $MgCl_2$ concentration to reduce non-specific amplification was unsuccessful (data not shown) and these primers were not tested further.

The next approach used to develop an ECoV assay was to align published ECoV conventional PCR primers (Guy *et al.*, 2000; Oue *et al.*, 2011) and qPCR primers for detection of coronaviruses in other species, such as dogs (Mitchell *et al.*, 2009) and cattle (Cho *et al.*, 2010), with the available ECoV sequences in GenBank (accession numbers NC_010327, AB555559 and AB671298) to see if any could be adapted. Unpublished primers, ECoV.F22-43, ECoVnest and ECoV.R549-70, from an in house nested PCR were also aligned (Figure 6-1).

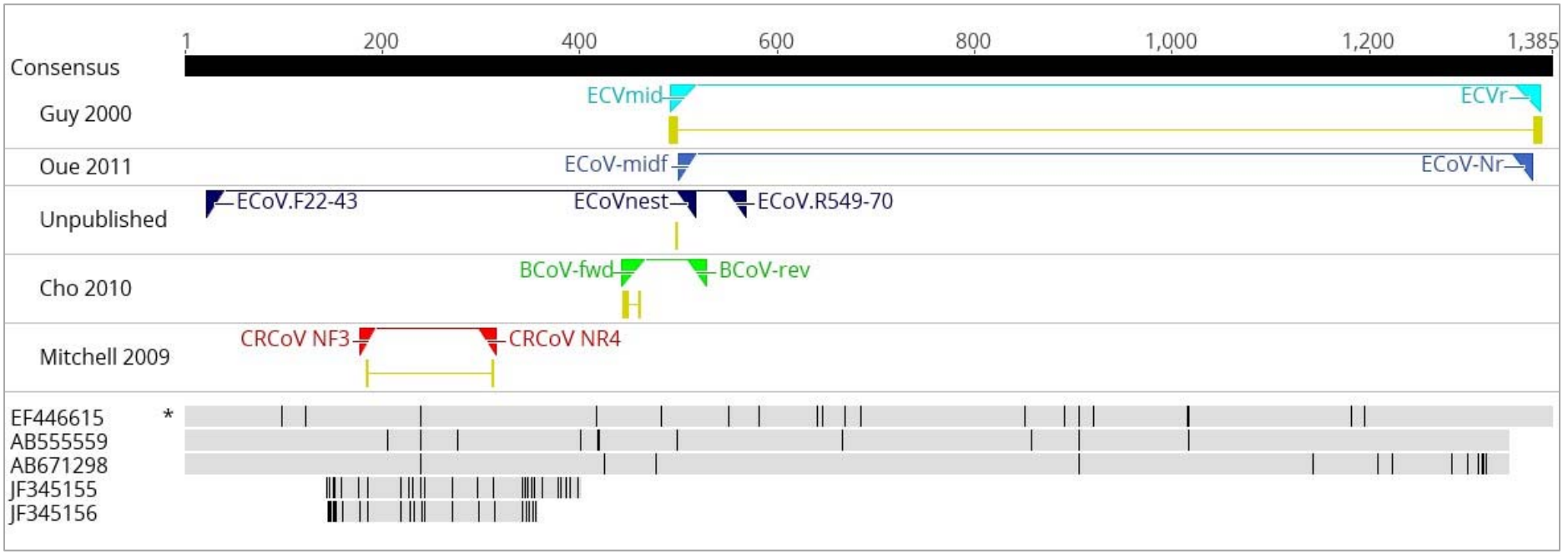

Figure 6-1 Nucleotide alignment of four ECoV N gene sequences (Genbank accession numbers displayed) and PCR primer binding sites. Primer pairs targeting ECoV include ECVmid and ECVr (Guy *et al.*, 2000), EcoV-midf and ECoV-NR (Oue *et al.*, 2011) and ECoV.F22-43, ECoVnest and ECoVR549-70 (unpublished nested PCR primers) are shown in shades of blue. Primers BCoV-fwd and BCoV-rev (Cho *et al.*, 2010) targeting BCoV are shown in green and primers CRCOv NF3 and CRCoV NR4 (Mitchell *et al.*, 2009) targeting canine respiratory CoV are shown in red. Primer sequence mismatches with ECoV nucleotide sequence are shown in yellow.

The reverse primer (BCoV-rev) targeting the gene encoding the nucleocapsid protein of bovine coronavirus (Cho *et al.*, 2010) matched the available ECoV sequences, while the corresponding forward primer (BCoV-fwd) had four nucleotide mismatches. The mismatches were consistent in all 3 available ECoV sequences covering this region of the gene encoding the N protein and so this primer sequence was adapted to match the ECoV sequence and was referred to as BCoV-fwd alt (see Figure 6-2).

Figure 6-2 Alignment of BCoV forward (A) and reverse (B) primers with the sequences of the gene encoding the nucleocapsid protein of three ECoV (GenBank accession numbers NC_010327, AB671298 and AB555559). Identical nucleotides are indicated by dots (.) and nucleotide differences in colour. The nucleotide sequence of primer BCoV-fwd has been modified to match the ECoV sequences and re-named BCoV-fwd alt.

The same archived foal faecal sample (# 219) was used to test the adapted bovine coronavirus primer pair BCoV-fwd alt and BCoV-rev. Nucleic acids were extracted from the foal faeces (Section 2.2.1) and the reverse primer was used to reverse transcribe cDNA (Section 2.2.8) and then 5µl of cDNA was tested in duplicate in a SYTO® 9 qPCR assay. A product was amplified that generated a single dissociation peak with a melting temperature of 83.4°C from both replicates, while there was no amplification of the no template controls (NTC).

An expected 87 bp band was visualised (not shown) when the positive control qPCR product was separated by electrophoresis on a 1.5% agarose gel (Section 2.2.5). The band was then excised from this gel, purified (Section 2.2.6) and cloned into a plasmid vector for use as a DNA standard (pGEM-T.ECoV.219) (Section 2.4).

The DNA standard (pGEM-T.ECoV.219) was then used as template while the assay conditions were optimised. Parameter variations that were tested included forward and reverse primer concentrations between 0.2 µM and 1.0 µM per reaction, $MgCl_2$ concentrations from 1.5 mM to 3.0 mM per reaction and annealing temperatures between 57°C and 64°C.

The optimal assay conditions were selected based on the concentrations and annealing temperature that produced the lowest Ct value and a single dissociation curve peak (data not shown). This corresponded to a forward and reverse primer concentration of 0.35 µm, a $MgCl_2$ concentration of 2 mM and an annealing temperature of 60°C (Section 2.3.2 and 2.3.3).

A standard curve was generated by testing 10-fold serial dilutions of the pGEM-T.ECoV.219 plasmid DNA in triplicate (Figure 6-3). Amplification was detected in all three replicates over a linear range of 6 orders of magnitude, from 10^2 to 10^7 DNA copies per reaction with high amplification efficiency (94.7%).

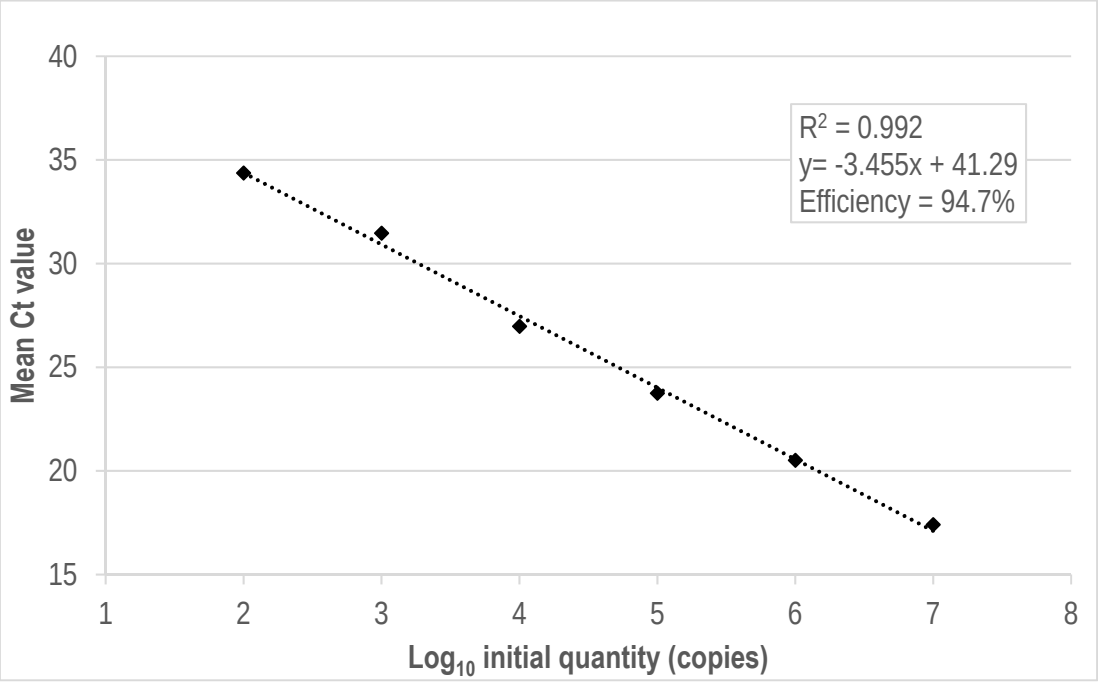

Figure 6-3 ECoV qPCR assay standard curve produced from 10 fold serial dilutions of pGEM-T.ECoV.219 plasmid DNA

To assess the reproducibility of the ECoV qPCR assay, the intra-assay and inter-assay variation was determined. The intra-assay variation was determined by calculating the CV% (Section 2.3.5) between Ct values of the replicates of a 10-fold serial standard dilution series with between 10^2 and 10^7 DNA copies/reaction (Table 6-1). The inter-assay variation was determined by calculating the CV% of the mean Ct values of two replicates of a 10-fold standard dilution series with between 10^2 and 10^7 DNA copies/reaction performed in five independent assay runs (Table 6-2). Both the intra-assay and inter-assay variation were low (≤3.67 CV% and ≤2.99 CV%, respectively), which indicated that the assay was both precise and reproducible.

Table 6-1 Intra-assay variation of the ECoV RT-qPCR assay

Copies[a]	Ct Value			Mean	SD	CV%
	Replicate					
	1	2	3			
7	17.6	17.1	17.6	17.4	0.31	1.78
6	20.8	20.6	20.3	20.5	0.24	1.17
5	24.1	23.5	23.7	23.8	0.31	1.30
4	25.9	27.8	27.3	27.0	0.99	3.67
3	31.3	31.6	31.5	31.5	0.17	0.54
2	34.6	33.8	34.7	34.4	0.48	1.40

[a]$\log_{10}$ DNA copies per reaction

Table 6-2 Inter-assay variation of the ECoV RT-qPCR assay

Copies[a]	Ct Value[b]					Mean	SD	CV%
	Assay							
	1	2	3	4	5			
7	17.3	17.5	18.3	17.3	17.1	17.5	0.49	2.81
6	20.4	20.4	21.6	20.4	20.0	20.6	0.61	2.99
5	23.3	23.6	24.9	23.6	23.6	23.8	0.62	2.60
4	27.4	27.3	28.6	27.6	27.2	27.6	0.59	2.14
3	30.6	31.0	31.2	31.6	30.2	30.9	0.54	1.73
2	35.4	34.6	35.1	34.3	34.1	34.7	0.54	1.57

[a]$\log_{10}$ DNA copies per reaction

[b]Mean of 2 replicates

6.3.2 Detection of ECoV in foal faeces

When the ECoV qPCR assay was used to screen foal faecal samples collected in this study, both specific and non-specific products were amplified despite optimisation of assay conditions to detect a single specific dissociation product. Therefore, foal faecal samples were considered potentially positive for ECoV if there was an ECoV qPCR amplification product with an appropriate dissociation melt peak (Tm 80.1-84.2°C), and an appropriate sized band could be visualised by gel electrophoresis. To confirm the amplification of ECoV, gel excision and purification (Section 2.2.6) was attempted on these samples but the small amplicon was hard to separate from the primer band even when a 3% w/v agarose gel was used. Together with the opacity of the gel and low level of fluorescence of some of the bands, excision and sequencing was unsuccessful.

To further elucidate whether these were true positives and to dilute any potential faecal inhibitors, the cDNA from 30 potentially qPCR positive samples was diluted 1:10 and retested in triplicate with the ECoV qPCR. If there was an appropriate dissociation product in at least two out of three replicates these qPCR products were sequenced to confirm whether they were true positives. If only one of the three replicates had an appropriate dissociation product the result was classified as negative and sequencing was not performed. Using these criteria, amplification with an appropriate dissociation product in at least two out of three replicates occurred in 16 samples. These qPCR products were then separated slowly at 60V on a 2.5% agarose gel (Section 2.2.5), the DNA bands excised and purified (Section 2.2.6), before being cloned into pGEM-T for sequencing (Section 2.4 and 2.5). Fifteen out of 16 of these were confirmed to be the expected ECoV sequence, including seven age matched case foals, four age matched control foals and four unmatched case foals. ECoV was not detected in any hospital case foals (Table 6-3).

Due to non-specific amplification in foal faecal samples, the Ct values did not accurately represent the ECoV viral load present in samples confirmed as ECoV positive and so comparison between viral load and the presence of clinical disease could not be performed.

Table 6-3 Tm and amplicon sequencing results of foal faecal samples potentially ECoV positive

Foal ID #	Sample type	ECoV qPCR Tm	Replicates with appropriate Tm[a]	ECoV+ve sequence
86	Case	82.5	+ + +	Y
123	Control	82.5		Np
124	Case	82.5		Np
142	Hospital	83.2		Np
151	Control	83.6	+ + +	Y
156	Unmatched case	83.1	+ + +	Y
159	Control	82.6	+ +	Y
165	Unmatched case	83.0	+ +	Y
173	Case	82.0	+ + +	Y
188	Unmatched case	83.0	+ + +	Y
193	Unmatched case	84.1	+	Np
207	Control	83.1		Np
222	Case	83.1		Np
228	Control	82.7		Np
230	Unmatched case	83.1	+ + +	Y
235	Control	82.1		Np
236	Case	84.2	+ +	Y
237	Control	81.7	+ + +	Y
238	Case	83.2	+ + +	Y
239	Control	83.7	+ + +	Y
241	Control	84.2	+	Np
243	Case	82.6	+ + +	Y
245	Case	82.6	+ + +	Y
245b	Case	82.7	+ + +	N
249	Control	83.2	+	Np
262	Control	80.1		Np
279	Case	80.7	+	Np
307	Case	82.7	+ + +	Y
308	Control	82.7	+	Np
311	Case	83.7	+	Np

[a]cDNA diluted 1:10 and tested in triplicate; +, Amplification product with appropriate Tm; Y, Yes; N, No; np, sequencing not performed if <2/3 triplicates had appropriate Tm

6.3.3 Analysis of ECoV detected in age matched case control foals

In the age-matched case control series, ECoV was detected by RT-qPCR in 5.9% (7/117) of case foals and 3.4% (4/117) of control foals. To determine if there was a significant association between the detection of ECoV and the presence of diarrhoea, the ECoV status of each age matched case-control pair was determined (Table 6-4). A matched analysis of discordant pairs showed the odds of detecting coronavirus in a foal with diarrhoea were 2.5 times that of detecting coronavirus in a foal without diarrhoea, however this was not statistically significant (95% CI 0.409 – 26.3, P=0.453).

Table 6-4 Matched analysis of case control pairs in relation to ECoV status

Case-control pair exposure	ECoV
Case + Control +	2
Case + Control −	5
Case – Control +	2
Case − Control −	108
Total number of case-control pairs	117

ECoV was detected in foals from all five farms. The age of all positive foals ranged from 1 to 92 days with a median age of 21 days. Age-matched case foals positive for ECoV ranged in age from 1-92 days with a median age of 21 days (Figure 6-4 A). Control foals positive for ECoV ranged in age from 5-26 days with a median age of 15.5 days (Figure 6-4 B). The median age of case and control foals positive for ECoV was not significantly different (P=0.927).

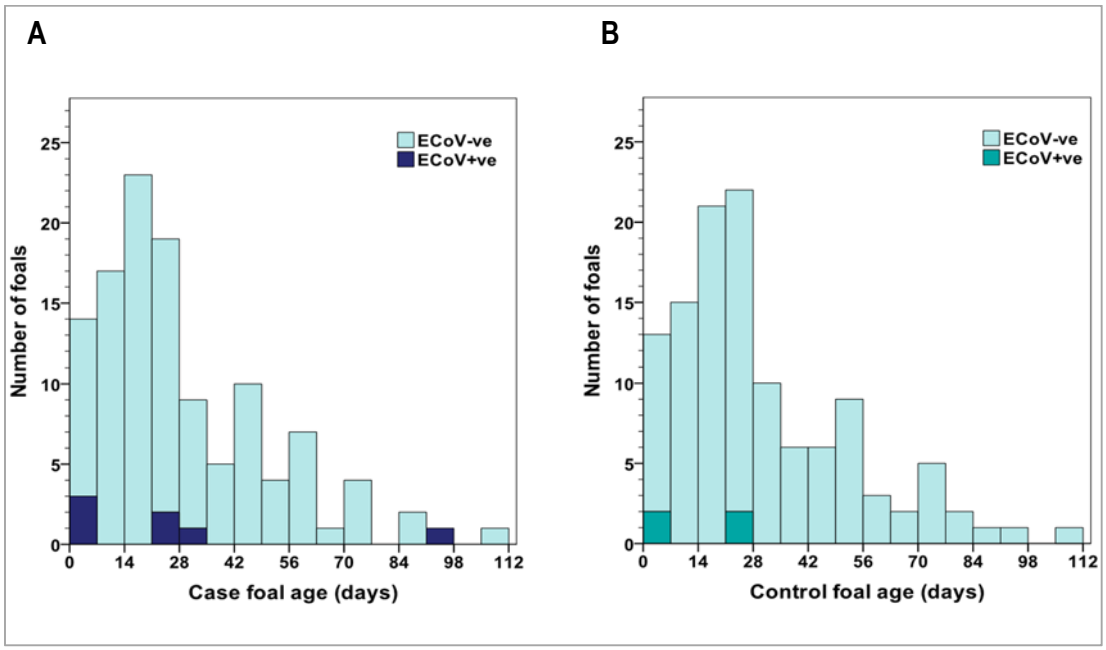

Figure 6-4 Age distribution of age-matched (A) cases and (B) controls and showing those that had an ECoV positive sample

ECoV was detected in foal faecal samples collected in October, November and December with the peak detection in age-matched foals occurring in November (Figure 6-5).

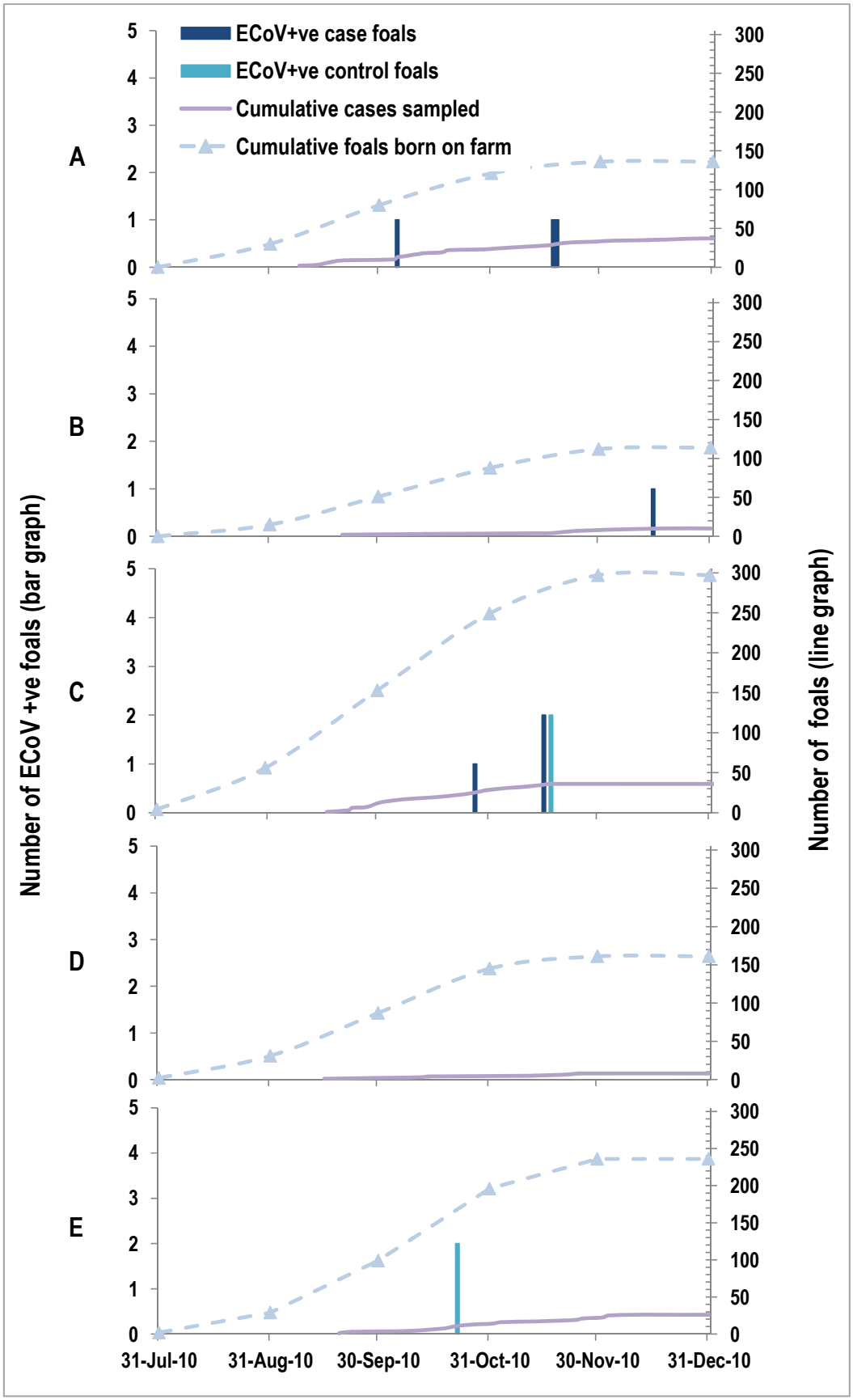

Figure 6-5 Temporal distribution of ECoV positive case and control foals in relation to the number of cases sampled and the number of foals born on each farm (A-E) in 2010

6.4 DISCUSSION

This is the first study to investigate ECoV in Australia. In the age-matched case control samples ECoV was detected more frequently in foals with diarrhoea (6%) than those without diarrhoea (3%), but ECoV was not significantly associated with disease. These results are consistent with a recent study in thoroughbred foals in Japan that only detected ECoV at a low prevalence in healthy foals and therefore was not associated with disease (Nemoto *et al.*, 2015b). Although at a higher prevalence of nearly 30%, a study in Kentucky in the USA also found an equal likelihood of detecting ECoV in foals with and without gastrointestinal disease (Slovis *et al.*, 2014). In the US study gastrointestinal disease was defined as foals with diarrhoea, but also encompassed foals with ultrasonographic evidence of excessive fluid in the small intestine and/or colon, and a history of fever, inappetance, or colic. Thus, despite a different case definition meaning a direct comparison cannot be made between studies, there was still no association identified between ECoV and disease. There have been case reports of ECoV in foals with severe disease, however these foals have had other complicating factors such as combined immunodeficiency syndrome or concurrent infections with other pathogens such as cryptosporidium and coccidiosis (Mair *et al.*, 1990; Solis and Foreman, 2010). In Kentucky, foals with gastrointestinal disease and coronavirus infections were associated with co-infections with other enteric infectious agents, while infections in healthy foals were more likely to be mono-infections (Slovis *et al.*, 2014). This may suggest ECoV infection alone in foals is not of clinical significance but requires further investigation. In the current study coronavirus co-infection was detected in one age-matched case foal with *Salmonella* and in one unmatched case foal with EqRV. Co-infection results will be discussed in more detail in 0.

In the current study, ECoV was not detected in hospitalised foals with diarrhoea. Similarly, a previous study of enteric infectious agents in hospitalised foals found a low prevalence (1%) of ECoV in foals with diarrhoea (Frederick *et al.*, 2009). This suggests that disease due to ECoV infection alone does not generally result in a requirement for the foal to be hospitalised for treatment.

Coronaviruses cause enteric, respiratory and neurological disease in a number of different animal species and humans (Balasuriya, 2013). In cattle, BCoV is considered an enteric pathogen in neonatal calves and is associated with winter dysentery in adult

cattle (Benfield and Saif, 1990; Saif, 1993). With the recent outbreaks of clinical disease and the detection of ECoV in adult horses there has been an increase in investigation of the prevalence of ECoV in adult horses with diarrhoea as well as healthy animals and those with respiratory disease. From these studies it has been ascertained that ECoV is shed in faeces and to a lesser extent in nasal secretions and that disease is more common in the cooler months of the year (Miszczak *et al.*, 2014; Nemoto *et al.*, 2014; Oue *et al.*, 2011; Pusterla *et al.*, 2015; Pusterla *et al.*, 2013; Pusterla *et al.*, 2016). Disease in adults has been experimentally reproduced in adult draft horses, but, experimental infection of adult thoroughbred horses did not produce disease (Nemoto *et al.*, 2014). A seroprevalence study of ECoV in the United States found a prevalence of 9.6% in healthy horses with a significant association between draft horses and seropositivity (Kooijman *et al.*, 2017). This suggests there may be a difference in breed susceptibility to ECoV infection. Similar future studies in foals, including experimental infection with ECoV alone and in combination with other enteric infectious agents would be valuable in establishing the pathogenic significance of ECoV infection in foals.

Outbreaks of ECoV in adult horses have generally resulted in low mortality rates (Oue *et al.*, 2011; Oue *et al.*, 2013; Pusterla *et al.*, 2013). However, a report of more severe disease with a higher fatality rate reported a significantly higher viral load in those horses that died (Fielding *et al.*, 2015). In the current study, despite optimization of the BCoV adapted ECoV RT-qPCR assay conditions to produce a specific amplicon, testing of foal faecal samples resulted in non-specific amplification. This made interpretation of qPCR results challenging, in particular whether samples were truly positive, and it also meant the viral load could not be inferred from the Ct value. With the subsequent description of an ECoV RT-qPCR assay by Pusterla *et al.* (2013) future screening of the samples from the current study with this assay may clarify if samples were truly positive and enable determination of the faecal viral load and the significance of ECoV detection in foals with and without diarrhoea.

Whether virulence varies in different strains of ECoV remains to be determined as very little is known about circulating ECoV strains due to the difficulty involved in isolation of ECoV in cell culture. Currently there are only four ECoVs that have been isolated and propagated in cell culture (Guy *et al.*, 2000; Nemoto *et al.*, 2015c; Oue *et al.*, 2011). Future isolation in cell culture of the ECoV detected in the current study and

comparison with the three Japanese ECoV isolates from adult horses and the American NC99 isolate from a diarrhoeic foal may be beneficial. Furthermore, surveillance of ECoV in adult horses in Australia and comparison of strains circulating in adult horses and foals may contribute valuable information in the future.

In summary, ECoV were detected in foals in Australia at a relatively low prevalence and there was no association between the presence of diarrhoea and the detection of ECoV. Co-infection with ECoV and another infectious agent was detected in only two foals. The pathogenic significance of ECoV in foal diarrhoea remains unclear and requires further investigation, including exploration of the interaction of co-infections in relation to clinical disease.

CHAPTER 7 DETECTION OF *SALMONELLA*

7.1 INTRODUCTION

Salmonella spp. infection is known to cause disease in horses of all ages, both in individual animals and outbreak scenarios (Bryans *et al.*, 1961; Edwards, 1934; Peet *et al.*, 1980; Walker *et al.*, 1991). *Salmonella* is not the most common infectious cause of diarrhoea in foals, however when it does occur the disease course can be severe and rapidly fatal (Bryans *et al.*, 1961; Mahaffey, 1952; Morse *et al.*, 1976). Other forms of clinical disease in foals due to salmonellosis with the potential to lead to mortality also occur, such as septicaemia and localised infections (e.g. septic arthritis, physitis, meningitis). In addition, clinically normal foals may shed *Salmonella* spp.in their faeces (Carter *et al.*, 1979). As salmonellosis is a zoonotic disease, infected animals pose a threat to the health of humans and other animal species in contact with them. Therefore, rapid detection of *Salmonella* infection is important to enable targeted treatment of individual animals, improve management of an outbreak and ensure appropriate biosecurity measures to protect human health.

Detection of *Salmonella* spp. by bacterial culture often requires selective enrichment followed by sub-culture on selective media, as isolation from primary culture is poor unless the organism is present in large numbers (Smith *et al.*, 1979). This leads to a delay of 3-5 days for culture results (Hyatt and Weese, 2004). In addition to this, faecal shedding can be intermittent making detection challenging (Roberts and O'Boyle, 1981). To be confident a horse is *Salmonella* negative by culture the testing of 5 faecal samples collected 24 hours apart has been recommended (Smith *et al.*, 1978). Detection of *Salmonella* infections by PCR and qPCR is reportedly more sensitive and more rapid than bacterial culture (Amavisit *et al.*, 2001; Cohen *et al.*, 1996; Cohen *et al.*, 1995; Pusterla *et al.*, 2010; Slovis *et al.*, 2014). As qPCR assays are based on detection of nucleic acid, viability of the bacterium is not required, rather, only the target nucleic acid sequence needs to be intact. Due to the sensitivity of PCR, the potential to also detect non-viable organisms (Cangelosi and Meschke, 2014; Josephson *et al.*, 1993), and detection of *Salmonella* in horses without clinical disease,

interpretation of positive PCR results can be difficult. However, PCR results are still useful for screening foals to exclude *Salmonella* infection as the cause of diarrhoea.

At the time this study was initiated there was no qPCR assay routinely available to detect *Salmonella* in horses in Australia. This chapter describes the detection of *Salmonella* in foal faecal samples by both bacterial culture and qPCR assay.

7.2 APPROACH TO THE DETECTION OF *SALMONELLA* SPP. IN FOAL FAECAL SAMPLES

Bacterial culture for *Salmonella* spp. (Section 2.6.1 & 2.6.2) was performed on foal faecal samples stored in glycerol broth (Section 2.1.1). Any *Salmonella* isolates cultured were then further characterised by PFGE (Section 2.7.2), antimicrobial sensitivity (Section 2.6.3) and serotyping (Section 2.7.4). While performing bacterial culture for *Salmonella* from foal faeces an aliquot of selenite enrichment broth from each sample was collected and stored at -70°C. Nucleic acids were then extracted from 200 µl of thawed selenite broth (Section 2.2.3). A TaqMan probe qPCR assay targeting the *invA* gene (Table 2-1 and Table 2-3) was then used to screen foals for the presence of *Salmonella*.

7.3 RESULTS – CULTURE

7.3.1 Isolation of *Salmonella* spp. in foal faeces

Salmonella spp. were isolated by bacterial culture from the faeces of four foals, including three age-matched case foals and one control foal (Table 7-7). No *Salmonella* was detected in the hospital case series.

7.3.2 *Salmonella* confirmation and further characterisation

Acid negative and H_2S positive bacterial colonies on XLD agar from four foal faecal samples, that were negative when inoculated on a urease slope, were identified as presumptive *Salmonella* spp. by latex agglutination (Section 2.6.2). An Enterotube II was inoculated and interpreted for each *Salmonella* isolate. All four isolates resulted in an Enterotube code of 27071, which was consistent with *Salmonella* spp.

The antimicrobial sensitivity profile of each *Salmonella* isolate was determined by CDS method (Section 2.6.3) and found to be identical for all four isolates. The

Salmonella isolates were susceptible to all antimicrobials tested except methicillin and sulfafurazole (Table 7-1).

Table 7-1 Antimicrobial sensitivity of *Salmonella* isolates

Antibiotic	Concentration (µg)	Zone of inhibition annular radius (mm)	Susceptibility
Ampicillin	25	11	Sensitive
Tetracycline	30	9	Sensitive
Sulfafurazole	300	2	Resistant
Trimethoprim	5	11	Sensitive
Enrofloxacin	5	12	Sensitive
Gentamicin	10	7	Sensitive
Ceftiofur	30	10	Sensitive
Methicillin	5	0	Resistant
Ticarcillin	75	11	Sensitive

For further characterisation, PFGE was used to compare the Xba1 restriction endonuclease digestion pattern of these four *Salmonella* isolates (Section 2.7.2). The isolates from foals #195, #196 and #238 were all from the same farm and had the same Xba1 digestion pattern, while the isolate from foal sample #311b, from another farm a few months later, had a different Xba1 digestion pattern (Figure 7-1).

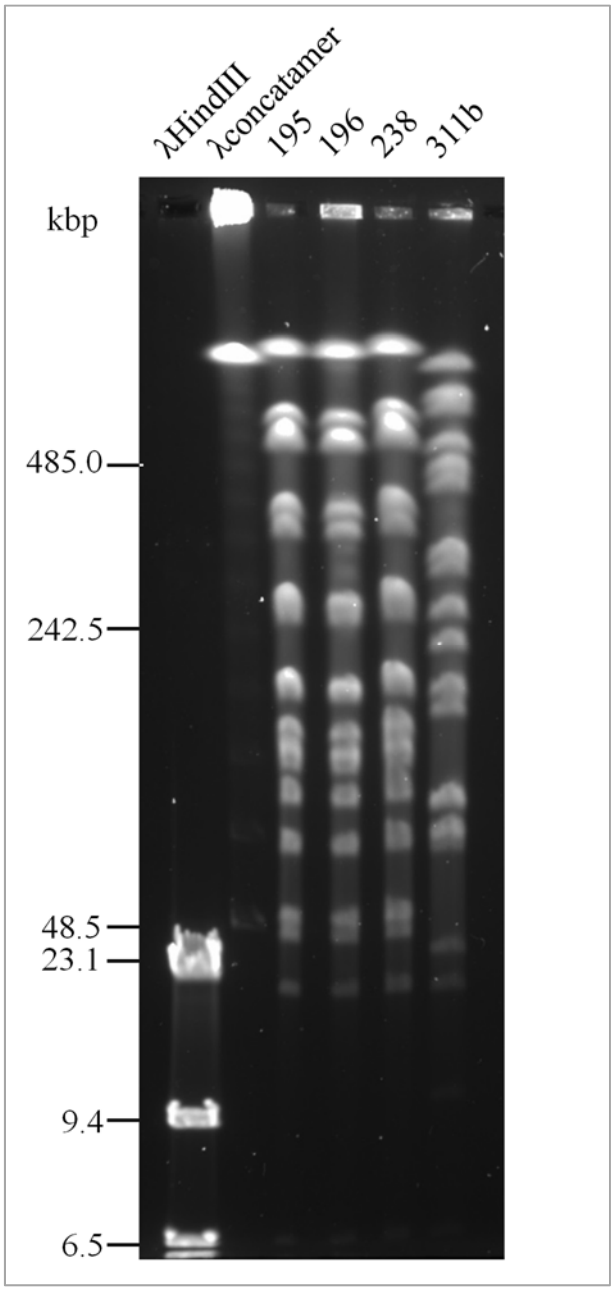

Figure 7-1 PFGE Xba1 digestion patterns of *Salmonella* isolates. Lane 1-lambda DNA digested with HindIII, lane 2-lambda concatamers, lanes 3-6 *Salmonella* isolates from foals 195, 196, 238 and 311.

The *Salmonella* isolate from foal #195, representative of the PFGE Xba1 digestion pattern for farm C, and the isolate from foal sample #311b, on farm A, were sent to the MDU for *Salmonella* serotyping (Section 2.7.4) and were found to be *Salmonella* enterica subsp. enterica serovar Mbandaka and *Salmonella* enterica subsp. enterica serovar Potsdam, respectively.

7.4 RESULTS –QPCR

7.4.1 Adaptation and validation of a qPCR to detect *Salmonella* in foal faeces

To detect *Salmonella* spp. in foal faeces forward primer *invA*-156f, reverse primer *invA*-288r and probe *invA*-189p sequences (Table 2-1) were selected from a published study on the detection of *Salmonella* spp. in horse faecal samples (Pusterla *et al.*, 2010). These primers target the *invA* gene on the pathogenicity island 1 of invasive *Salmonella* spp. that encodes the type III secretion system protein.

DNA template from an isolate of *Salmonella Typhimurim* (provided by the Asia Pacific Centre for Animal Health, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne) was prepared by selection of a single colony from sheep blood agar with a flamed metal loop and immersion in 50 µl of sterile water, which was then heated at 99°C for 5 mins. Five µl of this template was used to test the *invA* primers, without the TaqMAn probe, in a SYTO® 9 assay, generating a qPCR amplicon of the expected size, with a single dissociation peak and a melting temperature of 80.7°C (not shown). The ability of the assay to detect a range of *Salmonella* spp. was assessed by testing 16 other *Salmonella* isolates from 7 serotypes including: *Salmonella* Agona (1), *Salmonella* Dublin (1), *Salmonella* Enteritidis (4), *Salmonella* Havana (1), *Salmonella* Hessarek (3), *Salmonella* Muenchen (1), *Salmonella* Typhimurium (5) of avian, canine, equine, feline, ovine, and seal origin. Isolates were provided by the Laboratory of Clinical Microbiology, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne. All isolates produced a specific dissociation product with a melting temperature range of 80.1-81.1°C (Figure 7-2) and the expected 132 bp band when separated on an agarose gel by electrophoresis (Figure 7-3). Two sheep intestinal isolates of Yersinia psuedotuberculosis (data not shown) and a beta haemolytic *E. coli* isolate (provided by the Laboratory of Clinical Microbiology, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne) were also tested to assess the specificity of the assay. There was no amplification detected.

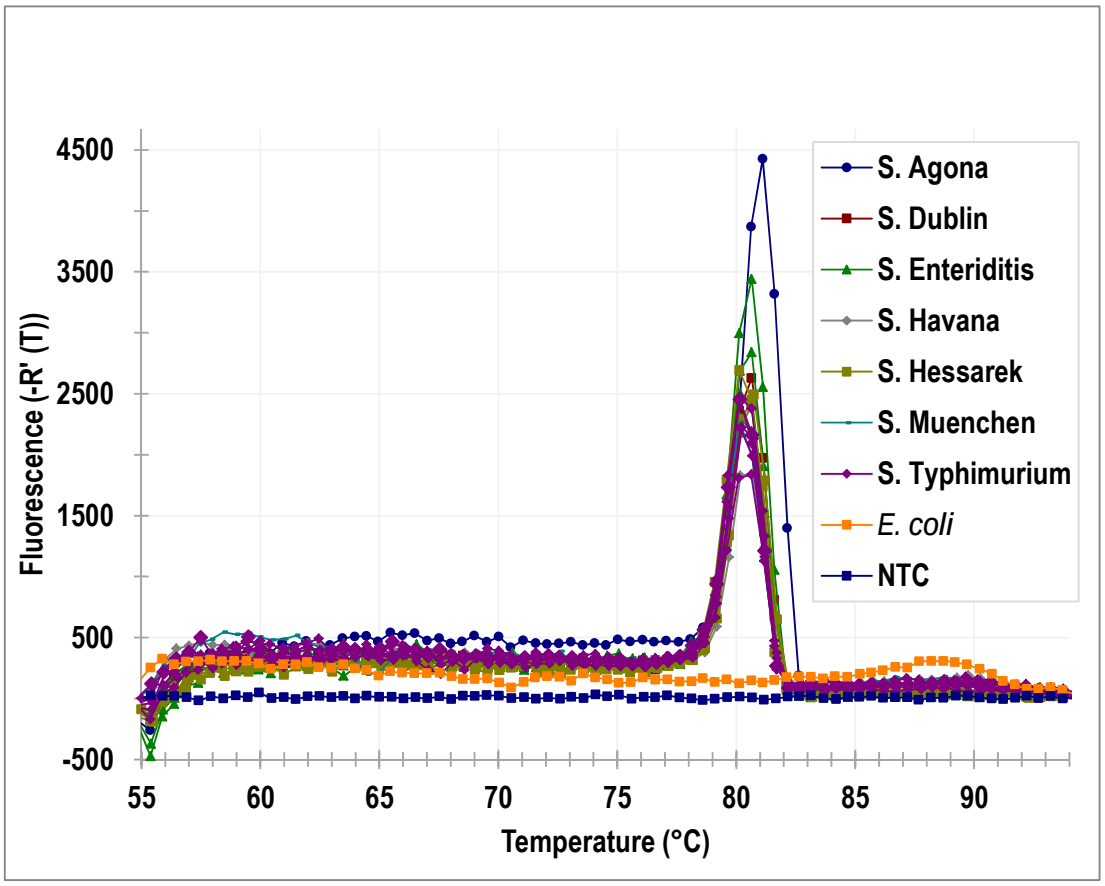

Figure 7-2-*Salmonella invA* qPCR assay dissociation curves of 16 *Salmonella* isolates from seven serotypes, one *E. coli* isolate and a NTC. Tm range 80.1-81.1°C

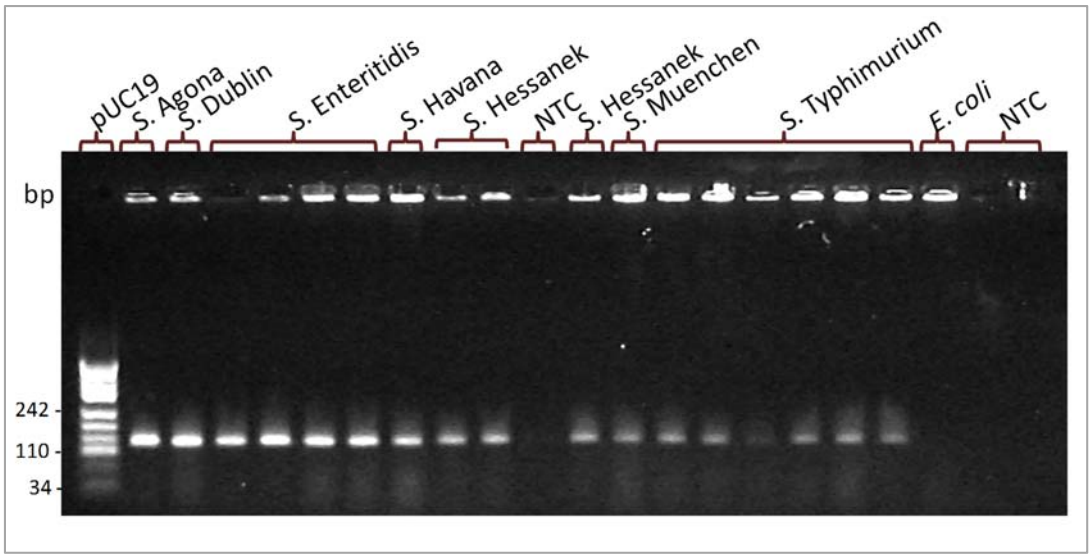

Figure 7-3 Gel electrophoresis of *invA* qPCR products from seven *Salmonella* serotypes (shown above the respective lanes). A 501 bp DNA molecular weight marker (pUC19 MspI), an *E. coli* isolate and NTCs are also shown.

A positive DNA standard (pGEM-T.311b) was prepared by cloning the *invA* qPCR amplicon into a plasmid vector (Section 2.4). A standard curve was then generated by testing 10-fold serial dilutions of the pGEM-T.311b plasmid DNA in triplicate (Figure 7-4) with the *invA* qPCR TaqMan probe assay (Section 2.3.4). Amplification of the DNA standard was detected consistently in all three replicates over a linear range of 6 orders of magnitude from 10^2 to 10^7 DNA copies per reaction with an amplification efficiency of 93.5%.

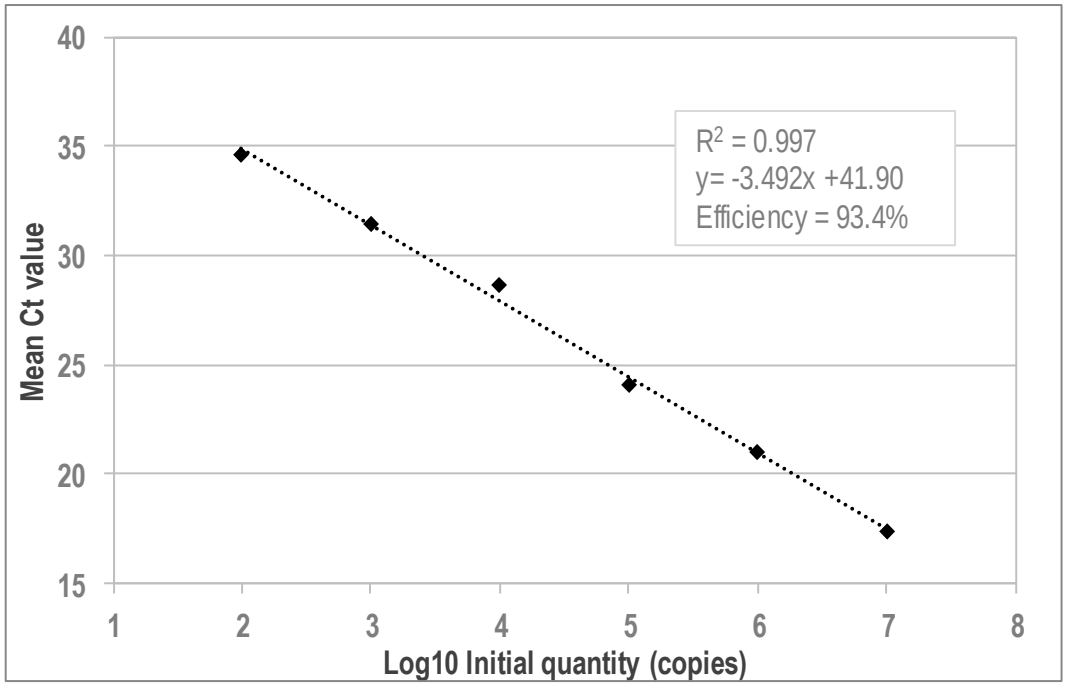

Figure 7-4 *Salmonella invA* **qPCR TaqMan probe assay standard curve produced from 10-fold serial dilutions of pGEM-T.311b plasmid DNA**

To evaluate the reproducibility of the *Salmonella invA* qPCR TaqMan probe assay, the intra-assay and inter-assay variation was determined. The intra-assay variation was determined by calculating the CV% (Section 2.3.5) between Ct values of three replicates of a 10-fold serial standard dilution series (Table 7-2). The inter-assay variation was determined by calculating the CV% of the Ct value of standard dilutions representing 10^7, 10^5 and 10^2 plasmid copies from 4 independent assay runs (Table 7-3). Both the intra- and inter-assay variation were low (≤7.20 CV% and ≤4.57 CV%, respectively) indicating the assay was precise and reproducible.

Table 7-2 Intra-assay variation of the *Salmonella invA* qPCR TaqMan probe assay

Copiesa	Ct value			Mean	SD	CV%
	Replicate					
	1	2	3			
7	18.0	15.9	18.2	17.4	1.25	7.20
6	20.9	21.0	21.1	21.0	0.10	0.48
5	23.6	24.1	24.6	24.1	0.46	1.91
4	29.4	28.3	28.2	28.7	0.65	2.27
3	31.0	31.7	31.7	31.4	0.40	1.27
2	34.7	34.6	34.6	34.6	0.10	0.29

[a]$\log_{10}$ DNA copies per reaction

Table 7-3 Inter-assay variation of the *Salmonella invA* qPCR TaqMan probe assay

Copies[a]	Ct value				Mean	SD	CV%
	Assay						
	1	2	3	4			
7	17.1	16.2	15.4	16.7	16.4	0.75	4.57
5	24.0	24.1	22.3	23.9	23.6	0.84	3.56
2	35.6	34.5	33.8	35.0	34.7	0.76	2.18

[a]$\log_{10}$ DNA copies per reaction

7.4.2 *Salmonella* qPCR results

Nucleic acids extracted from foal faeces enriched in selenite broth were screened for *Salmonella* with the *invA* qPCR TaqMan probe assay. There were 13 foals with Ct values <40, including five aged matched case foals, two matched control foals, three hospital foals and 3 unmatched case foals (Table 7-4). The four culture positive samples had the lowest Ct values, ranging between 17.0 and 22.3, and corresponding to between 2.51×10^5 to 7.21×10^6 *invA* gene copies. The remaining foal samples in which probe fluorescence was detected had high Ct values (>36), corresponding to <24 *invA* gene copies which was below the limit of detection of this assay.

Table 7-4 *Salmonella invA* **qPCR TaqMan Probe assay results**

	Foal ID	Farm	Number of faecal samples			Ct value	Calculated *invA* gene copies
			Total collected	Culture +ve	Ct <40		
Matched case foals	72	C	1		1	39.4	6.99
	195	C	1	1	1	17.0	7.21×10^6
	233	B	1		1	37.1	12.3
	238	C	1	1	1	22.3	1.25×10^5
	311	A	2	1	1	17.6	5.63×10^6
Matched control foals	196	C	1	1	1	22.3	2.51×10^5
	235	C	1		1	38.8	4.06
Unmatched case foals	64	B	1		1	39.8	4.76
	66	B	1		1	39.8	4.64
Hospital foals	17		2		1	39.1	6.28
	247		3		1	38.9	4.00
	167	C	1		1	36.9	23.4
	190	A	2		1	39.7	3.95

7.4.3 Analysis of *Salmonella* detected in age matched case control foals

In the age-matched case control faecal samples, *Salmonella* was detected by bacterial culture in 2.5% of case foals and <1% of control foals (Table 7-5). A matched analysis of discordant pairs showed there was no significant association between the detection of *Salmonella* and the presence of diarrhoea (Table 7-6). A matched analysis of age-matched case control faecal samples with qPCR probe fluorescence also showed no significant association.

Table 7-5 *Salmonella* **detection in age matched case control foals**

Sample	*Salmonella* culture +ve	*Salmonella invA* qPCR+ve
Case	3/117 (2.5%)	5/117 (4.3%)
Control	1/117 (0.85%)	2/117 (1.7%)

Table 7-6 Matched analysis of case-control pairs in relation to *Salmonella* status

Case-control pair exposure	*Salmonella* culture	*Salmonella* qPCR fluorescence
Case +ve Control +ve	1	1
Case +ve Control −ve	2	4
Case –ve Control +ve	0	1
Case −ve Control −ve	114	111
Total number of case-control pairs	117	117
Odds ratio (95% CI) P value	5 (0.2401, 104.1) P =0.2482[a]	4 (0.3958, 197) P =0.3750[b]

[a]As a cell contains a 0, 0.5 added to each cell for calculations and McNemar P value used

[b]P value Fisher's exact

Salmonella was cultured from age matched case foals ranging in age from one to 91 days, with a median of three days, and in one six day old control foal (Table 7-7). Due to the small number of culture positive samples there was no association between age and *Salmonella* detection.

Three foals were from farm C, including one matched case control pair (#195, #196) and one other case foal (#238). The matched control foal of case foal #238 was negative for *Salmonella*, however, this control foal (sampled at 5 days of age) had been treated with trimethoprim sulphonamide antibiotics on the day of birth and the following two days.

The forth foal (#311) from which *Salmonella* was isolated was from farm A. This foal was a case foal that had an episode of colic, fever, inappetance and diarrhoea at 83 days of age. Unfortunately no faecal sample was collected at that time. A faecal sample of normal consistency was collected at 85 days of age that was negative for *Salmonella.* On the same day, lung abscesses and a large amount of echogenic peritoneal fluid were seen during ultrasound examination. The foal was started on daily injections of penicillin and gentamicin as it was thought to have had an iatrogenic enterocentesis two days previously. *Salmonella* was isolated from a faecal sample collected at 91 days of age. This foal was matched with a control foal (#315), with no prior history of diarrhoea or antibiotic treatment. No *Salmonella* was cultured from this control foal.

Table 7-7 Age-matched case control pair data for foals with *Salmonella* isolated

Foal ID	Farm	Age (days)	Case/Control	*Salmonella* isolated	Date sampled	Previous antibiotics
195	C	3	Case	Y	30/10/2010	TMS day of first sample
196	C	6	Control	Y	3/11/2010	-
238	C	1	Case	Y	15/11/2010	TMS day of first sample
239	C	5	Control	N	17/11/2010	TMS 12-14/11/2010
311	A	91	Case	Y	20/12/2010 and 27/12/2010	Pen/Gent 21-27/12 Rif/Azith 27/12/10-6/1/11
315	A	90	Control	N	28/12/2010	-

Y, yes; N, no; TMS, Trimethoprim sulphonamide; Pen, Penicillin; Gent, Gentamicin; Rif, Rifampicin; Azith, Azithromycin

Salmonella was isolated from these age-matched case control foal faecal samples collected in October, November and December (Figure 7-5).

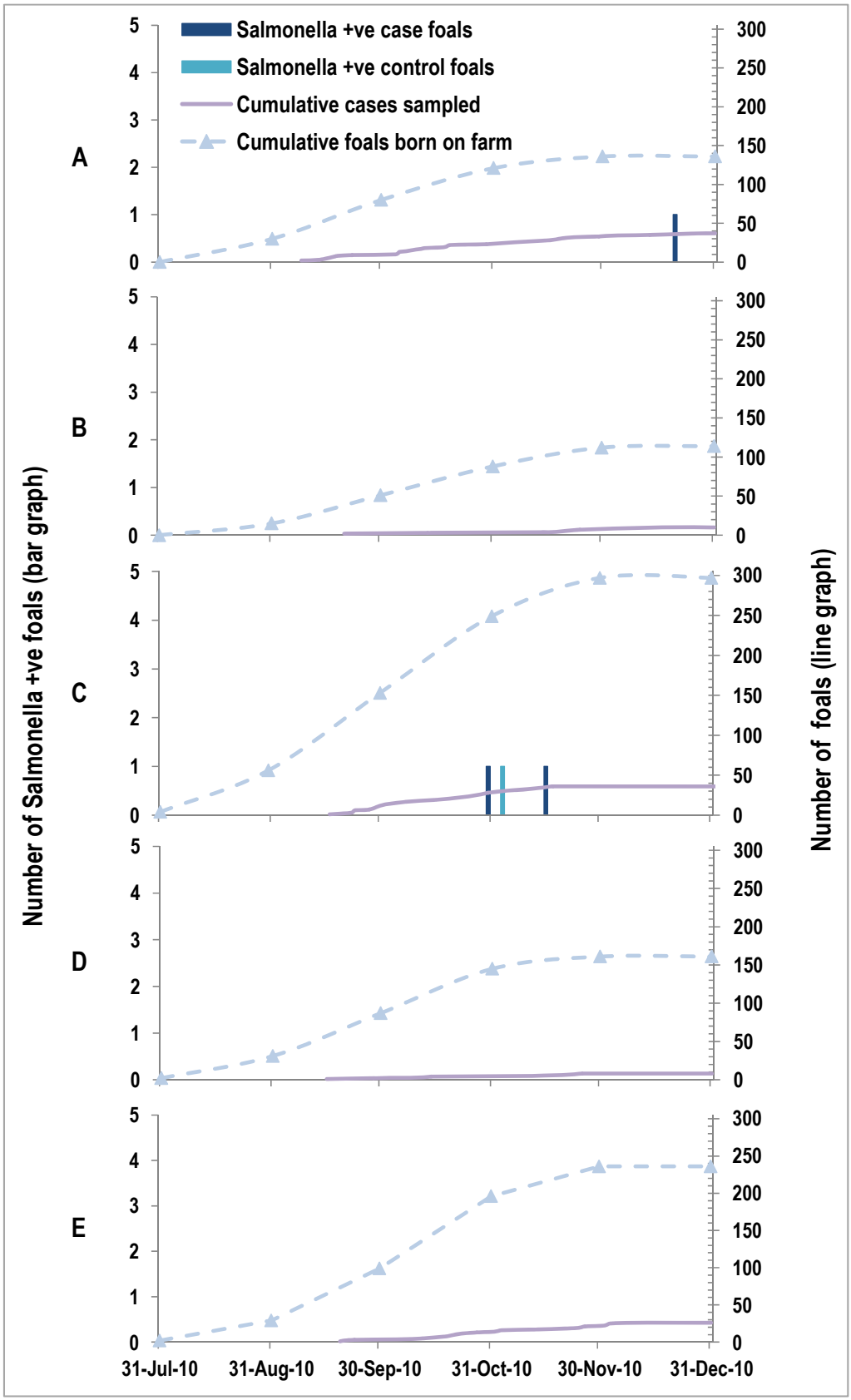

Figure 7-5 Temporal distribution of *Salmonella* culture positive age-matched case-control foals in relation to the number of cases sampled and foals born on each farm (A, B, C, D & E)

7.4.4 Analysis of *Salmonella* detected in hospital foals with diarrhoea

Of the 26 hospitalised foals with diarrhoea, *Salmonella* was detected by *invA* qPCR in four foals but not by bacterial culture. Three of the four *invA* qPCR positive foals had more than one faecal sample collected (Table 7-4), but were only positive in the first sample collected. One hospital foal (#167) was also positive for EqRV.

Salmonella was detected by *invA* qPCR in hospital foals ranging in age from 2 to 81 days, with a median age of 8 days. These foals originated from four farms, including farm A and C, with samples collected in September, October and November (Figure 7-6).

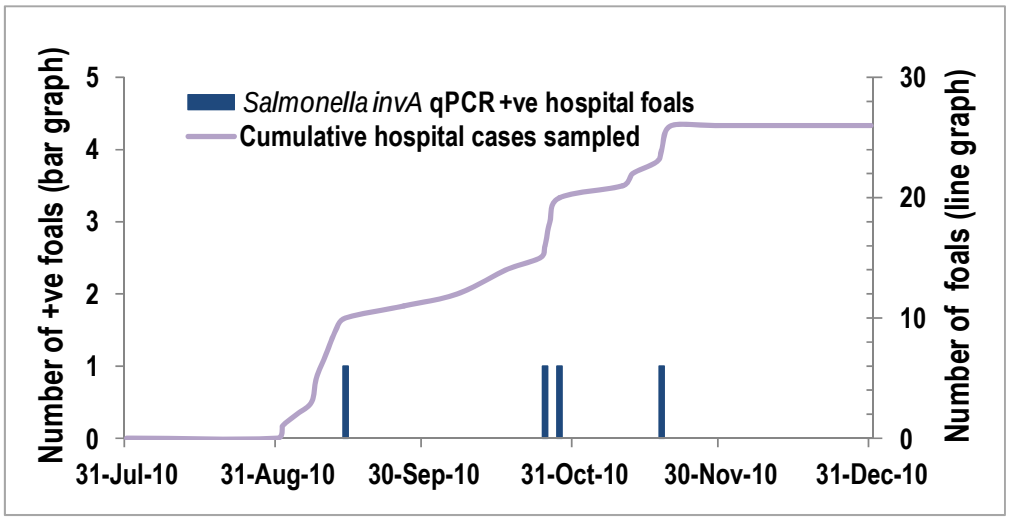

Figure 7-6 Temporal distribution of *Salmonella invA* qPCR positive hospital foals in relation to the number of hospital cases sampled

7.5 DISCUSSION

This is the first age-matched case control study to investigate the detection of *Salmonella* spp. in foals with and without diarrhoea in Australia. No association was found between the detection of *Salmonella* spp. in foals and the presence of diarrhoea. A likely explanation for this may be the prevalence of *Salmonella* spp. infection in the age-matched case and control foals was too low (2.5% and <1%, respectively) to give statistical significance. The prevalence of *Salmonella* in this study is similar to the prevalence reported in previous foal diarrhoea studies (Browning *et al.*, 1991b; Netherwood *et al.*, 1996b). In one study that investigated foals with diarrhoea and

control foals separated into those in contact and those not-in-contact with cases, there was also no association found between the detection of *Salmonella* spp. and the presence of diarrhoea (Netherwood *et al.*, 1996b). However, that study did find an association between the detection of *Salmonella* spp. and fatal diarrhoea. In the current study, there was no mortality of foals in which *Salmonella* spp. were detected.

A more recent study that looked at foals with gastrointestinal disease and healthy foals, did find an association between the detection of *Salmonella* spp. (by both culture and qPCR) and gastrointestinal disease (Slovis *et al.*, 2014). This study did have a higher prevalence of *Salmonella* spp. detection, 8% by culture and 14% by qPCR than other studies, with no *Salmonella* spp. detected in healthy foals. However, the healthy foals were sourced from five farms while the case foals came from 32 different stud farms and may represent different exposure conditions.

In the current study *Salmonella* spp. were detected by *invA* qPCR in 15% of hospital foals with diarrhoea, but no *Salmonella* spp. were detected by bacterial culture. In other studies of hospitalised foals with diarrhoea *Salmonella* spp. have been isolated by culture at frequencies between 3% and 12% (Frederick *et al.*, 2009; Holland *et al.*, 1991).

Salmonella organisms are shed in the faeces of either clinical cases or healthy carriers. In places where large numbers of horses are gathered, for example, veterinary clinics and studs, it is of great importance to identify the source of an infection quickly to prevent a disease outbreak. *Salmonella* spp. can be difficult to detect in faecal samples due to overgrowth of contaminating organisms, intermittent shedding and insensitivity of bacterial culture (Cohen *et al.*, 1996; Smith *et al.*, 1978). To compensate for the poor sensitivity of *Salmonella* spp. detection by bacterial culture, it has been recommended to culture multiple faecal samples. The culture of 5 faecal samples has been reported to increase the sensitivity of *Salmonella* spp. detection from 55% in a single faecal sample from a horse to >98% (Duijkeren *et al.*, 1995; Ward *et al.*, 2005). A study comparing conventional PCR and bacterial culture for detection of *Salmonella* found that 3 samples/horse enabled detection of all PCR positive horses while 5 samples/horse were required to detect all culture-positive horses (Cohen *et al.*, 1996).

In the current study, an attempt was made to collect more than one faecal sample per episode of diarrhoea. However, due to logistical reasons discussed in Section 3.5, only

a single faecal sample was collected from 84% of age-matched case foals and 69% of hospital case foals. This could mean the prevalence of *Salmonella* spp. is underestimated in this study. Of the foals that did have multiple faecal samples collected, nine foals had three or more faecal samples collected. None of the foals with multiple faecal samples collected had *Salmonella* spp. detected in more than one sample.

Another factor that may have influenced the prevalence of *Salmonella* spp. detected in this study was the treatment of foals with antibiotics on Farm C. After *Salmonella* spp. were detected in foals with diarrhoea, this farm elected to treat every foal with a trimethoprim sulphonamide antimicrobial for 3 days after birth as a disease prevention and management strategy. The isolates of *Salmonella* spp. from this study were all sensitive to trimethoprim, and while there was resistance to sulfafurazole, there is often synergy with trimethoprim sulphonamide combinations. Therefore, the use of this antimicrobial in the first few days of life may have influenced the detection by qPCR of *Salmonella* spp. in these foals. Depending on the timing of antimicrobial dosing in relation to sampling, there may have been no effect on DNA detection by PCR as the PCR could detect organisms killed by the antibiotic. However, if antibiotics had eliminated viable *Salmonella* spp. and the dead bacteria had transited out of the gastrointestinal tract then this would have impacted DNA detection.

All *Salmonella* spp. culture positive samples were also strongly qPCR positive when nucleic acids extracted from selenite enrichment broth were tested. The significance of the samples with high Ct values (>35) in the qPCR assay is hard to determine. These samples could contain low numbers of *Salmonella* organisms that are below the limit of detection by culture. Alternatively, they could represent non-viable organisms or simply be false positives. The enrichment of faecal samples in selenite broth prior to DNA extraction does delay qPCR results up to 24 hours (Stone *et al.*, 1994), however, this procedure is still more rapid than bacterial culture which may require between 2 to 5 days (Hyatt and Weese, 2004; Steneroden *et al.*, 2010). In addition, if the qPCR assay result is positive, bacterial culture can be completed from the enrichment broth. This is advantageous as it provides an isolate that can be genotyped for epidemiological tracing using whole genome sequencing, and tested for antimicrobial sensitivity.

In summary, *Salmonella* spp. were detected at a low prevalence in age-matched case control foals in this study and detection was not associated with the presence of diarrhoea. All the samples that were positive by bacterial culture were also strongly positive by qPCR. Therefore, this qPCR assay may be used as a rapid screening test for the detection of *Salmonella* spp. in foals allowing early instigation of appropriate and targeted therapy

CHAPTER 8 DETECTION OF *CLOSTRIDIUM DIFFICILE*

8.1 INTRODUCTION

The obligate anaerobic bacterium *Clostridium difficile* causes intestinal disease in humans and animals. While first isolated from the faeces of healthy new born infants in 1935 (Hall and O'Toole, 1935), it wasn't until the 1970's with the advent of new antimicrobials that antibiotic associated pseudomembranous colitis due to *C. difficile* emerged in humans (Bartlett *et al.*, 1978; Hall and O'Toole, 1935). In horses, *C. difficile* was first detected in foals with diarrhoea (Jones *et al.*, 1987a) and has since been detected in horses of all ages, both asymptomatic and with intestinal disease (Baverud *et al.*, 2003; Cosmetatos *et al.*, 1994; Medina-Torres *et al.*, 2011; Perrin *et al.*, 1993). Clinical disease in foals occurs either sporadically or in outbreaks, and ranges from mild diarrhoea, to severe and possibly fatal haemorrhagic necrotising enterocolitis (Jones *et al.*, 1987b; Weese *et al.*, 1999).

C. difficile is a ubiquitous organism that has been detected in the faeces of humans and numerous animal species, in soil on stud farms, farms with mature horses, public parks, playgrounds and gardens, in water from rivers, lakes, oceans, swimming pools and tap water, in retail meat and some vegetables, as well as from the environment in small animal, large animal and human hospitals (Al Saif and Brazier, 1996; Baverud *et al.*, 2003; Rodriguez-Palacios *et al.*, 2007; Songer *et al.*, 2009b; Weese *et al.*, 2000a). A disruption of the normal protective enteric flora, overgrowth of *C. difficile* and production of toxins is thought to be required for disease due to *C. difficile* to occur. These toxins include two large clostridial toxins, toxin A and toxin B, which are the main virulence factors of toxigenic strains. These toxins are encoded by the *tcdA* and *tcdB* genes located in a 19 kb pathogenicity locus (PaLoc) on the chromosome. The PaLoc also includes three accessory genes (*tcdR*, *tcdC* and *tcdE*) involved in regulating toxin production and secretion. However, not all *C. difficile* strains are toxigenic. In

non-toxigenic strains PaLoc has been replaced with a unique 115 bp sequence (Braun *et al.*, 1996).

Another virulence factor of *C. difficile* is binary toxin. This toxin is homologous to the iota toxin from *Clostridium perfringens* and causes disruption of the actin cytoskeleton of enteric cells (Popoff *et al.*, 1988). The enzymatic component of binary toxin is encoded by the *cdtA* gene and the binding component by the *cdtB* gene, located in a region of the chromosome known as the CdtLoc (Perelle *et al.*, 1997). Binary toxin negative strains either have functional deletions in this region or the whole CdtLoc is replaced by a unique 68 bp sequence (Carter *et al.*, 2007).

Diagnosis of disease due to *C. difficile* is therefore not merely dependant on detection of the organism but also determination of its toxigenicity. Isolation of *C. difficile* is slow and requires selective media and anaerobic conditions that are not available at all diagnostic laboratories. Alternative methods of detecting the organism include an immunoassay to detect the *C. difficile* antigen glutamate dehydrogenase (GDH) (Barbut *et al.*, 2000) or PCR assays (Lemee *et al.*, 2004b). Toxins can be detected by tissue culture cytotoxin assays or enzyme immunoassays (Medina-Torres *et al.*, 2010; Silva *et al.*, 2014). Alternatively, molecular typing methods and PCR assays can be used to determine if strains are toxigenic (Barbut *et al.*, 1993; Kato *et al.*, 1991; Lemee *et al.*, 2004a; Persson *et al.*, 2008).

Two commonly used methods to type *C. difficile* strains are toxinotyping and PCR-ribotyping. Toxinotyping classifies *C. difficile* strains into groups (toxinotypes) according to changes in the PaLoc region based on PCR- RFLP patterns compared to a reference strain VPI 10463 (Rupnik *et al.*, 1998). Strains with a PaLoc identical to VPI 10463 are considered non-variant strains and are designated toxinotype 0. Variant strains typically have deletions or insertions in the *tcdA* gene and point mutations in the *tcdB* gene (Rupnik and Janezic, 2016). Binary toxin genes are also more likely to be present in variant strains with significant changes in PaLoc (Stubbs *et al.*, 2000). As of January 2016, 34 variant toxinotypes (I-XXXIV) have been reported (Rupnik and Janezic, 2016). Production of toxins correlates well with the presence of toxin genes (Rupnik *et al.*, 2001).

PCR-ribotyping is a typing method that compares DNA fragment patterns of the intergenic spacer region between the 16S and 23S rRNA genes (Bidet *et al.*, 1999).

Good correlation has been reported between PCR ribotypes and variant toxinotypes (Rupnik *et al.*, 2001; Stubbs *et al.*, 2000).

Some PCR ribotypes, that are more frequently associated with *C. difficile* outbreaks and severe disease in people, are called hypervirulent strains. These include, ribotype 027 (RT027) which has been associated with hospital acquired disease and outbreaks of disease, and ribotype 078 (RT078) which has been associated with community acquired disease and sporadic disease (Goorhuis *et al.*, 2008). An increase in the incidence and severity of *C. difficile* disease in humans, as well as an increase in community acquired infection rather than the typical hospital acquired infection associated with antibiotic therapy, has led to an increased focus on strains circulating in animal populations as a potential public health concern. While the main focus has been on livestock as a source of *C. difficile* infection, close contact with horses that are often considered companion animals could result in transmission of strains to humans and vice versa.

The majority of studies into *C. difficile* in horses have been conducted in North America and Europe (Medina-Torres *et al.*, 2011; Ossiprandi *et al.*, 2010; Rodriguez *et al.*, 2014; Schoster *et al.*, 2012a; Schoster *et al.*, 2012b; Songer *et al.*, 2009a; Uzal *et al.*, 2012). Little is known about the prevalence of *C. difficile* and the strains circulating in horses in Australia, with only one preliminary study previously conducted involving predominantly healthy adult horses (Thean *et al.*, 2011). This chapter describes the development and validation of a qPCR assay to screen foal faecal samples for the presence of *C. difficile* and characterisation of the *C. difficile* strains isolated from this population.

8.2 APPROACH TO THE DETECTION OF *CLOSTRIDIUM DIFFICILE* IN FOAL FAECAL SAMPLES

To screen foal faecal samples for the presence of *C. difficile,* previously described primers targeting the triose phosphate isomerase (*tpi*) gene in a touchdown multiplex PCR assay (Lemee *et al.*, 2004b) were adapted for use in a singleplex qPCR assay. *C. difficile* bacterial isolates (Section 2.6.4) were used to optimise the assay conditions and generate a qPCR standard. Nucleic acids were extracted from foal faeces stored in RNAlater® (Section 2.1.2.1) with the PowerSoil®-htp 96 Well Soil DNA Isolation kit (Section 2.2.2) and were screened by *tpi* qPCR using touchdown cycling conditions

(Section 2.3.3). Anaerobic bacterial culture for *C. difficile* (Section 2.6.5) of foals positive by *tpi*-qPCR and 15 randomly selected control foals was performed on faeces stored anaerobically in CMB (Section 2.1.2.3). Isolates of *C. difficile* were characterised for the presence of PaLoc (Section 2.7.7.3), the gene encoding binary toxin (Section 2.7.7.1), toxinotype (Section 2.7.5) and ribotype (Section 2.7.6).

8.3 RESULTS

8.3.1 Development and validation of a qPCR to detect *C. difficile* in foal faeces

Primers *tpi*-F and *tpi*-R (Table 2-1) were selected from a published multiplex touchdown PCR assay (Lemee *et al.*, 2004b) for adaptation for use in a qPCR assay for the detection of *C. difficile* in foal faeces. These primers target a 230 bp fragment of the *C. difficile tpi* gene and were reported to have successfully amplified 72 different *C .difficile* strains (from a variety of hosts and geographic origins) without amplifying 11 other Clostridium species, including closely related *C. sordellii* and *C. bifermentans* (Lemee *et al.*, 2004b).

To test the *tpi* primers for use in a qPCR assay, five *C. difficile* isolates were obtained for use as template (Section 2.6.4). Template from the *C. difficile* cultures was prepared by diluting 5 µl of broth in 100 µl of sterile water followed by boiling at 99°C for 5 mins. Five µl of template was tested in a SYTO® 9 assay (Section 2.3.3) that incorporated the touchdown cycling conditions from the original published multiplex qPCR assay. Specifically, the cycling conditions involved a 1°C decrease in annealing temperature per cycle for the first 11 cycles from 65°C to 55°C, followed by 29 cycles with a consistent annealing temperature of 55°C with fluorescence data collected at the end of cycles 29-40, followed by generation of a dissociation melt curve (Table 2-2). The *tpi* qPCR assay generated a single dissociation peak with a Tm of 78.1°C for *C. difficile* isolates VPI10462, M7404, CD37 and CM09-0928, and 79.1°C for *C. difficile* isolate CM09-0930, with no amplification of the NTC (Figure 8-1).

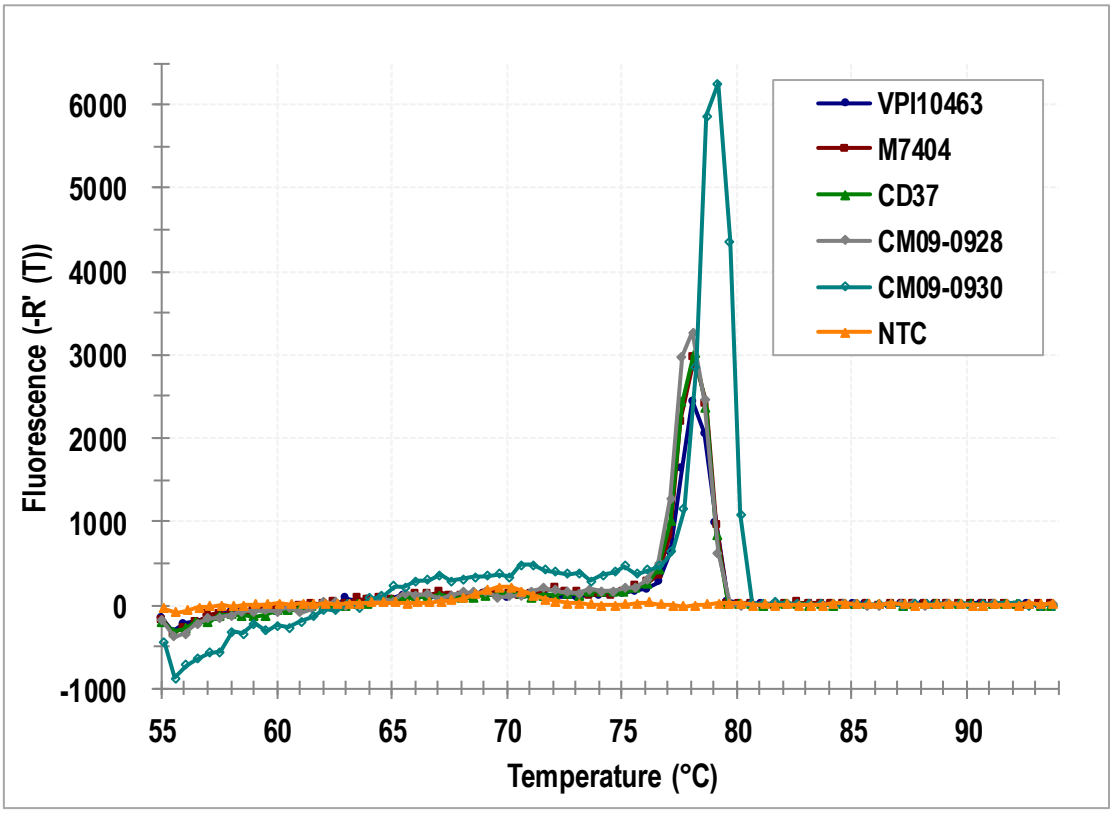

Figure 8-1 Dissociation curves of five *C. difficile* isolates representing A+B+CDT- (VPI10463), A+B+CDT+ (M7404), non-toxigenic (CD37) and unknown toxin profiles (CM09-0928 & CM09-0930) and a NTC. Tm range 78.1-79.1°C.

An expected 230 bp band was visualised when the *tpi* qPCR products were separated on an agarose gel by electrophoresis (not shown). The amplicon for the VPI10462 *C. difficile* isolate was excised from this gel and cloned into a plasmid vector for use as a DNA standard (pGEM-T.VPI10462) (Section 2.4).

To further optimise the assay efficiency and specificity, the DNA standard was then used as template while variations of forward and reverse primer concentrations between 0.2 µM to 0.5 µM and $MgCl_2$ concentrations of 1.5 mM, 2.0 mM, 2.5 mM and 3.0 mM per reaction were tested (Table 8-1). The conditions that gave a low Ct value without any non-specific amplification were then selected as the optimal assay conditions. Except for reactions with $MgCl_2$ at 1.5 mM, there was very little variation in Tm between various assay conditions. In regards to Ct value, there was quite a lot of variation, with no amplification occurring with F:R primer concentrations of 0.2:0.2 µM and 0.4:0.2 µM. The lowest Ct values were very close for F:R primer ratios 0.4:0.5, 0.2:0.5 and 0.5:0.5 and $MgCl_2$ concentrations 2.0 mM and 2.5 mM. An assay with

these primer and $MgCl_2$ concentrations was repeated to confirm the optimal assay conditions were a $MgCl_2$ concentration of 2.0 mM, forward primer concentration of 0.4 µM and reverse primer concentration of 0.5 µM per reaction.

Table 8-1 Comparison of Ct and Tm values with variations in assay conditions for optimisation of the *tpi* qPCR assay using the pGEM-T.VPI10462 plasmid as template.

Assay condition variable	Optimisation assay 1		Optimisation assay 2	
	Average Ct value[a]	**Average Tm value[a]**	**Average Ct value[b]**	**Average Tm value[b]**
Primer concentration (µM) F:R[c]				
0.2:0.2	36.2	78.3°C		
0.2:0.4	34.3	78.3°C		
0.4:0.4	32.3	77.8°C		
0.4:0.2	35.4	78.3°C		
0.4:0.5	31.0	78.3°C	23.3	78.1°C
0.2:0.5	31.1	78.3°C	25.4	78.1°C
0.5:0.2	34.7	78.3°C		
0.5:0.3	32.7	77.8°C		
0.5:0.5	30.0	78.4°C	25.1	78.1°C
0.3:0.5			24.9	78.1°C
$MgCl_2$ concentration per reaction[d]				
1.5 mM	34.9	76.8°C		
2.0 mM	31.0	77.7°C	24.4	78.1°C
2.5 mM	30.0	78.4°C	25.1	78.1°C
3.0 mM	31.9	78.3°C		

[a] Average of three replicates using 10^4 copies of pGEM-T.VPI10462 plasmid as template

[b] Average of three replicates using 10^5 copies of pGEM-T.VPI10462 plasmid as template

[c] [$MgCl_2$] 2.5 mM

[d] F:R primer concentration 0.5:0.5 µM

Using the optimised *tpi* qPCR assay conditions a DNA standard curve was generated by testing 10-fold serial dilutions of pGEM-T.VPI10462 plasmid DNA in triplicate. Template was detected in all three replicates over a linear range of 7 orders of magnitude including 10^2 to 10^8 DNA copies per qPCR reaction, with an amplification efficiency of 107.6% (Figure 8-2).

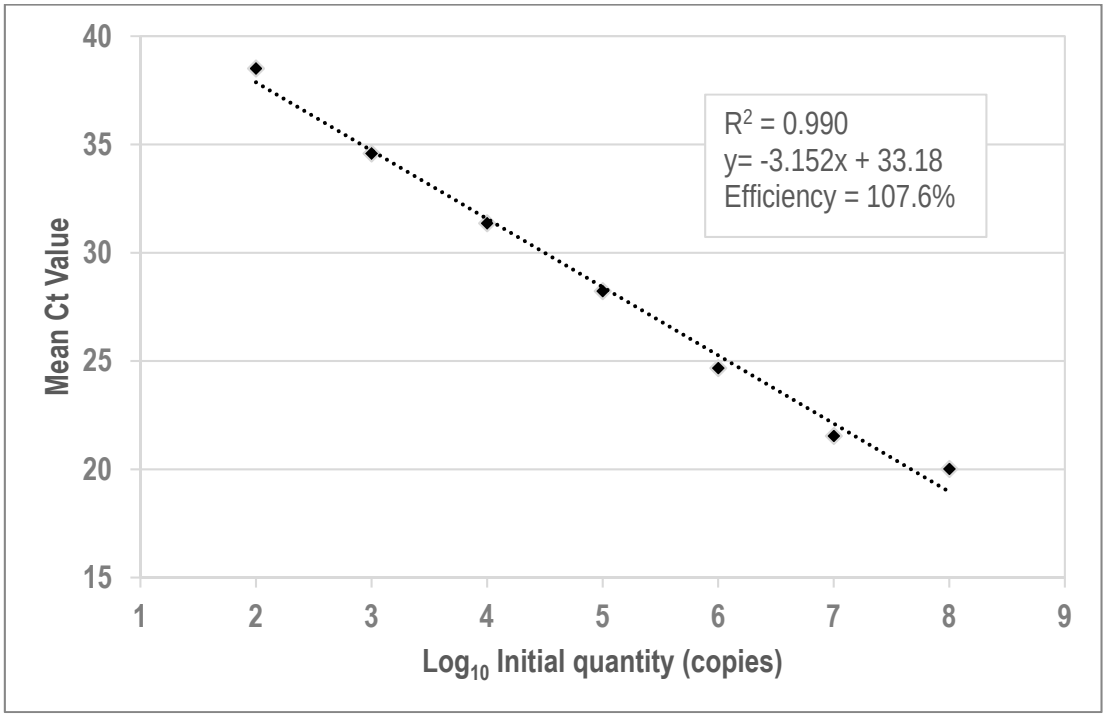

Figure 8-2 *C. difficile tpi* **qPCR assay standard curve produced from 10 fold serial dilutions of pGEM-T.VPI10462 plasmid DNA**

Precision and reproducibility of the *C. difficile tpi* qPCR assay was assessed by determining the within assay (intra-) and between assay (inter-) variation. The intra-assay variation was determined by calculating the CV% (Section 2.3.5) between Ct values of a standard dilution series performed in triplicate in a single assay run (Table 8-2). Similarly, the inter-assay variation was determined by calculating the CV% between the mean Ct values of 2 replicates of a standard dilution series performed in 5 independent assay runs (Table 8-3) including an inter-run calibrator. The intra- and inter-assay variation were low (≤1.90 CV% and ≤3.47 CV%, respectively) indicating the assay was precise and reproducible.

Table 8-2 Intra-assay variation of the *C. difficile tpi* qPCR assay

Copies[a]	Ct value			Mean	SD	CV%
	Replicate					
	1	2	3			
8	19.9	20.2	19.9	20.0	0.20	0.98
7	21.1	21.7	21.9	21.5	0.41	1.90
6	24.7	24.4	24.9	24.7	0.27	1.11
5	27.8	28.5	28.4	28.2	0.36	1.28
4	31.4	31.4	31.3	31.4	0.10	0.31
3	34.8	34.4	34.6	34.6	0.16	0.46
2	39.0	37.8	38.7	38.5	0.63	1.63

[a]$\log_{10}$ DNA copies per reaction

Table 8-3 Inter-assay variation of the *C. difficile tpi* qPCR assay

Copies[a]	Ct value[b]					Mean	SD	CV%
	Assay							
	1	2	3	4	5			
8	17.1	18.5	17.2	18.0	18.2	17.8	0.62	3.47
7	18.9	19.5	18.3	19.3	19.7	19.1	0.56	2.95
6	23.0	22.6	22.7	22.7	22.5	22.7	0.17	0.74
5	25.8	25.8	25.2	25.7	26.2	25.8	0.36	1.38
4	29.2	29.0	29.1	29.1	28.9	29.1	0.12	0.41
3	31.8	32.2	31.9	32.1	32.1	32.0	0.19	0.59
2	35.2	35.7	35.1	35.6	35.6	35.4	0.28	0.78

[a]$\log_{10}$ DNA copies per reaction

[b]Mean of two replicates

Fluorescent threshold determined using an inter-run calibrator (Well E11- 10^4 plasmid copies)

8.3.2 Detection of *Clostridium difficile* in foal faeces

Foal faecal samples were screened for *C. difficile* with the *tpi* qPCR assay. Foal faecal samples were considered potentially positive if there was a *tpi* qPCR amplification product with an appropriate dissociation melting curve (Tm 75.9-78°C). For confirmation that these samples did in fact amplify a product of the expected size, amplification products were separated and visualised by gel electrophoresis (Section 2.2.5). A sample with no amplification product and a plasmid standard from each

qPCR plate were also separated by gel electrophoresis as negative and positive controls (Figure 8-3). A *tpi* qPCR amplification product was visualised in 20 samples from 17 foals.

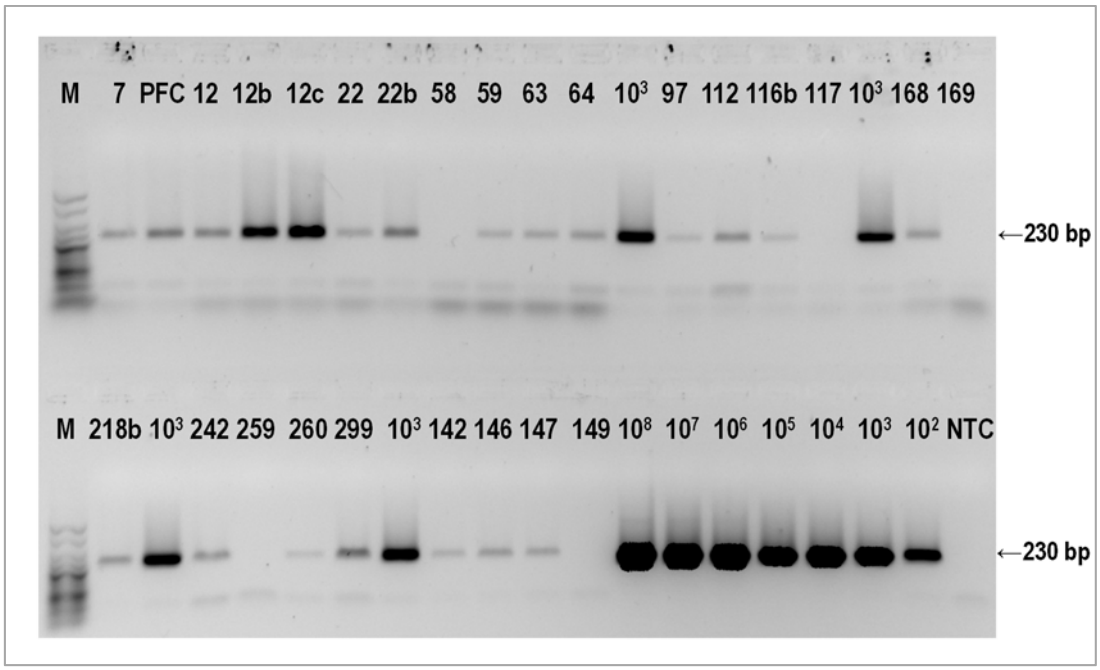

Figure 8-3 Gel electrophoresis of *C. difficile tpi* qPCR products. Sample numbers are shown above the lane. A 500 bp DNA molecular weight marker (M), samples without amplification (58, 117, 169, 259, 149), a NTC, a positive faecal control (PFC) and positive copy number controls (10^8 to 10^2) are also shown. Arrow indicates *tpi* qPCR amplicon size.

Anaerobic culture to isolate *C. difficile* (Section 2.6.5) was performed on the 20 samples positive by qPCR and from 15 randomly selected control foals that were negative by qPCR. Anaerobic bacterial culture isolated *C. difficile* from 14 faecal samples from 12 foals that were positive by *tpi* qPCR and from none of the control foals that were negative by *tpi* qPCR. Identification of these bacterial isolates was confirmed as *C. difficile* by colony PCR targeting the *spo0A* gene (Section 2.6.6).

Of the 12 foals positive by *tpi* qPCR, *C. difficile* was cultured from 3/3 age matched case foals, 2/3 matched control foals, 2/5 unmatched case foals and 5/6 hospital foals (Table 8-4).

Table 8-4 Results of *C. difficile* detection in foals by *tpi* qPCR and anaerobic culture

	Foal ID	Number of samples			Number of foals Culture +/qPCR+
		Total collected	qPCR+	Culture+	
Matched case foals	22	5	2	2	3/3
	59	2	1	1	
	146	1	1	1	
Matched control foals	7	1	1	0	2/3
	97	1	1	1	
	147*	1	1	1	
Unmatched case foals	63	1	1	0	2/5
	64	1	1	0	
	112	1	1	1	
	116b**	2	1	0	
	299	1	1	1	
Hospital foals	12	3	3	2	5/6
	142	1	1	0	
	168	1	1	1	
	218b	2	1	1	
	242	2	1	1	
	260	2	1	1	
Total			20	14	12/17

*This control foal developed diarrhoea 7 days later and was sampled as a case (#175)

**This case foal was sampled earlier in the study as a case foal (#22)

8.3.3 Molecular characterisation of *C. difficile* isolates

Molecular characterisation of these isolates by toxinotyping (Section 2.7.5), ribotyping (Section 2.7.6), and PCR assays to detect the *cdtB* gene which encodes for the binding component of binary toxin and the absence of PaLoc (Section 2.7.7) are summarised in Table 8-5.

Table 8-5 Molecular characterisation of foal *C. difficile* isolates

	Sample ID	*tcdA* PCR (A3)	*tcdB* PCR (B1)	*cdtB* PCR	toxinotype	ribotype	Predicted toxin profile
Age matched case foals	22	-	+	+	XXX	KB2	A-B+CDT+
	22b	-	+	+	XXX	KB2	A-B+CDT+
	59	+	+	-	0	012	A+B+CDT-
	146	+	+	-	0	012	A+B+CDT-
Age matched control foals	97	-	-	-	-	KB3	Non-toxigenic
	147	+	+	+	V	078	A+B+CDT+
Unmatched case foals	112	+	+	-	0	A	A+B+CDT-
	299	+	+	-	0	012	A+B+CDT-
Hospital foals	12b	+	+	+	V	078	A+B+CDT+
	12c	*	+	*	*	078	A+B+CDT+
	168	-	+	+	XXX	KB2	A-B+CDT+
	218b	+	+	-	0	KB4	A+B+CDT-
	242	+	+	-	0	012	A+B+CDT-
	260	+	+	+	V	078	A+B+CDT+

A3, *tcdA* A3 fragment; B1, *tcdB* B1 fragment; +, positive; -, negative; *, inconsistent; A, toxin A; B, toxin B; CDT, binary toxin.

One *C. difficile* isolate from an age matched control foal (#97) was classified non-toxigenic as there was no amplification of the A3 fragment of the *tcdA* gene or the B1 fragment of the *tcdB.* An additional PCR using primers Lok3/Lok1 was performed on this isolate to confirm the absence of the PaLoc (Section 2.7.7.3).

The other 13 isolates were classified as toxigenic with detection of the A3 fragment of the *tcdA* gene encoding for toxin A in 9/13 isolates, and detection of the B1 fragment of the *tcdB* gene encoding for toxin B in all 13 isolates.

The *cdtB* gene encoding for the binding component of binary toxin was detected in 6 isolates of *C. difficile*. Isolate 12c behaved inconsistently in these PCRs and could not be toxinotyped but was ribotyped.

Based on the restriction enzyme digestion patterns of the A3 and B1 fragments there were three toxinotypes, 0, V and XXX, identified in the toxigenic isolates of *C. difficile* in this study. The predicted toxin profile of toxinotype 0 strains were toxin A and B positive and binary toxin negative (A+B+CDT-). Toxinotype V strains were toxin A and B positive and binary toxin positive (A+B+CDT+) while toxinotype XXX strains were toxin A negative, toxin B positive and binary toxin positive (A-B+CDT+).

Six different PCR-ribotypes were identified in the *C. difficile* isolates from this study. Two ribotypes were internationally recognised ribotypes 078 and 012, one was a ribotype previously observed at the University of Guelph designated as ribotype A and two ribotypes did not correspond to any reference strains in the laboratory's collection and were assigned as ribotypes KB2-4.

The toxinotype V strains were all ribotype 078 while the toxinotype 0 strains were either ribotype 012, ribotype A or ribotype KB4. The toxinotype XXX strains were ribotype KB2. The non-toxigenic strain was designated as ribotype KB3.

8.3.4 Analysis of *C. difficile* detected in age matched case control foals

In the age-matched case control faecal samples, *C. difficile* was detected by *tpi* qPCR in 2.5% of case foals and 2.5% of control foals, and by culture in 2.5% of case foals and 1.7% of control foals (Table 8-6).

Table 8-6 *C. difficile* detection in age matched case control foals

Sample	*C. difficile tpi* qPCR+ve	*C. difficile* culture +ve
Case	3/117 (2.5%)	3/117 (2.5%)
Control	3/117 (2.5%)	2/117 (1.7%)

Based on the *C. difficile* status of each age matched case-control pair, a matched analysis of the discordant pairs showed there was no significant association (P>0.05) between the detection of *C. difficile* and the presence of diarrhoea (Table 8-7).

Table 8-7 Matched analysis of case-control pairs in relation to *C. difficile* status

Case-control pair exposure	*C. difficile qPCR*	*C. difficile culture*
Case +ve Control +ve	1	1
Case +ve Control −ve	2	1
Case –ve Control +ve	2	2
Case −ve Control −ve	112	113
Total number of case-control pairs	117	117
Odds ratio (95% CI) P value	1 (0.072, 13.8) >0.999	0.5 (0.008, 9.605) >0.999

C. difficile was cultured from age matched case foals ranging in age from one to 15 days, with a median of 14 days, and in control foals ranging in age from four to 11 days, with a median of 7.5 days (Figure 8-4). Due to the small number of *C. difficile* positive samples there was no association between age and *C. difficile* detection

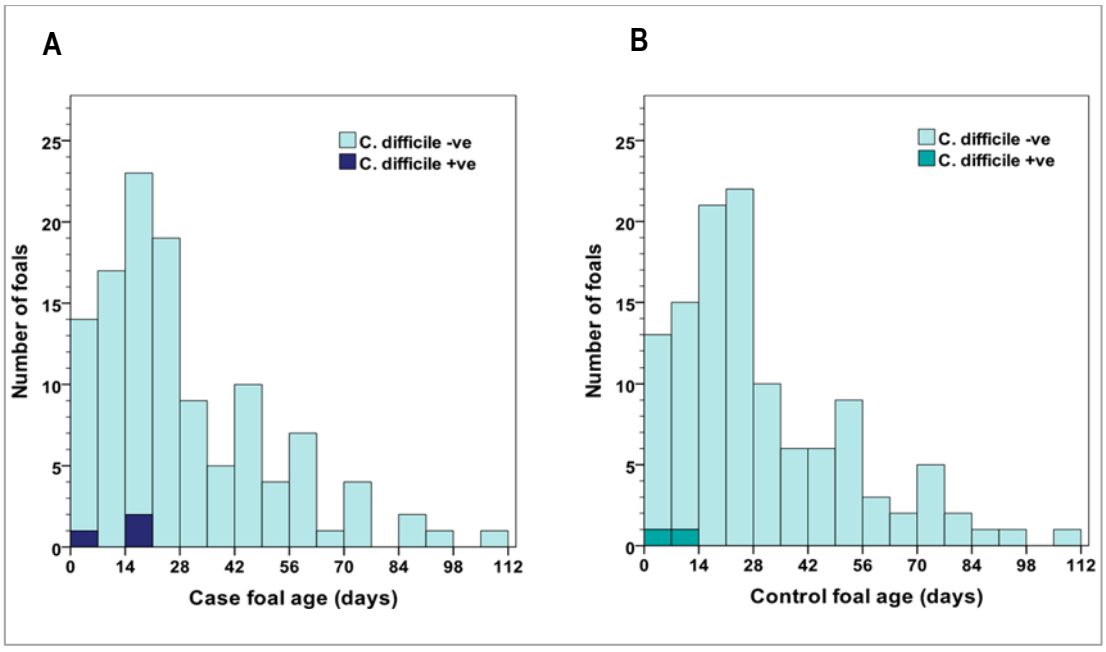

Figure 8-4 Age distribution of age-matched (A) cases and (B) controls and those positive by culture for *C. difficile*

C. difficile was detected in matched case control foals on farms A and C in September and October only (Figure 8-5).

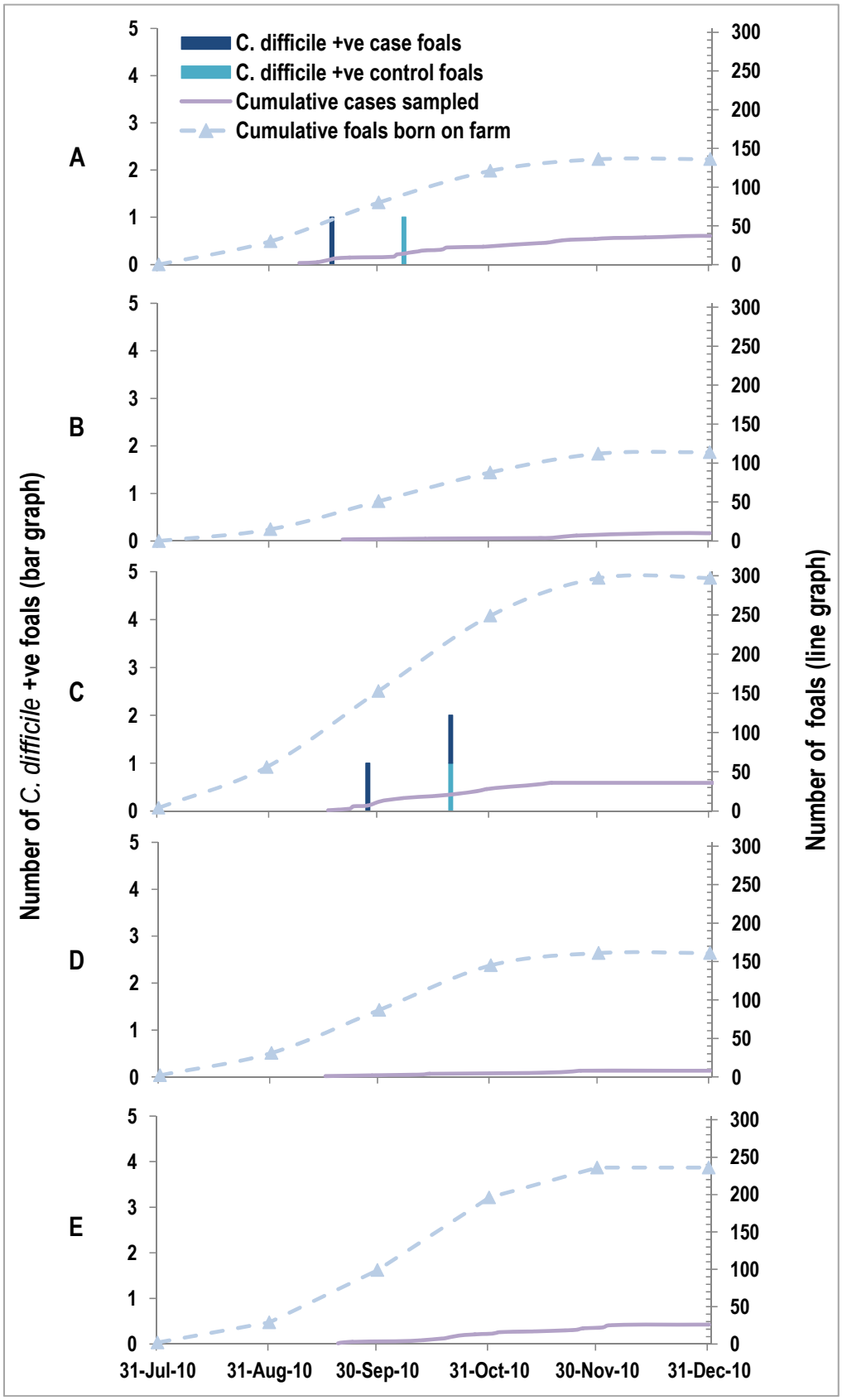

Figure 8-5 Temporal distribution of *C. difficile* culture positive age-matched case-control foals in relation to the number of cases sampled and foals born on each farm (A, B, C, D & E)

None of the age-matched case control foals that were *C. difficile* culture positive received antibiotic treatment prior to faecal sampling.

One of the case foals (#22) that had 5 faecal samples collected during an episode of diarrhoea was positive for *C. difficile* by qPCR and culture in the first two samples collected. This foal was then sampled as a case later in the study period and the second of two faecal samples (#116b) collected during this second episode of diarrhoea was positive by qPCR, but negative by culture for *C. difficile*. This second episode of diarrhoea was excluded from the matched case-control analysis because it was unmatched. This foal received treatment with the antimicrobial ceftiofur the day prior to collection of the diarrhoeic sample in the second episode of diarrhoea that was positive by *tpi* qPCR but negative by culture for *C. difficile*.

Control foal #147 was qPCR and culture positive for *C. difficile.* This foal was sampled seven days later as a case foal (#175) when it developed diarrhoea. There was no antibiotic therapy administered between sampling as a control foal and sampling as a case foal. The case sample was negative by qPCR for *C. difficile*, however EqRV was detected in this diarrhoeic faecal sample (#175).

Foal #59 was initially sampled as a control foal, but developed diarrhoea four days later and so was sampled again as a case foal. The first faecal sample was excluded as a control sample but was still tested and found *C. difficile tpi* qPCR and culture positive while the second diarrhoeic sample was *tpi* qPCR negative. This foal did not receive any antibiotic therapy prior to collection of either faecal sample.

8.3.5 Analysis of *C. difficile* detected in hospital foals with diarrhoea

Of the 26 hospital foals, six foals were qPCR positive and *C. difficile* was detected by culture in 5 of these six foals. One hospital foal (#12) had three faecal samples collected and was positive for *C. difficile* by qPCR in all three samples but only culture positive in the second and third faecal sample.

C. difficile was isolated by culture from hospital foals ranging in age from three to 25 days, with a median age of 4.5 days. *C. difficile* was detected in hospital foals originating from five farms, including farm C in samples collected in September, October and November (Figure 8-6).

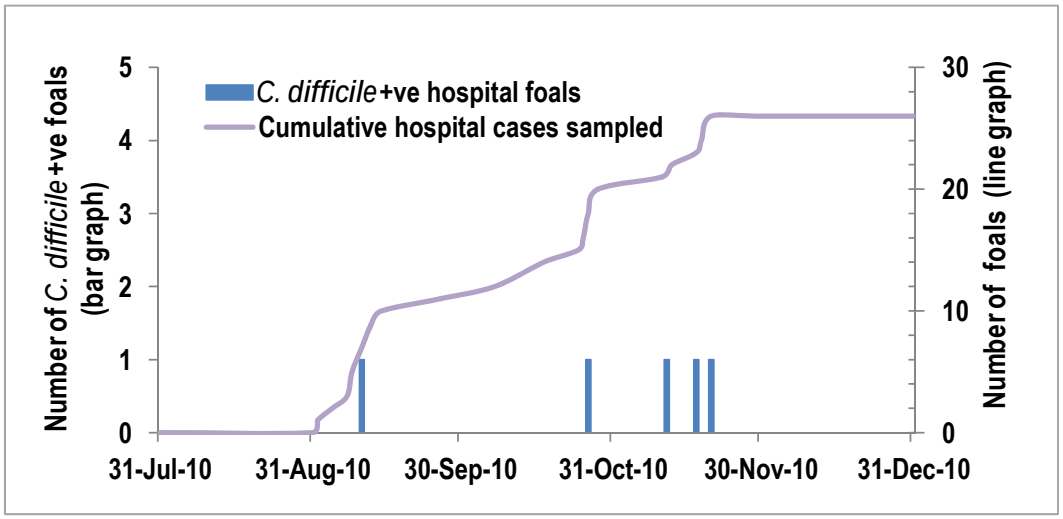

Figure 8-6 Temporal distribution of *C. difficile* culture positive hospital foals in relation to the number of hospital cases sampled

The presenting complaint at hospital admission for 3/5 of the *C. difficile* culture positive hospital foals was diarrhoea alone (Table 8-8). One foal presented with a combination of diarrhoea, sepsis and hypoxic ischaemic syndrome (HIS), while the other foal initially presented for septic arthritis and developed diarrhoea five days after hospital admission. Antibiotics had been administered 1-5 days prior to faecal sampling in four *C. difficile* culture positive hospital foals and on the day of sampling in one foal.

Table 8-8 Presenting complaint and antibiotic treatment history of hospitalised foals in which *C. difficile* was detected

Foal ID #	Number of samples			Foal age (days)	Presenting complaint	Antibiotics administered	
	Total	qPCR +ve	Culture +ve			Days before +ve sample	Type
12	3	3	2	2-4	Diarrhoea	0	Penicillin, Gentamicin
142	1	1	0	7	Diarrhoea	4	Penicillin, Gentamicin, TMS
168	1	1	1	5	Diarrhoea	3	Penicillin, Gentamicin
218b	2	1	1	4	Diarrhoea	1	TMS
242	2	1	1	25	Septic arthritis[a]	5	Oxytetracycline, Ceftiofur, Amikacin
260	2	1	1	4	Diarrhoea, HIS, Sepsis	3	TMS, Penicillin

[a]Diarrhoea developed 5 days after hospital admission
TMS, Trimethoprim sulphonamide; HIS, Hypoxic ischaemic syndrome;

8.4 DISCUSSION

This is the first report of *C. difficile* hypervirulent strain ribotype 078 (RT078) in horses in Australia. This ribotype is of public health significance as it has been increasingly associated with community-associated disease in humans in the northern hemisphere (Goorhuis *et al.*, 2008; Mulvey *et al.*, 2010; Saara *et al.*, 2016). Along with RT027, it is one of the most common ribotypes found in people in Europe (Barbut *et al.*, 2007; Freeman *et al.*, 2015; Hensgens *et al.*, 2009). It is also a prominent ribotype reported in pigs and cattle in North America and Europe (Keel *et al.*, 2007; Koene *et al.*, 2012) which has led to concerns of zoonosis and food borne transmission through meat contamination at slaughter. In horses, RT078 has been detected in North America, the Netherlands and Japan amongst a variety of other ribotypes with no clear predominant strain (Keel *et al.*, 2007; Koene *et al.*, 2012; Medina-Torres *et al.*, 2011; Niwa *et al.*, 2013; Schoster *et al.*, 2012b). In Australia, RT078 has been detected at low prevalence in humans (Bull *et al.*, 2012; Cheng *et al.*, 2016; Mackin *et al.*, 2015) but not in pigs, cattle, sheep or horses, until now (Knight and Riley, 2013; Knight *et al.*, 2015; Knight *et al.*, 2013; Squire *et al.*, 2013; Thean *et al.*, 2011).

The other internationally recognised ribotype detected in foals in this study was RT012. This was also the most common ribotype detected in this study and the only other study of *C. difficile* ribotypes in horses in Australia (Thean *et al.*, 2011). In people, RT012 was the seventh most common ribotype detected across Australia in 2010, while the three most common ribotypes were RT014/020, RT002 and RT054 (Cheng *et al.*, 2016).

Isolates from foals designated as ribotype KB2 were all toxinotype XXX. This is a relatively new toxinotype that was first described in *C. difficile* strains isolated from humans in Australia between 2005 and 2010 (Elliott *et al.*, 2011). Phylogenetic analysis of these human isolates has grouped them into the same clade as RT078 (Elliott *et al.*, 2014). The changes in PaLoc in toxinotype XXX isolates include deletion of the entire *tcdA* gene resulting in an $A^-B^+CDT^+$ toxin profile. The absence of the *tcdA* gene has implications for detection of these strains if a diagnostic immunoassay test based on the detection of toxin A alone is used. Given this toxinotype circulates in humans and foals in Australia, if immunoassays are used for *C. difficile* diagnosis, it would be prudent to use those based on the detection of both toxin A and toxin B to avoid false negatives.

In regards to ribotypes and toxinotypes it appears there are different strains of *C. difficile* circulating in both animals and humans in Australia compared to the northern hemisphere (Elliott *et al.*, 2014; Foster *et al.*, 2014; Lim *et al.*, 2014; Mackin *et al.*, 2015; Squire *et al.*, 2013). Whether Australian horses are a true reservoir of RT078 remains to be determined. As horses can be considered companion animals and commonly have close contact with their owners and carers, both human to animal, and animal to human transmission is theoretically possible. While the prevalence of RT078 is low in humans in Australia, global travel of people could introduce RT078, or other strains, into Australian horses (Neuberger *et al.*, 2013). Similarly, the global travel of horses, including that of shuttle stallions back and forth between the northern and southern hemisphere could result in the introduction of strains not previously circulating in Australia to Australian horses. This may explain the lack of RT078 detection in livestock species in Australia, as livestock tend to only travel out of the country, while the importation of genetic material rather than live animals is used to improve livestock breeding genetics.

It is interesting to note that the only non-toxigenic strain of *C. difficile* isolated in this study came from a control foal, although hypervirulent RT078 was also isolated from a control foal. The detection of the hypervirulent RT078 in Australian foals serves as a timely reminder to horse owners, stud workers and veterinarians to practice high standards of biosecurity, especially personnel receiving antimicrobial therapy.

Hypervirulent RT027 was not detected in this study. This ribotype typically features resistance to fluoroquinolones and has been associated with increased disease severity and hospital disease outbreaks in people in the northern hemisphere, but, has only been reported at low prevalence in people in Australia (Bull *et al.*, 2012; Cheng *et al.*, 2016; Richards *et al.*, 2011; Riley *et al.*, 2009). Treatment with fluoroquinolone antibiotics has the potential to inadvertently select for and enhance the ability of this strain to proliferate in the gut. Treatment of disease due to *C. difficile* in humans relies heavily on metronidazole and vancomycin, which poses challenges due to increasing antimicrobial resistance in *C. difficile*. As metronidazole resistant strains of *C. difficile* have previously been detected in horses (Jang *et al.*, 1997; Magdesian *et al.*, 2006), surveillance of strains present in animal populations is valuable from a public health perspective. This highlights the importance of judicious and appropriate use of antibiotics in both human and veterinary medicine.

In the age matched case control study there was no significant association between the detection of *C. difficile* and the presence of diarrhoea. Given the low prevalence of *C. difficile* in this sample population, this was not unexpected. In neonatal children, piglets and calves, high rates of *C. difficile* colonisation (14-71%, 67-74% and 56% respectively) that are not associated with diarrhoea status have been detected followed by a rapid decline with age (Khalaf *et al.*, 2012; Knight *et al.*, 2015; Knight *et al.*, 2013; Weese *et al.*, 2010). It is speculated that the decline in *C. difficile* colonisation is due to the development of colonisation resistance with maturation of the gut microflora. In foals, multiple studies have reported detection of *C. difficile* in foals and horses with and without diarrhoea, but only two reported higher colonisation rates in the first two weeks of life (11-29%) that declined to <4% by 6 weeks of age (Baverud *et al.*, 2003; Schoster *et al.*, 2015). Of the foals with *C. difficile* in the current study, all except two foals were aged ≤ 14 days.

Interestingly, the prevalence of *C. difficile* was higher in hospital foals than the matched farm foals. A similar finding was reported in Brazil where foals with diarrhoea sampled in a veterinary hospital were 16 times more likely to be positive for *C. difficile* than foals with diarrhoea on a farm (Silva *et al.*, 2013).

It is possible that antimicrobial treatment prior to sample collection of hospital foals may have contributed to the higher prevalence of *C. difficile* by disrupting the gastrointestinal flora. Antimicrobial therapy has been established as a risk factor for *C. difficile* disease in adult horses, however less is known about the role it plays in foals (Baverud *et al.*, 1997; Cosmetatos *et al.*, 1994; Gustafsson *et al.*, 2004). In a study of adult horses administered erythromycin, colitis developed 3-4 days after the start of antimicrobial therapy, although *C. difficile* was detected on the second day of therapy (Gustafsson *et al.*, 1997). While prevalence rates of *C. difficile* in healthy foals treated with erythromycin, gentamicin and rifampicin were as high as 44% (Baverud *et al.*, 2003).

In the current study, two foals were qPCR positive but culture negative on the day of hospital admission and commencement of antimicrobial therapy and became culture positive in samples collected on the following one to two days. This corresponded to increased bacterial load as detected by qPCR. Whether the increase in faecal bacterial load was due to the antimicrobial therapy or simply the natural progression of the infection is uncertain. The effect of antimicrobial therapy on *C. difficile* bacterial loads

in foal faeces would be better assessed in a separate study specifically focused on collection of daily faecal samples both before and after initiation of antimicrobial therapy.

C. difficile in people has increased in incidence and severity of disease over the last two decades and is the major cause of antibiotic associated diarrhoea in hospitalised people. However, community-associated *C. difficile* infection, defined as infection detected within 48 hours of hospital admission (Foster *et al.*, 2014), is also being increasingly recognised. Two foals were positive for *C. difficile* by qPCR and/or culture in samples collected within 48 hours of hospital admission suggesting possible community acquired *C. difficile* infection. These foals were also the only two foals that did not receive antibiotics prior to collection of the first diarrhoeal faecal sample. The other four hospital foals all received antibiotics 3 to 5 days before collection of the first diarrhoeal faecal sample and cannot be classified into hospital acquired or community acquired *C. difficile* infections as faecal samples were not collected and tested within 48 hours of admission. One foal did not develop diarrhoea until 5 days after admission, so hospital acquired *C. difficile* infection is possible and quite likely. Together with the detection of *C. difficile* in farm foals, this would suggest that community acquired *C. difficile* infection and possibly hospital acquired *C. difficile* infection occurred in this study population.

In summary, the detection of *C. difficile* in this study has highlighted the presence of a hypervirulent strain of public health significance in the Australian foal population that warrants ongoing surveillance. This would also aid further investigation of the role of *C. difficile* in diarrhoea in foals and whether antibiotics are as big a risk factor as in adult horses. Other future areas to investigate include the antimicrobial sensitivity patterns of the isolates from this study and whole genome sequencing to evaluate the relatedness to other *C. difficile* strains in Australia and overseas.

CHAPTER 9 GENERAL DISCUSSION

The main focus of this thesis was to establish a collection of faecal samples for investigation of the presence of potential diarrhoea pathogens in age-matched foals with and without diarrhoea to determine whether detection of any of these infectious agents was associated with the presence of diarrhoea. The major finding was that EqRV was significantly associated with the presence of diarrhoea in foals.

Although associations between EqRV and diarrhoea have been identified in other studies (Browning *et al.*, 1991b; Netherwood *et al.*, 1996b), this is the first Australian study to do so and also the first study to include age, temporal and spatial matching of case and control foals in an effort to select control foals representative of the same population, exposure factors and time at risk as case foals. In previous studies, control foals have been selected from different properties or from a relatively small subset of the farms from which case foals were sampled (Browning *et al.*, 1991b; Slovis *et al.*, 2014), therefore the absence of an infectious agent in these control foals could be due to the absence of the infectious agent on these properties.

At least one infectious agent was detected in 32% (37/117) of matched case foals, 13% (15/117) of matched control foals and 46% (12/26) of hospital foals. Of the four infectious agents investigated, EqRV was the most common infectious agent detected in the age matched foals. This finding is also consistent with previous studies (Browning *et al.*, 1991b; Dwyer *et al.*, 1990a; Frederick *et al.*, 2009; Netherwood *et al.*, 1996b; Slovis *et al.*, 2014). However, apart from one study published during the study period of this PhD project (Slovis *et al.*, 2014), the method of detection is different. As group A rotaviruses are an important cause of diarrhoea in children there are many ELISAs and latex agglutination tests available for detection in humans. These tests are based on antibodies directed against the VP6 protein which is common to group A rotaviruses and have been used in studies of group A rotaviruses in foals (Browning *et al.*, 1991b; Dwyer *et al.*, 1990b; Holland *et al.*, 1991; Netherwood *et al.*, 1996b). Availability of these tests has likely influenced the ability to investigate

rotaviruses, and as a result these are the most commonly assessed viruses in foal diarrhoea studies. If studies investigated other viruses, this was largely by electron microscopy, and not all samples collected were tested (Browning *et al.*, 1991b; Dwyer *et al.*, 1990b; Frederick *et al.*, 2009; Traub-Dargatz *et al.*, 1988). Therefore, the frequency of the detection of other viruses was not necessarily representative of the actual prevalence of those viruses in the study population. In the current study, all samples were tested for the presence of EqRV and ECoV with the same type of assay (RT-qPCR), and EqRV was detected more frequently than ECoV in both the case and control foals in this study. There is only one other study that has used molecular tests to investigate a range of infectious agents in foals with diarrhoea, including EqRV and ECoV, which also found EqRV to be the most common infectious agent detected in foals with gastrointestinal disease, but found ECoV to be the most common infectious agent in control foals (Slovis *et al.*, 2014). The results of these two studies cannot be directly compared due to different case definitions. However, these two studies highlight the fact that EqRV is still a significant and common cause of disease in separate foal populations. This suggests that prevention and management strategies for EqRV require improvement. There are three inactivated EqRV vaccines that have been in use globally since the late 1990's. EqRV vaccines are administered parenterally to pregnant mares to boost colostral antibodies against rotavirus and enhance passive immunity in foals. Two of these vaccines are monovalent based on G3P[12] strains of EqRV and licensed for use in the USA, Australia, New Zealand, Europe (Zoetis Inc); and Japan (Nisseiken Co., Ltd). The third vaccine licensed in Argentina is a polyvalent vaccine containing an EqRV G3P[12] strain, a simian G3P[2] rotavirus strain and a bovine G6P[1] rotavirus strain (Biogénesis Bagó). Previous studies of these vaccines have demonstrated significant increases in serum neutralising antibodies of vaccinated mares and their foals, and IgG, but not IgA, immunoglobulins in milk and colostrum of vaccinated mares (Barrandeguy *et al.*, 1998; Imagawa *et al.*, 2005; Imagawa *et al.*, 1998; Powell *et al.*, 1997; Sheoran *et al.*, 2000). Field studies and one small experimental challenge study of these vaccines have demonstrated partial protection of foals against rotavirus with a reduction in disease duration, severity and viral shedding (Barrandeguy *et al.*, 1998; Imagawa *et al.*, 2005; Imagawa *et al.*, 1998; Powell *et al.*, 1997). In the current study, one farm did not vaccinate mares against EqRV until an outbreak of disease occurred. This farm also had the highest incidence

of rotavirus infection. However, this study was not designed to measure the effect of vaccination on the incidence of EqRV, so conclusions cannot be drawn regarding vaccine efficacy. Therefore, whether the persistence of EqRV as a significant diarrhoeal pathogen in foal populations is due to lack of vaccine efficacy, poor uptake of the vaccine or other management practices requires further investigation.

In contrast to the farm case control foals, *C. difficile* was the most common infectious agent detected in the case series of hospitalised foals with diarrhoea. The clinical significance of the presence of *C. difficile* in these case foals cannot be determined due to the lack of control foals. For *C. difficile* associated disease to occur, disruption of the normal enteric flora is thought to be required, along with colonisation and toxin production. However, in infants, colonization with both toxigenic and non-toxigenic *C. difficile* strains occurs commonly in the absence of disease (Stark *et al.*, 1982; Tullus *et al.*, 1989). In a study of *C. difficile* in hospitalised adult horses and foals, with and without diarrhoea, *C. difficile* was isolated more frequently from foals with diarrhoea than from adult horses with diarrhoea, but the presence of *C. difficile* toxins was only associated with mortality in adult horses and not in foals (Weese *et al.*, 2001). A longitudinal study to characterise the foal intestinal microbiota may help determine whether the microbiota composition influences asymptomatic carriage or diarrhoeal disease.

The detection of the hypervirulent ribotype 078 in hospitalised foals with diarrhoea and a control foal is concerning in regards to its zoonotic potential. Future surveillance of *C. difficile* ribotypes circulating in foals and imported horses would provide important information that could influence public health with particular implications for horse owners, veterinary hospital staff and stud farm staff. Monitoring of antimicrobial sensitivity patterns would also be beneficial.

Two infectious agents were detected in a sample from the same foal in 4% (5/117) of matched case foals and 4% (1/26) of hospital foals with diarrhoea. The most frequent co-infection was EqRV and *Salmonella* which occurred in 3 matched case foals. Other co-infections in individual foals were EqRV and *C. difficile*, ECoV and *Salmonella* and *C. difficile* and *Salmonella*. There were no co-infections detected in control foals. These results are similar to the Kentucky molecular study that found coinfections were more likely in GI-diseased foals than healthy foals. The number of co-infections in the

current study were too small to draw any conclusions about the significance of particular combinations of infections and the presence of disease.

No infectious agents were detected in 68% (80/117) of case foals, 54% (14/26) of hospital foals with diarrhoea and 87% (102/117) of control foals. This is similar to other survey studies where no pathogen was identified in 44% to 78% of foals with diarrhoea (Dwyer *et al.*, 1990b; Frederick *et al.*, 2009; Holland *et al.*, 1991). A major advantage of the current study is the methods used to store the faecal samples have enabled them to be archived, providing a valuable resource of carefully and appropriately age matched case control samples for future studies to investigate other infectious agents. As there are numerous bacterial, viral, protozoan and parasitic agents that may play a role in foal diarrhoea, an alternative to investigating specific infectious agents one by one is the use of high throughput sequencing to characterise differences in the composition of the gastrointestinal microbiome in foals with and without diarrhoea. With the ongoing progress of molecular methods, these techniques are becoming more accessible and affordable, with the additional potential benefit of identifying novel and/or uncultivable infectious agents. Given the multifactorial nature of disease, a shift of focus from individual organisms to interactions between multiple microorganisms and changes in the composition of the gastrointestinal microbiome as a whole, will improve our future understanding of the infectious causes of diarrhoea.

APPENDIX 1

MO BIO Laboratories, Inc. Experienced User Combination Vacuum and Centrifugation Protocol

1. **BEFORE THE FIRST USE ONLY, Solution C5-D must be prepared. Add an equal volume of 100% Ethanol to Solution C5-D (for the 4 prep kit = 120 ml, or for the 12 prep kit = 360 ml).**

Mix well. Put a check mark in the "ethanol added" box on the bottle cap label.

2. Remove the Square Well Mat from the PowerSoil-htp™ Bead Plate and set aside. Add 0.25 grams of soil sample.

Note: This is an appropriate stopping point and you can store the PowerSoil-htp™

Bead Plate at 4°C covered with the Square Well Mat.

This is the most time consuming step of the protocol. Care must be taken to avoid cross contamination between sample wells.

3. Add 750 µl of PowerSoil-htp™ Bead Solution to the wells of the PowerSoil-htp™ Bead Plate.

4. **Check Solution C1.** If Solution C1 has precipitated, heat the solution at 60°C until the precipitate has dissolved. Mix gently before using.

Solution C1 contains SDS. If it gets cold, it will precipitate. Heating at 60°C will dissolve the SDS. Solution C1 can be used while it is still warm.

5. Add 60 µl of Solution C1. Secure the Square Well Mat (from step 2) tightly to the PowerSoil-htp™ Bead Plate.

A proper seal of the mat is critical to prevent loss of sample and leakage that might cause damage to your shaker.

6. Place PowerSoil-htp™ Bead Plate with Square Well Mat securely fastened in the 96 Well Plate Shaker (MO BIO Laboratories catalog number 11996).

Note: The final order of assembly is;Aluminum Plate, Square Well Mat, 96 Well PowerSoil-htp™ Bead Plate, and second Aluminum Plate.

7. Shake at speed 20 for 20 minutes. Centrifuge at room temperature for 10 minutes at 2500 x *g*.

8. Remove the Bead Plate from the centrifuge. Carefully and without splashing remove and discard the Square Well Mat and transfer the supernatant to a clean 1ml Collection Plate.

Note: The supernatant may still contain some soil particles.

9. Add 250 µl of Solution C2 to each well and apply Sealing Tape to 1 ml Collection Plate. Vortex for 5 seconds and incubate at 4°C for 10 minutes. Centrifuge the 1ml Collection Plate at room temperature for 10 minutes at 2500 x *g*. Remove and discard Sealing Tape.

10. Avoiding the pellet, transfer entire volume of supernatant to a new 1 ml Collection Plate.

11. Apply new Sealing Tape to the 1 ml Collection Plate and centrifuge at room temperature for 10 minutes at 2500 x *g*. Transfer entire volume of supernatant to a new 1 ml Collection Plate.

12. Add 200 µl of Solution C3 and apply Sealing Tape to the 1 ml Collection Plate. Vortex for 5 seconds and incubate at 4°C for 10 minutes. Centrifuge at room temperature for 10 minutes at 2500 x *g*. Remove and discard Sealing Tape.

13. Avoiding the pellet, transfer entire volume of supernatant to a new 1 ml Collection Plate.

14. Apply Sealing Tape to 1 ml Collection Plate. Centrifuge the 1 ml Collection Plate at room temperature for 10 minutes at 2500 x *g*.

15. Transfer no more than **650 µl** of supernatant to a 2 ml Collection Plate avoiding any residual pellet.

16. Add 650 µl of Solution C4 to each well of the 2 ml Collection Plate.

17. Add a second 650 µl of Solution C4 to each well of the 2 ml Collection Plate. **Note:** It is safe to stop the protocol at this step and store the samples covered with Sealing Tape, at 4°C.

18. Remove the top portion of the vacuum manifold and place a new 2 ml Collection Plate in the bottom of the vacuum manifold.

19. Replace the top of the manifold and place a Spin Plate on top of the manifold and turn the vacuum pump on.

20. Test that you have a good seal with the manifold and the Spin Plate by gently lifting up on the Spin Plate. You should be able to lift the entire unit without the Spin Plate separating from the manifold.

21. Pipet samples from step 17 “up and down” to mix.

22. Load approximately 650 µl into the wells of each row of the Spin Plate until all the samples have been processed. Allow the liquid to pass through the wells.

23. Once the liquid has passed through the wells, continue loading 650 µl volumes into the wells of each row of the Spin Plate until the entire volume of each sample is processed. Turn off the vacuum.

24. Remove the Spin Plate from the manifold and set aside, discard the flow through from the 2 ml

Collection Plate in the bottom of the manifold and then place the 2 ml Collection Plate back in the manifold.

25. Reassemble the manifold with the Spin Plate back on top of the manifold and turn the vacuum on. Test the seal by lifting gently.

26. **Confirm that ethanol has been added to Solution C5-D (see step 1).** Add 500 µl of Solution C5-D to each well of the Spin Plate. After the entire volume of Solution C5-D has passed through the wells of the Spin Plate, turn off the vacuum.

27. Apply Centrifuge Tape to the Spin Plate to cover the wells.

28. Place a 0.5 ml Collection Plate under the Spin Plate. Centrifuge at room temperature for 10 minutes at 2500 x *g*.

29. Carefully place Spin Plate onto a Microplate. Remove Centrifuge Tape and discard.

30. Add 100 µl of Solution C6 to the center of each well of the Spin Plate and apply Centrifuge Tape.

31. Centrifuge at room temperature for 5 minutes at 2500 x *g*. Remove Centrifuge Tape and discard.

32. Cover wells of Microplate with the Elution Sealing Mat provided. DNA is now ready for any downstream application. No further steps are required.

Prolonged storage at 4°C will result in the evaporation of eluted DNA. We recommend storing DNA frozen (-20°C or -80°C). Solution C6 does not contain EDTA. To concentrate the DNA see the Additional Information Section.

Thank you for choosing the PowerSoil-htp™ 96 Well Soil DNA Isolation Kit.

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