Department of Primary Industries

final report

Project code: B.AHE.0025

Prepared by: Alison Gunn, John House, Paul Sheehy, Andrew Thompson, Deborah Finlaison and Peter Kirkland
The University of Sydney and NSW Department of Industry and Investment

Date published: 23 August 2016

PUBLISHED BY
Meat and Livestock Australia Limited
Locked Bag 991
NORTH SYDNEY NSW 2059

Molecular methods for detection of calf scour pathogens

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

This publication is published by Meat & Livestock Australia Limited ABN 39 081 678 364 (MLA). Care is taken to ensure the accuracy of the information contained in this publication. However MLA cannot accept responsibility for the accuracy or completeness of the information or opinions contained in the publication. You should make your own enquiries before making decisions concerning your interests. Reproduction in whole or in part of this publication is prohibited without prior written consent of MLA.

Abstract

Calf scours is an ongoing issue for beef producers and a major cause of economic loss. Calf scours is a multifactorial disease caused by a complex interaction between viral, bacterial and protozoal pathogens, the calves' environment and immune status. This project has developed multiplex qRT–PCR assays with good analytic sensitivity for all major calf scour pathogens, providing the capacity to rapidly identify multiple pathogens contributing to disease in a single step. Loop mediated Isothermal Amplification (LAMP) assays were also developed for all major pathogens, but ongoing challenges with robustness and repeatability led to priority being directed at the PCR assays. The qRT-PCR successfully detected a high prevalence of infection with rotavirus, *Cryptosporidium* spp. and *Giardia* spp, and the complex interaction between these pathogens and clinical disease in large scale prospective on-farm studies. These studies also verified the effectiveness of the environmental sampling techniques to demonstrate reservoirs of these pathogens, confirming the application of these tests for subsequent epidemiological studies. The ability to identify the pathogens involved; the sources of infection and the limitations and value of management interventions, supported the allocation of resources to control and subsequently prevent ongoing disease, demonstrating that these assays can provide significant farm level value.

List of Acronyms and abbreviations

AMV	Avian Myeloblastosis Virus
Bst	*Bacillus stearothermophilus*
BCoV	Bovine Coronavirus
BVDV	Bovine Viral Diarrhoea Virus
cfu	colony-forming unit
Ct	Cycle threshold = the number of qPCR cycles required before the organism is detected. A low Ct indicates that the organism is present in large numbers.
EM	Electron microscopy
EMAI	Elizabeth Macarthur Agriculture Institute
FIP/BIP	Forward/backward inner primer(s)
GIT	Gastro-intestinal tract
LAMP	mediated
mL	Millilitre
MMLV	Moloney Murine Leukemia Virus
PBGS	Phosphate Buffered Gelatin Saline
PI	Persistently infected
PVPP	Polyvinyl polypyrrolidone
qPCR	Real-time (quantitative) polymerase chain reaction
qRT-PCR	Real-time (quantitative) reverse transcription polymerase chain reaction
S.T.A.R.	Stool transport and recovery
µL	Microlitre
xg	Times gravity
XIPC	Exogenous internal positive control

Executive Summary

Neonatal calf diarrhoea or calf scours in beef calves has been an ongoing issue for producers for many years (Dickson 1966; Gunn 2003; Lievaart et al. 2013). Overseas studies have shown that calf scours is a major cause of economic loss and have detailed preventive strategies. Despite this, there is little Australian research that documents the impacts of this disease on beef enterprises, or demonstrates preventive and treatment strategies to minimise the impact.

The current project was a direct result of the recommendations from the previous three “Calf Scours in southern Australian beef enterprises” projects (B.AHW.0026, B.AHW.0057 and B.AHW.0106). The initial study demonstrated that neonatal calf scours is a significant and time-consuming disease problem on many of the suckler beef enterprises surveyed, that producers and veterinarians poorly understand the predisposing factors, causes and management of calf scours in suckler beef enterprises and there was little or no extension material available. Consequently, affected producers have been frustrated by the lack of reliable and practical advice. A whole of industry approach was recommended to ensure clear and consistent advice, together with structured systems to minimise the impact of this problem. The second phase of the project included a comprehensive literature review that outlined the latest research and opinions on all aspects of scours in neonatal beef calves and used this information to compile best practice information modules targeted at veterinarians and farmers. The third phase of the project consulted widely with producers and farm consultants and produced Tips and Tools for producers on the prevention and control of calf scours and a technical document with supporting material for veterinarians and producers.

All projects recommended that further epidemiological studies should be carried out. However, with the rapid evolution of diagnostic tools, it was proposed that effective tests for calf scour pathogens should initially be developed to support a thorough epidemiological investigation. The objectives for the current project were to develop molecular tools including multiplex and singleplex qPCR and LAMP for cost effective, rapid, reliable and quantitative detection of the major recognized calf scour pathogens and to demonstrate if this technology can be adapted and applied to testing of environmental samples of various types, so that sources of infections can be identified rapidly and mitigation strategies assessed for effectiveness.

This project has developed qRT–PCR assays with good analytic sensitivity for all major calf scour pathogens, providing the capacity to rapidly identify pathogens contributing to disease. However, the *Salmonella* qPCR had reduced sensitivity compared to traditional selective culture methods. This was considered to be a result of the large difference in volumes of sample used for culture compared to the very small amount used for qPCR. The suite of qPCR assays were each successfully used to test a range of environmental samples.

Multiplexing involves running two or more qRT–PCR assays in the same reaction for the concurrent detection of multiple pathogens. The individual qPCR/qRT-PCR singleplex assays were progressively combined to achieve the following triplex combinations:

- <u>Bacterial and protozoal qPCR</u>: *Salmonella spp*, *E. coli* K99 / F5 and *Cryptosporidium spp.*
- <u>Viral multiplex qPCR</u>: *Rotavirus A* , bovine coronavirus (BCoV) and BVDV.

These triplex assays performed well when detecting plasmid DNA, with very little noticeable change in qPCR reaction efficiency. However, when an extra assay was added for a quadriplex combination, there was a reduction in sensitivity for the detection of one or more

pathogens. As optimisation of quadriplex assays involves a complex of interactions between many different reactants and the benefits were often marginal, further optimisation was not undertaken. Instead, research was directed at the impact on diagnostic sensitivity, particularly as a reduction in analytical sensitivity was mostly observed when high concentrations of more than 2 pathogens were present.

Loop mediated Isothermal Amplification (LAMP) assays were also developed for all major pathogens. LAMP is an alternate DNA amplification protocol to PCR and has been used for the detection of DNA or RNA from infectious organisms. A benefit of LAMP is that sophisticated laboratory facilities are not required to run the assay. A significant challenge was the need to assess multiple primer sets for each assay which markedly increases the amount of work required to develop each test. This challenge with LAMP primer design is not reported in detail in the literature but acknowledged and observed anecdotally. Generally, the assays developed all demonstrated specificity for the target organism although on occasion there was amplification observed with non-target DNA. The sensitivity of the assays varied significantly with the *Salmonella*, *E. coli*, *Giardia* and BVDV assays demonstrating a sensitivity comparable to an optimized PCR assay yet sensitivity of *Cryptosporidium*, *Rotavirus A* and BCoV were typically 10-100 fold less sensitive which may impact on their utility for use with clinical samples.

Throughout the studies to optimize the LAMP assays there were ongoing challenges with robustness and repeatability. All assays were impacted with amplification sporadically observed in negative control samples. This was initially considered to be potential contamination of reagents and was thoroughly investigated with assessment of new reagents and confounded by the intermittent nature of observations. There were also challenges with positive control samples intermittently not amplifying. At various stages DNA or RNA extracted from clinical samples was tested but the results were variable and there was a clear need for further refinement.

One of the desirable features of LAMP is the potential field use of the assays. Point of care diagnostics are an emerging area and recently this has extended to point of care PCR technology. Given the limitations of LAMP and the robustness of the PCR platform and continuing development of delivery options for PCR assays priority was directed at the PCR assays.

Two large scale prospective studies were carried out to determine the shedding patterns of calf scour pathogens from clinically affected calves and also to determine if pathogens could be detected in the environment. These studies monitored housed dairy calves for 3-4 weeks from birth collecting information on their clinical signs, pathogens shed in faeces and pathogens detected in their environment on a very regular basis. The aim was firstly to demonstrate the application of PCR technology in the field, both for epidemiological investigations and for diagnostic purposes, and secondly to provide guidance in determining the clinical relevance of results for faecal samples collected at a single time point in the field and submitted by a veterinarian as part of a calf scours diagnostic investigation.

These studies highlighted the dynamics of neonatal calf disease in a farm situation. Frequent monitoring with a sensitive diagnostic test demonstrated a high prevalence of infection with rotavirus, *Cryptosporidium* and *Giardia*. A feature of these studies was the lack of association between clinical disease and individual PCR values, suggesting that several pathogens were contributing to clinical disease at any one time. On Farm A there was a strong association between the appearance of *Cryptosporidium* in the faeces and the clinical score. The pathogen profile on farm B was modified by treatment for *Cryptosporidium* and

disease was associated with peak rotavirus shedding. These studies also demonstrated a more complex picture behind the routine clinical investigation of a scour outbreak, where samples are generally only taken at a single time point. Detection of pathogens at a single time point only indicates that those pathogens are likely to be contributing to disease on that farm. The contribution of each pathogen needs to be determined by knowledge of the clinical presentation and also response to specific targeted preventive measures.

These two studies also demonstrated that the environmental sampling techniques used were highly effective in detecting rotavirus. Environmental sampling techniques were also useful in implicating sources of *Cryptosporidium*, BCoV and *Salmonella*. However, it should also be realised that the study has only demonstrated potential sources of pathogens in the environment. The limitation of the PCR is that it does not provide any indication of infectivity of the agent detected but studies in humans implicate viruses, including rotavirus, detected on environmental surfaces in maintaining disease outbreaks. Significant farm level value was derived from identifying: the pathogens involved; sources of infection and the limitations and value of management interventions. These insights were used to support the allocation of resources to control and subsequently prevent ongoing disease.

Field epidemiological studies depend on efficient and reliable methods of collection and storage to ensure that results reflect the pathogen levels in samples at the time of sampling. Swabs have not usually been used for sampling faecal pathogens in calves and there are no publications that evaluate comparative data. For pathogens with sufficient comparative data, this study demonstrated that there was a small decrease in the sensitivity of detection with swabs. Despite this, from a clinical and laboratory perspective, swabs offer many advantages, particularly for the detection of non-bacterial pathogens and for environmental sampling. Further research is required to determine the impact of various transport media on the detection of bacterial pathogens from swabs. While storage of faeces at 4°C for several days is unlikely to have a significant alteration on pathogen load for some agents, BVDV, *Giardia* and potentially BCoV are more labile. The current study confirmed that for BVDV survival it is necessary to keep faecal samples cool at all times.

Calf scour outbreaks frequently involve multiple pathogens. Historically the tools available to manage and prevent calf scours have been limited. In recent years vaccines for rotavirus and BCoV, and halofuginone for the treatment and prevention of *Cryptosporidium* have become available. Despite the availability of these tools, calf scours continues to be a difficult problem to manage on farm. The first step in the process of investigating disease outbreaks is to define the problem. This requires identification of causal pathogens, sources of infection and stressors that contribute to compromised host immunity. This will support pathogen specific interventions, reduce the risk of pathogen challenge, and mitigate stressors contributing to compromised host immunity.

The diagnostic assays developed in this project will facilitate the disease investigation and management process. qPCR assays have enhanced sensitivity and quantitative output, and will allow detection of pathogens in the environment to determine areas with an increased infection risk. Effective and efficient on farm implementation of disease management / prevention plans requires allocation of resources to minimize disease risk by targeting procedures and products that address the most significant risks. Conversely, it is also useful to be able to evaluate the effectiveness of the intervention. The tools developed in this project will allow for significantly improved risk assessment and intervention on farm and expand the diagnostic options available by offering the capacity to detect several pathogens simultaneously.

Table of Contents

1 Background 10
2 Project objectives 11
3 Methodology 11
3.1 Development of qPCR/qRT-PCR assays 11
3.1.1 Assay design and validation 11
3.1.2 Bacterial pathogens 12
3.1.3 Protozoa 13
3.1.4 Viruses 14
3.1.5 Sample processing 15
3.1.6 Internal positive control (XIPC) 16
3.1.7 Nucleic acid extraction 16
3.1.8 Real time PCR parameters 16
3.1.9 Standards and control samples for qPCR 17
3.1.10 Assessment of analytical sensitivity of singleplex RT-qPCR assays 19
3.1.11 Bacterial cultures & quantification of microorganisms 19
3.1.12 Virus detection & virus isolation 19
3.2 Multiplex qPCR 20
3.3 Loop-mediated Isothermal Amplification (LAMP) 21
3.3.1 LAMP assay design: 22
3.3.2 Lamp assay development: 22
3.3.3 LAMP optimization protocols 23
3.4 Reference and clinical samples 24
3.4.1 Reference materials 24
3.4.2 Specimen collection and storage methods 25
3.4.3 Longitudinal animal studies 26
3.5 Environmental monitoring 29
3.5.1 Laboratory testing of spiked samples 30
3.5.2 Environmental samples from on farm longitudinal calf studies 32
3.6 Statistical analysis 33
4 Results 33
4.1 Real-time PCR assays 33
4.1.1 Assay design and validation 33
4.1.2 Bacterial pathogens 33

4.1.3 Protozoa ... 35
4.1.4 Viruses ... 36
4.1.5 XIPC ... 37
4.2 Multiplex PCR ... 37
4.2.1 Optimisation of multiplex real-time PCR panel ... 37
4.3 Loop-mediated Isothermal Amplification (LAMP) ... 39
4.3.1 Development of LAMP primer sets ... 39
4.3.2 Optimal LAMP reaction characteristics and parameters ... 40
4.4 Animal specimens ... 41
4.4.1 Specimen collection and storage methods ... 42
4.4.2 Longitudinal animal studies ... 42
4.5 Environmental monitoring ... 46
4.5.1 Laboratory testing of spiked samples ... 47
4.5.2 Environmental samples from on farm longitudinal calf studies ... 49
5 Discussion ... 52
5.1 Development and optimisation of qPCR/qRT-PCR assays ... 52
5.2 Multiplex qPCR ... 54
5.3 Loop-mediated Isothermal Amplification (LAMP) ... 55
5.4 Animal specimens ... 57
5.4.1 Sample collection and storage ... 57
5.4.2 Experimental challenge trials ... 58
5.4.3 On farm studies of calf shedding patterns and interpretation in the field ... 59
5.4.4 Environmental samples ... 62
5.5 Practical implications for industry; ... 64
5.6 Unanswered questions/additional research recommended ... 65
5.6.1 *Salmonella* qPCR ... 65
5.6.2 Coronavirus ... 65
5.6.3 Evaluation of risk factors for calf scours in beef cattle ... 66
5.6.4 Environmental sampling ... 66
5.6.5 Use of pooled samples ... 66
5.6.6 Extension ... 66
5.6.7 Rotavirus ... 66
5.7 Draft extension messages ... 67
5.8 What could have been improved in the project delivery ... 67
5.9 Extent to which each specific project objective was met ... 67
6 Conclusions/recommendations ... 68

6.1 General conclusions 68
6.2 Future studies 70
7 Key messages 71
8 Bibliography 72
9 Appendices 80
9.1 Appendix 1: Primer and probe sequences for singleplex assays 80
9.2 Appendix 3: LAMP target DNA/RNA sequence for which LAMP primer sequences were designed 81
9.3 Appendix 4: LAMP Primer sets chosen for assay development following initial functional assessment 82

1 Background

Neonatal calf diarrhoea or calf scours in beef calves has been an ongoing issue for producers for many years. A 1966 study of beef cattle diseases in Victoria noted that over 80% of properties experienced some form of white scour, and this was regarded as a problem on just under 50% of properties (Dickson 1966). In the mid-1990's there were two Meat and Livestock Australia (MLA) funded Producer Initiated Research and Development projects that studied aetiology of calf scours and the efficacy of an enterotoxigenic *E. coli* vaccine, but yielded few answers to the producers involved. More recent studies this century have continued to portray an ongoing problem (Gunn 2003; Sloane 2006; Lievaart et al. 2013).

The major pathogens associated with neonatal calf scours (calves < 6 weeks of age) are *Salmonella* spp., K99 *E. coli*, rotavirus, bovine coronavirus (BCoV) and *Cryptosporidium. Giardia* and a number of viruses including bovine torovirus have also been implicated, but not established as major pathogens. Studies from overseas have also shown that calf scours is a major cause of economic loss to beef producers and have detailed preventive strategies. Despite this, there is little Australian research that documents the impacts of this disease on beef enterprises, or demonstrates preventive and treatment strategies to minimise the impact. MLA projects B.AHW.0026 and B.AHW.0129 demonstrated that calf scours has a significant impact on affected properties, and should be considered an important industry problem in southern Australia. Diagnosis of calf scours has traditionally been expensive due to the need to test for viral, protozoal and bacterial causes in multiple samples. Although B.AHW.0026 surveyed and obtained veterinary laboratory data to determine the common pathogens causing this disease in beef calves, in many cases diagnostic testing was incomplete either due to the submitter's not requesting full pathogen panels or laboratories not offering a complete panel of tests.

A recent survey established the common pathogens causing diarrhoea in Australian dairy calves (Izzo et al. 2011), however, the distribution of major pathogens associated with neonatal calf diarrhoea in the Australian suckler beef industry and their epidemiology remains undefined. Robust advice on prevention and control of calf scours has been produced by MLA (B.AHW.0106). These recommendations were based on the application of principles derived from overseas studies due to the paucity of Australian studies on the subject. Producers often experience frustration when they implement "best practices" and continue to experience problems with calf scours. Veterinarians' capacity to problem solve and refine management recommendations was limited by the lack of tests that provide a quantitative assessment of pathogen shedding and of the environmental pathogen load, hence the need to develop better diagnostic tools prior to further epidemiological investigation. Testing procedures used in many veterinary laboratories provide a qualitative but not quantitative result. In the absence of quantitative data, relative significance and importance of individual pathogens, and indeed degree of contamination of the environment cannot be determined. At the commencement of the study, PCR techniques had been published for some of the major calf scour pathogens, but diagnostic quantitative assays that could detect multiple diarrhoea pathogens simultaneously (multiplex real-time PCR) had not been published. It was proposed that modification of existing methods utilizing newer technology to provide quantitative diagnostic methods would facilitate the early diagnosis of calf scour pathogens and further provide a useful tool for investigating the epidemiology of

calf scours and a means to assess the effectiveness preventive strategies on reducing pathogen load and disease.

2 Project objectives

To develop reliable, quantitative and cost-effective methods for diagnosis and evaluation of pathogens in calf scour outbreaks, including evaluation of environmental sampling techniques to determine pathogen load, assess risk, and to assess the effectiveness of management interventions.

1. Utilise previously characterized samples to develop and validate multiplex PCR techniques for diagnosis of rotavirus, coronavirus, bovine torovirus, bovine viral diarrhoea virus (BVDV) or pestivirus, *Salmonella*, K99 *E. coli*, *Cryptosporidia* and *Giardia* in faeces. This is likely to be 2 multiplex PCRs; one for viruses and the other for bacteria and protozoa.
2. Report on the prevalence of bovine torovirus in the faecal samples.
3. Demonstrate the variation in faecal shedding with time, and the relationship between faecal shedding and both calf morbidity / mortality and GIT pathology.
4. Investigate the potential to use these techniques to detect pathogens in samples from the environment: soil, water, paddocks, plants and man-made surfaces.
5. Develop LAMP technique for use on farm for same pathogens and samples.

3 Methodology

A major aspect of this project was to undertake the development and evaluation of a range of laboratory methods for the detection of common pathogens that can cause scours in calves. These assays would then be applied to answer practical questions related to the investigation and control of neonatal diarrhoea in cattle. The following section describes the approaches that were taken while the outcomes are documented in the subsequent “Results” section. As the technology applied in this project is rapidly evolving, there were some relevant methods published in the scientific literature during the course of the project. These were taken into consideration and included in these studies, whenever practical.

3.1 Development of qPCR/qRT-PCR assays

3.1.1 Assay design and validation

While an overall objective was to minimise the number of assays that needed to be undertaken for the detection of likely pathogens, preferably by the use of a multiplex real time PCR, in the first instance assays for each pathogen were developed and evaluated separately to define the performance characteristics. This would then allow the individual reactions to be combined as duplex and subsequent multiplex assays, in a manner that aims to optimise the kinetics of individual assay reactions. Published assays that appeared to be thoroughly designed were initially evaluated and new assays (either published or designed

as part of this study) were introduced as required. Some of the virus assays had been previously developed in the Virology Laboratory at EMAI.

All of the primers and probes were purchased from Biosearch Technologies, Inc., Petaluma, CA, USA. Real-time PCR amplified products were cloned and sequenced to check assay fidelity. This cloning was repeated for the entire gene of interest using conventional PCR reactions (with primers developed in this project), to enable the construction of control DNA that could be utilized for both real-time PCR and LAMP reactions. This would allow a direct comparison of the performance of different molecular assays to be made. These cloned real-time and conventional PCR products were also used to assess assay sensitivity.

Multiple faecal pathogens and normal microbiota from calf faeces were obtained, cultured, extracted and tested to ensure no false positive results were obtained when tested in any of the non-homologous assays. Similarly, the positive control samples for each individual assay were tested in each other assay being developed/evaluated, to ensure there was no cross-reactivity. When acceptable individual (singleplex) assays were available, the approach was to combine these in various combinations into a multiplex format to support concurrent/simultaneous detection of pathogens of interest. The development of the singleplex assays is described below and the multiplex assays in Section 3.2. The nucleic acid sequences for primers and probes used in the singleplex assays are summarised in Section 9.1. (Appendix 1).

3.1.2 Bacterial pathogens

Salmonella

Although there are over 2600 serotypes of *Salmonella*, typically 80% of the disease observed in calves in a region is limited to less than 20 serotypes. However, the makeup of the most common serotypes varies across regions. For *Salmonella enterica*, it was considered important to select a qPCR assay that would detect as many serotypes as possible to capture all serotypes that may cause disease in calves. The gene that encodes an enterotoxin (stn), was selected from the published literature (Moore and Feist 2007) as it has previously been shown to detect all serotypes of *Salmonella enterica* and *Salmonella bongori* (Makino, Kurazono et al. 1999). This qPCR was originally developed in order to detect all *Salmonella* species in food, with very few false positives. In the published method, the assay was used to detect bacteria cultured on selective media, and for this project, we have explored the usefulness of this assay, when applied directly to testing of faecal samples. The benefit of this approach is that the turnaround time would be markedly reduced (from around 5 days for traditional methods to isolate and identify *Salmonella* organisms in faecal samples) to 1 day for qPCR.

The analytical sensitivity, specificity, and limit of detection of the assay were determined using characterised, stored isolates at EMAI as well as DNA plasmid constructs. Briefly, serial dilutions of known numbers of bacteria were tested by qPCR and by culture using PBS and calf faecal samples that had been spiked with serial dilution of organisms harvested from broth culture. To allow the number of genome copies to be calculated at the limit of detection, the concentration of extracted DNA was measured using a Nanodrop spectrophotometer, serial dilutions were made for qPCR and an end-point determined. This experiment was carried out in triplicate.

E. coli

A diversity of *E. coli* is normally shed in the faeces of calves. Enterotoxigenic *E. coli* (ETEC) typically cause disease in calves less than 3 days of age. The age predilection of this pathogen reflects bacterial attachment to immature enterocytes mediated by bacterial pilins. The virulence attributes associated with pathogenicity are the pilin and enterotoxin production that induces secretory diarrhoea. The plasmid-borne F5 gene, which encodes the pilin was selected as an appropriate molecular diagnostic target for this pathogen. A qPCR assay detecting the F5 gene has previously been developed by West et al. to detect ETEC in bacterial cultures (West et al. 2007). This assay was originally designed, using multiplex qPCR , to detect important virulence genes of a range of *E. coli* isolates of veterinary importance, using pure *E. coli* cultures. The primer sequences from this assay were selected for incorporation in the suite of molecular assays used in this project for the direct detection of ETEC in faecal and environmental samples. The use of an ETEC specific assay will allow enterotoxigenic *E. coli* to be distinguished from other *E. coli* which represent normal flora of the gastrointestinal tract. Additionally, this assay will be particularly useful as conventional laboratory methods to detect the F5 (or K99) fimbrial antigen, can yield false negative results (due to the fact that expression of this plasmid-borne gene only occurs under very specific environmental conditions).

This assay was evaluated by comparing the qPCR results with traditional laboratory test results (either bacterial culture or Bio-X E. Coli F5 (Bio K 154) lateral flow test strips, Bio-X Diagnostics, Jemelle, Belgium) for routine diagnostic investigations undertaken at EMAI over several years. Comparison was also made with a bank of samples previously tested with an ELISA (Pourquier® ELISA Calves Diarrhoea; Institut Pourquier®, Montpellier, France) and had been stored at -80°C. The analytical sensitivity, specificity, and limit of detection of the assay were assessed by testing of characterised *E. coli* F5 isolates and DNA plasmid constructs. Briefly, serial dilutions of known numbers of bacteria were added to either phosphate buffered saline or faecal samples. The DNA was then extracted and tested by qPCR and the results compared with quantitative cultures.

3.1.3 Protozoa

Cryptosporidium spp

Cryptosporidium spp. are an important protozoal parasitic cause of neonatal calf diarrhoea and also has a widespread distribution. Until recently it was considered that *Cryptosporidium parvum* was the main species to infect cattle, but it is now recognised that cattle are infected with at least five *Cryptosporidium* species*: C. parvum, C. bovis, C. andersoni, C. ryanae* (previously called deer-like genotype) and *C. suis* (Ng et al. 2011). Most of these are morphologically indistinguishable. *Cryptosporidium parvum* is usually predominant in calves <3 months of age, whereas *C. bovis* and *C. ryanae* are more common in post-weaned calves and *C. andersoni* is more prevalent in older calves and adult cattle (Abeywardena et al. 2015). All of these species are initially detected using the PCR methods described in this section and further speciation is carried out by amplification of genes specific to each species.

A published assay designed to detect the *Cryptosporidium* oocyst wall protein gene (COWP) was initially selected as unique to this organism, conserved across a wide variety of

isolates and important in the biology of the organism in maintaining the integrity of the oocyst wall, to support long-term survival of this stage within the environment (Guy et al. 2003). The initial primers and probe were from a published assay (Cho et al. 2010).

Cryptosporidium positive samples were identified using the BioX rapid direct faecal antigen test kits and a subsample confirmed by faecal flotation (Kuczynska & Shelton 1999). A series of positive faecal samples were characterized by quantifying the number of *Cryptosporidium* oocysts using the method described by Kuczynska and Shelton (Kuczynska & Shelton 1999).

Comparison with known positive samples demonstrated that the initial assay had very limited analytical sensitivity. Further study of the original source of this assay (Guy et al. 2003) demonstrated that one of the primers described by Cho (Cho et al. 2010) had been published incorrectly. Once this was rectified the sensitivity improved. However, subsequent testing of clinical samples demonstrated that this assay still had sub-optimal sensitivity. Consequently, the assay was changed to one amplifying the 18 small subunit rRNA (18s) gene, a high copy target that has been demonstrated to improve sensitivity and reliability (Stroup et al. 2006; Stroup et al. 2012). This target has been previously included in a multiplex PCR that also detects *Rotavirus A* and bovine coronavirus (BCoV) (Schroeder et al. 2012). Use of this assay improved both the number of copies detected and the number of samples in which *Cryptosporidia* were isolated.

Giardia duodenalis

A published real-time PCR assay (Verweij et al. 2003) based on the conserved small subunit ribosomal RNA gene of *Giardia duodenalis* was chosen and is specific for *G. duodenalis*. This gene has been shown to be the most sensitive for detection of *Giardia* as it has a high copy number (Nantavisai et al. 2007). Traditionally, the “gold standard” diagnostic test has been to use a zinc sulphate flotation technique and stain oocysts with iodine, followed by microscopic examination.

Similar to *Cryptosporidium*, two plasmid constructs were developed – one based on the qPCR amplicon and the other one based on PCR performed on the whole small subunit ribosomal RNA gene. This was then used to evaluate assay analytical sensitivity and primer and probe optimization. Previously characterized and purified positive control DNA extracts were obtained from two other laboratories (St Vincent’s Hospital, Sydney and Murdoch University, WA) and the DNA concentration in these extracts measured for calculation of qPCR reaction efficiency.

3.1.4 Viruses

Bovine Coronavirus (BCoV) - The qRT-PCR assay used for this project was developed at EMAI and had been evaluated in a previous project (Izzo et al. 2012). The primers and probe sequences were identified after alignment of sequence data of an Australian strain of BCoV with sequences from Genbank. Testing of diagnostic samples from adult cattle affected with a winter dysentery syndrome which identified coronavirus-like particles by electron microscopy and differences of results compared with a BCoV ELISA raised concerns that some strains of coronavirus may not be detected. Two other published assays (Decaro et al. 2012; Cho et al. 2010) were included in these studies in an attempt to resolve this perceived discrepancy. Samples obtained from an outbreak of winter dysentery were

examined by electron microscopy, virus isolation, and nucleic acid sequencing in an attempt to establish whether current assays were failing to detect some strains of enteric coronavirus.

Rotavirus – Primer and probe sequences were available from published assays (Logan et al. 2006) for both Group A and Group C rotaviruses. These had been evaluated in a previous project (Izzo et al. 2012) and had been found to have high sensitivity. However, as group C rotavirus strains were rarely detected in previous research, only the Group A assay was used in this project.

Bovine Viral Diarrhoea Virus – a published pan-reactive qRT-PCR assay (Decaro et al. 2008) that has been in routine use in the EMAI Virology Laboratory for pestivirus diagnosis was employed in this project. This pan-reactive assay is used in preference to a BVDV-specific assay to ensure that all strains of pestivirus are detected.

Torovirus - The PCR assay was designed at EMAI and is directed at a sequence of the M gene (nucleotides 25758-26459) based on published sequence data (Draker et al. 2006). The assay that was used for this project was developed by converting a published conventional RT-PCR into qRT-PCR format. However, during initial validation undertaken by testing of a large panel of samples from calves with neonatal diarrhoea, and by examination of electron microscopy (EM) records at EMAI, it was found that there were very few positive samples and so further refinement of this assay was not undertaken.

3.1.5 Sample processing

Prior to nucleic acid extraction, preliminary processing of crude faecal samples was required. Unless otherwise described in a specific experiment faecal samples were diluted in phosphate-buffered saline (PBS) in the ratio of 3:7 (i.e. 300 µl faeces to 700 µl PBS) in a sterile 1.5 ml microcentrifuge tube and homogenized by mixing on a vortex mixer for 30 seconds. The suspension was then briefly centrifuged (100 xg for one minute) to pellet any large particulate material.

For some pathogens, additional physical treatment to disrupt the cell wall was investigated. Six hundred µL of the clarified supernatant was pipetted into a 2 ml screw topped tube (Sarstedt ™), which had been previously loaded with 0.5 grams of 0.1mm silica zirconia beads (Biospec products, Inc, Bartlesville, OK USA) and 300µl of 0.01 M phosphate buffered saline (pH 7.4). The loaded bead tubes were then shaken for 5 minutes at 20 Hz, on maximum speed, using a Mini Bead Beater (Bio-Spec products, Inc.) prior to centrifuging for 5 minutes at approximately 14000 xg to pellet any remaining particulate material and the beads.

The clarified supernatant was then used immediately for nucleic acid extraction or was stored below -20 °C for later extraction as a batch with other samples.

Rectal swabs were collected into 3mL of Phosphate Buffered Gelatin Saline (PBGS) in 5mL screw topped tubes. The tubes were briefly shaken for approximately 5 seconds to wash the swabs and suspend the particulate faecal matter (and longer if required to break up large particulate material) using a vortex mixer. After allowing the tubes to stand for at least 15 mins, 600µl was removed for bead beating (if required) or 50 µL of PBGS was removed for direct nucleic acid extraction.

3.1.6 Internal positive control (XIPC)

An exogenous nucleic acid sample was used as a control to monitor the efficiency of nucleic acid extraction, the possible presence of inhibitors of the PCR reaction and to ensure that sample transfer had been carried out efficiently both during nucleic acid extraction and during set up of the PCR. This control consisted of a cryptic synthetic nucleic acid sequence that is not known to occur in nature (10). It was included at a known, low concentration in the sample lysis buffer that is used in the first step of the nucleic acid extraction. Consequently, if all stages of the nucleic acid extraction and the PCR are carried out efficiently, the exogenous internal positive control (XIPC) should be detected at a level similar to what was included in the lysis buffer prior to commencement of the extraction. The XIPC is available as both DNA and RNA but was routinely used as RNA, allowing the same preparation to be used for bacteria, protozoa, and viruses. The PCR result for the XIPC included with the assay (target) negative control was used to set the bench-mark for the entire plate. If the cycle-threshold (Ct) value of a sample was >4 units higher than the XIPC, it was considered that the PCR result was suboptimal and required further investigation. Usually, a satisfactory result is obtained by using a ¼ to $^1/_{10}$ dilution of the sample.

3.1.7 Nucleic acid extraction

High through-put real time PCR has been used at EMAI for DNA viruses (qPCR) or, with the inclusion of a reverse transcription step, for RNA viruses (qRT-PCR) for almost 10 years. A major component of the success with this technology has been the streamlining of what were tedious and time consuming manual methods used for nucleic acid extraction from samples and subsequent purification. A magnetic bead based, semi-automated system has been shown to be highly effective for almost all sample types for viral PCR assays. The efficiency of this system has been improved further by the extraction of total nucleic acid (i.e. both DNA and RNA), rather than a selective enrichment of either DNA or RNA. This approach facilitates concurrent detection of both DNA and RNA viruses (or other organisms) in a sample. Consequently, this method was utilised as the default for this project, with evaluation of alternative methods for difficult sample types.

Total nucleic acid was extracted from 50µL of sample suspension in PBS using the commercially available MagMax™ Viral RNA Isolation Kit (Cat AM 1836-5, Ambion, Austin, Texas) in accord with the manufacturer’s instructions. The magnetic beads were manipulated using an automated magnetic particle handling system (Kingfisher® 96, Thermo Fisher Scientific Inc., Finland). Purified nucleic acids were eluted in 50µL of kit elution buffer and either tested immediately or frozen at -20 °C for later use in 96 well elution plates sealed with adhesive foil. In each nucleic acid extraction plate two positive controls and one negative control were included. One well was left empty for later addition of a ‘no template’ PCR control. When samples were being tested for rotavirus, the nucleic acid was denatured by heating at 95°C for 5 min then tested immediately. For testing of diagnostic samples, denaturation was carried out routinely because of the potential need to detect rotavirus.

3.1.8 Real time PCR parameters

To make greatest use of the capacity of real-time PCR to support rapid high through-put testing of a wide range of specimens, as well as the extraction of total nucleic acid from

specimens, a single assay format was used for the PCR. Although the target for the detection of bacteria, protozoa and some viruses by PCR is DNA, many viruses, including all of the major enteric pathogens, utilise RNA as their genetic material. By using an assay formulated to detect RNA samples, it is also possible, without changing the reaction components, to also detect DNA with equal efficiency. This provides not only the capacity to detect both DNA and RNA concurrently but also provides efficiencies in the use and purchase of reagents and simplifies laboratory protocols.

All of the essential chemicals and enzymes needed for the PCR are obtained from commercial sources as a 'ready to use mastermix' (AgPath-ID™ One-Step RT-PCR kit - Life Technologies, Foster City, California). As this is a mastermix originally designed for the detection of RNA, during an assay for the detection of both DNA and RNA, the reverse transcriptase first converts any RNA in a sample to DNA. During this time, DNA from microorganisms remains unchanged. The heat stable polymerase (Taq) then amplifies all DNA, including the DNA that has been transcribed from RNA. The amplified DNA is detected during each amplification cycle by a fluorophore labelled probe and the fluorescent signal is detected by the thermocycler. All assays were run under the same reaction conditions using an ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, California) run in standard mode for a total of 45 cycles, following the manufacturer's recommended times and temperature profiles.

Each assay was run in a 96 well plate format. Each well in which an assay was being run contained 20 µL of PCR reaction mix containing 12.5 µL 2x RT buffer, 2 µL tRNA, 1 µL 25x RT enzyme mix, primers and probes at a concentration of 0.2µM and water to a volume of 20 µL. Five µL of purified nucleic acid (from both samples and controls) was then added to each well by transferring from the nucleic acid purification plate and 5µL of nuclease free water was added to the 'no template' control well. The use of standard reaction conditions enabled all assays to be performed on the same machine, at the same time. This also facilitated the development of multiplex assays (see section 3.2).

During the analysis of data, background reactivity was adjusted automatically and the reaction threshold was set manually at 0.05. Results for qPCR or qRT-PCR assays were reported as 'cycle-threshold' (Ct) values, the number of amplification cycles at which the signal for a positive sample crossed the 0.05 baseline. Ct values less than 37 were considered to be consistent with specific amplification of the target genome. Ct values between 37 and 40 were usually considered to be inconclusive.

3.1.9 Standards and control samples for qPCR

In addition to the use of an internal positive control (XIPC) with each sample (section 3.1.6), each assay run included 2 negative and 2 positive controls to provide both a measure of quality control and standardisation from plate to plate. One negative control consisted of tRNA which was included at the time of nucleic acid extraction and, when combined with the result for the XIPC, monitors both the efficiency of nucleic acid extraction and possible cross contamination through all steps of the assay. The second negative control uses nuclease free water which is added after the completion of the extraction and monitors for any cross contamination during the set-up and running of the PCR. The two positive controls are samples that should be detected consistently, but are moderate to weak positives with Ct values of about 30 and 33. Failure to detect these samples, or obtaining Ct values that are

more than 2-3 Ct units higher than the expected range, would suggest that the PCR has lower than expected sensitivity.

The type of material that was used for the positive control varied with each assay. A short segment of DNA that included the target sequence for the individual assay was prepared for most agents as a cloned bacterial plasmid (see below). In some instances, the control consisted of a very high titred positive field sample or cultured material. These controls were usually stored as concentrates that were diluted to give the required Ct value and stored in single use aliquots to minimise the deleterious effects of repeated freezing and thawing.

Preparation of plasmid standards

To provide a supply of positive controls for long term use, a set of DNA sequences for the target regions of each qPCR/qRT-PCR assay (with the exception of torovirus) were constructed as cloned bacterial plasmids. The design of these plasmid standards was based on the LAMP target sequence, which also contained the shorter qPCR target within it, thereby creating a multipurpose standard. These plasmid standards were also useful for calculating analytical sensitivity of an assay because a defined number of copies of the gene sequence could be estimated much more readily than in field samples or cultured whole organisms.

Primers for conventional PCR assays were designed for the target regions of each of the microorganisms or viruses (*Rotavirus A*, BCoV, Bovine Viral Diarrhoea Virus, *Escherichia coli* K99, *Salmonella enterica, Cryptosporidium spp.* and *Giardia duodenalis*). DNA or RNA template was extracted from clinical samples containing the agent of interest or from culture stocks held at EMAI.

The best success was achieved with these conventional PCR assays using “Touch-down” thermocycling parameters (Don et al. 1991). Only limited optimization of each reaction was required. All PCR products were visualized on a 1.5 or 2% agarose gel, using “gel-red” DNA intercalating dye and a 100bp plus DNA ladder (Fermentas, Thermofischer Scientific Waltham MA). The DNA band of the expected size was excised from the gel then dissolved and prepared for ligation reactions. PCR products were incubated with Taq polymerase for 10 minutes at 72°C to ensure that A overhangs (or “sticky-ends”) were present before cleaning the PCR products for ligation.

A commercial cloning kit (Invitrogen TA cloning kit Carlsbad, CA, USA) with pCR2.1 cloning vector was used for PCR product ligations and was used according to the manufacturer’s instructions.

PCR products of expected size were selected and stored at -20°C prior to sequencing. Bacteria containing sequences of interest were cultured in a selective broth until the culture had reached the stationery phase (approximately 18 hours). DNA was purified from the broth using a Mini-prep kit (Qiagen, Venlo Netherlands). The DNA concentration was estimated by testing dilutions of the purified DNA in a Nanodrop 2000 spectrophotometer (Thermofisher Scientific Waltham MA). A stock solution of purified plasmid DNA was prepared by diluting 1/100 in tRNA and stored at -80°C.

3.1.10 Assessment of analytical sensitivity of singleplex RT-qPCR assays

The theoretical analytical sensitivity of each qPCR/qRT-PCR was established by testing ten-fold dilutions of plasmid DNA. The end point was determined as the lowest number of copies of genome sequence that could be detected in the real-time PCR assay. The number of genome copies had been previously determined for each agent from the DNA concentration using the Nanodrop spectrophotometer. The dilution series was prepared in PBS to ascertain the optimal theoretical limit in the absence of any substances that may interfere with the efficiency of the qPCR. In contrast, a similar evaluation was conducted for some agents by spiking faecal samples with dilutions of whole organisms. Nucleic acid was then purified from these spiked samples in the same manner as normal diagnostic samples but without bead beating.

3.1.11 Bacterial cultures & quantification of microorganisms

The number of bacteria shed in faeces changes during the course of infection. In the case of *Salmonella,* the number of organisms shed during the period of peak clinical disease is in the order of 10^7 to 10^9 cfu per gram of faeces. Disease recovery is associated with the shedding of lower numbers of cfu per gram as is chronic and or intermittent shedding by active and passive carrier animals. To understand the relevance of a qPCR Ct value it is useful to be able to relate the Ct value to the number of cfu per gram in the sample. To truly understand test sensitivity and specificity it is also important to define the type of sample that is been evaluated. For example test sensitivity is likely to be higher for samples derived from diseased animals as compared to samples from subclinical infections.

The relationship between qPCR Ct values and the number of CFU per gram of faeces was determined for *Salmonella* and K 99 *E. coli* using faecal samples spiked with known numbers of organisms. *Salmonella enterica* serovar Typhimurium was grown in Trypticase soy broth and K 99 *E. coli* in Minca broth for 12 hours. The broth cultures were subsequently centrifuged and the bacteria suspended in phosphate buffered saline. The number of *Salmonella* and *E. coli* present in each suspension was determined using serial dilution and plating on agar. An aliquot of each dilution was added to separate faecal samples providing a known number of organisms per gram of faeces. Following homogenisation the spiked faecal samples were extracted and assayed using the respective qPCR assays.

3.1.12 Virus detection & virus isolation

The sensitivity and specificity of each of the qRT-PCR assays that were used in this project had previously been assessed by comparison with other virus detection methods, either at EMAI or by others (as published and described previously). Although virus isolation is often considered a ‘gold standard’ for virus detection, it is infrequently successful for the detection of the major causes of neonatal diarrhoea. Both corona and rota viruses are extremely difficult to isolate in cell culture systems and require specialised cell lines, pre-treatment of specimens with enzymes and then culture in the presence of various enzymes. Microbial contamination is also an issue. Consequently, indirect detection methods such as electron microscopy (EM), ELISA, latex agglutination and other antigen-antibody based assays are usually used for diagnostic applications. Each of these also has limits in terms of sensitivity and specificity, as has been shown in the previous studies where the BCoV and rotavirus qRT-PCR assays were evaluated (Izzo et al. 2012). In contrast, the pestiviruses are amenable to comparative studies involving both virus isolation and ELISA methods because

these viruses can be found in many specimen types other than faeces. However, it is well recognised that qRT-PCR still has higher analytical sensitivity. In general, there are differences of at least 10-100 fold in favour of qRT-PCR for the detection of RNA in viral infections because of the capacity of qRT-PCR to detect residual RNA as well as RNA from both infectious and non-infectious virus.

The analytical sensitivity for the 3 main viral assays (BVDV, BCoV and rotavirus A) were assessed using serial dilutions of plasmid DNA as described previously. As these viral assays were often used in singleplex format for diagnostic or research purposes, the greater interest was to investigate the impact on analytical and diagnostic sensitivity when these assays were run in various multiplex combinations.

3.2 Multiplex qPCR

Multiplexing involves running two or more assays in the same reaction to support concurrent detection of multiple pathogens. Although the running of multiple reactions in parallel can place demands on the mastermix and associated chemistry, the main limitation is the fluorescent detection system of the thermocycler and its capacity to reliably detect and separate signals of different wavelengths together with accurate quantification. Consequently, assays are at present limited to the detection of 4 pathogens though duplex and triplex assays are more common. When there is consideration of the inclusion of an exogenous internal control (XIPC – see section 3.1.6 above) the number of available channels for pathogen detection is further compromised. After development of efficient singleplex assays, the development and evaluation of a multiplex assay involves a series of systematic studies to establish that:

- the primers and probe for one pathogen do not interfere with the function of reagents for another pathogen;
- varying concentrations of one pathogen do not affect the sensitivity of assays for other pathogens;
- the fluorescent signal for each assay is accurately detected without any “cross talk” to other channels.

Recognising the limitations of the extent to which assays could be multiplexed, it was decided that, in the first instance separate multiplex assays would be investigated for the detection of bacteria (*E. coli* K99 and *Salmonella*) and protozoa (*Cryptosporidia* and *Giardia*) and for the viruses (BCoV, pestiviruses and *Rotavirus A*). For each of these multiplex investigations, one assay was selected as the primary target and primers and probes for the other assays were sequentially added to the reaction (initially as various duplex combinations then later additional triplex combinations were assessed). In these studies, a moderate to low concentration of the nucleic acid of the primary target was used. Later, as usable combinations of reagents were established, a series of log10 dilutions of nucleic acid were used. These dilutions ranged from the highest potential concentration of nucleic acid likely to be found in a diagnostic sample to beyond the limit of detection for that assay. These titrations were then tested in various combinations – with parallel testing of high concentrations of each agent through to low concentrations of each and also the reverse – whereby high to declining concentrations of one pathogen were tested in the presence of low to increasing concentrations of the other. In the event that assay performance is suboptimal

compared to the ‘parent’ singleplex assay, alternative concentrations of primers and probes were evaluated.

Although specialised “high performance” mastermixes are available commercially, throughout these studies the AgPath-ID™ One-Step RT-PCR kit was used extensively. In other studies, this mastermix has been shown to support multiplex assays within the limits of the thermocycler, i.e. up to fourplex. In some instances a ‘high performance specialised multiplex mastermix was included in assay evaluations.

3.3 Loop-mediated Isothermal Amplification (LAMP)

Loop-mediated Isothermal Amplification (LAMP) is an alternate DNA amplification protocol to PCR and is commonly used in molecular diagnostic assays for the detection of DNA or RNA from infectious organisms/ pathogens. The technique uses a strand displacement strategy for DNA amplification from the genome of the organism to be detected (or reverse transcribed DNA copy of RNA genomes from viruses) which results in efficient production of amplified DNA from a target template. The turbidity is generated by precipitated magnesium pyrophosphate due to the hydrolysis of the triphosphate nucleotides liberated during DNA synthesis which may be utilised as a means of detection of amplification.

Basic Principle:

At 60-65°C DNA is in a dynamic state of denaturation which allows the primers to anneal to target sequences and initiate amplification by Bst (*Bacillus stearothermophilus*) DNA polymerase which has strand displacement activity and therefore has no requirement for heat to denature DNA as is required for conventional PCR. Similarly, the Bst DNA polymerase functions efficiently at this same temperature range thus enabling the isothermal amplification.

A combination of the target binding sites identified through primer design, as well as the primers themselves result, through the first stages of amplification, in the formation of looped DNA amplicons rather than linear amplicons synthesised in PCR. The looped amplicons allow, through subsequent amplification, the formation of linked multimers of the original target region of DNA which significantly increases the amount of template DNA in early stages of amplification and thus the significant final yield of amplified DNA. LAMP amplification can result in 10^{9}-10^{10} copies of DNA as opposed to 10^{8}-10^{9} copies in PCR.

The specificity of amplification comes from the 6-8 target regions that primers bind to as opposed to the 2-3 sites for primer and probe annealing during qPCR or conventional PCR.

There are a number of key differences to PCR which include:

- Reactions occur at a single temperature rather than cycling temperatures which arguably can facilitate utilization of less sophisticated equipment with potentially lower establishment costs.
- Speed of reactions is typically quicker in general terms than PCR (15-60 minutes) compared to several hours for PCR.
- There may be opportunities for application in the field where there may be limited resources, not yet fully realized.

- There are 6-8 primers used in a reaction as opposed to 2 primers utilised in PCR.
- While some multiplexing protocols have been proposed typically LAMP can facilitate only singleplex assays.
- There are a number of different detection/ observation methodologies from visual observation of precipitation, colour change, through to gel electrophoresis, fluorescent detection and real-time detection techniques using a turbidimeter for LAMP.
- The technique is reportedly less impacted by PCR inhibitors such as those observed in faeces and so may conceivably have an advantage over PCR for detection of pathogens in calf scours.

Some of the identified challenges of the technique include that the amount of DNA synthesized is significantly greater (10 fold +) than PCR which may increase risk of contamination impacting analysis, the complexity of the reactions in comparison to PCR can make assay optimization difficult, primer design software is less sophisticated than that for PCR leading to greater challenges in assay design and development and the infrastructure and technical support offered by commercial providers is limited.

3.3.1 LAMP assay design:

Assays were developed for the detection of each of the following pathogens: *Salmonella*, *E. coli* K99, *Giardia*, *Cryptosporidium* (LAMP), *Rotavirus A*, BCoV & BVDV (RT-LAMP).

3 or 4 sets of primers were designed for each pathogen to account for the imprecise nature of primer design. Primer sets were designed for the target sequences (Appendix 3) using Primer Explorer V4 (http://primerexplorer.jp/e/; Eiken Chemical Company Ltd, Japan) (set 1) or LAMP Designer (Premier Biosoft) (set 2 and 3).

3.3.2 Lamp assay development:

LAMP reactions (*Salmonella*, *E. coli* K99, *Giardia, Cryptosporidium*):

The LAMP reactions were performed within PCR tubes (Scientific Specialties Inc., CA) in a Loopamp real-time turbidimeter (model LA-320C, Eiken Chemical Co, Ltd, Japan) in 24µl of a mixture composed of 0.8 M betaine; 40 pmol FIP/BIP, 5 pmol F3/B3 and 20 pmol LF/LB primers per reaction; 1x detergent free PCR master mix (6mM MgSO4; 1.4 mM of each dNTPs; 8U Bst polymerase; DF Thermopol, New England BioLabs, Arundel, Queensland, Australia), 0.25 µL 10% Tween 20; BSA 0.25ul (2.5mg/ml) 8 µl DNA quality water; and 1 µl DNA or RNA template in elution buffer or water. Water (1 µl) was used instead of DNA template as a no template control in each reaction.

Genomic DNA isolated from control cultures and plasmids as outlined above for PCR reactions, at a concentration dependant on the initial culture concentration, was initially used for the evaluation of primer sets.

The reaction was initially carried out at 64 °C (or other temperatures assessed) for 60 min followed by 2 min at 80 °C to terminate the reaction. The time and temperature were optimized subsequently as part of the primer set selection for each assay. A sample was

considered positive when the characteristic amplification curves were observed on real time monitoring not just detected turbidity.

$MgCl_2$ and primer concentrations in reactions were also titrated at various times when assay variability was observed but ultimately not altered from standard conditions.

RT-LAMP Reactions (Rotavirus, BCoV, BVDV)

The assays that require reverse transcription of the viral RNA genome to facilitate DNA amplification are similar to those outlined above for standard LAMP reactions but also include the addition of AMV (Avian Myeloblastosis Virus) reverse transcriptase (10u/µl) at 0.1µl/ reaction or at 1 unit/ reaction (optimized from an initial concentration of 0.5units/ reaction).

MMLV (Moloney Murine Leukemia Virus) reverse transcriptase was also assessed for utility in these assays but was not functional in this context.

3.3.3 LAMP optimization protocols

Each primer set was assessed with standard reagent conditions outlined but assessed for performance at 64°C, 63°C, 62°C and 61°C using extracted DNA control samples – the primer set chosen based on level of turbidity, amplification curve and minimal background turbidity (as defined by minimal turbidity in the water blank or No Template Control).

At the optimal temperature determined for the chosen primer sets, an 8 fold dilution series was conducted in triplicate on the same instrument run to determine assay sensitivity with respect to DNA concentration. Plasmids used in PCR analysis and described elsewhere in these assessments were used to accurately estimate the DNA concentration in reactions. The sensitivity analysis was not assessed with respect to organism counts, CFU or PFU as methodologies for these techniques were not sufficiently advanced at this stage of assay development.

Specificity analysis was also conducted on extracted DNA for each of the selected primer sets from a variety of organisms including:

For bacterial LAMP primer sets: *Enterococcus faecalis, Salmonella dublin, E. coli (non-pathogenic), Yersinia enterocolitica, Erysipeles rhusiopathia, Listeria monocytogenes A. E. coli K99, Fusobacterium necrophorum, Bacteroides fragilis, Trueperella pyogenes, Proteus mirabilis, Clostridium perfringens,* and *Klebsiella pneumonia.*

For Viral LAMP primer sets: *Rotavirus A,* BVDV, *Salmonella* spp, *Cryptosporidium, E. coli K99, Enterococcus faecalis, Mycobacterium paratuberculosis, Giardia*, and BCoV.

For protozoan LAMP primer sets: BCoV, BVDV*, E. coli K99*, *Salmonella*, *Rotavirus A*, *Enterococcus faecalis, Giardia*, *Cryptosporidium, Fusobacterium necrophorum, Bacteroides fragilis, Trueperella pyogenes, Proteus mirabilis, Clostridium perfringens,* and *Klebsiella pneumonia.*

As there were a number of *Salmonella* species isolates available the specificity of this assay between species was also investigated. The amplification of *S.* Newport, *S.* Typhimurium, *S.* Mbandaka, *S.* Amsterdam, *S.* Orion & *S.* Infantis was investigated.

A measure of repeatability for each assay was also assessed by using a set concentration of extracted DNA or RNA from control cultures and was repeated 8 times within each block over 3 blocks of the turbidimeter to give a total of 24 samples. A coefficient of variation of the final turbidity was calculated based on the final turbidity measured at the conclusion of the run (endpoint).

Electrophoresis of products and sequencing was attempted from each assay including a product from amplification from a control culture sample as well as from a known clinically positive sample. The products were purified using the Qiagen Qiaquick PCR purification kit as per manufacturer's instructions. LF primer was used as the sequencing primer using 20pmol primer, 1µl of 5M Betaine, 8µl of water and 50ng of LAMP product.

3.4 Reference and clinical samples

3.4.1 Reference materials

A collection of reference materials was assembled for use as controls in the various qRT-PCR and LAMP assays or for use in preliminary evaluation of assays. Whenever possible, cultured pathogens were used to ensure the long-term availability of material. Otherwise, clinical samples containing high levels of an agent were retained so that the material could be diluted to meet ongoing needs. In some instances, samples that had been archived from an earlier research project were also used as reference material (Izzo et al. 2011). The specific materials used were as follows:

3.4.1.1 Bacteria

Standard reference cultures of *E. coli* (K99) (EQC0004), *S.* Dublin (EQC0018) and *S.* Typhimurium (EQC0008) from the diagnostic bacteriology laboratory at EMAI were used as needed.

3.4.1.2 Protozoa

Purified positive control DNA extracts were obtained from Murdoch University and live oocysts (*C. parvum*) of a known concentration from Waterborne Inc, and these were used as standards and controls for assay development and copy number quantification. Additionally, plasmid constructs, based on both the qPCR amplicon and also the whole COWP gene, were developed to use as quantifiable positive control material. *Giardia duodenalis* control DNA was obtained from Dr. Damian Stark at St Vincent's Hospital, Sydney and Dr. Andrew Thompson, Murdoch University WA.

3.4.1.3 Viruses

The Virology Laboratory at EMAI holds a cell culture propagated Australian BCoV isolate which was used for reference purposes. Similarly, a large collection of cell culture isolates of BVDV were available. Unfortunately, no cultured strains of bovine rotavirus were available. Consequently, an archival collection of field samples known to contain varying levels of *Rotavirus A* and BCoV were also used during the evaluation of qRT-PCR assays. Additionally, bacterial plasmids were prepared for target sequences of viral assays and used for experiments to determine the analytical sensitivity of some assays.

3.4.2 Specimen collection and storage methods

During testing for viral infections, swabs have commonly been used to collect samples from various mucosal surfaces. The cotton or polyester swabs are usually placed into a liquid transport medium to minimise deterioration of any viruses that may have been collected. For virus testing, phosphate buffered gelatin saline is used. This solution maintains a stable pH that can affect infectivity and also antibiotics to prevent growth of bacteria and fungi. Swabs have also been used extensively for avian influenza surveillance where "environmental" samples are collected. Effectively, these are swabs collected from faecal samples on the ground. Swabs have many advantages in that sample collection is very easy, samples are small (facilitating both transport and storage) and minimal processing of the sample is required (see Section 3.1.5 above). Consequently, a study was initiated to compare the collection of rectal swabs from scouring calves with the testing of faecal samples.

For collection of swabs for detection of bacteria, it was recognised that the standard PBGS solution would potentially have limitations (e.g. contained antibiotics which would affect culture) but as this transport medium was readily available, it was used in the first instance.

To validate the use of cotton-tipped swabs stored in PBGS as a sampling method simultaneous collections were made of faeces and a rectal swab from calves with diarrhoea during the longitudinal studies on farms A and B documented below. Rectal swabs were collected by placing a cotton-tipped swab in the rectum and rotating it gently against the rectal mucosa at about 5cm depth. All swabs were immediately placed into 3ml of PBGS. Faeces were collected per rectum by rectal stimulation. The samples were evaluated for all pathogens using the methods described elsewhere, then the results for both sampling method were compared.

An important consideration for the detection of micro-organisms and viruses is the effect of temperature on the stability of the target organism during both transport and storage. Whilst this has been established for rotavirus and cryptosporidium there is less information on the stability of the other pathogens. When using cultural methods maintaining the correct temperature is extremely important because warm temperatures can encourage replication of non-target bacteria and fungi (possibly reducing the chance of detecting a pathogen), or inactivate viruses. During storage in the laboratory, ultralow temperatures (below -70°C) provide maximum stability for viruses and bacteria but may not be optimal for viability and detection of protozoa depending on diagnostic method (Fayer & Nerad 1996; Kuhnert-Paul et al. 2012). However, the effects of lower temperatures are generally considered to be less important when detecting pathogens by PCR. Further, low temperature freezer capacity is usually a premium, is limited and is not practical under field conditions or during transport. As holding samples at approximately 4°C in transport media such as PBGS for short to medium periods (days to weeks) has also been shown to provide good results with viruses (P.D. Kirkland and R.J. Davis, unpublished data), investigations were undertaken to establish whether samples could be transported and held in the laboratory in a refrigerator or cold room at approximately 4°C.

In a pilot experiment, the stability of BVDV was examined by obtaining a large volume of faeces from several cattle that were persistently infected with BVDV. This material was divided into many small subsamples that were held at 4°C, 24°C and 37°C for up to 7 days to reflect the possible time from sample collection to receipt at a laboratory. Each day, a

subsample was removed from the respective holding temperatures and placed at -80°C, to allow all samples to be tested concurrently at the end of the trial.

3.4.3 Longitudinal animal studies

The very high analytical sensitivity of real-time PCR and its capacity to detect nucleic acid regardless of whether the pathogen is still infectious are both potential advantages and limitations with this technology. There is a need to establish a relationship between nucleic acid levels detected by qPCR and clinical signs. Originally, an approach considered for at least some of the pathogens was to undertake experimental infections of colostrum deprived calves. However, maintaining a disease-free status of colostrum deprived calves during a period of acclimatisation to housing presented unacceptable health concerns and this approach was abandoned on welfare grounds. Consequently, an alternative approach was adopted by intensive monitoring of two groups of calves under field conditions which allowed the shedding patterns of pathogens in a natural outbreak to be determined. Both faecal swabs and faeces were collected from calves at regular intervals for 3 to four weeks after birth.

Farm A

This was a prospective study of all heifer calves that were born over a 4 week period. The facility had previously had scours at that time of year.

Calves

Fifty-four Holstein female dairy calves (bodyweight 22-59kg) were monitored daily for the first month of life. Calves were fed 3 litres of colostrum by teat or oesophageal feeder within the first 2 hours of life and another 2 litres 8-12 hours later. They continued to be fed lower quality colostrum for the next three to seven days. After this, they were fed whole milk plus commercial calf pellets, and had free access to water.

Housing

Calving cows were housed in a clean maternity pen bedded with straw for a few days prior to calving. Calves were transferred into a group pen of 4-5 calves in the calf shed within 2-6 hours of birth. They remained there for three to seven days, before moving outside to individual calf pens where they remained in the same pen for approximately 8 weeks. The individual calf pens were in 2 opposing rows of approximately 50 pens constructed on a concrete pad. The pens had a wooden frame, corrugated iron exterior and sawdust bedding that was retained behind a concrete lip. At the front of the pen was a small yard where feed and water was provided, this area was separated from the laneway and adjacent pens by iron bars. The design allowed calves to touch the neighbouring calf and also allowed runoff between the pens. Dirty sawdust was removed 3 times a week and then topped up. At that time the outside yards were also hosed out. All pens were completely cleaned of sawdust and hosed down with a pressure washer as soon as calves were removed, sprayed with Virkon S®(Dupont, Lexington KY USA) and left empty for a minimum of 3 days (generally > 7) before another calf was housed in that pen. New sawdust was put in pens approximately 24 hours before calves were introduced.

Clinical observations

Faecal consistency, appetite, demeanour and degree of dehydration were evaluated daily and given a numerical score from 0 (normal) to 3 or 4 (markedly abnormal) using scores

adapted from Walker (Walker et al. 1998) as shown in Table 1. The duration of diarrhoea was considered to be the duration of a calf having a faecal score ≥2. Where the faecal score reduced to one for a period of no more than 3 days and then returned to 2 or greater this was considered the same episode of diarrhoea. Calves that were observed to have a faecal score >1 were given electrolytes by the dairy staff. Severely affected calves were treated with Scourban® (Bayer Anima Health, Leverkusen, Germany) as directed by the farm veterinarian.

Table 1: Clinical score definition

	0	1	2	3	4
Appetite Score	Normal	Slightly decreased (<50%)	Decreased (>50%)	Anorexic	
Faecal Score	Normal	Soft/Runny	Blood or Casts/watery with solid parts	Diarrhoea (Watery/increased frequency)	
Attitude Scores	standing to be fed	stands w/stimulus	stands w/assistance	unable to stand/ sternal recumbency	unable to stand/ lateral recumbency
Hydration Score	normal hydration Skin tent< 2	Moderate dehydration, eyeball slightly sunken, and skin tent >2 s but <4 s	Obvious dehydration, eyes sunken, dry nose, skin tent >5 s	Severe dehydration, eyes very sunken with an easily perceptible distance between the eyeball and socket	

Calves were also given a total clinical score which was the sum of all the observed scores

Sample collection

Rectal swabs were collected 3 times a week commencing from 24 hours of age and placed into 3ml of PBGS. Faeces were also collected where available.

All samples were collected by researchers wearing protective clothing that was disinfected between calves. When a calf had active diarrhoea and for 3 days after recovery, disposable shoes and overalls were discarded after that calf to minimise the risk of researchers transferring pathogens between calves.

Sample storage

Prior to freezing 150µL of faeces was suspended in 850µL of PBS in a sterile 1 ml microcentrifuge tube and homogenised by mixing on a vortex mixer for 30 seconds. Additional faeces was put in a 5ml screw topped tube. Samples containing faecal swabs briefly shaken using a vortex mixer, then 850 µL of PBGS was removed and placed in a 1mL screw top vial. All vials containing environmental and faecal samples were stored at -80°C until processing for qPCR assays.

Sample processing

Samples were processed and analysed for rotavirus, BCoV, *Salmonella*, *Cryptosporidium* and *Giardia* using individual qPCR/qRT-PCR assays as described in Section 3.1. Samples

were not tested for BVD as serum samples had shown that there was no PI in the study calves. They were not tested for K99 *E. coli* as the dams were vaccinated against this and calves were receiving protective immunity in colostrum and milk.

Rectal swabs and faeces were tested for all calves for the first collection date after diarrhoea was observed and 1 week later. These samples were tested for all the pathogens listed above. After these results were analysed a subset of 19 calves that had a range of clinical presentations were selected for additional sample analysis. All samples in the first 3 weeks, plus the final sample, were processed for rotavirus and *Cryptosporidium*, and weekly samples were processed for all pathogens described above to determine the variation of pathogen shedding in the first 4 weeks of life.

<u>Farm B</u>

Farm B was a year round calving dairy farm that presented with a calf scour problem. The diagnostic tools developed by this project were applied to investigate the problem and evaluate the practical application of the tests. The ongoing problem resulted in morbidity in 80% of the calves and a mortality rate of approximately 5%. Recovered calves had reduced growth rates and high labour inputs were required to manage sick calves.

Calf Rearing Facility

Immediately prior to calving cows were placed in a clean maternity pen bedded with straw. Following birth calves were removed from the maternity pen, tube fed 4 litres of colostrum using an oesophageal feeder and placed in a clean calf collection pen. Twelve hours following birth calves were administered a further 2 litres of colostrum and each morning the calves were picked up from the collection pen and transferred to the calf shed using a trailer. Each pen in the calf shed was bedded with straw and used to house 25 calves. Between each batch of calves, all bedding was removed and the concrete floor and walls steam cleaned and disinfected with Virkon S® (Dupont, Lexington KY USA), before being bedded with fresh straw. Calves were fed *ad libitum* whole milk using a robotic calf feeder. Calves could consume up to 2.5 litres of pasteurised milk every 2 hours and also had calf meal and water available *ad libitum*.

Disease history

Farm B had installed a new calf rearing facility with robotic calf feeders 12 months previously. For the first 3 months of use calf health was very good with minimal disease and excellent calf growth. Calf scours had emerged as a continuing problem with high morbidity (80%) and low mortality (5%) over the subsequent 6 months. The emergence of diarrhoea in calves was not associated with any changes in the health or production of mature cows. A diagnostic investigation conducted prior to the application of qPCR had identified shedding of *Cryptosporidium*, rotavirus, and *Salmonella* by sick calves. Management interventions implemented to prevent disease included the reinforcement of strict hygiene practices and work flow routines as well as vaccination of pregnant cows with Rotavec Corona (Vaccine to prevent rotavirus, BCoV and enterotoxigenic *E. coli*) and Bovilis S (*Salmonella* Dublin and *Salmonella* Typhimurium bacterin). At 48 hours of age, all calves were also started on a 7 day course of halofuginone as a prophylaxis for *Cryptosporidium*. Effectiveness of passive transfer was monitored using serum proteins and milk quality monitored with quantitative bacterial cultures. Despite what appeared to be excellent hygiene, robust vaccination, and

sound work flow practices (working from youngest to oldest), scours continued to be a problem with calves getting sick between 8 – 10 days of age. Treatment of affected calves included administration of oral electrolytes and in the case of more severely affected calves systemic antibiotics and anti-inflammatories.

Diagnostic Investigation

The primary diagnostic question coming from farm management and calf rearing staff was the source of the infection. Potential sources included cows, other calves, facilities, equipment and staff. The onset of disease at 8 – 10 days of age suggested the possibility for cross contamination within the calf-raising facility. To investigate this question a longitudinal study of calves was initiated. The sampling protocol called for 10 calves to be sampled 3 times per week from birth through to 25 days of age.

Calf attitude was monitored by visual observation. Calves had *ad lib* access to milk delivered via a robotic feeding system that recorded milk volume consumed per day and drinking speed. Calves failing to drink 2.5 litres of milk by 7 am were presented to the robotic feeder. Calves failing to drink and calves with scours and dehydration were administered 2 litres of electrolytes 3 times per day. If dehydration was accompanied by fever (rectal temperature > 39.5^{o}C) calves were also administered systemic antibiotics.

Sample collection

Rectal swabs were collected every 3 days from 12-24 hours of age and placed into 3ml of PBGS. Faeces, when available, were also collected per rectum using a new disposable glove. The calves were housed in a single pen so the staff did not change clothes between calves.

Sample storage

Swabs and faeces were placed in the refrigerator following collection and picked up weekly for transport to the NSW State Diagnostic Laboratory at EMAI. Prior to freezing 150µL of faeces was suspended in 850µL of PBS in a sterile 1 ml microcentrifuge tube and homogenised by mixing on a vortex mixer for 30 seconds. Additional faeces was put in a 5ml screw topped tube. Samples containing faecal swabs were briefly shaken using a vortex mixer, then 850 µL of PBGS was removed and placed in a 1mL screw top vial. All vials containing environmental and faecal samples were stored at -80°C until processing for qPCR assays.

Sample processing

All samples were processed and analysed for rotavirus, BCoV, *Salmonella* and *Cryptosporidium*. A multiplex qPCR/qRT-PCR rotavirus, BCoV and cryptosporidia assay was initially used to screen samples. The extracted samples were also analysed using singleplex cryptosporidium and *Salmonella* qPCR assays. Faecal samples were also cultured for *Salmonella spp*. Samples were not tested for BVD or *Giardia* as prior samples collected from diseased calves and post-mortems had failed to detect these pathogens. They were not tested for K99 *E. coli* as the age of affected calves was inconsistent with this pathogen and the herd vaccinated against this disease.

3.5 Environmental monitoring

The capacity of qPCR to detect and quantify pathogen nucleic acid levels without any need for culture, when combined with the use of swabs, provides a unique tool for the

investigation of outbreaks of diarrhoea in calves. There is potential to test a wide range of materials and components of the environment to identify the presence of a pathogen and levels of contamination that could be present. Similarly, these tools could be employed to monitor the efficacy of any decontamination or other hygiene measures that might be undertaken following a disease outbreak.

Initially, a brief assessment of published literature was carried out to determine the range of calf scour pathogens that could already be detected from environmental samples, the sample types that had been proven to be successful, the methods of detection and any known limitations.

3.5.1 Laboratory testing of spiked samples

A number of different environmental sample types and sampling strategies were investigated for this part of the project. Consideration was given to those samples that would be most useful for investigative purposes and sampling strategies were chosen for practicality. Various sample types were spiked with faecal material containing each of the pathogen types, allowing an evaluation of sampling, processing and subsequent testing for bacteria, viral and protozoal pathogens.

Bedding material – Pine wood shavings representative of calf bedding, were collected and spiked with a small volume of faecal material containing one of each class of pathogen of interest, and dried in an incubator, to mimic warm environmental conditions that could be encountered in the field. Samples were washed with a solution of PBS/ 0.2% Tween (or PBS alone) by adding 1 gram of shavings to 30mL of wash solution in a 50 ml centrifuge tube which was placed on a rotary mixer (Ratek RSM7, Ratek Instruments Pty Ltd, Boronia, Victoria, Australia) for 10 minutes on medium speed. This was compared with sonication of the sample suspended in PBS/Tween for 6 minutes, setting 5 using a laboratory sonicator or by immersion of the tube in a sonicating water bath.

Milk – Faecal samples containing each pathogen class were spiked into whole unpasteurized cow's milk or milk replacer. The samples were then processed and tested using the same methods as used for faecal suspensions and the results compared with the faecal sample without the addition of milk.

Pasture and Feed – To assess the capacity to detect pathogens on the surface of pasture or in feed samples (chaff, pellets) samples were spiked in the same manner as used for bedding. Samples were mixed with faecal material containing a target pathogen and stored for a period of time in the laboratory to mimic field conditions. The subsequent sample processing methods were the same as used for the testing of bedding except for the quantity of sample that was washed. These consisted of 1.5gm of pasture, 2gm of chaff or 10gm of pellets.

Soil – Soil is well recognised as a difficult sample from which to extract and amplify nucleic acid. The aim was to develop a method for soil sample processing which makes automated nucleic acid extraction possible and is similar or, preferably, the same as the methods which had already been evaluated for processing of faecal samples or other environmental samples, based on ease of use and application of high throughput methods. Several different types of collection and storage buffers, methods of processing and nucleic acid extraction techniques were investigated. Methods tested included: the use of chloroform

isoamyl alcohol for separation and manual extraction; chemical flocculation using aluminium potassium sulphate (Dong, Yan et al. 2006); skim milk (Takada-Hoshino and Matsumoto 2004); PVPP (polyvinylpolypyrrolidone) (Lakay et al. 2007; Picard et al. 1992; Sutlovic et al. 2007) and the addition of a proprietary commercial preparation (InhibitEx, Qiagen, Hilden, Germany) to the soil sample to attempt to remove inhibitors, such as humic acid, prior to automated nucleic acid extraction. All nucleic acid was extracted using the Kingfisher magnetic particle handling system. During the course of these experiments a newly developed commercial automated nucleic acid extraction kit (PowerMag TM DNA Isolation Kit, MO BIO Laboratories Inc, Carlsbad, CA) was also tested and several Kingfisher extraction protocols were compared.

Surface swabs – Studies were undertaken to investigate methods to detect pathogens on the types of surfaces that calves contact, and could harbour faecal contamination and infectious pathogens. The surfaces assessed included concrete, steel mesh and stainless steel. Several different types of swabs were also evaluated including standard sterile cotton-tipped swabs and commercially available swabs for environmental sampling (Enviroswab, 3MTM and Quick swab 3MTM). All swabs were placed in either PBS or PBGS for comparison. The preferred method was then used to sample calf pens that had housed calves known to be shedding *Salmonella sp.* , or that were contaminated with faecal material containing high levels of rotavirus. Samples were collected from calf pens pre- and post-cleaning and disinfection allowing for an assessment of both detection of pathogen nucleic acid and also the effectiveness of cleaning procedures.

Water – As there is already a large body of work describing detailed and specific preparation, processing and NA extraction from water samples for specific pathogens, a simplified approach was utilised to determine whether a universal method could be used to detect the pathogens of interest at clinically significant levels. Spiking water samples with faeces containing these pathogens (to mimic faecal contamination of calf water supply) was monitored over time. Nucleic acid was extracted in the same way as used for faecal samples. However, no sample concentration step was used to compensate for the dilution of pathogens in large volumes of water.

While there are published methods available for handling large volumes of water, these do not concentrate both DNA and RNA in one step, or are time-consuming, very costly or require expensive equipment which is not routinely available in diagnostic veterinary laboratories (e.g. ultracentrifugation or ultrafiltration equipment to concentrate viral nucleic acid).The published methods mostly target a single agent and are usually specific for that agent or group of agents and hence of limited use for this study. For example, filtration methods to capture *Cryptosporidium* oocysts would likely not be effective for collecting viral RNA or DNA, but may be applicable to *Giardia*. A significant amount of further experimental work would be required in an attempt to identify a single practical protocol for concentrating DNA and RNA from all bacterial, viral and protozoal organisms of interest from a large volume of water and was considered beyond the scope of this project.

Nucleic acid extraction and qPCR

Wherever possible, standard total nucleic acid extraction methods were used (see Section 3.1.7). However, during these environmental studies, two different magnetic bead kits were evaluated (AM1836 and AM1840), to ensure that the extracted DNA and RNA was of the

highest purity possible, to maximise the chance of detection by successfully amplifying the pathogen NA by RT-qPCR. Some environmental samples also required processing steps that deviated somewhat from the original processing protocol for faeces. The qPCR/RT-qPCR assays were run in the standard assay format, usually in singleplex rather than multiplex format, as individual agents were being investigated and maximal sensitivity was desirable during the evaluations of these sampling procedures.

3.5.2 Environmental samples from on farm longitudinal calf studies

The studies to evaluate shedding of calf scour pathogens in the field were utilised as an ideal opportunity for also collecting environmental samples and identify sources of infection as well as evaluate the efficiency of cleaning procedures for facilities and equipment..

The source of infection was a question raised by the management of farm B prior to the investigation. Possible sources included the calving area, calf pen, equipment and people. During the period in which calves were being sampled, swabs were collected from staff boots, colostrum harvesting equipment, colostrum feeding equipment and a trailer used to transport calves to the calf pens. Bedding samples (sawdust or straw) were also collected.

Sampling strategy

Calves were housed as described in Section 3.4.3 above. Environmental samples were collected prior to the calf entering each pen and then weekly for the duration of the studies. The areas sampled were the walls of the pens, the feeding equipment, water troughs and the bedding. Samples were also collected from the calving area on Farm B and from colostrum harvest and administration equipment and farm staff's protective clothing and boots.

Sample collection

Dry cotton-tipped swabs were used to wipe the walls in 4 to 8 different areas on each wall, including under bars and in corners. The feeding equipment was sampled by wiping the swab around the inside rim. Swabs taken of the same sample – i.e. from different walls of a pen, were all placed into a single vial containing 3ml PBGS. Bedding material was collected in a grid pattern from 9 sites in each pen and placed in a zip-lock bag. For pens with sawdust bedding a heaped teaspoon was collected at each site and for straw bedding a few pieces of straw were collected.

All samples were chilled at the collection point and transferred to the laboratory for freezing on the same day for farm A and weekly for farm B.

Sample storage

All vials containing environmental and faecal samples were frozen at -80°C with no additional processing prior to qPCR extraction. Bedding samples were processed as described below and also stored at -80°C prior to qPCR extraction.

Sample processing

The bedding samples from Farm A were mixed well on farm and 4g removed and placed in a 50ml conical centrifuge tube. The tube was transferred to the laboratory on ice and 25mls of PBS containing 0.2% Tween added to each vial which was then shaken using an orbital

shaker at 200rpm for 5 minutes at 23°C. The sample rested for 10 minutes to allow the bedding material to precipitate or float. Approximately 2ml of clear fluid from the middle of the sample was then removed with a transfer pipette, taking care to minimise removal of any solid particles, placed in a 5ml screw top vial and stored at -80°C.

These samples were individually tested for the presence of rotavirus and *Cryptosporidium* nucleic acid as these were the pathogens most frequently detected in calves exhibiting clinical disease. The liquid supernatant retrieved from each sample was extracted and tested in individual qPCR/qRT-PCR assays as described in Section 3.1.

All samples from Farm B were stored at 4°C on farm prior to weekly pick up. Samples were transported to the NSW State Diagnostic Laboratory where they were processed using the method described above. A few pieces of straw from each bedding sample, weighing approximately 4 grams, were placed in the 50ml conical centrifuge tube for processing. Bedding samples were also placed in buffered peptone and cultured for *Salmonella* in addition to performing qPCR for rotavirus, *Cryptosporidium*, and BCoV.

3.6 Statistical analysis

A Paired T-test was used to compare means in the faecal swab vs faeces comparison.

Regression analysis and Chi-squared test was used to explore the relationship between rotavirus levels in faecal samples and clinical presentation.

4 Results

4.1 Real-time PCR assays

4.1.1 Assay design and validation

As a preliminary step in the validation of the individual qPCR assays, the DNA from the PCR was separated using agar gel electrophoresis and the size determined against DNA size standards. All assays generated amplification products that were of the expected size. Nucleic acid sequence analysis was then undertaken to confirm that the PCR products were homologous for each assay. Subsequently, the analytical and diagnostic sensitivity and specificity of the qPCR assays were determined in singleplex format. Multiplex assay evaluations are described separately.

4.1.2 Bacterial pathogens

Evaluation of the qPCRs by testing a dilution series of the plasmid DNA showed that both the *E. coli* (K99) and the *Salmonella* assays had high analytical sensitivity, detecting 1 and approximately 5 copies of the respective DNA targets. Examination of the amplification profiles towards the limit of detection suggested that the *Salmonella* assay has sub-optimal efficiency when detecting low numbers of copies of DNA, with a calculated efficiency of approximately 89%.

The specificity of the qPCR assay was confirmed by testing organisms frequently detected in faecal samples (including BCoV, BVDV, *Cryptosporidium* spp, *Enterococcus faecalis*, S*almonella* spp, *Escherichia coli* K99 (x4 isolates), *Giardia duodenalis, Klebsiella spp., Pseudomonas* spp*, Rotavirus A* and *Yersinia enterocolitica*).

Table 2: Results of qPCR quantitative and enrichment culture results.

Faeces average cfu/gm	qPCR average cfu in extract	Faecal qPCR Quantitative Ct	Faecal qPCR Qualitative	Enrichment Culture	PBS qPCR Quantitative Ct	PBS qPCR Qualitative
20,000,000	37,500.00	18.99	+++	+++	18.18	+++
2,000,000	3,750.00	22.44	+++	+++	22.33	+++
200,000	375.00	26.56	+++	+++	26.41	+++
20,000	37.50	29.71	+++	+++	29.84	+++
2,000	3.75	32.99	+++	+++	32.69	+++
200	0.38	35.66	++-	+++	35.24	+++
20	0.04	36.51	+--	+++	ND	---
2	0.0038	ND	---	+++	ND	---

cfu = colony forming unit, Ct = cycle threshold, ND – not detected, + = weak reaction, ++ = moderate reaction, +++ = strong reaction

The limit of detection for the *Salmonella* spp. qPCR was explored through the evaluation of spiked samples as described in section 3.1.11. The relationship between quantitative PCR and enrichment culture was evaluated for the range of 2 to 2 x 10^7 cfu/gm of faeces. Sample processing for qPCR involved adding 300 µl faeces to 700 µl PBS and subsequent extraction of 50µl of the diluted sample. Five microliters of the NA extract is tested in the assay. With this sample processing 666 cfu/gm of faeces would need to be present for the nucleic acid from one organism to consistently be detected by the PCR reaction to simply account for the dilution factor. The average number of cfu calculated to be in the extract is presented in the second column of Table 2. As expected the average Ct value increased as the number of cfu decreased. Consistent with the dilution factor calculations above, once the number of cfu in the faeces dropped below 2 x 10^3, negative qPCR results were observed with one of three replicates negative at 200 cfu/gm, 2 replicates negative at 20 cfu/gm and all replicates negative at 2 cfu/gm. The qPCR assay performed concurrently on the PBS dilutions containing the same number of cfu as faeces yielded similar results suggesting that faecal inhibitors were not a significant limitation of the assay. All enrichment cultures were positive in the tested range indicating that enrichment culture (1 gm of faeces added to 10 ml of enrichment media) was more sensitive for detecting *Salmonella* than qPCR. The limit of detection for the qPCR assay effectively reflects the dilution factor introduced through sample preparation.

A paucity of samples submitted to the diagnostic laboratory from calf scour outbreaks involving calves less than 3 days of age, resulted in limited samples available for comparative testing of the *E. coli* qPCR. Of 10 samples that were positive using the Bio-X Diagnostics lateral flow assay, 2 were negative by qPCR. There was agreement for 4 samples negative in the lateral flow. In contrast the qPCR results of sixteen frozen samples that had previously tested positive using the Pourquier® ELISA were in complete agreement – with all samples positive in both assays.

A limit of detection experiment was conducted similarly to the *Salmonella* sp. assay. In this experiment, the performance of the qPCR assay was evaluated in spiked faecal samples containing known numbers of cfu derived from serial dilutions of K 99 *E. coli* harvested from broth culture (Table 3). The sensitivity of the K99 *E. coli* qPCR from the perspective of limit of detection was lower than that observed with the *Salmonella* qPCR. This result was in contrast to the apparently better sensitivity of the assay in comparison to the serological tests utilised to evaluate clinical samples submitted to EMAI. Disease associated with enterotoxigenic *E. coli* is associated with the shedding of large numbers of organisms. The improved sensitivity of the assay in this regard, therefore, is likely to reflect the high number of cfu present in the samples submitted. The limit of detection for the qPCR assay was similar the limit of detection reported by the manufacturer of the ELISA assay (10^5 cfu/ml).

The limit of detection for the *E. coli* qPCR assay was significantly lower when bacteria were diluted in PBS as opposed to faeces suggesting that inhibitors present in the faecal material reduced the efficiency of the assay (Table 3). This result contrasted with the *Salmonella* qPCR where the sample processing and extraction process appeared to negate the impact of faecal inhibitors.

Table 3: Quantitative and qualitative assessment of the limit of detection for the *E. coli* q PCR

Faeces average cfu/gm	qPCR average cfu in extract	qPCR Faeces Quantitative	qPCR Qualitative	qPCR PBS Quantitative	qPCR Qualitative PBS
413,333,333	623,333	30.87	+++	18.01	+++
41,333,333	62,333	33.74	+++	21.54	+++
4,133,333	6,233	37.17	+++	25.38	+++
413,333	623	37.17	++-	28.98	+++
41,333	62	ND	- - -	32.41	+++
4,133	6.23	ND	- - -	36.08	+++
413	0.623	ND	- - -	36.21	++-
41	0.0623	ND	- - -	37.98	+ - -

cfu = colony forming unit, Ct = cycle threshold, ND – not detected, + = weak reaction, ++ = moderate reaction, +++ = strong reaction

4.1.3 Protozoa

Cryptosporidium

The *Cryptosporidium* qPCR assay did not appear to be very sensitive in the early stages of the evaluation. However, it was later discovered that an incorrect primer sequence had been published. After running the test with the correct primer sequence, an improvement in analytical sensitivity of 3-4 $\log_{10}$ was confirmed. Nevertheless, when compared with oocyst counts in a small number of diagnostic samples, there were still concerns about the sensitivity of this qPCR. Consequently, an alternative qPCR was investigated (Schroeder et al. 2012), this proved to be markedly more sensitive and was used for the remaining components of this project. This assay was used to test a number of bacterial and protozoal species found in the gastrointestinal tract of cattle, yielding no false positive results.

Giardia

Forty-six faecal samples were tested by qPCR for the presence of *Giardia* DNA and 31 faecal samples submitted from clinical calf diarrhoea cases were found to be positive and 15

were found to be negative. There is currently no alternative *Giardia* test available at EMAI at the time of writing.

4.1.4 Viruses

The singleplex BCoV and rotavirus A assays in use at EMAI have been evaluated in a previous study (Izzo et al. 2012). However, the pan-pestivirus assay that was used for BVDV detection is not used routinely for testing of faecal samples as faeces are not a preferred sample type for BVDV diagnosis. This study is probably the first instance in which a pestivirus assay has been used extensively for testing of faecal samples. It was included in the panel of assays because of the likelihood that some young calves would be either persistently infected (PI) or become infected from a PI animal soon after birth. As BVDV is recognised as an agent that causes both enteric infections and immunosuppression, it is likely that under some circumstances it will contribute to neonatal diarrhoea directly or potentiate the effects of other agents. The analytical sensitivity of the qRT-PCR assays for these viral infections was confirmed by testing of the bacterial plasmids that had been constructed to contain a set of DNA sequences for the target regions of each qPCR/qRT-PCR assay. Both the BVDV and rotavirus assays were shown to have very high sensitivity, being able to detect approximately 1 and 6 copies of cloned DNA respectively. As could be expected, the rotavirus qRT-PCR was shown to have considerably higher diagnostic sensitivity (96%) than the Bio-X calf enteric lateral flow test kit (82%) that is now routinely used for diagnostic investigations.

In contrast, the sensitivity of the BCoV assay was sub-optimal, detecting about 400 copies of the genome. In some instances, the BCoV qRT-PCR also has inferior performance in comparison to ELISA or EM. The lower sensitivity of the BCoV assay has been the subject of ongoing investigation and the EMAI assay was compared with two other published assays (Decaro et al. 2008; Cho et al. 2010). These were shown to have similar sensitivity but the EMAI assay was slightly superior. Examination of faecal samples by EM during both past and very recent diagnostic investigations have confirmed the presence of virus particles consistent with coronavirus and in some instances, in large numbers. Further, a published conventional PCR that is considered to be broadly reactive against North American coronaviruses has also been used with negative results. Collectively, these observations would suggest that there may be genetically variant strains of bovine coronavirus in Australia. In order to establish the RNA sequence of an Australian coronavirus that is not detected by the current assay, virus isolation (which can be very problematic for enteric coronavirus) was attempted on several apparently high titred samples without success. RNA has been extracted from these samples and will be subjected to virus detection attempts using “Next generation” sequencing. Until this issue has been resolved, use of the current EMAI assay will continue as it has significantly improved sensitivity and specificity to other commercially available diagnostic methods of detection (Izzo et al. 2012).

Each of the viral qRT-PCR assays has very high specificity, showing no evidence of cross-reactivity with other viruses. Previous and published investigations have shown that there are usually greater concentrations of RNA in specimens than infectious virus, contributing to the high sensitivity of qRT-PCR when compared to ELISA, EM or virus isolation. This occurs because specimens also contain non-viable and dead viruses that would not be detected by virus isolation, nor appear as an identifiable particle on EM. This discrepancy identifies a need to establish clinically relevant levels of RNA as detected by qRT-PCR.

Samples collected from calf scour outbreaks across Australia were screened for torovirus. Of the 585 samples tested, torovirus was detected in 13 (2.2%). Examination of EM records at EMAI also revealed very few positive samples. Because of this low prevalence, further testing for torovirus was not undertaken.

4.1.5 XIPC

During the latter stages of the project, an exogenous internal control was used successfully in combination with each of the individual assays. It was also possible to include the XIPC in the multiplex assays. Inclusion of the XIPC provides an excellent tool to monitor the quality of the nucleic acid extract and the efficiency of the PCR. However, use of an internal control reduces the number of pathogen targets that can be included in a multiplex assay because it utilises one of the possible 4 fluorophore channels that are available. To reduce this limitation, when samples are being tested for multiple pathogens, it is only essential to include the XIPC when the first batch of assays is run. This provides assurance that the NA extract is suitable for further testing. On the other hand, it could be argued that the ongoing inclusion of the internal control is beneficial for monitoring the performance of the PCR, also providing a check for manipulation errors during setting up of the assay.

4.2 Multiplex PCR

For the development of multiplex assays, the nucleic acid sequences for the primers and probes were the same as those utilised for the singleplex assays. If required, depending on the combinations of pathogens, the only modification was to use a different fluorophore. This was necessary to ensure that there was the greatest possible separation of the wavelengths for the fluorophore on each of the probes. In some instances, the concentrations of primers and probes were modified for optimal performance.

4.2.1 Optimisation of multiplex real-time PCR panel

The qPCR/qRT-PCR singleplex assays were progressively consecutively combined to achieve a triplex combination as follows:

Bacterial and protozoal qPCR: *Salmonella* spp., *E. coli* K99 / F5 and *Cryptosporidium* spp

Viral multiplex qPCR: *Rotavirus A* , BCoV and BVDV.

When these combinations were evaluated with the respective plasmids, the triplex assays performed well, with very little noticeable change in qPCR reaction efficiency, without the use of a specialised multiplex mastermix. The positive controls (nucleic acid from weak and strongly reactive clinical samples) were detected with the expected level of reactivity and there was no evidence of false positive reactivity in the negative control wells. As there was a potential need for quadriplex assays (e.g. the 2 bacterial and 2 protozoal assays together or the 3 viruses combined with the exogenous internal control, or various other combinations of these assays), experiments were undertaken to investigate the impact on assay sensitivity. From these investigations it was shown that there was a loss of analytical sensitivity. For a quadriplex reaction which included *E. coli* K99, *Salmonella*, *Cryptosporidium* and *Giardia* qPCR assays (which precludes the use of an internal control), there was one $\log_{10}$ reduction in sensitivity with the assay detecting to 100 attograms of *E. coli* DNA compared to the limit of detection being as little as 10 attograms of DNA in an *E. coli* singleplex assay. Further, in a quadriplex assay that included *Rotavirus A,* BCoV, BVDV

and the exogenous internal control, there was a 2 $\log_{10}$ loss of sensitivity in the *Rotavirus A* and BCoV assays.

From these investigations, it was evident that there can be a reduction in analytical sensitivity. In limited investigations, it was shown that adjustment of the concentrations of primers and probe for some assays could improve the sensitivity in a triplex/quadriplex assay. Use of a specialised multiplex mastermix did also result in improvement of some assays, though this was not universal. As optimisation of quadriplex assays involves a complex of interactions between many different reactants and the benefits were often marginal, further optimisation was not undertaken. Rather, research was directed at the impact on diagnostic sensitivity, particularly as a reduction in analytical sensitivity was mostly observed when high concentrations of more than 2 pathogens were present.

As there is very high analytical sensitivity achieved with single or duplex qPCR assays, the impact on diagnostic sensitivity of testing sample panels with triplex assays based on standard reagent concentrations and mastermix was investigated. Different combinations of pathogens were included in the multiplex combination depending on the purpose of testing or by taking previous results or history into consideration. A collection of 192 samples that had been characterised in a previous project were tested in two multiplex assay combinations (BVDV, BCoV, Rotavirus and *Cryptosporidium*, *E. coli* and *Giardia*). The original results for the singleplex BCoV and *Rotavirus A* were compared with the results for these same agents in the multiplex assay. There was an acceptable level of agreement between the rotavirus results with 72.9% of samples reacting in the singleplex assay and 62.5% in the multiplex assay. Closer examination of these data suggests that these differences are probably of little diagnostic significance in that the differences are largely limited to samples with low levels of reactivity that would be of doubtful clinical relevance. In order to explore this difference further, as the original tests had been done about 7 years ago and the samples had been frozen and thawed on several occasions, 20 samples with varying reactivity were selected for retesting in the singleplex assay. There was a good level of agreement between the current datasets, with a mean difference of 2.4 Ct units (range 0.8-4.5). This would not result in misclassification of the results. The level of agreement for the BCoV assay was not as good with 29.2% of samples reacting in the singleplex assay when originally tested compared to 14.6% in the multiplex assay. When the Ct values were compared, the differences were much greater (10-12 units), indicating a much poorer efficiency of the BCoV assay in multiplex format. Use of a specialised multiplex mastermix may improve this performance although it is recognised that the BCoV assay *per se* probably needs refinement. The results of the bacterial assays were not compared because a variety of methods had been used originally. An interesting finding was the detection of BVDV in a number of these samples. This sample panel had not previously been tested for BVDV. Weak positive reactivity was detected in 36 (18.7%) samples and there were 2 (1.04%) definite positives. These nucleic acid extracts were retested in the singleplex BVDV assay, with confirmation of the 2 positives at slightly lower Ct values than in the multiplex assay and at levels consistent with persistently infected animals. None of the weak positive reactions were confirmed but the possibility that these represent acutely infected animals must be considered as it is not uncommon for weak positive reactors to give a negative result on retest unless a new RNA extract is prepared. This finding requires further investigation.

An entire collection of samples (373 faecal and swab samples) from Farm B was also tested in a triplex assay combination (rotavirus, BCoV and *Cryptosporidium*). This combination was selected on the basis that all samples had previously been tested in a singleplex *Salmonella* assay and *E. coli* vaccination of cows was practised. Rotavirus was detected at varying reactivity in 77.7% of samples and *Cryptosporidium* was detected in 26.5% of samples. Weak BCoV reactivity was detected in 9 (2.4%) samples. The findings from this analysis were interesting and illustrated the usefulness of the assays for investigating calf scour disease outbreaks.

4.3 Loop-mediated Isothermal Amplification (LAMP)

4.3.1 Development of LAMP primer sets

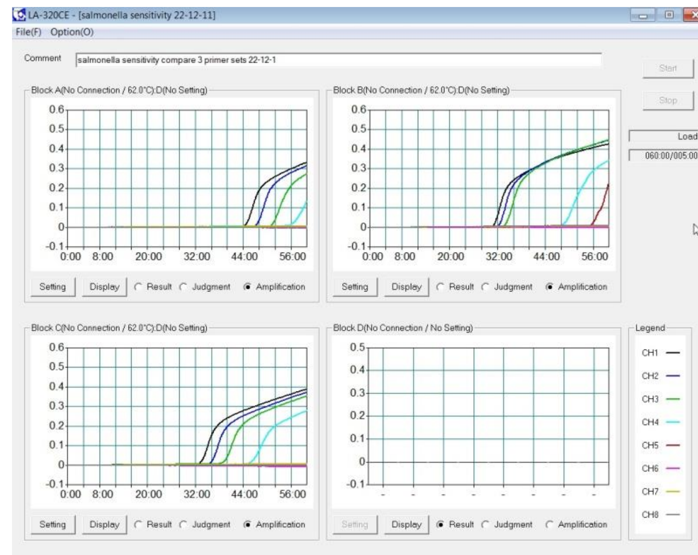
Progress in developing these assays was initially slow until the addition of bovine serum albumin (BSA) to the standard reaction mix. This addition enhanced the amplification in these assays and was included in all subsequent development protocols. An additional challenge was the functional assessment of multiple primer sets for each assay which significantly increases the amount of work required to develop each assay. This is typically required due to the imprecise nature of primer design and the complexity of the design process for the development of 4-6 primers for each assay. This challenge with LAMP primer design is not reported in detail in the literature but acknowledged and observed anecdotally.

Optimal primer sets chosen

Of the 3 primer sets designed for each of the gene targets, a final primer set was chosen based on the characteristics under general conditions outlined in section 3.3. The choice was determined by the shape of the amplification curve, the level of turbidity observed and minimal turbidity in the ‘No Template’ water blank (See Figure 1 below).

Each primer set was assessed under differing amplification temperatures as part of this assessment. The primer sets chosen for assay development are listed in the appendix section 9.3. In general, the primer design software ‘Lamp Designer’ appeared superior to Primer Explorer software with the primers generally being the ones chosen and the design process simpler with fewer steps as the Loop primers can be designed simultaneously where the Primer Explorer process required multiple steps. The Lamp Designer software also provided a visual interface representing the primers designed. LAMP Designer also has an integrated BLAST feature to assess primer specificity which is not available in the other LAMP Primer Design software.

A



B

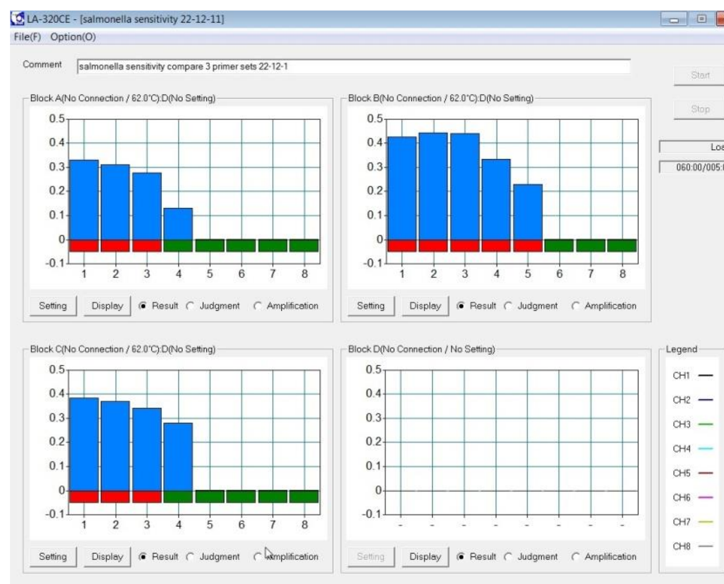


Figure 1: Example of a) real-time amplification curves (measured as turbidity at 650nm) for 3 different primer sets as part of the validation process depicting serial dilutions of control DNA and b) final turbidity (endpoint) at 60 minutes (measured as turbidity at 650nm) view of the same analysis.

4.3.2 Optimal LAMP reaction characteristics and parameters

Despite significant assessment of assay temperature, primer concentrations and $MgCl_2$ concentrations in different assays, ultimately the initial reaction components and concentrations were retained and used in subsequent analysis of assay sensitivity, specificity and repeatability for all assays and detailed below in Table 4. With respect to the RT-LAMP protocols, it was only the AMV reverse transcriptase that worked within these assay systems and was initially included at 0.5U/reaction and subsequently increased to 1 unit/ reaction following optimisation in the viral RT-LAMP assays.

Table 4: Assay characteristics for chosen LAMP primer sets for each target pathogen.

Assay	Primer set	LAMP parameter	Analytical Sensitivity	Coefficient of variation (CV) within block (repeatability)
Salmonella	#2	62 ^{0} C for 1 hour	~1.2 pg	0.5%
E. coli K99	#3	63 ^{0} C for 1 hour	~34 pg	1%
Giardia	#3	61 ^{0} for 1 hour	24.5 - 245 pg	1%
Cryptosporidium	#2	62 ^{0} for 1 hour	>735 pg	4.5%
Rotavirus A	#3	63 ^{0} for 1 hour	~570 pg	0.8%
Coronavirus	#2	61 ^{0} for 1 hour	>490 pg	4%
BVDV	#2	62 ^{0} for 1 hour	~50 pg	2.9%

LAMP products have been stored and the DNA separated using agarose gel electrophoresis. The banding pattern generated from LAMP products can be used as a guide only to assess assay specificity. All results obtained were in agreement with the size of the expected DNA amplification product for the respective assay. Additionally, all LAMP products have been sequenced to check assay specificity. BLAST searches were then conducted on products to ensure homology with target sequences. All sequencing results obtained supported the expected amplicon from the target sequence.

Generally, the assays all demonstrated specificity for the target organism although on occasion there was amplification observed with non-target DNA as described previously. The *Salmonella* assay detected all of the different *Salmonella* species analysed with differing levels of amplification. Importantly, the same *Salmonella* species did not amplify when assessed with the *E. coli* assay.

The one-hour duration of each of the assays was used which is the upper limit of the proposed or published LAMP run times. The sensitivity of the assays varied significantly with the *Salmonella*, *E. coli*, *Giardia* and BVDV assays demonstrating a sensitivity comparable to what would be expected with a well-developed and optimized PCR assay yet sensitivity of *Cryptosporidium*, *Rotavirus A* and BCoV were typically 10-100 fold less sensitive which may impact on their utility for application to clinical sample assessment. This reduced sensitivity may result from additional time required for reverse transcription to occur with the viral assays and also varied with target copy number.

The coefficient of variation for all assays was quite acceptable although only conducted on undiluted control DNA and may vary at lower or higher concentrations of template DNA such as those that might be found in clinical samples.

4.4 Animal specimens

4.4.1 Specimen collection and storage methods

Collection Methods

Results of the faeces vs rectal swab comparison showed a statistical but small decrease in the levels of pathogen detected by swabs. The level of the decrease was similar for both *Cryptosporidium* and rotavirus

Table 5: Results of comparison between rectal swab and faecal samples to evaluate the level of pathogen detected

Pathogen	Number of paired samples	Test used	Mean Difference* in Ct (+/-SD)	P Value
Rotavirus	107	Multiplex PCR	1.32 (+/- 6.29)	<.05
Rotavirus	23	Singleplex PCR	2.81 (+/- 4.08)	<.01
Cryptosporidium	65	Multiplex PCR	1.36 (+/- 5.02)	<.05
Cryptosporidium	30	Singleplex PCR	3.05 (+/- 4.35)	<.01
Giardia	8	Singleplex PCR	2.63 (+/- 2.85)	<.05

*All swabs had higher Ct values than faecal samples

Sample storage evaluation

The trial that was undertaken to investigate the stability of BVDV in faecal samples at different temperatures confirmed that this virus could be held at 4°C for at least 1 week and still be readily detected by qRT-PCR. After one week at 24°C about half of the sample replicates gave negative results. As expected, no virus was detected in samples held at 37°C and there was evidence of fungal growth in the samples. Examination of the long-term stability of rotavirus showed that there was little change in reactivity after 1 month at 4°C. The stability ranges shown by these viruses probably cover what could be expected of other viruses found in faeces as BVDV is a relatively labile enveloped virus and rotavirus is a very stable double-stranded RNA virus.

4.4.2 Longitudinal animal studies

Farm A

Ninety-four percent of calves (n=51) developed diarrhoea, defined as semiliquid or liquid faeces (with or without blood or casts), with the initial observation between 7 and 20 days of age (mean 10.5). The majority of calves started scouring between 8 and 11 days of age and the mean duration of a scouring episode was 4.3 days. Most calves remained bright and were only treated by adding electrolyte solution to the water. Only 4 calves were inappetant during the diarrhoea episode and three had noticeable dehydration. Whilst many calves were a little lethargic, only 11 required encouragement to stand.

Cryptosporidia were detected from every calf in the study, rotavirus was detected from 53/54 calves. Only 3 samples were processed from the calf that did not shed, so it is likely that it also shed rotavirus at some point during the study. *Giardia* was detected in 44/54 calves. Similarly, only one of the calves from which *Giardia* was not detected was included in the longitudinal study and hence, it is likely that many of the others were infected with *Giardia* at a stage later than the 3 samples were taken (mean age 12.5 days). A low level of BCoV was detected from one calf at 23 days of age. *Salmonella* was shed by 9 calves, with the highest proportion identified in the first week. In 7 calves *Salmonella* was only detected at a single time point. It is possible that more calves were shedding *Salmonella* , or that *Salmonella* was shed more frequently by the calves from which *Salmonella* was detected, but the sensitivity of the *Salmonella* PCR using faecal swabs may have resulted in reduced detection. Nevertheless, the clinical presentation of almost all calves was not consistent with *Salmonella* infection, consequently it is not considered that this is a significant limitation of this study. *E. coli* K 99 was not found but this was expected as the dams had been vaccinated. All blood samples from calves were negative for pestivirus and consequently the samples were not tested for bovine viral diarrhoea.

The proportion of calves infected with these pathogens each week is in Table 6.

Table 6: Proportion of calves shedding each pathogen per week

Age in weeks	1	2	3	4	5
Number of calves sampled	20	52	51	28	15¥
Rota +ve % (number)	70% (14)	94% (49)	86% (44)	50% (14)	33% (5)
***Cryptosporidium* +ve % (number)**	30% (6)	100% (52)	73% (37)	25% (7)	20% (3)
***Salmonella* +ve % (number)**	20% (4)	2% (1)	2% (1)	7% (2)	14% (2)
***Giardia* +ve % (number)**	0% (0)	15% (8)	65% (33)	86% (24)	100% (14)

¥ PCR for *Salmonella* and *Giardia* was only carried out on 14 samples

Pathogens were identified in all samples taken closest to the day of diarrhoea. Ninety-one percent of the samples contained *Cryptosporidia*, 70% contained rotavirus and 11% contained *Giardia*. *Salmonella* was not detected in any of these samples and *E. coli* and BCoV were not detected in any of the study calves throughout the trial. As would be expected from these results the number of calves with multiple infections at any one time was high.

Results from calves in the longitudinal study

The longitudinal study demonstrated the pattern of pathogen shedding in the first month of life. It was also used to explore the relationship between clinical disease and detection of pathogens in faeces. Rotavirus was detected in calves from day 2 and cryptosporidia from day 3, with the highest proportion of calves shedding both of these pathogens occurring at 12-14 days of age (Figure 2). The proportion of calves shedding cryptosporidia declined rapidly, whereas more than 80% of calves were still shedding rotavirus at three weeks of age. *Giardia* was initially detected at day 12 and by the end of the study it was detected in all calves sampled. *Salmonella* was detected intermittently, mainly in the first few days of life

and towards the end of the study. The mean Ct values for calves shedding rotavirus, *Cryptosporidium* and *Giardia* over the duration of the study is presented in Figure 3.

Figure 2: Variation in proportion of calves shedding calf scour pathogens with age

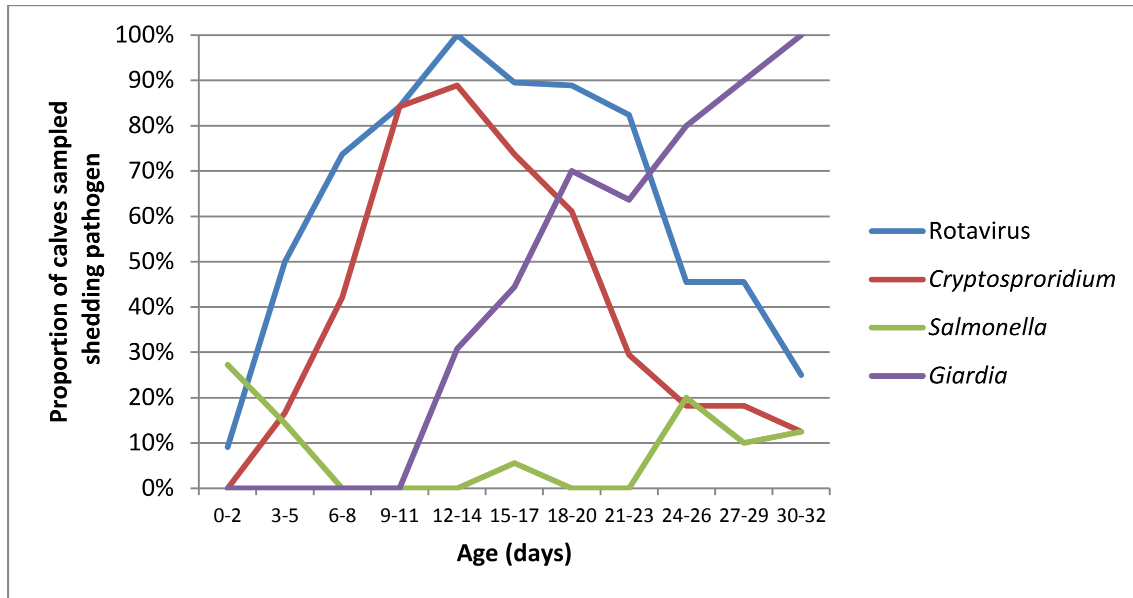
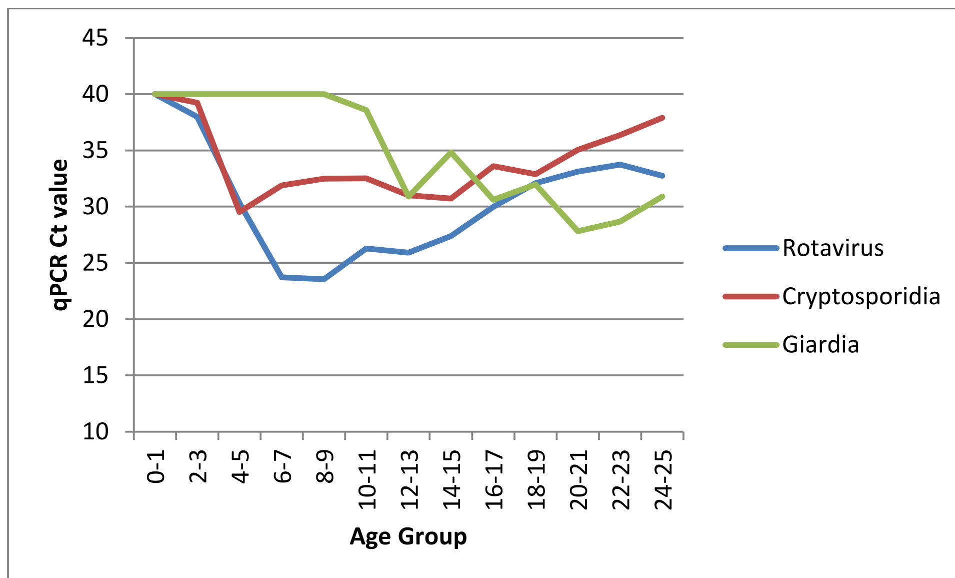


Figure 3: Farm A - Average qPCR Ct values for calves shedding Rotavirus, Cryptosporidia and *Giardia* over time∂.



∂: Where no calves were detected to be shedding a specific pathogen the Ct value in the graph has been assigned a value of 40.

Determination of the relationship between the onset of clinical disease as defined by clinical score ≥ 2 and pathogen shedding was complicated due to calves shedding multiple pathogens. The onset of clinical disease was most closely related to the onset of shedding of cryptosporidia with symptoms occurring in 14 of the 19 calves +/- 1 day from the date cryptosporidium was detected (Mean 0.75 (+/-0.93), n=19). Rotavirus was detected in the faeces 3.74 (+/-4.2) days prior to the onset of clinical disease and in some calves, the onset

of clinical disease was also associated with peak rotavirus shedding. The time interval for peak rotavirus shedding to onset of clinical disease = 0.58 days (+/- 3.55) days.

The relationship between the amount of pathogen detected in faeces and the clinical score was investigated with the aim to determine whether the PCR cycle at which the pathogens were detectable had any relationship with the severity of the disease or could be used as a prognostic indicator. Variables considered included presence of pathogens, number of pathogens on day of scour, and cycle thresholds for each pathogen. None of these variables were significantly associated with the duration of scours, maximum faecal score or maximum clinical score.

Farm B

Ten of the eleven calves had reduced milk intake for 2 or more days during the observation period (mean 4.2 +/- 2.9 days). Three calves required treatment with electrolytes and antibiotics. Rotavirus was detected in all calves in the first few days of life (mean age 2 +/-1 .8 days). *Salmonella* was cultured from 8 calves, in 3 calves the initial detection was within 24 hrs of birth, in 3 calves the initial detection was between 6-7 days of age and in 2 calves it was first detected between 15-17 days of age. *Cryptosporidia* were detected in all calves commencing from 11 days of age (mean age 13.7 +/- 1.8 days). BCoV was detected in 2 calves aged 2- 3 weeks of age. The proportion of calves shedding pathogens at each time point is shown in Figure 4.

Figure 4: Farm B - Proportion of calves shedding enteric pathogens by age.

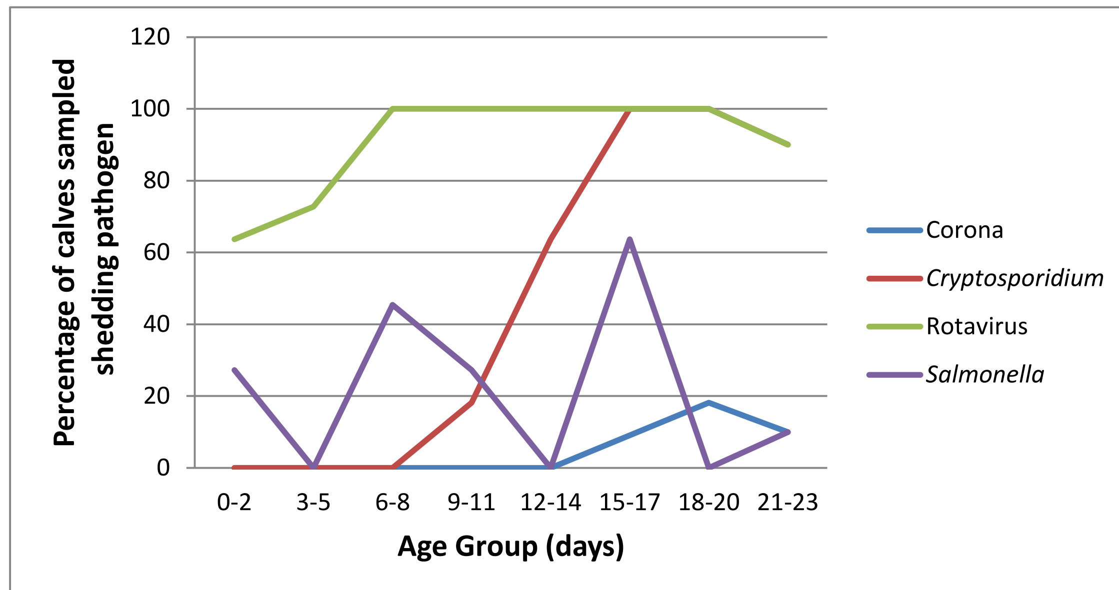
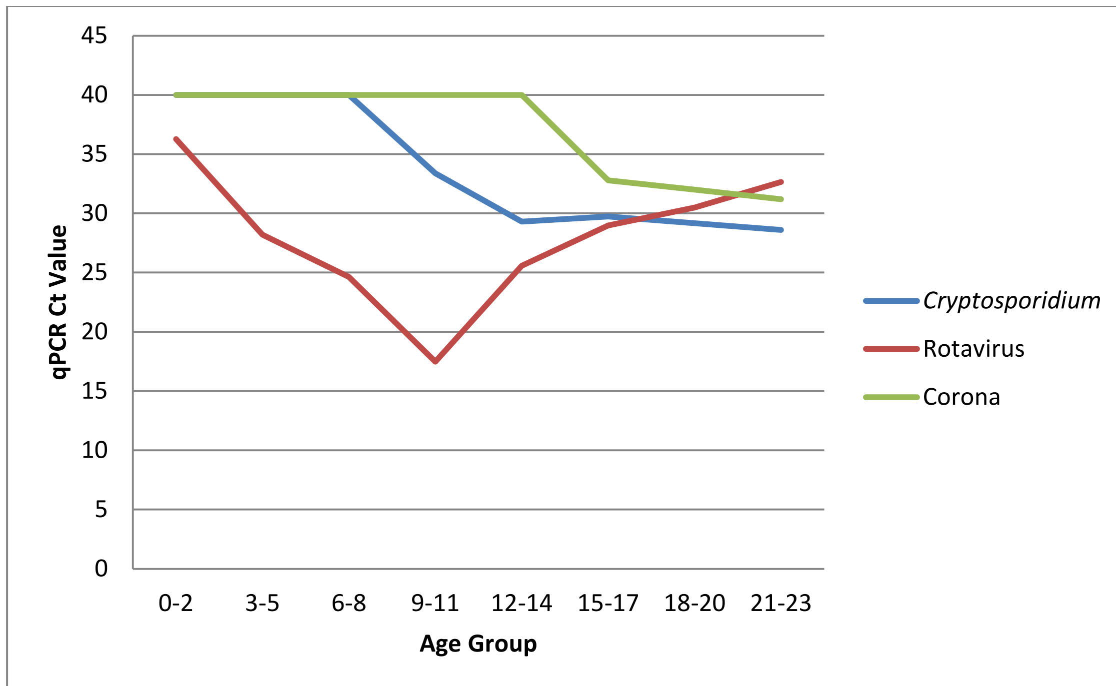


Figure 5: Farm B - Average qPCR for calves shedding Cryptosporidia, Rotavirus and BCoV over time∂.



∂ For BCoV and *Cryptosporidia* where no calves were detected to be shedding the Ct value in the graph has been assigned a value of 40.

Eleven calves were sampled in each age bracket except for the 21-23 day age group where there were only 10. Peak shedding of rotavirus occurred around 10 – 11 days of age and corresponded with the onset of shedding of *Cryptosporidium*. This timing corresponded with clinical disease. The mean Ct values for calves shedding rotavirus, *Cryptosporidium* and BCoV over the duration of the study is presented in Figure 5.

4.5 Environmental monitoring

The potential to detect pathogens in environmental samples was initially evaluated by assessing current literature. In the past decade, much research has focused on the use of molecular based methods for detecting microbes in various samples, including those from the environment. Much of this work concentrates on microbial community analysis for ecological investigations, but pathogen detection using environmental samples has also been reported. Rotavirus has been detected in water, sewerage and swabs from contaminated surfaces (Lyman et al. 2009; Schlindwein et al. 2010; Sdiri-Loulizi et al. 2010; Prado et al. 2011; Yang et al. 2011). BCoV has been shown to survive on lettuce leaves for at least 14 days (Mullis et al. 2012) and human and avian coronaviruses have been detected in swabs from contaminated surfaces and in sewerage (Wang et al. 2005; Booth et al. 2005; Otter et al. 2015) There is no research published on the detection of Bovine Viral Diarrhoea Virus (BVDV) in environmental samples.

There is a large volume of work published on the detection of *Salmonella* in water, soil and animal environments using PCR and *Giardia* and *Cryptosporidium* in water and soil, as they are important zoonotic pathogens (van Blerk et al. 2011; Jacobsen & Bech 2012; Smith & Nichols 2010; Singh et al. 2012; Baque et al. 2011; Liang & Keeley 2011). The reliability of PCR for detecting and quantifying pathogens in complex environmental matrices is largely

dependent on the quality of DNA and RNA extracted, and will vary with the nature of the environmental sample (e.g. soil type), conditions and type of microorganism. Detection of DNA has been shown to be more reliable than RNA (Marsh et al. 1998; Bech et al. 2010; García et al. 2010). Known inhibitory compounds such as humic acid are found in environmental samples such as soil and is often extracted with nucleic acid. Furthermore, the quality of nucleic acid extracted will depend upon sample storage and preparation prior to extraction. Enterotoxigenic *E. coli* has also been detected on soil, water and surface swabs (Omar et al. 2010).

The literature review demonstrated that there are established methods for detection of many of the pathogens included in this study, but that efficacy of detection is variable depending on the pathogen, environment and sample preparation used. Further, there have been significant improvements both in nucleic acid extraction technology and the changes from conventional gel based PCR to real time PCR methods have each improved the sensitivity of pathogen detection. Consequently, the published studies were reviewed in this context prior to undertaking the research described below.

4.5.1 Laboratory testing of spiked samples

Bedding – Washing wood shavings on a rotary mixer or sonicating the mixture in a jewellery cleaning water bath, both successfully detected the nucleic acid from the respective pathogens and returned very similar results (Table 7).

Milk – Pathogen DNA and RNA was reliably detected in unpasteurized cow's milk which had been spiked with faecal samples containing *Salmonella*, *Rotavirus A* and *Cryptosporidium* and stored at 4°C for 12 days. This contaminated milk sample was subsequently placed in both PBS and stool transport and recovery (S.T.A.R.) buffer (Roche Diagnostics) prior to further processing and extraction, yielding similar results.

Pasture - For cut and dried pasture which had been spiked with known levels of pathogens in faeces, washing of the sample on a rotary platform (as used for other sample types) successfully detected all pathogens present and with higher sensitivity than other methods (i.e. lower Ct values – Table 7) and to a level comparable with the control faecal samples.

While there was little or no difference between some of the methods of sample treatment to recover pathogen nucleic acid, overall, washing samples on a rotating platform is a simple universal method that can be utilised to detect pathogen DNA or RNA from these sample types.

It was apparent that a combination of water/Tween was deleterious to all pathogens but particularly to the bacteria. Similar trends could be expected with enveloped viruses such as BVDV and coronavirus.

Soil –The most reliable method for testing of soil samples involved sample preparation and semi-automated extraction using the commercially available PowerMag (Mo-Bio) soil DNA isolation kit in combination with the Kingfisher automated magnetic particle processing system. The results obtained with the PowerMag kits were very similar to those obtained for the extraction of faecal samples that were used as positive controls. The manual extraction kits based on chemical flocculation (Powersoil and Powerlyzer, Mo Bio Laboratories, Inc, Carlsbad, CA), extraction using isoamyl alcohol, the addition of PVPP or the standard

magnetic bead based processing methods used for faecal samples were all ineffective in removing inhibitors of the qPCR reaction, such as humic acids, to a level where the assay could be reliably used. The addition of skim milk improved the detection of *Rotavirus A* RNA to a limited degree. Placing soil samples in MagMax sample lysis buffer (Ambion®) or in S.T.A.R buffer did not improve the results compared with soil in PBS.

Surface swabs – Concrete and steel mesh surfaces of calf houses and pens were sampled by swabbing a 30cm x 30cm area extensively in a zigzag pattern using either standard plain sterile cotton tipped swabs, "Quick swabs" or Enviroswabs (3M), and compared with *Salmonella* enrichment culture. The pen was known to have held a calf infected with *Salmonella*, quite some time prior. A stainless steel surface contaminated with faeces containing *Salmonella* was also assessed. All swabs were stored in either PBS or PBGS after collection and yielded similar qRT-PCR results (compared using the Ct value), except in the case of 'Quick swabs" which completely inhibited the RT-qPCR. For the detection of *Salmonella*, there was improved qPCR sensitivity after the overnight incubation of samples collected on cotton-tipped swabs and placed in mannitol selenite broth compared with direct extraction of the swab washings. In general, for environmental sample matrices, it was found that the standard (original) Kingfisher protocol and extraction kit (AM1836) used on most of the faecal samples in this project still delivered superior results.

Consequently, for environmental sampling that was undertaken during the longitudinal studies, nucleic acid was extracted from samples that had been collected with standard cotton swabs and placed in PBS or PBGS then washed in PBS on a rotary platform.

Water –DNA and RNA were successfully detected in the faecal contaminated water samples and results were comparable to the control faecal samples after allowing for the dilution factor. The original faecal sample gave a Ct value of 12 for rotavirus and when 1ml of faecal suspension was added to 100mL of water, rotavirus Ct values of 16.5-17.5 were detected. RNA was also detected in water at a similar level after being stored at 4°C for 4 weeks.

Further results of environmental sampling are described in the respective longitudinal studies.

Table 7: Results of spiked environmental samples

Environmental or feed samples were contaminated with either rotavirus, *Cryptosporidium* or *Salmonella*. These pathogens were used at a uniform concentration for each material. The starting solution was used as a control. A standard volume was used to wash each material prior to nucleic acid extraction. The control was diluted by a factor equivalent to the volume of wash solution used. The results are presented as cycle-threshold (Ct) values.

		Rotavirus PCR		
Sample	**Wash**	**Sonicate**	**Cleaner**	**Rotary**
Pasture	PBS-Tween	19.68	19.90	NT
Pasture	Water-Tween	21.67	20.60	NT
Shavings	PBS-Tween	NT	20.67	20.34
Pellets	PBS-Tween	NT	18.99	18.41
Chaff	PBS-Tween	NT	28.40	18.85
Control		18.49	18.38	18.56
		Crypto PCR		
Sample	**Wash**	**Sonicate**	**Cleaner**	**Rotary**
Pasture	PBS-Tween	31.37	31.37	NT
Pasture	Water-Tween	38.46	36.73	NT
Shavings	PBS-Tween	NT	33.63	32.10
Pellets	PBS-Tween	NT	32.78	30.15
Chaff	PBS-Tween	NT	Neg	31.31
Control		31.55	31.31	31.37
		Salmonella PCR		
Sample	**Wash**	**Sonicate**	**Cleaner**	**Rotary**
Pasture	PBS-Tween	21.64	20.30	NT
Pasture	Water-Tween	32.71	32.96	NT
Shavings	PBS-Tween	NT	23.52	22.77
Pellets	PBS-Tween	NT	23.96	20.33
Chaff	PBS-Tween	NT	Neg	21.26
Control		21.11	21.23	20.47

NT= Not tested.

4.5.2 Environmental samples from on farm longitudinal calf studies

<u>Farm A</u>

Samples were processed from the 19 calves for which a complete set of faecal samples had been processed. Rotavirus was detected in 93% (81/87) of the swabs from the pen walls including 89% (16/18) of the samples taken after cleaning and before the calves entered the

pen. It was detected in 56 of the 68 bedding samples with a strong relationship between detection in the bedding and level of shedding by the calf (Figure 6). Three of the 4 negative samples taken when calves were in the pens were taken in the group pens when the calves were 2-3 days old, the other sample was taken at 26 days. It was not detected in any bedding samples taken in the pens prior to the calf entering the pen. Rotavirus was also detected in 66% of feeding equipment samples. From days 7-15 there were some very high levels of rotavirus detected in bedding samples (Figure 9) coinciding with the maximum amount of rotavirus shed by calves (Figure 3).

Cryptosporidium was less prevalent in the environmental samples. It was detected in 2 bedding samples prior to calves entering, but was not detected again in bedding samples until day 12 at the time of peak shedding by the calves. *Cryptosporidium* was only detected in one of the 18 pen wall swabs taken prior to the calves entering the pen and only 11% of the total pen wall samples and 10% of the feeding equipment samples (Figure 8). Similar to the bedding, it was not detected on either the pen walls or the feeding equipment before peak shedding by the calves.

Figure 6: Variation in the number of positive and negative bedding samples with calf age

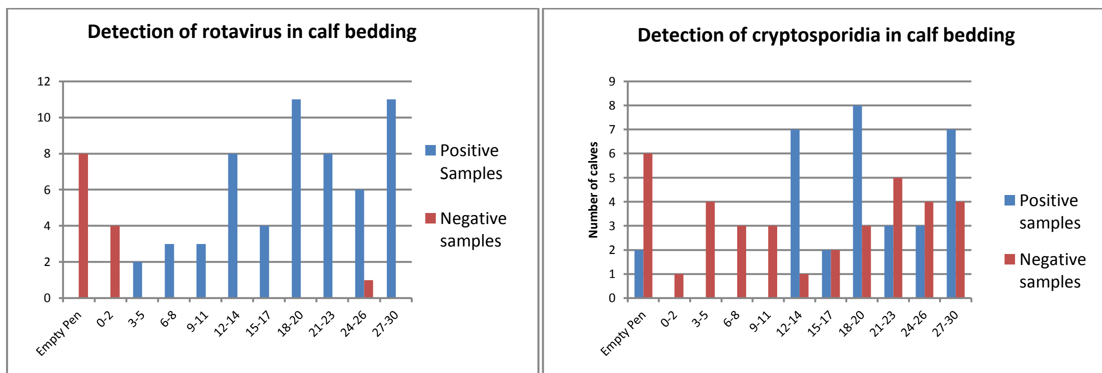


Figure 7: Variation in the number of rotavirus positive and negative pen wall and environmental samples with calf age

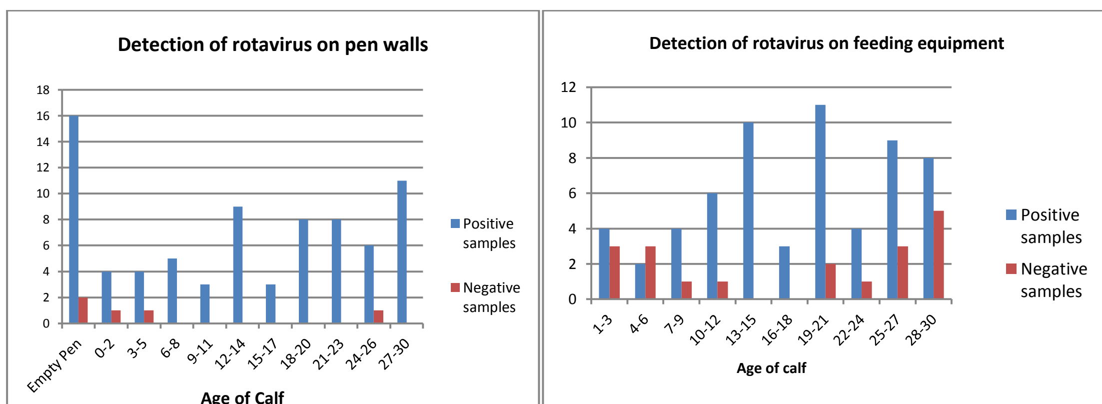


Figure 8: Variation in the number of *Cryptosporidium* positive and negative pen wall and environmental samples with calf age

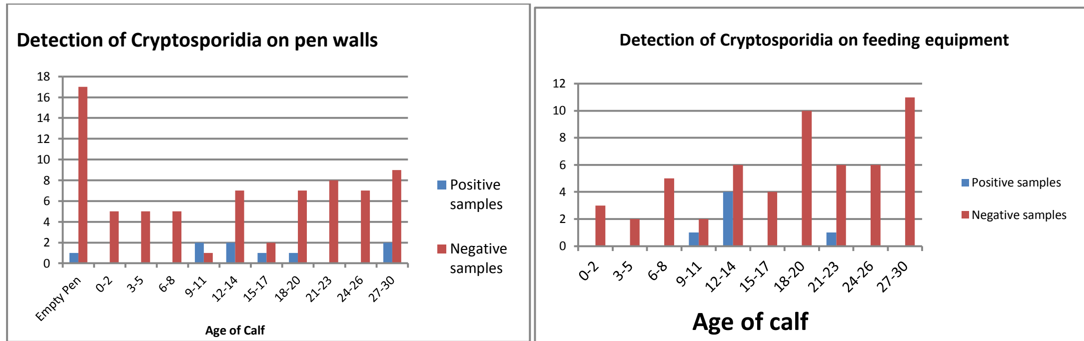


Figure 9: Variation in levels of rotavirus (Ct values) detected in pen bedding

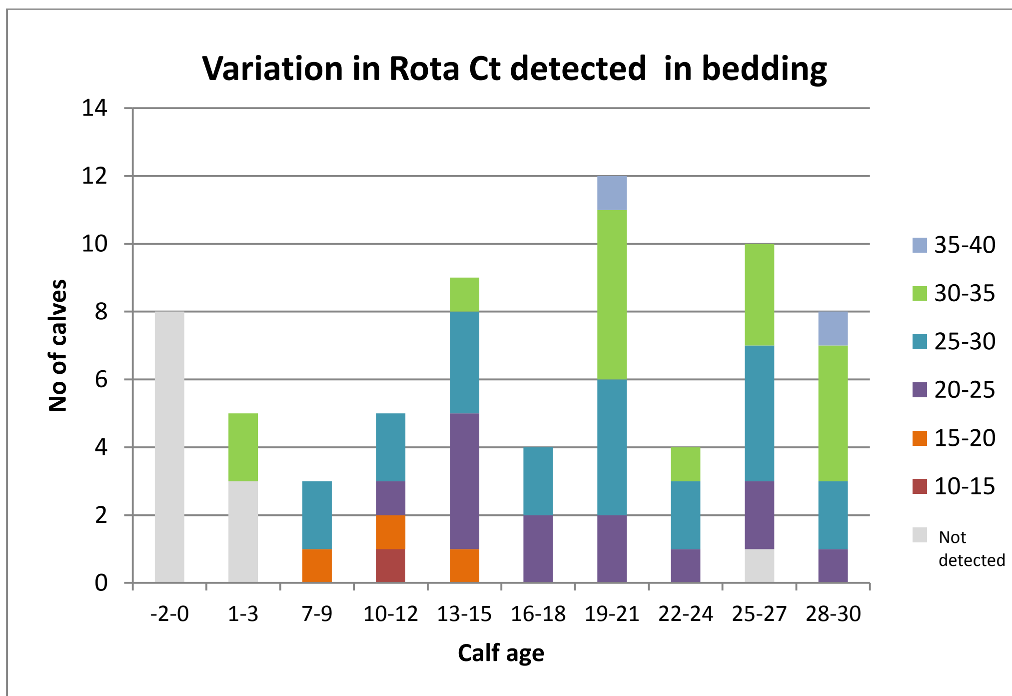
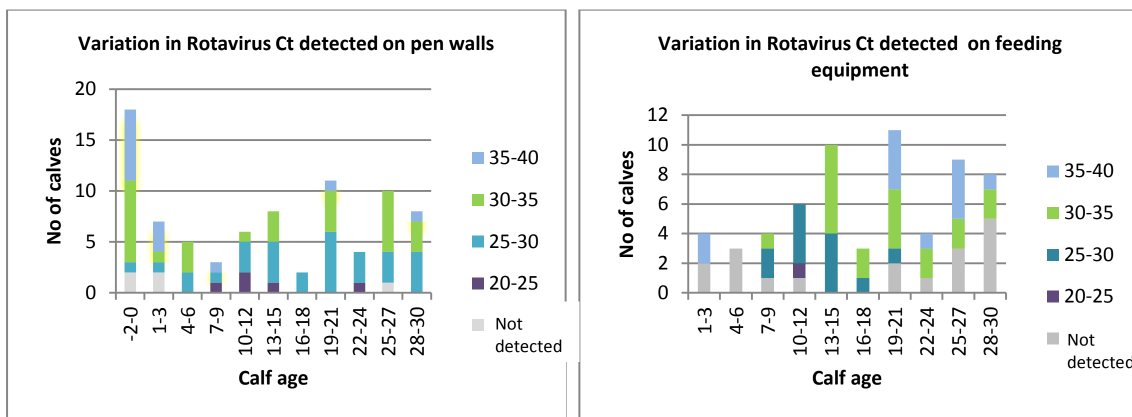


Figure 10: Variation in levels of rotavirus (Ct values) detected on pen walls and feeding equipment



Farm **2**

The results of the environmental sampling revealed significant contamination with rotavirus in the calving area and associated with colostrum harvesting and feeding equipment. It was also detected on the trolley used to move the calves from the maternity pen to the calf collection pen. The straw bedding in the calf collection area was also identified as been contaminated with *Salmonella*. BCoV was detected in bedding although these samples were taken several days after the birth of the study calves. Interestingly *Cryptosporidium* was not detected in the maternity area bedding.

Despite the apparently clean appearance of the calf rearing area, it was evident that the pathogen exposure in this area was significant. Prior to the calves entering the calf pen it had been cleaned. Swabs of the clean pen revealed low levels of rotavirus throughout the pen. Higher levels were detected on the gate at the entrance to the robotic feeder, on the water trough, and on the teat of the robotic feeder.

Cryptosporidium was also detected on the teat of the robotic feeder on the day that the calves entered the pen. It was not detected again until calves had commenced shedding oocysts in the faeces, when it was detected in the bedding and on pen surfaces. Similar to Farm A, cryptosporidia were much less ubiquitous than rotavirus.

Salmonella was isolated from the calf meal and the canola meal used to make the calf meal, as well as on the bedding. Surface swabs of the pen and maternity area did not detect *Salmonella.* These were preserved in PBGS (contain antibiotics) so they were not cultured. The failure to detect *Salmonella* may reflect the lower sensitivity of the *Salmonella* PCR.

BCoV was detected on the floor of the calf pen and the trolley used to move the calves on the first day of the study. It is likely that the trolley was contaminated by a cow in the maternity area and she may have infected her calf. BCoV was then detected on a water trough 3 days later, but then not detected again until there was bedding contamination on and after day 15 of the study coinciding with shedding by calves

5 Discussion

5.1 Development and optimisation of qPCR/qRT-PCR assays

The practical application of real-time PCR and related technologies for the detection of nucleic acids for animal health applications has been expanding exponentially in recent years. During the course of this project, there was considerable international progress with the use of qPCR for the detection of infectious diseases. Similarly, there have been further new developments with nucleic acid extraction methods. This has resulted in the availability of new publications in areas relevant to this project. This was complicated further by the fact that a number of bacterial, protozoal and viral pathogens were included – providing greater potential for new developments to be encountered. The rate of change in these fields has to some extent compromised the systematic approach that was needed for this project. On one hand, it was not always possible or practical to “back-track” to undertake additional research to evaluate additional variables but on the other did allow some problems to be resolved. An example was the publication of an alternative assay for *Cryptosporidium* that significantly improved analytical sensitivity.

While BCoV was readily detected in some samples, the results of previous research (Izzo et al. 2012) suggest that some strains of coronavirus are not being detected by PCR. During the course of the project, two additional assays were published and were evaluated but gave no change in diagnostic sensitivity. It is possible that the virus particles observed by EM may not be coronaviruses but are morphologically indistinguishable and can be readily differentiated from torovirus particles. Attempts to obtain nucleic acid sequence for these particles have not been successful.

An assay for torovirus was developed but the prevalence detected was extremely low and suggests that this virus has limited clinical relevance in Australia. The proportion detected was similar to that found in Korea (Park et al. 2008), but less than detected in Japan, Europe or S America (Kirisawa et al. 2007; Haschek et al. 2006; Nogueira Diaz et al. 2009) and significantly less than the 36.4% of clinical samples reported from the USA (Duckmanton et al. 1998). Hence this test should only be considered when investigating calf scours once the common pathogens have been excluded from the aetiology.

The qPCR for *Salmonella* has good analytical sensitivity when used to test reference cultures or faecal samples spiked with plasmid preparations. However, in many instances, when clinical samples were examined, the qPCR gave negative results despite bacteria being detected by enrichment culture methods. It is considered that this difference is a function of the quantity of material being cultured (10-15 gm) compared to the volume of sample from which DNA is extracted and then included in the qPCR. DNA is usually extracted from approximately 100 mg of sample and then due to the volume constraints of qPCR, only 10% of this is tested. These volumes represent a difference of approximately 1000 fold and can clearly have a marked impact on sensitivity. Nevertheless, the combination of qPCR with a simple overnight enrichment step has been shown to overcome most of these differences and would still provide considerable benefits through reduced time and cost compared to culture and should be investigated further. The disadvantage of this approach is that it is not possible to quantify the result. Assays that amplify RNA using reverse transcriptase have recently been described and have proven to be more sensitive than assays directly detecting DNA, however, it is unlikely that this would compensate for the amplification gained through use of an enrichment culture. Hence, currently enrichment culture needs to be used to reliably detect and diagnose *Salmonella*, but more sensitive PCR technology should be developed to quantify the levels of Salmonella from potential reservoirs of infection in the environment, as in this case low levels of *Salmonella* would not be clinically relevant.

In some instances, the clinical relevance of such low levels of *Salmonella* in diagnostic samples may also be questioned and the possibility that the qPCR is identifying clinically affected animals compared to those with a subclinical infection cannot be discounted. Defining the limits that are clinically relevant remains an outstanding issue for the use of all of the real-time PCR assays, which in general have extremely high analytical sensitivity. Rotavirus provides an excellent example where there is a difference of 10^6-10^7 between the limit of assay sensitivity and the levels of viral RNA commonly detected in scouring calves. Defining the boundaries between clinical infections, infections in which organisms are either on the rise or decline (but associated with disease) and nucleic acid levels that are the residue from a resolved infection remain a challenge, particularly as there are generally multiple pathogens present in the same animal at the same time.

One of the goals of this project was to establish both simple uniform nucleic acid extraction and real-time PCR protocols that could be applied to samples for testing for bacteria, protozoa and viruses. In general, this was achieved and multiplex assays were able to be run on a common nucleic acid preparation. There were, however, instances where there may be need for further research. When processing faecal samples to test for *Cryptosporidium*, published protocols recommended disruption of organisms/mixing of the sample by “bead beating”. There were some instances where this gave superior results but was an additional processing step, and at times gave inferior results for other pathogens. Conversely, there were times where good results were obtained for all pathogens, including *Cryptosporidium* without bead beating. These differences warrant further systematic study with a wide range of clinical samples to establish whether there is a need to bead beat samples.

5.2 Multiplex qPCR

The selection of combinations of pathogens for multiplexed assays is complex. Currently, thermocyclers used for real-time PCR have a limit of 5 different channels that can be used. As there are potentially at least 7 pathogens of interest, it is necessary to run at least 2 multiplexed assays. The recommendation to include an exogenous internal control adds another complication. The main reasons for using multiplexed assays are to reduce costs and potentially save time, though the latter is less of an issue when small numbers of samples are being tested. For example, approximately 40 samples could be tested concurrently in 2 parallel multiplex assays on a single test plate. While the set-up time is longer, there are still significant savings in reagents and time compared to individual assays. While there may be debate about the use of real-time PCR technology for detection of less frequently encountered pathogens, some agents may be under-recognised due to the lack of suitable assays or inclusion of a test in a diagnostic panel. Consequently, for optimal understanding of the role of some less common pathogens multiplexed assays offer obvious advantages.

The choice of practical combinations will vary depending on the purpose of testing and the local herd situation. The needs for diagnostic applications will differ from those for detailed epidemiological investigations where the main pathogens may have been identified and testing is being undertaken to provide advice to assist disease control. It is essential during a diagnostic investigation to cover the range of possible agents. Options for assay combinations for diagnostic investigations could include a combination of the most frequently encountered agents where laboratory testing is required (e.g. BCoV, rotavirus, *Cryptosporidium* plus an internal control) and use conventional assays for other pathogens (e.g. *E. coli, Salmonella* spp) but this overlooks BVDV and *Giardia*. However, there is debate about the merits of using real-time PCR to confirm *E. coli* during disease investigations and the role of *Giardia* is currently unclear. Similarly, BVDV screening is not usually undertaken using faecal samples but opportunistic inclusion of BVDV could be advantageous as it is immunosuppressive and may predispose calves to infection with other agents. Therefore, other possible assay combinations could be a virus mix (BVDV, BCoV, rotavirus plus an internal control) and then a bacterial/protozoal combination (*Cryptosporidium*, *E.coli*, *Giardia* and *Salmonella*). With further optimisation, it is likely that most pathogen combinations could be multiplexed to provide combinations for herd investigations after an initial diagnosis has been made. Current investigations of assay combinations have not been exhaustive due to

the application of specific combinations during the herd longitudinal studies, where the multiplexed assays were shown to be extremely useful.

While a modest reduction in analytical sensitivity has been observed with some pathogen combinations, this may often have little diagnostic impact due to the very high levels of nucleic acid that are detected in scouring animals. For most pathogens, the very low levels of nucleic acid that are sometimes detected in a single-plex assay are probably not diagnostically relevant. However, this requires further investigation and may need to be taken into consideration with the use of different sample collection methods (e.g. rectal swabs) where there may be lower levels of nucleic acid than in faecal samples. Further refinements to improve sensitivity will probably centre on the evaluation of recently developed multiplex mixes and fine tuning of reagent concentrations.

5.3 Loop-mediated Isothermal Amplification (LAMP)

Throughout the development process for optimization of assays there were ongoing challenges with respect to robustness and repeatability. All assays were impacted to a lesser or greater degree with amplification sporadically observed in negative control samples (no-template control) or amplification in control negative DNA (i.e. DNA from another pathogen). This was initially considered to be potential contamination of reagents and was thoroughly investigated with assessment of new reagents and confounded by the intermittent nature of observations. There were also challenges with respect to the positive control samples intermittently not amplifying, resulting in analysis being repeated as required. Protocols for physical set-up of the assays were also modified. For example, tubes were left capped unless reagents were added to the specific tube to limit the possibility of aerosol contamination and the Bst was added as the final reagent just prior to the placement on the real-time turbidity meter to limit the potential of non-specific amplification at reaction set up. While these were good practices that were maintained, they did not provide a solution for the intermittent challenges observed.

Subsequent investigations focused on the possibility that different primers degraded at different rates with an assumption that the larger forward and backward inner primers (FIP/BIP) may be more susceptible to degradation. HPLC purified primers were assessed to see if this may be a means of enhancing stability of the primer sets, yet did not provide a clear resolution and lot- to -lot variability with respect to primer stability was observed. It became standard practice to replace the FIP/BIP if the assay failed and that often resolved the issue.

At various stages through the assay development process, some DNA or RNA extracted from clinical samples was assessed. The results were variable and there was a clear need for further assay development and as such the utility of the assays on clinical samples was not progressed. It may be that the elution buffer or other sample characteristics had a negative effect on the LAMP reaction.

Although there are numerous reports of LAMP assays for the detection of enteric pathogens, it is challenging to compare outcomes with the assays developed here. This is mainly due to the different ways in which sensitivity has been established in different assays, although specificity is typically reported as being very high for LAMP assays predominantly due to the complexity of primer design. There is also significant diversity with respect to the use of the

loop primers (not necessary but increases the rate of amplification) as well as diversity in the gene targets for detection.

Salmonella spp. have been detected from faeces by others, e.g. Zhuang et al. (Zhuang et al. 2014), which targeted the bcfD gene and was able to detect down to 5 cfu/reaction from clean cultures but observed 1000 fold decrease in sensitivity when DNA was isolated from faeces. Importantly, this assay was able to detect in as short as 25 minutes when loop primers were added utilising a real-time turbidity meter similar to that utilised in our experiments. Interestingly, a more contemporary assay developed by Fan et al., (Fan et al. 2015) for the detection of *Salmonella* spp. utilised a RT-LAMP assay as well as a LAMP assay for detection and was able to detect down to 30 cfu/ml in faeces using the STY1607 gene and using AMV RT enzyme. These researchers also conducted the assay at 65°C for 60 minutes but identified that the RT-LAMP was ~ 50 fold more sensitive than the comparable LAMP assay, culture and RT-PCR using the same target gene.

Similar assays for the detection of *E. coli* in milk (Yang et al. 2014) identified that a LAMP assay targeting the heat-labile enterotoxin A subunit and B subunit encoding gene was up to 1000 fold more sensitive than a similarly targeted PCR assay and used precipitation, Sybr Green 1 and electrophoresis as the detection methodology. This assay was also at 63°C for 60 minutes comparable to the assay for *E. coli* described in the current study. It is important to note that while the LAMP assays reported here are generally comparable to PCR, in sensitivity, others have developed LAMP primer sets that are more sensitive. Plutzer and Karanis (2009) describe a LAMP assay which includes loop primers targeted to the Ef1α gene of *Giardia duodenalis* that can detect down to 0.548pg/reaction or the equivalent of 4 oocysts.

Other assays for rotavirus using RT-LAMP have also been developed and applied to detection of virus in calf faeces (Xie et al. 2012) or human samples (Malik et al. 2013). Both assays were 60 minutes in duration. While it is difficult to compare assay performance with our assay, it is interesting that, while MMLV was not effective in our assays, it was utilised by Malik et al., (Malik et al. 2013) to great effect. These researchers identified that that their RT-LAMP assay was 10 fold more sensitive in the detection of human rotavirus using hydroxynapthol blue discoloration, than a comparable RT-PCR assay.

Other researchers have also developed RT-LAMP assays for other pathogens outlined in this study including BCoV (Qiao et al. 2012) and BVDV (Fan et al. 2012; Aebischer et al. 2014; Zhang et al. 2014) yet based on the literature reviewed to date this current study represents the most comprehensive development of assays to investigate the single yet complex disease state of calf diarrhoea.

The level and complexity of the challenge to develop, optimize and characterize this number of LAMP assays is quite significant, particularly considering the limited resource and technology base that is available locally for this technology.

Isothermal DNA amplification technology has been demonstrated in the literature to be an effective diagnostic tool for the detection of pathogens in a variety of clinical samples. The results of the current project support this assertion. However, a number of limitations of LAMP were encountered during the assay development process which prompted efforts to be focused on the qPCR assays.

1. Limited local technical support.
2. Limited local distribution network for reagents.
3. Primer instability and subsequently assay instability.
4. Difficulty licensing developed assays for commercial use.

One of the desirable features of LAMP is the potential field application of the assays due the isothermal nature of the reaction. Point of care diagnostics are an emerging area of interest and recently this has extended to point of care PCR technology. Given the limitations of LAMP and the robustness of the PCR platform and continuing development of delivery options for PCR assays priority was directed at the PCR assays.

5.4 Animal specimens

5.4.1 Sample collection and storage

In order to perform field epidemiological studies efficient and reliable methods of collection and storage are needed. Swabs have not commonly been used for sampling faecal pathogens in calves and there are no publications that evaluate comparative data. For pathogens with sufficient comparative data, this study demonstrated that there was a small decrease in the sensitivity of detection by using swabs. Despite this, from a clinical and laboratory processing perspective, there are many advantages of using swabs: it is sometimes not possible to collect a clean representative faecal sample from a scouring calf due to the absence of faeces in the rectum at the time of sampling; a PBGS swab is smaller to transport; prevents bacterial overgrowth and requires less processing in the laboratory. Swabs also proved valuable for detection of rotavirus in the environment, but the use of swabs for investigating areas with high levels of *Cryptosporidium* requires further attention. It is possible that the efficacy of using swabs will vary depending on both the pathogen and the type of swabs and transport medium used a as studies in other species and with other pathogens have demonstrated some variation with swab type (Warnke et al. 2014; Tan et al. 2014). Nevertheless, the advantages of using swabs for these investigations are sufficient that this area should be further investigated.

The limited sensitivity of the current qPCR assay for *Salmonella* required incubation of samples in enrichment buffer prior to performing the qPCR assay. The antibiotics currently included in PBGS (developed primarily for virus detection) preclude the use of enrichment methods for *Salmonella* but future studies could explore options for the collection of rectal or faecal swabs without antibiotics.

Sample storage

For quantitative field epidemiological and diagnostic studies, the pathogen level in samples needs to be representative of those at the time of sampling when processed by the laboratory. This means that, as a minimum, the sample should not degrade in transit or during storage which typically equates to stability at 4-10°C (refrigeration and transport on ice). Storage methods that facilitate survival at room temperature or higher are preferable. Whilst PCR does not require live pathogen, it is still important that the nucleic acid is intact.

The survival of viruses in faeces will vary with the lability of the virus. Rotavirus is a stable virus known to survive in fresh water for more than 2 weeks at 23°C and for months in water or soil < 5°C (Smith & Gerba 1980). Hence it is not surprising that rotavirus particles can

survive storage in ambient tropical temperatures for more than 2 months (Fischer et al. 2002). BCoV can survive for several months at 4°C with faecal contamination, but is inactivated after several days at 37°C (Mullis et al. 2012), hence it is likely that it will survive in refrigerated faecal samples for the desired length of time. Further studies are required to determine its survival at room temperature.

A range of studies has shown that the faecal environment is not conducive to BVDV survival (Ridpath et al. 2009; Ridpath et al. 2014; Bøtner & Belsham 2012) and it is necessary to cool faecal samples soon after collection and during transport to the laboratory. It is possible that the smaller quantity of faeces collected on swabs and effectively diluted by placing in a buffer such PBS or PBGS will increase the survival rate under suboptimal transit conditions, but this requires further evaluation.

There is a paucity of studies that specifically measure the survival of *Salmonella* and *E. coli* in faeces for an extended duration. Moreover, with *Salmonella* it is currently necessary to ensure bacterial survival for culture or enrichment prior to PCR. Nevertheless, there are studies that show acceptable stability of both *E. coli* and *Salmonella* for short periods at room temperature and for at least 6 days in a refrigerator (O'Carroll et al. 1999; Shimoda et al. 1991; Himathongkham et al. 1999; Echeverry et al. 2006). Where some estimate of the number of bacteria in the sample is required, transport at 4°C is preferable to enrichment medium.

Cryptosporidia are very stable in whole faeces at 4°C (Amar et al. 2005) but storage of faeces at 35°C resulted in loss of infectivity after 4 weeks (Olson et al. 1999). *Giardia* cysts are more labile with a 95% reduction in the proportion of detectable cysts after 4 weeks of refrigeration and a total loss of cysts in the sample when stored at room temperature for 2 weeks (Olson et al. 1999).

For most calf scour pathogens, elevated temperatures, including the variable temperatures encountered in vehicles, are likely to have an adverse impact on pathogen viability and perhaps nucleic acid stability. Conversely storage of faeces at 4°C for up to 1 week is unlikely to have a significant alteration in pathogen load and influence analytical sensitivity. For *Salmonella* detection, until PCR assays that do not require an enrichment step are available, collection, storage and transport conditions must be optimised to maintain viability of organisms. Consequently, for all pathogens, it is recommended that samples should be kept chilled from collection through to receipt at the laboratory. However BVDV, *Giardia* and potentially BCoV are the more labile pathogens and further evaluation of methods to ensure stability of the nucleic acid are warranted. Options may be to evaluate nucleic acid stabilising solutions such as RNAlater® or specialised card based sample collection systems for DNA and RNA. However, in most cases these are designed for samples tested with PCR not culture, and so there is not a "one method suits all" solution. If specialised media are required this would also increase the cost of the test and require veterinary practitioners to keep additional sampling media in their vehicle at all times.

5.4.2 Experimental challenge trials

The initial plan for characterizing the shedding profile of each pathogen and its relationship to pathology was to conduct experimental challenge studies using colostrum deprived calves. On farm pathogen exposure during the calf collection phase of the study prior to

experimental challenge proved a limitation to this approach. Calves developed scours prior to experimental challenge reflecting the pathogen profile of the source farm. Given that the outcome from a pathogen perspective was confounded by natural pathogen exposure precluding singular examination of individual pathogens this approach was revised to longitudinal studies of naturally occurring calf scour outbreaks. The longitudinal studies reflected the diversity of pathogens that are frequently encountered in calf scour outbreaks and provided opportunity for environmental sampling that was relevant to real life situations where identification of sources of infection were relevant to disease prevention. Studying naturally occurring disease also avoided experimental induction of disease in healthy calves and allowed a greater number of calves to be included in the study.

5.4.3 On farm studies of calf shedding patterns and interpretation in the field

The field studies highlighted the complexity of neonatal calf disease in a farm environment. Both of the calf units in these studies were extremely well run, calves were fed adequate colostrum within 2 hours of birth, and there were high standards of cleanliness both between and within batches of calves. Yet despite this, multiple pathogens were detected resulting in disease in the majority of calves. Consistent with other studies the highest prevalence of diarrhoea was in the second week of life (Trotz-Williams et al. 2007; Bartels et al. 2010; Smith 2012). There are few prospective longitudinal calf studies and those that are available do not sample as frequently as in this study and often focus on a single or limited number of pathogens (Heckert et al. 1990; Coura et al. 2015; Rieux et al. 2013). Consequently, they do not give insights into the dynamics of infection by the different pathogens, the interaction between infection and clinical disease, or interaction between management interventions and pathogen shedding as was shown by this study. Significant levels of rotavirus and *Cryptosporidium* were isolated from calves from both properties, *Salmonella* was a contributing factor to disease in Herd B and was detected in calves in Herd A, however no cultures were carried out in Herd A so it is possible that it may have had a more significant contribution to disease in some calves in this herd. *Giardia* was detected in significant numbers in samples from calves in Herd A, however, this pathogen was not tested for in Herd B. BCoV was detected in herd B only and did not contribute significantly to disease in that herd. Significant but unsuccessful efforts were made to find calves that were actively shedding BCoV in order to characterise the relationship between shedding patterns and clinical disease. *E. coli* K99 was not tested for in either herd as both farms vaccinated against this disease and the history of scours on the farms did not fit with the age distribution of enterotoxigenic *E. coli* infection.

Previous studies have shown that rotavirus and *Cryptosporidium* are the most common pathogens detected in faecal samples from calf scour outbreaks in suckler beef herds in southern Australia (Gunn 2003). The frequent monitoring with a sensitive diagnostic test demonstrated the high prevalence of infection by both rotavirus and *Cryptosporidium* in these herds. Whilst PCR for these pathogens is more sensitive than traditional diagnostic tests (Izzo et al. 2012; Operario et al. 2015), the 100% cumulative prevalence of *Cryptosporidium* and greater than 98% cumulative prevalence of rotavirus on both farms is significantly greater than demonstrated either by single time point studies, or by longitudinal studies using less sensitive diagnostic tests or less frequent sampling (Bendali et al. 1999; De La Fuente et al. 1999; Reynolds et al. 1986; Al Mawly et al. 2014; Izzo et al. 2011; Trotz-Williams et al. 2005). This study does concur with a longitudinal study by Santín et al. (2008)

that demonstrated a 100% cumulative prevalence of *Cryptosporidium* in a 2 year study of 30 dairy heifers from birth to 24 months of age.

Studies in beef calves have demonstrated a lower prevalence of *Cryptosporidium* than found in dairy calves (Kváč et al. 2006; Geurden et al. 2007). It is likely that the increased chance of calves in confined spaces licking surfaces contaminated with faecal-oral material contributes to this variation (Winkworth et al. 2008; Becher et al. 2004). However few studies in suckler beef calves have characterised the presence of pathogens using a longitudinal study, due to the practicality of carrying out such a study. Where this was done *Cryptosporidium* was isolated from 92% of the calves within 1 month of age (Rieux et al. 2013). It should be noted that the cows and calves in that study were housed when the calves were less than 1 month of age, whereas another study of range beef calves in Canada demonstrated a much lower prevalence (Ralston et al. 2003).

It is well documented that prophylactic administration of halofuginone delays shedding of *Cryptosporidium* oocysts in treated calves, with a lower prevalence of shedding in the first week of life. (Trotz-Williams et al. 2011; Almawly et al. 2013; Silverlås et al. 2009). However in most studies, halofuginone failed to completely prevent shedding in the treated calves in the first week, and only delayed shedding for the first week of life in the majority of calves. The complete absence of shedding until calves were 10 days of age demonstrated on Farm B can be explained by De Waele et al (2010), where a study of calves in individual pens treated with halofuginone had a mean onset of oocyte shedding of 16 days, compared to the first week of life in control calves treated with a placebo. Only 30% of calves shed any oocysts before 14 days of age. In that same study, oocyst shedding in treated calves kept in a group pen was compared to shedding in placebo treated calves in a group pen. Treated calves in the group pen started shedding from 6 days of age and 80% were shedding by 10 days of age. Moreover, the number of oocysts shed by both the control and treated animals in the group pens was significantly higher than the level shed by calves housed individually. That study demonstrated that the effect of halofuginone is significantly greater when the *Cryptosporidium* challenge is lower. On farm B the environmental tests showed that there was a very low level of exposure to *Cryptosporidium* oocysts in the maternity area as *Cryptosporidium* was not detected on any of the environmental samples. Once the calves entered the group pen the meticulous cleaning procedure resulted in an environment in which *Cryptosporidium* was also not detected, apart from one sample on the teat of the automatic calf feeder. As *Cryptosporidium* oocysts are highly resistant to disinfection it is probable that there was a low level of contamination of this area, but the exposure of the calves to oocysts was reflective of the cleanliness of the individual pens in the Der Waele study. This resulted in the superior efficacy of the halofuginone administration, not demonstrated in many other studies.

There are insufficient longitudinal studies characterising rotavirus infections in suckler beef calves to determine if the prevalence is similar to housed dairy calves. However, this study has demonstrated the extent to which rotavirus can be distributed in the environment by infected calves and hence it is likely that the prevalence would be equally as high when the challenge was high.

Rotavirus strains are known to have a range of virulence (Bridger 1994) and this is demonstrated by the variation in clinical presentation with rotavirus infection between the 2 farms. Without further investigating the strains causing disease in calves on these properties

it is difficult to establish if the variation between herds can be purely attributed to strain variation or if there is also a variation in immunity between herds.

Giardia was identified in the majority of calves in herd A. The significance of *Giardia* as a cause of diarrhoea in calves is debated in the published literature. *Giardia* is often found in concurrent infection with *Cryptosporidium* or *Eimeria* in older calves (O'Handley et al. 1999; Gillhuber et al. 2014). *Giardia* are found in the jejunum of calves and causes inflammation including villous atrophy and distortion of the crypts (Ruest et al. 1997). Our clinical studies did not demonstrate a relationship between detection of *Giardia* and diarrhoea, however where this has been demonstrated the mean age of onset is > 30 days (O'Handley et al. 1999), which is outside of the monitoring period of the current study. Recent studies have demonstrated that *Giardia duodenalis* is a species complex, whose members show little variation in their morphology, yet can be assigned to at least eight distinct genetic groups or assemblages (A to H) based on protein or DNA polymorphisms (Ryan & Cacciò 2013) Assemblage E has been associated with disease in cattle (Gillhuber et al. 2014). To determine the significance of the level of *Giardia* found in clinical specimens a future diagnostic test should include primers specific to assemblage E.

Differences in virulence are observed within and between different *Salmonella* serotypes (Heithoff et al. 2012). The frequency of *Salmonella* shedding by calves may be high, with the relationship of shedding to both morbidity and disease dependent on host immunity and pathogen virulence (House et al. 2001). Causality is usually based on evidence of clinical disease and isolation of *Salmonella* from tissues at necropsy (Mohler et al. 2009). *Salmonella* was detected on both farms. In Herd A it was only detected by PCR and for many calves, it only was detected at a single time point and did not result in severe disease. In Herd B *Salmonella* had been isolated from tissues of calves at necropsy immediately prior to the longitudinal study suggesting that it was contributing to morbidity and mortality on this farm.

A feature of the current studies was the lack of association between clinical disease and individual PCR values. There are several reasons for this including a potential lag between pathogen replication and clinical effects, the extreme dilution effects due to faecal consistency that could affect the amount of pathogen detected at the time of severe disease, and the interaction between several pathogens contributing to clinical disease at any one time. On farm A there was a strong association between the appearance of *Cryptosporidium* in the faeces and the clinical score. The pathogen profile on farm B was modified by the use of halofuginone and clinical disease was associated with peak rotavirus shedding. These observations concur with a recent study on New Zealand dairy farms that demonstrated that liquid faeces was associated with the isolation of *Cryptosporidium*, rotavirus and the presence of multiple pathogens (Al Mawly et al. 2015). That study had a much greater sample size. Examination of the results at a group level over time illustrated good correlation between prior pathogen exposure, the relationship between pathogen shedding and disease, and the influence of management interventions on pathogen shedding. As is common with calf scours both disease outbreaks studied involved multiple pathogens.

The rationale behind the longitudinal studies was firstly to demonstrate the application of PCR technology in the field, both for epidemiological investigations and for diagnostic purposes, and secondly to provide guidance on the clinical relevance of faecal sample results taken at a single time point in the field and submitted by a veterinarian as part of a calf scours diagnostic investigation. Hence this study has demonstrated that there is a much

bigger picture behind the routine clinical investigation of a scour outbreak, where samples are generally only taken at a single time point. Detection of pathogens at a single time point only indicates that those pathogens are likely to be contributing to disease on that farm. The contribution of each pathogen needs to be determined by knowledge of the clinical presentation and also response to specific targeted preventive measures. Significant farm level value was derived from identifying the pathogens involved, sources of infection and limitations/value of management interventions. This information was pertinent to allocation of resources to control and subsequently prevent ongoing disease.

5.4.4 Environmental samples

The two 'on farm' longitudinal studies demonstrated that the environmental sampling techniques used were efficacious at detecting rotavirus in the environment. Both studies were carried out in well run facilities with very good hygiene and thorough cleaning practices between calves, yet despite this rotavirus was commonly found on pen walls before calves entered. As the proportion of calves shedding rotavirus increased, the virus became ubiquitous throughout the calves' environment. In farm A the maternity area and vehicles used to transport the calves to the rearing facility were not sampled. Thus it is not clear as to whether the calves entered the facility having already been exposed, or if the source of the infection was other calves in the same airspace or pathogens remaining in the pens. What is clear is that rotavirus is shed prolifically resulting in a high level of environmental contamination once calves become infected. There is no doubt that the application of this technology could be refined further to aid in a diagnostic investigation and provide advice on prevention and control of calf scours by focussing on areas where it is detected in the highest concentration. In a suckler beef outbreak this may be around calf camps or the calving paddock. It is known that rotavirus can survive in the environment for many months in cooler moister climates, so this tool potentially could be used to assess the risk of infection in paddocks used for calving cows and associated handling facilities It should also be realised that the study has only demonstrated potential sources of rotavirus in the environment, the limitation of the PCR is that it only detects the nucleic acids and there is no indication of infectivity. However several studies in humans implicate viruses, including rotavirus, detected on environmental surfaces in maintaining disease outbreaks (Butz et al. 1993; Wu et al. 2005). The relationship observed on farm B between prior pathogen exposure and subsequent pathogen shedding suggested that at least a portion of the virus was viable and infective.

Cryptosporidium was also frequently detected in the environment; however, the level of detection was lower. Although *Cryptosporidium* was shed by all the calves in the longitudinal study on Farm A it was not detected in the environment of 2 calves. It is likely that the small volumes collected by the cotton tip swabs and perhaps the relatively larger size of the *Cryptosporidium* oocyst, compounded by the small amount of sample used in the PCR, reduced the sensitivity of this sampling method. Improved results were obtained with the bedding samples where a much larger sample was collected. On farm B a direct correlation was observed between faecal shedding by calves and detection of *Cryptosporidium* in bedding in the calf pen. Whilst this tool has significant potential, it requires further refinement to determine if significant areas of infection are being accurately represented by the sampling techniques. Currently most published methods using PCR to detect *Cryptosporidium* in the environment have been optimised for detection in water, however it is

recognised that the technology cannot reliably pick up the low levels of oocysts detected in drinking water (Staggs et al. 2013). Experiments to detect *Cryptosporidium* with other substrates utilise oocyst counts that are time-consuming and costly (McLaughlin et al. 2013; Santamaria et al. 2012).

The incidence of BCoV infection in the calves was small, but the usefulness of environmental sampling techniques was demonstrated by the ability to trace BCoV from the maternity area on farm A to the calf area where it produced a transient infection in 4 calves. The fact that only transient shedding was detected suggests the calves had adequate immunity to BCoV. Cows on this property were vaccinated to control BCoV.

Whilst the development and environmental sampling has been highly successful, further work is required to improve the sensitivity of this approach for detection of *Salmonella*. Overnight pre-incubation of environmental samples in buffered peptone water increased the sensitivity of detection and provided a more rapid result than culture. This combination of pre-enrichment with buffered peptone water and qPCR was useful in defining environmental contamination in bedding on Farm B.

The source of infection was a question raised by the management of Farm B prior to the investigation. Possible sources included the calving area, calf pen, equipment and people. During the period in which calves were being sampled, swabs were collected from staff boots, colostrum harvesting equipment, colostrum feeding equipment and a trailer used to transport calves to the calf pens. Bedding samples (straw) were also collected.

The calving area was identified as a significant area of pathogen exposure with the pathogens identified subsequently shed by calves. Rotavirus was detected by qPCR in the bedding, on the colostrum feeding equipment, and on the calf transport trailer. BCoV was also detected on the calf transport trailer. *Salmonella* was detected by culture and using the qPCR on maternity bedding samples enriched by overnight incubation in buffered peptone water. Interestingly cryptosporidia were not detected, potentially reflecting a low level in this environment, which is consistent with the efficacy of halofuginone as discussed above.

Prior to the calves entering the calf pen all bedding had been removed and the pen steam cleaned with a commercial steam cleaner. Following cleaning all surfaces had been sprayed with Virkon S® (Dupont, Lexington KY USA). Swabs of the calf rearing area collected prior to calf entry included: boots, floors, water trough, feed trough, gate on entry to robotic feeder, teat on robotic feeder, and milk mixing bowl on robotic feeder. Rotavirus was detected on all surfaces with highest levels associated with the robotic feeder. *Cryptosporidium* was also detected on the teat of the robotic feeder. None of the pathogens were detected in samples of bedding (straw) collected prior to calf entry. This pathogen profile of the environmental contamination in the calf rearing facility identified prior to entry of the calves into the rearing facility corresponded with the subsequent pathogen excretion by calves in the facility.

Following calf entry rotavirus was consistently detected throughout the calf rearing area. *Salmonella* was detected on calf bedding 24 hours following calf entry and in 5 of the 6 bedding samples collected during the rearing period. *Cryptosporidium* was detected in 2 samples initially and then from day 12 onward in the bedding samples, consistent with the observed faecal shedding pattern of the calves.

The data collected utilising the qPCR assays on Farm B illustrated how these tests can translate to practical management decision making.

1. Assessment of the calving area revealed significant contamination that had not been anticipated according to the routines and physical appearance of the facility. From a management perspective the testing provided evidence for more emphasis to be placed on cleaning routines in the calving area to avoid infecting calves at birth.
2. In regards to the cleaning of the calf pen the highest level of contamination was observed at the robotic calf feeder indicating that the protocol for cleaning the device between groups of calves needed to be revised.
3. The farm was vaccinating cows to control rotavirus, BCoV, *S.* Dublin and *S.* Typhimurium and enterotoxigenic *E. coli*. The data indicated that the vaccination and calf feeding protocol was not producing the desired outcome. A management strategy that could be adopted to improve the efficacy of the vaccine is the feeding of surplus colostrum (200 ml per head per day) to calves less than 10 days of age. This strategy has been reported to enhance efficacy by providing local protection of the gut. The farm reviewed colostrum and milk feeding practices to maximise vaccine efficacy and protection.
4. Prior to the onset of the diagnostic investigation, the farm had implemented a prophylactic strategy for *Cryptosporidium* utilising daily administration of halofuginone for 7 days starting at 48 hours of age. The cost of this strategy is significant but the absence of *Cryptosporidium* shedding until day 10 – 11 suggests that the halofuginone is efficacious and in this context the expense is justified. Prior to the implementation of halofuginone prophylaxis, *C*
5. *ryptosporidium* had been detected in younger scouring calves.
6. The results of the qPCR *Salmonella* assay were disappointing. This assay failed to detect environmental contamination using environmental swabs. However, the sensitivity of detection of *Salmonella* in environmental samples was significantly improved by overnight incubation in buffered peptone (see later discussion).

A potential limitation of the information obtained related to the inability of the assay to determine pathogen viability. This was most pertinent to the calf rearing area which had been thoroughly disinfected. Following this investigation the farm modified cleaning protocols, particularly in the calving area. After 6 months of 80% calf scour morbidity, the morbidity dropped to < 10% with some groups not experiencing scours.

5.5 Practical implications for industry;

Calf scour outbreaks frequently involve multiple pathogens. Historically the tools available to manage and prevent calf scours have been limited with efficacious vaccines available for enterotoxigenic *E. coli* and *Salmonella* vaccines for a limited range of *Salmonella* serovars. In recent years the number of tools available has increased with the introduction of vaccines for rotavirus and BCoV and halofuginone for the treatment and prevention of cryptosporidia. Despite the availability of these tools, calf scours continues to be a difficult problem to manage on farm.

The first step in the process of investigating disease outbreaks is to define the problem. This requires identification of causal pathogens, sources of infection and stressors that contribute to compromised host immunity. The logic of the process is to determine which pathogens

are involved, initiate pathogen specific interventions (e.g. vaccination and or treatment), reduce the risk of pathogen challenge, and mitigate stressors contributing to compromised host immunity.

The diagnostic assays developed in this project will facilitate the disease investigation and management process. Historically the diagnostic assays available for the detection of pathogens had limited sensitivity, were qualitative, not quantitative and were limited to samples from affected calves. The advantage of the qPCR assays is their enhanced sensitivity and quantitative output, as well as their application in detecting pathogens in the environment to determine areas with an increased infection risk .

On farm implementation of disease management and prevention plans requires allocation of resources to minimize disease risk. For this process to be efficient and effective the resource allocation should be targeted to procedures and products that address the most significant risks. Conversely, following the implementation of management interventions to control disease, it is useful to be able to evaluate the outcome of intervention in regards to pathogen exposure / shedding to verify the effectiveness of the intervention.

The herd scenario presented on Farm B illustrated how the tests developed in this project can facilitate disease investigation and management. On Farm B sources of infection were identified. Failures in cleaning processes were highlighted and the effectiveness / ineffectiveness of different interventions were illustrated. The quantitative nature of the data delivered facilitated interpretation of the clinical picture and identified areas of greatest risk. Changes implemented in response to these findings reduced the incidence of disease.

5.6 Unanswered questions/additional research recommended

5.6.1 Salmonella qPCR

The qPCR assay developed in this project for *Salmonella* had poorer sensitivity than selective culture. This largely reflected sample dilution during sample processing and has been reported with other *Salmonella* PCR assays. Recently published research in this area suggests that RT-PCR assays for *Salmonella* may be more sensitive, presumably because there are more copies of RNA produced than DNA. It would be useful to investigate the improvement in sensitivity that this assay might give in comparison with a ‘pre-PCR” enrichment step. This is particularly important to allow for quantification of the levels of *Salmonella* when monitoring the environmental for potential sources of infection.

5.6.2 Coronavirus

The literature suggests that the different strains of coronavirus are closely related. In this study we identified coronavirus like particles in faeces that were not recognized by three different PCR assays. One of these assays is reported to pick up all strains of coronavirus. This finding suggests there is an untyped coronavirus, or a morphologically similar virus in Australia. Further work is ongoing with the diagnostic material collected to determine the nature of this virus.

5.6.3 Evaluation of risk factors for calf scours in beef cattle

The purpose of these studies was to develop tools to facilitate the diagnosis and control of calf scours. The tools produced provide a means of quantifying environmental contamination and pathogen shedding. The tools would also be useful for conducting epidemiological studies to better characterise the risk factors for calf scours on Australian beef farms and subsequently conduct studies to identify effective strategies to mitigate these risks.

5.6.4 Environmental sampling

The environmental sampling conducted in these studies was found to be effective for identifying pathogens on solid surfaces such as feed and water troughs, equipment, steel and concrete. Investigations into detection of pathogens in soil proved to be more complex with soil type influencing test performance. Further field testing and refinement of soil and pasture environmental sampling techniques is warranted.

5.6.5 Use of pooled samples

The current study demonstrated that the relationship between the presence of pathogens causing calf scours on farm, the level and timing of shedding of these pathogens by neonatal calves and the relationship between the level of pathogen in the faeces and clinical disease is highly variable from farm to farm. This is potentially due to many factors including herd immunity, the level of contamination that the newborn calf is exposed to and treatment and vaccination protocols within the herd. Consequently from a diagnosis and control perspective, it is more important to determine which pathogens are present on a property, than to know which calves were shedding a specific pathogen at a specific time point. Whilst the use of multiplex PCR is likely to significantly reduce the cost of running a complete suite of diagnostic tests for each calf, it still remains costly to run these tests on a representative number of calves in the herd. Consequently, further research should investigate the reliability and interpretation of tests carried out on pooled samples. The biggest challenge to achieve meaningful results from pooling is likely to be the additional dilution of pathogens that would occur.

5.6.6 Extension

The current Tips and Tools developed for industry were written prior to availability of rotavirus and BCoV vaccines and registration of halofuginone for treatment and prophylaxis of *Cryptosporidium*. It would be useful for producers for the Tips and Tools to be updated. Similarly, it would be helpful to include details in the Tips and Tools regarding the application of the diagnostic tools developed for veterinary advisors.

5.6.7 Rotavirus

Previous and current studies indicate that rotavirus is the most common cause of calf scours in Australia. Rotavirus is recognized as a resilient virus. While there are disinfectants available on the market that are reported to have activity against the virus there is a paucity of practical evidence based cleaning protocols for managing facilities and equipment on farm. The quantitative nature of the PCR assay developed here provides a means for evaluating cleaning procedures. This work would be most relevant to dairy and bobby calf producers.

5.7 Draft extension messages

In recent years new tools have become available to prevent and manage calf scours. Effective application of these tools is facilitated by identifying which pathogens are involved and targeting prophylactic and therapeutics to the relevant organisms. It is common for multiple pathogens to be involved requiring a combination of management strategies and interventions to manage and prevent disease. The newer diagnostic tests available are useful for investigating disease outbreaks as they can be applied to environmental samples to identify sources of infection and they provide a quantitative estimate of pathogen numbers which helps to clarify which pathogens are contributing most to the disease problem. Managing calf scours is confounded by pathogen interactions and producers are often frustrated by apparent failures of “Best Practice” interventions. The diagnostic tools developed provide a means of more specifically evaluating the impact of interventions on environmental contamination and shedding to verify their effectiveness or lack thereof.

5.8 What could have been improved in the project delivery

A difficult aspect of this project was the compromised health of the PhD student undertaking the work and providing the bulk of the technical input. The health problem developed during the course of the project and progressed with variable severity over the following two years, eventually resulting in cessation of work. In retrospect, it would have been better if different staffing arrangements had been organised at an earlier time. This was difficult at the time as the student would appear to recover only to relapse weeks to months later. Naturally, the student was keen to continue with the work and our inclination was to support this course to avoid compromising a PhD program. The outcome of this is that the project fell well behind on schedule and had to be completed by technical staff with limited familiarity with day to day activities previously undertaken.

What worked well in the project was the application of a robust assay delivery platform. Conducting the assay development in a diagnostic laboratory setting was a plus to the project as it provided a working perspective to the process. The bigger picture of the assay development process was that the assays needed to be robust and utilise similar sample processing techniques that are compatible with automation. The reality of diagnostic laboratories is that labour is the biggest cost. A practical combination of automation (such as use of a magnetic bead based system for nucleic acid extraction) and technical expertise helps to contain costs but also supports a rapid turn-around system. This standardised system can be enhanced over time by the inclusion of additional/improved assays without the need for any changes in nucleic acid extraction or PCR formats.

5.9 Extent to which each specific project objective was met

1. Utilise previously characterized samples to develop and validate multiplex PCR techniques for diagnosis of rotavirus, BCoV, bovine torovirus, bovine viral diarrhoea virus (BVDV) or pestivirus, *Salmonella*, K99 *E. coli*, *Cryptosporidium* & *Giardia* in faeces This is likely to be 2 multiplex PCRs; one for viruses and the other for bacteria and protozoa.
    - A number of multiplex qPCR assay combinations were developed. The initial two assays were designed to detect viruses (BVDV, BCoV, rotavirus) and bacteria/protozoa (K99 *E. coli*, *Salmonella*, *Cryptosporidium* and *Giardia*).

However, a several other combinations were also developed and utilised during the field studies, including BCoV/rotavirus/ *Cryptosporidium* and BVDV/ K99 *E.coli*/*Cryptosporidium*.

2. Report on the prevalence of bovine torovirus in the faecal samples
   - A very low prevalence of bovine torovirus was detected

3. Demonstrate the variation in faecal shedding with time, and the relationship between faecal shedding and both calf morbidity / mortality and GIT pathology.

   - Faecal shedding was monitored over time in two longitudinal studies.
   - The relationship between pathogen exposure and subsequent shedding was established as was pathogen shedding over time.
   - The case studies illustrated the impact of both effective and ineffective intervention strategies (halofuginone administration and vaccination).
   - No mortality was observed in these studies hence mortality and gastrointestinal pathology were not evaluated.

4. Investigate the potential to use these techniques to detect pathogens in samples from the environment: soil, water, paddocks, plants and man-made surfaces.

   - Potential use investigated for all of these environmental samples.
   - The practical application of environmental sampling was demonstrated in two longitudinal studies. These studies evaluated contamination of man-made surfaces and bedding for rotavirus, BCoV, *Cryptosporidium*, and *Salmonella*.

5. Develop LAMP technique for use on farm for same pathogens and samples

   - The development of a number of LAMP assays was achieved and the assays were extensively characterised. The utility of the LAMP platform has been demonstrated and constraints identified. Specific LAMP assays for pathogens would require further development and validation to be reliably used on farm for pathogen detection. The assay development and application is challenging and complex and it is a platform which is underdeveloped with respect to support. We concluded that the limitations of the technology outweighed the potential benefits and subsequently focused on the real-time PCR assays.

6 Conclusions/recommendations

6.1 General conclusions

This project has developed qRT–PCR assays with good analytic sensitivity for all major calf scour pathogens. However, the *Salmonella* qPCR had poorer sensitivity than traditional selective culture methods. This was considered to be a result of the large difference in volumes of sample used for culture compared to the very small amount used for qPCR. The suite of qPCR assays were each successfully used to test a range of clinical and

environmental samples. The PCR assays were successfully combined to achieve the following triplex combinations:

- Bacterial and protozoal qPCR: *Salmonella* spp, *E. coli* K99 / F5 and *Cryptosporidium* spp.
- Viral multiplex qPCR: *Rotavirus A* , BCoV and BVDV.

LAMP assays were also developed for all major pathogens. Generally, the assays developed all demonstrated specificity for the target organism although on occasion there was amplification observed with non-target DNA. The sensitivity of the assays varied significantly with the *Salmonella*, *E. coli*, *Giardia* and BVDV assays demonstrating a sensitivity comparable to an optimized PCR assay yet sensitivity for *Cryptosporidium*, *Rotavirus A* and BCoV were typically 10-100 fold less, which may impact on their utility for use with clinical samples. Hence whilst the LAMP platform has a level of utility in identifying pathogens in samples, the specific assays developed would require additional development and validation before they could be reliably utilised for diagnosis of pathogens that are impacting on calf health. Point of care diagnostics are an emerging area and recently this has extended to point of care PCR technology. Given the limitations of LAMP and the robustness of the PCR platform and continuing development of delivery options for PCR assays, priority was directed at the PCR assays.

The large scale prospective clinical studies demonstrated that the qPCR/qRT-PCR assays could determine shedding patterns of calf scour pathogens from clinically affected calves and establish where they were present in the calves' environment. These studies demonstrated the application of PCR technology in the field, both for epidemiological investigations and for diagnostic purposes. However, it was not possible to demonstrate a consistent association between the Ct value for a specific pathogen and any of the clinical disease parameters measured. This means that the clinical relevance of results for faecal samples collected at a single time point, as part of a calf scours diagnostic investigation, needs to be determined by understanding of the epidemiology and clinical pathology of the pathogens detected. Hence diagnostic investigations need to be thorough and complete to ensure that the contribution of all pathogens is understood.

The two field studies demonstrated that the environmental sampling techniques used were highly effective in detecting rotavirus. Environmental sampling techniques were also useful in implicating sources of *Cryptosporidium*, BCoV, and *Salmonella*. However, it should also be realised that the study has only demonstrated potential sources of pathogens in the environment. The limitation of the PCR assay is that it does not provide any indication of infectivity of the agent detected but studies in humans implicate viruses, including rotavirus, detected on environmental surfaces in maintaining disease outbreaks. Significant farm level value was derived from identifying: the pathogens involved; sources of infection and the limitations and value of management interventions. These insights were used to support the allocation of resources to control and subsequently prevent ongoing disease.

The diagnostic assays developed in this project will facilitate the disease investigation and management process. qPCR assays have enhanced sensitivity and quantitative output, and will allow detection of pathogens in the environment to determine areas with an increased infection risk. Effective and efficient on farm implementation of disease management / prevention plans requires allocation of resources to minimize disease risk by targeting

procedures and products that address the most significant risks. Conversely, it is also useful to be able to evaluate the effectiveness of the intervention. The tools developed in this project will allow for significantly improved risk assessment and intervention on farm and expand the diagnostic options available by offering the capacity to detect several pathogens simultaneously.

6.2 Future studies

This study was designed as an interim step to improve diagnostic capacity before carrying out a full epidemiological investigation into the prevention and control of neonatal calf diarrhoea in Australia suckler beef herds. The tools designed and tested in this project have significantly advanced the ability of researchers to detect and quantify both pathogens contributing to a scours outbreak and reservoirs of pathogens in the environment. As such they would be useful for conducting epidemiological studies to better characterise the risk factors for calf scours on Australian beef farms and subsequently conduct studies to identify effective strategies to mitigate these risks.

Currently, these tools are most useful for identifying pathogens on solid surfaces such as feed and water troughs, equipment, steel, and concrete. Investigations into detection of pathogens in soil proved to be more complex with soil type influencing test performance. Further field testing and refinement of soil and pasture environmental sampling techniques is warranted.

The current study demonstrated that the relationship between the presence of pathogens causing calf scours on farm and clinical disease is highly variable from farm to farm. Hence from a disease diagnosis and control perspective it is more important to determine which pathogens are present on a property, than to know which calves were shedding a specific pathogen at a specific time point. Whilst the use of multiplex PCR is likely to significantly reduce the cost of running a complete suite of diagnostic tests for each calf, it would still be costly to run these tests on a representative number of calves in the herd. Consequently, further research should investigate the reliability and interpretation of tests carried out on pooled samples.

An unexpected finding of this study was the identification of coronavirus like particles in faeces that were not recognized by three different PCR assays. One of these assays is reported to pick up all strains of coronavirus. This finding suggests there is an untyped coronavirus, or a morphologically similar virus in Australia. Further work is ongoing with the diagnostic material collected to determine the nature of this virus.

Previous and current studies indicate that rotavirus is the most common cause of calf scours in Australia. Rotavirus is recognized as a resilient virus. While there are disinfectants available that are reported to have activity against this virus there is a paucity of practical evidence based cleaning protocols for managing facilities and equipment on farm. The quantitative nature of the PCR assay developed here provides a means for evaluating cleaning procedures. This work would be most relevant to dairy and bobby calf producers, but would also apply to equipment used to treat sick calves and the protective clothing of farm workers on suckler beef properties.

The qPCR assay developed in this project for *Salmonella* had reduced sensitivity compared with selective culture. This largely reflected sample dilution during sample processing and

has been reported with other *Salmonella* PCR assays. Recently published research in this area suggests that RT PCR assays for *Salmonella* may be more sensitive, presumably because there are more copies of RNA produced than DNA. It would be useful to investigate the improvement in sensitivity that this assay might give in comparison with a ‘pre-PCR” enrichment step.

7 Key messages

The rapid turnaround of diagnostic tests submitted to the laboratory that results from the use of PCR should be of significant benefit to producers, particularly those facing a sudden case of calf scours with high morbidity or mortality.

This project was designed as an interim step to a full epidemiological study, focussing on diagnosis rather than farm level change. The key messages reinforce some of those published in the Tips and Tools and other extension material produced by B.AHW.0106 in 2006.

Specifically:

1. The complexity of neonatal calf diarrhoea means that diagnostic investigations need to be thorough and complete. Diagnostic tests should be run for all of the major pathogens that could be involved, as it is likely that there is multi-pathogen aetiology.

    - This recommendation is even more pertinent as in the last few years the range of pathogens that can be controlled by vaccination has significantly increased.

2. The dam at calving, older calves, and the environment are significant reservoirs of pathogens. In the first 4 weeks of life it is important to minimise exposure of calves to calving cows, keep the age range of calves within a group at less than 3 weeks and isolate calves from areas where there is likely to be a significant build-up of pathogens.

3. Pathogens may often be present without causing disease. Disease is more likely to occur if there is a concurrent reduction in immunity or if the pathogen load in the environment is high. Therefore producers should continue to implement strategies to minimise infection and maximise calf immunity as recommended by current extension material.

The clinical studies in this project confirmed that calf scours is a complex disease and the pathogens, predisposing factors and presentation vary from property to property. The tools developed in this project will allow producers to better define the pathogens present on the property and the likely sources of those pathogens. Using this information they can work with their advisors to develop a tailored approach to minimising calf scours. This will result in significant benefit on those properties where calf scours has been a significant and ongoing financial and emotional challenge.

8 Bibliography

Abeywardena, H., Jex, A.R. & Gasser, R.B., 2015. *A Perspective on Cryptosporidium and Giardia, with an Emphasis on Bovines and Recent Epidemiological Findings*, Elsevier Ltd. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0065308X1500010X.

Aebischer, A. et al., 2014. Rapid genome detection of Schmallenberg virus and bovine viral diarrhea virus by use of isothermal amplification methods and high-speed real-time reverse transcriptase PCR. *Journal of Clinical Microbiology*, 52(6), pp.1883–1892.

Al Mawly, J. et al., 2014. Prevalence of endemic enteropathogens of calves in New Zealand dairy farms. *New Zealand veterinary journal*, 0169(September 2014), pp.1–18. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25237728.

Al Mawly, J. et al., 2015. Risk factors for neonatal calf diarrhoea and enteropathogen shedding in New Zealand dairy farms. *The Veterinary Journal*, 203(2), pp.155–160. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1090023315000283.

Al Mawly, J. et al., 2013. Utility of halofuginone lactate for the prevention of natural cryptosporidiosis of calves, in the presence of co-infection with rotavirus and *Salmonella typhimurium. Veterinary Parasitology*, 197(1-2), pp.59–67. Available at: http://dx.doi.org/10.1016/j.vetpar.2013.04.029 [Accessed November 21, 2013].

Amar, C.F.L. et al., 2005. Detection of viral, bacterial, and parasitological RNA or DNA of nine intestinal pathogens in fecal samples archived as part of the english infectious intestinal disease study: assessment of the stability of target nucleic acid. *Diagnostic molecular pathology : the American journal of surgical pathology, part B*, 14(2), pp.90–96.

Baque, R.H. et al., 2011. A real-time RT-PCR method to detect viable *Giardia lamblia* cysts in environmental waters. *WR*, 45(10), pp.3175–3184. Available at: http://dx.doi.org/10.1016/j.watres.2011.03.032.

Bartels, C.J.M. et al., 2010. Prevalence, prediction and risk factors of enteropathogens in normal and non-normal faeces of young Dutch dairy calves. *Preventive veterinary medicine*, 93(2-3), pp.162–169. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19819574 [Accessed December 18, 2013].

Bech, T.B. et al., 2010. Transport and distribution of *Salmonella enterica* serovar typhimurium in loamy and sandy soil monoliths with applied liquid manure. *Applied and Environmental Microbiology*, 76(3), pp.710–714.

Becher, K. a et al., 2004. Molecular epidemiology of *Giardia* and *Cryptosporidium* infections in dairy calves originating from three sources in Western Australia. *Veterinary parasitology*, 123(1-2), pp.1–9.

Bendali, F. et al., 1999. Pattern of diarrhoea in newborn beef calves in south-west France. *Veterinary Research*, 30(1), pp.61–74.

van Blerk, G.N. et al., 2011. Rapid and specific detection of *Salmonella* in water samples using real-time PCR and High Resolution Melt (HRM) curve analysis. *Water science and technology : a journal of the International Association on Water Pollution Research*, 64(12), pp.2453–9.

Booth, T.F. et al., 2005. Detection of airborne severe acute respiratory syndrome (SARS) coronavirus and environmental contamination in SARS outbreak units. *The Journal of infectious diseases*, 191(9), pp.1472–1477.

Bøtner, A. & Belsham, G.J., 2012. Virus survival in slurry: Analysis of the stability of foot-and-mouth disease, classical swine fever, bovine viral diarrhoea and swine influenza viruses. *Veterinary Microbiology*, 157(1-2), pp.41–49.

Bridger, J.C., 1994. A definition of bovine rotavirus virulence. *Journal of General Virology*, 75(10), pp.2807–2812.

Butz, a M. et al., 1993. Prevalence of rotavirus on high-risk fomites in day-care facilities. *Pediatrics*, 92(2), pp.202–205.

Cho, Y.-I. et al., 2010. Development of a Panel of Multiplex Real-Time Polymerase Chain Reaction Assays for Simultaneous Detection of Major Agents Causing Calf Diarrhea in Feces. *Journal of Veterinary Diagnostic Investigation*, 22(4), pp.509–517.

Coura, F.M. et al., 2015. Longitudinal study of *Salmonella* spp., diarrheagenic Escherichia coli, Rotavirus, and Coronavirus isolated from healthy and diarrheic calves in a Brazilian dairy herd. *Tropical Animal Health and Production*, 47(1), pp.3–11.

Decaro, N. et al., 2008. Detection of bovine coronavirus using a TaqMan-based real-time RT-PCR assay. *Journal of virological methods*, 151(2), pp.167–71.

Decaro, N. et al., 2012. Experimental infection of cattle, sheep and pigs with “Hobi”-like pestivirus. *Veterinary microbiology*, 155(2-4), pp.165–71.

De La Fuente, R. et al., 1999. *Cryptosporidium* and concurrent infections with other major enterophatogens in 1 to 30-day-old diarrheic dairy calves in central Spain. *Veterinary Parasitology*, 80(3), pp.179–185.

Dickson, J., 1966. *A survey of beef cattle diseases in Victoria*. University of Melbourne.

Don, R.H. et al., 1991. “Touchdown” PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Research,*, 19, p.4008.

Draker, R. et al., 2006. The complete sequence of the bovine torovirus genome. *Virus Research*, 115(1), pp.56–68.

Duckmanton, L. et al., 1998. Detection of bovine torovirus in fecal specimens of calves with diarrhea from Ontario farms. *Journal of Clinical Microbiology*, 36(5), pp.1266–1270.

Echeverry, A., Loneragan, G.H. & Brashears, M.M., 2006. Survival of Escherichia coli O157 : H7 in Bovine Feces over Time under Various Temperature Conditions. *Journal of Food Protection*, 69(12), pp.2851–2855.

Fan, F. et al., 2015. Rapid and Sensitive *Salmonella* Typhi Detection in Blood and Fecal Samples Using Reverse Transcription Loop-Mediated Isothermal Amplification. *Foodborne Pathogens and Disease*, 12(9), pp.778–786.

Fan, Q. et al., 2012. A reverse transcription loop-mediated isothermal amplification method for rapid detection of bovine viral diarrhea virus. *Journal of virological methods*, 186(1-2), pp.43–8.

Fayer, R. & Nerad, T., 1996. Effects of low temperatures on viability of *Cryptosporidium parvum* oocysts. *Applied and environmental microbiology*, 62(4), pp.1431–1433.

Fischer, T.K., Steinsland, H. & Valentiner-Branth, P., 2002. Rotavirus particles can survive storage in ambient tropical temperatures for more than 2 months. *Journal of Clinical Microbiology*, 40(12), pp.4763–4764.

García, R. et al., 2010. Influence of temperature and predation on survival of *Salmonella enterica* serovar Typhimurium and expression of invA in soil and manure-amended soil. *Applied and environmental microbiology*, 76(15), pp.5025–31. Geurden, T. et al., 2007. Molecular epidemiology with subtype analysis of *Cryptosporidium* in calves in Belgium. *Parasitology*, 134(Part 14), pp.1981–1987.

Gillhuber, J. et al., 2014. Giardiosis and other enteropathogenic infections: a study on diarrhoeic calves in Southern Germany. *BMC research notes*, 7, p.112. Gunn, A.A., 2003. *Calf scours in southern Australia (Phase 1)*, Meat and Livestock Australia.

Guy, R.A. et al., 2003. Real-Time PCR for Quantification of *Giardia* and *Cryptosporidium* in Environmental Water Samples and Sewage. *Applied and Environmental Microbiology*.69 5178–5185

Haschek, B. et al., 2006. Detection of bovine torovirus in neonatal calf diarrhoea in lower Austria and Styria (Austria). *Journal of Veterinary Medicine Series B*, 53(4), pp.160–165.

Heckert, R. a et al., 1990. A longitudinal study of bovine coronavirus enteric and respiratory infections in dairy calves in two herds in Ohio. *Veterinary microbiology*, 22(2-3), pp.187–201.

Heithoff, D.M. et al., 2012. Intraspecies variation in the emergence of hyperinfectious bacterial strains in nature. *PLoS Pathogens*, 8(4).

Himathongkham, S. et al., 1999. Survival of Escherichia coli O157:H7 and *Salmonella typhimurium* in cow manure and cow manure slurry. *FEMS Microbiology Letters*, 178, pp.251–257.

House, J.K. et al., 2001. Evaluation of an autogenous *Salmonella* bacterin and a modified live *Salmonella* serotype choleraesuis vaccine on a commercial dairy farm. *American journal of veterinary research*, 62(12), pp.1897–902.

Izzo, M.M. et al., 2012. Comparison of three diagnostic techniques for detection of rotavirus and coronavirus in calf faeces in Australia. *Australian Veterinary Journal*, 90(4), pp.122–129.

Izzo, M.M. et al., 2011. Prevalence of major enteric pathogens in Australian dairy calves with diarrhoea. *Australian Veterinary Journal*, 89(5), pp.167–173

Jacobsen, C.S. & Bech, T.B., 2012. Soil survival of *Salmonella* and transfer to freshwater and fresh produce. *Food Research International*, 45(2), pp.557–566.

Kirisawa, R. et al., 2007. Detection of bovine torovirus in fecal specimens of calves with diarrhea in Japan. *Journal of Veterinary Medical Science*, 69(5), pp.471–476.

Kuczynska, E. & Shelton, D.R., 1999. Method for Detection and Enumeration of *Cryptosporidium* parvum Oocysts in Feces, Manures, and Soils. *Applied and environmental microbiology*, 65(7), pp.2820–2826.

Kuhnert-Paul, Y. et al., 2012. Cryptosporidiosis: comparison of three diagnostic methods and effects of storage temperature on detectability of cryptosporidia in cattle faeces. *Parasitology Research*, 111(1), pp.165–171.

Kváč, M., Kouba, M. & Vítovec, J., 2006. Age-related and housing-dependence of *Cryptosporidium* infection of calves from dairy and beef herds in South Bohemia, Czech Republic. *Veterinary Parasitology*, 137(3-4), pp.202–209.

Lakay, F.M., Botha, A. & Prior, B.A., 2007. Comparative analysis of environmental DNA extraction and purification methods from different humic acid-rich soils. *Journal of Applied Microbiology*, 102(1), pp.265–273.

Liang, Z. & Keeley, A., 2011. Detection of viable *Cryptosporidium parvum* in soil by reverse transcription-real-time PCR targeting hsp70 mRNA. *Applied and Environmental Microbiology*, 77(18), pp.6476–6485.

Lievaart, J.J. et al., 2013. Incidence of calf scours and associated risk factors in southern New South Wales beef herds. *Australian veterinary journal*, 91(11), pp.464–8.

Logan, C., O'Leary, J.J. & O'Sullivan, N., 2006. Real-time reverse transcription-pcr for detection of rotavirus and adenovirus as causative agents of acute viral gastroenteritis in children. *Journal of Clinical Microbiology*, 44(9), pp.3189–3195.

Lyman, W.H. et al., 2009. Prospective study of etiologic agents of acute gastroenteritis outbreaks in child care centers. *The Journal of pediatrics*, 154(2), pp.253–257.

Malik, Y.S. et al., 2013. Rapid detection of human rotavirus using NSP4 gene specific reverse transcription loop-mediated isothermal amplification assay. *Indian Journal of Virology*, 24(2), pp.265–271.

Marsh, P., Morris, N.Z. & Wellington, E.M.H., 1998. Quantitative molecular detection of *Salmonella typhimurium* in soil and demonstration of persistence of an active but non-culturable population. *FEMS Microbiology Ecology*, 27(4), pp.351–363.

McLaughlin, S.J., Kalita, P.K. & Kuhlenschmidt, M.S., 2013. Fate of *Cryptosporidium parvum* oocysts within soil, water, and Plant environment. *Journal of Environmental Management*, 131, pp.121–128.

Mohler, V.L., Izzo, M.M. & House, J.K., 2009. *Salmonella* in Calves. *The Veterinary clinics of North America. Food animal practice*, 25(1), pp.37–54.

Mullis, L. et al., 2012. Stability of bovine coronavirus on lettuce surfaces under household refrigeration conditions. *Food Microbiol*, 30(1), pp.180–186.

Nantavisai, K. et al., 2007. Evaluation of the sensitivities of DNA extraction and PCR methods for detection of *Giardia duodenalis* in stool specimens. *Journal of Clinical Microbiology*, 45(2), pp.581–583.

Ng, J. et al., 2011. Molecular characterization of *Cryptosporidium* and *Giardia* in pre-weaned calves in Western Australia and New South Wales. *Veterinary Parasitology*, 176(2-3), pp.145–150.

Nogueira Diaz, M.A. et al., 2009. Chemical composition and biological activity of some essential oils from traditional medicinal plants against clinical isolates of Staphylococcus aureus from bovine mastitis. *International Journal of Essential Oil Therapeutics*, 3(2-3), pp.119–124.

O'Carroll, J.M. et al., 1999. Effects of sample storage and delayed secondary enrichment on detection of *Salmonella* spp in swine feces. *American Journal of Veterinary Research*, 60(3), pp.359–362.

O'Handley, R.M. et al., 1999. Duration of naturally acquired giardiosis and cryptosporidiosis in dairy calves and their association with diarrhea. *Journal of the American Veterinary Medical Association*, 214(3), pp.391–6.

Olson, M.E. et al., 1999. *Giardia* Cyst and *Cryptosporidium* Oocyst Survival in Water, Soil,

and Cattle Feces. *Journal of Environment Quality*, 28(6), p.1991.

Omar, K.B., Potgieter, N. & Barnard, T.G., 2010. Development of a rapid screening method for the detection of pathogenic Escherichia coil using a combination of Colilert Quanti-Trays/2000 and PCR. *Water Science & Technology: Water Supply*, 10(1), pp.7–13.

Operario, D.J. et al., 2015. Correlation Between Diarrhea Severity and Oocyst Count via Quantitative PCR or Fluorescence Microscopy in Experimental Cryptosporidiosis in Calves. *American Journal of Tropical Medicine and Hygiene*, 92(1), pp.45–49.

Otter, J.A. et al., 2015. Transmission of {SARS} and {MERS} coronaviruses and influenza virus in healthcare settings: the possible role of dry surface contamination. *Journal of Hospital Infection*, 44, pp. 1-16

Park, S.I.S.J. et al., 2008. Molecular epidemiology of bovine toroviruses circulating in South Korea. *Veterinary Microbiology*, 126(4), pp.364–371.

Picard, C. et al., 1992. Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction. *Applied and environmental microbiology*, 58(9), pp.2717–22.

Plutzer, J. & Karanis, P., 2009. Rapid identification of *Giardia duodenalis* by loop-mediated isothermal amplification (LAMP) from faecal and environmental samples and comparative findings by PCR and real-time PCR methods. *Parasitology Research*, 104(6), pp.1527–1533.

Prado, T. et al., 2011. Quantification and molecular characterization of enteric viruses detected in effluents from two hospital wastewater treatment plants. *Water Research*, 45(3), pp.1287–1297.

Qiao, J. et al., 2012. Rapid detection of Betacoronavirus 1 from clinical fecal specimens by a novel reverse transcription loop-mediated isothermal amplification assay. *Journal of Veterinary Diagnostic Investigation*, 24(1), pp.174–177.

Ralston, B.J., McAllister, T.A. & Olson, M.E., 2003. Prevalence and infection pattern of naturally acquired giardiasis and cryptosporidiosis in range beef calves and their dams. *Veterinary Parasitology*, 114(2), pp.113–122.

Reynolds, D.J. et al., 1986. Microbiology of calf diarrhoea in southern Britain. *Veterinary Record*, 119(2), pp.34–39.

Ridpath, J.F. et al., 2014. Stability of Bovine viral diarrhea virus 1 nucleic acid in fetal bovine samples stored under different conditions. *Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc*, 26(1), pp.6–9.

Ridpath, J.F. et al., 2009. Stability of Bovine Viral Diarrhea Virus Antigen in Ear Punch Samples Collected from Bovine Fetuses. *Journal of Veterinary Diagnostic Investigation*, 21(3), pp.346–349.

Rieux, a. et al., 2013. Dynamics of excretion and molecular characterization of *Cryptosporidium* isolates in pre-weaned French beef calves. *Veterinary Parasitology*, 195(1-2), pp.169–172.

Ruest, N. et al., 1997. Morphological changes in the jejunum of calves naturally infected with *Giardia* spp. and *Cryptosporidium* spp. *Veterinary Parasitology*, 69(3-4), pp.177–186.

Ryan, U. & Cacciò, S.M., 2013. Zoonotic potential of *Giardia*. *International Journal for*

Parasitology, 43(12-13), pp.943–956.

Santamaria, J. et al., 2012. Transport and Retention of *Cryptosporidium parvum* Oocysts in Sandy Soils. *Journal of Environmental Quality*, 41(4), pp.1246–1252.

Santin, M., Trout, J.M. & Fayer, R., 2008. A longitudinal study of cryptosporidiosis in dairy cattle from birth to 2 years of age. *Veterinary Parasitology*, 155(1-2), pp.15–23.

Schlindwein, a D. et al., 2010. Detection of enteric viruses in sewage sludge and treated wastewater effluent. *Water science and technology : a journal of the International Association on Water Pollution Research*, 61(2), pp.537–44.

Schroeder, M.E. et al., 2012. Development and performance evaluation of calf diarrhea pathogen nucleic acid purification and detection workflow. *Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc*, 24(5), pp.945–53.

Sdiri-Loulizi, K. et al., 2010. Detection and molecular characterization of enteric viruses in environmental samples in Monastir, Tunisia between January 2003 and April 2007. *Journal of Applied Microbiology*, 109(3), pp.1093–1104.

Shimoda, K. et al., 1991. Stability of pathogenic bacteria from laboratory animals in various transport media. *Lab Anim*, 25(3), pp.228–231.

Silverlås, C., Björkman, C. & Egenvall, A., 2009. Systematic review and meta-analyses of the effects of halofuginone against calf cryptosporidiosis. *Preventive veterinary medicine*, 91(2-4), pp.73–84.

Singh, G. et al., 2012. Bio-capture of S. Typhimurium from surface water by aptamer for culture-free quantification. *Ecotoxicology and environmental safety*, 78, pp.320–6.

Sloane, R., 2006. *Calf Scours: A Survey of Producers in Southern Australia*, Meat & Livestock Australia.

Smith, D.R., 2012. Field disease diagnostic investigation of neonatal calf diarrhea. *The Veterinary clinics of North America. Food animal practice*, 28(3), pp.465–81.

Smith, E.M. & Gerba, C.P., 1980. Survival and detection of rotaviruses in the environment. In *Third International Symposium on Neonatal Diarrhoea*. University of Saskatchewan, pp. 67–81.

Smith, H. V & Nichols, R.A.B., 2010. *Cryptosporidium*: Detection in water and food. *Experimental Parasitology*, 124(1), pp.61–79.

Staggs, S.E. et al., 2013. The applicability of taqman-based quantitative real-time PCR assays for detecting and enumerating *Cryptosporidium* spp. oocysts in the environment. *PLoS ONE*, 8(6).

Stroup, S.E. et al., 2012. Dual probe DNA capture for sensitive real-time PCR detection of *Cryptosporidium* and *Giardia*. *Molecular and Cellular Probes*, 26(2), pp.104–106.

Stroup, S.E. et al., 2006. Real-time PCR detection and speciation of *Cryptosporidium* infection using Scorpion probes. *Journal of Medical Microbiology*, 55(9), pp.1217–1222.

Sutlovic, D., Gojanovic, M.D. & Andelinovic, S., 2007. Rapid extraction of human DNA containing humic acid. *Croatica Chemica Acta*, 80(1), pp.117–120. Available at: <Go to ISI>://000247410300017.

Tan, T.Y. et al., 2014. Evaluation of bacterial recovery and viability from three different swab transport systems. *Pathology*, 46(3), pp.230–233.

Trotz-Williams, L. a et al., 2011. Efficacy of halofuginone lactate in the prevention of cryptosporidiosis in dairy calves. *The Veterinary record*, 168(19), p.509.

Trotz-Williams, L.A. et al., 2007. Calf-level risk factors for neonatal diarrhea and shedding of *Cryptosporidium parvum* in Ontario dairy calves. *Preventive Veterinary Medicine*, 82(1-2), pp.12–28.

Trotz-Williams, L.A. et al., 2005. Prevalence of *Cryptosporidium parvum* infection in southwestern Ontario and its association with diarrhea in neonatal dairy calves. *The Canadian veterinary journal. La revue vétérinaire canadienne*, 46(4), pp.349–51.

Verweij, J.J. et al., 2003. Real-time PCR for the detection of *Giardia lamblia*. *Molecular and Cellular Probes*, 17(5), pp.223–225.

De Waele, V. et al., 2010. Control of cryptosporidiosis in neonatal calves: Use of halofuginone lactate in two different calf rearing systems. *Preventive Veterinary Medicine*, 96(3-4), pp.143–151.

Walker, P.G. et al., 1998. A reliable, practical, and economical protocol for inducing diarrhea and severe dehydration in the neonatal calf. *Canadian Journal of Veterinary Research*, 62(3), pp.205–13.

Wang, X. et al., 2005. Concentration and detection of SARS coronavirus in sewage from Xiao Tang Shan Hospital and the 309th Hospital of the Chinese Peoples Liberation Army. *Water Science & Technology*, 52(8), pp.213–221.

Warnke, P., Warning, L. & Podbielski, A., 2014. Some are more equal--a comparative study on swab uptake and release of bacterial suspensions. *PloS one*, 9(7), p.e102215.

West, D.M. et al., 2007. Rapid detection of Escherichia coli virulence factor genes using multiplex real-time TaqMan® PCR assays. *Veterinary Microbiology*, 122(3-4), pp.323–331.

Winkworth, C.L., Matthaei, C.D. & Townsend, C.R., 2008. Prevalence of *Giardia* and *Cryptosporidium* spp in calves from a region in New Zealand experiencing intensification of dairying. *New Zealand veterinary journal*, 56(1), pp.15–20.

Wu, H.M. et al., 2005. A Norovirus Outbreak at a Long-Term–Care Facility: The Role of Environmental Surface Contamination •. *Infection Control and Hospital Epidemiology*, 26(10), pp.802–810.

Xie, Z. et al., 2012. Reverse transcription loop-mediated isothermal amplification assay for rapid detection of Bovine Rotavirus. *BMC Veterinary Research*, 8(1), p.133. Available at: BMC Veterinary Research.

Yang, M. et al., 2011. Detection of rotaviruses in river water and sewage water in Shanghai by RT-PCR. *ISWREP 2011 - Proceedings of 2011 International Symposium on Water Resource and Environmental Protection*, 3, pp.2007–2009.

Yang, W. et al., 2014. Detection methods for milk pathogenic bacteria by loop-mediated isothermal amplification. *BioScience Trends*, 8(6), pp.316–321.

Zhang, S.Q. et al., 2014. Comparison of conventional RT-PCR, reverse-transcription loop-mediated isothermal amplification, and SYBR green I-based real-time RT-PCR in the rapid detection of bovine viral diarrhea virus nucleotide in contaminated commercial

bovine sera batches. *Journal of Virological Methods*, 207, pp.204–209.

Zhuang, L. et al., 2014. Detection of *Salmonella* spp. by a loop-mediated isothermal amplification (LAMP) method targeting *bcfD* gene. *Letters in Applied Microbiology*, 59(6), pp.658–664.

9 Appendices

9.1 Appendix 1: Primer and probe sequences for singleplex assays

Pathogen (target gene)	Primer or probe (5'/3' labels)	Sequence (5'-3')
Escherichia coli K99^{+} (K99 / F5)	K99-fwd	GCTATTAGTGGTCATGGCACTGTAG
	K99-rev	TTTGTTTTCGCTAGGCAGTCATTA
	K99-probe (FAM/BHQ1)	ATTTTAAACTAAAACCAGCGCCCGGCA
Salmonella (Stn)	Stn-fwd	GCCATGCTGTTCGATGAT
	Stn-rev	GTTACCGATAGCGGGAAAGG
	Stn-probe (CAL Fluor Gold 540/BHQ-1	TTTTGCACCACMGCCAGCCC
Cryptosporidium (COWP)	*Cryptosporidium*-fwd	CAAATTGATACCGTTTGTCCTTCTGT
	Cryptosporidium-rev	GGCATGTCGATTCTAATTCAGCT
	Cryptosporidium-probe (Quasar 670/BHQ2)	TGCCATACATTGTTGTCCTGACAAATT
Cryptosporidium (18srRNA)	CVN F	GGTTGTATTTATTAGATAAAGAAC
	CPN R	AGGCCAATACCCTACCGTCT
	CPN pb	ACATATCATTCAAGTTTCTGACCTATCAGC
Giardia (18s rRNA)	*Giardia*-80F	GACGGCTCAGGACAACGGTT
	Giardia-127R	TTGCCAGCGGTGTCCG
	Giardia-105T (TAMRA/BHQ2)	CCCGCGGCGGTCCCTGCTAG
Bovine *Rotavirus A* (NVP3)	GA rota NSP3-F	ACCATCTACACATGACCCTC
	GA rota NSP3-R	GGTCACATAACGCCCC

Pathogen (target gene)	Primer or probe (5'/3' labels)	Sequence (5'-3')
	Rota A-probe (TAMRA/BHQ2)	ATGAGCACAATAGTTAAAAGCTAACACTGTCAA
BCoV	EMAIBC-F	GCGTCCAAAGGCTATATTGCTAA
	EMAIBC-R	CCCAACATTTGGATTCTGACATAA
	EMAIBC-P (Quasar670/BHQ2)	TGCCTTTCAACAGGTATT
Bovine Torovirus	BToV_M F1 BToV_M F2	GCTTACACGCGGTCCACTACT GCCTATTCGCGATCAACTACT
	BToV_M R	AAGATGGTATTTTGCAGTGCTGTTA
	BToV_M P (FAM-BHQ1)	CCAGCGCCGCTAAAGTAGCTGCA
Bovine Viral Diarrhoea Virus (BVDV)	BVD 190 F	GRA GTC GTC ART GGT TCG AC
	V326 (R)	TCA ACT CCA TGT GCC ATG TAC
	TQ pesti (FAM-BHQ1)	TGC YAY GTG GAC GAG GGC ATG C

9.2 Appendix 3: LAMP target DNA/RNA sequence for which LAMP primer sequences were designed

Target Organism	Target DNA/RNA sequence	Accession Number
Salmonella	enterotoxin gene (stn)	L16014.1
E. coli K99	K99 / F5 plasmid (K99)	M35282
Giardia duodenalis	ssrRNA	M54878
Cryptosporidium	*Cryptosporidium* oocyst wall protein (COWP)	AF248743
Rotavirus A	VP6	GU984757
BCoV	polyprotein ORF 1a/b	FJ938066.1
BVDV	complete genome	M31182.1

9.3 Appendix 4: LAMP Primer sets chosen for assay development following initial functional assessment

Target Organism	Primer sets chosen after functional assessment	
Salmonella	Stn F3 (2)	5' TCGGTAACAGTGATGATAACG 3'
	Stn B3 (2)	5' CGAATTGCTCGAACTGGTA 3'
	Stn FIP (2)	5' GCTGACTCAGGTGCTGTTGAGCCTCTACGCTAATCGTTC 3'
	Stn BIP (2)	5' GTCTCATTCGCCGTGAATCTCATGAATCTGGTCAGTCAGGAT 3'
	Stn LF (2)	5' CGCTATTCATGCGATTGGC 3'
	Stn LB (2)	5' ACTGAATCTCTCTGCTTTGGG 3'
E. coli K99	K99 F3 (3)	5' GCGACTACCAATGCTTCT 3'
	K99 B3 (3)	5' GGTATCCTTTAGCAGCAGTATT 3'
	K99 FIP (3)	5' AATCCACTACAGTGCCATGACCCCTGAGGTCAATGGTAATCG 3'
	K99 BIP (3)	5' CGCCCGGCAGTAATGACTGTTCATAGAACCAGACCAGTC 3'
	K99 LF (3)	5' CAGCCTGCCCAAGATCTATAG 3'
	K99 LB (3)	5' CTAGCGAAAACAAATGCTCGT 3'
Giardia duodenalis	GF3 (3)	5' GATGATCCCGCCGAGA 3'
	G B3 (3)	5' GATTGAGCCGCAGACTC 3'
	GFIP (3)	5' CCGTTTACGGCCGGGAATCCGCCTCTGTCAATCAAG 3'
	GBIP (3)	5' AGGGAAACCGGGAGGCTTGCCTTCAAGTTTCAGCC 3'
	G LF (3)	5' GTGGTGTCTGATCGCCTT 3'
	GLB (3)	5' GGGAGTATGGCCGCAA 3'
Cryptosporidium	Cr F3 (2)	5' ACACAAAAGGCCAGGTAC 3'
	Cr B3 (2)	5' TCGTATCCTGGTGGACAT 3'
	Cr FIP (2)	5' GGACACAAACTCCATTCTCCATTAAACGTATGTTGAAACTCCGC 3'
	Cr BIP (2)	5' AGGTTCCTCCTATGCCTTTCTGTGCTTTAGTGGTGCTGTT 3'
	Cr LF (2)	5' CCTGGAGGGCAGCTTTCTA 3'
	Cr LB (2)	5' TCAGGAACCAGCAATCTATCACG 3'
Rotavirus A	R F3 (3)	5' ACAATCATAGCTAGAAACTTTGA 3'
	R B3 (3)	5' CACATTTGCTAGCATCGTT 3'
	R FIP (3)	5' CGGAAATAATGCCGCTACCGATAAGATTGTCGTTCCAGTTGA 3'
	R BIP (3)	5' CACAGCCATTTGAACATCATGCTACCTTGCATCGGCAAGTACG 3'
	R LF (3)	5' TGGTGTCATATTTGGTGGTCTCA 3'
	R LB (3)	5' AGTGGGACTCACACTTAGAATTGA 3'
BCoV	Co F3 (2)	5' GGGTTGGGATTATCCTAAGTG 3'
	Co B3 (2)	5' GTAGTTGCATCACCACTACTAG 3'
	Co FIP (2)	5' TTGCGAACAACATGCCTCATGATCGTGCTATGCCAAACAT 3'
	Co BIP (2)	5' ATCGACTTGCGAATGAATGCGGCCACCAGGCTTAACATAA 3'
	Co LF (2)	5' CGAGCCAAAACCAGACTACT 3'
	Co LB (2)	5' TGTTATGTGTGGTGGCTGTTA 3'
BVDV	P F3 (2)	5' ATCTAGCCACCGATATAGAACT 3'
	P B3 (2)	5' CTCTAGCTTGTGTTACCACG 3'
	P FIP (2)	5' TCCACTCATGGCGTTGAAGTCCAATTCATGGTATGATGGATGC 3'
	P BIP (2)	5' ATGGTTGGTGCAACTGGTACAACTCAGTGAGATTGGCTTGG 3'
	P LF (2)	5' ACGTGTAGTTGGTCTTCTCAC 3'
	P LB (2)	5' TGAACCCTGGATTCTAGTCATG 3'