Development of Multiplex Real-Time PCR Screens for the Diagnosis of Feline and Canine Infectious Disease

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Abstract

Infectious disease is a significant cause of morbidity and mortality in cats and dogs. The diagnosis of the causative agent is essential to allow for the appropriate clinical intervention, to reduce infection spread, and also to support epidemiological studies which in turn will better the understanding of infectious disease transmission and control. The polymerase chain reaction (PCR) has replaced traditional detection methods as the gold standard diagnostic technique for many pathogens, however replacing traditional detection methods with panels of internally controlled multiplex real-time PCR screens designed along syndromic lines is not widespread. The aim of this thesis is to undertake the development phase of a wider project that intends to develop a broad panel of syndrome based real-time PCR multiplex assays for infectious diseases in cats and dogs.

In order to achieve this, appropriate extraction and real-time PCR platforms and reagents were chosen. The aim of this project was to begin the development of five syndrome based panels of multiplex screens:- feline respiratory disease, feline conjunctivitis, feline anaemia, feline gingivitis, and feline and canine gastroenteritis. The multiplex assays were optimised and then evaluated through a series of experiments to determine the endpoint sensitivity, specificity and robustness of each multiplex in comparison to the single assays. The full optimisation and pre-clinical validation assessment of five multiplex assays was completed in the timescale of this project; the feline respiratory screen, the feline conjunctivitis screen and the feline/canine gastroenteritis 1, 2 and 3 screens.

This study highlighted some of the difficulties that can be encountered when developing in-house multiplex real-time PCR assays. The main limitations were the lack of readily available positive control material, published assays and sequence data. The results of this study highlight that the development of an in-house multiplex real-time PCR diagnostic service can be at times difficult and occasionally time-consuming. A significant finding of this study was that PCR may not be as sensitive as virus isolation in the detection of feline calicivirus (FCV). It is hoped that further work including additional sequencing will aid in the development of a more sensitive FCV PCR assay. In-house multiplex real-time PCR should bring many advantages over current veterinary diagnostic assays. This will in turn aid in the treatment and clinical management of the animal, and increase our understanding of infectious disease in cats and dogs.

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Abbreviations

AAD: Antibody associated diarrhoea

ABCD: Advisory Board on Cat Diseases

BLAST: Basic Local Alignment Search Tool

CoV: Co-efficient of variation

CCoV: Canine coronavirus

cDNA: Circular DNA

CMt: *Candidatus Mycoplasma haemomintum*

CMhm: *Candidatus Mycoplasma turicensis*

CPV: Canine parvovirus

Ct: Cycle threshold

DNA: Deoxyribonucleic acid

EM: Electron microscopy

FCV: Feline calicivirus

FCoV: Feline coronavirus

FECV: Feline enteric coronavirus

FeLV: Feline leukemia virus

FHV: Feline herpesvirus

FIPV: Feline infectious peritonitis virus

FIV: Feline immunodeficiency virus

FPV: Feline parvovirus

FRET: Fluorescence resonance energy transfer

F Primer: Forward primer

GE: Gastroenteritis

GVDS: Glasgow Veterinary Diagnostic Service

HAI: Haemagglutination inhibition

HIV: Human immunodeficiency virus

IC: Internal control

MGB:	Minor groove binder
Mhf:	*Mycoplasma haemofelis*
mRNA:	Messenger RNA
NOAH:	National Office of Animal Health
NGS:	Next generation sequencing
ORF:	Open reading frame
PCR:	Polymerase chain reaction
QC:	Quality control
RNA:	Ribonucleic acid
RT:	Reverse transcriptase
R Primer:	Reverse primer
***Taq*:**	*Thermus aquaticus*
VSD:	Virulent systemic disease
WoSSVC:	West of Scotland Specialist Virology Centre
WSAVA:	World Small Animal Veterinary Association
ZN:	Ziehl-Neelsen

CHAPTER 1
Introduction

CHAPTER 1

1. Introduction

1.1 Importance of feline and canine infectious disease

Infectious disease is a significant cause of morbidity and mortality in cats and dogs. Viruses are the major cause of disease although bacteria, parasites and fungi are commonly implicated. To combat these infectious agents, anti-bacterial, anti-viral and anti-parasitic medicines and vaccines have been developed and marketed for companion animal use. Figures published by the National Office of Animal Health (NOAH) who represent companies that research, develop, manufacture and market animal medicines in the UK, show that £554 million was spent over the 12 month period between September 2012 and 2013 on animal medicines, 53.5% of which has been spent on companion animals such as cats and dogs (NOAH, 2014). This is a rise of 32% since 2005 (£374 million). When established in 1986, NOAH reported that the majority of animal medicine costs (70%) were for livestock animals, therefore the data above highlights a significant shift in the market. These figures can be partially attributed to the fact that 77% of UK companion animal owners regard their pet's health as important as, or more important than, their own, with 16% claiming to visit their vet more than their own doctor (NOAH, 2014).

Despite the willingness of owners to invest in their pets' health, infectious agents remain a significant cause of disease. Vaccines are not available for all known pathogens; in any event, no vaccine is 100% protective, and vaccine breakthrough infections can occur (Chalmers, 2006). Many human anti-viral agents are not suitable for veterinary use, whereas only feline recombinant interferon omega (rfeIFN-ω), Virbagen Omega (Virbac, Bury St. Edmunds, UK), is licensed for use in veterinary species. The movement towards multi-animal and/or indoor households and the rise in shelter accommodation readily facilitates the transmission of many infectious agents. Some infections can rapidly become enzootic within these multi-animal environments, such as feline enteric coronavirus infection.

Rapid, sensitive and specific detection of the causative agent is vital to allow for the appropriate clinical intervention (Greiner and Gardener, 2000; Zarlenga and Higgins, 2001; Dahlhausen, 2010; Belak *et al,* 2013). It is also important for infection control, and for epidemiological studies, to improve our understanding of infectious disease transmission

and prevalence in cats and dogs. Detection of veterinary infectious agents has traditionally relied upon techniques such as virus isolation, bacterial culture, serological assays, electron microscopy and others. These traditional techniques can be hugely labour intensive and time consuming. These detection methods can also be insensitive and occasionally non-specific. The polymerase chain reaction (PCR) is now being used more widely in veterinary diagnostics, especially with the advent of real-time PCR which provides rapid results, from receiving the sample into the laboratory a result can be obtained in as little as two to three hours. Since PCR assays can be designed to detect DNA or RNA (RT-PCR), viruses, bacteria, parasites or fungi, multiple pathogens can be targeted from a single sample. A further benefit to real-time PCR is the ability to multiplex more than one single PCR assay together to allow for the simultaneous detection of multiple pathogens in one reaction well.

1.2 Important feline and canine infectious diseases

1.2.1 Feline respiratory disease

Respiratory disease in cats is common and presents particular management problems in multi-cat environments such as shelters, breeding colonies and boarding catteries. The most significant pathogens are feline herpesvirus (FHV) and feline calicivirus (FCV); however the bacterium *Bordetella bronchiseptica (B. bronchiseptica)* is also considered a primary respiratory pathogen (Table 1.1). Feline herpesvirus and FCV infections can result in severe disease, which can be fatal in young, immunocompromised or unvaccinated animals. The prevalence of all three pathogens generally increases proportionally to the number of cats housed in a group. The prevalence of FHV is around 20%, with increased risk in cat shelters (reviewed by Thiry *et al,* 2009). The prevalence of FCV has been found to range between 25% and 40%, with prevalence within individual colonies ranging from 50 to 90% (reviewed by Radford *et al,* 2009). For *B.bronchiseptica,* as with FHV and FCV, prevalence is variable between studies and the size of group of cats, prevalence has been found to be between 0.4% and 19% in cats (reviewed by Egberink *et al,* 2009).

Table 1.1 Important veterinary pathogens

Syndrome	Pathogen	Vaccine available?*		Traditional Gold standard
		Cat	**Dog**	
Respiratory	**Feline herpesvirus**	Core	n/a	Virus isolation
	Feline calicivirus	Core	n/a	Virus isolation
	Bordetella bronchiseptica	Non-core	Non-core	Culture
Conjunctivitis	**Feline herpesvirus**	Core	n/a	Virus isolation
	Mycoplasma felis	n/av	n/a	Culture
	Chlamydophila felis	Non-core	n/a	Culture
Gastroenteritis	**Parvovirus**	Core	Core	Electron microscopy on faeces
	Salmonella	n/av	n/av	Culture
	Campylobacter	n/av	n/av	Culture
	Coronavirus	n/l	Non-core	None
	Cryptosporidium parvum	n/av	n/av	Ziehl-Neelsen smear
	Giardia lamblia	n/av	n/av	Zinc sulphate floatation
	Tritrichomonas foetus	n/av	n/av	Microscopy
Anaemia and Gingivitis	**Feline leukaemia virus**	Non-core	n/a	Virus isolation
	Feline immunodeficiency virus	n/l	n/a	Virus isolation
	Feline infectious peritonitis (FCoV)	n/l	n/a	None, histopathology
	Feline calicivirus	Core	n/a	Virus isolation
	Haemoplasmas	n/av	n/a	Microscopy

* only applicable to the UK. n/a: not applicable; n/l: not licenced; n/av: not available; FCoV: feline coronavirus

This table summarises important veterinary pathogens of cats and dogs, the syndromes that they cause, the vaccines available, and the traditional gold standard method for diagnosis.

1.2.1.1 Feline herpesvirus

Feline herpesvirus is an alpha-herpesvirus containing double stranded (ds) DNA with a glycoprotein-lipid envelope and little strain variation, antigenically all FHV strains belong to one serotype (Maggs, 2005; Thiry *et al*, 2009). Feline herpesvirus causes "cat-flu", often accompanied by systemic signs of disease such as fever, depression and anorexia. Following recovery, the virus remains latent within the nerve ganglia of the head and is periodically shed at times of stress. During reactivation, few particles are shed and for a very short time. Frequently, reactivating infection is associated with single presentations such as conjunctivitis, corneal ulceration, rhinitis or sinusitis. The gold standard detection method for FHV has been virus isolation; however this has widely been replaced with PCR (Burgesser *et al*, 1999). Treatment for cats is mainly supportive; however antiviral treatments such as famciclovir have been shown to be effective in resolving clinical signs (Malik *et al*, 2009; Thomasy *et al*, 2011). A vaccine for FHV is available (Table 1.1) and recommended (Thiry et al, 2009; Day, Horzinek and Schultz, 2011); this vaccine is given as a trivalent vaccine with FCV and feline parvovirus (FPV).

1.2.1.2 Feline calicivirus

Feline calicivirus is a small, non-enveloped, single stranded (ss) RNA virus, and a member of the vesivirus genus of the *Calicivirus* family. Infection with FCV causes a range of clinical signs due to differences of tropism and virulence in different strains (Gaskell, Dawson and Radford, 2011). Some strains are non-pathogenic whereas others are more virulent. Many vaccinated cats can become infected due to variation of strains as the vaccine strains do not cross-protect against all strains or the virulent strains (Radford *et al*, 2007). FCV has a well characterised carrier state (Radford, 1998) and asymptomatic cats can shed virus. In addition, vaccinated cats can become infected with field strains without showing clinical signs. Feline calicivirus infection results in less severe respiratory disease than FHV infection. In recent years there have been an increasing number of reports of highly virulent FCV haemorrhagic strains causing systemic infections with high mortality (Meyer *et al*, 2011), these strains can cause virulent-systemic disease (VSD) and can infect vaccinated cats. There has been no consistent molecular difference between haemorrhagic strains and other strains found, and there is no consistent genetic motif that differentiates between haemorrhagic and non-haemorrhagic strains, therefore VSD cannot be identified by molecular typing methods (Hurley *et al*, 2003). However these strains are genetically different from vaccine and non-haemorrhagic strains and differ from outbreak to outbreak suggesting that these strains evolve independently (Coyne *et al*, 2006; Porter *et al*, 2008).

The gold standard for FCV detection is virus isolation. Treatment for FCV-related respiratory disease could include antivirals (i.e. rfeIFN-ω), however no large properly controlled trials have not been carried out to demonstrate their efficacy. Prophylaxis is available as a trivalent vaccine with FHV and FPV (Table 1.1) and is recommended (Radford *et al*, 2010; Day, Horzinek and Schultz, 2011).

1.2.1.3 *Bordetella bronchiseptica*

Bordetella bronchiseptica is a bacterial pathogen of cats and dogs that can cause a wide range of respiratory signs including mild illness, fever and coughing, to severe pneumonia and death, especially in the young and immunocompromised. *Bordetella bronchiseptica* is an aerobic Gram-negative coccobacillus. The gold standard detection method is bacterial culture. Animals with severe *B. bronchiseptica* infection require antimicrobial therapy, supportive therapy and intensive nursing care. It should be noted that *B.bronchiseptica* can be an opportunistic pathogen in humans therefore infection in animals may have public health implications. Treatment with antibacterial therapy is indicated to prevent infection progressing to colonise the lower respiratory tract (Egberink *et al*, 2009). A vaccine is available (Table 1.1) but is generally recommended only if a cat is to be boarded in a cattery (Day, Horzinek, and Schultz, 2011).

1.2.2 Feline conjunctivitis

Feline herpesvirus together with *Chlamydophila felis* (*C. felis*) are the most common causes of conjunctivitis in cats; infection is particularly severe in young kittens, *Mycoplasma felis (M. felis*) is associated with feline conjunctivitis; however the importance of *M. felis* as a primary agent is not yet fully understood. Feline herpesvirus has already been discussed in Section 1.2.1.1.

1.2.2.1 *Chlamydophila felis*

Chlamydophila felis (formally *Chlamydia psittaci*) is a Gram-negative rod-shaped bacterium. It causes unilateral to bilateral ocular disease, with an initial watery discharge. Transmission is by close contact with infected cats, and ocular secretions are the most likely route of transmission as the bacterium is unable to survive outside the host (Sykes and Greene, 2011). A study by Di Franceso, Piva and Baldelli (2004) found that *C .felis*

was detected by PCR in 12 to 20% of cats with conjunctivitis, with detection low in healthy cats at two to three percent. The traditional detection method for *C. felis* was bacterial culture using McCoy's cells (Wills, Johnson and Thompson, 1984) but PCR is now the preferred method for diagnosis since culture was difficult and relatively insensitive. Treatment with antibiotics is recommended, and systemic treatment is more effective than topical treatment (Dean *et al*, 2005). A vaccine is available (Table 1.1) and recommended only in at-risk cats (Day, Horzinek and Schultz, 2011).

1.2.2.2 *Mycoplasma felis*

Mycoplasma spp. are prokaryotes, the smallest free-living microorganisms. *Mycoplasma felis* is suspected to be a significant pathogen in conjunctivitis of cats (Greene and Chalker, 2011), however little data is available on the prevalence of *M. felis*. The gold standard method traditionally used to detect *M.felis* is bacterial culture, although molecular techniques are now available (Chalker *et al*, 2004). *Mycoplasma felis* can be treated with systemic antibiotics, and no vaccine is available (Table 1.1) (Day, Horzinek, and Schultz, 2011).

1.2.3 Feline anaemia

Several infectious causes of anaemia in cats have been identified and characterised, such as feline leukaemia virus (FeLV), feline immunodeficiency virus (FIV), feline coronavirus (FCoV) and the haemoplasmas (Table 1.1).

1.2.3.1 Feline leukaemia virus

Feline leukaemia virus (FeLV) is a retrovirus that infects cats worldwide. The virus is an enveloped single stranded (ss) RNA gamma retrovirus that infects domestic cats and other exotic felidae. Over the past 25 years, the prevalence and importance of FeLV infection in Europe has greatly decreased - thanks to reliable tests, 'test-and-removal' programmes of viraemic carriers, an improved understanding of FeLV pathogenesis and the introduction of effective vaccines (Lutz *et al*, 2009).

Transmission occurs mainly through friendly contacts, such as mutual grooming, but also through bites. Infection results in several outcomes: - (1) abortive infection; (2) regressive

infection; (3) progressive infection; and (4) focal or atypical infection (Hartmann, 2011). Feline leukaemia virus can cause varying clinical signs as the clinical course of disease is determined by a combination of host and viral factors. Most persistently infected cats die within three and a half years of infection, usually as a result of immunosuppression. Feline leukaemia virus is also aetiologically associated with tumours, haematological disorders and immune-mediated disease (Hartmann, 2011). Many types of anaemia can be caused by FeLV, approximately 10% are regenerative, however the majority are non-regenerative and caused by the virus suppressing the bone marrow (Hartmann, 2011). Infection with FeLV-C infection is a very rare cause of aplastic anaemia, arising due to mutation of the envelope gene in individual animals (Onions *et al*, 1982). Cats infected with feline leukaemia virus can be diagnosed by detection of FeLV p27 antigen using ELISA, proviral DNA using PCR, or whole infectious virus by virus isolation (Hartmann, 2011). Different therapies, including antivirals are available for cats with FeLV. The therapy is largely dependent on the disease caused by the virus, however it should be noted that no properly controlled trials of antivirals have been carried out. Many vaccines are marketed for FeLV however these are not considered core vaccines (Table 1.1), and none are 100% effective (Day, Horzinek and Schultz, 2011).

1.2.3.2 Feline immunodeficiency virus

Feline immunodeficiency virus (FIV) is a retrovirus of the genus *lentivirus* that is closely related to human immunodeficiency virus (HIV), sharing a similar structure, life cycle and pathogenesis (Sellon and Hartmann, 2011). Infected cats generally remain free of clinical signs for several years, and some cats never develop disease. Typical manifestations are chronic gingivostomatitis, chronic rhinitis, lymphadenopathy, weight loss and immune-mediated glomerulonephritis. Additional clinical signs are the consequence of the cat being immunosuppressed and more susceptible to secondary infection.

Since FIV was discovered in 1986, serological studies have demonstrated that the virus is enzootic in domestic cat populations worldwide; the seroprevalence of FIV is highly variable between regions, with estimates of one to 14% in cats with no clinical signs and up to 44% in sick cats (Sellon and Hartmann, 2011). The major route of natural transmission is via biting. The clinical stages of disease are not fully defined, with different research groups having different findings. The recognised phases are like HIV; an acute phase, an asymptomatic phase and a terminal phase (AIDS). Other studies have found up to six stages (Sellon and Hartmann, 2011). Overall the clinical signs of FIV

infection are non-specific. Like FeLV, FIV suppresses the bone marrow which can result in the cat becoming anaemic. As with FeLV, therapies are available for FIV including antiviral chemotherapy and immunomodulatory therapy. The most common diagnostic assay for FIV is antibody testing. Virus isolation is time consuming, and technically challenging with limited commercial availability. A vaccine is available for FIV (Table 1.1), but is not licensed in the UK due to questionable efficacy (Day, Horzinek, and Schultz, 2011).

1.2.3.3 Feline Coronavirus

Feline coronavirus can be divided into two biotypes: feline enteric coronavirus (FECV) and feline infectious peritonitis virus (FIPV). Approximately one to three percent of FECV-seropositive cats develop lethal FIPV, with stress predisposing to the development of disease (Addie, 2011). More than one theory exists on how FIPV develops; one is that it is a result of mutations of FECV during intestinal replication, the 'in vivo mutation' theory. An alternative hypothesis is the 'circulating virulent/avirulent' theory, where both virulent and avirulent strains circulate in the cat population and susceptible cats exposed to the virulent strains develop FIPV (Pedersen, 2009). Non-effusive FIPV is the more chronic form of the disease and is associated with a non-regenerative anaemia (Addie, 2011). FIP is a fatal condition, with a median survival of nine days after diagnosis (Truyen *et al*, 2009). Some studies have been carried out to detect messenger (m)RNA in the blood (Simons *et al*, 2005; reviewed by Pedersen, 2009). However a subsequent study found that mRNA testing may not be specific (Can-Sahna *et al*, 2007), therefore the reliability of this method is still unclear and studies are ongoing. Vaccination is available in the USA and some European countries, but not in the UK (Table 1.1) (Day, Horzinek, and Schultz, 2011). Vaccination is ineffective in seropositive cats.

1.2.3.4 Feline haemoplasmas

Three haemoplasmas are known to infect cats: *Mycoplasma haemofelis* (Mhf), "*Candidatus Mycoplasma haemomintum*" (CMt), and "*Candidatus Mycoplasma turicensis*" (CMhm). Similarity between the three organisms is only approximately 83% (Messick and Harvey, 2011).

The haemoplasmas are cell wall-free bacteria that attach to red blood cells. Infections in domestic cats can induce acute haemolysis, and the disease is characterized by anorexia, lethargy, dehydration, weight loss, and sudden death (Willi *et al*, 2007). The immune system of the cat may also cause the death of red blood cells as it tries to kill the parasite attached. Anaemia is generally regenerative and can be severe. The prevalence of CMt was found to be 0.5% to 10% in sick cats, and CMhm which is thought to have low pathogenic potential (Peters *et al*, 2008) has a similar prevalence rate. *Mycoplasma haemofelis* is the least prevalent, found in 0.5% to 6% of sick cats (Sykes, 2010). The haemoplasmas cannot be cultured in artificial media and the low numbers of organisms present result in blood smear examination being an insensitive diagnostic test. PCR is used to detect the bacteria in blood when too few organisms are present to make a diagnosis using blood smears (Messick and Harvey, 2011). The haemoplasmas can be treated with doxycycline (Sykes, 2010).

1.2.4 Feline Gingivostomatitis

Despite its common occurrence, the aetiology of chronic gingivostomatitis in cats remains uncertain. Feline calicivirus, FeLV and FIV are all thought to be associated with gingivostomatitis in cats (Healey *et al*, 2007) (Table 1.1), however direct causation has not been proven (Greene and Marks, 2011). Studies have found varying prevalence of FCV, FeLV, and FIV in affected cats (reviewed in Healey *et al*, 2007). Gingivostomatitis is the most common syndrome in FIV infected cats, and FCV concurrent infection is often detected (Hartmann, 2012). However, a recent study by Belgard *et al* (2010) suggests that in fact FCV is the only significant cause of gingivostomatitis in cats. Nonetheless, the cause of gingivostomatitis in cats is controversial, highlighting the need for a rapid screening assay that can simultaneously detect FCV, FIV and FeLV in symptomatic cats, to study the prevalence of these viruses and so better the understanding the infectious causes of gingivostomatitis in cats. The recommended vaccination for these viruses is described above and in Table 1.1.

1.2.5 Feline and canine gastroenteritis

Many pathogens, including viral, bacterial, and parasitic organisms, can cause gastroenteritis in cats and dogs, with mixed infections being common (Paris *et al*, 2014). Given the large overlap in clinical signs, it is difficult to distinguish pathogens clinically. It is therefore important to correctly identify the causative pathogen(s) in order to use the

correct treatment and apply appropriate infection control measures. The key pathogens that cause feline and canine gastroenteritis are canine and feline parvovirus (CPV and FPV), *Salmonella* serovars, *Campylobacter jejuni (C.jejuni)*, feline and canine enteric coronaviruses (FCoV and CCoV), *Cryptosporidium parvum (C.parvum), Giardia lamblia* and *Tritrichomonas foetus (T.foetus).* In addition, enterotoxigenic strains of *Clostridium perfringens (C.perfringens), Clostridium difficile (C.difficile) and Escherichia coli (E.coli)* can also cause gastroenteritis in dogs, and occasionally cats (Table 1.1).

1.2.5.1 Parvovirus

Canine and feline parvovirus (CPV and FPV) are small non-enveloped DNA viruses that require rapidly dividing cells for replication. The viruses are extremely stable and resistant in the environment for up to one year (Greene, 2011[a]). Transmission of the virus occurs as a result of contact with infected faeces in the environment. The viruses primarily attack the gastrointestinal tract leading to sloughing of intestinal epithelium; the virus also reduces white blood cell numbers therefore compromising the immune system. Feline parvovirus is also known as feline panleucopenia virus; clinical signs can include high fever, depression, lack of appetite, diarrhoea and vomiting. Peracute infection causes sudden death, usually in kittens but occasionally in adults. A study by Paris *et al* (2014) found 22.1% of diarrheic cats to have FPV by real-time PCR. Transmission is usually indirect as FPV persists in the environment for up to one year. Transplacental transmission leads to cerebellar hypoplasia in kittens.

CPV-1 was discovered in 1967, and a new variant CPV-2 was discovered in 1978. By 1979 another more aggressive variant CPV-2a was discovered, and over the last 30 years, CPV-2b (the most common type) and CPV-2c (Goddard and Leisewitz, 2010; Greene and Decaro, 2011[b]) were discovered. CPV-2 is presumed to have originated from FPV; CPV-2a, b and c can infect cats, however FPV does not infect dogs (Goddard and Leisewitz, 2010). CPV infection has been associated with three main tissues, the gastrointestinal tract, bone marrow and myocardium. There is a marked variation in clinical signs including parvovirus enteritis, neurological disease, cutaneous disease, myocarditis, and thrombosis. The virulence of CPV-1 in dogs is uncertain, CPV-1 was thought to be non-pathogenic before 1985 (Lamm and Rezabek, 2008, Goddard and Leisewitz, 2010). Vaccines against FPV/CPV are available (Table 1.1) and recommended in cats and dogs (Day, Horzinek and Schultz, 2011). The gold standard for diagnosis of CPV or FPV

infection was electron microscopy (EM) on faeces and haemagglutination inhibition serological assays (HAI), however PCR has become increasingly available. Cage side tests (e.g. SNAP® Parvo Test, IDEXX Laboratories, UK) are also available. Treatment of cats and dogs is generally supportive; and rfeIFN-ω is licensed for treating CPV and FPV infection.

1.2.5.2 Feline coronavirus

Feline coronavirus is a large spherical, enveloped, positive sense single stranded (ss) RNA *alphacoronavirus* belonging to the family *Coronaviridae*. As described above FCoV can be divided into two biotypes: FECV and FIP; FECV causes a mild self -limiting gastroenteritis mainly in kittens. A recent study by Paris *et al* (2014) found a FECV prevalence of 56.9% in cats with clinical signs of gastroenteritis using real-time PCR. Prevalence of FECV has increased with more cats kept indoors, increasing the length of exposure to infected faeces (which would have been buried outdoors), and increased breeding of pedigree cats, which reduces genetic diversity and so results in a loss in immunity (Addie, 2011).

Immunofluorescence to detect serological exposure to FECV can be carried out on blood; however titres are frequently slow to fall following viral clearance. Consequently, RT-PCR is increasingly used for detection of viral RNA in faeces. Treatment of FECV is generally supportive. Vaccine availability for FCoV is described above (Table 1.1).

1.2.5.3 Canine coronavirus

Canine coronavirus (CCoV) and FCoV are closely related, both of the *alphacoronavirus* genus, subgroup B. A study by Decaro *et al*, 2010 found that recombinant CCoVs are circulating in dogs in different European countries. Canine coronavirus can affect all dogs of all ages, which differs from CPV which usually affects dogs under two years. The clinical signs of CCoV vary significantly; infected dogs usually have sudden onset diarrhoea preceded by some vomiting (Greene and Decaro, 2011[a]). There is no sensitive gold standard diagnostic method for CCoV detection. As with FCoV, treatment of CCoV is supportive and a vaccine is available (Table 1.1) however it is not recommended for routine use (Day, Horzinek and Schultz, 2011).

1.2.5.4 *Cryptosporidium parvum*

Cryptosporidium parvum is an ubiquitous coccidian genus in the phylum Apicomplexa, family Cryptosporidiidae (Scorza and Lappin, 2011). *Cryptosporidium parvum* is a zoonotic pathogen and is an important cause of gastroenteritis in a variety of animals; young and immunocompromised animals are more susceptible. In immunocompromised humans *C.parvum* can be life-threatening (Higgins, *et al* 2001; Fontaine and Guillot, 2002; Tanriverdi *et al*, 2002). Prevalence can be high, 24.4% of diarrheic cats were found to be infected by *C.parvum* by real-time PCR detection (Paris *et al*, 2014). The traditional method of detection is a ZN (Ziehl-Neeslsen)-stained faecal smear, however PCR is now often used for diagnosis. Treatment of *C.parvum* is generally supportive, with good nursing care.

1.2.5.5 *Giardia lamblia*

Giardia (also known as *Giardia intestinalis*, *Giardia lamblia*, or *Giardia duodenalis*) is the most commonly diagnosed gastrointestinal protozoan in the world, yet is poorly understood (Thompson, 2000). *Giardia* infection can show no clinical signs, however younger, immunosuppressed animals, and those living in crowded environments can show clinical signs such as chronic diarrhoea and weight loss (Scorza and Lappin, 2011). In cats the parasite can damage the small intestinal tract which can lead to maldigestion as well as malabsorption. A recent study detected *Giardia* in 20.6% of diarrheic cats by real-time PCR (Paris *et al*, 2014). In dogs there have been several studies using different diagnostic techniques giving a range of 13.0% to 19.04% (Itoh et al, 2001; Carlin et al, 2006; Olson *et al*, 2010). Fenbendazole or metronidazole are regarded as the treatments of choice.

1.2.5.6 *Tritrichomonas foetus*

Tritrichomonas foetus is a highly motile flagellate protozoan parasite that resides in the large intestine of cats, and is distinct from other *Tritrichomonas* species and not considered to be zoonotic. In the last 10 years it has emerged as a new and important cause of feline diarrhoea worldwide (Xenoulis *et al,* 2013). Infection is most common in young cats from multi-cat households, particularly pedigree breeding catteries. Clinical signs include frequent fetid diarrhoea, often with mucus, fresh blood and straining, but generally this is not severe. Traditional diagnosis of infection is usually based on direct microscopic examination of culture (Gookin *et al*, 2002) of freshly voided faeces, however PCR testing is used more widely now (Gookin, 2011). The treatment of choice is ronidazole, which

should be used with care as it is an unlicensed drug for cats with a narrow safety margin. Clinical signs are generally self-limiting in untreated cases, but may take months to resolve.

1.2.5.7 *Salmonella*

Salmonella spp. is a Gram-negative bacillus of the family *Enterobacteriaceae*. *Salmonella* is an ubiquitous pathogen that infects a variety of mammals, birds, reptiles and insects, and is a potential zoonotic infection. The clinical signs of *Salmonella* spp. are variable, diarrhoea can be watery to mucoid, with fresh blood present in severe cases, weight loss and dehydration are also common signs (Greene, 2011[b]). Complications are rare but can occur; less than 10% of cats and dogs will die due to acute disease (Greene, 2011[b]). The prevalence of the bacterium has been reported to be from one to 36% in healthy or hospitalised dogs, and one to 18% in healthy cats (Greene, 2011[b]). Paris *et al* (2014) found that 0.8% of diarrheic cats were real-time PCR positive for *Salmonella*. It is thought that cats may have natural immunity to *Salmonella*. Kittens are more likely to be clinically affected than adult cats. The standard detection method for *Salmonella* is bacterial culture. Treatment is generally supportive, antibacterial therapy may be indicated in severe illness.

1.2.5.8 *Campylobacter jejuni*

Campylobacter is a microaerophilic Gram-negative curved bacillus that causes gastroenteritis and is spread through contaminated food and water. *Campylobacter jejuni* is the strain commonly associated with disease in cats, dogs and humans. Infections in cats and dogs can often be asymptomatic, often seen with other infectious agents, and are usually seen in cats and dogs that are less than six months old (Fox, 2011). There is a wide clinical spectrum of signs from mild loose faeces to bloody mucoid diarrhoea for five to 15 days. *Campylobacter jejuni* has been isolated from 21% of cats, and 29% of dogs with diarrhoea, but only in four percent of clinically healthy animals. However prevalence data varies somewhat, some studies have found ranges of zero to 50% in healthy and ill cats (Fox, 2011). The traditional method of detection is bacterial culture. The effectiveness of antibacterial therapy in cats and dogs is unknown, however in severe disease it may be warranted (Fox, 2011).

1.2.5.9 Enterotoxigenic bacteria

Clostridium perfringens and *Clostridium difficile* can cause canine diarrhoea. These bacteria are normal constituents of the indigenous intestinal microflora and isolation rates are often similar in healthy and sick cats and dogs, therefore presenting great difficulties in determining their clinical significance (Marks, 2011). Clinical signs are extremely variable, the severity can be mild and self limiting to potentially fatal.

Clostridium perfringens is a Gram-positive spore-forming anaerobic rod. *Clostridium perfringens* enterotoxin (CPE) is an important virulence factor for *C. perfringens* type A gastrointestinal disease in humans and dogs; however, the data implicating CPE in other animal diseases remains ambiguous. PCR would be a useful tool in *C.perfringens* diagnosis as it can target the specific disease-causing toxin CPE gene. Antibacterial therapy is indicated (Marks, 2011).

Clostridium difficile is an anaerobic spore-forming Gram-positive rod. *Clostridium difficile* is the most serious cause of antibiotic-associated diarrhoea (AAD) in humans and can lead to pseudomembranous colitis. *Clostridium difficile* is resistant to most antibiotics and can therefore thrive when common antibiotics are used to treat other dog diseases. Toxins A and B (TcdA and TcdB) are thought to be the main virulence factors involved in canine *C. difficile* infection (Marks, 2011). Toxin A has been shown to cause histological damage; in contrast TcdB shows no tissue damage. Studies have suggested a synergistic effect of TcdA and TcdB, where the mucosal damage of TcdA enables the cytotoxic activity of TcdB (Marks, 2011). Treatment is usually supportive, metronidazole is highly effective with little resistance reported (Marks, 2011).

Escherichia coli (*E.coli)* is a Gram-negative rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms (endotherms). The harmless strains of *E.coli* are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K2, and by preventing the establishment of pathogenic bacteria within the intestine. There are five known groups of diarrhoeagenic *E. coli*, of which enteropathogenic *E. coli* (EPEC), and enterotoxigenic *E. coli* (ETEC) have been associated with enteric disease in dogs, with samples being ST1 and ST2 (heat stable enterotoxin) positive but LT (heat labile enterotoxin) negative (Beutin, 1999). As with *C.perfringens*

and *C.difficile* the ability of PCR to target the disease causing toxin genes ST1 and ST2 is highly advantageous. Antibacterial therapy is indicated to treat dogs infected with enterotoxic *E.coli.*

1.3 Polymerase Chain Reaction (PCR)

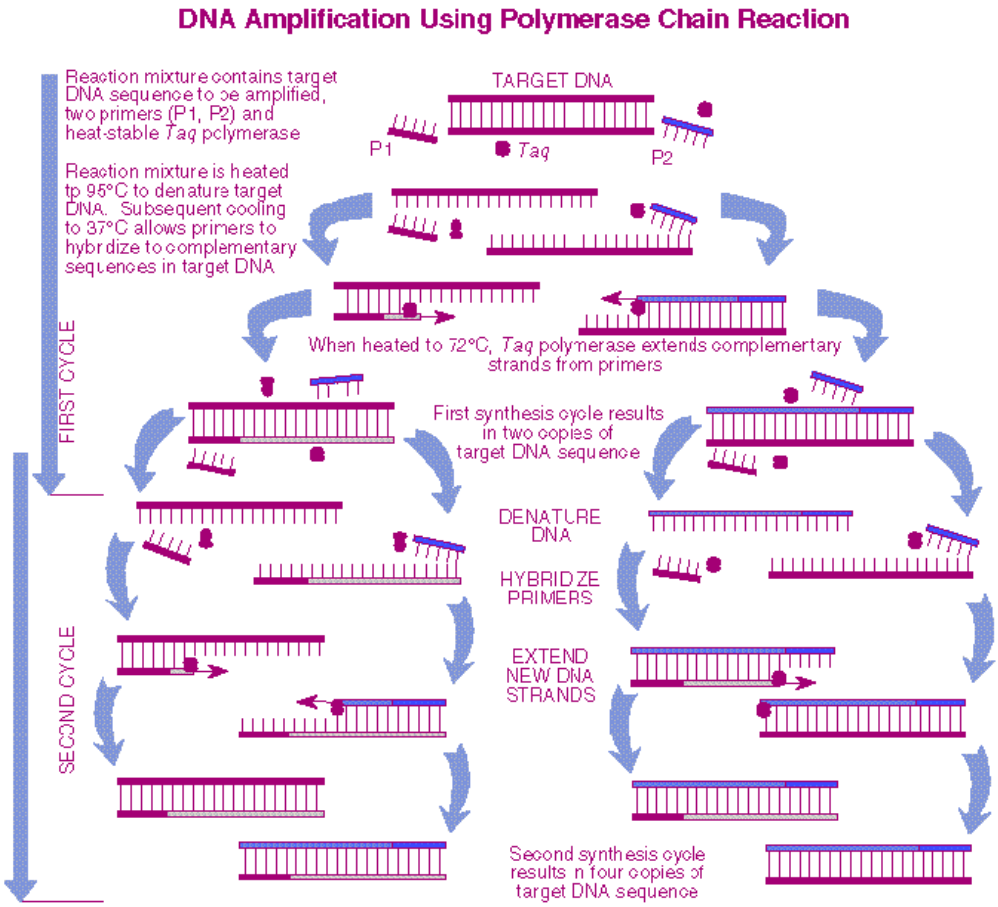
The polymerase chain reaction (PCR) is a sensitive and specific method of detecting small amounts of nucleic acid in a biological sample, enabling a targeted sequence to be exponentially amplified (Mullis and Faloona, 1987). The method of PCR has undergone many changes since its advent (Holland *et al,* 1991) and today the technique is used not just as a research tool, but as a diagnostic tool.

The polymerase chain reaction requires the design of two oligonucleotides known as primers, a forward primer and a reverse primer, which are complementary and specific to the target sequence to be amplified; these primers should be designed from a conserved region of the target pathogen genome. The PCR reaction requires several components; a heat-stable DNA polymerase enzyme such as *Thermus aquaticus* (*Taq*) polymerase, nucleotides, buffer, magnesium, primers and the template DNA (Figure 1.1). PCR can only be carried out on DNA, so for RNA pathogens the RNA must first be transcribed into cDNA in a reverse transcriptase (RT) step. The PCR reaction is carried out in a thermal cycler containing a thermal block that can be rapidly heated and cooled down. Upon heating, double stranded DNA is denatured and split into single strands. The temperature is then reduced to allow the specific primers to anneal to the single stranded DNA. The *Taq* polymerase then enzymatically adds nucleotides, using the primers as template to assemble and extend a complementary strand of DNA. Each copy of DNA can serve as a template for further amplification. The cycle of heating and cooling is repeated 30 to 40 times, doubling the PCR product with every cycle. This is described in Figure 1.1. Therefore as each copy of DNA can act as a template in the next cycle, one copy of DNA can by multiplied into billions of copies over 30 to 40 cycles. The PCR product is visualised by gel electrophoresis; DNA is separated by size using an electric current and viewed using ethidium bromide which fluoresces under ultraviolet light. The sample PCR product is run with a DNA ladder (containing a series of DNA fragments of known base-pair size). A band at the expected base-pair size comparable to the positive control indicates a positive result, provided positive and negative controls have behaved as expected.

Polymerase chain reaction is more sensitive and rapid in comparison to traditional methods of detection, PCR is preferred for the detection of fastidious pathogens, therefore increasing overall detection rate, and is also useful to screen for newly discovered pathogens. As well as more cases being associated to an aetiological cause, a new understanding of clinical syndromes has been developed, as previously undiagnosed or under-diagnosed pathogens are detected (Templeton *et al*, 2005; Gunson *et al*, 2008; Wolffs *et al*, 2011).

In addition to using PCR to clinically diagnose the causative pathogen of the disease, the method can also be used to determine if the animal is "free from infection", therefore detecting subclinical carriers, which can be important when introducing a cat or dog to a new home, prior to breeding, or for infection control when boarding, etc (Evermann, Sellon and Sykes, 2011). Due to the high sensitivity of PCR, the presence of pathogen nucleic acid does not necessary indicate ongoing disease; therefore any PCR results must be interpreted carefully along with clinical signs of the animal.

Figure 1.1 The polymerase chain reaction



The reaction is heated causing double stranded DNA to denature and split into single strands. The specific primers are able to anneal to the single stranded DNA when the temperature is reduced. Taq polymerase then enzymatically adds nucleotides, using the primers as template to assemble and extend a complementary strand of DNA. Each copy of DNA can serve as a template for further amplification. The cycle of heating and cooling is repeated 30 to 40 times, doubling the PCR product with every cycle.

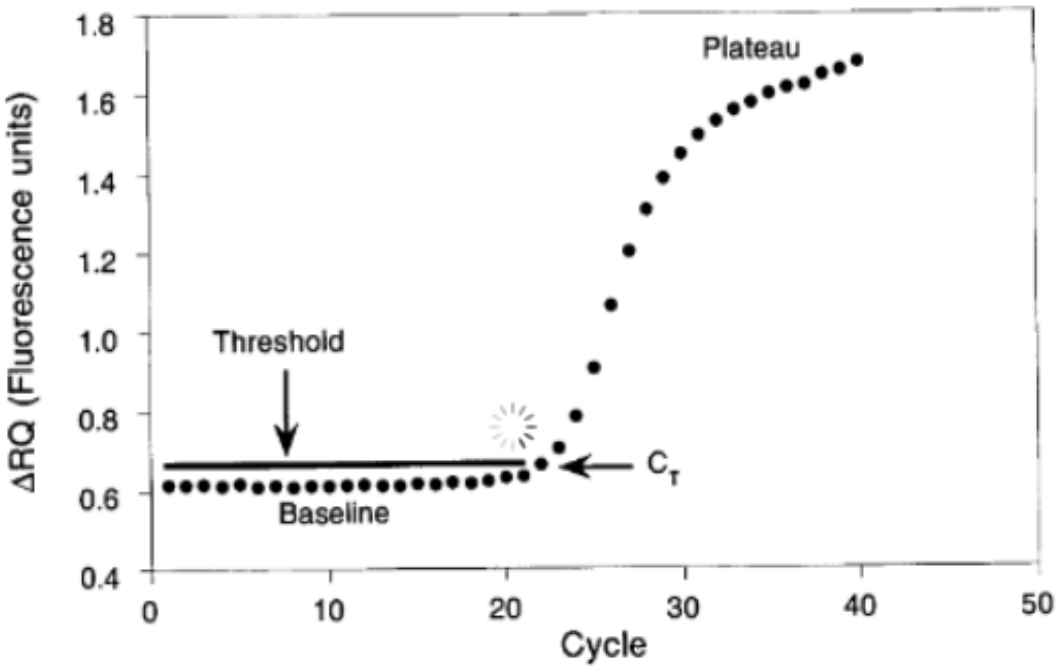
Image taken from University of Maine, 2013.

1.3.1 Real-time PCR

Over the last 20 years PCR methods have been revolutionised, mostly so with the advent of real-time PCR, where the amplification of the PCR product is observed in real-time. The exponential increase in product is seen as an increase in fluorescence, measured by a photo-detector in a real-time PCR instrument. The PCR reaction can take place and be measured in a single tube with no need for further analysis; referred to as a 'closed tube technique'. Several real-time PCR chemistries exist; some use a specific probe in addition to specific primers, which increases the specificity of the reaction. This probe is labelled with a fluorescent dye and a quencher dye and is designed to bind to an internal region of the PCR product.

In real-time PCR, fluorescence increases every PCR cycle, when and how this occurs varies with the chemistry used. The increase in fluorescence released every cycle results in an overall exponential increase in fluorescence which can be plotted in a graph (Figure 1.2). The point at which the sample becomes positive is called the cycle threshold (Ct), this is when the fluorescence becomes detectable above the background. As the Ct value is directly related to the starting target copy number, the Ct value is semi-quantitative. A low Ct value indicates a strong positive (less cycles required for the fluorescence to rise appreciatively above the background), whereas a high Ct values indicates a weak positive (with many cycles required before fluorescence is appreciatively increased above the background). The assay can be made fully quantitative by running a set of known standards, a 10-fold dilution series of standards of known quantity, alongside the test samples creating a standard curve. The Ct values of unknown samples can be measured against the standard curve of the standards giving a quantification value for that sample. This is particularly useful when the viral load may help with clinical diagnosis, disease progression and treatment, for example the stage of FIV infection (Diehl *et al* 1996).

Figure 1.2 An exponential amplification curve.



The point at which the sample becc ositive is called the cycle threshold (Ct), this is when the fluorescence becomes detectable above the background.

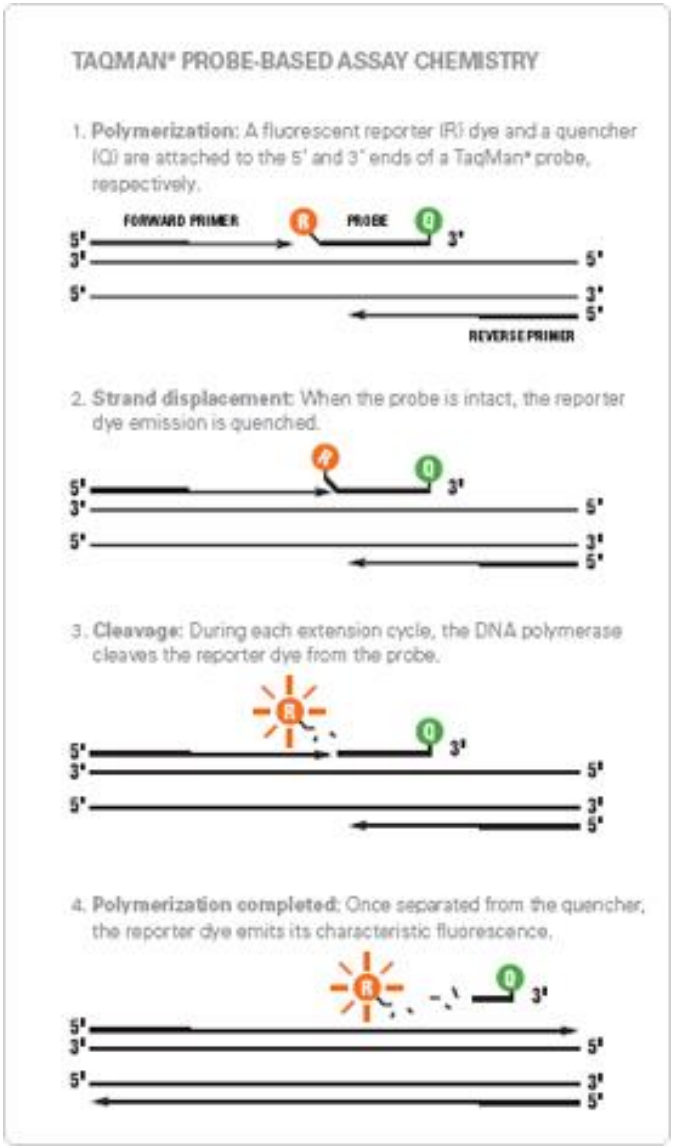
Image taken from Heid *et al*, 1996.

As mentioned above there are a number of chemistries available for real-time PCR, specific methods include dual-labelled probes such as TaqMan probes (also known as hydrolysis or 5' nuclease probes), molecular beacons and minor groove binders (which can be TaqMan probes or Eclipse probes), dual hybridisation probes, fluorescent labelled primers such as Scorpion primers and Lux primers, and DNA binding dyes such as SYBR green.

1.3.1.1 Dual labelled probes

Dual labelled probes are designed to hybridise to an internal region of a PCR product. TaqMan probes are dual labelled probes which have a fluorescent dye attached to the 5' end and a quencher attached to the 3' end of the probe. While the probe is intact, the fluorescent dye is quenched by the quencher dye. TaqMan PCR utilises the 5'→3' exonuclease activity of *Taq* polymerase (Figure 1.3). As with traditional PCR the DNA is denatured by heating, when the reaction is cooled the primers and probe then anneal to the single stranded DNA, as the *Taq* polymerase extends the strand 5'→3', it cleaves the probe, separating the fluorescent dye and quencher dye, causing the probe to fluoresce. Fluorescence increases in each cycle, proportional to the rate of probe cleavage. Minor groove binder (MGB) probes have a fluorescent dye at the 5' end and MGB on the 3'end, a MGB is a non-fluorescent quencher which allows the real-time PCR instrument to measure the reporter dye more precisely. In addition the MGB increases the melting temperature of the probe, allowing the use of shorter probes. Although MGB probes offer advantages, they are much more expensive than non-MGB probes, and they are available with limited fluorescent dyes.

Figure 1.3 Overview of TaqMan probe based chemistry



TaqMan PCR utilises the 5'→3' exonuclease activity of *Taq* polymerase. The DNA is denatured by heating, the primers and probe then anneal to the single stranded DNA when the temperature is reduced. As the *Taq* polymerase extends the strand 5'→3', it cleaves the probe, separating the fluorescent dye and quencher dye, causing the probe to fluoresce. Fluorescence increases in each cycle, proportional to the rate of probe cleavage.

Image taken from Life Technologies, 2014[a]

1.3.1.2 Molecular beacons

Molecular beacons like TaqMan probes also have a fluorescent and quencher dye attached, however unlike TaqMan probes, molecular beacons are designed to remain intact during the amplification reaction. A molecular beacon has a hairpin like structure with fluorescent and quencher attached, when the molecular beacon binds to the target DNA the probe becomes linear and so the fluorescent and quencher dyes become separated causing the fluorescent probe to fluoresce.

1.3.1.3 Dual-hybridisation probes

Dual-hybridisation probes have two specific primers, and also two specific probes – a donor and an acceptor probe. The probes are labelled with a pair of dyes that exhibit fluorescence resonance energy transfer (FRET). The donor dye is attached to the 3' end of the first probe, while the acceptor dye is attached to the 5' end of the second probe. They bind the template DNA adjacent to each other, during this annealing step when the probes are fully hybridised FRET occurs from the donor to the acceptor probe and fluorescence is released.

1.3.1.4 Fluorescent-labelled primers

Fluorescent-labelled primers exist in several forms, for example Scorpion primers, Lux primers. Scorpion Primers have a reporter and quencher dye and the primer is in a stem-loop formation. Unlike molecular beacons, the primer anneals to target DNA with the stem-loop intact, it is during the extension step that the quencher is separated from the reporter and fluorescence is released.

1.3.1.5 DNA-binding dyes

DNA-binding dyes used in real time PCR, such as SYBR GREEN is a real time method in which the dye binds to all double stranded DNA in a sample, therefore it may bind to non-specific dsDNA. As the PCR progresses, more PCR product is created. SYBR® dye binds to all double-stranded DNA, so the result is an increase in fluorescence intensity proportioned to the amount of PCR product produced. Good design of primers and melt curve analysis improves specificity, as the melting temperature should be specific to the target sequence.

1.3.2 Benefits and limitations of real-time PCR

Real-time PCR is an extremely sensitive and specific method of pathogen detection and the use of a specific probe further increases the specificity. The closed system of real-time PCR lends the technology to automation (extraction and plate set-up) which increases rapidity, leading to reduced turn-around times and overall costs. The standard parameters of real-time PCR also allows several PCR assays to be multiplexed, as all types of pathogens, DNA or RNA can be tested using the same cycling conditions and PCR kit (Gunson, Collins, and Carman, 2006).

As mentioned above a benefit of replacing traditional detection methods with real-time PCR is that it lends itself to automation, so making it easier for the laboratory to become automated, from sample receipt, to sample extraction, through to PCR set-up, analysing of results and uploading of results onto a laboratory database for reporting. Unlike traditional methods, PCR does not require live virus for detection, therefore samples are easily transported, and samples can be added to lysis buffer (lysis of the cells rendering the virus non-infectious) and kept at room temperature. Samples can arrive in the laboratory in the correct vial to be loaded directly onto the extraction platform; if these samples are also labeled with a unique barcode then the extraction platform can scan and record the samples extracted. The tests required for the samples can be inputted into a laboratory database (laboratory information management system (LIMS), allowing automated real-time PCR setup of the appropriate samples with the requested test. Samples are then amplified and once results are analysed (which can be manually done or automatically carried out), then results can be uploaded to the laboratory database for reporting. This greatly reduces hands-on time, turnaround times, costs, and the opportunity for human error.

As mentioned above, one of the major advantages of real-time PCR is that it is highly sensitive; however this can be at times a disadvantage. Due to real-time PCR being able to amplify and detect just one copy of DNA, it is possible that a pathogen may be detected that is not causing disease, for example low level residual DNA from a past infection may be detectable if an animal is shedding virus. Also, if an animal has been recently vaccinated, this should be taken into account when interpreting results as recent vaccination may lead to a PCR signal due to residual nucleic acid from the vaccine.

Therefore a weak positive result, for example a Ct greater than 35 (very weak positive), must be considered along with clinical signs as this may not be the cause of disease.

1.3.3 Multiplex real-time PCR

Although all PCR methods can be multiplexed, real-time PCR tends utilises the same reaction conditions (more so than traditional PCR protocols) and so lends itself readily to multiplexing. Assays can be multiplexed in order to detect more than one target simultaneously in one reaction well. For example the availability of five different channels on the ABI Prism 7500 SDS real-time platform (Applied Biosystems, Warrington, UK) can allow 96 samples/controls to be screened for four to five pathogens in one test. Multiplex assays can be designed along syndromic lines, offering users a single test to investigate a specific set of clinical signs, for example. respiratory disease. This prevents sequential testing and offers all results at the same time. For human diagnostics these 'menus' of screens have improved rapidity, cost, ease of use and throughput of the diagnostic service (Gunson *et al*, 2008).

The ability to detect the main causes of a disease syndrome in one sample, whether viral, bacterial, parasitic or fungal, is a tremendous benefit to a veterinary practitioner for rapid, "all in one" results. This will allow the treating veterinary practitioner to make a clinical decision in a timely manner that could influence the outcome for the animal. In addition, multiplexing would be greatly advantageous to the veterinary laboratory to reduce costs and turnaround times.

Few veterinary laboratories offer multiplex real-time PCR, most use single assays for each pathogen, and currently no commercial companies supply multiplex kits. The use of multiplex real-time PCR in the veterinary laboratory would allow for simultaneous testing of multiplex infectious pathogens from one sample in as little as three hours. As outlined above in section 1.2.5 possible causes of feline gastroenteritis include FPV, FCoV, *Salmonella, C.jejuni, C.parvum, Giardia* and *T.foetus*. In order to detect these pathogens by current methods, virus isolation, bacterial culture, ZN staining, zinc sulphate floatation and PCR would need to be carried out. Each of these methods requires a different set of specialist technical skills, and setting up each method can be labour intensive and costly. However this panel of seven pathogens can be tested rapidly and simultaneously using one

method: multiplex real-time PCR. In addition, multiplex real-time PCR easily allows the incorporation of an internal control (IC) assay. An IC is the ideal positive control as it allows for monitoring of sample inhibition, extraction failure and PCR set-up failure. Nucleic acid extraction and screening of the sample using two or three multiplex real-time PCR assays can be done in less than three hours to determine the causative agent or agents, allowing for timely clinical intervention (Gunson *et al*, 2008). In addition, several probes can be labeled with the same dye to increase the level of detection further, for example in human diagnostics coronaviruses NL63, 229E and OC43 can be labeled with the same dye, leaving other channels available for different pathogens, for example rhinovirus and adenovirus.

As well as being semi-quantitative, real-time PCR can be designed to quantify, subtype, or even resistance test a particular pathogen. Consequently novel in-house methods can be designed to diagnose, type and quantify. Therefore as well as replacing "gold standards" that may not be sensitive or specific, multiplex real-time PCR can also replace the need for traditional nucleic acid sequencing, which significantly reduces turnaround times and costs. Multiplex PCR can also be used for large scale prevalence and epidemiology studies of cats and dogs, and for testing of archived samples, allowing retrospective analysis of samples.

However developing multiplex assays is not just a case of adding assays together. The assay must be carefully optimised to ensure that multiplexing does not result in a loss of sensitivity or specificity in comparison to the single test. A multiplex assay also requires careful optimisation to ensure that no crosstalk is evident; the ABI 7500 real-time PCR instrument has five channels therefore theoretically five different targets can be detected in one well (Figure 1.1). However, this is not always achievable, as it is possible for the fluorescence increase with one dye to 'spill over' into another channel, resulting in 'crosstalk'. This leads to what appears to be two positive results, one of which is a false positive. Crosstalk can be eliminated by carefully optimising the probe concentrations in the multiplex, for example by reducing the concentration of the probe that is crosstalking into the other channel. In addition to crosstalk, cross-reaction can affect the sensitivity and specificity of a multiplex assay. This is because cross-reaction between primers and probes from different assays can occur; this is also called primer-dimer formation.

Another way in which multiplexing can result in a loss of sensitivity is through competition for PCR reagents. Multiplexing can create competition in a mixed infection between targets where a stronger positive out-competes a weaker positive, resulting in a false negative result. The stronger target in the sample has more DNA to begin with and so uses up all of the PCR reagents quickly, the target with less starting material is not able to amplify enough to show a rise in fluorescence due to the lack of reagents available in the presence of a stronger target. In many sample types, including respiratory and faecal, mixed infections can be expected, therefore it is important to be able to detect all potential disease-causing pathogens in a sample. In any sample containing an IC, a positive sample is effectively a mixed infection; therefore competition could result in a sample being falsely inhibited, or the IC could outcome a positive sample, resulting in a sample being false negative. Competition can be overcome by careful optimisation, by reducing one or both of the primers and/or probes that are cross-reacting, and by the use of PCR kits specially designed for use with multiplex primer and probe pools. However care must be taken to ensure that any changes to the concentration of each component of the assay does not affect the overall efficiency and sensitivity of the assay. There are now commercially available PCR kits specifically designed for use with multiplex assays, which can help reduce crosstalk, cross-reaction and competition.

Once a multiplex assay has been designed, optimised, and evaluated to assess the endpoint detection limit, specificity and robustness, the assay must the be evaluated to determine the clinical sensitivity and specificity of the assay. Ideally, clinical validation comparing the multiplex assay to a "gold standard" for each pathogen should be carried out. This is a large scale evaluation where the multiplex assays are run in parallel to a gold standard and the results compared. This gives the clinical sensitivity and specificity of the multiplex and if planned properly can determine negative and positive predictive values with prevalence data. It should be noted that completing the clinical validation of the feline and canine multiplex assays is beyond the timescale of this research project, however preparations to carry out the validations have been made. This project aims to assess the analytical components of the multiplex assays as a measure of the test performance of the multiplex panels.

Although introducing a streamlined, automated multiplex real-time PCR diagnostic service will significantly reduce turn-around times, time, labour time and overall costs, I should be noted that the initial implementation of such a service is expensive. In addition staff must be trained to develop and run the service, including troubleshooting assay problems, and importantly to ensure quality control of the service.

1.4 Aim of research

Although PCR is becoming more readily available, the use of real-time PCR in veterinary diagnostic laboratories is not currently widespread, and many laboratories still use traditional detection methods. The implementation of an internally controlled real-time multiplex PCR would allow for rapid detection of a variety of pathogens, which will in turn aid in the treatment and clinical management of the animal, and increase our understanding of infectious disease in cats and dogs. The aim of this project is to begin the development of a panel of internally controlled in-house designed syndrome based multiplex real-time PCR assays for the detection of infectious disease in cats and dogs. The panels developed will screen for feline respiratory disease in throat swab samples, feline conjunctivitis in eye swab samples, feline anaemia in blood samples, feline gingivitis in blood and swab samples, and feline and canine gastroenteritis in faecal samples. The assays will be optimised and assessed as single real-time PCR assays and then multiplexed together. The evaluation of the multiplexes will include assessing the robustness of the multiplexes by direct sensitivity comparison to the single assays, the endpoint detection limit of each target in the multiplex, the specificity, and the repeatability and reproducibility of the assay.

This project aims to assess the analytical components of the multiplex assays as a measure of the test performance of the multiplex panels. If successful, the in-house designed multiplex real-time PCR assays will replace conventional methods currently available and enable the veterinary clinician to screen a clinical sample for multiple pathogens causing a specific clinical syndrome with minimal tests.

CHAPTER 2
Methods

CHAPTER 2

2. Methods Introduction

This chapter details the methods used to develop the in-house designed multiplex assays. The MIQE guidelines (Bustin *et al*, 2009) for the development of in-house molecular assays provide recommendations on what is required in order to validate in-house assays. These guidelines were used in this study as a basis for the evaluation of each assay. Section 2.1 describes the selection of a nucleic acid extraction platform, a real-time PCR kit, a real-time PCR instrument, the selection of significant pathogen targets, real-time PCR assay selection and the design of the multiplex assays. Section 2.1 also includes the general laboratory methods used in the development of the multiplex assays. In Section 2.2, the methods used to optimise each multiplex primer-probe pool are described, together with the assessment of the sensitivity, specificity and robustness of each multiplex assay.

2.1 Methods common to all multiplex assays

2.1.1 Nucleic acid extraction platform

The first stage in developing an in-house real-time multiplex panel of tests is to choose a nucleic acid extraction method. There are many methods available for the extraction of nucleic acid from clinical samples, including manual methods, semi-automated and fully automated extraction platforms. These methods can extract DNA or RNA, and in some cases total nucleic acid, from numerous sample types.

Manual extraction is highly labour intensive and not suitable for high-throughput laboratories; in addition, the separate washing and spinning/vacuum steps present an increased risk of contamination. For these reasons manual extraction was not considered for this project. Many automated and semi-automated platforms are available. Automated platforms offer rapid extraction with high throughput and little hands-on time, which although expensive to implement initially, can significantly reduce overall testing costs. In the laboratory where the project is being carried out, the West of Scotland Specialist Virology Centre (WoSSVC, Glasgow), the MDX (Qiagen, Germany), M2000 (Abbott, USA) and the easyMag (BioMérieux, France) extraction platforms are in use.

The Qiagen MDX and the Abbott M2000 can extract 96 samples in two to three hours, which is exceptionally fast and ideal for high through-put laboratories. However, there are some limitations with these platforms. Firstly, different extraction kits are recommended for viruses and bacteria (MDX), or for RNA and DNA (M2000). Secondly, all samples on an extraction run must have the same input and output volumes, and a dead volume for each sample, for example the MDX requires a 500µl sample volume for nucleic acid extraction however the instrument only uses 263µl of the sample. For this study, as the multiplex panels contain both RNA and DNA pathogens, a mix of viruses, bacteria and parasites, and a variety of sample types, a platform that enables complete nucleic extraction from all sample types is required. Therefore the bioMerieux easyMag was chosen as the extraction platform (Figure 2.1). This is a semi-automated extraction platform for the purification and concentration of total nucleic acids (RNA and DNA) from all types of biological specimens. Different sample volumes can be accommodated, negating the requirement for a dead volume, and the nucleic acid can be eluted into different volumes on the same run, making this platform ideal for variable sample types. Moreover, it has been shown to work for viruses, bacteria and parasites without needing multiple diverse extraction kits. The throughput of 24 samples/controls per run in less than 45 minutes is ideal for the throughput of the intended laboratory (Glasgow Veterinary Diagnostic Services (GVDS), School of Veterinary Medicine, University of Glasgow). The method is based on a generic method for binding nucleic acids from complex biological samples to magnetic silica (Boom *et al*, 1990). The sample is mixed with a lysis buffer containing a chaotropic agent (guanidinum thiocyanate). Any cellular material, viruses, fungi, parasites or bacteria present in the sample will be lysed and the nucleic acid released. The lysis buffer inactivates any nucleases present in the sample. The isolation process is initiated by the addition of magnetic silica to the lysed sample. Nucleic acids present in the lysate will bind to the magnetic silica under the high salt conditions. The magnetic silica is then washed several times using two wash buffers to improve purification. Next, the nucleic acids are released (eluted) from the magnetic silica and concentrated in a specified volume of the elution buffer. This elution process is accelerated by flushing the magnetic silica in the elution buffer at an elevated temperature. Finally the magnetic silica is separated from the elution buffer before the concentrated nucleic acid solution is available for collection. For the samples used in the assessment of the multiplexes the standard easyMag protocol was followed according to the manufacturer's instructions. Nucleic acids were extracted from 200µl sample, and eluted into a final volume of 110µl. All reagents were supplied by BioMérieux.

2.1.2 Real-time RT-PCR kit

The next stage is to choose an appropriate real-time PCR kit. Many kits are commercially available for real-time PCR. These kits contain all the reagents needed to carry out PCR (e.g. reaction buffer, *Taq* polymerase enzyme, RT enzyme if RT-PCR) with the exception of the pathogen specific primers and probes. RT-PCR was originally a two-step reaction with the RT step being carried out separately prior to PCR, now one-step RT-PCR kits are available where the RT enzyme is added to the PCR mastermix (containing reaction buffer, magnesium, etc.), and the RT step occurs prior to the PCR, but in the same reaction. A one-step reaction is optimal for reduced hands-on time and reduced risk of contamination. Usually, separate kits are used for DNA and RNA targets, although some RT-PCR kits can also be used to detect DNA pathogens (Gunson *et al,* 2008), which is ideal for multiplex assays that contain both RNA and DNA targets. Recently PCR kits have been designed to use specifically with multiplex assays. These are RNA or DNA kits and aim to reduce cross-reactions between primers and probes, crosstalk between dyes, and competition between targets. Some kits have been assessed internally at the WoSSVC and it was found that these kits were prone to non-specificity, resulting in false positive results (Rory Gunson, personal communication, 2010). These kits were not considered in this study.

As most of the multiplexes are likely to contain at least one RNA pathogen (Table 1.1), for ease of use, a one-step RT-PCR kit was chosen as the basis for all assays. The kit chosen to assess the multiplex development was the AgPath-ID One-step RT-PCR kit (Ambion/Life Technologies, Paisley, UK), that is suitable for both RNA and DNA. This kit has been assessed in the WoSSVC with human diagnostic multiplex screens. When compared to a similar one-step RT-PCR kit by Invitrogen (SuperScript® III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase) that has been in use in the laboratory for 10 years, the Ambion kit was found to be as sensitive as the Invitrogen kit at the endpoint of detection (Rory Gunson, personal communication, 2010). In addition the Ambion kit was found to perform better than the Invitrogen kit when a mixed infection or IC was present in a sample; therefore competition was reduced with the Ambion kit (Rory Gunson, personal communication, 2010). Based on these data, the Ambion kit was chosen. In this study, RT-PCR was performed on 6µl of template nucleic acid in a 15µl reaction and the following thermal profile: 50ºC for 15 minutes (RT step); 95ºC for 10 minutes; and then 40 amplification cycles of 95ºC for 8 seconds and 60ºC for 34 seconds. Reaction volumes were modified in-house (Gunson, Collins, and Carman, 2006).

Figure 2.1 The BioMérieux easyMag extraction platform

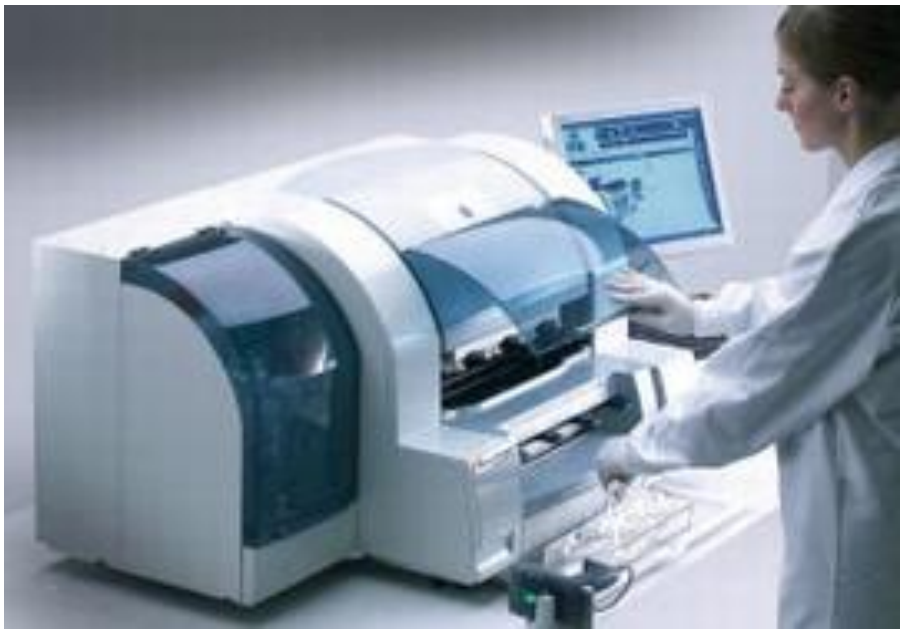


Image taken from BioMérieux, 2014.

2.1.3 Real-time PCR instrument

The RT-PCR was carried out on an ABI 7500 real time PCR instrument as these were the established instruments in the laboratory (Figure 2.2). The ABI Prism 7500 platform has five channels which can detect different fluorescent dyes; this allows the multiplexing of PCR assays by labelling each probe with a different detector dye (Figure 2.3).

2.1.4 Pathogen selection

The next step was to determine which pathogens to include in each panel. Pathogens of importance in feline and canine infectious disease were identified by searching the literature and consulting with veterinary practitioners and veterinary microbiologists at the School of Veterinary Medicine, University of Glasgow. The pathogens selected are outlined in Table 2.1.

2.1.5 Real-time PCR assay selection

The next step was to choose assays from the literature that could detect the targets outlined in Table 2.1. When developing a multiplex assay a researcher can choose to either design their own assays from scratch or choose a published assay from the literature. Designing a real-time assay from scratch requires significant experience and can be costly and time consuming. Knowledge and experience of the different software programs required for real-time PCR design is also essential (such as BLAST® (Altschul *et al*, 1990), and Primer Express® (Life Technologies, Foster City, USA). In addition, an in-depth knowledge of the genome of the pathogen of interest is required, to know where to best target a new assay. The newly designed assay will also have to be extensively evaluated as there will be no data to support its performance. Choosing a published assay has a great number of advantages over designing an assay from scratch. It is likely that any published real-time PCR assays have been developed and evaluated by research groups that specialise in a particular pathogen and therefore the assay will have already been validated extensively. As a result the researcher can choose a published assay with confidence of its sensitivity and specificity. Although it is important to note that not all published assays are well validated and so care should be taken when selecting published assays. For this project the assays came from two sources: assays already in use at GVDS, and assays published in the literature.

Figure 2.2 The ABI Prism 7500 SDS real-time platform

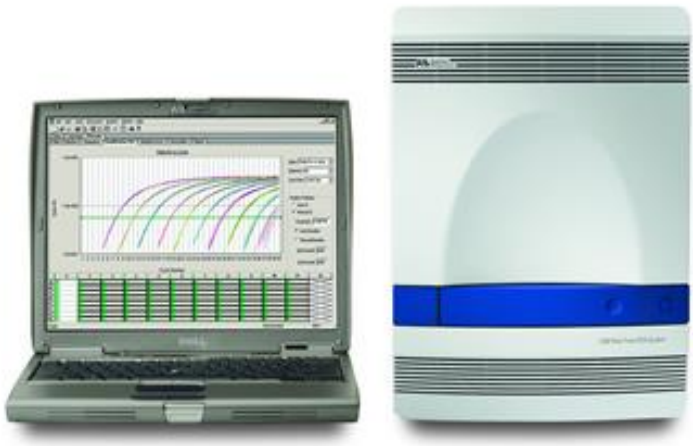
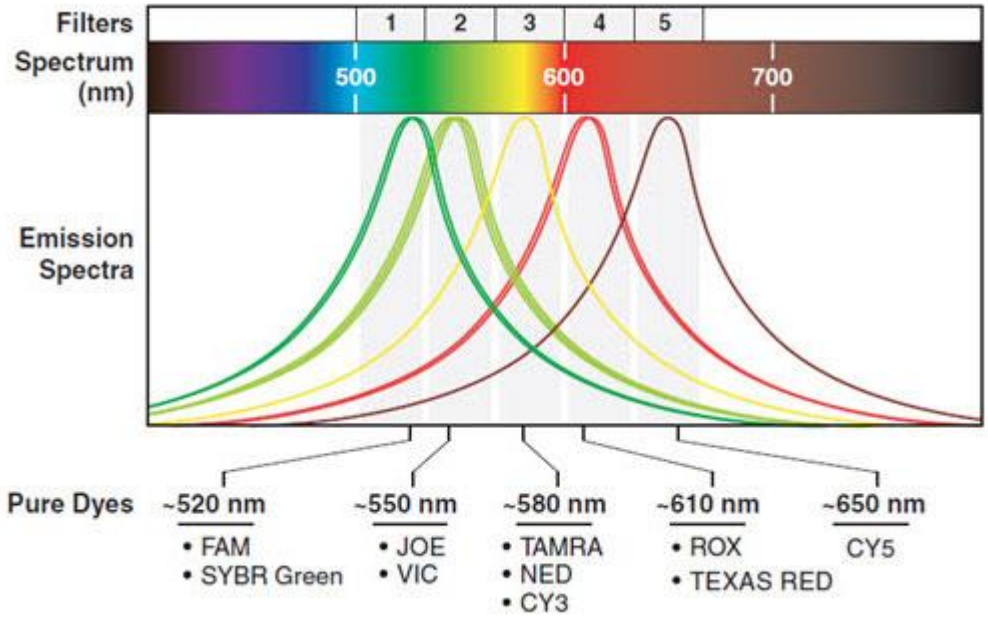


Image taken from Life Technologies, 2014[b]

Figure 2.3 The ABI 7500 Prism spectra



The ABI 7500 has five channels which can detect up to five different fluorescent dyes; FAM, VIC/HEX, TAMRA, ROX and Cy5. Probes can be labelled with different dyes and so can be distinguished from each other in a multiplex.

Table 2.1 Selected pathogens and in-house multiplex design

Screen		**Multiplex components**
Feline Respiratory		feline herpesvirus, feline calicivirus, *Bordetella bronchiseptica*, internal control (mumps virus)
Feline Conjunctivitis		feline herpesvirus, *Chlamydophila felis*, *Mycoplasma felis*, internal control (herpes simplex virus-1)
Feline Anaemia	**Anaemia 1**	feline leukaemia virus, feline immunodeficiency virus, feline coronavirus
	Anaemia 2	haemoplasmas, internal control (mumps virus)
Feline Gingivitis		feline leukaemia virus, feline immunodeficiency virus, feline calicivirus, internal control (mumps virus)
Feline/Canine Gastroenteritis	**Gastroenteritis 1**	parvovirus, internal control (mumps virus)
	Gastroenteritis 2	coronavirus, *Salmonella*, *Campylobacter jejuni*
	Gastroenteritis 3	*Cryptosporiduim parvus*, *Giardia*, *Tritrichomonas foetus*
	Gastroenteritis 4*	*Clostridum perfringens* (toxin CPE), *Escherichia coli* (toxin ST1), *Clostridium difficile* (toxins A and B)

* Canine only.

2.1.5.1 Existing assays in use at Glasgow Veterinary Diagnostic Service

The GVDS currently use validated singleplex real-time PCR assays for FHV, *M. felis*, and FCoV. The *M. felis* assay is based on a conserved region of the genome identified by Chalker *et al* (2004), the primers and probe were designed at the GVDS; the FCoV assay was modified from the assay published by Gut *et al* (1999) and the FHV assay was designed in-house. These assays have been fully validated and were found to be at least as sensitive as the traditional method (data not shown). The primer and probe sequences for these assays are given in Table 2.2

2.1.5.2 Published assays in the literature

The literature was searched for real-time TaqMan PCR assays for the remaining targets. Where more than one assay was published, the validation data was compared and the most validated assays (i.e. sensitivity and specificity assessed) were chosen. The primer and probe sequences and published sources for the selected assays are given in Table 2.2. For each primer and probe, the theoretical sensitivity and specificity was confirmed using BLAST. BLAST allows the comparison of a sequence, such as a primer or probe sequence, to a library of sequences that have been uploaded onto the database by research groups from around the world.

Table 2.2 Selected real-time PCR assay primer-probe sequences with dyes

Target	Forward primer	Reverse primer	Probe	Reference
Feline herpesvirus	TGG TGC CTA TGG AAT AGG TAA GAG TT	GTC GAT TTT CAT CCG CTC TGA	**FAM-**AAC GGC GAA GTA CC-MGB	GVDS
Feline calicivirus	GTA AAA GAA ATT TGA GAC AAT	TAC TGA AGW TCG CGY CT	**HEX-** CAA ACT CTG AGC TTC GTG CTT AAA-BHQ	Abd-Eldcum *et al* , 2009
Bordetella bronchiseptica	ACT ATA CGT CGG GAA ATC TGT TTG	CGT TGT CGG CTT TCG TCT G	**CY5-**CGG GCC GAT AGT CAG GGC GTA G-BHQ	Helps, Lait, and Damhuis, 2005
Mycoplasma felis	GTG GGG ATG GAT TAC CTC	GGA CTA TTA TCA AAA GCA CAT AAC	**HEX-**CTA CGG AGT ACA AGT TAC AAT TCA-BHQ	GVDS
Chlamydophila felis	GAA CTG CAA GCA ACA CCA CTG	CCA TTC GGC ATC TTG AAG ATG	**CY5-**CGC TGC CGA CAG ATC AAA TTT TGC C-BHQ	Helps, Lait, and Damhuis, 2005
Parvovirus	ACT GCA TCA TTG ATG GTT GCA	GGT ATG GTT GGT TTC CAT GGA	**FAM-** CCC AAT GTC TCA GAT CTC ATA GCT GCT GG-BHQ	Meli *et al* , 2004
Salmonella	CTC ACC AGG AGA TT AC AA CA TGG	AGC TCA GAC CAA AAG TGA CCA TC	**HEX-** CAC CGA CGG CGA GAC CGA CTT T-BHQ	PC
Campylobacter jejuni	TGG TGG TTT TGA AGC AAA GAT T	AAT ACC AGT GTC TAA AGT GCG TTT AT	**CY5-**TTG AAT TCC AAC ATC GCT AAT GTA TAA AAG CCC TTT-BHQ	PC
Coronavirus	GAT TTG ATT TGG CAA TGC TAG ATT T	AAC AAT CAC TAG ATC CAG ACG TTA GCT	**FAM-**TCC ATT GTT GGC TCG TCA TAG CGG A-BHQ	GVDS (modified from Gut *et al* 1999)
Giardia	GAC GGC TCA GGA CAA CGG TT	TTG CCA GCG GTG TCC G	**ROX-**CCC GCG GCG GTC CCT GCT AG-BHQ	PC
Tritrichomonas foetus	GCG GCT GGA TTA GCT TTC TTT	GGC GCG CAA TGT GCA T	**VIC**-ACA AGT TTC GAT CTT TG-MGB	McMillen and Lew, 2006
Cryptosporidium parvum	CTT CAC GTG TGT TTG CCA AT	CCT TTT CAT GAC TTG TCT TAT CAG G	**CY5-**CCA ATC ACA GAA TCA TCA GAA TCG ACT GGT ATC-BHQ	PC

Target	Forward primer	Reverse primer	Probe	Reference
Clostridium perfringens toxis	AAC TAT AGG AGA ACA AAA TAC AAT AG	TGC ATA AAC CTT ATA ATA TAC ATA TTC	**FAM**-TCT GTA TCT ACA ACT GCT GGT CCA-BHQ	Gurjar *et al* 2008
Escherichia coli toxin	CTG GTT TTG ATT CAA ATG TTC GTG	TTC TGA GGG AAA GGT GAA AAA GAC	**HEX**- TTG ATT TCT TCA TAT TAC CTC CGG ACA TGG CA-BHQ	Hardegen *et al* 2010
Clostridium difficile (Toxin B only)	GAA AGT **Y**CA AGT TTA CGC TCA AT	GCT GCA CCT AAA CTT ACA CCA	**Cy5**-ACA GAT GCA GCC AAA GTT GTT GAA TT-BHQ	Van den berg *et al* 2007
Feline leukaemia virus	AAC AGC AGA AGT TTC AAG GCC	TTA TAG CAG AAA GCG CGC G	**FAM**- CCA GCA GTC TCC AGG CTC CCC A-BHQ	Tandon *et al* , 2005
Feline immunodeficiency virus	CCA TCG AAC GTC TGC CCT A	TCA CCC GTG GTC ACC ATG	**HEX**- CGA TGG TGG TCG CCG TGC CTA-BHQ	Ryan *et al* , 2003
Mycoplasma haemofelis	GTG CTA CAA TGG CGA ACA CA	TCC TAT CCG AAC TGA GAC GAA	**FAM**- TGT GTT GCA AAC CAG CGA ATG GT-BHQ	Peters *et al* , 2008
Mycoplasma haemominutum	TGA TCT ATT GTK AAA GGC ACT TGC T	TTA GCC TCY GGT GTT CCT CAA	**HEX**- TTC AAT GTG TAG CGG TGG AAT GCG T-BHQ	Peters *et al* , 2008
Mycoplasma turicensis	AGA GGC GAA GGC GAA AAC T	CTA CAA CGC CGA AAC ACA AA	**Cy5**-CGT AAA CGA TGG GTA TTA GAT GTC GGG AT-BHQ	Peters *et al* , 2008
RNA internal control (mumps virus)	TCT CAC CCA TAG CAG GGA GTT ATA T	GTT AGA CTT CGA CAG TTT GCA ACA A	**ROX**-AGG CGA TTT GTA GCA CTG GAT GGA ACA-BHQ	Uchida *et al* , 2004
DNA internal control (human herpesvirus-1)	TCC TSG TTC CTM ACK GCC TCC C	GCA GIC AYA CGT AAC GCA CGC T	**ROX**- CGT CTG GAC CAA CCG CCA CAC AGG T-BHQ	Van Doornum *et al* , 2003

PC: Personal communication with Miriam Steiner, 2010. GVDS: Glasgow veterinary diagnostic service.

2.1.6 Internal control selection

The next step is to select an internal control (IC) to use in each multiplex. An IC is the ideal positive control in real-time PCR. The IC should be suited to the target i.e. an RNA IC should be used if the target is an RNA pathogen, so that the IC is also controlling the RT step. Internal controls exist in different forms, some controlling the whole extraction and PCR process whereas some only control the PCR stage. The IC used in the multiplex panels will assess the whole process from extraction through to PCR. The IC will be added to each sample at the extraction phase, and will be a pool of a DNA and RNA IC. The IC will therefore monitor inhibition in the sample, PCR inhibition and PCR set-up error for DNA and RNA pathogens.

In this study we used herpes simplex virus-1 (HSV1) as a DNA IC. For the RNA IC, mumps virus was used. Both viruses are ideal ICs as they will not be present in the sample population being tested, and BLAST searches were carried out to ensure that the primers and probes did not cross-react with any of the feline or canine assays. Since both are readily cultured, including at the WoSSVC, large batches of IC can be manufactured locally at a low cost in comparison to a commercially available IC. Each syndromic panel has an IC assay incorporated into the multiplex assay. If there was more than one multiplex assay for a particular syndrome (e.g. the gastroenteritis panel has four multiplex assays), an IC was added to one of the multiplexes, e.g. GE1 for the gastroenteritis panel (Table 1.1).

In practice, the pooled HSV1/mumps virus IC was added to each sample prior to extraction by adding a set volume to each easyMag reaction vessel. The Ct of the IC in each sample was then compared to the expected Ct to determine whether inhibition/error had taken place. Several extraction runs of samples containing the IC are assessed to give a reliable expected output Ct value prior to routine use.

2.1.7 Multiplex panel design and choice of fluorescent dyes for probes

The multiplexes were designed based on the chosen pathogens and combinations outlined in Table 2.1, and fluorescent dyes were applied to each probe. The dyes ascribed to each probe are outlined in Table 2.2. The preferred dyes for triplex assays are FAM, VIC/HEX and Cy5 (Figure 2.1). These dye combinations work on most assay platforms and are

commonly used by researchers. For fourplex PCR we used the dye ROX. The ROX dye is often used as a background/reference dye with some PCR kits. However this dye can be used as a fluorescent dye for a probe like FAM, VIC etc, as long as the PCR kit contains no ROX as a reference dye. The combination of FAM, VIC, Cy5 and ROX were chosen because the excitation and emission wavelengths are sufficiently different to allow accurate detection of each. This reduces the risk of crosstalk, which is described in more detail in Sections 2.1.9.3. All probes had black hole quenchers (BHQ) as quenchers with the exception of FHV and *Tritrichomonas foetus* which had minor groove binders (MGB).

2.1.8 Laboratory methods

2.1.8.1 Reconstitution of primers and probes

Primers and probes were reconstituted according to the manufacturer's instructions, in pH 7 TE buffer (Ambion, Life Technologies, Paisley, UK) for all primers and Cy5 probes, and pH 8 TE buffer (Ambion, Life Technologies, Paisley UK) for all other probes. The reagents were reconstituted to stock concentrations of 100µM for probes and 1000µM for primers.

2.1.8.2 Sample panels used for multiplex assessment

A strong positive control was used to manufacture a 10-fold dilution series to assess the multiplex assay over a range of Ct values, to determine the endpoint detection limit, and to assess the trace quality in comparison to single assays. The positive control was a strongly positive clinical sample, an isolate or plasmid DNA. The preparation of clinical samples used is described in section 2.1.8.3.

To assess the specificity of the multiplex assays, pooled positive controls containing a range of human pathogens for human diagnostic assays was assessed, these controls included:- adenovirus; human herpes virus 6 and 7; parvovirus B19; *Pneumocystis pneumonia;* norovirus GI and GII; herpes simplex virus 2; varizella zoster virus *Syphilis; Chlamydia trachomatis*; cytomegalovirus; Epstein Barr virus; measles virus; rubella virus; astrovirus; rotavirus; sapovirus; influenza A, B, H1N1sw; H1N1sw(H275Y); coronaviruses 229E, OC43, NL63 & HKU1; parainfluenza 1 to 4; rhinovirus; respiratory syncytial virus A and B; human metapneumovirus; and *Mycoplasma pneumonia*. In addition, no-template

controls were tested. No-template controls are negative controls which should contain no target nucleic acid, instead nuclease-free water is added to the PCR plate (plus mastermix) during set-up. Large scale clinical validation will be required to fully validate these tests, however small panels (~20) of positive and negative clinical samples determined by "gold standards", were tested where available. At this stage, clinical samples have only been assessed for the feline respiratory, feline conjunctivitis, and GE 1 screens (FPV only). These panels assess the sensitivity and specificity of the assays.

2.1.8.3 Sample preparation

Clinical samples submitted to the GVDS laboratory for routine diagnostic testing were used in this study. The type of sample assessed was dependent on the multiplex panel. Throat swabs were used for the respiratory panels, conjunctival swabs were used for the conjunctivitis panel, faecal samples were used for the gastroenteritis panel, blood samples collected into EDTA anticoagulant for the feline anaemia panel, and EDTA and gingival swabs were used for the feline gingivitis panel. Swabs were placed into a tube containing lysis buffer (Buffer AL, Qiagen, Germany) which lyses the cells and preserves the DNA/RNA. Blood samples were spun and separated; the serum or plasma was used for extraction. Faecal samples required pre-extraction processing. A small amount of faecal material was added to a cryovial and 1ml of lysis buffer was added. The tube was then vortexed and left for 10 minutes at room temperature. Then 200µl of the sample/lysis buffer mix was added to 1ml of lysis buffer and this was used for extraction.

2.1.9 Optimisation of primer and probe concentrations

As described in Chapter 1, the multiplex panels have been designed along syndromic lines offering users a single test to investigate clinical signs. Each multiplex must be carefully optimised to ensure that multiplexing does not result in either a loss of sensitivity at the endpoint of detection or a loss in specificity as a result of crosstalk, cross reaction or competition in comparison to the single test. The various stages involved in optimisation are outlined below.

2.1.9.1 Initial single assay probe and primer optimisation

Prior to multiplexing the primers and probe of each assay were optimised as a single test. The optimal concentration of each primer and probe is when the trace quality is optimal and the Ct value is lowest (so most sensitive) (Gunson *et al*, 2008). In order to determine the optimal primer concentrations a range of concentrations of the forward primer were run against a range of concentrations of the reverse primer in a primer chessboard (6.25 to 100µM) (Gunson, Gillespie, and Carman, 2003). These combinations of forward and reverse primers were tested with a positive control. For the primer assessment, a probe concentration of 5µM is used. Once the optimal primer concentrations were determined, a range of probe concentrations were assessed with the pre-determined primer optimal concentrations to determine the optimal probe concentration.

2.1.9.2 Optimisation of the multiplexed assay

The optimised single assays were then multiplexed together. A 10-fold dilution series of a strong positive control was used to compare the multiplex to the single assays. This was carried out to ensure that there is no loss in sensitivity at the endpoint of detection. If a loss in endpoint detection was evident then this could be optimised through careful adjustment of the primer and/or probe concentrations. If the issue cannot be rectified then the multiplex will not be developed further. Following this, the traces were examined to ensure results are easily interpretable and ensure no cross-reaction or crosstalk was evident.

2.1.9.3 Assess multiplex for crosstalk

Crosstalk is caused by the fluorescence output from one dye 'spilling over' into another channel to create what appears to be two positive traces, one of which is false. To eliminate crosstalk the probe concentrations need to be optimised, usually by reducing the concentration of the probe that is "spilling over" into the other channel. This has to be done carefully to ensure that the probe concentration would not have a detrimental effect on the performance of that assay. In order to do this several primer probe pools are made, each with a different concentration of the crosstalking probe (for example 10µM, 5µM, 2.5µM). These pools are then assessed with a 10-fold dilution series to determine if crosstalk is evident. If reducing the concentration of the crosstalking probe does not completely eliminate the crosstalk, it may be necessary to increase the concentration of the

probe that is detected in the channel in which the crosstalking probe is spilling over. In this way any crosstalk can be easily differentiated from a real trace.

It is important to remember that for every change made to a probe concentration, the endpoint detection limit of that component needs to be re-assessed against the single assay to ensure that the re-optimisation does not result in a loss in sensitivity or specificity for any component in the multiplex.

2.1.9.4 Assess multiplex for cross-reaction

Cross-reaction of primers or probes within an assay, or the formation of primer-dimers, is a result of primers or probes hybridising to each other because of strings of complementary bases in the sequence. When designing a new assay, primer design software helps to reduce these issues. Cross-reaction can inhibit or reduce the sensitivity of a reaction by using up the available reagents, it can also cause non-specific amplification traces if a probe were to cross-react with a primer. To assess if such a problem exists in a multiplex, no-template controls (water) can be run through the multiplex. If cross-reaction is occurring you would expect to see false positive/non-specific traces in these wells. To determine if cross-reaction is reducing the performance of the assays, the multiplex assay should be compared to the single assays. Cross-reaction can be eliminated with careful optimisation, by determining which components are cross-reacting and optimising the concentration of these components. This can be assessed by carrying out a 'plus/minus' experiment, where the multiplex assay is made up, minus one assay, e.g. FHV/FCV/*B.bronchiseptica* no IC; FHV/FCV/IC no *B.bronchiseptica* and so on to determine which part is causing the false positives or reduction in performance. Once the assay causing the issues is determined, a further plus/minus experiment is carried out removing each primer and probe of that assay to determine which primer or probes are cross-reacting. Reducing the concentration of one or both of these components may then eliminate cross-reaction. As with optimising crosstalk, the endpoint detection limit of that component needs to be re-assessed against the single assay to ensure that the re-optimisation does not result in a loss in sensitivity or specificity for any component in the multiplex.

2.1.9.5 Assess multiplex for competition

Multiplexing can create competition between targets where a stronger positive out-competes a weaker positive by using up available reagents, resulting in a false negative result for the out-competed target. Therefore it is important to ensure that mixed infections are detected effectively, and also to ensure that the internal control is not out-competed leading to a false inhibited results, or indeed that the internal control does not out-compete a genuine positive, resulting in a false negative result.

To assess if there was any competition between the internal control and any target within the multiplex, a 10-fold dilution series of each target was compared with and without IC in the sample and tested in duplicate.

Where mixed infections are expected, panels of simulated samples were generated using eight dilutions manufactured from a strong positive control to assess if any competition existed between the targets in a multiplex. For example, one panel contained various concentrations of FCoV and *Salmonella*, one contained FCoV and *C.jejuni* and a third contained *Salmonella* and *C.jejuni*. The dilution series from which these panels was made were tested in duplicate using the multiplex and singleplex assays to assess if the multiplex missed any positives due to competition that would be detected by a singleplex assay. Mixed infections are likely in respiratory samples and gastroenteritis samples. Mixed infections were assessed for the gastroenteritis multiplexes, but have not yet been investigated for the respiratory multiplex as work was focused on improving the FCV assay in the timescale of the study.

2.1.10 Evaluation of the endpoint sensitivity of the multiplex in comparison to the single assays

The endpoint sensitivity of the optimised multiplex was compared to the optimised single assay by using a 10-fold dilution series of a strong positive control for each target. The dilution series was tested in duplicate through the single and multiplex assays on at least four PCR runs.

2.1.11 Inter-assay and intra-assay variability

The inter-assay and intra-assay variability of the multiplex should be assessed to determine the robustness of the multiplex assay. The intra-assay (repeatability) variability was assessed by testing a positive control in 20 wells on one PCR run. The inter-assay variability (reproducibility) was not assessed in the timescale of this study. To assess the inter-assay variability positive run controls are monitored controls over approximately 20 PCR runs. These experiments assess how well the assay performs over time, on different ABI instruments and with different users to assess the whole system reproducibility of the multiplex. The co-efficient of variation (CoV) for the intra-assay variability was calculated for each assay, this is the standard deviation divided by the mean Ct value of the positive controls tested, and so shows the extent of variation in relation to the mean of the controls. A low CoV value indicates low variation which suggests good inter-assay and intra-assay variability, suggesting that the assay is repeatable and reproducible and therefore robust.

2.1.12. Evaluation of the sensitivity and specificity of the multiplex

Ideally, clinical validation comparing the multiplex assay to a "gold standard" for each pathogen should be carried out. This can be a large scale prospective evaluation where the multiplex assays are run in parallel to a gold standard and the results compared, or retrospectively testing a panel of positive and negative samples previously assessed by a gold standard. This gives the clinical sensitivity and specificity of the multiplex, and if planned properly can determine the negative and positive predictive values with prevalence data. Care must be taken when comparing PCR to a "gold standard" as PCR is often more sensitive than traditional methods, therefore if PCR is compared to a gold standard that is of poor sensitivity, the specificity of the PCR may falsely appear to be poor, as the PCR will detect more positives than the "gold standard" method. Therefore the quality of the "gold standard" should be taken into account when determining clinical sensitivity and specificity. It should be noted that completing the clinical validation of the feline and canine multiplex assays was beyond the timescale of this research project, however preparations to carry out the validations have been made. Small panels of known positive and negative clinical samples have however been assessed using the multiplex assays for FHV, FCV, *B.bronchiseptica*, *M.felis*, *C.felis*, FPV and CPV, which gives a limited comparison to the gold standard methods.

2.2 Methods specific to each multiplex

2.2.1 Feline respiratory multiplex

The feline respiratory panel consists of FHV, FCV, *B.bronchiseptica* and an IC (mumps virus). FHV was labelled with a FAM dye, FCV with a HEX dye, *B.bronchiseptica* with a Cy5 dye, and the IC was labelled with ROX. Primers and probes were optimised as described in Section 2.1.8.

2.2.1.1 Clinical samples

The performance of the multiplex assays was assessed using a panel of known positive and negative clinical samples for each pathogen; for the feline respiratory panel throat swabs were tested. The FHV samples tested were previously detected as positive by a singleplex FHV real time PCR and virus isolation, and the FCV and *B.bronchiseptica* samples were previously determined positive by virus isolation and bacterial culture respectively. Eleven known FHV-positive, initially 10 known FCV-positive, and 10 known *B.bronchiseptica*-positive throat swabs were tested through the multiplex. In addition, 15 throats swabs known to be negative for all three pathogens were tested. Testing this retrospective sample panel gives a limited comparison to the gold standard and assesses specificity, by ensuring each positive sample is correctly detected by the relevant component of the multiplex, but not by the other components of the multiplex. Not all FCV positive clinical samples were detected by the multiplex therefore to further investigate the sensitivity of the FCV PCR, an additional 27 known positive clinical samples were tested, along with typed FCV isolates (FCV virus isolate(VI)1, FCV VI2, FCV VI3) and 13 un-typed field isolates from around the UK.

2.2.2 Subsequent Methods following unexpected FCV PCR assay results

The initial PCR results obtained for the FCV clinical samples suggested that the FCV PCR was insensitive, which led to further sample panels being tested. Further assessment was also carried out by using a two-step RT-PCR kit designed for use with multiplex assays, by further concentrating the nucleic acid extract, and by assessing other extraction platforms available in the laboratory. Alternative FCV assays published in the literature were also assessed, and furthermore an in-house FCV real-time PCR assay was designed and assessed to see if sensitivity could be improved.

2.2.2.1 Assessment of FCV PCR with a two-step RT-PCR kit

A two-step RT-PCR kit was assessed:- the Quanta qScript cDNA kit/Quanta multiplex qPCR supermix. To assess if the PCR kit improved sensitivity, five virus isolation positive samples found to be negative by the multiplex assay previously were tested using this kit. Studies have suggested that two-step RT-PCR protocols are more sensitive than one-step due to secondary structures in RNA.

The cDNA mastermix for each sample consists of the following: 4.95µl of qScript reaction mix plus was added to 1.2375µl qScript reverse transcriptase. 5.625µl of cDNA mastermix was then added to 16.875µl of extracted RNA. The RT step was carried out on an ABI 9700 PCR machine using the following parameters: 22ºC for 5 minutes, 42ºC for 30 minutes and 85ºC for 5 minutes.

For PCR 5.5µl of the primer probe pool was added to 27.5µl of 2x Quanta qPCR mastermix, PCR was then carried out on the ABI 7500 with the following parameters: 2 minutes at 50°C, 95ºC for 2 minutes, followed by 40 cycles of 95ºC for 8 seconds and 60ºC for 34 seconds.

2.2.2.2 Concentration of the nucleic acid extraction and evaluation of other extraction platforms

The extraction of FCV-positive clinical samples on the easyMag was assessed. Concentrating the nucleic acid may improve sensitivity by increasing the amount of RNA in the extract. Several extraction and elution volumes were compared raging from a 1.81 times concentration (current) to a 40 times concentration. Other available automated extraction platforms available were also assessed (Qiagen MDx, Abbot M2000). The standard viral protocol for each of these platforms was used followed as per manufacturer's instructions.

2.2.2.3 Alternative published assays

The literature was searched for alternative FCV assays to assess if an alternative assay may be more sensitive that the assay original used in the multiplex, published by Abd-Eldain *et al* (2009). Two further assays were found (Helps *et al*, 2005; Chander *et al*, 2007) and assessed. All three assays are shown in Table 2.3; two assays (Abd-Eldain *et al*, 2009; Helps *et al*, 2005) target different areas of ORF1, one assay targets ORF2 (Chander *et al*, 2007).

2.2.2.4 Design of an in-house FCV assay

FCV is a member of the same family as norovirus and sapovirus, and studies on these viruses have found the open reading frame (ORF)1/2 breakpoint region to be a conserved region of the genome (Kageyama *et al* 2003, Chan *et al* 2005), therefore based on these previous findings we designed an assay that targets this breakpoint region.

Fifteen FCV strains sequenced at the ORF1/ORF2 region were found on BLAST, and a small conserved region was identified (Figure 2.4). A conserved sequence of 163 bases (5229-5391) was used for Primer-Express analysis, and 50 possible combinations of primers and probes were found. These primers and probes were aligned against the 15 sequences on BLAST and the primers and probe with the best match were selected. The forward primer contained the most mis-matches therefore three different sets of forward primer were ordered and assessed (Table 2.4). This assay was assessed using the panel of typed and un-typed isolates.

Table 2.3 Feline calicivirus TaqMan assays assessed in this study

Assay	Target		5'-3' sequence
Abd-Eldaim *et al* (2009)	OFR1 0-120	Forward primer	GTA AAA GAA ATT TGA GAC AAT
		Reverse primer	TAC TGA AGW TCG CGY CT
		Probe	CAA ACT CTG AGC TTC GTG CTT AAA
Helps *et al* (2005)	ORF1 2410-2540	Forward primer	GTT GGA TGA ACT ACC CGC CAA TC
		Reverse primer	CAT ATG CGG CTC TGA TTT GGC CTG
		Probe	TCG GTG TTG ATT TGG CCT G
Chander *et al* (2007)	Capsid protein gene 5320-5470	Forward primer	CAA CCT GCG CTA ACG
		Reverse primer	TCC CAC ACA GTT CCA AAT T
		Probe	CTT AAA TAY TAT GAT TGG GAY CCC CA
In-house	ORF1/2 BP junction 5245-5335	Forward primer	RCG CGG WBY GAH CAK A*
		Reverse primer	ATG TGC TCA ACC TGC GCT AA
		Probe	MCG MYC THC ACT GYG ATG TKT TCR AA

*see table 2.4 also

Table 2.4 In-house designed feline calicivirus assay: possible forward primer combinations

1 primer covering all mismatches RCG CGG WBY GAH CAK A
(a) 1 primer covering majority, 2 degenerate primers covering rest F1a: GCG CGG TCC GAT CAG A F2a: GCG CGG WCY GAY CAG A F3a: RCG CGG TBY GAW CAK A
(b) 8 primers covering all strains F1b: GCG CGG TCC GAT CAG A F2b: GCG CGG TCT GAC CAG A F3b: GCG CGG ACT GAC CAG A F4b: GCG CGG TTC GAT CAG A F5b: ACG CGG TCT GAT CAG A F6b: GCG CGG TGC GAT CAG A F7b: GCG CGG TCT GAA CAT A F8b: GCG CGG TCC GAC CAG A

The green letters highlight where degenerate bases have been added, these represent a position in the sequence that can have multiple possible bases

Figure 2.4 Conserved region of the feline calicivirus genome

2.2.3 Feline conjunctivitis multiplex

The feline conjunctivitis panel consists of FHV, *C.felis*, *M.felis* and an IC (HSV-1). FHV was labelled with a FAM dye, *M.felis* with a HEX dye, *C.felis* with a Cy5 dye, and the IC (HSV-1) was labelled with ROX. Primers and probes were optimised as described in Section 2.1.8.

2.2.3.1 Clinical samples

The performance of the multiplex assays was assessed using a panel of known positive and negative clinical samples for each pathogen; for the feline conjunctivitis panel eye swabs were tested. Eleven known FHV-positive (by real-time PCR and virus isolation) 13 known *C.felis* positive (by PCR), and 12 known *M.felis* positive (by real-time PCR and bacterial culture) eye swabs were tested through the multiplex. In addition 15 eye swabs known to be negative for all three pathogens were tested. Testing this retrospective sample panel gives a limited comparison to the gold standard and specificity, by ensuring each positive sample is correctly detected by the relevant component of the multiplex, but not by the other components of the multiplex.

2.2.4 Feline anaemia panel

The feline anaemia panel consists of two multiplex assays, Anaemia 1 (An1) and Anaemia 2 (An2). The An1 multiplex consists of FeLV which was labelled with a FAM dye, FIV which was labelled with a HEX dye, and FCoV which was labelled with a ROX dye. Primers and probes were optimised as described in Section 2.1.8.1. Anaemia 2 consists of the feline haemoplasmas (Mhf, CMt and CMhm) and an IC (mumps virus). The An2 assay was unable to be assessed as positive control material could not be obtained for the three haemoplasmas in the timescale of the project.

2.2.5 Feline gingivitis multiplex

The feline gingivitis multiplex consists of FeLV which was labelled with a FAM dye, FIV which was labelled with a HEX dye, FCV which was labelled with a Cy5 dye, and an IC (mumps virus) labelled with a ROX dye. Primers and probes were optimised as described in Section 2.1.8.

2.2.6 Feline and canine gastroenteritis panel

The feline and canine gastroenteritis panel consists of four multiplex assays, GE1 consists for FPV/CPV labelled with a FAM dye and an IC labelled with a ROX dye, GE2 consists of FCoV/CCoV labelled with a FAM dye, *Salmonella* labelled with a HEX dye and *C.jejuni* labelled with a Cy5 dye, GE3 consists of *Giardia* labelled with ROX, *T.foetus* labelled with HEX, and *C.parvum* labelled with Cy5, and GE 4 (for canine only) consisting of *E.coli* toxin ST1, *C.difficile* toxins A and B and *C.perfringens* toxin CPE. It was not possible to source an assay that specifically detected both *C.difficile* toxins A and B, during the timescale of the study on a specific toxin B assay could be sourced. Primers and probes were optimised as described in Section 2.1.8. The GE4 assays was initially assessed with positive controls, however the controls were not detected, suggesting that the assays may not be sensitive or specific for the toxins. This assay was not able to be further assessed in the timescale of this project.

2.2.6.1 Clinical samples

The performance of the GE1 multiplex assay was assessed using a panel of known positive and negative clinical samples for each pathogen, to date only FPV positive and negative clinical samples (faecal samples) have been assessed, these samples had been tested by traditional gel based PCR. Fourteen known positive faecal samples and 28 known negative faecal samples were assessed. Testing this retrospective sample panel gives a limited comparison to the gold standard and specificity, by ensuring each positive sample is correctly detected by the relevant component of the multiplex, but not by the other components of the multiplex.

CHAPTER 3
Results

CHAPTER 3

3. Results

This chapter outlines the results of the optimisation of each multiplex. Following this, the results of the evaluation of each multiplex are presented, which includes the endpoint detection limit of each multiplex in comparison to the single assays, the intra-assay variability of each multiplex, and if carried out the sensitivity and specificity of each multiplex with a small panel of clinical samples. Not all stages of the multiplex evaluation were completed for all multiplexes in the timescale of the project.

3.1 Feline respiratory multiplex

3.1.1 Initial single assay primer probe optimisation

The FHV, FCV and *B.bronchiseptica* assays were optimised first as single assays. Each primer and probe set was individually optimised and the final concentrations are given in Table 3.1.

3.1.2 Multiplex primer probe optimisation

The single assays were then multiplexed together; a 10-fold dilution series of a strong positive control and “no template” controls (water) were used to assess the performance and trace quality of the multiplex in comparison to the single assays. At this stage any signs of crosstalk, cross-reaction and competition were investigated. The results are described below.

Table 3.1 Optimal probe concentrations (µM)

Multiplex Screen		Optimal concentration (µM)	
		Single	Multiplex
Feline respiratory	**Feline herpes virus**	5	0.5
	Feline calicivirus	10	10
	Bordetella bronchiseptica	5	0.5
	Internal control	5	5
Feline conjunctivitis	**Feline herpes virus**	5	2.5
	Chlamydophila felis	20	30
	Mycoplasma felis	20	20
	Internal control	5	5
Feline Anaemia 1	**Feline leukaemia virus**	10	-
	Feline immunodeficiency virus	30	-
	Feline coronavirus	10	-
Feline Gingivitis	**Feline leukaemia virus**	10	-
	Feline immunodeficiency virus	30	-
	Feline calicivirus	10	-
	Internal control	10	-
GE 1	**Parvovirus**	10	10
	Internal control	10	10
GE 2	***Salmonella***	10	10
	Campylobacter jejuni	25	25
	Coronavirus	10	10
GE 3	***Cryptosporidium .parvum***	10	10
	Giardia	10	10
	Tritrochomonas foetus	10	10

The primers and probes of each assay was optimised as a single assay and, if necessary, within the multiplex assay. GE: gastroenteritis

3.1.2.1 Crosstalk

On initial assessment of the multiplex, crosstalk from the FAM (FHV) channel into HEX (FCV) was evident. Several concentrations of the FHV probe were assessed, and it was found that reducing the FHV probe concentration to 0.5µM eliminated the crosstalk. The endpoint sensitivity of each assay in the multiplex was not affected by the re-optimisation of the FHV and FCV probes.

3.1.2.2 Cross-reaction

Evidence of cross-reaction was seen in the "no template" control wells, resulting in false positive *B.bronchiseptica* traces. A 'plus/minus' experiment was carried out and the results suggested that the *B.bronchiseptica* probe was cross-reacting with more than one component in the multiplex (Figure 3.1). Several concentrations of the *B.bronchiseptica* probe were assessed in the multiplex and it was found that by reducing the *B.bronchiseptica* probe concentration to 0.5µM the cross-reaction was eliminated without any detrimental effect on the *B.bronchiseptica* sensitivity (Figure 3.2).

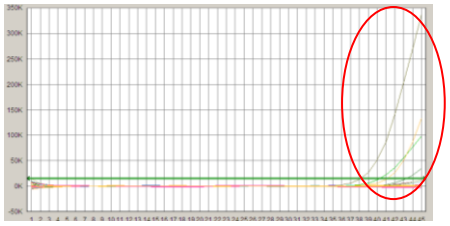
3.1.2.3 Competition

Since an IC is included in the multiplex, the possibility of competition was studied. A dilution series of each target with and without the IC in the samples was compared. For FHV, when the IC was excluded, the assay detected the target down to a dilution of 10^{-6}, when the IC was included the assay detected the target down to 10^{-7}. For FCV, the assay detected the target down to a dilution of 10^{-6} both with and without the IC. Similarly for *B.bronchiseptica*, the endpoint detection limit was 10^{-7} with and without the IC (Table 3.2). The Ct values of the IC were consistently around 25 (the exact values for each dilution are given in Table 3.2). These results suggest that no significant competition was evident. The final optimised concentrations of the respiratory multiplex are given in Table 3.1. The assessment of mixed respiratory infections has not yet been investigated.

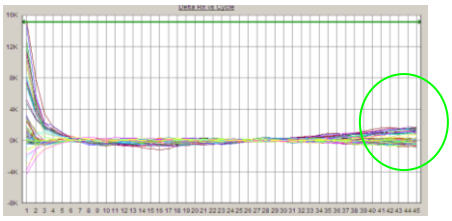
Figure 3.1 Cross-reaction observed in the respiratory multiplex.

A

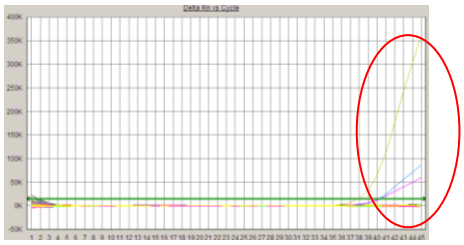
Minus IC assay



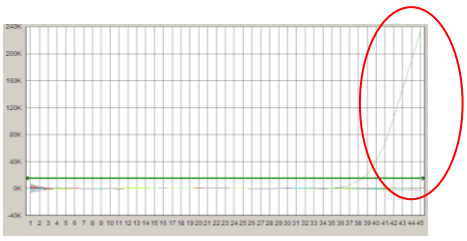
Minus *B.bronchiseptica* assay



Minus FCV assay

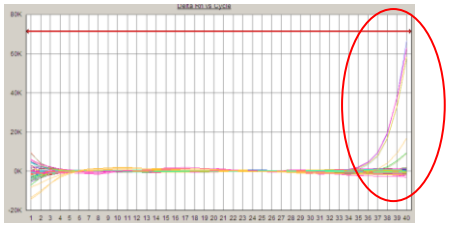


Minus FHV assay

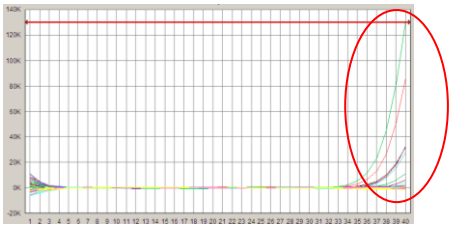


B

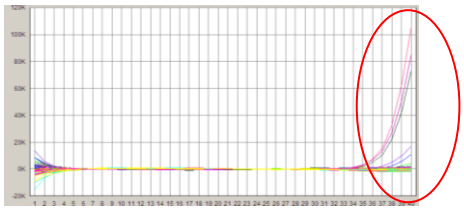
Multiplex



Minus *B.bronchiseptica* forward primer



Minus *B.bronchiseptica* reverse primer



Minus *B.bronchiseptica* probe

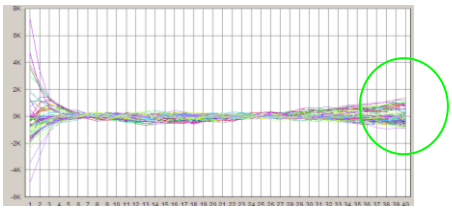
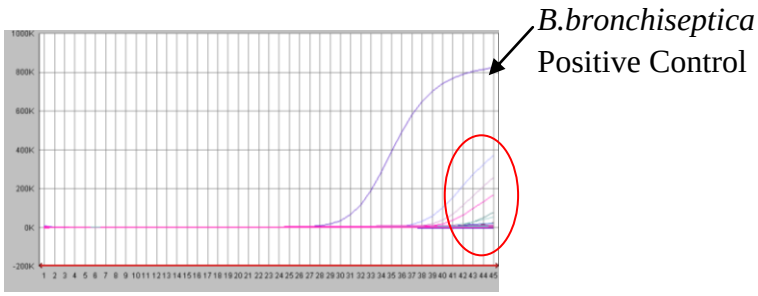
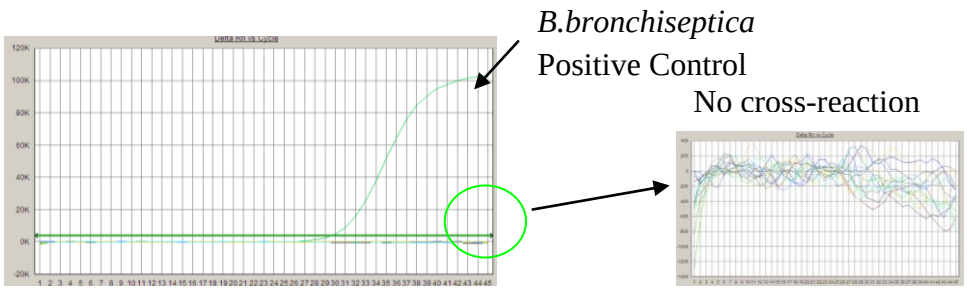


Figure 3.2 Elimination of Bordetella bronchiseptica probe cross-reaction.

A



B



C

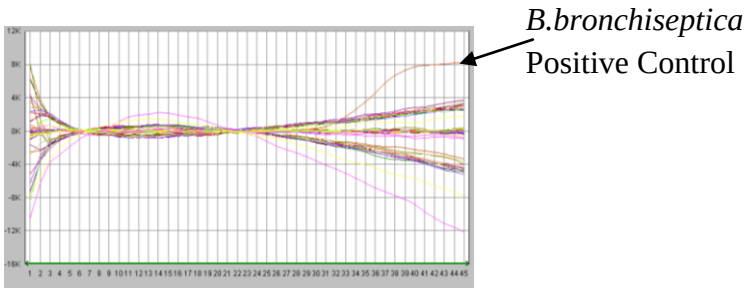


Table 3.2 Competition of each target with internal control in the respiratory screen

Dilution	Feline herpesvirus			Feline calicivirus			*Bordetella bronchiseptica*		
	No IC	With IC		No IC	With IC		No IC	With IC	
10^{-1}	21.55	21.27	25.29	19.57	17.25	25.14	19.03	19.12	25.33
10^{-2}	24.97	24.40	25.37	23.70	21.00	25.26	22.75	22.26	25.00
10^{-3}	28.32	27.45	25.16	27.58	24.41	25.32	24.85	25.15	25.22
10^{-4}	31.27	30.29	25.16	30.66	28.33	25.40	28.05	28.22	25.35
10^{-5}	35.19	32.74	25.19	34.51	32.26	25.24	31.17	30.79	25.23
10^{-6}	N	36.50	25.23	N	N	25.07	34.97	32.78	25.32
10^{-7}	N	37.77	25.28	N	NT	NT	N	NT	NT

A 10-fold dilution series of each pathogen was assessed with and without an internal control in the sample to assess if there was any competition between the internal control and the target pathogens. N: Negative; NT: Not tested; IC: internal control. IC Ct given in faded text under "with IC" column

3.1.3 Evaluation of the endpoint sensitivity of the multiplex in comparison to the single assays

The endpoint sensitivity of the multiplex was assessed in comparison to the single assays. In order to do this the endpoint detection limit of each multiplex component was compared to that of the single assays using a 10-fold dilution series of a positive control. Testing was done in duplicate wells. When testing the dilution series of FHV the single assay detected the 10^{-8} dilution in one out of two wells, whereas the multiplex detected the target down to 10^{-8} in both wells. For FCV the single test detected the 10^{-8} dilution in both wells, and the multiplex detected down to 10^{-8} in one out of two wells. When testing the dilution series of *B.bronchiseptica* the single assay detected down to the 10^{-5} dilution in one out of two wells, whereas the multiplex detected down to 10^{-5} in both wells (Table 3.3). In addition, due to the variability of FCV, three typed FCV isolates (FCV VI1, VI2, VI3) and 13 un-typed field isolates were assessed at the endpoint detection. For all samples, similar endpoints were found between single and multiplex assays (data not shown). These results suggest that the endpoint sensitivity of the multiplex assay is comparable to that of the single assays.

Table 3.3 Endpoint detection limit of the respiratory multiplex in comparison to the single assay

Dilution	Feline herpesvirus		Feline calicivirus		*Bordetella bronchiseptica*	
	Single	**Multiplex**	**Single**	**Multiplex**	**Single**	**Multiplex**
10^{-1}	16.06	15.32	13.90	14.38	26.09	24.86
10^{-2}	19.29	18.49	17.61	17.70	29.09	28.19
10^{-3}	21.99	21.65	20.83	21.32	32.02	32.21
10^{-4}	25.31	24.69	24.95	24.82	35.72	34.06
10^{-5}	28.39	27.91	28.82	28.94	37.30/N	36.93
10^{-6}	29.36	31.58	32.35	32.92	N	N
10^{-7}	33.57	34.73	37.39	36.77	N	N
10^{-8}	35.66/N	37.07	39.58	38.70/N	N	N
10^{-9}	N	N	N	N	NT	NT
10^{-10}	N	N	N	N	NT	NT

A 10-fold dilution series of each pathogen was assessed using a single assay for each pathogen and the multiplex assay. N: negative; NT: not tested

3.1.4 Intra-assay variability

The intra-assay variability was assessed and the co-efficient of variation (CoV) values are shown in Table 3.4. The CoV values were low for each component; FHV CoV 0.003, FCV CoV 0.004 and *B.bronchiseptica* CoV 0.006. The results suggest that the intra-assay variability of the assay i.e. precision, is good for each component as little variation was observed when the positive control was repeatedly tested. The assessment of the inter-assay variability of the assay is ongoing.

3.1.5 Evaluation of the sensitivity and specificity of the multiplex

The multiplex was evaluated using a small panel of known positive and negative clinical samples. The FHV component of the assay detected successfully the 11 samples that contained FHV. The *B.bronchiseptica* component of the assay correctly detected the 10 *B.bronchiseptica* positive samples present in the panel. No false positives were detected in the 15 known negative samples and no cross-reaction was observed for FHV and *B.bronchiseptica.*

For FCV on initial screening not all of the 10 known positive throat swab samples were detected by the single and multiplex assays. One clinical sample that was highly positive for FHV (Ct 16) was false negative for FCV in the multiplex but positive in the single test. This sample was consistently positive in the FCV single test (Ct ~22) and negative in the multiplex, therefore the false negative result for FCV is likely due to competition, i.e. the strong FHV sample out-competed the weaker FCV sample resulting in a false negative result for the FCV sample. The other false negative samples were not mixed infections. Therefore an additional panel of FCV positive samples was assessed. The panel was assessed using the respiratory multiplex assay and the single FCV assay. Eight out of 24 of the additional clinical samples were negative on initial screening. The false negative samples were repeated in triplicate and gave plus/minus results (i.e. 1/3 or 2/3 wells negative) for all but two samples, which were consistently negative. In addition the mean Ct value of the clinical samples was 31.71 by the multiplex (29.84 by single assay). FCV is detected traditionally by virus isolation; therefore PCR would be expected to detect FCV in clinical samples at low Ct values (strong positives). The results suggest that the single and multiplex are comparable and that the loss in sensitivity is not a result of multiplexing.

The FCV component of the respiratory screen was further investigated and is detailed below in section 3.2.

Table 3.4 Intra-assay variability

Multiplex	**Target**	**Mean** (20)	**CoV**
Feline Respiratory	**Feline herpesvirus**	29.35	0.003
	Feline calicivirus	27.99	0.004
	Bordetella bronchiseptica	28.01	0.006
Feline Conjunctivitis	**Feline herpesvirus**	29.93	0.007
	Myoplasma felis	27.90	0.004
	Chlamydophila felis	28.64	0.006
GE1	**Parvovirus**	27.41	0.009
GE2	**Coronavirus**	26.91	0.005
	Salmonella	24.42	0.004
	Campylobacter jejuni	26.39	0.005
GE3	***Tritrichomonas foetus***	26.93	0.013
	Giardia	30.48	0.008
	Cryptosporidium parvum	28.07	0.003

The intra-assay variability is presented as a co-efficient of variation (CoV) value, a low value indicates low variability and so good repeatability for the assay. GE: gastroenteritis

3.2 Feline calicivirus further results

3.2.1 Assessment of FCV PCR with a two step RT-PCR kit

A two-step RT-PCR was assessed to determine if sensitivity could be improved. The results suggest that using a two step RT-PCR kit may improve the sensitivity of the FCV PCR marginally as the Ct was lower (stronger), however no significant improvement was seen at the endpoint of detection (Table 3.5). These results suggest that the loss in sensitivity is not RT-PCR kit related.

3.2.2 Concentration of the nucleic acid extraction and evaluation of other extraction platforms

The result of assessing different nucleic acid concentrations suggested that increasing the concentration of the sample five-fold at extraction improves the sensitivity 10-fold (by 1 log) therefore adjusting the elution volume of respiratory samples may improve the sensitivity of the FCV assay (Table 3.6). Other automated extraction platforms available were also assessed (Qiagen MDx, Abbot M2000) and results were similar to the original easyMag results (data not shown).

3.2.3 Alternative published assays

The three alternative assays were assessed using the panel of 24 culture-positive clinical samples (Table 3.7) and 15 isolates (Table 3.8). The Abd-Eldaim assay detected 14 out of the 24 samples as positive, the Chander assay detected 18 samples positive, and the Helps assay detected 23 samples positive (Table 3.7). Four samples were false negative in both the Abd-Eldaim and Chander assays, however the other false negatives, and the one false positive by the Helps assay were not identical across the three assays.

Table 3.5 Comparison of a one and two step RT-PCR kit for Feline calicivirus

FCV		One-step Ambion	Two-step Quanta
FCV1		33.07	33.70
FCV2		32.02	34.85
FCV3		N	37.17
FCV4		31.63	31.51
FCV15		N	37.98
FCV VI1	**10^{-1}**	12.95	14.33
	10^{-2}	16.74	17.76
	10^{-3}	20.73	20.70
	10^{-4}	24.04	24.24
	10^{-5}	26.97	27.42
	10^{-6}	NT	NT
	10^{-7}	34.71	33.97
	10^{-8}	36.91	N
FCV VI2	**10^{-1}**	16.12	13.58
	10^{-2}	20.38	16.96
	10^{-3}	23.63	20.40
	10^{-4}	27.41	23.26
	10^{-5}	30.71	27.39
	10^{-6}	34.45	30.69
	10^{-7}	36.69/N	34.24
	10^{-8}	N	38.69
FCV VI3	**10^{-1}**	15.63	17.42
	10^{-2}	20.33	21.97
	10^{-3}	24.86	25.39
	10^{-4}	29.08	29.34
	10^{-5}	33.04	33.31
	10^{-6}	36.68	37.31
	10^{-7}	N	N
	10^{-8}	N	39.93/N

A panel of feline calicivirus (FCV) samples were tested using a one and two step RT-PCR kit (multiplex kit) and the results compared.

N: negative; NT: not tested

Of note there were several significant differences in Ct values between the FCV assays, for example sample FCV6 was negative by the Abd-Eldaim assay and positive by the other assays, however there was an 8 Ct difference between the Chander assay (Ct 19.89) and Helps assay (Ct 28.09). Similarly sample FCV23 was negative by the Abd-Eldaim assay and positive by the other assays; however this time the Chander assay had a higher Ct value (Ct 37.71) than the Helps assay (Ct 25.60). There was no pattern where for example one test consistently detected samples at a higher Ct than the other tests; the results were variable from sample to sample suggesting that the assays may detect some strains better than others. Overall the Helps assay performed the best on this panel of samples.

On assessment of the isolate panel the Abd-Eldaim and Helps assays detected all the isolates in the panel, whereas the Chander assay failed to detect two of the un-typed isolates (Table 3.8).

An interesting observation was that the Chander assay produced different types of exponential curve (Figure 3.3). The majority of positive traces were of a similar type of curve; however three clinical samples and three isolates, one the typed FCV VI3, had a noticeably different type of curve.

3.2.4 Comparison of the in-house FCV assay to other published assays

The in-house assay was compared to the other FCV assays using the 13 isolates from around the UK and the three typed isolates (Table 3.8). The assay was significantly less sensitive than the other assays, detecting all isolates at high Ct values (weakly positive) and therefore the in-house assay not assessed further.

Table 3.6 Concentration of feline calicivirus nucleic acid elute on easyMag

Concentration	FCV Ct
x 40	25.67
x 20	26.03
x 10	26.60
x 5	26.34
x 2	29.14
Standard (x 1.81)	29.40

A feline calicivirus (FCV) positive sample was concentrated to various degrees on the easyMag and the results compared. Ct: cycle threshold; x: times concentrated

Table 3.7 Comparison of the two alternative published feline calicivirus assays to the original selected published assay (Abd-Eldaim *et al*, 2009)

Clinical samples (24)	Abd-Eldaim	Chander	Helps
FCV1	N	37.28	36.61
FCV2	N	N	34.59
FCV3	N	36.65	34.00
FCV4	N	N	31.09
FCV5	29.08	26.31*	23.83
FCV6	N	19.89	28.09
FCV7	25.23	N	20.34
FCV8	25.81	24.99	27.17
FCV9	20.38	25.56	27.49
FCV10	25.09	24.48	22.10
FCV11	29.80	22.92	22.60
FCV12	27.51	25.18	N
FCV13	25.18	22.57	38.04
FCV14	29.48	20.19*	24.78
FCV15	N	26.04	29.85
FCV16	28.91	35.41	23.19
FCV17	N	N	34.04
FCV18	21.72	N	21.20
FCV19	23.65	19.05	22.01
FCV20	29.16	28.64*	30.86
FCV21	N	N	28.65
FCV22	25.01	24.12	30.32
FCV23	N	37.71	25.60
FCV24	N	23.15	26.25

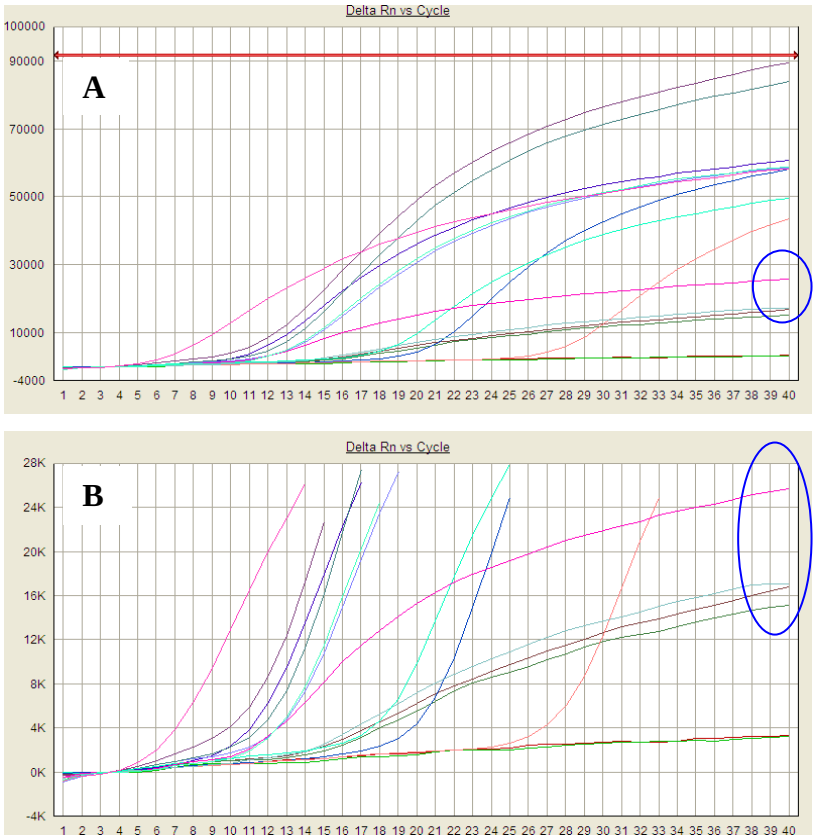
The feline calicivirus (FCV) assay originally chosen and assessed with the multiplex was the Abd-Eldaim *et al* (2009) assay. In the literature two alternative assays are published (Chander *et al* (2007) and Helps *et al* (2005)), these assays were assessed alongside the Abd-Eldaim *et al* (2009) assay with a panel of FCV positive samples and isolates. N: negative; * strange traces

Table 3.8 Comparison of the in-house designed feline calicivirus assay to the published assays

Isolates (15)	Abd-Eldaim	Chander	Helps	In-house
FCV VI untyped 1	13.91	N	15.22	26.20
FCV VI untyped 2	23.38	17.44*	15.81	29.34
FCV VI untyped 3	29.32	20.70	14.99	27.39
FCV VI untyped 4	15.99	13.25	13.37	25.51
FCV VI untyped 5	14.64	13.18	12.17	27.54
FCV VI untyped 6	19.40	15.42*	13.71	29.83
FCV VI untyped 7	11.67	18.77	11.29	29.92
FCV VI untyped 8	14.20	13.13	17.99	25.33
FCV VI untyped 9	17.25	N	15.11	28.89
FCV VI untyped 10	18.38	17.81*	20.13	34.52
FCV VI untyped 11	14.19	11.36	NT	27.21
FCV VI untyped 12	15.05	28.83	15.59	31.50
FCV VI1	10.22	10.53	9.43	25.19
FCV VI2	10.96	7.41	7.80	24.71
FCV VI3	13.47	11.28*	12.63	25.45

The three published assays were then compared to an assay designed in-house using a panel of positive FCV isolates. N: negative; FCV VI: feline calicivirus virus isolate; *strange traces

Figure 3.3 Strange curves observed with the Chander et al (2007) assay.



3.3 Feline conjunctivitis multiplex

3.3.1 Initial single assay primer probe optimisation

The FHV, *C.felis* and *M.felis* assays were optimised as single assays. Each primer and probe was individually optimised, the optimal concentrations are given in Table 3.1.

3.3.2 Multiplex primer probe optimisation

The optimised assays were then multiplexed together and a 10-fold dilution series of a strong positive control was used to assess the performance of the multiplex in comparison to the single assays. At this stage any signs of crosstalk, cross-reaction and competition were investigated. The results are detailed below.

3.3.2.1 Crosstalk

On initial assessment, cross-talk from the FAM (FHV) channel into HEX (*M.felis*) was evident. Several concentrations of the FAM probe was assessed, it was found that reducing the probe concentration to 2.5µM significantly reduced the crosstalk. To eliminate the crosstalk, the HEX probe concentration was boosted to 30µM. The sensitivity of the FHV and *M.felis* components of the multiplex were not affected by changing the probe concentration.

3.3.2.2 Cross-reaction

No cross-reaction was evident in this multiplex pool

3.3.2.3 Competition

Since an IC is included in the multiplex, the possibility of competition was studied. A dilution series of each target, with and without the IC, was compared. For FHV the assay detected the target down to a dilution of 10^{-7} both with and without the IC. For *M.felis* the assay detected down to 10^{-7} when no IC was present and 10^{-6} with IC. Similarly for *C.felis* the endpoint detection limit was 10^{-7} without IC and 10^{-8} with IC (Table A-1). The Ct values of the IC are given in Table A-1. These results show that no significant competition was evident. The assessment of competition in mixed infections is yet to be carried out. The final optimised concentrations of the respiratory multiplex are given in Table 3.1.

3.3.3 Evaluation of the endpoint sensitivity of the multiplex in comparison to the single assays

The endpoint detection limit of each component of the multiplex was compared to the single assays using a 10-fold dilution series of a positive control tested in duplicate wells. When testing the dilution series of FHV the multiplex assay detected the 10^{-6} dilution in one out of two wells, the single test detected down to 10^{-6} in both wells. For *M.felis*, both assays detected down to the 10^{-9} dilution one out of two wells. When testing the dilution series of *C.felis* the single and multiplex assays both detected down to the 10^{-6} (Table A-2). These results suggest that the endpoint detection limit of the multiplex assay is comparable to that of the single assays.

3.3.4 Intra-assay variability

The intra-assay variability was assessed and the CoV values are given in Table 3.4. The CoV values were low for each component; FHV CoV 0.007, *M.felis* CoV 0.004 and *C.felis* CoV 0.006. The results suggest that the repeatability of the assay is good for each component as little variation was observed. The inter-assay variability assessment is ongoing.

3.3.5 Evaluation of the clinical sensitivity and specificity of the multiplex

The multiplex was evaluated using a small panel of known positive and negative clinical samples. The FHV component of the assay detected successfully the 11 samples that contained FHV. The *M.felis* component of the assay correctly detected the 12 *M.felis* positive samples present in the panel. The *C.felis* component of the assay successfully detected the 13 samples that contained *C.felis*. No false positives were detected in the known negative samples and no cross-reaction was observed.

3.4 Feline anaemia panel - Anaemia 1: FeLV, FIV and FCoV

3.4.1 Initial single assay primer probe optimisation

The FeLV, FIV and FCoV assays were optimised as single assays. Each primer and probe was individually optimised and the optimal concentrations are given in Table 3.1.

3.4.2 Multiplex primer probe optimisation

The optimised assays were then multiplexed together and a 10-fold dilution series was used to assess the performance of the multiplex in comparison to the single assays. At this stage any signs of crosstalk, cross-reaction and competition are investigated.

From this initial assessment of sensitivity the FCoV component of the multiplex was found to be less sensitive in comparison to the single assay. For FeLV the single assay detected the 10^{-5} dilution, whereas the multiplex assay detected to 10^{-4}. For FIV the single assay detected down to both the 10^{-5} and 10^{-6} dilutions in one out of two wells, the multiplex detected down to 10^{-5} in one out of two wells. For FCoV the single assay detected the 10^{-6} dilution in one out of two wells, the multiplex detected the 10^{-4} dilution (Table A-3). Therefore the sensitivity of the FCoV was greatly reduced in the multiplex in comparison to the single test. In addition to the loss in endpoint detection limit for the assays, the trace quality was poor, with traces flattening out. This experiment was repeated, and the multiplex endpoints were more comparable to the single tests (Table A-4), however flattening of traces was still evident. Therefore a two-step RT-PCR kit designed for use with multiplex PCR assays was assessed; the Quanta qScript cDNA kit and Quanta multiplex qPCR supermix (Table A-5). The endpoint detection limit and trace quality were not improved, in fact for FeLV in particular the multiplex endpoints were worse than previous results. Overall the results suggested that the assay is not robust as a multiplex.

A ‘plus/minus’ experiment was carried out to determine if one assay was responsible for the poor endpoint sensitivity and trace quality. The results suggest that when FeLV is removed from the multiplex, the FIV traces improve, and FCoV traces slightly improve. FeLV did not improve whether FIV or FCoV were removed from the multiplex. Attempts were made to optimise the FeLV assay within the multiplex by adapting probe concentration (assessing concentrations from 1.25µM to 20µM) and primer concentration

(assessing mixtures of concentrations of forward and reverse primers from 25µM to 100µM), but these measures failed to improve the FeLV assay, or the FIV and FCoV assays within the multiplex. Overall the results suggest that these assays do not work together as a triplex assay.

3.5 Feline gingivitis multiplex

3.5.1 Initial single assay primer probe optimisation

The FeLV, FIV and FCV assays were optimised as single assays. Each primer and probe was individually optimised using a strong positive control; the optimal concentrations are given in Table 3.1.

3.5.2 Multiplex primer probe optimisation

The optimised assays were then multiplexed together and a 10-fold dilution series was used to assess the performance of the multiplex in comparison to the single assays. At this stage any signs of crosstalk, cross-reaction and competition were investigated.

As with the anaemia 1 assay, issues with endpoint sensitivity were evident in this multiplex assay. The FCV dilutions failed to amplify in the multiplex pool (Table A-6). The experiment was repeated using a PCR kit specifically designed for use with multiplex assays, the Quanta qScript cDNA kit and Quanta multiplex qPCR supermix (Table A-7). The results suggested that there was a significant loss in sensitivity for the FeLV component of the multiplex in comparison to the single test. In addition endpoint sensitivity of the FCV component of the multiplex was not improved, and trace quality was poor. No further work was carried out on this assay in the timescale of the project.

3.6 Feline/canine gastroenteritis panel

The GE panel consists of 3 multiplex screens; GE1: FPV/CPV, IC, GE2: FCoV, *Salmonella, C.jejuni*, and GE3: *C.parvum, Giardia, T.foetus.* The optimisation and evaluation of these multiplex assays are described below.

3.6.1 GE1: FPV/CPV and Internal control

3.6.1.1 Initial single assay primer probe optimisation

The parvovirus assay was optimised as a single assay. Each primer and probe was individually optimised and the optimal concentrations are given in Table 3.1.

3.6.1.2 Multiplex primer probe optimisation

The optimised assays were then multiplexed together and a 10-fold dilution series was used to assess the performance of the multiplex in comparison to the single assays. At this stage any signs of crosstalk, cross-reaction and competition are investigated.

3.6.1.3 Crosstalk and cross-reaction

No crosstalk or cross-reaction was observed in the multiplex.

3.6.1.4 Competition

As an IC is included in the assay, the possibility of competition was studied. A dilution series of parvovirus was compared with and without IC in the sample (Table A-8) and the endpoint detection limit is similar with and without IC in the sample, and the IC had a consistent Ct value of ~28. It is known that faecal samples positive for parvovirus can have a Ct of less than five, therefore strong positive samples will always out-compete the internal control. However since this assay is qualitative and not quantitative, if a sample is found to be parvovirus positive and IC negative, then inhibition/competition is not significant.

3.6.1.5 Evaluation of the endpoint sensitivity of the multiplex in comparison to the single assays

The endpoint detection limit of parvovirus in the duplex was compared to the single assay using a 10-fold dilution series of positive control. The single assay detected the 10^{-5} dilution in one out of two wells, whereas the duplex detected down to the 10^{-5} dilution in both wells (Table A-9). These results suggest that there is no loss in sensitivity when the assay is multiplexed in comparison to the single assay.

3.6.2 Intra-assay variability

The robustness of the assay was determined by assessing the intra-assay variability of the assay. The intra-assay variability (Table 3.4) of the parvovirus assay was found to be low (CoV 0.009). The assessment of the inter-assay variability is ongoing.

3.6.2.1 Evaluation of the clinical sensitivity and specificity of the multiplex

The sensitivity and specificity of the duplex was evaluated using a panel of known positive and negative clinical samples. The duplex and single test successfully detected the 14 parvovirus samples in the panel; in addition the assays detected another three parvovirus positive samples at higher Ct values (weak positive). Contamination was not observed in either the negative controls extracted with the samples, or in the no-template controls; therefore these samples may be true positives and low level shedders. There was no evidence of cross-reaction occurring in the duplex, and the single assay also detects the samples as positives. Of the 17 samples negative for parvovirus by conventional gel based PCR (detection by gel electrophoresis), 14 were found negative by duplex and single PCR. A further 11 samples found to be negative by traditional PCR (total 28 negatives) were tested through the multiplex and were found to be negative. The discrepant samples were retested by the GVDS and found to negative on repeat, this may suggest that the real-time PCR assay is more sensitive than traditional PCR, however this must be further investigated.

3.6.3 GE2: Coronavirus, *Campylobacter jejuni* and *Salmonella*

3.6.3.1 Initial single assay primer probe optimisation

The coronavirus, *C.jejuni* and *Salmonella* assays were optimised as single assays. Each primer and probe was individually optimised and the optimal concentrations are given in Table 3.1.

3.6.3.2 Multiplex primer probe optimisation

The optimised assays were then multiplexed together and a 10-fold dilution series was used to assess the performance of the multiplex in comparison to the single assays. At this stage any signs of crosstalk, cross-reaction and competition are investigated.

3.6.3.3 Crosstalk and cross-reaction

No crosstalk or cross-reaction was observed in the multiplex.

3.6.3.4 Competition

Mixed infections can occur in gastroenteritis cases therefore competition was assessed. In total three panels of simulated samples were generated using eight dilutions manufactured from a strong positive control: one containing various concentrations of FCoV and *Salmonella*, one containing FCoV and *C.jejuni* and a third containing *Salmonella* and *C.jejuni*. The dilution series from which these panels were manufactured were then tested using the singleplex assays. The results are presented as a mean (unless they were positive/negative) in Tables A-10, A-11 and A-12. Overall, the sensitivity of the FCoV assay is not affected when *Salmonella* or *C.jejuni* is present at any concentration. The *Salmonella* and *C.jejuni assays* are both affected at the endpoint of detection in the presence of another strong pathogen. However weak detection (Ct >35) of these pathogens may not be clinically significant. If another pathogen is present at a higher level than it would be likely that this pathogen is causing illness, therefore the multiplex primer probe pool was not further optimised.

3.6.3.5 Evaluation of the endpoint sensitivity of the multiplex in comparison to the single assays

The endpoint detection limit of each component of the multiplex was compared to the single assays using a 10-fold dilution series of a positive control tested in duplicate. For FCoV both the multiplex and the single assay detected the 10^{-3} dilution. For *Salmonella* the single and multiplex assays detected down to 10^{-7}. For *C.jejuni* the single assay detected the 10^{-8} dilution in one out of two wells, the multiplex detected the 10^{-7} dilution in one out of two wells (Table A-13). Therefore there was no significant loss in sensitivity observed when the three assays were multiplexed.

3.6.3.6 Intra-assay variability

The robustness of the assay is determined by assessing the intra-assay variability of the assay. The CoV values were low for each component; coronavirus CoV 0.005, *C.jejuni* CoV 0.005 and *Salmonella* CoV 0.004. The intra-assay variability of the assay were found to be low (Table 3.4). The inter-assay variability assessment is ongoing.

3.6.4 GE3: *Giardia*, *T.foetus* and *C.parvum*

3.6.4.1 Initial single assay primer probe optimisation

The *Giardia*, *T.foetus* and *C.parvum* assays were optimised as single assays. Each primer and probe was individually optimised and the optimal concentrations are given in Table 3.1.

3.6.4.2 Multiplex primer probe optimisation

The optimised assays were then multiplexed together and a 10-fold dilution series was used to assess the performance of the multiplex in comparison to the single assays. At this stage any signs of crosstalk, cross-reaction and competition are investigated.

3.6.4.3 Crosstalk and cross-reaction

No crosstalk or cross-reaction was observed in the multiplex.

3.6.4.4 Competition

Mixed infections can occur in gastroenteritis cases therefore competition should be assessed. It was not possible to assess competition in mixed infections as a strong enough *T.foetus* positive control could not be sourced in the timescale of the project.

3.6.4.5 Evaluation of the endpoint sensitivity of the multiplex in comparison to the single assays

The endpoint detection limit of each component of the multiplex was compared to the single assays using a 10-fold dilution series of a positive control tested in duplicate. For *Giardia* both the multiplex and the single assay detected the 10^{-4}. For *C.parvum* the single assay detected down to 10^{-4} in one out of two wells, and the multiplex detected down to 10^{-3}. For *T.foetus* the single assay detected the 10^{-6} dilution in one out of two wells, the multiplex detected the 10^{-5} dilution (Table A-14).

A panel of clinical samples was not assessed for this multiplex in the timescale of the project.

3.6.4.6 Intra-assay variability

The robustness of the assay is determined by assessing the intra and inter-assay variability of the assay. The CoV values were low for each component; *T.foetus* CoV 0.013, *Giardia* CoV 0.008 and *C.parvum* CoV 0.003 (Table 3.4). The inter-assay variability assessment is ongoing.

CHAPTER 4
Discussion

CHAPTER 4

4. Discussion

The diagnosis of the causative agent in infectious disease in cats and dogs is essential to allow for the appropriate clinical intervention (Greiner and Gardener, 2000; Zarlenga and Higgins, 2001; Dahlhausen, 2010; Belak *et al*, 2013), reduce infection spread, and also for epidemiological and prevalence studies which in turn will better the understanding of the causes of infectious disease (Templeton *et al*, 2005; Gunson *et al*, 2008; Wolffs *et al*, 2011). In recent years PCR has replaced traditional detection methods as the gold standard diagnostic technique for many pathogens. However replacing traditional detection methods with panels of internally controlled multiplex real-time PCR screens designed along syndromic lines is not widespread in veterinary laboratories. This thesis describes the first phase of a project which aims to develop in-house multiplex assays for the detection of infectious diseases in cats and dogs. It is hoped that in time the implementation of such assays may lead to significant service and clinical benefits as has been seen in human diagnostics, particularly for clinical virology (Gunson *et a*l, 2008).

After choosing the appropriate nucleic acid extraction platform, RT-PCR kit, real-time PCR platform, and internal control for the service, 17 real-time PCR assays were chosen with the aim of developing five syndrome based multiplex panels consisting of a total of nine multiplex assays (Table 1.1). The multiplex assays were optimised and assessed for crosstalk, cross-reaction and competition, and then evaluated through a series of experiments to determine the endpoint sensitivity, specificity and robustness of each multiplex in comparison to the single assays, in some cases small panels of known positive and negative clinical samples were also assessed (Bustin *et al*, 2009).

In the timescale of this project, the full optimisation and pre-clinical validation assessment of five multiplex assays were completed; feline respiratory screen, feline conjunctivitis screen and the feline/canine GE 1, 2 and 3 screens. These multiplex assays were optimised to remove any issues of crosstalk and cross-reaction, and the multiplex assays performed well in comparison to the single assays when evaluated with dilution series and specificity panels. The multiplex assays were found to be robust by assessment of the intra-assay variability, each multiplex had a low CoV value (less than 0.05), which suggests little variation was evident within a PCR run. An IC was also successfully included in each

multiplex panel. As described previously an IC is the ideal positive control and monitors for sample inhibition, PCR inhibition and PCR set-up error, therefore is essential for reliable and accurate diagnostics. In this study the IC in each multiplex was not found to out-compete a positive target which would result in a false negative result, and the IC was not out-competed by a strong target which would result in a falsely inhibited result. The possibility of competition was assessed for the feline conjunctivitis and the gastroenteritis 2 screen and no significant competition was observed in these assays. Competition in mixed infections is to be assessed for the feline respiratory multiplex and the GE 3 multiplex. Some of these multiplex assays were assessed with a limited panel of clinical samples and it should be noted that some issues arose with the feline respiratory screen and the GE 1 screen which is described in detail below.

It should be noted that various issues arose during the optimisation and development of some of the multiplex assays, and although most of these issues were overcome by appropriate optimisation, laboratories intending to implement in-house multiplex real-time PCR should be aware of these potential issues and so plan and budget accordingly. Firstly, access to strong positive control material to enable the optimisation of the primer probe pool was difficult at times. This was a particular problem for pathogens that were not tested for at the GVDS (e.g. *T.foetus*), pathogens that had not previously been detectable by traditional methods (e.g. haemoplasmas), or pathogens of low prevalence. Collaboration with other centres worldwide allowed some positive control material to be sourced however these delays prevented the complete optimisation of some assays in this study (Anaemia 2 screen, GE 3 (*T.foetus*) and GE 4 screens). For some assays plasmids were bought to allow the primers and probes of the single and multiplex assays to be optimised, however plasmids are expensive and so significantly increase the cost of optimising the assays. In addition plasmid controls can increase the risk of test contamination resulting in false positive results therefore it is advisable to store stocks of plasmids in a different location to where testing is carried out. It should be noted that the lack of available positive samples will have a significant impact on the ability to clinically validate these assays as access to positive material is essential.

Veterinary molecular diagnostics is in its infancy and there are few relevant real-time assays published in the literature. In some cases tests for particular pathogens were not

available which in turn resulted in some of the multiplex assays not being developed, for example in the proposed GE 4 screen a specific toxin real-time assay for *C.difficile* Toxin A could not be found. A solution to this issue would be to design in-house assays, but with an additional lack of sequence data available to be able to design an assay, this was not possible. Lack of published assays and sequence data will also affect future developments. For example a major issue with the ongoing expansion of the canine infectious disease panels is that no published real-time assay can be found for canine respiratory coronavirus or canine parainfluenza virus-1, and only one published assay for canine adenovirus-1 can be found.

As described above many of the screens developed in this study were multiplexed with minimal optimisation, however some issues arose during optimisation. Crosstalk was evident in both the feline respiratory and conjunctivitis multiplexes and although in both assays FAM was found to be crosstalking into the HEX channel, each multiplex had to be optimised differently to fully eliminate the crosstalk without resulting in a detrimental effect on any component in the multiplex. This demonstrates that although the same probe dyes can be used in different multiplex assays, the optimisation required can be very different for each multiplex, highlighting again the experience and skill required to successfully optimise a multiplex assay.

In addition to crosstalk, cross-reaction was observed in the feline respiratory screen. Cross-reaction such as in the case of the *B.bronchiseptica* can result in non-specific false positive traces, therefore it is essential that issues such as cross-reaction are assessed and overcome. This was overcome by determining which components were cross-reacting and optimising these components. To do this each primer and probe was removed from the respiratory multiplex and it was determined that cross-reaction did not occur when the *B.bronchiseptica* probe was removed. Therefore it could be concluded that this probe was cross-reacting with not one but several components of the assay. Reducing the *B.bronchiseptica* probe concentration eliminated the cross-reaction without having a detrimental effect on any component of the multiplex. This again highlights the skill and experience needed to determine what is causing cross-reaction, and to optimise the multiplex assays to eliminate cross-reaction.

In some cases, assays were unable to be multiplexed at all. An example of this was with the feline anaemia and gingivitis multiplex assays. In both assays the FeLV assay could not be multiplexed with the other assays without significantly affecting sensitivity. This highlighted that even with experience and careful optimisation there are occasions when assays simply will not be able to be multiplexed together. Occasionally this can be overcome by using PCR kits specially designed for use with multiplex assays. As mentioned in the introduction, RT-PCR kits specifically designed for multiplex assays which aim to reduce some of the issues caused by multiplexing such as crosstalk, cross-reaction and competition are now available. One such kit, the Quanta qScript cDNA kit/Quanta multiplex qPCR supermix kit was assessed with the FeLV containing multiplexes. However as described in the results section the use of this kit did not improve the assay. In the anaemia and gingivitis multiplexes components of the FeLV assay may have been inhibiting the other assays by cross-reacting with the other primers and/or probes. Despite various attempts to optimise and reduce this effect, no improvement was seen. Therefore as described previously, cross-reaction can cause false positives but can also cause false negatives by cross-reacting with other components in the multiplex and so inhibiting the assays. One solution would be to use a single FeLV assay alongside a FIV/FCoV and FIV/FCV duplex assay, as the FIV, FCoV and FCV endpoint detection limits and trace quality improved when FeLV was removed from each multiplex. However an alternative to removing FeLV from the multiplexes would be to source an alternative FeLV assay which may not cross-react with the other assays. An alternative FeLV real-time assay was sourced from the literature (Torres *et al* 2008); however it was not possible to assess this assay with the anaemia and gingivitis assays in the timescale of this project.

Some of the optimised multiplex assays underwent limited further evaluation using small panels of positive and negative clinical samples. Most performed as expected, detecting the appropriate samples as positive and failing produce false positives, however a notable exception was FCV. Prior to the assessment of the respiratory multiplex with a panel of positive and negative respiratory clinical samples, the FCV component of the assay was found to be sensitive and specific for the target pathogens detecting all the expected controls. However, on testing a further panel of FCV positive clinical samples the assay did not detect all the expected clinical samples. This suggests that FCV PCR may be less sensitive than virus isolation. In total three published real-time TaqMan FCV RT-PCR assays were assessed using a panel of known virus isolation FCV positive samples and

positive viral isolates. Each FCV assay performed differently, failing to detect different samples and also detecting the same samples at variable Ct values. The IC was positive and in the expected Ct range for all the samples indicating that no sample inhibition, PCR inhibition or PCR set-up error was causing this loss in sensitivity. In an attempt to increase the performance of FCV PCR an in-house assay was then designed targeting the ORF1/2 breakpoint region and was evaluated using the same panel of FCV isolates. This region was chosen as a target as this region been successfully used as a target for norovirus assays. The in-house designed assay was significantly less sensitive in comparison to the three published assays. There was a lack of sequence data available to design the assay therefore a reason for the reduced sensitivity could be that there are in fact many more mismatches in this region than observed during development.

It may be that FCV is so variable that a PCR has not yet been designed to sensitively detect all variants of the virus, and that the assays assessed here perform differently with different variants of the virus. An interesting observation was that the Chander *et al* (2007) assay appeared to produce two distinct sets of amplification curves, one of the typed isolates FCV VI3 fitted in with the minority type of trace suggesting that these samples could be the same strain and that the assay does not amplify this strain as efficiently as others, sequencing of these samples would allow to further investigate these differences. Multiplexing the three published FCV assays together may improve overall FCV detection, however some virus isolation positive clinical samples were still undetected by any of the PCR assays.

When looking at the Ct values of the clinical samples detected by the three published assays, the Ct values were high (weakly positive), and so it may be that the reduced sensitivity of PCR is clinically useful. For example perhaps the PCR assay will only detect clinically significant infection i.e. cats shedding high levels of virus, whereas cats that are carriers shed low levels of virus, however further investigation is needed to determine the significance of the Ct value. PCR usually improves sensitivity in comparison to traditional methods and so can detect DNA or RNA at low levels that may not be clinically significant. However, in the case of FCV, the reduced sensitivity compared to virus isolation, may have some clinical value.

Ideally in order to assess and improve the FCV PCR a larger number of typed isolates and clinical samples, both virus isolation positive and negative, need to be assessed over a longer time period. In addition the use of sequencing would be useful to further investigate discrepant samples and inform better test design. The FCV component of the respiratory assay will remain in the multiplex however work will continue to improve the sensitivity of the FCV component.

Another interesting outcome from the limited clinical sample evaluations related to the GE 1 multiplex assay. The GE 1 multiplex assay performed well when assessed with a small panel of positive and negative clinical samples detected by gold standard methods (traditional gel based PCR). However an additional three positive samples were detected by the GE 1 screen. These results could reflect an increase in sensitivity for the real-time PCR assay however these could also be false positive results. These samples will require further analysis with alternative detection methods to determine if they are true parvovirus positives. Real-time PCR can be more sensitive than traditional PCR, as stated above, care must be taken when comparing real-time PCR to other methods, even traditional PCR methods, as real-time PCR is often more sensitive (Mackay *et al*, 2002; Gunson *et al*, 2008). It is likely that the real-time PCR is more sensitive than traditional PCR at the endpoint of detection for parvovirus however this is yet to be proven.

Overall this study demonstrates some of the pitfalls that may be encountered when developing an in-house multiplex real-time PCR diagnostic service. Although it has been demonstrated that with careful optimisation most issues can be corrected, this process can incur costs and requires in depth experience of in-house multiplex real-time PCR. It is important the laboratories considering implementing such assays realise that optimisation and test development issues are also frequently encountered post implementation once the assays are in routine use. This process can also be costly and requires experience but is essential for a robust and quality service (Gunson *et al*, 2008). Some examples of the expected ongoing issues are discussed below.

When manufacturing new lots of multiplex PCR reagents (i.e. the multiplex primer and probe pools), the sensitivity and specificity of the newly developed reagents must be

compared to the current "in-use" reagents. In order to do this, again the endpoint sensitivity (10-fold dilution panels of a positive control tested in duplicate) and the specificity (testing of no-template controls) of each new lot of reagents must be assessed and compared to the "in-use" lot to ensure that the new lot is as sensitive and specific as the previous lot. If the new lot is found to be less sensitive or specific then the new primer probe pool will have to be re-optimised. It is useful to manufacture primer probe pools in large lots as this reduces overall work load and limits the variation of the reagents over time. This also applies to positive extraction controls and may also extend to different lots of PCR kit and extraction kit reagents.

To ensure an assay is reliable and robust over time, the sensitivity and specificity must be monitored in each extraction and PCR run (Bustin *et al*, 2009). In order to do this negative controls and positive controls for each target must be extracted alongside clinical samples and no-template controls should be tested on each PCR run. The positive controls should be monitored over time to ensure any loss in sensitivity is detected. If a loss in performance is evident, this assay can then be repeated, preventing the wrong results being reported. If problems continue with the assay, then the assay must be troubleshooted to determine what component is failing, for example the primer probe pool, the positive control, the extraction, etc. Negative extraction controls and no-template controls monitor for contamination in the system, in addition these controls may highlight other issues with reagents such as degradation of a primer probe pool which can also cause low level positive traces, which are false positive traces, and so could result in false positives being reported (Bustin *et al*, 2009). Extensive record keeping of lot numbers of each reagent used in each real-time PCR run and the user carrying out the test is also useful as it can inform any troubleshooting of an assay. For example, if a test performs badly i.e. the control is out of range, by looking back at record keeping it may be simple and easy to determine if a new primer probe pool lot had been used, or a new PCR kit lot etc.

Awareness of the literature is also important as part of QC, novel types or variants of pathogens may evolve, therefore the sensitivity and specificity of the assays can change over time. A good example in human diagnostics is influenza. Influenza is a virus that evolves constantly (known as antigenic drift) with the emergence of new strains a common result. In addition, influenza also re-assorts (known as antigenic shift) and so novel

influenza subtypes can emerge (Bennett *et al*, 2011). Laboratories have to constantly ensure that tests are able to detect these newly emerging strains. External QC panels are also useful for monitoring test performance; these panels contain a variety of relevant types and subtypes of a pathogen over a range of Ct values. External QC panels are useful to help identify if the in-house assay is performing poorly, they also allow comparison of the in-house assay to methods used in different laboratories nationwide.

For the reasons described some laboratories may not be keen to implement in-house molecular methods and so would wait for commercial solutions. Currently there are no commercially available real-time multiplex PCR panels available for feline and canine infectious disease however these are likely to be available in the near future. Commercial assays remove the need for extensive in-house quality control as the kit would contain a fully optimised assay with positive and negative control material and pre-determined quality control ranges (Bustin *et al*, 2009). Reagents would therefore not be manufactured and assessed lot to lot in-house, and importantly a commercial company would be responsible for troubleshooting any issues with the multiplex primer probe pools, removing the need and cost of a highly experienced technician running the service. However once a molecular service is implemented and the expertise is available an in-house service will be less costly, and future additions could be made to panels, and new panels developed with little extra cost.

To summarise, this thesis describes the beginning phase of the development of a panel of in-house multiplex real-time PCR assays for the detection of feline and canine infectious disease. Viruses are the major cause of infectious disease in cats and dogs although bacteria, parasites and fungi are commonly implicated. Since these pathogens can present with indistinguishable clinical signs, it is essential to be able to determine the cause of infectious disease to enable appropriate clinical intervention, for example determining if the cause of disease is viral or bacterial changes the clinical intervention and disease management. The real-time PCR assays described here will enable the simultaneous detection of DNA or RNA (RT-PCR), viruses, bacteria, parasites or fungi from a single sample. These assays are envisaged to bring significant benefits to veterinary practices.

As found in human diagnostics traditional methods of detection can be insensitive and so many feline and canine pathogens may be under-diagnosed (e.g. rhinovirus in human infectious disease), real-time PCR can improve the overall detection and be particularly useful for fastidious pathogens (Templeton *et al*, 2005; Gunson *et al*, 2008; Wolffs *et al*, 2011). Therefore as well as associating more cases with an aetiological cause, a new understanding of clinical syndromes can develop as previously undiagnosed or under-diagnosed pathogens may be more readily detected. Furthermore the significance and clinical impact of mixed infections may become apparent; the use of sensitive multiplex real-time PCR assays at the WoSSVC highlighted that mixed infections were commonly detected in humans. Using such sensitive assays in cats and dogs will enable the clinical importance of mixed infections to be investigated in these species. The use of multiplex PCR also standardises the detection method for the different pathogens in contrast to using several different detection methods to screen for a panel of pathogens, this in turn significantly reduces the turn around times from the receipt of a sample to the result being reported, which has significant clinical benefits.

The use of sensitive detection techniques such as the real-time PCR multiplexes described here will be extremely valuable for use in epidemiological studies to determine more accurate figures on the actual prevalence of disease causing pathogens. As described in the introduction, prevalence studies for most of the pathogens described in this study are published in the literature, however these studies have been carried out using various detection methods including traditional methods that may be insensitive, and so variable ranges of prevalence have been reported. For example as detailed in the introduction the prevalence of *B.bronchiseptica* has been reported to range from as low as 0.4% to as much as 19% (Egberink et al, 2009) which is a vast range, making it difficult to truly determine the importance of this pathogen in feline respiratory disease. The use of a sensitive detection method such as real-time PCR for epidemiology studies will provide more accurate prevalence data, in addition the use of multiplex real-time to detect several pathogens from one sample in one reaction will be advantageous as this generates more data rapidly for epidemiology studies. These data can also inform public health where relevant, and if indeed the use of sensitive techniques such as real-time PCR results in certain pathogens being found to be more significant in causing disease than previously thought then this may potentially indicate the case for new vaccines to be developed for these pathogens, if not currently available. In addition more sequencing data could result

in new variants being identified, and this new data could be used to improve current vaccines, making them more efficient in preventing disease.

4.1 Future work

Future work will include completing the full validation of the multiplex screens. As mentioned above, not all aspects of the multiplex validation were completed in the timescale of the project. Other aspects yet to be carried out as part of the multiplex evaluation include the assessment of the inter-assay variability of each assay. The intra-assay variability has been carried out and each assay was found to have good repeatability within a PCR run. The inter-assay variability must be carried out to demonstrate the robustness of the assay over time, and so gives an indication of the reproducibility of the assay on different PCR runs, extraction runs and with different users.

In addition the assessment of mixed respiratory infections has yet to be carried out to assess if any competition is evident between the targets. Mixed infections are expected in respiratory samples, in fact data from the GVDS suggests approximately 25% of FCV positive samples will also be positive for FHV therefore the possibility of one positive target out-competing another weaker positive in a sample which would result in a false negative result must be evaluated. One mixed FCV and FHV infection was tested by the respiratory multiplex and single assays and the FHV positive did out-competed the FCV positive resulting in a false negative FCV result by the multiplex, highlighting that competition may be an issue with this assay, and further optimisation or use of a multiplex RT-PCR kit may be necessary for this multiplex. Mixed infections were not investigated in the timescale of this study as the FCV component of the multiplex was being further optimised and investigated.

The anaemia and gingivitis assays will be multiplexed and assessed with an alternative FeLV assay, this will involve starting again from scratch to optimise the assay and so will increase the costs and time for the development of these screens, and if again the multiplex is unsuccessful, as stated above a single FeLV assay may be the only solution at this point in time, which would be more costly as samples would need to be tested by two assays.

Each assay must be clinically validated with a panel of relevant positive and negative clinical samples for each pathogen; this will be the last stage of evaluation and if the assays are comparable to the "gold standard" methods of detection then the assays can be implemented. The molecular assays could be run alongside the current traditional methods for a time period to collect this data. It is possible that problems will be encountered at this stage, as found with FCV, in which case the development of assays may have to go back to the drawing board and alternative assays assessed if available, which again would increase the time and costs for the development.

While completing the validation of the five multiplex panels in this study, there are also ongoing plans are to develop other multiplex screens to this feline and canine infectious disease panel. Work has begun on a canine respiratory screen which will screen for *B.bronchiseptica*, canine parainfluenza virus, canine adenovirus-2, canine herpesvirus, canine respiratory coronavirus, influenza A and *Angiostrongylus vasorum*. Several other screens including a panel of ocular, neurological, hepatitis and abortion/fading pup screens for canines will be developed. As well as continuing to develop the assays described here, and extending the panels, the assays can be further developed to be able to quantify and even type as well as diagnose, this may be useful for FIV were the viral load may be clinically significant.

Future collaborations with other research groups may provide the control materials and sequence data required to fully develop and improve the multiplex assays. The GVDS plan to use Next Generation Sequencing (NGS) to further investigate and analyse clinical positive and negative samples, generating the sequence data required to continue the development of the molecular diagnostic service as well as bettering the understanding of the circulating pathogens causing feline and canine infectious disease. In addition, further analysis of the clinical samples screened negative by the multiplex screens using NGS could result in novel pathogen discovery.

As mentioned above commercial methods for the molecular detection of veterinary pathogens may become available in the near future. Another future option may be to use point-of-care (POC) testing of cats and dogs where the veterinarian can rapidly screen the

animal in their practice, or indeed where tests can be used at home by the owner. In humans, various POC assays are available at the bedside in wards, at local GP practices and at home. Point of care assays do not require expertise staff to operate and therefore are easy to use and implement. These POC assays provide rapid diagnosis that is sensitive and specific, and can be used for the detection antibody, antigen and molecular applications, and POC assays for antibody and antigen detections are currently available for cats and dogs. In the future, it may be possible that the in-house PCR assays developed here will be adapted to POC assays. Such testing methods would be of great advantage in shelters and boarding houses to enable the rapid screening of animals prior to entering, allowing infection control to be set up, preventing the rapid spread of infectious disease throughout the population of animals.

4.2 Conclusions

This thesis describes the initial phase of the development of an internally controlled syndrome based multiplex real-time PCR diagnostic service at the GVDS that will eventually replace traditional detection methods. The results of this study highlight that the development of an in-house multiplex real-time PCR diagnostic service can be at times difficult and occasionally time-consuming. Often these aspects are not discussed in the literature and as a result laboratories considering implementing/developing in-house multiplex assays may not be aware of these issues. As this study has shown, most issues can be overcome by using the approaches described in this thesis but this requires experience of in-house multiplex techniques and can increase the costs of a development project. However, despite this it is envisaged that in-house multiplex techniques will bring many advantages over current veterinary diagnostics; similar to what has been seen in human diagnostics - especially clinical virology.

Appendix

Appendix 1

Table A-1 Competition of each target with internal control in the conjunctivitis screen

Dilution	Feline herpesvirus			*Mycoplasma felis*			*Chlamydophila felis*		
	No IC	**With IC**		**No IC**	**With IC**		**No IC**	**With IC**	
10^{-1}	**18.76**	**19.74**	N	19.29	20.11	30.06	**17.75**	**18.66**	**33.65**
10^{-2}	22.22	22.54	28.66	22.35	23.18	29.16	20.46	21.43	28.52
10^{-3}	25.86	25.90	28.78	25.68	26.02	29.01	24.03	24.71	28.50
10^{-4}	29.03	28.50	28.99	29.02	28.94	28.90	27.35	27.67	28.68
10^{-5}	32.05	30.80	29.02	32.18	31.61	29.14	31.00	30.82	29.04
10^{-6}	35.23	34.77	28.87	34.33	33.87	29.08	33.29	33.00	28.91
10^{-7}	36.58	36.26	29.20	38.96	N	29.14	36.04	36.06	29.15
10^{-8}	N	N	29.47	N	N	NT	N	38.23	28.98

A 10-fold dilution series of each pathogen was assessed with and without an internal control in the sample to assess if there was any competition between the internal control and the target pathogens. N: Negative; NT: Not tested; IC: internal control. IC Ct given in faded text under "with IC" column

Table A-2 Endpoint detection limit of the conjunctivitis multiplex in comparison to the single assay

Dilution	Feline herpesvirus		*Mycoplasma felis*		*Chlamydophila felis*	
	Single	**Multiplex**	**Single**	**Multiplex**	**Single**	**Multiplex**
10^{-1}	22.13	22.09	17.19	16.08	20.74	20.45
10^{-2}	24.77	24.63	20.07	19.27	24.08	23.92
10^{-3}	28.26	28.04	23.45	22.81	27.61	27.29
10^{-4}	31.48	31.34	26.63	25.75	30.83	30.78
10^{-5}	35.82	36.45	29.64	29.43	34.31	35.07
10^{-6}	37.11	38.10/N	33.00	32.16	37.7	28.19
10^{-7}	N	N	35.08	35.04	N	N
10^{-8}	N	N	32.62	33.61	N	N
10^{-9}	NT	NT	31.45/N	36.88/N	NT	NT
10^{-10}	NT	NT	N	N	NT	NT

A 10-fold dilution series of each pathogen was assessed using a single assay for each pathogen and the multiplex assay. N: negative;

NT: not tested

Table A-3 Endpoint sensitivity of the anaemia 1 multiplex in comparison to the single assay

Dilution	Feline leukaemia virus		Feline immunodeficiency virus		Feline coronavirus	
	Single	**Multiplex**	**Single**	**Multiplex**	**Single**	**Multiplex**
10^{-1}	23.26	23.56	20.22	20.21	24.46	24.45
10^{-2}	26.36	26.42	23.60	23.98	28.07	28.04
10^{-3}	29.57	29.69	26.76	27.32	31.41	31.41
10^{-4}	33.08	39.69/N	30.17	30.41	34.57	N
10^{-5}	35.67	N	39.23/N	37.14/N	38.15	N
10^{-6}	N	N	36.32/N	N	38.29/N	N
10^{-7}	N	N	N	N	N	N
10^{-8}	N	N	N	N	N	N

A 10-fold dilution series of each pathogen was assessed using a single assay for each pathogen and the multiplex assay. N: Negative

Table A-4 Repeat testing of the endpoint sensitivity of the anaemia 1 multiplex in comparison to the single assay

Dilution	Feline leukaemia virus		Feline immunodeficiency virus		Feline coronavirus	
	Single	**Multiplex**	**Single**	**Multiplex**	**Single**	**Multiplex**
10^{-1}	26.83	26.26	26.59	27.18	25.31	25.49
10^{-2}	30.05	29.97	29.50	30.92	28.64	28.74
10^{-3}	32.39	36.68	37.06/N	33.62	31.54	31.56
10^{-4}	N	N	N	N	37.15	38.40/N
10^{-5}	N	N	N	N	N	N
10^{-6}	N	N	N	N	N	N
10^{-7}	N	N	N	N	N	N
10^{-8}	N	N	N	N	N	N

A 10-fold dilution series of each pathogen was assessed using a single assay for each pathogen and the multiplex assay. N: negative

Table A-5 Endpoint detection limit of the anaemia 1 multiplex in comparison to the single assay using the Quanta RT-PCR mastermix kit

Dilution	Feline leukaemia virus		Feline immunodeficiency virus		Feline coronavirus	
	Single	**Multiplex**	**Single**	**Multiplex**	**Single**	**Multiplex**
10^{-1}	25.96	28.17	25.48	28.06	24.17	26.37
10^{-2}	29.39	31.67	29.11	30.27	27.38	30.10
10^{-3}	31.84	N	37.23	35.50/N	30.14	32.87
10^{-4}	35.17	N	38.46/N	N	32.85	35.80/N
10^{-5}	37.20/N	N	N	N	35.22	N
10^{-6}	N	N	N	N	37.17	N
10^{-7}	N	N	N	N	34.98	N
10^{-8}	N	N	N	N	N	N

A 10-fold dilution series of each pathogen was assessed using a single assay for each pathogen and the multiplex assay using a PCR kit designed for use with multiplex PCR. N: negative

Table A-6 Endpoint detection limit of the gingivitis multiplex in comparison to the single assay

Dilution	Feline leukaemia virus		Feline immunodeficiency virus		Feline coronavirus	
	Single	**Multiplex**	**Single**	**Single**	**Multiplex**	**Single**
10^{-1}	16.16	16.38	23.86	24.25	30.58	N
10^{-2}	19.15	19.35	27.11	27.00	33.96	N
10^{-3}	22.32	22.55	29.51	30.20	37.77	N
10^{-4}	25.56	25.84	32.56	34.35	38.37/N	N
10^{-5}	28.85	28.28	N	35.16/N	N	N
10^{-6}	31.96	34.72	N	N	N	N

A 10-fold dilution series of each pathogen was assessed using a single assay for each pathogen and the multiplex assay. N: negative

Table A-7 Repeat of the endpoint detection limit of the gingivitis multiplex in comparison to the single assay

Dilution	Feline leukaemia virus		Feline immunodeficiency virus		Feline coronavirus	
	Single	**Multiplex**	**Single**	**Single**	**Multiplex**	**Single**
10^{-1}	28.26	27.36	24.22	24.07	25.96	24.86
10^{-2}	32.29	N	27.30	27.13	29.39	N
10^{-3}	35.31	N	30.79	30.58	32.7	N
10^{-4}	N	N	N	N	38.58	N
10^{-5}	N	N	N	N	N	N
10^{-6}	N	N	N	N	N	N
10^{-7}	N	N	N	N	N	N
10^{-8}	N	N	N	N	N	N

A 10-fold dilution series of each pathogen was assessed using a single assay for each pathogen and the multiplex assay. N: negative

Table A-8 Competition of each target with internal control in the gastroenteritis 1 screen

Dilution	Parvovirus (PV)		
	No IC	With IC	
		PV	IC
10^{-1}	21.15	20.43	28.22
10^{-2}	24.24	23.71	28.26
10^{-3}	27.41	26.45	28.43
10^{-4}	30.87	29.79	28.34
10^{-5}	N	N	28.32
10^{-6}	N	N	28.21
10^{-7}	N	N	28.32
10^{-8}	N	N	28.45

A 10-fold dilution series of parvovirus was assessed with and without an internal control in the sample to assess if there was any competition between the internal control and the target pathogen. N: negative; IC: internal control

Table A-9 Endpoint detection limit of the gastroenteritis 1 (parvovirus and internal control) screen in comparison to the single assay

Dilution	Single	Multiplex
10^{-1}	17.62	17.35
10^{-2}	21.42	21.54
10^{-3}	23.61	23.88
10^{-4}	31.82	27.95
10^{-5}	37.82/N	34.63
10^{-6}	N	N
10^{-7}	N	N
10^{-8}	N	N

A 10-fold dilution series of parvovirus was assessed using a single assay and the multiplex assay. N: negative; NT: not tested.

Table A-10 Competition between targets in the gastroenteritis 2 screen: coronavirus against *Salmonella*

	***Salm* -1**	***Salm* -2**	***Salm* -3**	***Salm* -4**	***Salm* -5**	***Salm* -6**	***Salm* -7**	***Salm* -8**
	24.09[a]	**27.22**	**30.67**	**33.58**	**34.92**	**N**	**N**	**N**
corona -1	24.83	28.02	31.16	34.89	N	N	N	N
24.07[b]	24.86	25.18	24.98	25.05	25.25	25.12	25.10	25.25
corona -2	24.84	28.32	31.68	36.05	N	N	N	N
27.31	28.33	28.03	28.64	28.18	28.21	28.30	28.71	28.39
corona -3	24.94	28.45	31.61	36.01	N	N	N	N
30.21	32.05	32.01	31.71	31.39	32.10	31.56	31.26	31.25
corona -4	24.98	28.64	32.32	36.74	N	N	N	N
34.24	34.18	N	34.12	34.49	34.03	35.23	N	34.24
corona -5	24.76	28.19	31.97	36.21	N	N	N	N
N	N	N	N	35.13	35.20	N	N	N
corona -6	24.67	28.56	31.57	N	N	N	N	N
35.38	N	N	N	N	N	N	N	N
corona -7	24.76	28.35	31.33	34.55	N	N	N	N
N	N	N	N	N	N	N	N	N
corona -8	24.97	28.51	31.24	33.95	N	N	N	N
N	N	N	N	N	N	N	N	N

Panels of simulated samples were generated using eight dilutions manufactured from a strong positive control and tested to assess if any competition existed between targets in a multiplex. *Salmonella* Ct values are in blue[a], and coronavirus Ct values are in green[b]. N: negative; *salm: salmomella*; corona: coronavirus

Table A-11 Competition between targets in the gastroenteritis 2 screen: coronavirus against *Campylobacter jejuni*

	C.jejuni **-1** **25.98**[a]	***C.jejuni*** **-2** **28.81**	***C.jejuni*** **-3** **32.64**	***C.jejuni*** **-4** **37.77**	***C.jejuni*** **-5** **N**	***C.jejuni*** **-6** **N**	***C.jejuni*** **-7** **N**	***C.jejuni*** **-8** **N**
corona-1	26.70	30.41	33.20	N	N	N	N	N
24.29[b]	25.46	25.61	26.00	25.61	25.31	25.69	25.57	25.56
corona-2	26.35	30.17	34.18	34.35	N	N	N	N
28.06	28.99	28.75	29.06	29.01	29.23	N	29.09	29.20
corona-3	26.58	29.60	33.71	35.47	N	N	N	N
30.98	31.80	32.34	32.32	32.07	N	31.84	32.27	31.90
corona-4	27.02	30.06	34.16	36.71	37.31	N	37.80	N
34.28	34.11	34.73	35.12	N	35.75	34.16	35.65	36.03
corona-5	26.48	29.90	34.38	N	N	N	N	N
N	N	N	N	N	N	N	N	N
corona -6	26.31	29.86	33.41	N	N	N	N	N
N	N	N	N	N	N	N	N	N
corona -7	26.71	29.57	33.85	36.63	N	N	N	N
N	N	N	N	N	N	N	N	N
corona -8	26.39	30.08	33.12	37.15	N	N	N	N
N	N	N	N	N	N	N	N	N

Panels of simulated samples were generated using eight dilutions manufactured from a strong positive control and tested to assess if any competition existed between targets in a multiplex. *Campylobacter* jejuni Ct values are in blue[a], and coronavirus Ct values are in green[b]

N: negative; corona: coronavirus*; C.jejuni*: *Campylobacter jejuni*

Table A-12 Competition between targets in the Gastroenteritis 2 screen: *Campylobacter jejuni* against *Salmonella*

	***C. jejuni* -1**	***C. jejuni* -2**	***C. jejuni* -3**	***C. jejuni* -4**	***C. jejuni* -5**	***C. jejuni* -6**	***C. jejuni* -7**	***C. jejuni* -8**
	25.39[a]	**29.47**	**32.34**	**34.66**	**37.07**	**N**	**N**	**N**
Salm -1	26.61	30.12	33.24	36.67	N	N	N	N
24.01[b]	24.88	24.96	25.00	24.77	24.93	25.22	25.14	25.02
Salm -2	26.96	30.11	33.16	N	N	N	N	N
27.43	28.32	28.65	28.64	28.70	28.80	28.44	28.46	28.51
Salm -3	27.12	30.33	32.92	N	N	N	N	N
30.93	32.42	32.17	32.05	N	32.09	32.38	31.75	32.72
Salm -4	26.95	30.32	32.84	36.16	N	N	N	N
34.18	N	N	36.03	36.02	N	34.37	36.51	34.11
Salm -5	27.09	30.18	33.98	36.32	N	N	N	N
N	N	N	N	N	N	N	N	N
Salm -6	27.22	30.47	33.65	36.42	N	N	N	N
N	N	N	N	N	N	N	N	N
Salm -7	27.24	30.75	34.04	N	N	N	N	N
N	N	N	N	N	N	N	N	N
Salm -8	27.08	30.28	33.51	37.70	N	N	N	N
N	N	N	N	N	N	N	N	N

Panels of simulated samples were generated using eight dilutions manufactured from a strong positive control and tested to assess if any competition existed between targets in a multiplex. *Campylobacter* jejuni Ct values are in blue[a], and *Salmonella* Ct values are in green[b] N: negative; *Salm: Salmonella; C.jejuni*: *Campylobacter jejuni*

Table A-13 Endpoint detection limit of the gastroenteritis 2 multiplex in comparison to the single assays

Dilution	**Coronavirus**		***Salmonella***		***Campylobacter jejuni***	
	Single	**Multiplex**	**Single**	**Multiplex**	**Single**	**Multiplex**
Neat	25.99	26.47	21.01	21.07	13.75	13.73
10^{-1}	29.21	29.51	24.39	24.28	16.03	16.39
10^{-2}	37.71	32.63	27.75	27.77	19.21	19.39
10^{-3}	34.92	35.78	31.42	31.45	23.16	22.23
10^{-4}	N	N	35.05	34.81	26.28	26.38
10^{-5}	N	N	N	N	30.40	29.08
10^{-6}	N	N	N	N	33.76	32.03
10^{-7}	NT	NT	NT	NT	35.16	34.56/N
10^{-8}	NT	NT	NT	NT	37.29/N	N
10^{-9}	NT	NT	NT	NT	N	N
10^{-10}	NT	NT	NT	NT	N	N

A 10-fold dilution series of each pathogen was assessed using a single assay for each pathogen and the multiplex assay. N: negative; NT: not tested

Table A-14 Endpoint detection limit of the gastroenteritis 3 screen in comparison to the single assays

Dilution	*Cryptosporidium parvum*		*Giardia*		*Tritrichomonas foetus*	
	Single	**Multiplex**	**Single**	**Multiplex**	**Single**	**Multiplex**
10^{-1}	22.26	22.24	23.04	24.00	26.64	26.44
10^{-2}	26.99	26.93	25.85	27.59	28.29	27.23
10^{-3}	30.98	30.87	29.26	30.85	31.11	28.83
10^{-4}	35.62/N	N	33.33	37.94	33.36	30.89
10^{-5}	N	N	N	N	37.08	35.66
10^{-6}	N	N	N	N	38.74/N	N
10^{-7}	N	N	N	N	N	N
10^{-8}	N	N	N	N	N	N

A 10-fold dilution series of each pathogen was assessed using a single assay for each pathogen and the multiplex assay. N: negative;

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