A re-evaluation of known and potential pathogens in canine and feline infectious respiratory disease

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Abstract

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Respiratory infections represent an important and frequent cause of disease in cats and dogs worldwide. Many pathogens have been associated with feline (FIRD) and canine infectious respiratory disease (CIRD) including feline calicivirus (FCV) and herpesvirus (FeHV-1) in cats and canine distemper virus (CDV), parainfluenza virus (CPiV), adenovirus type 2 (CAV-2), herpesvirus (CHV) and *Bordetella bronchiseptica* in dogs. All these pathogens are currently included in either core or non-core vaccines, leading to a reduction in overall disease burden.

Despite widespread use of vaccines, disease is still present at significant levels. In dogs, several new pathogens, potentially associated with respiratory disease, have recently emerged including canine respiratory coronavirus (CRCoV) and canine pneumovirus (CnPnV). In cats, it has been theorised that the high rates of evolution of viruses such as FCV may lead to a reduction of vaccine efficacy. Finally, even though respiratory disease of suspected infectious origin is frequent, the culprit often remains unidentified. This thesis aims to build understanding of canine and feline infectious respiratory disease epidemiology, re-evaluate the roles of known and suspected pathogens in this syndrome and to aid in informing the development of new disease prevention strategies.

During a cross-sectional study across 6 European countries, samples were collected from 1521 veterinary practice attending cats. The prevalence of FCV in this population was 9.2%. Phylogenetic analysis of these field isolates showed high viral variability with a radial phylogeny. In vitro viral neutralisation suggested that antibodies raised to the FCV-F9 vaccine strain (which has been widely in use for decades) are still broadly cross-reactive to contemporary field isolates.

A retrospective serosurvey of 200 canine and 179 feline samples screened for influenza A and B viruses suggested that UK dogs and cats have been rarely exposed to influenza viruses in recent years with only 1.5% of canine samples being seropositive for equine H3N8 and 0.56% of feline samples being seropositive to human pandemic 2009 H1N1.

Two case control studies were conducted in British veterinary practices in order to re-appraise the role of known and potential upper respiratory pathogens in pet dogs and cats. In the feline study, FCV was detected in 21.1% of cases and 4.7% controls. FeHV-1 was detected in 10.5% cases and 1.6% controls. *Bordetella bronchiseptica* was identified in 5.3% cases and 9.4% controls. Finally, 65.8% cases and 48.8% controls were positive for *M. felis.* In the canine study, CRCoV was identified in 6.1% of cases, CnPnV in 4.4% cases and 1.2% controls, *M.cynos* in 4.4% cases and 2.3% controls, and *B.bronchiseptica* in 6.7% cases and 20.7% controls. No samples tested positive for CAV-2, CPiV, CHV, CDV, influenza A, *Streptococcus equi subsp. zooepidemicus* or *C.felis.*

A cross-sectional sampling of dogs and cats was conducted in four British shelters with one of these also taking part in a 10-week longitudinal study. FCV, FeHV-1 and *M. felis* were detected in 7.4%, 4.6% and 16.8% feline samples. Cross-sectional dog samples tested negative for all pathogens included in the study. In the longitudinal study, *M. felis* was the most frequently encountered microorganism followed by FCV in cats. In dogs, CnPnV was identified during two weeks that coincided with reports of canine respiratory disease by members of the shelter staff.

Together, findings confirm the importance of CIRD and FIRD as multifactorial syndromes in both pet and unowned shelter populations where pathogen shedding is often observed without clinical signs. In FIRD, FCV is reaffirmed as a majorly important disease causing pathogen. Finally, where CIRD is concerned, findings suggest that recently emerged pathogens such as CRCoV are of increasing importance in this syndrome whereas classic pathogens seem to be less and less present, likely due to widespread vaccination.

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This thesis is a work of love and, like any good Horcrux, is a piece of my soul. If anyone ever feels compelled to read it, I hope they find what they are looking for.

Declarations and contributors

Chapter 2: MSD Animal Health staff members did practice recruitment and questionnaire translation in The Netherlands, France, Germany, Sweden and Italy. Dr. Shirley Bonner (Research Assistant, University of Liverpool) performed a significant number of viral isolations, PCR testing and viral neutralisation testing.

Chapter 3: The University of Liverpool's Small Animal Hospital and clinical pathology laboratory provided the samples and related clinical details used in this study. The laboratory work described was carried out at the University of Nottingham by the author with help and supervision from Dr. Janet Daly. Laboratory testing was performed by the author with the exception of fifty samples for which testing was performed by Bethany Gerrard as part of an undergraduate degree research project under the supervision of Dr. Janet Daly.

Chapter 4, 5 and 6: Dr. Shirley Bonner (Research Assistant, University of Liverpool) performed a significant number of PCR tests and viral isolation tests for all the pathogens explored in these chapters. The author and Dr. Shirley Bonner administerd the questionnaires to participating pet owners. The author, Dr. Shirley Bonner and Professor Alan Radford (University of Liverpool), performed sampling at shelter sites.

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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1 Introduction to infectious respiratory disease and its aetiology – a review of the literature

1.1 Introduction to infectious respiratory disease in dogs and cats

Infectious diseases of the respiratory tract are a major cause of morbidity in most animal species. Dogs and cats are no exception, and despite widespread vaccination, infectious respiratory disease is still a major reason for veterinary visits (Arsevska et al., 2018). In both species, it is recognized that these syndromes are multifactorial in nature. In the past and in many instances to this day, they are presumptively diagnosed and treated based mostly on clinical signs. However, the advent and widespread availability of molecular diagnostic testing (i.e. polymerase chain reaction - PCR) is making it possible to increasingly diagnose recognised infectious causes of these clinical signs (Mitchell et al., 2017; Mitchell and Brownlie, 2015; Priestnall et al., 2014).

A wide range of viral and bacterial pathogens are recognised as causative agents of feline and canine infectious respiratory disease, with some degree of overlap between the two species (Afonso et al., 2017b; Priestnall, 2017). Despite this, in many instances, the aetiology remains unknown. In some cases, this is due to a clinical choice on the part of the attending veterinary surgeon that can be driven by financial restrictions or the lack of pathogen-specific treatment to justify the undertaking of expensive diagnostic tests, especially in the case of viral disease. In other cases, where diagnostic testing is undertaken, frequently no pathogen is identified. This could be due to a series of factors such as untimely sampling, sampling errors, inadequate sample storage or processing but possibly also the presence of only previously unidentified pathogens. Fortunately, the rapid evolution of metagenomics approaches such as next generation sequencing, signifies that this is an exciting time for new pathogen discovery (Radford et al., 2012).

Below we will briefly review characteristics relating to canine and feline respiratory disease, respectively and summarize the most common pathogens potentially involved in disease.

1.2 An overview of canine infectious respiratory disease (CIRD)

Canine infectious respiratory disease (CIRD), also referred to as infectious tracheobronchitis or “kennel cough” is a common, acute and highly contagious syndrome of dogs with a worldwide distribution. CIRD is often seen in shelter and other multi-animal environements. It is typically characterized by paroxysmal cough and, in some cases, may be accompanied by other signs including serous or purulent nasal discharge, dyspnoea, pyrexia, conjunctivitis, anorexia and lethargy. On clinical exam, submandibular lymphadenopathy and congestion of mucous membranes may be present (Opperman and Brownlie, 2018). It can persist for weeks and may lead to severe disease such as bronchopneumonia and may even result in euthanasia or natural death (Mitchell et al., 2017).

CIRD is a complex multifactorial clinical syndrome in which pathogen, host and environmental factors act together influencing disease susceptibility and severity (Mitchell et al., 2017). While occurring throughout the year, recent evidence shows seasonal prevalence fluctuations in CIRD (and, to a less extent, in FIRD), with peaks of disease occurring around Autumn in the UK and certain pathogens, such as CRCoV, being more common during cooler months (Erles and Brownlie, 2005, Arsevska et al,. 2017, Maboni et al., 2019, Singleton et al., 2019). Despite widespread vaccination, CIRD remains an important threat to susceptible animals especially those that are housed in multi-animal environments such as shelters and boarding kennels where outbreaks are an important cause of morbidity (Chalker et al., 2003b). CIRD has often been considered a mild syndrome of relatively limited clinical importance especially in the pet dog. However, in the past two decades, due to the emergence and re-emergence of several pathogens, this paradigm has begun to shift, and a surge of interest in this disease has occurred (Priestnall et al., 2014).

In CIRD, infections of clinical significance include bacterial and/or viral pathogens that can act alone as primary pathogens, or synergistically in combination, to cause disease (Mitchell and Brownlie, 2015; Priestnall et al., 2014). Traditionally, canine herpesvirus type-1, canine parainfluenza virus, canine adenovirus type 2 and *Bordetella bronchiseptica* have been considered the main causes of CIRD (Appel, and

Percy, 1970; Bemis, 1992; Binn et al., 1967). Vaccination is the primary prevention and control strategy used for these pathogens but despite its widespread use, CIRD is still a major problem especially in more sensitive environments such as multi-animal households, kennels and shelters (Erles et al., 2004). In recent years the emergence and re-emergence of other pathogens associated with upper respiratory tract disease has shed further light on the multifactorial nature of this syndrome and the possibility that unknown pathogens may be involved in disease pathogenesis. Viruses such as canine respiratory coronavirus, influenza viruses and others are now considered as potential aetiologies in suspected cases of CIRD (Decaro et al, 2016; Erles et al, 2003; Mitchell et al., 2017; Priestnall et al., 2014; Renshaw et al., 2010). Table 1.1 presents a summary of prevalences of these pathogens in dogs with CIRD-like disease found in relevant studies published within the past 15 years.

Table 1.1 Summary of prevalences of pathogens involved in CIRD-like disease. Pet populations include privately owned animals and kennelled populations refer to multi-animal environments including shelters, and breeding and research kennels.

Pathogen	*Prevalence % (n)*	*Population*	*Region*	*References*
CAV-2	0% (0/110)	Kenneled	UK	Erles et al 2004
	0% (0/61)	Pet + kennelled	Germany	Schulz et al 2014
	0% (0/78)	Pet + kennelled	Italy	Pecoraro et al 2014
	2.5% (14/559)	Pet	USA	Maboni et al 2019
	2.9% (2/68)	Pet	Japan	Mochizuki et al 2008
CPiV	7.4% (5/68)	Pet	Japan	Mochizuki et al 2008
	19% (21/110)	Kennelled	UK	Erles et al 2004
	20.5% (16/78)	Pet + kennelled	Italy	Pecoraro et al 2014
	29% (33/144)	Pet	USA	Maboni et al 2019
	37.7% (23/61)	Pet + kennelled	Germany	Schulz et al 2014
	42% (27/64)	Pet	Canada	Joffe et al 2016
CHV-1	0% (0/61)	Pet + kennelled	Germany	Schulz et al 2014
	1.6% (1/64)	Pet	Canada	Joffe et al 2016
	18.8% (20/110)	Kennelled	UK	Erles et al 2004
CDV	0% (0/61)	Pet + kennelled	Germany	Schulz et al 2014
	0% (0/78)	Pet + kennelled	Italy	Pecoraro et al 2014
	1.5% (1/68)	Pet	Japan	Mochizuki et al 2008
	1.6% (1/64)	Pet	Canada	Joffe et al 2016
	2% (14/559)	Pet	USA	Maboni et al 2019
CRCoV	1.5% (1/68)	Pet	Japan	Mochizuki et al 2008
	7.7% (43/559)	Pet + kennelled	Europe	Mitchell et al 2017
	9% (7/78)	Pet + kennelled	Italy	Pecoraro et al 2014
	9.4%	Pet	Canada	Joffe et al 2016
	9.8% (6/61)	Pet + kennelled	Germany	Schulz et al 2014
CnPnV	6.4% (5/78)	Pet + kennelled	Italy	Pecoraro et al 2014
	14.2% (19/134)	Kennelled	UK	Mitchell et al 2013
	23.4% (130/555)	Pet + kennelled	Europe	Mitchell et al 2017
Influenza A	0% (0/68)	Pet	Japan	Mochizuki et al 2008
	0% (0/78)	Pet + kennelled	Italy	Pecoraro et al 2014
	0% (0/61)	Pet + kennelled	Germany	Schulz et al 2014
	0% (0/64)	Pet	Canada	Joffe et al 2016
	0% (0/511)	Pet + kennelled	Europe	Mitchell et al 2017
	11.2% (63/559)	Pet	USA	Maboni et al 2019
B.bronchiseptica	9% (51/559)	Pet	USA	Maboni et al 2019
	10.3% (7/68)	Pet	Japan	Mochizuki et al 2008
	10.3% (8/78)	Pet + kennelled	Italy	Pecoraro et al 2014
	78.7% (48/61)	Pet + kennelled	Germany	Schulz et al 2014
S.equi	0% (0/78)	Pet + kennelled	Italy	Pecoraro et al 2014
	0% (0/559)	Pet	USA	Maboni et al 2019
M.cynos	0.9% (5/566)	Pet + kennelled	Europe	Mitchell et al 2017
	7.7% (6/78)	Pet + kennelled	Italy	Pecoraro et al 2014
	23.9% (33/105)	Kennelled	UK	Chalker et al 2004
	24.5% (28/144)	Pet	USA	Maboni et al 2019
	81% (52/64)	Pet	Canada	Joffe et al 2016

1.2.1 Canine adenovirus type 2 (CAV-2)

Canine adenovirus type 2, a member of the *Adenoviridae*, is a non-enveloped double stranded DNA virus. It was originally identified in 1961, in Canada in dogs with laryngotracheitis (Ditchfield et al., 1962).

CAV-2 is considered as one of the classic causes of CIRD with acute onset cough being its dominant characteristic (Ditchfield et al., 1962). Infection usually leads to mild or inapparent disease when the virus is acting alone. However, when other viral or bacterial causes are present, it can be associated with more severe disease. Young puppies and immunosuppressed animals are also at higher risk of developing bronchopneumonia which, in some cases, can be fatal (Decaro et al., 2008). Transmission is via the oronasal route, with replication peaking at 3 to 6 days post-infection and occurring mostly in the nasal, pharyngeal and non-ciliated bronchiolar epithelium (Buonavoglia and Martella, 2007).

Vaccination against CAV-2 provides cross-protection against canine adenovirus type 1 infection and is a part of the World Small Animal Veterinary Association (WSAVA) - recommended core vaccination schedule using live-attenuated vaccines (Day et al., 2016). Widespread vaccination has been associated with significantly reduced viral circulation, and isolation of the virus is now rare within Europe. In a longitudinal study conducted over a period of two years in a vaccinated British shelter population with endemic respiratory disease, CAV-2 was not isolated (Erles et al., 2004) and, more recently in another study conducted in Southern Germany, this virus was only isolated from 1.1% of healthy participants (n=90) with no diseased dogs testing positive for CAV-2 (Schulz et al., 2014).

1.2.2 Canine parainfluenza virus (CPIV)

Canine parainfluenza virus (CPIV) is classically considered one of the most common viruses involved in the CIRD complex. It is a member of the *Paramyxoviridae* and a single stranded negative sense RNA virus with a lipid envelope of host cell origin (Appel, and Percy, 1970; Priestnall, 2017).

CPiV is present worldwide and is highly contagious (Buonavoglia and Martella, 2007; Priestnall, 2017). This pathogen is able to spread quickly especially in high-density populations, and is transmitted through contact with infectious aerosols. Dogs may start shedding viral particles before the onset of clinical signs and remain infectious up to 10 days post infection (Buonavoglia and Martella, 2007).

Clinical signs typically start 2 to 8 days post infection, and are generally mild in the uncomplicated forms. A dry, hacking cough lasting for 3 to 6 days is the most common presentation of disease. Other clinical signs include modest pyrexia, mucous nasal discharge, pharyngitis and tonsillitis (Love, 1972). In most cases, dogs appear healthy and maintain normal activity levels. When coinfections with other viruses or bacteria are observed, the clinical presentation tends to be more severe; a complicated form which can occur in young unvaccinated puppies and immunocompromised animals leads to more severe signs such as lethargy, inappetence and pneumonia (Buonavoglia and Martella, 2007; Priestnall, 2017).

Diagnosis can be made using viral isolation from nasopharyngeal and laryngeal swabs and CPiV is able to grow in cell lines from several mammal species. However, with the widespread use of molecular diagnostic techniques it is now more common to use reverse-transcription PCR (RT-PCR) for CPiV identification in respiratory secretions, swabs and tracheal or lung tissue samples (Erles and Brownlie, 2005; Mitchell et al., 2017). Vaccination is used worldwide, commonly combined with general core vaccines, as well as in CIRD specific vaccines, and is efficient in reducing severity of disease (Day et al, 2016). Although not considered core itself, vaccination is frequent with some 70% of vaccinated dogs being vaccinated against CPiV (Sánchez-Vizcaíno et al., 2018).

1.2.3 Canine herpesvirus type 1 (CHV-1)

Canine herpesvirus 1 is an enveloped, double stranded DNA virus belonging to the Alphaherpesvirus subfamily of the *Herpesviridae*. As typical of herpesviruses, CHV-1 has a large genome of approximately 125kbp (Papageorgiou et al., 2016; Sarker et

al., 2018) and is thought to be a monotypic virus with a close relationship to feline herpesvirus type 1 (Buonavoglia and Martella, 2007; Poste et al., 1972).
This virus is present worldwide in domestic and wild dogs and was originally described in the mid-1960s as the causative agent of fatal septicemia in young puppies (Carmichael et al., 1965).

The involvement of CHV-1 in CIRD is still controversial. The virus leads to high mortality in newborn and very young puppies and is described as causing subclinical to mild respiratory and genital disease without systemic involvement in pups older than 2 weeks-of-age and adult dogs (Carmichael et al., 1965). In experimentally infected dogs, mild clinical signs of rhinitis, pharyngitis or tracheobronchitis have been observed (Appel et al., 1969). Transmission of CHV-1 occurs via direct oronasal contact with infected respiratory or genital secretions and transplacental transmission from bitch to pup in the uterus (Appel et al., 1969; Wright et al., 1974). The incubation period ranges from 6 to 10 days. After primary infection, and as is typical for other herpesviruses, recrudescence can occur after periods of stress and immunosuppressive disease or therapy. In between disease episodes the virus becomes latent in the trigeminal, lung and lumbosacral ganglia, retropharyngeal lymph nodes, tonsils and parotid salivary gland (Burr et al., 1996; Miyoshi et al., 1999).

Laboratory diagnosis has historically been achieved by using viral isolation in cell culture (in primary or secondary kidney or testicular cell lines, for example) and now more commonly, PCR of oronasal, ocular or genital swabs which significantly increase diagnostic sensitivity. Serology can be useful but does not confirm active infection. Vaccination against this virus is not done routinely but can be recommended in pregnant bitches to prevent infections of newborn pups and is currently licensed for use in Europe (Buonavoglia and Martella, 2007 Day et al., 2016).

1.2.4 Canine distemper virus (CDV)

CDV is an enveloped, single-stranded RNA virus belonging to the *Paramyxoviridae*. It is known to be able to infect a range of members of the order Carnivora including

canids, mustelids, procyonids, large felids, giant pandas and red pandas (Appel and Summers, 1995; Jin et al., 2017; Martella et al., 2008). The virus has also shown propensity for wider species transmission and has been described in members of the Rodentia, Artyodactila, Proboscidea and Primates orders (Martinez-Gutierrez and Ruiz-Saenz, 2016). The virus leads to systemic disease with neurologic manifestations and a high mortality rate in both dogs and other carnivores. For the purposes of this work we will focus mainly on respiratory disease caused by CDV.

Transmission is via the oronasal route, through exposure to contaminated bodily fluids such as aerosols generated by coughing and sneezing. Viral incubation ranges from one to three weeks with shedding typically starting about 7 – 10 days post exposure. Infection severity differs based on a variety of factors including, but not limited to, the animal's age and immune status as well as the involved viral strain (Summers et al., 1984). While older dogs are more likely to develop subclinical or mild forms, puppies are more prone to severe courses of disease with serious sequels and high mortality (Blixenkrone-Møller et al., 1993; Summers and Appel, 1994).

In addition to becoming lethargic and anorexic, many dogs develop respiratory and ocular signs, including cough and nasal and ocular discharge, which can range from serous to mucopurulent. In cases of mild respiratory signs without other systemic signs, it is impossible to clinically distinguish CDV infection from other causes of CIRD. Infection of the lower respiratory tract can also occur with associated pneumonia, predisposing the animal to secondary bacterial infections.

Vaccination against this pathogen has been available for over five decades and is recommended as part of WSAVA's "core" vaccination schedules (Day et al., 2016). However, due to the diverse wildlife reservoirs, CDV still poses a serious threat to canines in several countries.

Diagnosis can be achieved by using molecular techniques such as RT-PCR and serology but most commercially available diagnostic tests do not distinguish between

vaccine and field strains in recently vaccinated dogs (up to 3 weeks post-vaccination) (Elia et al., 2006).

1.2.5 Canine respiratory coronavirus (CRCoV)

A large enveloped virus, CRCoV belongs in the *Betacoronavirus* genus of the *Coronaviridae*. It is a single-stranded RNA virus and is closely related to human coronavirus OC43 and bovine coronavirus (Erles et al., 2003; Erles and Brownlie, 2008). In fact, it shares 97.3% sequence homology with the latter and it has been postulated that it may have originally been transmitted from cattle to canines (Erles et al., 2003). CRCoV is serogically and genetically distinct from canine coronavirus, which has a primarily gastrointestinal tropism (Erles et al., 2003; Stavisky et al., 2012b).

CRCoV was initially reported in 2003, in the UK, in a study of kennelled dogs vaccinated against the classical causes of CIRD that still exhibited upper respiratory disease signs. The original report showed a strong association between viral exposure and the development of CIRD signs (Erles et al., 2003).

When present as a single pathogen, CRCoV primarily causes mild clinical disease with non-specific upper respiratory tract signs such as cough, sneezing and nasal discharge. However, as with human respiratory coronaviruses, CRCoV can lead to reversible and irreversible damage of the respiratory ciliated epithelia predisposing the animal to secondary infections and more severe clinical presentations (Erles and Brownlie, 2008). It is not uncommon to find CRCoV associated with other CIRD pathogens such as CPIV or CHV-1 (Mitchell et al., 2017; Schulz et al., 2014).

Viral shedding has been observed up to 6 to 10 days after infection and viral particles are detected in a range of respiratory and lymphoid tissues. CRCoV is most commonly found in the trachea and nasal cavity, as well as other upper and lower respiratory tract tissues (Mitchell et al., 2013a). However, it has also been detected in the gastrointestinal tract which may indicate that this virus, as with some bovine coronavirus strains, may have the potential for dual tropism (Park et al., 2007).

Evidence of CIRD caused by CRCoV has been reported worldwide and the virus is now considered a cause of upper respiratory tract disease in dogs. There is currently no vaccine available against CRCoV and diagnosis, due to the non-specific nature of the clinical signs, cannot be based solely on clinical presentation. RT-PCR of oropharyngeal or nasal swabs is the most reliable diagnostic technique for this virus and serological assays are also becoming more readily available (Erles et al., 2003; Mitchell et al., 2009).

1.2.6 Canine pneumovirus (CnPnV)

Canine pneumovirus (CnPnV) is a single stranded, negative sense, enveloped RNA virus within the *Pneumovirus* genus and sub-family of the *Paramyxoviridae* (Decaro et al., 2014; Renshaw et al., 2010). It was first identified in a study of respiratory disease outbreaks in closely confined dogs in two related canine shelters in the USA. Nasal and oropharyngeal samples from 200 dogs collected between 2008 and 2009 were analysed. After a series of passages in cell culture, a cytopathic effect with a pattern not similar to those seen with other viruses regularly isolated from dogs was observed. The virus was subsequently identified as closely related to murine pneumovirus, a common rodent pathogen observed in commercial and research colonies. CnPnV can replicate in mice lung tissue causing disease of varying severity (Glineur et al., 2013; Percopo et al., 2011; Renshaw et al., 2010).

Since the publication of the original report of this novel pathogen, the virus was also identified in samples from eight further states in the USA (Priestnall et al., 2014), in the UK (Mitchell et al., 2013b), Italy (Decaro et al., 2014; Mitchell et al., 2017), Greece, Hungary, France, Spain and The Netherlands (Mitchell et al., 2017). Seropositive samples were also identified in Ireland (Mitchell et al., 2013b).

While Koch's postulates have not yet been fulfilled for this pathogen, very strong evidence suggests that it is an emerging cause of CIRD. CnPnV stands within a family of viruses side by side with various relevant pathogens causing severe respiratory disease in other mammal and avian species, and murine pneumovirus has been

proven to cause respiratory disease in mice. This is further strengthened by the fact that it is commonly present in dogs with CIRD signs and recently, in an epidemiological retrospective study by Mitchell et al (2017), it was found to be strongly associated with respiratory disease.

1.2.7 Influenza viruses in dogs

Influenza viruses belong to the *Orthomyxoviridae* and are single stranded RNA viruses. Until fairly recently there were only sporadic reports of influenza virus in dogs, and they were not considered important pathogens of this species. Canine influenza virus (CIV) H3N8 was originally detected in racing hounds with respiratory disease in the USA in 2004 (Crawford et al., 2005). It subsequently infected pet dogs, developed transmissibility between dogs, and became endemic in the USA. The virus is most closely related to equine influenza H3N8 suggesting a direct transmission event from horses and subsequent adaptation to the canine species (Crawford et al., 2005; Payungporn et al., 2008). As with other pathogens causing upper respiratory tract disease, CIV was found to be more prevalent in multi-animal environments (Anderson et al., 2012; Dubovi, 2010). Outside of the USA, there is little evidence for H3N8 with only sporadic limited outbreaks of suspected equine to dog transmission being reported in the literature, with no evidence that these have established endemic infection in dogs (Daly et al., 2008; Kirkland et al., 2010).

A second canine influenza virus, CIV H3N2, was identified in South Korean dogs exhibiting signs of respiratory disease (Song et al., 2008). This virus is thought to have been transmitted from avian reservoirs to dogs through ingestion of contaminated poultry. In 2015, CIV H3N2 emerged in the USA causing upper respiratory tract disease, similar to CIV H3N8. It has been postulated that the virus was introduced to the USA through rescue and rehoming networks of dogs from Asia (Voorhees et al., 2017).

Other influenza subtypes have also been identified in the canine population worldwide showing that dogs are susceptible to influenza viruses of equine, avian and human origin. These include highly pathogenic avian H5N1, which has been

associated with generally subclinical infection in the canine species, although the virus has been reported as the cause of death in a dog in Thailand (after ingestion of duck carcasses infected with the virus) (Maas et al., 2007; Songserm et al., 2006). In another report, pandemic H1N1 was found in two dogs presenting with respiratory disease signs in China; samples were collected from both animals and researchers established that experimental infection with this subtype of influenza virus leads to mild disease signs and low transmissibility between dogs (Lin et al., 2012). In 2009 in China, a dog with signs of respiratory disease was identified as being infected by a novel influenza virus H5N2 generated by the reassortment of viral genome segments (Zhan et al., 2012). Dogs experimentally infected with this virus developed mild signs of influenza-like disease (Song et al., 2012). Canine influenza H3N1 is another example of a reassortant virus, in this case between human pandemic H1N1 and canine H3N2 that was detected in asymptomatic dogs in South Korea (Song et al., 2012). The cases above, and others not mentioned in this brief summary, show that dogs can potentially play an important role in the evolution, spread and interspecies transmission of influenza viruses (Na et al., 2016; Pulit-Penaloza et al., 2017; Song et al., 2013).

Vaccines against H3N8 and H3N2 CIV are available in the USA and considered "lifestyle" vaccines (i.e: not core) which are recommended to dogs at greater risk of exposure (Ford et al., 2017).

1.2.8 Bordetella bronchiseptica (*B. bronchiseptica)*

The aerobic coccobaccilus *B. bronchiseptica* is another pathogen frequently associated with the CIRD complex. Originally isolated in the early 20th century (in a study that wrongly implicated it in the pathogenesis of canine distemper) (Ellis, 2015; Ferry, 1910), it is known to be a commensal of the nasal mucosa of healthy animals (Bemis et al., 1977a, 1977b). This Gram-negative bacterium is both capable of acting as a primary agent of upper respiratory tract infection as well as an opportunistic pathogen associated with other causative agents of CIRD such as CPiV and CRCoV leading to more severe infections (Priestnall, 2017; Schulz et al., 2014). In the past it has been referred to as a "disease of puppyhood" similar to whooping cough in

children, but it is often isolated from adult animals with and without respiratory signs (Bemis et al., 1977a, 1977b; Chalker et al., 2003b).

B. bronchiseptica is also known to infect and cause disease in other species such as cats, rabbits, pigs and birds (Binns et al., 1998; Dawson et al., 2000, Kadlec and Schwarz, 2017). While immunocompetent humans do not seem to usually develop clinical disease after exposure to *B. bronchiseptica*, it is a seemingly infrequent, zoonosis of immunocompromised people (Mani and Maguire, 2009, Register et al., 2012). Mild to severe disease has been reported in children post lung transplantation, as well as in patients with HIV, cystic fibrosis and lymphoma (Mani and Maguire 2009, Szvalb et al 2019, Ackerman et al 2014). *B.bronchiseptica* has also been found to cause disease in cancer patients in contact with animals (dogs, horses and cattle) post haematopoietic stem cell transplantation (Szvalb et al 2019). In immunocompromised humans, it is recommended that contact with animals recently vaccinated with modified life attenuated vaccines is avoided (Mani and Maguire, 2009) and *B.bordetella* associated disease has been reported post-kidney transplantation in a patient in close contact with recently vaccinated dogs (Gisel et al., 2010). Additionally, it has been hypothetised that B. bronchiseptica may have given origin to human pathogens Bordetella pertussis and parapertussis (Bjornstad and Harvill, 2005).

The incubation period for *B. bronchiseptica* has been observed to range from 2 to 14 days post infection (Thrusfield et al., 1991) and shedding can persist for over 6 months in some cases (Bemis et al., 1977b, 1977b; Ellis, 2015). To improve its ability to infect and colonise the respiratory tract, it produces several toxins including cytotoxins which induce ciliostasis (Bemis, 1992).

Similarly to other classic agents of CIRD, *B. bronchiseptica* leads to upper respiratory tract clinical signs such as coughing and nasal discharge as well as systemic signs, which are present in a lower number of animals, such as an elevated temperature and anorexia (Thrusfield et al., 1991). Isolation of *B. bronchiseptica* is the gold standard for diagnosis of infection. Specimens from nasal or oropharyngeal swabs

are often used due to their convenience but are not as sensitive as samples collected by bronchioalveolar lavage (BAL).

Parenteral and, more commonly, intranasal vaccination against *B. bronchiseptica* has been used for decades. Intranasal live-modified *B. bronchiseptica* vaccines are available in Europe, often in combination with CPiV, and are recommended annually for all at risk dogs such as those being kennelled (Day et al., 2016; Ellis, 2015; Jacobs et al., 2007). They are not considered core vaccines and are only used in about one third of vaccinated animals (Sánchez-Vizcaíno et al, 2018). Due to the difficulty in administering intranasal vaccines to some animals, an oral vaccination is now available in the USA but is not presently commercialised in Europe (Ellis, 2015). There is some controversy relating to the efficiency of different routes of administration of *B. bronchiseptica* vaccines at the time of the writing of this thesis (Ellis, 2015).

1.2.9 Streptococcus equi subs. zooepidemicus (*S. zooepidemicus)*

Streptococcus equi subs. zooepidemicus (S. zooepidemicus) is a Lancefield group C, beta-haemolytic bacterium causing acute necrotising haemorrhagic pneumonia (Chalker et al., 2003a; Priestnall et al., 2014). As the name implies, it is a zoonotic pathogen and at least one instance of human disease has been directly linked to contact with a dog with pneumonia (Abbott et al., 2010; Arsevska et al., 2018).

While sporadic disease in dogs has been described since the 1970s, in recent years this pathogen seems to have re-emerged and several outbreaks of pneumonia associated with *S. zooepidemicus* have been described. As with other respiratory pathogens, *S. zooepidemicus* is frequently associated with disease in multi-animal environments such as rescue shelters and racing kennels (Pesavento and Murphy, 2014; Priestnall et al., 2014).

The clinical course of *S. zooepidemicus*-associated disease often starts similarly to other CIRD pathogens including cough and serous to mucous nasal discharge. However, more severe signs often quickly develop which include pyrexia and epistaxis, with sudden death sometimes reported within 24 to 48 hours of disease

onset. The high morbidity and mortality associated with *S. zooepidemicus* infection is likely associated with the production of bacterial exotoxins that are thought to act as superantigens (Priestnall et al., 2014).

Diagnosis can be confirmed via bacterial culture or molecular techniques such as real time PCR (Arsevska et al, 2018). At the time of writing there is no vaccine available against this bacterium and treatment in confirmed cases is largely supportive (intravenous broad spectrum antibiotics and fluid therapy). Prevention is therefore focused on good hygiene and quarantine of suspected cases (Arsevska et al, 2018).

1.2.10 Mycoplasma cynos *(M. cynos)*

Mycoplasmas, members of the class Mollicutes, are bacteria lacking a cell wall and are the smallest living organisms capable of existing independently (Priestnall et al., 2014). *Mycoplasma spp.* are thought to be commensal inhabitants of the canine upper respiratory tract and a diverse range of these organisms are frequently isolated from healthy and diseased dogs (Bemis, 1992; Chalker et al., 2004).

M. cynos was first isolated from the lungs of a dog with pneumonia in the early 1970s (Priestnall, 2017). In a study published in 2004, this Mycoplasma species was the only one found to be associated with respiratory disease (Chalker et al., 2004). However, its role in CIRD is still not fully understood (Priestnall et al., 2014). Furthermore, the role played by other *Mycoplasma spp.* in respiratory disease also remains to be clarified and experimental infection with other Mycoplasmas such as *M. canis, M. gateae* and *M. spumans* have not reproduced respiratory disease in the canine species (Chalker, 2005).

The pathogenicity of canine mycoplasmas is still poorly understood. *M. cynos* persists in the lungs of infected animals for up to 3 weeks after infection and it can also be isolated from the tonsils and conjunctiva of infected dogs (Chalker, 2005; Chalker et al., 2004). Due to their unique structure, *Mycoplasma spp.* are resistant to beta-lactam antibiotics, and if considered clinically necessary, doxycycline is often considered the drug treatment of choice (Chalker, 2005).

1.2.11 Other pathogens of possible clinical significance in CIRD

A positive-sense RNA virus tentatively named canine hepacivirus (genus *Hepacivirus,* family *Flaviviridae)* was identified, using high-throughput sequencing, in respiratory tract samples of dogs with CIRD signs and in liver tissues of dogs with gastrointestinal disease in US shelters (Kapoor et al., 2011). This virus was found to be genetically very closely related to human hepatitis C virus (HCV). This discovery intrigued researchers since, up until then, all known hepaciviruses had, as their name perhaps suggests, a strict hepatic tropism. However, subsequent studies attempting identification of Canine hepacivirus RNA or seroconversion in additional dogs have been unsuccessful (Bexfield et al., 2014; Burbelo et al., 2012; Lyons et al., 2012). The virus has since been reclassified as a non-primate hepacivirus (NPHV) due to the finding of CHV-like viruses in horses (Burbelo et al., 2012). The newly named NPHV were subsequently also found in commercially available equine sera for cell culture which raises the question of whether the finding of NPHV in dogs may be the consequence of contamination rather than true infection, as can occur with pathogen discovery projects based on next-generation sequencing (Postel et al., 2016).

Canine enteric coronavirus (CECoV) usually leads to intestinal tract infections which are normally self-limiting and characterised by mild clinical signs. Infection is most common in kennelled dogs with a prevalence ranging from 5 - 13.5% in boarding kennels and 13.8% - 33.3% in rescue kennels (Stavisky et al., 2012b). The prevalence of CECoV in dogs attending veterinary practices was found to be much lower (approximately 3%) and around 8% in pet dogs with severe diarrhoea (Godsall et al., 2010; Stavisky et al., 2010). In 2005, an outbreak of fatal disease in puppies in Italy was caused by a highly pathogenic variant of CECoV that was isolated from several organs including the lungs, where severe lesions were observed; these variants of CECoV with tropism outside the enteric tissue have been since termed pantropic coronaviruses (Buonavoglia et al., 2006). Whilst they remind us of the potential for coronaviruses to infect enteric and respiratory tissues, this particular virus is not generally considered an important respiratory pathogen.

Mammalian orthoreoviruses (MRV; genus *Orthoreovirus*, family *Reoviridae*) have occasionally been isolated from dogs with respiratory disease in association with other viruses (including CDV and canine parvovirus type-2) but their role in CIRD is unclear (Buonavoglia and Martella, 2007).

A range of bacteria can also be isolated and may be involved in lower respiratory tract infections (often as a secondary or opportunistic pathogen). These include but are not limited to *Escherichia, Pseudomonas, Pasteurella* and *Staphylococcus spp* (Bemis, 1992). Due to this work's focus being majorly on CIRD within the context of upper respiratory tract disease, these organisms are not further considered.

1.3 An overview of feline infectious respiratory disease (FIRD)

The following section is based on work published by the author: Afonso, M.M., Radford, A., Gaskell, R.M., 2017. Chapter 229: Feline Upper Respiratory Infections in: Textbook of Veterinary Internal Medicine: Diseases of the Dog and Cat. Elsevier, Missouri, pp. 1013-1016.

Similarly to CIRD, feline infectious respiratory disease (FIRD; often referred to as "cat flu") is common and manageable in individual cats (Bannasch and Foley, 2005). However, it is more prevalent and challenging in environments where cats are housed together such as catteries and shelters. In these environments a number of factors including but not limited to host susceptibility, stress, concomitant disease, current and previous exposure to pathogens, shelter design and biosecurity protocols, endemic disease and shelter density act synergistically creating the perfect conditions for disease spread (Willby, Radford and Afonso, 2018). FIRD is characterized by sneezing, oculonasal discharge, conjunctivitis and, in some cases, hypersalivation and coughing. More severe presentations including pneumonia and systemic disease may occur, especially in young or immunocompromised animals (Bannasch and Foley, 2005; Binns et al., 2000).

Feline herpesvirus-1 (FeHV-1) and feline calicivirus (FCV) are the two main viral causes of FIRD. FCV seems to be more commonly isolated than FeHV-1 but the latter often leads to more severe disease (Radford et al., 2009; Thiry et al., 2009). *Bordetella bronchiseptica, Chlamydophila (Chlamydia) felis* and possibly *Mycoplasma felis* are also involved in the FIRD disease complex (Litster et al., 2015). Like dogs, cats are also increasingly recognised as being susceptible to Influenza A virus infection including highly pathogenic and zoonotic avian influenza H5N1 and canine H3N2 and some interesting reports have emerged in recent years (Jeoung et al., 2013; Thiry et al., 2009). Table 1.2 presents a summary of prevalences of the above mentioned pathogens in cats with CIRD-like disease found in relevant studies published within the past 15 years.

Table 1.2 Summary of prevalences of pathogens involved in FIRD-like disease. Pet populations include privately owned animals and catteries refer to multi-animal environments including shelters, and breeding and research kennels. Number of animals reported alongside percentages where available in original research papers.

Pathogen	*Prevalence % (n)*	*Population*	*Region*	*References*
FCV	45%	Pet	Switzerland	Berger et al 2005
	34.2%	Pet	Europe	Hou et al 2016
	48% (61/127)	Pet	Spain	Fernandez et al 2017
FeHV-1	8%	Pet	Europe	Hou et al 2016
	20%	Pet	Switzerland	Berger et al 2005
	28.3% (36/127)	Pet	Spain	Fernandez et al 2017
	98% (51/52)	Catteries	USA	Veir et al 2008
C. felis	8%	Pet	Switzerland	Berger et al 2005
	20.5% (26/127)	Pet	Spain	Fernandez et al 2017
B.	4%	Pet	Switzerland	Berger et al 2005
bronchiseptica	5.1% (3/59)	Catteries	USA	Veir et al 2008
Mycoplasma	46.5% (59/127)	Pet	Spain	Fernandez et al 2017
spp.	47% (	Pet	Switzerland	Berger et al 2005
	80.9% (34/42)	Catteries	USA	Veir et al 2008

1.3.1 Feline herpesvirus – 1 (FeHV-1)

Feline herpesvirus type 1 is an enveloped, double-stranded DNA virus (alpha-herpesvirus) of which only one serotype exists with all isolates being genetically similar (Gaskell and Willoughby, 1999; Willoughby et al., 1991). Cats and other members of the *Felidae* are susceptible to infection. Infection tends to be associated with relatively severe signs of FIRD. In addition, FeHV-1 is also associated with severe ocular disease such as ulcerative keratitis (Thiry et al 2009).

FeHV-1 is primarily transmitted by direct contact between cats, although indirect transmission may also occur in the short term through contact with infectious discharges. In addition to clinical signs previously mentioned for FIRD, FeHV-1 replication can more rarely lead to osteolytic damage of nasal turbinate bones which is believed to predispose animals to more chronic and debilitating forms of rhinitis (Gaskell et al., 2007).

Whilst acutely infected cats are a source of virus, transmission also commonly occurs from clinically recovered carrier and persistently infected cats. It is generally believed that all cats recovering from acute infection develop a lifelong latent infection. In such cats, the virus persists in a latent or quiescent form largely in trigeminal ganglia, though other tissues may also be involved (Gaskell et al., 2007; Townsend et al., 2013). During this latent phase, infectious virus is generally not detectable from oro-nasal secretions. Periodically, particularly after a stressful event, virus reactivates in such carriers and they can then infect other animals. Stresses that may induce virus shedding include a change of housing (including going into a cattery), kittening and lactation, and corticosteroid treatment (Gaskell and Povey, 1977). Some cats may show clinical signs during a reactivation episode, which can be a useful indicator that they are likely to be infectious (Gaskell et al., 2007; Thiry et al., 2009).

FeHV-1 diagnosis (as well as FCV) has long been performed by virus isolation in cell culture from oropharyngeal and conjunctival swabs. Currently, PCR is the most commonly used technique for diagnostic purposes as it is more sensitive than traditional methods (Poste et al., 1972; Thiry et al., 2009).

Although several topical and systemic antivirals and other putative treatments have been investigated for use against FeHV-1, none of these are licensed for FIRD, their use being limited to ocular disease (Thiry et al., 2009).

1.3.2 Feline calicivirus (FCV)

FCV (family *Caliciviridae,* genus *Vesivirus)* is a small non-enveloped RNA virus. Although it is quite variable, it is generally considered that there is only one genotype and one serotype of the virus (Geissler et al., 1997; Glenn et al., 1999; Radford et al., 2009). There is some evidence of two genetic clusters/genotypes in Japan, although the wider significance of this remains uncertain (Ohe et al., 2007; Sato et al., 2002).

Most strains of FCV are closely related enough antigenically to induce some degree of cross-protection and this has been utilized in developing vaccines. FCV affects both domestic cats, and some non-domestic *Felidae.* Interestingly, FCV-like viruses have also been isolated from dogs; however, their clinical significance in dogs, and their ability to transmit between these species is uncertain (Binns et al., 2000; Helps et al., 2005). In addition, epidemiologic evidence of a possible association between dogs and FCV infection in cats is conflicting (Binns et al., 2000; Helps et al., 2005).

FCV transmission mostly follows direct contact with infected cats through the oro-nasal route. The virus may also be shed in urine (Larson et al., 2011) and faeces (Zhang et al., 2014) of cats, as well as being found in fleas (Mencke et al., 2009), although these are not thought to be of major relevance to transmission.

Usually cats shed FCV for up to a month, and at this point an infected animal is considered to be a carrier. In a small number of cats, the FCV carrier state can be lifelong (Radford et al., 2009). However, most carriers appear to eliminate virus at some point, remaining susceptible to reinfection. During the carrier phase, and in contrast to FeHV-1, cats shed virus more or less continuously, and are therefore likely to be infectious to other cats. The virus persists in the tonsils and other oropharyngeal tissues. FCV carriers appear to be common, with approximately 10% of cats in the general population shedding FCV, rising to almost 100% in some more densely populated environments such as rescue shelters and larger colonies (Bannasch and Foley, 2005; Coyne et al., 2006a, 2007a; Helps et al., 2005). From studies of endemically infected colonies, it appears that only a minority of carrier cats

are true persistent shedders; the majority seemingly undergoing cycles of reinfection from other cats in the colony (Coyne et al., 2007a).

Following FCV infection, considerable strain diversity may lead to some variation in clinical signs. The most characteristic sign is oral ulceration, typically on the tongue, but lesions may also occur elsewhere in the mouth or on the skin. Classic upper respiratory tract disease signs, such as sneezing, ocular and nasal discharges and conjunctivitis, also commonly occur, but these are generally milder than those seen with FeHV-1. With some strains of FCV, lameness and pyrexia may be a feature, with or without respiratory/oral disease (Dawson et al., 1994); other strains may induce an interstitial pneumonia with infection of alveolar macrophages (Monné Rodriguez et al., 2014), and some appear apathogenic (Rong et al., 2014). In addition, FCV infection is associated with chronic stomatitis, although its precise role in the condition is not clear and other factors are likely to be involved (Radford et al., 2009).

More recently, hypervirulent strains (virulent systemic FCV; VS-FCV) have emerged across North America and in several European countries. In addition to URT disease, affected cats show, to varying degrees, pyrexia, cutaneous oedema, ulcerative dermatitis, anorexia, and jaundice, with a high mortality rate (Coyne et al., 2006b; Pesavento et al., 2004; Schulz et al., 2011). Adult cats are frequently affected more severely than kittens, and in the field, disease is seen in both vaccinated and unvaccinated individuals. Each outbreak appears to be caused by a distinct strain; so far none of these VS-FCVs appears to have become widely established in the population (Ossiboff et al., 2007).

Currently, the most common diagnostic technique applied for FCV detection is RT-PCR. In some cases this may be less sensitive than viral isolation in cell culture largely because of the variability between strains and the difficulty of finding suitably cross-reactive primers. However, more recent RT-PCR assays appear to be highly sensitive (Litster et al., 2015). In addition to diagnosis, RT-PCR followed by sequencing is useful for differentiating between FCV strains in investigating the epidemiology of infection

and disease, and in particular, can help pinpoint where an individual cat became infected from (Coyne et al., 2007a, 2012; Radford et al., 1997).

Both modified live and inactivated vaccines are available for FCV prevention and are reasonably effective at protecting against disease, but none are able to prevent infection or development of carrier status (Pedersen and Hawkins, 1995; Radford et al., 2009). Various strains of FCV are used in commercial vaccines, such as FCV-F9 or FCV-255 in isolation, or two strains such as FCV-431 and FCV-G1 (Poulet et al., 2005). These seem to be generally cross-reactive against most recent FCV isolates when assessed *in vitro* (Addie et al., 2008; Porter et al., 2008) and there are some publications that point to their continued ability to induce heterologous cross-protection following challenge (Almeras et al., 2017; Lesbros et al., 2013; Poulet et al., 2008). Partial efficacy has also been reported with some vaccines against some VS-FCV strains (Pedersen et al., 2000; Poulet et al., 2008; Rong et al., 2014). Because FCV is highly variable and able to evolve rapidly, there is a consensus that field efficacy should be regularly monitored.

1.3.3 Bordetella bronchiseptica

In domestic cats, *B. bronchiseptica* is considered a primary as well as secondary pathogen. Experimentally, *B. bronchiseptica* infection induces mild clinical signs of FIRD and submandibular lymphadenopathy (Jacobs et al., 1993). On the other hand, field infection may lead to more severe disease, especially in younger animals, including pneumonia, dyspnoea, cyanosis and death (Egberink et al., 2009; Willoughby et al., 1991). As previously mentioned, *B.bronchiseptica* has the ability to cause disease in immunocompromised humans and it has been isolated from cystic fibrosis patients in contact with cats suffering from acute respiratory illness (Register at al 2012).

Diagnosis of *B. bronchiseptica* is done by culture or PCR. Animals that have clinically recovered from infection can continue to shed the organism and thus test interpretation must be carefully done. As with dogs, vaccines are available, but are not frequently used (Sánchez-Vizcaíno et al, 2018).

1.3.4 Chlamydophila felis

Chlamydophila felis (C. felis) is an obligate intracellular Gram-negative rod-shaped coccoid bacterium (Gruffydd-Jones et al., 2009). Infection with *C. felis* is generally associated with both acute and chronic conjunctivitis, though upper and lower respiratory signs may sometimes also be seen. Cases that start in one eye can rapidly progress to become bilateral. Affected eyes can be markedly painful with profound conjunctival hyperemia, chemosis, blepharospasm, and watery followed by mucoid or mucopurulent ocular discharges. In some cases adhesions of the conjunctiva may develop. More severe signs such as keratitis and corneal ulcers, and other systemic illness, are not common, and this can help differentiate *C. felis* from FeHV-1. Whilst thought to be primarily an ocular pathogen, it is mentioned here as part of the differential diagnosis for FCV and FeHV-1, both of which can cause ocular as well as respiratory disease (Gruffydd-Jones et al., 2009; Sykes, 2012).

1.3.5 Mycoplasma spp.

As in dogs, the role of *Mycoplasma* species as primary pathogens in FIRD is still controversial as these have been isolated from both animals with respiratory disease and healthy cats, with studies suggesting that mycoplasmas are more common in cats with respiratory and/or ocular disease (conjunctivitis) than those without (Haesebrouck et al., 1991). Additionally, a recently published meta-analysis of field literature concluded that *Mycoplasma felis* is associated with upper respiratory tract disease (Le Boedec, 2017). Diagnosis can be achieved via culture, but PCR allows for faster results and speciation (Lee-Fowler, 2014). Antimicrobials are the main focus of treatment but any underlying or concurrent disease should also be addressed as Mycoplasmas are often associated with other pathogens (Bemis, 1992; Lee-Fowler, 2014).

1.3.6 Influenza viruses in cats

In parts of Asia where H5N1 infection is endemic in poultry, localized outbreaks of H5N1 with high mortality have been described in various felids including domestic cats, tigers and leopards (Keawcharoen et al., 2004; Kuiken et al., 2004). Occasional

asymptomatic infections have also been seen in domestic cats in Europe, where there was close contact with infected aquatic birds (Leschnik et al., 2007). Experimental studies have shown that cats can become infected both through contact with infected cats and by consuming infected poultry. Infected cats develop severe respiratory disease with high mortality, and can transmit infection directly to in-contact cats (Kuiken et al., 2004; Rimmelzwaan et al., 2006). Influenza H5N1 should be considered in cats in contact with birds where highly pathogenic H5N1 is currently circulating. Heterologous vaccination of cats has been shown to protect against lethal infection of cats with H5N1, and reduce shedding, leading some to suggest vaccines may have a role in reducing risk of onward transmission from cats (Vahlenkamp et al., 2008).

Recently, CIV H3N2 has also been reported in shelter housed cats with coughing and dyspnoea and was associated with high mortality (Jeoung et al., 2013). Population and experimental studies have also shown that cats are susceptible to a wide range of influenza viruses including H1N1, H3N8, H5N2 and H9N2 and in some cases developing clinical signs (Damiani et al., 2012; Hai-xia et al., 2014; Su et al., 2014; van den Brand et al., 2010; Zhao et al., 2014).

Cats (and dogs) are only recently being considered as part of the influenza-susceptible species and much more work needs to be done to clarify their role, both in transmission from aquatic birds, and for their potential to infect people.

Other pathogens of possible clinical significance in FIRD

Feline reovirus and cowpox virus have occasionally been reported in animals with respiratory and/or ocular disease (Muir et al., 1992; Schöniger et al., 2007). However, due to their primary association with other diseases, will not be discussed further here.

1.4 Conclusions and thesis aims

It is clear that upper tract respiratory disease of infectious origin remains an important health issue of different cat and dog populations both in the UK and worldwide. Many questions still remain unanswered regarding the aetiologies and risk factors involved in the pathogenesis of these multifactorial syndromes. Thus, the aims of this study were:

- Assess the diversity and epidemiology of FCV in the UK and wider European cat population and assess the efficacy of anti-FCV-F9 vaccine antisera to neutralise a panel of recent field isolates of FCV.

- Determine, through a retrospective sero-survey, whether there is evidence of exposure to Influenza A and B viruses in UK cats and dogs.

- Re-evaluate, through a case-control study, the role of known and potential FIRD and CIRD pathogens as well as risk factors for clinical disease, in cats and dogs attending UK veterinary practices.

- Re-evaluate, through a longitudinal and cross-sectional study, the presence of known and potential respiratory pathogens in CIRD and FIRD in UK rescue shelters.

2 A multi-national European cross-sectional study of feline calicivirus epidemiology, diversity and vaccine cross-reactivity

The contents of this chapter have been published in a peer-reviewed journal:
Afonso, M., Pinchbeck, G., Smith, S., Daly, J., Gaskell, R., Dawson, S. and Radford, A. (2017). A multi-national European cross-sectional study of feline calicivirus

2.1 Abstract

Feline calicivirus (FCV) is an important pathogen of cats for which vaccination is regularly practiced. Long-term use of established vaccine antigens raises the theoretical possibility that field viruses could become resistant. This study aimed to assess the current ability of the FCV-F9 vaccine strain to neutralise a randomly collected contemporary panel of FCV field strains collected prospectively in six European countries.

Veterinary practices (64) were randomly selected from six countries (UK, Sweden, Netherlands, Germany, France and Italy). Oropharyngeal swabs were requested from 30 (UK) and 40 (other countries) cats attending each practice. Presence of FCV was determined by virus isolation, and risk factors for FCV shedding assessed by multivariable logistic regression. Phylogenetic analyses were used to describe the FCV population structure. *In vitro* virus neutralisation assays were performed to evaluate FCV-F9 cross-reactivity using plasma from four vaccinated cats.

The overall prevalence of FCV was 9.2%. Risk factors positively associated with FCV shedding included multi-cat households, chronic gingivostomatitis, younger age, not being neutered, as well as residing in certain countries. Phylogenetic analysis showed extensive variability and no countrywide clusters. Despite being first isolated in the 1950s, FCV-F9 clustered with contemporary field isolates. Plasma raised to FCV-F9 neutralized 97% of tested isolates (titres 1:4 to 1:5792), with 26.5%, 35.7% and 50% of isolates being neutralized by 5, 10 and 20 antibody units respectively.

This study represents the largest prospective analysis of FCV diversity and antigenic cross-reactivity at a European level. The scale and random nature of sampling used gives confidence that the FCV isolates used are broadly representative of FCVs that cats are exposed to in these countries. The *in vitro* neutralisation results suggest that antibodies raised to FCV-F9 remain broadly cross-reactive to contemporary FCV isolates across the European countries sampled.

2.2 Introduction

Feline calicivirus (FCV) is a common pathogen of cats causing oral and upper respiratory tract disease (URTD) (Radford et al 2009). It has a single-stranded, positive-sense RNA genome (Afonso et al., 2017), the plasticity of which is important for antigenic evolution, viral persistence (Coyne et al., 2007a; Radford et al., 2009), recombination (Coyne et al., 2006a; Symes et al., 2015), and the sporadic outbreaks of highly virulent FCV strains causing severe disease (Radford et al., 2009). Despite high levels of variability, FCV strains are generally considered to comprise one diverse genogroup with a radial phylogeny and little evidence for sub-species clustering (Coyne et al., 2007a, 2012; Glenn et al., 1999; Hou et al., 2016; Sato et al., 2002).

This diverse genogroup is mirrored by a single diverse serotype; although individual strains are distinguishable antigenically, they generally show some cross-reactivity (Wardley et al., 1974) allowing the development of several FCV vaccines based on different antigens (Radford et al 2009). Whilst vaccines reduce clinical signs, none are licensed to reduce virus shedding post-challenge and FCV infection remains highly prevalent in both vaccinated and unvaccinated populations (Radford et al 2009). Most live vaccines include FCV-F9 (Pedersen and Hawkins, 1995) whereas inactivated vaccines commonly include strains FCV-255, or a combination of FCV-431 and FCV-G1 (Poulet et al., 2008, 2005). These vaccine antigens are chosen based on their ability to induce broadly cross-reactive antisera against contemporary isolates circulating at the time of vaccine development (Pedersen and Hawkins, 1995; Poulet et al., 2005). The widespread use of such vaccines together with the high adaptability of FCV raises the theoretical possibility that vaccine resistant strains may evolve over time. Whilst some studies have supported this hypothesis (Addie et al., 2008; Lauritzen et al., 1997; Wensman et al., 2015), others have not (Porter et al., 2008).

Here we describe the antigenic and genetic relationships between FCV-F9 and a representative panel of currently circulating FCV strains, obtained from randomly selected veterinary practices across six European countries.

2.3 Materials and methods

Ethical statement

Ethical approval was from the Veterinary Research Ethics Committee, University of Liverpool. Informed consent was obtained from participating owners.

Recruitment

Samples were collected between October 2013 and May 2014 from cats attending veterinary practices in the UK, France, Italy, Netherlands, Sweden and Germany.

In the UK, three Unitary Authorities (UAs) were randomly chosen from each of the nine regions of England, as well as from Wales, Scotland and Northern Ireland. Geographically remote islands were also selected (Western Isles, Orkney, Shetland, Anglesey, Isle of Wight, Scilly Isles, Isle of Man, Channel Islands) based on convenience. From each of these 44 regions, a small animal practice was randomly selected from the Royal College of Veterinary Surgeons database. The remaining five countries were chosen based on convenience, divided into five regions based on official divisions and/or local geography, and a single practice randomly selected from each. If chosen practices declined to participate, a further practice was randomly selected. This process was repeated up to three times until a practice in each region agreed to take part.

Field isolates

There is much debate regarding the most appropriate FCV isolates to use for assessment of *in vitro* neutralisation. Several studies have used isolates obtained by convenience from diagnostic laboratories to represent pathogenic viruses (Lauritzen et al 1997, Addie et al 2008, Wensman et al 2015); lack of random sampling means such results may not be generalizable to the wider population (Addie et al, 2008). Here we sample sick and healthy cats randomly to ensure our results are representative of the sampled population. The occasional description of non-pathogenic FCV strains (Povey and Hale, 1974) justifies the inclusion of isolates from healthy animals. In this regard, it should be noted that FCV isolates from healthy cats

can still be pathogenic: virulent FCV continues to be shed from cats recovered from acute disease (Povey and Hale 1974), and seropositive cats previously exposed to vaccine or field virus may shed virus in absence of clinical signs when subsequently challenged with virulent virus (Kahn and Hoover, 1976). Indeed, experimental challenge has confirmed that FCV from healthy cats can recreate typical disease (Povey and Hale 1974).

In each practice, veterinary surgeons were asked to collect oropharyngeal swabs from the next 30 (UK) or 40 (other countries) cats presented at their surgery regardless of reason for presentation (diseased or healthy). Random recruitment of practices and random sampling of cats based on attendance at these practices were used to ensure results could be generalised to the sampled population, and is in contrast to an earlier study by the authors where sampling was by convenience (Hou et al., 2016).

Swabs were collected into virus transport medium, stored at −20 °C before shipping to the laboratory. The veterinary surgeon and owner were asked to complete a short questionnaire capturing demographic data, vaccination history and information about current respiratory disease, mouth ulcers and chronic gingivostomatitis (CGS).

Viral isolation (VI)

Feline calicivirus was isolated using standard techniques (Jarrett et al., 1973) based on presence of typical cytopathic effect (CPE). Samples were only considered negative after two passages (Knowles et al., 1991).

RNA extraction and reverse transcription-PCR

Viral RNA was extracted from positive cell cultures (second passage or less) (Viral RNA mini-kit; Qiagen). One negative control (mock infected cells) was included for each three samples. Reverse transcription was performed using 200 ng random hexamers (Superscript III, Life Technologies). A 529-nucleotide region of the capsid gene, equivalent to residues 6406-6934 of FCV-F9 (GenBank M86379) and

incorporating immunodominant regions C and E (Radford et al., 1999; Seal et al., 1993), was amplified according to manufacturer's guidelines (Reddy-Mix; Thermo scientific) and published protocols using 25pmoles of each primer per 50 µl reaction (Hou et al., 2016). In addition, 486 nucleotides from the 3' end of the FCV polymerase gene were also sequenced as previously described (Hou et al., 2016).

Nucleotide sequencing and phylogenetic analysis

Amplicons were purified (QIAquick; Qiagen), quantified (Nanodrop; Genequant) and sequenced (Source Bioscience; Nottingham). Forward and reverse sequences were aligned (ChromasPro; Technelysium), and pairwise p-distances and neighbour-joining trees (1000 bootstrap replicates) calculated using MEGA7. A threshold of 20% uncorrected nucleotide distance was used to define distinct strains (Prikhodko et al., 2014; Radford et al., 1997).

Epidemiological analysis

Prevalence estimates with 95% confidence intervals were determined (Epitools; AusVet) based on results of VI. Data from questionnaires were used to examine risk factors and associations with FCV carriage. Univariable and multivariable multilevel logistic regression allowing for clustering within practice was conducted using MLwiN (v2.1, University of Bristol). Potential risk factors included country, cat's age, gender, breed, lifestyle, vaccination status, vaccine strain, neutering status, presence of mouth ulcers, URTD signs, CGS and number of cats in the household. Variables with P–values <0.25 in initial univariable analysis were considered in the multivariable model retaining variables with Wald *P*-values <0.05.

Isolates and plasma for viral neutralisation (VN) testing

Isolates for VN testing were randomly selected with stratification, approximately half were from the UK, the remainder from other participating countries. There is no approved standard for producing immune reagents for FCV neutralisation studies. Conventional FCV vaccination induces insufficient neutralisation titres (Dawson et al., 1993b), such that previous studies have used infection with vaccine viruses to

produce test sera (Addie et al., 2008; Lauritzen et al., 1997; Porter et al., 2008; Wensman et al., 2015). This will likely impact on both the quantity and range of any measured immune response compared to vaccination, especially when the tested vaccines often contain inactivated antigens. The plasma used in this study was collected from animals used in a standard vaccine safety study conducted by the funders. Four specific pathogen free cats were vaccinated subcutaneously with 10 commercial doses of Nobivac® TricatTrio (FCV-F9 live-attenuated vaccine) at 8–9 weeks of age, and again four weeks later. Blood samples were taken three weeks after the second vaccination. Whilst such a challenge regime will induce a quantitatively higher response than routine vaccination, the antigenic targets for the response should be broadly similar to those of routine vaccination. The plasma from all four cats was used as a pool for all tested isolates, and also separately for 10 randomly selected isolates.

Viral neutralisation assays

Virus neutralisation tests were performed using a constant virus, varying plasma method. Briefly, duplicate, serial twofold dilutions of plasma were incubated with 32–320 $TCID_{50}$ (Dawson et al., 1993a) of virus at 37 °C for 1 h before addition to FEA cells which had been plated 24hours previously at approximately 1×10^4 cells/well of a 96 well plate. Plates were observed for CPE at 48 h and 120 h. Antibody titres were expressed as 50% end points (Reed and Muench, 1938). An internal FCV-F9 homologous control was included in each experiment. As homologous antibody titres can vary between experiments, between serum from different cats and depending on the method of challenge, antibody units (AU) for each isolate were calculated using the titre of this internal control. One antibody unit (AU) is the highest plasma dilution neutralizing 100$TCID_{50}$ of homologous virus in 50% of cultures (Povey, 1974). AUs were also calculated using the mean FCV-F9 titre of all experiments (Porter et al., 2008), excluding those in which the internal homologous FCV-F9 titre was >2-fold either side of the mean FCV-F9 titre for all experiments (Dawson et al., 1993b)

2.4 Results

Fifty (27 UK, 23 mainland Europe) of the 64 recruited practices (78.1%) returned samples (Figure 2.1). Of the 2140 samples requested, 1521 (71%) were received.

Figure 2.1 Map showing the 64 recruited veterinary practices. Circles in red are the 50 practices that supplied samples during the study. Blue circles are the 14 recruited practices that did not send back samples during the study. (

A total of 140 of 1521 samples tested positive for FCV (9.2%, 95% confidence interval (CI) 7.8, 10.8), ranging from 5.4% in Italy to 16.2% in the Netherlands (Table 2.1).

Table 2.1 Summary of samples, isolates and strains identified in each country.

Country	***UK***	***France***	***Netherlands***	***Germany***	***Sweden***	***Italy***
Samples (n)	686	187	99	175	205	149
FCV Positive n (%, CI)	58 (8.4%, 0.06–0.1)	11 (5.8%, 0.03–0.09)	16 (16.2%, 0.09–0.2)	25 (14.2%, 0.09–0.19)	17 (8.3%, 0.05–0.12)	8 (5.4%, 0.02–0.09)
FCV Capsid Sequence	56	11	16	19	17	10
Strains (n)	48	11	14	14	14	10

Questionnaires were not received for 1.2% of samples and therefore analysis was performed using 1502 questionnaire-sample matches. Nine of twelve predictor variables were significantly associated with FCV isolation in univariable analysis (data not presented). Of these, five remained significant on multivariable analysis (Table 2.2): Cats sampled in France, Italy and the UK were at a lower risk of shedding FCV than those from the Netherlands. Entire (non-neutered) cats were 1.7 times more likely to shed FCV than neutered cats, regardless of gender. Cats in multi-cat households were 1.7 (2–3 cats) and 2.8 (4–10 cats) times more likely to shed FCV than cats living alone. Cats with CGS were 8.3 times more likely to shed FCV than those without. Finally, each additional year of a cat's age reduced FCV shedding likelihood by 12%. Vaccination was not significantly associated with risk of FCV infection in the final model.

Table 2.2 Multilevel multivariable logistic regression analysis (allowing for clustering within practice) of factors associated with FCV isolation in 1502 vet visiting cats from UK and mainland Europe. P-values below 0.05 considered significant.

Variable	*Odds ratio*	*95% CI lower*	*95% CI upper*	*P-value*
Country				
France	0.29	0.11	0.75	0.01
The Netherlands	Ref.			
Italy	0.234	0.087	0.631	0.004
Germany	0.72	0.31	1.65	0.44
Sweden	0.599	0.253	1.419	0.24
UK	0.434	0.213	0.88	0.02
Neutered status				
Yes	Ref.			
No	1.69	1.053	2.736	0.03
Chronic gingivo-stomatitis				
Yes	Ref.			
No	0.12	0.06	0.23	<0.001
No. cats/household				
1	Ref.			
2–3	1.75	1.10	2.79	0.02
4–10	2.82	1.49	5.31	0.001
>11	0.74	0.08	6.97	0.79
Age (month)	0.99	0.99	0.99	0.01
Level 2 variance (standard error)				0.035 (0.106)

A total of 128 partial capsid consensus sequences were obtained from the 140 FCV isolates (Figure 2.2; GenBank accession numbers KX257491–KX257617). The unamplified isolates typical of such experiments are presumed to be caused by primer mismatches (Coyne et al., 2012; Hou et al., 2016). In total, 110 strains (pairwise genetic distance>20%) were observed, ranging from 10 (Italy) to 48 (UK) (Table 2.1). Of these strains, only 10 were represented by more than one isolate (bootstrap values >80%; A to L on Figure 2.2). The largest cluster included FCV-F9-like isolates from the UK and Sweden (D in Figure 2.2). All other strains with more than one variant were restricted to individual practices, with no evidence for widespread

or international transmission. Similar phylogenetic results were obtained for the polymerase gene (Figure 2.3).

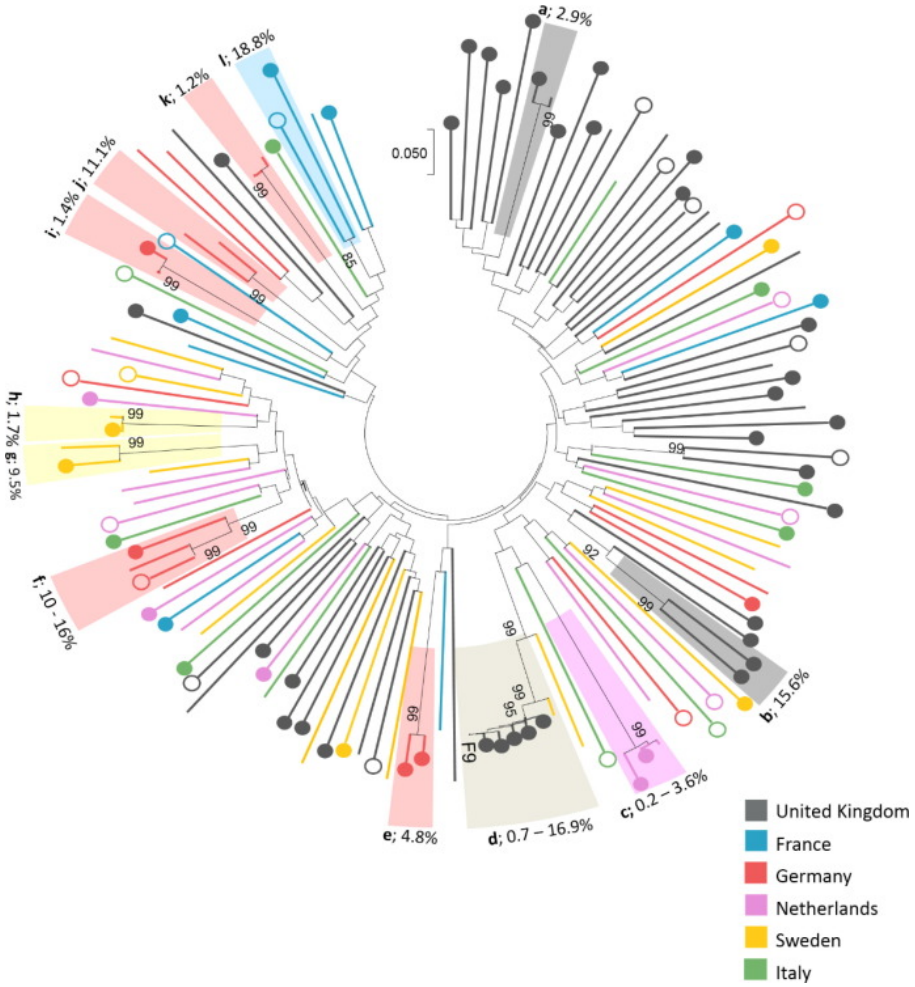

Figure 2.2 Unrooted Neighbor-Joining tree of 128 FCV partial capsid consensus sequences obtained from the national study (including the sequence of FCV vaccine strain F9 [GenBank accession No. M86379]). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown, only bootstrap values >80% are indicated. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree and relate to the distance bar. The evolutionary distances were computed using the Tamura-Nei method and represent the number of base substitutions per site. The rate variation among sites was modelled with a gamma distribution (shape parameter = 1). Codon positions included were 1st + 2nd + 3rd + Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 435 positions in the final dataset. The geographical origin of sequences is shown in different colours. Those strains represented by more than a single sequence (<20% capsid divergence) are boxed, additionally labelled A–L, and the intra-strain capsid diversity indicated next to the box; isolates in each box originate from the same country and veterinary practice with the exception of cluster D, where all sequences were collected in different practices and two different countries. Circles at the tip of the branch indicate isolates used in viral neutralisation testing: empty circles represent isolates from animals with either chronic or acute upper respiratory tract disease (URTD) and full circles represent isolates from animals either without URTD or no information in regards to clinical status.

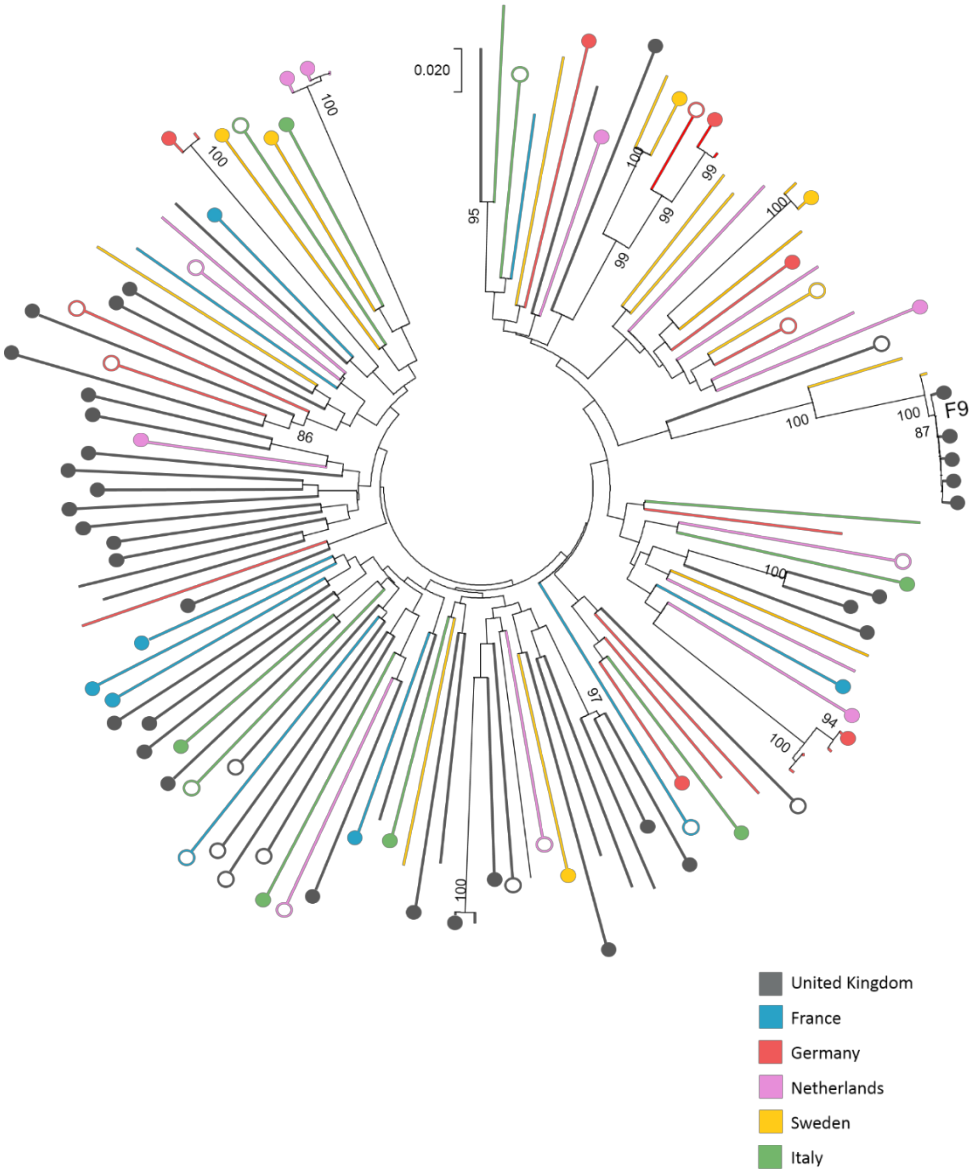

Figure 2.3 Unrooted Neighbor-Joining tree of 128 FCV partial polymerase consensus sequences obtained from the national study (including the sequence of FCV vaccine strain F9 [GenBank accession no. M86379]). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown, only bootstrap values >80% are indicated. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree and relate to the distance bar. The evolutionary distances were computed using the Tamura-Nei method and are in the units of the number of base substitutions per site. The geographical origin of sequences is shown in different colours. Circles at the tip of the branches indicate the isolates used in Viral Neutralisation testing: empty circles represent isolates from animals with either chronic or acute upper respiratory disease (URTD) and full circles represent isolates from animals either without URTD or no information in regards to clinical status.

The reproducibility of VN assays was assessed in two ways. Firstly, 10 field isolates were randomly repeated giving an average difference in neutralisation titres between repeats of 2.08, comparable to previous studies (Dawson et al., 1993a). In addition, the mean homologous titre for the internal FCV-F9 control across 19 experiments was 1 in 1658 ± 345 standard error (data not presented).

Viral neutralisation was attempted in 121 of the 140 FCV isolates. In total, 98 VN tests were successfully completed (48 UK, 6 Sweden, 9 France, 16 Germany, 10 the Netherlands and 9 Italy; Appendix 1A); the remaining 23 failed due to inability to regrow in cell culture, titration failure, or bacterial contamination.

Of these 98 FCV isolates, 95 (97%) were neutralized at titres ranging from 1:4 to 1:5792 (Figure 1.4a; Appendix 1A). Whilst group sizes precluded statistical analysis, the pattern of neutralisation appeared to be broadly similar when isolates from different clinical presentations were compared (Figure 1.5). The VN results based on different countries are shown in Figure 1.4b. When titres were standardised to homologous FCV-F9 titres derived within individual experiments, 26.5%, 35.7% and 50% of isolates were neutralized by 5, 10 and 20 AUs respectively. When using the same method as described previously (Dawson et al., 1993b), using only those experiments where the titre for the internal FCV-F9 control was within 2-fold of mean FCV-F9 titre across all experiments, 0%, 20% and 32% of 25 isolates were neutralized by 5, 10 and 20 AUs respectively (Table 2.3).

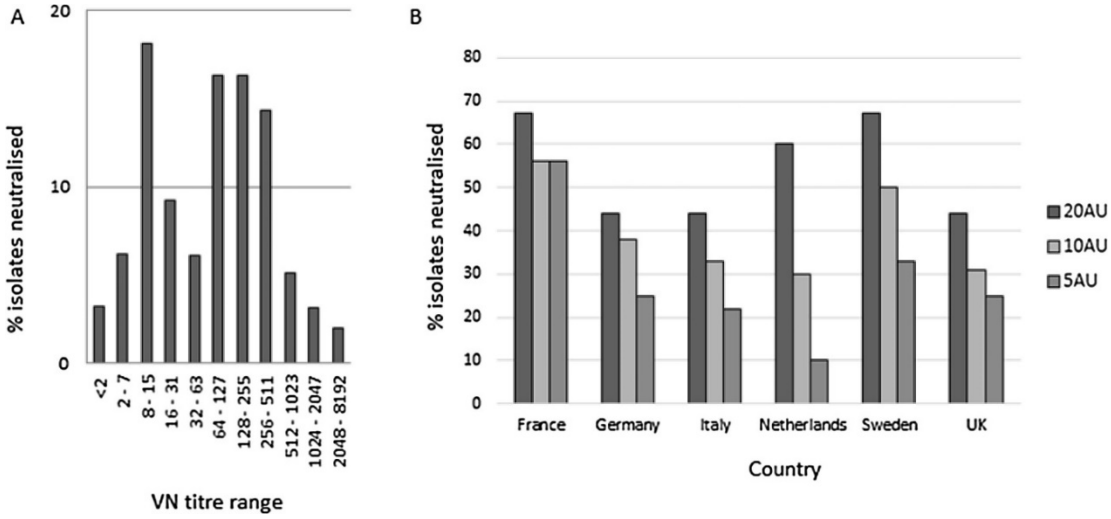

Figure 2.4 Results of viral neutralisation testing. A: VN titre ranges (% of isolates) for the current study. B: Percentage of isolates neutralized by 20, 10 and 5AU per country.

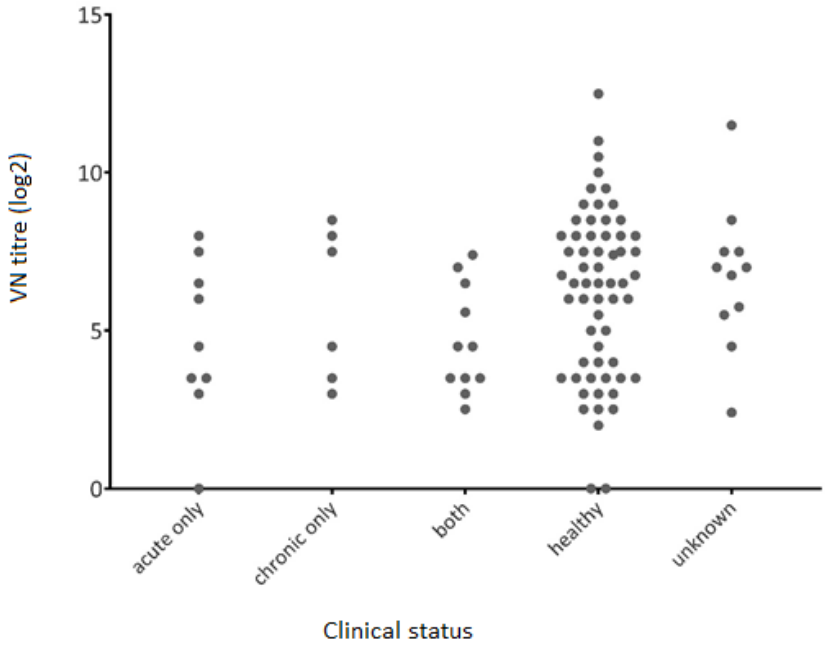

Figure 2.5 Scatterplot of log2 of VN titres (Y axis) for FCV isolates grouped based on the presence of respiratory and oral disease at time of sampling (X axis). Acute only: animals with ulcers and/or upper respiratory tract disease. Chronic only: cats with chronic gingivostomatis. Both: cats with both chronic and acute disease. Healthy: animals with neither acute nor chronic disease. Unknown: cats where information regarding clinical disease was not provided by the assisting veterinary surgeon through questionnaire at the time of sampling.

Table 2.3 Percentage of isolates neutralized by 20, 10 and 5AU (antibody units) for each different cut-off or analysis method used.

Cut-off	*Number of VN results*	*20AU*	*10AU*	*5AU*
AUs based on individual experiment FCV-F9 homologous control	98	50	36	27
AUs for those isolates where the titre for the internal F9 control was within 2-fold of mean F9 titre across all experiments	25	32	20	0

In order to analyse the variability of plasma from the four cats, viral neutralizations with single cat plasma were undertaken for 10 random field isolates and FCV-F9 (Table 1.4). The plasma from each cat had demonstrable neutralizing ability to each isolate. However, there was variation in the order of individual cat responses, with some cats' plasma seemingly neutralising some viruses particularly well, and others less well.

Table 2.4 Neutralizing titres for pooled plasma from four cats vs individual plasma. BT stands for back titration. Isolates were given a unique ID code using two letters to represent the country of origin (GB=United Kingdom, FR=France, DE=Germany, IT=Italy, NL=Netherlands, SE=Sweden) followed by two digits representing the practice where the sample was collected and the two next digits representing the individual animal.

Isolate	*pool*	*Cat 6071*	*Cat 6038*	*Cat 6480*	*Cat 6452*	*F9 titre*	*F9 BT*	*isolate BT*
NL0129	5.7	5.7	5.7	8	2	1448	31.6	215.4
S0436	42.2	32	84.4	128	215.3	1448	31.6	31.6
F9	4096	2048	4096	1024	2048	4096	46.4	46.4
GB1818	2.8	5.7	5.7	8	4	4096	46.4	100
SE0114	2	4	2.8	2.8	4	4096	46.4	215.4
IT0421	26.9	26.9	13.5	22.6	22.6	2896	100	31.6
GB0325	26.9	32	13.5	22.6	22.6	2896	100	316.2
NL0342	256	512	512	181	181	2896	100	100
GB1612	5.7	4	8	5.7	5.7	2896	31.6	215.4
GB1528	256	64	64	512	128	2896	31.6	31.6

2.5 Discussion and conclusions

Widespread use of individual vaccines is associated with a theoretical risk for the emergence of vaccine resistance strains, particularly for RNA viruses. Here we have undertaken the first multinational European study to assess the current *in vitro* cross reactivity of FCV-F9, first isolated over 40 years ago, and still one of the most frequently used vaccine antigens (Kalunda et al., 1975).

In order to maximise the generalizability of our findings to the European cat population, a cross-sectional survey sampling cats from randomly recruited veterinary practices was undertaken. This approach also provided an opportunity to assess the epidemiology and molecular epidemiology of FCV infection. Whilst every effort was made to sample in a random manner, we are unable to rule out that some practitioners may have selected for diseased patients. In a previous study looking into tick prevalence in dogs in the UK where veterinary surgeons were recruited on a voluntary basis it was postulated that over-reporting may have happened due to participants being particularly interested in ticks and tick-borne diseases, resulting in selection bias (Abdullah et al, 2016). Since veterinary surgeons participating in the present study also agreed to take part voluntarily it is likely that a similar bias may be present. Additionally, this study only sampled veterinary visiting cats which would seem appropriate as the project aimed to look at vaccine efficacy. However, results should not be generalised to non-veterinary visiting cats. It is likely that viruses circulating in the latter population are similar to those present in veterinary visiting cats but we cannot formally comment on that. In future studies, it would be interesting to compare viruses from both populations.

Consistent with previous studies, cats in multi-cat households, those with CGS, and younger cats, were more likely to shed FCV (Fernandez et al., 2017; Porter et al., 2008). Chronic gingivostomatitis affects 0.7% of the population (Healey et al., 2007), with most affected cats testing FCV positive (Fernandez et al., 2017; Knowles et al., 1991). Previous studies have shown that FCV prevalence increases from around 10% in single-cat households to over 50% in some larger colonies (Coyne et al., 2007b; Helps et al., 2005). These large colonies are believed to drive antigenic diversity as

strain variants evolve under positive selection within a variable population immunity (Coyne et al., 2007b; Radford et al., 2001). In addition, neutered cats were less likely to test positive for FCV regardless of age. This suggests behavioural changes associated with neutering, such as becoming less territorial, may lower FCV risk as has also been shown for feline immunodeficiency virus (Berger et al., 2015; Wardley et al., 1974). We also found that cats in some countries (the Netherlands) had a higher prevalence of FCV infection than those from others (France, Italy, UK). Whether this represents true population differences, or the relatively small sample sizes in some countries will need to be assessed further.

The phylogenetic analysis is broadly in agreement with previous national and international studies (Coyne et al., 2012; Glenn et al., 1999; Henzel et al., 2012; Hou et al., 2016), highlighting a radial phylogeny with little evidence for sub-species clustering except viruses sharing immediate temporal or spatial links. As previously (Abd-Eldaim et al., 2005; Coyne et al., 2012, 2007b), FCV-F9 variants were found in this population, five from the UK and two from Sweden, of which four had been vaccinated with FCV-F9 attenuated vaccines <25 days prior to sampling, one was un-vaccinated for at least three years, and one a rescue cat that was presumed unvaccinated. The only time such vaccine-derived viruses are not observed is when recently vaccinated cats are excluded from the sampled population (Hou et al., 2016). Our findings are consistent with experimental studies showing occasional shedding of vaccine virus following live-FCV vaccination (Dawson et al., 1991; Pedersen and Hawkins, 1995). Looking at the diversity within this FCV-F9 clade, six of the seven strains were <3.6% distant from the FCV-F9 published sequence, suggesting they had not been replicating for long in the cat, consistent with recent vaccination history of most of these cats. In contrast, a Swedish isolate from a vaccinated cat (unknown strains), was 16.9% different to FCV-F9, possibly representing a rare persisting and evolving strain of FCV-F9 or an unrelated strain. Taken together, this confirms that whilst live vaccine viruses are occasionally shed following vaccination, they only seem to have a limited potential to persist in the general cat population.

The balance between antibody- and cell-mediated immunity in FCV protection is

somewhat uncertain. Some cats exposed to previous FCV antigens show protection to heterologous challenge, even when there are no demonstrable *in vitro* antibodies to the new challenge (Knowles et al., 1991), suggesting that other factors including cellular immunity contribute to protection. That said, it is still believed that there is sufficient correlation between antibody levels and protection, for *in vitro* virus neutralisation tests to remain the accepted method of assessing cross-reactivity (Addie et al., 2008; Porter et al., 2008; Povey and Ingersoll, 1975; Wensman et al., 2015). Therefore, we have used a pool of plasma raised to 10 doses of FCV F9 vaccine, and demonstrated neutralising activity to the majority of this cross-sectional European panel of contemporary FCV isolates. These results are broadly similar to those observed in a similar cross-sectional study of FCV-F9 strain diversity in the UK in 2001 (Porter et al., 2008). When results are expressed as antibody units to try and control for variations in sera production (infection vs vaccination overdose), and the between-cat variation, the percentage of isolates neutralized by 20, 10 and 5 AUs was similar to, or higher than, that from the earlier study in 2001 (Porter et al., 2008). When taken together, this suggests antisera against FCV-F9 remains broadly cross-reactive against recently circulating FCV strain diversity. This is consistent with our observation that, despite its age, FCV-F9 remains an integral part of this contemporary phylogeny, and suggesting that FCV may not evolve in a linear ("clock-like") fashion, such as is typical for other rapidly evolving viruses (Gojobori et al., 1990; Kimura, 1987).

These conclusions are in contrast with other studies suggesting the levels of FCV-F9 cross-reactivity have reduced over time (Addie et al., 2008; Lauritzen et al., 1997; Wensman et al., 2015). However, two important methodological differences between studies make direct comparisons impossible. Firstly, previous studies used isolates collected by convenience from diagnostic laboratories; these should not be considered representative of those in the general population (Addie et al., 2008). Secondly, previous studies have used infection rather than vaccination to produce antisera of sufficient titre for testing; differences in viral replication and antigen presentation between virus replicating locally in target tissues of the upper respiratory tract as opposed to the subcutaneous tissues at the site of vaccination,

are likely to impact in albeit unknown ways, on the nature of the ensuing immune response, and this impact is likely to be greatest for viral antigens from inactivated vaccines. Here for the first time we used subcutaneous vaccination (albeit at 10× release dose), of a live vaccine, using a cross-sectional sample of contemporary FCV isolates to maximise the generalizability of our results. Clearly these *in vitro* results cannot be used to suggest the rate of cross-protection in the field. To facilitate better comparison between these studies in the future, we recommend the development of an internationally agreed study protocol as exists for other viral vaccines.

2.6 Appendix to Chapter 2

Appendix 2A Viral neutralisation data alongside questionnaire data for each tested isolate. Country names in this table use ISO codes. DSH stands for Domestic Short Hair, DLH stands for domestic long hair and DSLH stands for domestic semi-long hair (n=98).

Isolate	Back titration	Titre	Homologous f9 back titration	Homologous f9 titre	Country	Gender	Neutered status	Type/breed	Confinement	Age in months	Vaccinated	Time since last vaccine (months)	Vaccine strain	Number of cats in household	Ulcers?	Upper respiratory tract disease?	Acute disease	Chronic gingivostomatitis?
Fr0135	316.2	8	100	512	Fr	M	N	Dsh	Both	5	Y	31	Fcv-f9	4 to 10	N	N	N	N
Fr0201	316.2	256	46.4	4096	Fr	F	Y	Dsh	Both	72	Y	N/a	N/a	4 to 10	N	N	N	Y
Fr0204	316.2	<2	31.6	256	Fr	M	N	Dsh	Indoors	5	Y	N/a	N/a	2 to 3	N	Y	Y	N
Fr0208	46.4	256	31.6	4096	Fr	F	Y	Dsh	Outdoors	8	N	N/a	N/a	2 to 3	N	N	N	N
Fr0236	100	128	215.4	512	Fr	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a
Fr0321	46.4	181	31.6	512	Fr	F	Y	Dsh	Both	67	Y	N/a	Fcv-f9	1	N	N	N	Y
Fr0515	46.4	724	100	724	Fr	M	Y	Dsh	Both	9	N	N/a	N/a	2 to 3	N	N	N	N
Fr0538	46.4	362	100	512	Fr	M	Y	Pedigree	Unknown	N/a	N/a	N/a	N/a	2 to 3	N	N	N	N
Fr0637	46.4	362	31.6	512	Fr	M	N	Dsh	Both	5	Y	N/a	Fcv-431 + g1	2 to 3	N	N	N	N
De0104	170	5.7	31.6	512	Deu	M	Y	Pedigree	Outdoors	N/a	N	N/a	N/a	1	Y	N	Y	Y
De0130	316	11.3	316	512	Deu	F	Y	Dsh	Both	151	Y	362	Fcv-f9	2 to 3	N	N	N	N
De0209	316.2	8	215.4	512	Deu	F	Y	Pedigree	Both	110	Y	37	Fcv-f9	2 to 3	N	Y	Y	Y
De0214	31.6	168.9	100	724	Deu	F	Y	Dsh	Outdoors	N/a	N	N/a	N/a	11 to 30	Y	N	Y	Y
De0229	215.4	256	215.4	4096	Deu	M	Y	Cross	Both	161	Y	63	Fcv-f9	2 to 3	N	N	N	N

Isolate	Back titration	Titre	Homologous f9 back titration	Homologous f9 titre	Country	Gender	Neutered status	Type/breed	Confinement	Age in months	Vaccinated	Time since last vaccine (months)	Vaccine strain	Number of cats in household	Ulcers?	Upper respiratory tract disease?	Acute disease	Chronic gingivostomatitis?
De0232	316.2	22.6	100	512	Deu	F	Y	Pedigree	Both	115	Y	37	Fcv-f9	2 to 3	N	N	N	Y
De0234	316.2	107.6	46.4	2896	Deu	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a
De0235	100	5.7	31.6	724	Deu	F	Y	Pedigree	Both	132	Y	34	Fcv-f9	2 to 3	N	N	N	N
De0237	100	11.3	31.6	256	Deu	F	N	Dsh	Outdoors	N/a	N	N/a	N/a	11 to 30	Y	N	Y	Y
De0242	100	256	31.6	256	Deu	M	Y	Dsh	Outdoors	N/a	N	N/a	N/a	4 to 10	N	N	N	N
De0321	316.2	90.5	31.6	256	Deu	F	Y	Pedigree	Both	137	Y	379	Fcv-431 + g1	1	N	N	N	N
De0322	46.4	256	316.2	512	Deu	F	N	Dsh	Indoors	2	N	N/a	N/a	2 to 3	N	Y	Y	N
De0426	316.2	90.5	316.2	512	Deu	M	N	Dsh	Both	7	Y	N/a	N/a	2 to 3	N	N	N	N
De0502	100	22.6	46.4	1448	Deu	M	N/a	Pedigree	Indoors	89	Y	1874	Fcv-f9	2 to 3	Y	Y	Y	Y
De0520	100	90.5	100	724	Deu	M	Y	Dsh	Both	24	Y	380	Fcv-431 + g1	2 to 3	Y	N	Y	Y
De0538	316.2	64	46.4	2896	Deu	M	Y	Pedigree	Indoors	20	Y	454	Fcv-f9	1	N	N	N	N
It0107	316	64	316	512	Ita	M	N	Dsh	Both	18	N	N/a	N/a	2 to 3	N	Y	Y	N
It0201	215.4	16	316.2	512	Ita	M	Y	Dsh	Both	75	N	N/a	N/a	1	N	N	N	N
It0208	215.4	11.3	46.4	1448	Ita	M	N	Dsh	Both	48	Y	1043	Fcv-431 + g1	2 to 3	Y	Y	Y	Y
It0341	46.4	128	31.6	512	Ita	M	N	Dsh	Indoors	5	Y	16	Fcv-f9	1	N	Y	Y	Y
It0418	100	512	46.4	1448	Ita	M	Y	Dsh	Outdoors	15	N	N/a	N/a	1	N	N	N	N
It0421	46.4	32	100	724	Ita	F	Y	Dsh	Outdoors	10	Y	108	Fcv-f9	1	N	N	N	N
It0427	316.2	107.6	46.4	4096	Ita	M	N	Dsh	Outdoors	9	N	N/a	N/a	2 to 3	N	N	N	N
It0433	31.6	362	215.4	4096	Ita	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a

Isolate	Back titration	Titre	Homologous f9 back titration	Homologous f9 titre	Country	Gender	Neutered status	Type/breed	Confinement	Age in months	Vaccinated	Time since last vaccine (months)	Vaccine strain	Number of cats in household	Ulcers?	Upper respiratory tract disease?	Acute disease	Chronic gingivostomatitis?
It0539	316.2	128	100	4096	Ita	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a	N/a
Nl0129	100	8	31.6	1448	Nld	M	Y	Dsh	Both	6	Y	89	Fcv-f9	2 to 3	N	N	N	N
Nl0131	100	90.5	31.6	4096	Nld	M	Y	Cross	Indoors	12	Y	253	Fcv-f9	2 to 3	N	N	N	N
Nl0132	31.6	181	100	2896	Nld	F	Y	Dsh	Indoors	6	Y	127	Fcv-f9	1	N	N	N	N
Nl0135	316.2	512	46.4	4096	Nld	F	Y	N/a	Unknown	62	Y	13	Fcv-f9	1	N	N	N	N
Nl0342	31.6	181	100	724	Nld	M	Y	Dsh	Both	162	Y	779	Fcv-431 + g1	2 to 3	N	Y	Y	N
Nl0413	215.4	64	46.4	1448	Nld	F	N	Dsh	Both	7	Y	112	Fcv-f9	4 to 10	N	N	N	N
Nl0415	215.4	128	46.4	1448	Nld	M	N	Dsh	Both	86	Y	23	Fcv-f9	1	N	N	N	N
Nl0425	31.6	90.5	46.4	724	Nld	F	Y	Dlh	Both	122	Y	3002	Fcv-f9	2 to 3	N	Y	Y	N
Nl0426	316.2	362	46.4	4096	Nld	M	Y	Dsh	Both	20	Y	16	Fcv-f9	1	N	N	N	Y
Nl0434	316.2	8	46.4	1448	Nld	F	Y	Dlh	Both	83	Y	240	Fcv-f9	1	N	N	N	Y
Se0114	316	45.3	316	512	Swe	F	Y	Dsh	Indoors	36	Y	N/a	Fcv-f9	11 to 30	N	N	N	N
Se0216	31.6	1024	100	2896	Swe	F	Y	Dsh	Both	6	Y	78	Fcv-f9	2 to 3	N	N	N	N
Se0315	316.2	2896	100	4096	Swe	F	N/a	Dlh	Indoors	N/a	Y	N/a	Fcv-f9	1	N	N/a	N/a	N/a
Se0329	68.1	11.3	31.6	724	Swe	M	Y	Pedigree	Indoors	156	Y	1334	Fcv-f9	1	Y	N	Y	N
Se0404	46.4	16	31.6	512	Swe	F	Y	Pedigree	Indoors	63	Y	304	Fcv-f9	2 to 3	N	N	N	N
Se0436	100	64	215.4	512	Swe	M	Y	Dlh	Outdoors	N/a	N/a	N/a	N/a	2 to 3	N	N	N	N
Gb0110	46.4	168.9	31.6	256	Gbr	F	Y	Dsh	Both	5	Y	62	Fcv-431 + g1	1	N	N	N	N
Gb0111	100	256	31.6	256	Gbr	M	N	Dlh	Both	12	N	N/a	N/a	4 to 10	N	N	N	N

Isolate	Back titration	Titre	Homologous f9 back titration	Homologous f9 titre	Country	Gender	Neutered status	Type/breed	Confinement	Age in months	Vaccinated	Time since last vaccine (months)	Vaccine strain	Number of cats in household	Ulcers?	Upper respiratory tract disease?	Acute disease	Chronic gingivostomatitis?
Gb0116	215	11.3	31.6	512	Gbr	F	Y	Dsh	Both	135	N	N/a	N/a	2 to 3	N	N	N	Y
Gb0203	100	5792	46.4	2896	Gbr	M	Y	Dsh	Both	69	N	N/a	N/a	4 to 10	N	N	N	N
Gb0311	100	64	215.4	4096	Gbr	F	N	Pedigree	Indoors	2	N	N/a	N/a	4 to 10	N	N	N	N
Gb0325	316	22.6	316	512	Gbr	M	Y	Dsh	Outdoors	105	Y	296	Fcv-f9	1	Y	N	Y	N
Gb0331	170.1	512	316.2	512	Gbr	M	N	Dsh	Indoors	3	Y	21	Fcv-f9	1	N	N	N	N
Gb0424	316.2	48	31.6	724	Gbr	M	Y	Dsh	Both	166	Y	2190	N/a	1	Y	N	Y	Y
Gb0528	46	128	316	512	Gbr	F	Y	Dsh	Both	183	N/a	N/a	N/a	2 to 3	N	N	N	N
Gb0805	31.6	22.6	100	724	Gbr	F	Y	Dlh	Outdoors	79	Y	2562	Fcv-255	N/a	N/a	N/a	N/a	N/a
Gb0810	316	256	316	512	Gbr	F	Y	Dsh	Outdoors	12	N	N/a	N/a	N/a	N	N	N	N
Gb0814	46.4	181	46.4	2896	Gbr	M	N	Dsh	Both	180	N	N/a	N/a	2 to 3	N	N	N	N
Gb1504	316.2	2048	215.4	4096	Gbr	F	Y	Dsh	Indoors	N/a	Y	21	Fcv-f9	2 to 3	N	N	N	N
Gb1508	31.6	22.6	31.6	1448	Gbr	M	Y	Dsh	Both	144	N	N/a	N/a	1	Y	N	Y	Y
Gb1521	46.4	32	46.4	1448	Gbr	M	Y	Pedigree	Indoors	8	N/a	N/a	N/a	4 to 10	N	N	N	N
Gb1524	316	<2	31.6	512	Gbr	M	Y	Dlh	Both	28	Y	682	Fcv-f9	4 to 10	N	N	N	N
Gb1612	215.4	4	215.4	362	Gbr	M	Y	Dsh	Both	12	N/a	N/a	N/a	2 to 3	N	N	N	N
Gb1628	170.1	181	100	512	Gbr	F	N/a	Pedigree	Indoors	N/a	N/a	N/a	N/a	N/a	N	N	N	N/a
Gb1630	215.4	181	215.4	4096	Gbr	M	N	Dsh	Outdoors	96	N/a	N/a	N/a	N/a	N	N	N	N/a
Gb1811	100	45.3	100	724	Gbr	M	Y	Dsh	Both	43	Y	13	Fcv-431 + g1	2 to 3	N	N/a	N/a	N/a
Gb1818	31.6	11.3	100	724	Gbr	M	Y	Pedigree	Both	84	N	2510	Fcv-431 + g1	1	N	N	N	N

Isolate	Back titration	Titre	Homologous f9 back titration	Homologous f9 titre	Country	Gender	Neutered status	Type/breed	Confinement	Age in months	Vaccinated	Time since last vaccine (months)	Vaccine strain	Number of cats in household	Ulcers?	Upper respiratory tract disease?	Acute disease	Chronic gingivostomatitis?
Gb1823	31.6	22.6	31.6	724	Gbr	M	Y	Dsh	Indoors	162	Y	8	Fcv-431 + g1	1	N	N	N	N
Gb1925	316.2	11.3	215.4	4096	Gbr	F	Y	Dsh	Both	6	Y	N/a	N/a	2 to 3	N	N	N	N
Gb2128	170	11.3	31.6	724	Gbr	F	N	Dsh	Outdoors	12	N/a	N/a	N/a	N/a	N	N	N	N
Gb2207	316.2	181	215.4	362	Gbr	M	Y	Dsh	Both	132	N	N/a	N/a	2 to 3	N	N	N	N
Gb2210	146.7	256	215.4	512	Gbr	F	N	Dsh	Indoors	32	N	N/a	N/a	1	N	N	N	N
Gb2324	215.4	16	31.6	256	Gbr	F	Y	Dsh	N/a	N/a	Y	58	Fcv-f9	2 to 3	N	N	N	N
Gb2515	46.4	8	100	2896	Gbr	F	Y	Dsh	Both	60	Y	383	Fcv-f9	4 to 10	N	N	N	N
Gb2726	100	53.8	46.4	2896	Gbr	F	Y	Pedigree	Indoors	7	N/a	121	Unknown	2 to 3	N	N/a	N/a	N
Gb2803	316.2	181	31.6	1448	Gbr	F	Y	Dsh	Both	5	N	N/a	N/a	2 to 3	N	N	N	N
Gb2825	170	64	31.6	512	Gbr	M	Y	Dsh	Indoors	7	N	N/a	N/a	2 to 3	N	N	N	N
Gb2907	316.2	724	100	4096	Gbr	F	Y	Dsh	Indoors	N/a	N	N/a	N/a	4 to 10	N	N	N	N
Gb2926	215.4	11.3	316.2	512	Gbr	M	Y	Dsh	Both	84	N	N/a	N/a	4 to 10	N	N	N	N
Gb2927	46.4	5.7	46.4	4096	Gbr	F	Y	Dsh	Both	6	Y	179	Fcv-f9	2 to 3	N	N	N	N
Gb2929	316.2	362	46.4	4096	Gbr	F	Y	Dsh	Outdoors	5	Y	8	Fcv-f9	2 to 3	N	N	N	N
Gb3020	316.2	90.5	46.4	2896	Gbr	F	N	Dsh	Indoors	6	N/a	N/a	N/a	2 to 3	N	N	N	N
Gb3029	46.4	181	100	2896	Gbr	F	Y	Dsh	Both	36	Y	22	Fcv-f9	2 to 3	N	N	N	N
Gb3114	316.2	11.3	215.4	362	Gbr	F	Y	Pedigree	Both	79	Y	N/a	Fcv-f9	4 to 10	Y	N	Y	N
Gb3115	215.4	5.3	100	4096	Gbr	F	N	N/a	Indoors	3	Y	14	Fcv-f9	2 to 3	Y	N	Y	N/a
Gb3117	316.2	1448	100	2896	Gbr	F	Y	Dsh	Both	5	Y	21	Fcv-f9	4 to 10	N	N	N	N

Isolate	Back titration	Titre	Homologous f9 back titration	Homologous f9 titre	Country	Gender	Neutered status	Type/breed	Confinement	Age in months	Vaccinated	Time since last vaccine (months)	Vaccine strain	Number of cats in household	Ulcers?	Upper respiratory tract disease?	Acute disease	Chronic gingivostomatitis?
Gb3123	316.2	362	316.2	512	Gbr	F	Y	Dsh	Both	22	Y	229	Fcv-f9	2 to 3	N	N	N	N
Gb3130	316.2	90.5	100	4096	Gbr	M	Y	Dsh	Both	87	Y	243	Fcv-f9	4 to 10	N	N	N	N
Gb3132	68.1	11.3	31.6	724	Gbr	F	Y	Dlh	Both	15	Y	84	Fcv-f9	2 to 3	N	N	N	N
Gb3804	100	11.3	215.4	362	Gbr	M	Y	Dsh	Both	54	Y	266	Fcv-431 + g1	4 to 10	Y	N	Y	Y
Gb3807	316.2	<2	100	2896	Gbr	M	Y	Dsh	Both	73	N/a	N/a	N/a	1	N	N	N	N
Gb3812	316.2	8	215.4	4096	Gbr	M	Y	Pedigree	Both	16	Y	412	Fcv-431 + g1	4 to 10	N	Y	Y	N
Gb4211	31.6	107.6	31.6	4096	Gbr	F	Y	Dsh	Both	28	Y	N/a	Fcv-f9	2 to 3	N	N	N	N
Gb4216	215.4	5.7	100	512	Gbr	F	N	Dsh	Indoors	3	Y	N/a	Fcv-f9	2 to 3	N	N	N	N

3 A retrospective serosurvey of influenza A and B in dogs and cats in the United Kingdom

3.1 Abstract

There is historic evidence of sporadic infection of dogs and cats with influenza A and B viruses and, more recently, two influenza A subtypes (equine-origin H3N8 and avian-origin H3N2) have become established in dogs in the USA. In order to investigate the potential transmission and circulation of influenza viruses in dogs and cats in the UK, a retrospective serosurvey was carried out using archival serum samples collected from 2012 to 2014.

A total of 200 canine and 179 feline serum samples were screened for influenza A (human seasonal H3N2, human pandemic H1N1, equine H3N8) and influenza B (Yamagata and Victoria lineages), using haemagglutination inhibition (HI). Single radial haemolysis (SRH) was used to confirm any HI positive results.

Of the dogs, 1.5% (3 samples) tested positive for antibodies to an equine H3N8 subtype strain in both HI and SRH tests. One of the cat samples (0.56%) was positive for H1N1 antibodies, representing the first evidence of seroreactivity of cats to the human 2009 H1N1 pandemic virus in the UK.

These data suggest that dogs and cats in the UK sporadically come into contact with influenza A viruses. The risk of infection appears low, but we recommend that veterinary practitioners consider influenza virus as a differential diagnosis in pets with acute clinical signs of respiratory disease, especially if these coincide with influenza-like illness in owners or other in-contact species.

3.2 Introduction

Influenza A viruses are sporadically transmitted from aquatic birds to various mammalian species and, occasionally, certain subtypes (e.g. human H1N1 and H3N2 and equine H3N8) become established in the new host (Lipatov et al., 2004; Short et al., 2015; Webster et al., 1992). Influenza A viruses, including human seasonal H3N2, 2009 pandemic H1N1 and H5N1 strains, have occasionally been reported to infect cats and dogs (Harder and Vahlenkamp, 2010; Horimoto et al., 2015; Löhr et al., 2010; Short et al., 2015; Songserm et al., 2006; Yin et al., 2014). However, influenza A viruses were not reported to circulate endemically in dogs or cats until an H3N8 subtype virus of equine origin was isolated from racing greyhounds with respiratory disease in the USA in 2004 (Crawford et al., 2005). This virus became established in shelter and pet dog populations in the USA. In the UK, transmission of equine H3N8 influenza to foxhounds has been described (Daly et al., 2008), which is believed to occur sporadically (Newton et al., 2007). A similar occurrence took place in dogs in close proximity with horses during an outbreak of equine influenza in Australia in 2007 (Kirkland et al., 2010).

In Asia, an avian-derived H3N2 influenza virus has also been a source of acute respiratory disease in dogs (Li et al., 2010) and since 2015, this virus has been circulating in dogs in the USA (Abente et al., 2016; Jang et al., 2017; Voorhees et al., 2017). There are also reports of transmission of the avian-origin canine H3N2 influenza virus to cats in Asia (Jeoung et al., 2013; Song et al., 2011).

Evidence of influenza B virus infection in animal species other than humans and seals is very limited (Osterhaus et al., 2000). However, it has been suggested that dogs may be susceptible to these viruses (Todd and Cohen, 1968).

The aim of this study was to determine whether there is serological evidence of exposure of dogs and cats to influenza A or B viruses in the UK.

3.3 Materials and methods

Ethics

Ethical approval for the use of archived serum samples was obtained from the Veterinary Research Ethics Committee, University of Liverpool.

Viruses

Viruses used in this study were the human pandemic influenza A H1N1 strain A/California/7/2009 (A/H1N1), human seasonal influenza A H3N2 strain A/Texas/50/2012 (A/H3N2), A/equine/Newmarket/5/2003 (A/eq/H3N8), and human influenza B strains from the Yamagata lineage, B/Massachusetts/1/2012 (B/Mas), and the Victoria lineage, B/Brisbane/60/2008 (B/Bris). All human influenza viruses were obtained from the National Institute for Biological Standards and Control (NIBSC). Equine influenza virus was kindly provided by Dr. Debra Elton (Animal Health Trust). Virus stocks were cultivated in embryonated hens' eggs and stored at -80ºC.

Serum samples

Archived serum samples were available from 200 dogs and 179 cats, and represented leftover diagnostic samples collected from patients attending the University of Liverpool's Small Animal Teaching Hospital for various reasons between 2012 and 2014. All serum samples had been stored at -20ºC. The relevant positive control serum samples for the human influenza virus isolates were obtained from NIBSC. The equine influenza virus control serum was collected from a hyper-immune experimental pony that had been subjected to multiple viral exposures.

Serological testing

Haemagglutination inhibition (HI) testing was performed using an adapted protocol from the World Health Organization (World Health Organization, 2002). Briefly, test and positive and negative control serum samples were heat-inactivated at 56ºC for 30 minutes and pre-treated with receptor destroying enzyme (RDE, Sigma-Aldrich) resulting in a final dilution of 1:10. Serial two-fold dilutions of each sample were incubated for 30 minutes at room temperature (RT) with four haemagglutination

(HA) units of test virus. Subsequently, an equal volume of 0.5% chicken red blood cells (RBCs, TCS Biosciences Ltd.) in PBS was added, and HI titres determined as the highest serum dilution giving at least 50% inhibition of agglutination after incubation for 30 minutes at RT.

Single radial haemolysis (SRH) testing as described in the OIE Terrestrial Manual (OIE, 2015) was used as a confirmatory test in all samples that exhibited HI titres ≥1:10. Briefly, the different viral strains were coupled with 8% sheep RBCs with chromium chloride. Immunoplates containing 1% agarose were prepared using the sensitised sheep RBCs and guinea pig complement. Then, 10 µl test and control sera treated as previously described were pipetted into pre-punched 3 mm wells on the immunoplates and incubated at 34ºC in a humidified box for 20 hours. Haemolytic zones were measured with digital callipers and areas of haemolysis were calculated.

Confidence intervals for the proportion of positive samples (expressed as a percentage) were calculated using the Wilson method.

3.4 Results

Of 200 canine serum samples tested by HI, seven had HI titres ≥1:10 against A/H1N1 (Figure 3.1) and four samples tested positive for antibodies against A/eq/H3N8. Twenty-two samples tested positive for antibodies against both B/Mas and B/Bris, two of which were also positive for A/H1N1 as above. A further five samples tested positive for B/Mas alone and five more for B/Bris alone. No samples tested positive for A/H3N2. Single radial haemolysis was performed with all 41 samples exhibiting HI titres ≥1:10. Anti-influenza A/eq/H3N8 antibodies were confirmed in three samples (1.5%, 95% CI 0.5%, 4.3%); all remaining samples were negative by SRH to both influenza B strains and A/H1N1 (Table 3.1).

Of 179 feline serum samples tested by HI, three tested positive for antibodies against A/H1N1 with titres of 1:40 (n=2) to 1:320 (n=1). No samples tested positive for A/H3N2, B/Mas, B/Bris or A/eq/H3N8. The feline sample with an HI titre of 1:320 was confirmed positive by SRH testing (0.56%, 95% CI 0.1%, 3.1%; Table 3.1).

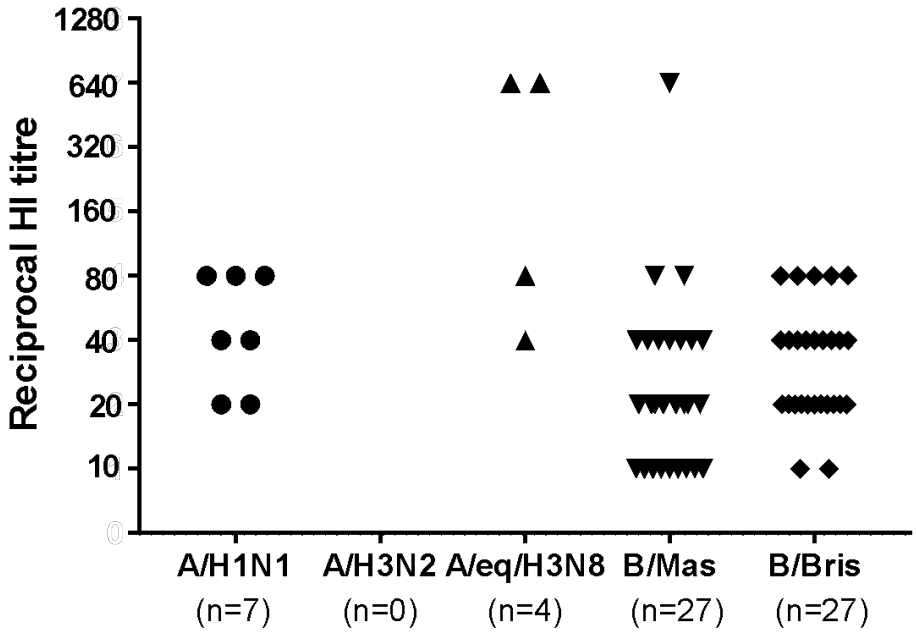

Figure 3.1 Numbers of canine serum samples (indicated in brackets) for which titres ≥1:10 were obtained in haemagglutination inhibition tests with influenza A/H1N1 = A/California/7/2009 (H1N1), A/H3N2 = A/Texas/50/2012 (H3N2), A/eq/H3N8 = A/equine/Newmarket/5/2003 (H3N8), B/Mas = B/Massachusetts/1/2012, and B/Bris = B/Brisbane/60/2008.

Table 3.1 Samples testing positive by both haemagglutination inhibition and single radial haemolysis.

	A/equine/Newmarket/5/03 (H3N8)		*A/California/7/2009 (H1N1)*	
Sample ID	**HI titre**	**SRH area (mm^2)**	**HI titre**	**SRH area (mm^2)**
Canine 1	1:640	62.3	-	-
Canine 2	1:640	82.8	-	-
Canine 3	1:40	60.1	-	-
Feline 1	-	-	1:320	56.5

3.5 Discussion and conclusions

The results of this study suggest a very low canine and feline level of seroreactivity of to influenza A viruses in the UK.

Initial screening of the serum samples by HI suggested that 41 canine and three feline sera were influenza antibody positive. However, the high number of samples that tested positive for both influenza B strains, with two of these samples also testing positive for A/H1N1 in HI, suggested non-specific inhibition of agglutination. This was confirmed by SRH testing, with only the feline sample with an HI titre of 1:320 against A/H1N1 and three canine samples with HI titres of 1:40 or 1:640 against A/eq/H3N8 also testing positive against the respective viruses.

The HI assays were performed using 0.5% chicken red blood cells and the sera heat-inactivated and treated with RDE to remove non-specific inhibitors of agglutination. The HI assay was shown to have high sensitivity (99.6%) and specificity (94.6%) for diagnosis of canine H3N8 influenza with a positive titre cut-off of 1:32 providing serum was pre-treated with RDE or periodate and 0.5% turkey or chicken RBCs and an antigenically-matched H3N8 virus strain was used (Anderson et al., 2012).

The results of the current study suggested that a positive titre cut-off of 1:40 was appropriate for the feline sera with all of the viruses tested, but that titres of up to 1:80 should be treated with caution for influenza A viruses with canine sera and all samples testing positive with influenza B viruses required confirmatory testing. An earlier study (Todd and Cohen, 1968) suggested that dogs could be experimentally infected with an influenza B virus. However, a more recent study showed that although dogs were susceptible to experimental infection with human influenza A 2009 pandemic H1N1 and H3N2 viruses, they were not infected with the same Victoria lineage influenza B strain (B/Brisbane/60/2008) used in this study (Song et al., 2015). Furthermore, there is only limited serological evidence of seroreactivity of dogs or cats to influenza B viruses with no reports of influenza B virus isolated from a naturally infected animal.

The low A/eq/H3N8 seroprevalence in canine samples obtained in this study contrasts with the generally higher seroprevalence reported in the US during a similar period that the samples in this study were taken (Anderson et al., 2013) but is in line with studies conducted in several other European countries. In an extensive study of canine infectious respiratory disease in Europe, Mitchell et al. (2017) found that 2.7% of dogs (6/220) were seropositive using two ELISA methods including one using A/equine/Miami/63 (H3N8) as antigen. In a serosurvey conducted in Italy, 0.03% (2 of 6,858) of samples of canine sera tested positive for H3N8 antibodies (De Benedictis et al., 2010). Interestingly, one of these two dogs had lived in Florida for three years and another was raised in close contact with horses, each of which are risk factors for exposure to H3N8 virus (De Benedictis et al., 2010). More recently, 0.36% (2 of 562) of canine serum samples from southern Italy tested positive for virus antibodies (Pratelli and Colao, 2014). The clinical records for the three dogs testing positive for antibodies against A/eq/H3N8 in this study did not show any indication of travel, vaccination against canine influenza virus, contact with horses or past acute infectious respiratory disease (data not shown). However, none of these factors can be excluded given the retrospective nature of the study and potential for such information not to be recorded in their clinical records.

In the present study, no samples of canine sera tested positive for human A/H1N1 or A/H3N2 viruses although other recent studies have demonstrated low seroprevalence levels in dogs in China, Germany and the USA (Damiani et al., 2012; Jang et al., 2017; Sun et al., 2014). However, the isolation of an H3N1 virus from a dog in South Korea that was evidently a reassortant with seven gene segments from human A/H1N1 and the haemagglutinin gene from canine A/H3N2 confirms that dogs can be infected with the human A/H1N1 virus (Song et al., 2012).

The finding of a very low seroprevalence of A/H1N1 in cats is in contrast to studies conducted with samples collected in the US during and shortly after the 2009 pandemic. In feline serum samples collected between 2009 and 2010, seropositivity of 22.5% for A/H1N1 was observed (Ali et al., 2011) and, in another study of samples dated from 2011 to 2012, seroprevalence was 33.6% (Ibrahim et al., 2016). However,

in these studies, heat-inactivation was the only method used to remove non-specific inhibitors after previously finding that treatment with RDE appeared to make no difference, which could potentially have had an impact on the results. The results of this present study are more in line with the low seroprevalence of 1.93% (8/414) found in Germany by testing feline serum samples collected from 2010 to 2011 by ELISA and confirming positive results using a virus neutralisation test (Damiani et al., 2012). Such sporadic infections of cats with H1N1 are consistent with occasional pathological demonstration of infection in naturally infected cats (Ozawa and Kawaoka, 2013). No antibodies to A/eq/H3N8, A/H3N2 or influenza B were detected in the feline sera in this study, although human seasonal H3N2 antibodies were detected in cats in a recent US study (Ibrahim et al., 2016).

The results from this study and the apparently increasing incidence of influenza A viruses in dogs in particular in some parts of the world support the need for continued surveillance of influenza in pet populations. In the future, the avian-origin H3N2 strain of canine influenza should be included in the panel of viruses against which canine (and to a lesser extent feline) sera are screened. It is important to take into account that retrospective studies such as the present one using convenience samples from a population of pets that were sampled as part of a diagnostic workup may have an inherent bias. Additionally, the population sampled here is a referral one attending a busy specialist veterinary hospital and possibly geographically limited to the surrounding areas of North West England and North Wales. The sampling bias present in this study design is quite clear due to the convenience nature of used samples and, therefore, a prospective study design would potentially hold different results. Signalment data as well as history was also not available prior to sample testing and therefore, conclusions are limited and cannot be generalised. Therefore, it would be useful to further evaluate this in the form of prospective studies alongside careful data collection. Although current evidence suggests cats and dogs are rarely affected by human influenza strain, it would be prudent not to exclude these species from the surveillance of these viruses (Ozawa and Kawaoka, 2013). Finally, veterinary practitioners should consider influenza as a potential

differential in animals showing acute signs of respiratory disease, particularly if these coincide with influenza-like illness in owners / in-contact animals

4 Pathogens associated with signs of respiratory disease in cats attending veterinary practices in the UK: a case-control study

4.1 Abstract

Feline infectious respiratory disease (FIRD) is a common syndrome in cats attending veterinary practices worldwide with many recognised potential viral and bacterial causes. This case-control study aimed to reappraise the role of feline calicivirus (FCV), feline herpesvires (FeHV-1), influenza A, *Mycoplasma felis (M.felis), Chlamydophila felis (C.felis), Bordetella bronchiseptica (B.bronchiseptica)* in FIRD-like disease as well as identify potential cat-level risk factors for infection in animals attending UK veterinary practices.

Veterinary practices (12) were recruited into the study and samples from a total of 38 cases and 64 controls were collected. Presence of pathogens was determined using bacterial culture and polymerase chain reaction (PCR). Telephone questionnaires were administered to participating pet owners to collect data relating to signalment, lifestyle and characteristics of respiratory disease and risk factors for case status were assessed using multivariable logistic regression.

FCV was detected in 10.8% of study samples (21.1% in cases and 4.7% in controls) and FeHV-1 was detected in 4.9% of samples (10.5% in cases and 1.6% in controls). In regards to bacterial pathogens, 7.8% of samples tested positive for *B. bronchiseptica* (5.3% in cases and 9.4% in controls) and 30.6% tested positive for *M.felis* (65.8% in cases and 48.4% in controls). No samples tested positive for influenza A or *C. felis*. Previous history of respiratory disease was found to be positively associated with case status.

Although recruitment rates were disappointing, this study reaffirms the importance of FCV as one of the major pathogens of FIRD in veterinary practice attending cats. Over a quarter of cases tested negative for all tested pathogens, suggesting other yet-to-be identified pathogens remain to be discovered.

4.3 Introduction

Infectious respiratory disease remains a common and important reason for clinical presentation in cats attending veterinary practices around the world despite widespread vaccination being common place for over three decades (Binns et al., 2000). While FIRD is a bigger issue in animal shelters, cats living in outdoor colonies and multi-animal households, it remains as an important reason for veterinary consultation. In a report by SAVSNET (Small Animal Veterinary Surveillance Network), 2.3 % of cats attending veterinary practices in the UK between January 2014 to December 2015 were presented for respiratory disease (Sánchez-Vizcaíno et al., 2016). More recently, SAVSNET reported that presentation due to respiratory disease had decreased to 1.3% of the feline veterinary practice attending population which still comprises an important number of animals (Arsevska et al., 2018).

Clinical signs associated with FIRD include sneezing, coughing, nasal and ocular discharges, conjunctivitis, keratitis, lethargy, pyrexia and difficulty breathing (Binns et al., 2000; Gerriets et al., 2012). In most cases, a presumptive diagnosis is made based on clinical examination and history alone, without resorting to further testing (Afonso et al., 2017b). This leads to a still poor understanding of the true prevalence of different pathogens and associated risk factors for infection especially in privately owned pet cat populations, since most studies have focused in high throughput, multi-cat environments.

Previous studies indicate that FIRD causes are commonly of viral nature with FCV and FeHV-1 being the two most commonly isolated pathogens (Afonso et al., 2017a; Binns et al., 2000; Litster et al., 2015; Wardley et al., 1974). FCV is typically associated with oral ulceration and relatively mild ocular and nasal discharges, although strain variants may also be associated with inapparent infection, lameness and even severe, life threatening systemic disease. FeHV-1 generally causes a more severe but consistent disease with purulent ocular and nasal discharges; some cats can also develop ocular complications including ulcerative keratitis (Gaskell et al., 2007; Thiry et al., 2009). Both pathogens can be found in healthy and diseased animals as they are capable of inducing carrier states (Afonso et al., 2017b; Radford et al., 2009; Thiry

et al., 2009; Wardley et al., 1974). Subclinically infected animals therefore present an extra challenge in the etiological diagnosis of FIRD since pathogen identification cannot be considered synonymous with clinical disease (Veir and Lappin, 2010). Bacterial pathogens such as *B. bronchiseptica, C. felis* and *M. felis* are also part of the infectious respiratory disease complex in cats (Afonso et al., 2017b; Dawson et al., 2000; Gruffydd-Jones et al., 2009; Le Boedec, 2017). Despite the importance of FIRD in clinical practice, there has been no recent systematic investigation of its aetiology and risk factors for its development.

The aim of this chapter was to use recent assays to reappraise FIRD. In particular, we performed a case-control study in cats attending veterinary practices in the UK to identify significant cat-level risk factors associated with the presence of FIRD, as well as the role of infection with known and/or potential respiratory pathogens (such as FCV, FeHV-1, *B.bronchiseptica*, influenza A viruses, *M felis* and *C felis*).

4.4 Materials and methods

Ethics and informed consent

Ethical approval was obtained from the Veterinary Research Ethics Committee, University of Liverpool (VREC233). Informed consent was obtained from participating cat owners (Appendix 4A).

Study design

In order to identify and quantify risk factors for cats with clinically suspected FIRD, a case-control study design was used. As one of the main causes of FIRD is FCV which has an expected prevalence of 10% (Chapter 2) and the frequency of exposure to other pathogens and risk factors was uncertain the sample size estimate was based on an exposure of 10% (Epi Info 7). For a study with 2 controls per case , to detect odds ratio of 3 or greater, with a confidence level of 95% and a power of 80%, the required sample size was 71 cases and 142 matched controls.

Recruitment

Samples were collected from July 2014 to December 2016 from cats attending UK veterinary practices. Practices were recruited based on convenience using a combination of phone calling and sharing on social media platforms (blogs, Twitter and Facebook; Appendix 4A). A total of 165 British veterinary practices were contacted to participate in this study. Several had participated in a previous study conducted as part of this PhD project while others were approached by using the RCVS veterinary practice register (Chapter 2). A total of 36 veterinary practices spread throughout the United Kingdom agreed to participate in the study. The period of participation of each practice ranged from approximately 3 months to 10 months, depending on time of recruitment. No incentives were offered to participating practices.

Case and control definitions

A case was defined as any cat presenting at a veterinary surgery which, to the best of the clinical judgement of the attending veterinary surgeon, was suffering from

infectious respiratory disease at the time of consultation and presented with two or more of the following clinical signs: oral ulcers, ocular discharge, facial ulceration, cough, sneezing, altered lung sounds, altered respiratory patterns, pyrexia. Animals were to be excluded if they suffered from any of the previously mentioned clinical signs due to a cause different than respiratory disease (for example: cough in animals with cardiac disease, epistaxis in animals with hypertension, sneezing in animals with nasal foreign bodies, etc).

Control cats were defined as the next two cats to be presented to each veterinary surgeon following a case, with the exclusion of any cats which suffered from respiratory disease within the previous fortnight or were showing any of the previously mentioned clinical signs of disease.

Sample collection

Veterinary staff at participating practices were asked to recruit up to 10 cases and 20 controls per practice (a mixture of cats and dogs). All practices had confirmed, upon recruitment, that they believed their caseload would allow for the sampling of these animal numbers in a period of 3 to 6 months. A pack containing 32 dry cotton swabs, 32 collection tubes containing viral transport media (VTM), 32 charcoal Amies swabs, 32 owner consent forms (Appendix 4B), a laminated owner information sheet (Appendix 4B), a sampling protocol (Appendix 4C), 10 plastic containers (with space for 3 samples in VTM; 1 case and 2 matching controls) and 10 pre-paid envelopes for returning samples to the University of Liverpool team at the Leahurst Campus was sent to practices. Practice staff were informed that VTM vials should be frozen (-20ºC) on arrival and again after sample collection until posting the samples back to the author. Veterinary staff were asked to simultaneously collect two oropharyngeal swabs (dry cotton swab placed into VTM and charcoal Amies swab) as explained in the sampling protocol (Appendix 4C).

On arrival at the University of Liverpool, samples were recorded and assigned an alphanumeric code to allow for all testing to be performed blinded to control or case categorization. Samples in VTM were aliquoted into two tubes labelled OPA and OPB

and frozen at -80ºC until testing. Charcoal Amies swabs were either immediately tested or refrigerated at 4ºC until test performance.

Staff at each of the veterinary practices explained the study to the owners of recruited cats using the previously mentioned owner information sheet. Owner contact details, preferred contact times and contact details (Appendix 4D) were collected alongside consent to participate.

Questionnaire design

The questionnaire for cases and control contained a core of identical questions, with additional details being collected for cases including questions regarding the nature of the respiratory signs (Appendix 4D). Data collected included signalment, vaccination and worming status, contact with other animals (including other cats, dogs and birds), exposure to potential risk factors, previous respiratory disease, current or past illnesses and medication. All data was owner reported.

Once the samples were returned to the author alongside owner contact details, telephone contact was attempted in order to administer the questionnaire. Participating owners were telephoned as soon as possible after receiving the samples and contact was attempted at least three times per participant. Questionnaires were administered either by the author or by Dr. Shirley Bonner, after briefing by the author. No incentives were offered to participating veterinary practices or owners.

Viral isolation

FCV and FeHV-1 were isolated using standard techniques as described in Chapter 2, using confluent monolayers of feline embryo A (FEA) cells and by observing typical CPE (Povey and Johnson, 1971). Samples were reported negative after two negative passages and positive isolates were stored at -80ºC for further analyses (Knowles et al., 1991).

Nucleic acid extraction

Viral nucleic acid (RNA and DNA) was extracted from oropharyngeal swabs or tissue

culture fluid using the same method as described in Chapter 2 (Viral RNA mini-kit; Qiagen).

Reverse transcription and PCR

Reverse transcription was performed using 200 ng random hexamers (Superscript III, Life Technologies). Table 4.1 shows details of diagnostic tests using PCR for detection of nucleic acids from oropharyngeal swabs. Conventional PCRs were performed for FCV, Influenza A (using cDNA obtained from reverse transcription), *M felis*, *C felis* and FeHV-1. Real-time PCRs were performed for *B. bronchiseptica* (in addition to bacterial culture). Positive controls for each pathogen were included in each PCR run.

Where viral isolation for FCV or FeHV-1 was positive but PCR from OP swab was negative, PCR was repeated using nucleic acid extracted from the second sample passage in cell culture (p2).

Table 4.1 Details of PCR diagnostics used for detection of pathogens. AT stands for annealing temperature.

Pathogen	*Test*	*Gene*	*Primer name*	*Primer*	*Methods*	*AT ºC*	*Cycles*	*Sample type*	*Product size (bp)*
FCV (polymerase)	RT-PCR	Polymerase	M13-53D T7-33D	CAGGAAACAGCTATGACGAYATGATGACYTAYGGKGAYGAYGG; TAATACGACTCTCAATAGGGCCGCGCYTCCACRCCRTTRAAYTG	See Chapter 2	55	45	OP swab or p2	505
FCV (capsid)	RT-PCR	Capsid	M13cap2F T7cap2R	CAGGAAACAGCTATGACCCCTTTGTCTTCCARGCHAAYCG; TAATACGACTCTCAATAGGGCCTCACCAATTCCNGTRTANCC	See Chapter 2	45	45	OP swab or p2	528
FeHV-1	PCR	gE gene	FHV1-1f FHV1-1r	GGTCATGTGTAATGTTGACG; GTCTTTGGTTCTGATGAGAG	(Townsend et al., 2013)	50	40	OP swab or p2	478
Influenza A	RT-PCR	Matrix (M)	M30F2/08 M264R3/08	ATGAGYCTTYTAACCGAGGTCGAAACG TGGACAAANCGTCTACGCTGCAG	World Health Organisation	50	40	OP swab	244
B. bronchiseptica	Real Time-PCR	FimA	BbronchisepticaF BbronchisepticaR Bbronchiseptica probe	ACTATACGTCGGGAAATCTGTTTG; CGTTGTCGCCTTTCCTCTG; FAMCGGGCCGATAGTCAGGGCGTAGBHQ1	(Helps et al., 2005)	60	45	OP swab	80
M.felis	PCR	16S/23S rRNA intergenic spacer	Myc1 m.felis	CACCGCCCGTCACACCA; GGACTATTATCAAAAGCACATAAC	(Chalker et al., 2004)	52	40	OP swab	238
C.felis	PCR	IGS of rRNA operon	Cfeli-F Chuni-4R	CGTTGTTAAGCGTGGGTT; AGACTAGGTTTCACGTGTCTAG	(Nordentoft et al., 2011)	60	40	OP swab	583

Isolation and identification of *Bordetella bronchiseptica*

Charcoal swabs were streaked onto charcoal selective media. Any small grey/white colonies growing within 24-48 hours were sub-cultured onto blood agar. Suspected colonies were gram stained and underwent an oxidase test (ProLab). Gram negative, oxidase positive colonies were subjected to the API 20 NE biochemical test to identify *B. bronchiseptica.*

Epidemiological analysis

All sample, diagnostic results and questionnaire data were entered onto a spreadsheet programme (Microsoft Excel for Mac). Visual verification of correct data input was verified for 5% of entries.

The outcome variable used was case or control status and the associations with predictor variable were assessed using univariable logistic regression using R Studio (version R 3.4.1 GUI 1.70 El Capitan build).

Sixteen predictor variables were assessed including gender (female or male), neutered status (entire or neutered), breed (re-classified from questionnaire responses as pedigree or crossbreed), number of cats in household (classified into three groups: 1 cat/household, 2 to 3 cats/household, 4 or more cats/household), contact with other animals (yes or no), lifestyle (classified as living indoors, outdoors or both), recent stay/visit to multi-animal household (eg.: shelter, cattery, veterinary surgery, etc. Re-classified from questionnaire data to yes or no), where cat goes to the toilet (uses litter tray, goes outdoors or both), vaccination history (classified into three groups: vaccinated within last year, over one year ago, never), vaccine antigen used (FCV-F9; FCV-431 + FCV-G1), worming and flea and tick control status (both classified into: within last three months, more than three months ago or never), reason for veterinary visit (classified into routine appointment, ill health – respiratory or ill health – not respiratory), previous history of respiratory disease (yes or no) and history of concurrent medical conditions (yes or no).

To build a multivariable analysis model, univariable analysis results were taken into account in combination with a causal web built specificically for FIRD (figure 4.1). Number of cats in household, vaccine status, previous respiratory disease and age were taken into account as variables for testing. All these, with the exception of age, had p-values < 0.1 on univariable analysis. Age has been previously found as a risk factor for respiratory disease in cats (chapter 2) and therefore was also considered. Roughly, one explanatory variable per 10 cases was considered. Variables were then eliminated from testing until only those with likelihood ratio p-value < 0.05 remained in the final model. This model was assessed for two-way interactions.

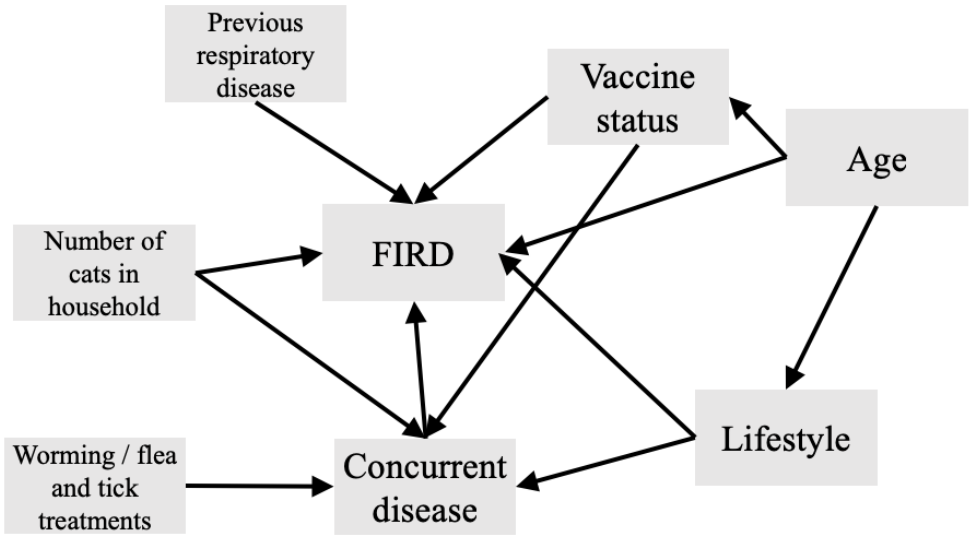

Figure 4.1 Causal web showing relationships between several independent variables (information collected through questionnaires) and dependent variable (i.e. case status, in this context, cases of feline infectious respiratory disease)

4.5 Results

Out of the 36 practices recruited into the study, only 12 submitted samples from feline patients. Figure 4.2 shows the approximate geographical location of these practices.

Figure 4.2 Map of the United Kingdom showing the approximate location of practices that returned samples to the study

The number of samples submitted by each practice was variable, ranging from 1 to 15 (median 7.5). A total of 38 cats meeting the case definition and 64 controls presenting to participating veterinary practices were recruited into the study. Despite the long period allowed for sample collection, the low number of samples, and the failure to collect matched case control sets in some practices meant that a matched analysis using conditional logistic regression was not possible Therefore,

further analysis was done using an unmatched analysis. Table 4.2 summarises study population signalment and pathogen detection rates.

Table 4.2 Study population signalment and pathogen detection rates. Age: kitten (1 – 12 months), young adult (13 – 60 months), adult (61 – 96 months), senior (over 96 months).

Variable		*n (%)*	*FCV*	*FeHV*	*Influenza A*	*B.b*	*M. felis*	*C.felis*
			n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Case		38 (37.2)	8 (21.1)	4 (10.5)	0 (0)	2 (5.3)	25 (65.8)	0 (0)
Control		64 (62.7)	3 (4.7)	1 (1.6)	0 (0)	6 (9.4)	31 (48.4)	0 (0)
Gender	Female	30 (46.2)		I				
	Male	35 (53.8)						
Neutered	Yes	57 (89)						
	No	7 (10.9)						
Breed	Pedigree	6 (9.4)						
	Crossbreed	58 (90.6)						
Age.	Kitten	17 (29.8)						
	Y.adult	15 (26.3)						
	Adult	6 (10.5)						
	Senior	19 (33.3)						

Telephone contact of owners for questionnaire administration was always attempted within the first week post sample reception (and up to three months after in cases where there was no answer) during a time selected by the participating owners when study participation was agreed to (morning, afternoon or evening). Despite each pet owner being contacted at least three times via phone, out of the total number of animals recruited into the study (n=102), questionnaire data were obtained for only 66.3% (n=65, 26 cases and 39 controls) of participating cats.

The association between reason for presentation to the veterinary practice with the outcome (case or control) were assessed in univariable models only as this is a consequence (rather than the cause of) FIRD.

Out of the 26 cases for which questionnaire data was obtained, 22 (57.9%) owners reported their cat had been sneezing, followed by 16 (42.1%) owners reported coughing and 13 (34.2%) reported nasal discharge in their animals. Ocular discharge

and breathing difficulties were each reported five times (13.2%), nose bleed was reported three times (7.9%) and mouth ulcers, conjunctivitis ("red eye") and pyrexia were each reported once (2.6%). All except one owner, reported two or more concomitant clinical signs. Two owners also added clinical signs not included in the questionnaire and reported their animal as having "difficulty swallowing" in one instance and a "crusty nose" in the second case.

Within the case group, 21 of the animals were brought into the veterinary practice for consultation due to respiratory illness. Of the remaining cases, four animals were presented for routine appointments and one animal had suffered from fighting wounds. These five animals were all deemed to be suffering from respiratory disease by the attending veterinary surgeon and also had signs of respiratory disease included in the case definition.

Regarding onset of clinical disease, 12 (46.2%) owners reported respiratory signs had been ongoing for 15 or more days, three (11.5%) cases between 8 - 14 days, four (15.4%) cases between 3 - 7 days, and four (15.4%) owners reported signs had started in the previous 48 hours. Three (11.5%) owners were unsure when clinical signs had first manifested.

Owners of cats belonging to the cases group were asked about any potential reasons why they thought their animal may be showing signs of respiratory nature. Some of the reasons mentioned were "a rough life" (mentioned once), contact with other infected cats (eg.: feral or in multi-cat households/breeder; mentioned twelve times), allergies (e.g.: to air freshener; mentioned three times) and a reaction to vaccination (mentioned once).

Univariable analysis of signalment and clinical data collected from questionnaires are shown in Appendix 4E and are briefly summarised here. No significant associations were shown between case status and gender, neutered status, breed, contact with other animals, stay in multi-animal environments or vaccination status.

Cases were 3.3 times more likely than controls to come from households with four or more animals (p-value 0.1). Compared to cats living indoors only, cases were less likely (OR = 0.4, p-value = 0.08) to have a mixed indoor and outdoor lifestyle and more likely to be fed a mixture of commercial and homemade food, than commercial food alone (OR = 2.4, p-value = 0.15). Additionally, cases were 4.2 times more likely to have been vaccinated over one year ago animals vaccinated over one year ago were 4.2 more likely to be cases (p-value = 0.1) and cats that were never wormed (OR = 9.3, p-value = 0.05) or treated for fleas and ticks (OR = 5.2, p-value = 0.16) were also more likely to be cases. Cases were more likely to have had a history of previous respiratory disease (OR = 5, p-value = 0.02) and were less likely to have concurrent disease (OR = 0.4, p-value = 0.16). Finally, animals undergoing treatment with antibiotics were more likely to be cases of FIRD (OR = 3.10, p-value = 0.1).

Variables with P<0.25 were carried forward to the multivariable model and included number of cats per household, lifestyle, food, vaccination, worming and flea/tick treatment statuses, concurrent medical conditions.

The final multivariable model is shown in Table 4.3. Animals with no history of previous respiratory disease were found to be less likely to belong in the cases group (OR = 0.19, p-value = 0.014). Additionally, animals not undergoing courses of antibiotic treatment were found to be less likely to be cases (OR=0.21, p-value = 0.05).

Table 4.3 Multivariable logistic regression of factors associated with respiratory cases in cats attending veterinary practices in the UK (n=65 questionnaires, 26 cases, 39 controls). P-values below 0.05 considered significant.

Variable	*Coefficient (Std. Error)*	*Odds Ratio*	*Lower 95%CI*	*Upper 95%CI*	*Wald P-value*
Previous respiratory disease					
Yes		Ref.			
No	-2.3 (0.8)	0.098	0.02	0.46	0.006
Age					
0 – 12m (kitten)	)	Ref.			
13-60m (young adult)	-2.6 (0.9)	0.07	0.009	0.4	0.005
61-96m (adult)	--3.3 (1.4)	0.04	0.001	0.4	0.02
>96m (senior)	-1.5(0.8)	0.23	0.05	1.2	0.06

The results of laboratory testing are shown in Table 4.3. Eleven samples (10.8%) tested positive for FCV, five (4.9%) tested positive for FeHV-1, eight (7.8%) tested positive for *B. bronchiseptica* by PCR and 56 (30.6%) tested positive for *M felis*. No samples tested positive for either Influenza A or *C felis.* All the samples positive for *Bb* by PCR were negative by bacterial culture. Mixed infections were present with FCV and *M. felis* being the most common (six occurrences, only cases), followed by FeHV-1 with *M. felis* (three cases) and *M. felis* with *B.bronchiseptica* (two controls). A significant proportion of cases tested negative for all tested pathogens (26.3%; n=10). Table 4.4 shows the results of univariable logistic regression of PCR testing results. FCV was the only pathogen significantly associated with case status (p-value < 0.05) with cats with FIRD being 5.4 times more likely to test positive for FCV. Cases were also more likely to be FeHV and *M. felis* positive but these associations were not significant.

Table 4.4 Univariable logistic regression results of PCR testing for pathogens associated with respiratory cases (n = 102 samples, 38 cases and 64 controls from 12 veterinary practices). P-values below 0.05 considered significant.

Pathogen	*Case*		*Control*		*Odds Ratio*	*Wald P-value*	*Lower 95%CI*	*Upper 95%CI*
	n	%	n	%				
FCV								
Positive	8	21.1%	3	4.7%	5.4	0.02	1.5	26.13
Negative	30	78.9%	61	95.3%	Ref.			
FeHV								
Positive	4	10.5%	1	1.6%	7.4	0.08	1.04	148.1
Negative	34	89.5%	63	98.5%	Ref.			
B. bronchiseptica								
Positive	2	5.3%	6	9.4%	0.54	0.5	0.08	2.48
Negative	36	94.7%	58	90.6%	Ref.			
M.felis								
Positive	25	65.8%	31	48.4%	2.05	0.09	0.9	4.8
Negative	13	34.2%	33	51.6%	Ref.			
Influenza								
Positive	0	0%	0	0%				
Negative	38	100%	64	100%				
C.felis								
Positive	0	0%	0	0%				
Negative	38	100%	64	100%				

4.6 Discussion and conclusions

Feline infectious respiratory disease is recognised as an important syndrome in clinical practice with many recognised potential viral and bacterial causes. Vaccination against FeHV-1 and FCV, which is widespread in practice, is generally considered to reduce clinical signs, but may not entirely prevent them, nor does it prevent infection. Vaccination for *B.bronchiseptica* and *C. felis* is available but not commonly practiced in cats (Sanchez-Vizcaíno et al., 2018). Here we used a case control study, to reappraise the role of cat-level risk factors and infections in FIRD.

In previous studies, severity scoring was used to assign animals into disease groups ranging from mild to moderate or severe (Bannasch and Foley, 2005). It was an overlook in our part not to include severity scoring as part of case definitions distributed to participating vets and should a similar study be performed in the future, we would consider doing this as it allows for more detailed risk factor analysis (especially where it relates to pathogens isolated and their potential to cause serious disease). However, given the small number of cases submitted to the study the simpler analysis of animals suffering with respiratory disease of suspected infectious origin of any severity (cases) versus controls seems more appropriate.

No previous history of respiratory disease was found to be correlated with control status. This is consistent with the fact that the two most common viruses involved in FIRD are both capable of inducing a life-long carrier status. FeHV-1, can recrudesce in periods of stress causing new episodes of clinical disease thereby making animals previously affected by FIRD more likely to suffer from the syndrome again in the future (Gaskell et al., 2007; Helps et al., 2005; Thiry et al., 2009). Previous respiratory infections with viruses such as FeHV-1, that in their acute phase can damage turbinates and reduce localised respiratory innate immunity, can also lead to chronic lesions therefore predisposing the animal to subsequent infections (Gaskell et al., 2007; Thiry et al., 2009). Other chronic respiratory diseases (such as feline chronic bronchitis) could potentially also be associated with FIRD (Schulz et al., 2014).

Age group was found to be significantly associated with case status in this sample with young adult, adult and senior cats being less likely to be cases. Previous studies, as well as our own findings in chapter 2, have also shown that as animals grow older they are less likely to be shedding FIRD pathogens as well as less likely to exhibit severe signs of disease (Porter et al, 2008, Afonso et al 2016, Fernandez et al 2017). This may be an age-driven immune-mediated mechanism (Coyne et al 2006). As animals grow older, they are more exposed to both field and vaccine strains of FIRD pathogens which can boost immunity. Additionally, even though vaccination is widespread (specifically for FCV and FeHV-1), it does not prevent the carrier state which in turn leads to a relatively boosted immunity as these infected animals grow older.

On multivariable analysis, the number of cats per household was not found to be associated with case or control status. In prior studies as well as in chapter 2, the opposite has been reported (Afonso et al., 2017a; Bannasch and Foley, 2005; Coutts et al., 1994; Helps et al., 2005; Wardley et al., 1974). Whether this observation represents the smaller than planned sample size of the current study, or some other difference in the study population is unknown.

Vaccination status was also not found to be significantly associated with case status which is in contrast with other studies where vaccination was found, perhaps not surprisingly, to have a protective effect against respiratory disease (Binns et al., 2000; Fernandez et al., 2017). Again, this could be due to the size of the sample in the present study and the fact that only 10 animals in total had reportedly never been vaccinated.

It is worth mentioning that for 12 cases (31.6%), no pathogen was identified. This is not uncommon in similar studies in cats (Binns et al., 2000). Failing to identify a causative known pathogen in diseased animals may be due to several factors including poor sample collection, timing of sampling (a lot of the animals had been suffering with respiratory signs for over two weeks before being taken to the veterinary surgery which decreases the likelihood of viral shedding being present

upon sampling), misdiagnosis due to broad case definitions (i.e: respiratory disease due to non-infectious causes or non-viral/bacterial causes such as parasites), poor sample handling, etc. Another possibility is that the actual aetiological agent was not among the panel of pathogens tested and may still be unknown to the veterinary community. Next generation sequencing offers an unbiased approach to identifying potential new pathogens in these cases (Radford et al., 2012).

On the other hand, out of the 64 controls, 26 (40.6%) were found to be positive to one or more tested pathogens. Of these 4.7% (n=3) tested positive for FCV and 1.6% (n=1) for FeHV-1. Given the ability of both viruses to induce subclinical carrier infection these numbers are not surprising (Coutts et al., 1994; Harbour et al., 1991; Wardley et al., 1974). Cats recovered from acute FCV disease shed virus more or less continuously for 30 days; thereafter an approximate 75-day half-life has been proposed with only a minority of cats shedding virus possibly for life (Povey, 1986; Radford et al., 2009). In contrast, FeHV-1 develops a classic herpesvirus latent infection, only shedding virus intermittently following stressors such as kittening, lactation and rehoming (Gaskell and Povey, 1977). As a result, in most studies the carriage rate of FCV is higher than that of FeHV-1, as shown here (Binns et al., 2000; Helps et al., 2005; Holst et al., 2005; Zicola et al., 2009). Of note is the fact that FCV was found in 10.8% of the total samples which is similar to the prevalence reported in similar populations (Chapter 2) of animals with and without respiratory signs.

Perhaps surprisingly, a higher percentage of controls than cases tested positive for *B. bronchiseptica* than cases. This is an interesting finding which contrasts with results from Binns et al., 1999, who showed *B. bronchiseptica* to be widespread in multi-animal environments but found no evidence of *B. bronchiseptica* infection in household pet cats. These contrasting results likely reflect differences in the sampled populations; clinical disease associated with *B. bronchiseptica* in cats is generally considered by most to be associated with stress and overcrowding, both likely to be less of an issue in pet cats than in rescue centre and catteries (Binns et al., 1999; Egberink et al., 2009; McArdle et al., 1994). Although vaccines are available for *B. bronchiseptica*, they are generally rarely used in the cat (Sánchez-Vizcaíno et al.,

2018). It is also interesting to note that PCR proved to be more sensitive than bacterial culture in detecting *B.bronchiseptica* (see also Chapter 6) which, again, could explain the contrasting results to those obtained by Binns et al., 1999.

M. felis was detected in high numbers in both cases and controls; although the percentage positive was higher in cases the differences between groups were not found to be statistically significant (p-value > 0.05) although this may be due to small sample size. Previous studies indicate that its presence is higher in animals with respiratory and/or ocular disease (Afonso et al., 2017b; Fernandez et al., 2017; Sykes, 2012). *M. felis* was also found in all of the cases that tested positive in this study for more than one potential pathogen, hinting at a potential role in mixed infections. It would be interesting to further study this and understand if *M. felis* perhaps is mostly an opportunistic pathogen of the upper respiratory tract.

Neither *C. felis* nor Influenza A viruses were detected by PCR in any of the collected samples. Influenza A is, to the author's knowledge, an uncommon finding in cats and reports of feline influenza are scarce (Chapter 3). *C. felis* is mostly associated with ocular disease especially conjunctivitis so it is somewhat surprising that no samples tested positive for this pathogen since ocular signs of disease were mentioned at least six times in questionnaire responses and the prevalence in other studies were found to be high (Fernandez et al., 2017). As well as the small sample size, this could possibly be explained by the fact that only oropharyngeal swabs were collected from cats in this study. Ocular swabs would be more appropriate for *C. felis* detection given the pathogen's tissue tropism (Fernandez et al., 2017; Gruffydd-Jones et al., 2009).

This study's conclusions are limited by the low sample numbers and therefore results should be interpreted with caution. It would be interesting to repeat this study in a larger sample population. Originally, the author decided to administer questionnaires to owners via the phone as feedback from previous studies (including the study conducted by the author in Chapter 2) as well as anecdotal evidence and discussion with veterinary researchers lead to the postulation that the time burden for questionnaire completion by veterinary surgeons would lead to low response

rate. However, telephone administration of questionnaires came with other challenges such as owners not answering the phone despite several contact attempts and possibly not recollecting information accurately. Despite efforts being made to reduce to time from sample collection to phone questionnaire administration as much as possible (with owner contact being attempted within 7 days of sample collection), recall bias is still likely. If another study was to be conducted, the author suggests that questionnaires are filled out directly by the attending veterinary surgeon during clinical history intake.

It is also worth mentioning that originally, this study was meant to be conducted as a matched case-control study with results analysed with clustering by participating practice. However, due to the fact that some practices only submitted cases and no controls and vice versa as well as the fact that in a lot of instances there were no matched controls for each case, the data was analysed using an unmatched case-control design.

This study was designed in an attempt to allow the investigation of risk factors for infectious disease affecting the upper airways. However, the broad case selection criteria could represent a range of aetiologies of non-infectious origin. This was partly mitigated by asking participating veterinary surgeons to exclude any cases where an obvious non-respiratory origin (such as cardiac disease) was suspected but the possibility still remains that other, non-infectious, causes of respiratory symptoms were present. It is also worth noting that, as is known with FCV and FeHV-1 ability to cause carrier statuses, an absence of clinically apparent signs does not rule out respiratory tract infections and the inclusion of controls with subclinical disease was not possible. Other potential limitations, which would be hard to circumvent in a study like this, is the reliance on multiple veterinary surgeons to classify recruited animals as cases or controls and the possibility of observer selection.

In conclusion, despite challenges around recruitment, this study was able to reaffirm the importance of FIRD in animals with prior history of respiratory disease and the importance of major vaccine preventable pathogens notably FCV. The high

proportion of cases that lacked a recognised pathogen despite using PCR tests general considered to be highly sensitive reiterates the need to look for new potential pathogens using more unbiased molecular techniques like next generation sequencing.

4.7 Appendix to Chapter 4

Appendix 4A Figure shared through social media platforms (Twitter, Facebook and blogs) for the purpose of veterinary practice recruitment.

Appendix 4B Owner information sheet and informed consent for participating cat owners.

Pet Health Study - understanding infectious causes of respiratory disease in dogs and cats

Dear pet owner,

This document aims to invite you to participate in a research study. Please take time to read the following information carefully and feel free to ask if you would like more information or if there is anything that you do not understand. We would like to stress that you do not have to accept this invitation and you should only agree to take part if you want to.

What is the purpose of this study?
Respiratory infection represents an important cause of disease in cats and dogs, sometimes leading to outbreaks of respiratory disease in sensitive environments like rescue shelters, kennels and veterinary hospitals. It can have severe and even fatal consequences for our pets and can be caused by many viruses and bacteria. The purpose of this project, which is funded by one of the main manufacturer's of pet vaccines (MSD Animal Health) is to understand the role of known and yet-to-be identified pathogens in respiratory disease in dogs and cats and to study which lifestyle and medical risks may be associated with disease in these cases.

Why have I been chosen to take part?
This study was designed as a case-control study. Therefore, there will be two types of participants: If your pet was selected as a case, it means that your dog or cat is currently suffering from respiratory disease according to strict criteria identified by your veterinary surgeon. On the other hand, if your pet was selected as a control, it means that your dog or cat is not currently suffering from respiratory disease but was one of two non-respiratory cases seen by your vet following a pet with respiratory disease.

Do I have to take part?
Participation is voluntary and you may withdraw from this project at anytime without explanation and without incurring any disadvantage.

What will happen if I take part?
Your veterinary surgeon will collect a saliva sample from your pet (a double-mouth swab). This process is very straightforward, not painful and should take no more than a minute to complete. The veterinary surgeon will place a cotton-wool swab inside

your pet's mouth, until the swab is coated in saliva (the swab is a bit like a long cotton bud). Your contact details will then be collected by your veterinary surgeon, so that we can contact you afterwards in order to ask you a few questions regarding your pet (this will be done in the form of a phone-administered questionnaire). The swabs and your contact details will be sent to the University of Liverpool and used to look for respiratory germs.

Are there any risks in taking part?

The sample collection is a very simple, harmless procedure that is routinely performed on many dogs and cats for diagnostic purposes. Some animals might chew on the swab as it is put in their mouth and very rarely, some have chewed off and swallowed the tip of the swab. In the very rare cases where this happens the involved animals never too our knowledge suffered any health problems. If, at any time, you are concerned about your pet's safety please contact your veterinary surgeon immediately.

Are there any benefits in taking part?

By taking part in this study, you will be helping us ensure we better understand respiratory disease in dogs and cats. Also, if you were selected as a case, you can receive the results of the tests, in order to find out what may be causing your pet's signs. In this case, results would be sent back to your veterinary surgeon (please note that we cannot provide results for control animals).

What if I am unhappy or if there is a problem?

Your veterinary surgeon will be happy to discuss the project with you. If you are unhappy, or if there is a problem, please feel free to let us know by contacting Dr Alan Radford the project lead (Telephone: 0151 794 6121; alanrad@liv.ac.uk) and we will try to help. If you remain unhappy or have a complaint which you feel you cannot come to us with then you should contact the Research Governance Officer on 0151 794 8290 (ethics@liv.ac.uk). When contacting the Research Governance Officer, please provide details of the name or description of the study (so that it can be identified), the researcher involved, and the details of the complaint you wish to make.

Will my participation be kept confidential?

Samples and the information obtained may be retained to up to seven years and possibly used in future projects. All data will be kept confidential and will be stored in a secure database accessible only by people working on the project. You may withdraw from the project at any time, without explanation and any information provided by you can be destroyed.

What will happen to the results of the study?

The anonymised results of this study will be sent for publication in veterinary journals and non-veterinary press. No participant will be identifiable from any published work. Any germs we isolate will be frozen, and may be used for ongoing research and development aimed at improving animal health.

What next?
If you are happy to allow your pet to become involved, please read, initial the boxes and sign the consent form. The veterinary surgeon will then collect the required sample from your pet and fill in a short contact detail form so that we can call you afterwards.

Many thanks,

Dr Alan Radford
Department of Infection Biology
University of Liverpool
Leahurst Campus
CH64 7TE
0151 794 6121
alanrad@liv.ac.uk

Please read the following information carefully. You may also request a copy for yourself.

Title of Research Project: Pet Health Study - understanding infectious causes of respiratory disease in dogs and cats

Researcher(s): Alan Radford/G Pinchbeck/Maria Afonso/S Bonner

1. I confirm that I have read and have understood the information sheet dated 11/06/2014 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐ Please **initial** box

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my rights being affected. If I do not participate this will **not** affect the care and treatment of my animal. ☐ Please **initial** box

3. I understand that, under the Data Protection Act, I can at any time ask for access to the information I provide and I can also request the destruction of that information if I wish. ☐ Please **initial** box

4. I allow participation of my animal in the above study. ☐ Please **initial** box

Owner's Name	Date	Signature

Principal Investigator:
Dr Alan Radford, Department of Infection Biology, University of Liverpool, Leahurst Campus, CH64 7TE; Tel: 0151 794 6121; email: alanrad@liv.ac.uk

Student Researcher:
Maria Afonso, Department of Infection Biology, University of Liverpool, Leahurst Campus, CH64 7TE; email: maria.afonso@liv.ac.uk

Appendix 4C Letter to veterinary surgeons with sampling protocol and study summary

A matched case-control study to evaluate the role of pathogens and other risk factors in respiratory disease in vet-visiting cats and dogs in the UK

Dear ,

Thanks for agreeing to take part in our study. Here we provide instructions for the project including

- What to find in the 3 parcels you receive
- What to do for the project
- Definitions of cases and controls
- Protocol for "double" oropharyngeal swabbing
- How to send the samples back to us

If you have any questions then please do not hesitate to contact us, using the details provided below.

Once again, many thanks for taking part in our study. We hope the findings will be published and expect that as a result of this study, some of the questions associated with infectious respiratory diseases will be answered.

Yours sincerely,

Maria Afonso	Alan Radford	Gina Pinchbeck	Shirley Bonner
PhD Student	Reader in Infection Biology	Reader in Epidemiology	Project Technician

Contact: Maria Afonso – Email: mmanuel@liverpool.ac.uk, Tel: 0151-794-6005

Dr Shirley Bonner – Email: shirley@liverpool.ac.uk, Tel: 0151-794-9

- 30 vials of virus transport medium (VTM) – please store at -20°C.
- 10 x Speci-safe packs for the return of 3 x VTM vials each

- 30 dry cotton swabs
- 30 Amie's charcoal swabs (please store at room temperature)
- 30 Sheets with owner details form and owner consent form, one to be filled out for each animal
- 2 Copies of the Owner Information Sheet
- 10 Prepaid and addressed envelopes for the return of samples and consent forms

What to do for the project

We would like you to collect samples from 5 cat cases with 2 control animals per case and 5 dog cases with 2 control animals per case. More or less is fine too!

For each animal please:
- Obtain informed consent (by asking the owner to read the Owner Information Sheet and then sign the owner consent form)
- Fill out the owners details on the reverse of the consent form
- Collect 2 swabs (see below)
- Send all forms and the swabs to us at the University via the postage paid padded envelopes

Definitions of cases and controls

Cases and controls should be recruited according to the following criteria:

Cats - A **case** is defined as any cat presenting at a veterinary surgery which, to the best of the clinical judgement of the attending veterinary surgeon, is suffering from infectious respiratory disease at the time of consultation AND presents with two or more of the following clinical signs: pyrexia, sneezing, nasal discharge, ocular discharge, conjunctivitis, mouth ulcers, facial ulceration, cough, altered lung sounds, altered respiratory patterns**. Control** cats are defined as the next two cats to be presented to the same veterinary surgeon following a case, with the exclusion of any cats which have suffered from respiratory disease within the previous two weeks or are showing any of the above clinical signs of disease.

NB – cats showing any of the previously mentioned clinical signs as a result of something other than infectious respiratory disease (e.g. cough in animals with cardiac disease, epistaxis in animals with hypertension, sneezing in animals with nasal foreign bodies), should also be excluded as controls.

Dogs - A **case** is defined as any dog presenting at a veterinary surgery which, to the best of the clinical judgement of the attending veterinary surgeon, is suffering from

infectious respiratory disease at the time of consultation AND presents with two or more of the following clinical signs: pyrexia, cough, sneezing, nasal discharge, ocular discharge, inflamed tonsils, epistaxis, altered lung sounds, altered respiratory patterns.

Control dogs are defined as the next two dogs to be presented to the same veterinary surgeon following a case, with the exclusion of any dogs which have suffered from respiratory disease within the previous two weeks or are showing any of the above clinical signs of disease.

NB – dogs showing any of the previously mentioned clinical signs as a result of something other than infectious respiratory disease (e.g. cough in animals with cardiac disease, epistaxis in animals with hypertension, sneezing in animals with nasal foreign bodies), should also be excluded as controls.

Protocol for "double" oropharyngeal swabbing

Store all virus transport medium (VTM) in a freezer (-20^{o}C). Thaw samples to be used as needed. Store Amies charcoal swabs in a dry cupboard.

Each case and each control should be swabbed after consent was given by the rightful person (owner in privately owned animals and a member of staff in case of stray animals).

You may *exclude* dogs or cats:

- that in your clinical judgement, it would not be appropriate to swab.
- that it may be dangerous to swab.

Two kinds of swab are provided for each case and each control animal - a dry swab and an Amies charcoal swab in its own container. The animals should be swabbed simultaneously with each type: Place both swabs in the animal's mouth. The animal is likely to chew on them. If possible, rub the swabs against the side and back of the pet's mouth. If taken correctly, the swabs should be moist with saliva. Place the swab from the Amies charcoal back into its container, and the other (previously dry) swab, into a vial of VTM (you will need to break off most of the shaft). If the VTM is still not thawed, that is acceptable. Place the VTM vial into the Speci-safe container provided and the Amies Charcoal Swab in its container, into a plastic bag. **Please ensure the pet's name, and owner's surname, are written on both the Amies Charcoal Swab tube and on the vial of VTM containing the other swab.** Many vets are finding the simultaneous swabbing difficult. These can be done consecutively if this is easier.

How to send the samples to us

Three sample vials of VTM will fit in the Speci-Safe packs - a case and 2 control VTM samples should be sent at the same time. Place the vials in the slots in the container and click the pouch shut. Do not peel the white material away. These containers mean that the samples meet requirements for proper mailing of biological samples.

Fill in the owner details on the forms provided (on the reverse of the consent form) and place it, together with the corresponding samples inside one of the pre-paid addressed envelopes we have sent you. The samples should be sent as soon as possible after being collected, preferably within the same day. If you are not able to collect two controls in the same day as the corresponding case please place the VTM samples in the freezer until you are ready to send them. The pre-paid envelope can go in a normal Royal Mail postbox.

Send back to us:

- 1 x Specisafe containing 3 x swabs in VTM vials (1 case, 2 controls)
- 3 charcoal swabs (1 case, 2 controls)
- 3 consent/owner detail forms
- All in a padded pre-paid envelope

Please note:

If the animal is a stray, the vet may consent for the study and could you please provide your contact details on the owner contact form.

We will process the samples for the presence of respiratory pathogens. We will also contact the owners for further information about their animal. The project has Ethical Approval and any details provided by the owners/vets will remain completely confidential. **Please note that results may not be returned to you in a diagnostically useful timescale.**

A matched case-control study to evaluate the role of pathogens and other risk factors in respiratory disease in vet-visiting cats and dogs in the UK

Summary of project for veterinary surgeons

Infectious respiratory disease represents an important and frequent cause of disease in both cats and dogs. Over the years, research interest on the topic has been intermittent and respiratory disease has often been subvalued frequently leading to outbreaks in sensitive environments like rescue shelters, kennels and veterinary hospitals. Further understanding of the panoplia of pathogens associated with respiratory disease in dogs and cats is therefore critical to ensure the usage of the best techniques for the prevention and treatment of these diseases.

This study aims to reassess the role of known and yet-to-be identified pathogens in respiratory disease in dogs and cats. In order to do so, two parallel matched case-control studies will be undertaken to explore risk factors including pathogens in respiratory disease. Cases presenting for respiratory disease (71 cases for each species) will be recruited from general practices which will be recruited based on convenience. Controls (142 controls for each species) will consist of two animals (two controls per case) seen for reasons other than respiratory disease. Each practice will be asked to submit samples from cases and controls they see over a period of 6 months, in this way we expect to get the desired number of samples (see above), as obtained by sample size calculation based on a pathogen exposure of 10% (e.g.: FCV prevalance).
A phone questionnaire will be administered by the University of Liverpool team to the participating pet owners in order to study risk factors associated with pathogen carriage.

In the lab, respiratory pathogens will be looked for by virus isolation, PCR/RT-PCR, bacterial culture and API. Results for each case will be sent to you at a later date. The pathogens isolated will help us understand the current panorama of respiratory pathogens circulating in the UK and the risk factors involved in infection of dogs and cats with these.

Appendix 4D Questionnaires administered to participating owners via telephone.

Pet Health Study
Understanding infectious causes of respiratory disease in dogs and cats

PHONE ADMINISTERED OWNER QUESTIONNAIRE - CATS

1. General information:

Pet name	
Owner surname	
Owner postcode (to AA00 level eg.: CH60)	
Veterinary surgery	

2. Pet details:

a) Age: ☐ years ☐ months (known / estimated – circle correct)

☐ Don't know

b) Gender: Male ☐ Female ☐ Don't know ☐

c) Is your pet neutered (castrated/spayed)? Yes ☐ No ☐ Don't know ☐

d) Is your pet a: Purebreed ☐ Crossbreed ☐ Don't know ☐

e) If you answered purebreed, which breed is it? ☐

3. Lifestyle questions:

a) How many cats are there in the house including this cat? ☐

b) Does your pet have contact with other animals? Yes ☐ No ☐ Don't know ☐

c) If your answer to 3.b) was yes, which species does your pet have contact with?

☐ Other cats
☐ Dogs
☐ Cattle/sheep
☐ Pigs

☐ Poultry
☐ Horses
☐ Pet birds
☐ Wild animals (eg. If cat hunts, please specify) ☐
☐ Others (please specify ☐

d) Does your cat live?

☐ Indoors
☐ Outdoors
☐ Both

f) During the last month has your cat stayed at or attended any of the following?

☐ Boarding cattery
☐ Vet surgery (excluding the one during which he/she was recruited for this study)
☐ Rescue shelter
☐ Kitten parties
☐ Groomers
☐ Other. Please specify: ☐

g) Your cat eats (choose all that apply):

☐ Commercial food (e.g: biscuits)
☐ Raw meat
☐ Home cooked meals
☐ Solely vegetarian
☐ Other. Please specify: ☐

h) Where does your cat alleviate him/herself?

☐ Litter tray
☐ Goes outside
☐ Both
☐ Other. Please specify: ☐

i) If you answered littered tray, what do you line the tray with?

☐ Clay litter
☐ Clumping litter
☐ Deodorant litter
☐ Crystal litter
☐ Pine/Wheat/Corn litter
☐ Recycled paper
☐ Other. Please specify: ☐

j) How long have you had your cat for?

4. Medical information:

a) Is your cat vaccinated? When was he/she last vaccinated?

- ☐ Yes, within the last year.
- ☐ Yes, within the last three years.
- ☐ Yes, more than three years ago.
- ☐ Yes, he has had his first vaccination but hasn't received his booster yet.
- ☐ No, he is not old enough to receive vaccination.
- ☐ No, he was never vaccinated.
- ☐ Don't know.

b) Do you know the name of the last vaccine used?

c) When was your cat last de-wormed?

- ☐ In the last 3 months.
- ☐ In the last year.
- ☐ Over a year ago.
- ☐ Don't know.
- ☐ Never.

d) When was your cat last treated for fleas and ticks?

- ☐ In the last 3 months.
- ☐ In the last year.
- ☐ Over a year ago.
- ☐ Don't know.
- ☐ Never.

e) What was the reason for your visit to the vet?

f) Before the vet visit you have just described, has your pet suffered from respiratory disease (example: coughing, sneezing, runny nose or eyes)? If yes, what was it? Did he/she receive any treatment?

g) Does your pet suffer from any other medical condition? If yes, explain.

h) Is your cat currently on antibiotics?

☐ Yes. Please specify: ☐
☐ No

i) Is your cat currently receiving any medical treatment?

☐ Yes
☐ No

j) If your answer to 4.c) was yes, what is the name of the product(s):

☐

k) Is there any other information about your cat's health that you feel might be relevant?

☐

l) Has your cat showed any of the following signs within the past 14 days?

☐ Coughing
☐ Sneezing
☐ Runny nose
☐ Runny eyes
☐ Nose bleed
☐ Red eye
☐ Trouble breathing
☐ Mouth ulcers
☐ Face/lip ulcers
☐ Fever

m) Has your cat had any other respiratory disease signs within the past 14 days?

☐ Yes. Please specify: ☐
☐ No
☐ Don't know

n) If you answered yes for any of these signs, when did they start?

☐ Last 48 hours
☐ Last 3 – 7 days
☐ Last 8 – 14 days
☐ 15 or more days ago

☐ Don’t know

o) Do you have any thoughts on the reason your cat is suffering from respiratory disease?

p) Have you suffered from any signs of respiratory disease or conjunctivitis (red eye) yourself within the past 14 days?

Thank you for taking part in this study!

Appendix 4E Univariable logistic regression results of questionnaire data associated with respiratory disease in cats attending veterinary practices in the UK. All variables with p-values < 0.25 are highlighted with an *.

Variable	*Case*	*Control*	*Odds Ratio*	*Wald P-value*	*Lower 95%CI*	*Upper 95%CI*
	n	n				
Gender						
Female	11	19	Ref.			
Male	15	20	1.29	0.6	0.5	3.6
Neutered status						
Yes	22	35	Ref.			
No	4	3	2.12	0.35	0.42	11.6
Breed						
Pedigree	3	3	Ref.			
Crossbreed	23	35	0.7	0.62	0.11	3.81
Number cats/household						
1	9	15	Ref.			
2 to 3	9	20	0.8	0.6	0.2	2.4
4 or more	8	4	3.3	**0.1***	0.8	15.7
Contact with other animals						
Yes	20	32	Ref.			
No	5	7	1.1	0.83	0.3	4.1
Lifestyle						
Indoors	13	11	Ref.			
Indoors+Outdoors	13	28	0.4	**0.08***	0.13	1.1
Stay in multianimal environment						
Yes	5	4	Ref.			
No	21	35	0.5	0.3	0.1	1.98
Food						
Commercial	18	33	Ref.			
Commercial + Home cooked	6	6	2.4	**0.15***	0.7	8.5
Where cat alleviates						
Litter	14	17	Ref.			
Outdoors	6	13	0.6	0.34	0.17	1.86
Litter + Outdoors	6	9	0.8	0.74	0.2	2.8
Vaccinated						
Within last year	17	29	Ref.			
>1 year ago	5	2	4.2	**0.1***	0.8	32.1
Never	3	7	0.7	0.7	0.1	3.02
Vaccine antigen						
FCV-F9	10	16	Ref.			
FCV-431+FCV-G1	2	3	1.07	0.948	0.12	7.57

Variable	Case	Control	OR	P-value	Lower 95%CI	Upper 95%CI
	n	n				
Worming						
Within last 3m	15	29	Ref.			
>3m ago	6	9	1.3	0.7	0.4	4.3
Never	5	1	9.7	**0.05***	1	194
Flea/tick treatment						
Within last 3m	19	33	Ref.			
>3m ago	2	4	1.7	0.47	0.4	8.1
Never	3	3	5.2	**0.16***	0.6	109.5
Reason for vet visit						
Routine	3	22	Ref.			
Ill (respiratory)	21	1	153	<0.001*	21	3357.6
Ill (not-respiratory)	2	16	0.9	0.9	0.1	6.2
Previous respiratory disease						
Yes	10	4	Ref.			
No	16	30	0.2	**0.02***	0.05	0.74
Concurrent medical conditions						
Yes	5	14	Ref.			
No	20	24	2.3	**0.16***	0.7	8.2
Current antibiotics						
Yes	7	4	Ref.			
No	19	34	0.32	**0.1***	0.08	1.19
Other current medication						
Yes	2	1	Ref.			
No	24	36	0.33	0.381	0.01	3.7
Age						
0 – 12m	6	11	Ref.			
13-60m	3	12	0.14	**0.015***	0.02	0.62
61-96m	1	5	0.11	**0.067***	0.005	0.88
> 96 m	9	10	0.49	0.299	0.12	1.85

5 Pathogens and risk factors associated with signs of respiratory disease in dogs attending veterinary practices in the UK: a case-control study

5.1 Abstract

Canine infectious respiratory disease (CIRD) is a major syndrome affecting dogs on a global scale. This case-control study aimed to reappraise the role of classic and emerging CIRD pathogens such as canine distemper virus (CDV), canine adenovirus type 2 (CAV-2), canine parainfluenza virus (CPiV), canine herpesvirus (CHV-1) and *Bordetella bronchiseptica (B. bronchiseptica),* canine respiratory coronavirus (CRCoV), canine pneumovirus (CnPnV), influenza A viruses, *Mycoplasma cynos (M.cynos)* and *Streptococcus equi subsp. zooepidemicus (S .equi subsp. zooepidemicus).* This study also aimed to identify potential dog-level risk factors for infection in animals attending UK veterinary practices.

Veterinary practices (11) were recruited into the study and samples from a total of 45 cases and 87 controls collected. Presence of pathogens was determined using bacterial culture and polymerase chain reaction (PCR). Telephone questionnaires were administered to participating pet owners to collect data relating to signalment, lifestyle and characteristics of respiratory disease. Risk factors for case status were assessed using multivariable logistic regression.

No samples tested positive for CAV-2, CPiV, CDV, Influenza A or *S.equi* subsp. *zooepidemicus.* However, CRCoV was detected in 6.1% of samples (all of which were cases) and this virus was found to be positively associated with case status. *M. cynos* was detected in 3.1% of samples (4.4% cases and 2.3% controls), 2.3% of samples tested positive for CnPnV (4.4% cases and 1.2% controls) and 15.9% tested positive for *B.bronchiseptica* (6.7% cases and 20.7% controls). Entire (not neutered) and older animals were found to be more likely to suffer from CIRD.

This study highlights the importance of newly emerged pathogens in CIRD as well as the need to re-evaluate the relevance of currently available CIRD vaccine antigens. CRCoV was identified as an important pathogen in CIRD in the UK for which vaccination is not currently available.

5.2 Introduction

Canine infectious respiratory disease (CIRD), classically referred to as "kennel cough", is an important multifactorial syndrome affecting dogs, especially those which are housed in groups, such as breeding and rescue kennels, as well as working dogs. In a recent report by SAVSNET, 1.1% of canine patients are brought into consults at UK veterinary practices due to respiratory disease (Arsevska et al., 2018). This number shows a slight decrease in more recent years in comparison to a similar study by the same group which reported 1.7% of consults with dogs were due to respiratory signs (Sánchez-Vizcaíno et al., 2016). Additionally, it has also been suggested that dogs present for this reason slightly less frequently than cats (1.3%; Arsevska et al., 2018).

While a range of clinical signs are associated with CIRD, the most commonly reported is coughing, often described as a dry honking cough. Other frequently associated signs include dyspnea, sneezing and nasal discharge (Arsevska et al., 2018; Buonavoglia and Martella, 2007; Mochizuki et al., 2008).

CIRD is an endemic respiratory syndrome of worldwide distribution. Although historically it has been perceived as a disease complex of relatively limited significance (especially in pet dog populations), it has received growing attention more recently due to the emergence of new pathogens. Classical pathogens associated with CIRD include canine parainfluenza virus (CPiV), canine distemper virus (CDV), canine adenovirus type 2 (CAV-2), canine herpesvirus 1 (CHV-1) and *B. bronchiseptica.* More recently, canine respiratory coronavirus (CRCoV) was identified as a causative agent of kennel cough-like disease. It is commonly identified in samples of dogs with respiratory disease, seeming to be one of the most prevalent viruses in association with the complex (Erles et al., 2003; Priestnall et al., 2007, 2006). In 2010, canine pneumovirus (CnPnV) was isolated in the USA from kenneled dogs with CIRD-like disease and has since been identified in several other countries (Decaro et al., 2014; Mitchell et al., 2017, 2013b; Renshaw et al., 2010). Canine influenza was first detected in 2004 in racing greyhounds in Florida. The first circulating subtype identified in association with CIRD was H3N8 which seemingly underwent a species jump from horses to dogs (Crawford et al., 2005). This virus has subsequently become

adapted for transmission between dogs, and has become a major issue in several states of the USA. Other influenza subtypes causing disease in dogs have also emerged including canine influenza H3N2, which circulates in several Asian countries and continues to be an issue in the USA, where it was imported with rescue dogs (Song et al., 2008, Voorhees et al., 2017). Interestingly, canine influenza does not seem to have been able to establish itself outside of North America and very little evidence of its presence in the UK exists (chapter 3; Mitchell et al., 2017). Finally, in recent years, *Streptococcus equi* subsp. *zooepidemicus* has re-emerged as a relevant pathogen causing occasional outbreaks of severe and fatal lower respiratory tract disease and *Mycoplasma cynos (M.cynos)* has also been frequently found in animals with CIRD. The role of these two last microorganisms in the pathogenesis of CIRD is still not fully understood (Priestnall, 2017).

While a myriad of studies looking into CIRD have focused on disease of animals housed in groups, few studies exist focusing solely on CIRD in pet dog populations (Mochizuki et al 2008, Joffe et al 2015) and, to the knowledge of the author, none focusing on CIRD in UK household dogs. The aim of this study was, therefore, to use recent assays to reappraise the role of CIRD where it relates to the UK veterinary-visiting pet dog population. Taking a similar approach to this dissertation's previous chapter, a case-control study was performed in dogs attending UK-based veterinary practices to identify risk-factors associated with the presence of CIRD and further understand the role of infection with classical and emerging/potential respiratory pathogens including CPiV, CDV, CAV-2, CHV-1, *B. bronchiseptica*, CRCoV, CnPnV, Influenza A, *S. equi* subsp. *zooepidemicus* and *M. cynos.*

5.3 Materials and methods

Ethics and informed consent

Ethical approval was obtained from the Veterinary Research Ethics Committee, University of Liverpool (VREC233; same as previous chapter). Informed consent was obtained from participating dog owners (Appendix 5A).

Study design

This study was done in conjunction with the previous chapter. Thus, in order to enable comparison between dogs with and without clinically suspected canine infectious respiratory disease (CIRD), a case-control study design was used. An expected prevalence of 10% (see chapter 4) was used to construct a sample size calculation (Epi Info 7) for a 1:2 matched case-control study.

Recruitment and sample collection

Samples were collected from July 2014 to December 2016 from dogs attending UK veterinary practices. Recruitment and sample collection methods used were similar as for the feline patients in chapter 4.

Case and control definitions

A case was defined as any dog presenting at a veterinary surgery which, to the best of the clinical judgement of the attending veterinary surgeon, was suffering from respiratory disease at the time of consultation and presented with two or more of the following clinical signs: cough, sneezing, altered lung sounds, pyrexia, nasal discharge, ocular discharge, inflamed tonsils, epistaxis, altered respiratory patterns. As with cats in Chapter 4, animals were to be excluded if any of the previously mentioned signs were likely to be caused by a reason other than respiratory disease (e.g. cardiac disease).

Control dogs were defined as the next two dogs to be presented to each veterinary surgeon following a case, with the exclusion of any dogs which suffered from

respiratory disease within the previous fortnight or were showing any of the above clinical signs of disease.

Sample collection and processing

The same methods were applied as previously described for feline samples in Chapter 4.

Questionnaire design

The questionnaires used for canine participants are shown in Appendix 5A. A core of identical questions was used for both cases and controls with additional questions for cases only. Questionnaire administration was performed in the same way as with feline patients (Chapter 4).

Nucleic acid extraction, reverse transcription and PCR

Viral nucleic acid (RNA and DNA) was extracted from oropharyngeal swabs using the same method as described in Chapter 2 (Viral RNA mini-kit; Qiagen). Reverse transcription was performed using 200 ng random hexamers (Superscript III, Life Technologies). Table 5.1 shows details of diagnostic tests using PCR for detection of nucleic acids from oropharyngeal swabs. Conventional PCRs were performed using cDNA from reverse transcription for Influenza A, CDV, CPiV, CRCoV and CnPnV; and using the nucleic acids extracted from oropharyngeal swabs for *M. cynos*, *B. bronchiseptica*, CAV-2 and CHV. Real-time PCRs were performed for *B.bronchiseptica* (in addition to bacterial culture) and CnPnV.

Table 5.1 Details of PCR diagnostic assays used for detection of potential pathogens associated with CIRD. AT stands for annealing temperature

Pathogen	*Test*	*Gene*	*Primer name*	*Primer (5-3)*	*Methods*	*AT ºC*	*Cycles*	*Sample*	*Product size (bp)*
CDV	RT-PCR	Nucleoprotein	P1 P2	ACAGGATTGCTGAGGACCTAT CAAGATAACCATGTACGGTGC	(Frisk et al., 1999)	58	40	OP swab	287
Influenza A	RT-PCR	Matrix (M)	M30F2/08 M264R3/08	ATGAGYCTTYTAACCGAGGTCGAAACG TGGACAAANCGTCTACGCTGCAG	World Health Organisation	50	40	OP swab	244
CPiV	RT-PCR (nested)	Nucleocapsid	PNP1 PNP2 PNP3 PNP4	AGTTTGGGCAATTTTTCGTCC TGCAGGAGATATCTCGGGTTG CGTGGAGAGATCAATGCCTATGC GCAGTCATGCACTTGCAAGTCACTA	(Erles et al., 2004)	58	35	OP swab	667, 182 (nested PCR)
CRCoV	RT-PCR (nested)	Spike	Sp1 Sp2 Sp3 Sp4	CTTATAAGTGCCCCCAAACTAAAT CCTACTGTGAGATCACATGTTTG GTTGGCATAGGTGAGCACTG GCAATGCTGGTTCGGAAGAG	(Erles et al., 2003)	55	35	OP swab	621, 442 (nested PCR)
CnPnV	Real Time RT-PCR	Nucleoprotein	CnPV-NF CnPV-NR Probe	GACCTGTTTGAAAGGAAGCCTTATT ACCAGAAAACAGCCCCTCAAC CTTCCATCACTTTTGGCCT GGCCCAG	(Mitchell et al., 2013b)	60	45	OP swab	104
CAV-2	PCR	E3 and flanking regions	HA-1 HA-2	CGCGCTGAACATTACTACCTTGTC CCTAGAGCACTTCGTGTCCGCTT	(Hu et al., 2001)	60	40	OP swab	1030
CHV	PCR	Thymidine kinase	CaHV1 CaHV2	TGCCGCTTTTATATAGATGA AGCGTTGTAAAAGTTCGT	(Schulze and Baumgärtner, 1998)	53	40	OP swab	493
B. bronchiseptica	Real Time-PCR	FimA	BbronchisepticaF BbronchisepticaR Bbronchiseptica probe	ACTATACGTCGGGAAATCTGTTTG; CGTTGTCGCCTTTCCTCTG; FAMCGGGCCGATAGTCAGGGCGTAGBHQ1	(Helps et al., 2005)	60	45	OP swab	80
M. cynos	PCR	16S/23S rRNA intergenic spacer	Myc1 m.cynos	CACCGCCCGTCACACCA GATACATAAACACAACATTATAATATTG	(Chalker et al., 2004)	56	45	OP swab	227

Isolation and identification of *Bordetella bronchiseptica* and *S. equi subsp. zooepidemicus*

For *B.bronchiseptica*, the methods used were described in Chapter 4. For *S. equi subsp. zooepidemicus,* charcoal swabs were streaked onto blood agar plates. Any β-haemolytic colonies were sub-cultured on to a further blood agar plate to ensure single colonies and gram stained. Any gram-positive colonies were tested using an APIstrep kit to identify *S. equi* subsp. *zooepidemicus.*

Data analysis

All sample, diagnostic results and questionnaire data were entered onto a spreadsheet programme (Microsoft Excel for Mac). Visual verification of correct data input was verified for 5% of entries.

The outcome variable used was case or control status and the associations with predictor variable were assessed using univariable logistic regression using R Studio (version R 3.4.1 GUI 1.70 El Capitan build).

Twenty-two predictor variables were assessed including gender (female or male), neutered status (entire or neutered), breed (re-classified from questionnaire responses as pedigree or crossbreed), number of dogs in household (classified into three groups: 1 dog per household, 2 dogs per household, 3 or more dogs per household), contact with other animals (yes or no), contact with other dogs excepting those in the household (yes or no), contact with cats (yes or no), access to a private garden (yes or no), access to public spaces (yes or no), access to woods (yes or no), access to water streams (yes or no), recent stay or visit to a multi-animal household (e.g. shelter, boarding kennel, veterinary surgery, etc., re-classified from questionnaire data to yes or no), food (re-classified into two categories commercial or home-cooked/mixture), vaccination history (re-classified into two groups due to small sample size: within the last year and over one year ago/never), kennel cough vaccinated (yes or no, reclassified from questionnaire data based on vaccine brand and type information given by owner), worming (categorised into within last 3 months, within last year or never) and flea and tick control status (classified into

within last three months and more than three months ago/never), reason for veterinary visit (classified into routine appointment, ill health – respiratory or ill health – not respiratory), previous history of respiratory disease (yes or no), antibiotic usage (yes or no), other current medication (yes or no) and history of concurrent medical conditions (yes or no).

Univariable logistic regression was also used to analyse the relationship between pathogen presence and case or control status.

To build a multivariable analysis model, univariable analysis results were taken into account in combination with knowledge of the CIRD clinical syndrome. Roughly, one explanatory variable per 10 cases was considered. The model was then built using the two variables with lower p-values on univariable analysis.

5.4 Results

Out of the 36 practices recruited into the study, only 11 submitted samples from canine patients. Figure 5.1 shows the approximate geographical location of these practices.

The number of samples submitted by each practice was variable ranging from 3 to 32 samples (median 9). A total of 45 dogs meeting the case definition and 87 controls presenting to participating veterinary practices were recruited into the study. Similarly to chapter 4, due to low number of samples, and the failure to always collect matched case control sets and questionnaire responses in some practices (with instances where no controls or no cases were collected in a participating practice) data were analysed using an unmatched case-control design. Table 5.2 summarises study sample signalment and pathogen detection.

Table 5.2. Study population signalment and pathogen detection rates.

Variable		*n (%)*	*CAV-2*	*CDV*	*CPiV*	*CHV*	*CRCoV*	*Influenza A*	*CnPnV*	*B.b*	*M.cynos*
			n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Case		45 (34)	0 (0)	0 (0)	0 (0)	0 (0)	8 (17.8)	0 (0)	2 (4.4)	3 (6.7)	2 (4.4)
Control		87 (65.9)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1.2)	18 (20.7)	2 (2.3)
Gender	Male	30 (46.2)									
	Female	35 (53.8)									
Neutered	Yes	49 (75.4)									
	No	16 (24.6)									
Breed	Pedigree	47 (72.3)									
	Crossbreed	18 (27.7)									
Age.	4 – 180 months										

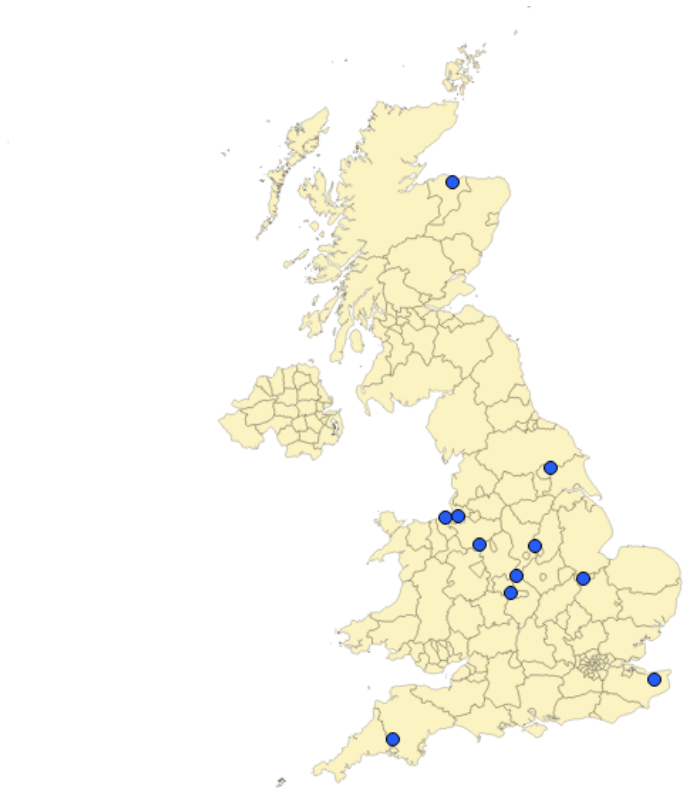

Figure 5.1 Map of United Kingdom showing the approximate location of practices that returned samples to the study.

Contact of participants was attempted at least three times via phone. Out of the total number of dogs recruited into the study (n=132), questionnaire data were obtained for only 50% (n=65) of participants.

Out of the 22 cases for which questionnaire data were obtained, 19 (86.4%) owners reported their dog had been coughing, followed by six (27.3%) owners reporting sneezing and the same number reporting a runny nose. Trouble breathing was reported four times (18.2%), fever was reported in three instances (13.6%). Eye discharge and conjunctivitis were each reported twice (9.1%). Eleven owners (50%) reported a single clinical respiratory sign during questionnaire administration (with the majority of these, n=9, reporting coughing as the only noticed clinical sign).

Within the case group, 18 animals were taken for a veterinary consultation due to exhibiting respiratory disease signs The four remaining dogs presented for routine consultations and were identified as suffering from respiratory disease and recruited

as cases by the veterinary surgeon at the time of consult; in these animals the signs of respiratory disease were reported by their owners during questionnaire administration.

Seven of the cases had started exhibiting respiratory signs within 48 hours of presenting to the veterinary surgery for consultation. Four animals had been suffering from respiratory disease for 3 to 7 days before their consultation, two animals for 8 to 14 days and seven animals for over 14 days. Two owners didn't know when their dog's respiratory signs had started. All dogs for which questionnaire data were obtained were pet dogs (no working or service dogs in the sample) and no animals were on antibiotics at the time of sampling.

When asked if they could identify any factors that could have led to their animals contracting respiratory disease, five owners mentioned their animal had recently attended "doggy day-care", four owners thought it had been contracted during walks out, two owners mentioned being aware of ongoing CIRD outbreaks in the area and two owners had taken their animals to dog shows. Five owners also mentioned having mild respiratory disease at the same time as their dogs.

Univariable analysis of signalment and clinical data collected from questionnaires are shown in Appendix 5B. The only variable with a p-value<0.05 on univariable analysis was neutered status with cases being 5.14 times more likely to be entire than controls. Age as a continuous variable was assessed for linearity using a generalised additive model (GAM) (figure 5.2) and was found not to have any significant non-linear relationship with case or control status.

To build the multivariable logistic regression model the following variables with p-values<0.2 in the univariable analysis were initially used: prior respiratory disease, vaccination status, worming status, flea and tick treatment status, currently medicated, access to water streams, neutered status and age. Table 5.3 shows the final multivariable model including variables with p-value<0.05.

In the final model, cases were almost 8 times more likely than controls to be entire and dogs were more likely to suffer from respiratory disease as they got older (OR=1.02, per month).

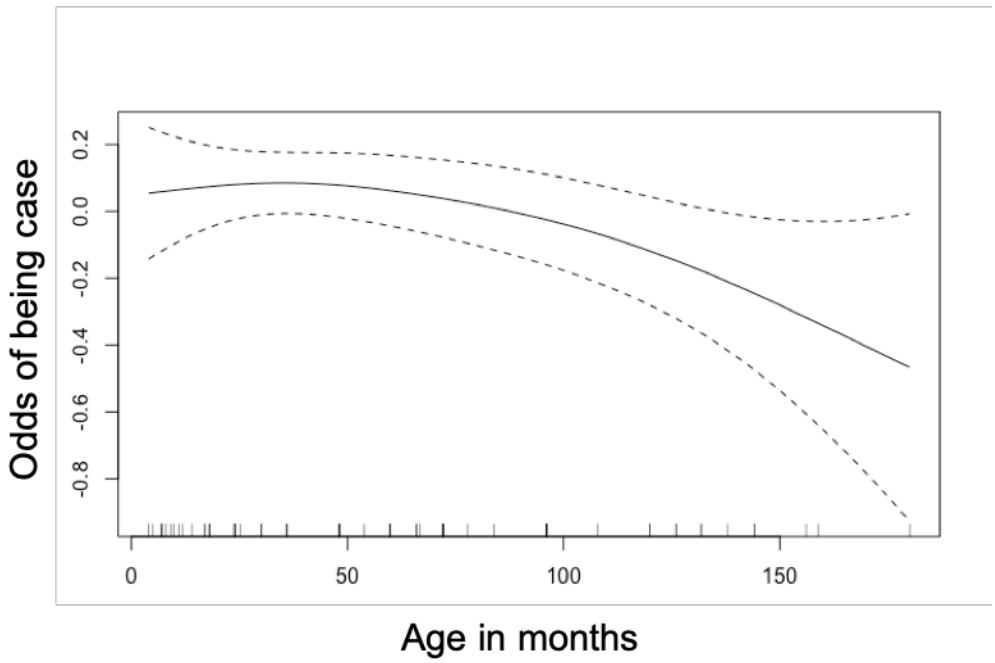

Figure 5.2 GAM plot of dog age against odds of being a case.

Table 5.3. Multivariable logistic regression of factors associated with respiratory cases in dogs attending 11 veterinary practices in the UK (n=132; 45 cases and 87 controls).

Variable		*Coefficient (Std. Error)*	*Odds ratio*	*Lower 95%CI*	*Upper 95%CI*	*Wald P-value*
Neutered status						
	Yes		Ref.			
	No	2.07 (0.69)	7.95	2.19	33.85	0.003
Age (in months)		0.02 (0.006)	1.02	1.003	1.029	0.02

Ref.= reference category

The results of laboratory testing and univariable logistic regression of PCR testing results of 132 animals (questionnaire data was only available for 65 of these animals) are shown in Table 5.4. No samples tested positive for CAV-2, CPiV, CDV, Influenza A or *S.equi* subsp. *zooepidemicus.* Eight samples (6.1%, all cases) tested positive for CRCoV (these belonged to four different practices: one practice had four positives and another had two positives, none of these animals being co-habitants; the remaining two practices had one positive each). Four samples (2 cases , 2 controls) (3%) tested positive for *M.cynos* (three positive samples belonged to the same practice – one case and two controls, with the two controls animals living together). Three samples (2.3%) tested positive for CnPnV with two of these having been collected in the same practice (1 case and 1 control, not co-habitants). Twenty one samples (15.9%) tested positive by PCR to *B.bronchiseptica* (only three of these were cases and none belonged to the same household). By bacterial culture, only five samples tested positive for *B.bronchiseptica* (one case and four controls). Only three (2.3%) mixed infections were identified, all of which were cases: one sample was positive for CnPnv and *B.bronchiseptica,* one was positive for CRCoV, *B.bronchiseptica* and *M.cynos* and the last one was positive for both CRCoV and *M.cynos.* A high proportion of cases tested negative for all tested pathogens (75.6%).

Cases were found to be 18.8 times more likely to test positive for CRCoV. Cases were also found to be less likely to test positive for *B.bronchiseptica* than controls in this sample. The differences between cases and controls for CnPnV testing results were not statistically significant.

Table 5.4 Univariable logistic regression results of PCR testing for pathogens associated with respiratory cases (n=132, 45 cases and 87 controls). Due to complete data separation, one extra data point (positive control) was added in order to run the logistic regression model.

Pathogen	*Case*		*Control*		*Odds Ratio*	*Wald*	*Lower 95%CI*	*Upper*
	n	*%*	*n*	*%*		*P-value*		*95%CI*
CAV-2								
Positive	0	0%	0	0%				
Negative	45	100%	87	100%				
CDV								
Positive	0	0%	0	0%				
Negative	45	100%	87	100%				
CPiV								
Positive	0	0%	0	0%				
Negative	45	100%	87	100%				
CHV								
Positive	0	0%	0	0%				
Negative	45	100%	87	100%				
*CRCoV**								
Positive	8	17.8%	0	0%	18.80	0.006	3.29	355.35
Negative	37	82.2%	87	100%	Ref.			
Influenza A								
Positive	0	0%	0	0%				
Negative	45	100%	87	100%				
CnPnV								
Positive	2	4.4%	1	1.2%	4.00	0.26	0.37	87.49
Negative	43	95.6%	86	98.9%	Ref.			
B. bronchiseptica *(PCR)*								
Positive	3	6.7%	18	20.7%	0.26	0.04	0.06	0.81
Negative	42	93.3%	69	79.3%	Ref.			
M. cynos								
Positive	2	4.4%	2	2.3%	1.98	0.5	0.23	16.9
Negative	43	95.6%	85	97.7%	Ref.			

5.5 Discussion and conclusions

CIRD is an important clinical syndrome in dogs, especially those housed in multi-animal environments. While in UK pet dog populations it may be perceived as less impactful, it is not uncommon to see regional, small-scale outbreaks of CIRD from time to time in these populations (authors unpublished observations). CIRD has historically been considered as a self-limiting condition leading largely to mild clinical signs. However, in recent years, this paradigm has slowly shifted with the emergence of pathogens for which vaccination is not available and which can lead to more severe disease such as canine influenza (CIV) in the USA, CnPnV and CRCoV (Priestnall et al., 2014). Despite its significance, few studies looking at vet visiting dogs have been conducted with most research focusing on multi-animal households (see Chapter 6). The author is not aware of any recent case-control studies focusing solely on CIRD in pet populations within the UK. Therefore, in this study, we sought to reappraise the role of known and potentially newer pathogens and dog-level risk factors in CIRD in a random population of UK veterinary visiting pet dogs.

As with FIRD, scoring has been used in prior studies to assign animals into disease groups based on observed clinical signs (Erles et al., 2003, Mitchell et al., 2017, Maboni et al., 2019). A recent study found that disease severity was associated with vaccination status for classic viral pathogens CPiV, CDV and CAV-2 and presence of CRCoV and CnPnV was associated with more severe disease presentations (Mitchell et al., 2017). Another recent study showed that co-infections lead to more severe disease and that animal age is an important predictor of disease severity (Maboni et al., 2019). As previously discussed, it would have been useful to take a similar approach of using clinical scoring for a more in-depth analysis of risk factors. However, grouping animals into cases or controls, without any further grouping, seems more appropriate given the small sample sizes.

In this case control study, entire dogs were found to be more likely to suffer from CIRD. This is an interesting finding similar to that of chapter two where entire cats were more likely to shed FCV, a FIRD pathogen. In cats, it is possible this increased risk is associated with the impact of neutering on behaviour, with entire cats tending

to roam more, which could lead to an increased likelihood of contact with other infected animals. For dogs, it is unclear why this would happen since, in most circumstances, their contact with other animals is likely controlled by their owners, with decisions to walk often revolving around owner needs not dog needs (Westgarth et al., 2017). Indeed, several studies have failed to find any association between neutering and dog walking (Westgarth et al., 2015, 2014). It is perhaps more likely that neutering itself is a marker for other factors not covered in the questionnaire. For example, it has been shown that owners of neutered animals may be more invested in the health care of their pets, and neutered dogs are more likely to be insured and vaccinated (Sánchez-Vizcaíno et al., 2018). Also, it is becoming increasingly clear that sex hormones have the ability to impact on immune reposes (Brown and Su, 2019). In dogs, hormonal changes associated with oestrus have been associated with reduced immune function, although in a bacterial infection (Sugiura et al., 2004). Understanding the basis of this apparent neutering effect on CIRD risk could provide new opportunities to enhance disease control. It is worth noting that these findings may be due to sampling bias. Similar biases have been identified in past studies where, for example, neutered cats were more likely to be registered with a veterinary practice in comparison with entire animals (Murray and Gruffydd-Jones, 2012).

Age was also found to be significantly associated with case status, with older animals tending to be more likely to have CIRD-like disease. This is also a curious finding since animals tend to have higher levels of immunity through both field exposure and vaccination as they grow older. These results could be down to the small sample size and a control selection bias as the controls were selected from the next veterinary visiting dogs following recruitment of a case. As with the previous chapter on feline infectious respiratory disease, the fact that recruited dogs were from a pool of animals registered and attending veterinary practices is known to introduce a selection bias in similarly designed studies (Murray and Gruffydd-Jones, 2012).

Vaccination status was not found to be significantly associated with case or control status whereas in prior studies occurrence and severity of CIRD was found to be lower in vaccinated animals (Mitchell et al., 2017).

In this study, 17.8% of all cases tested were positive for CRCoV. This virus was firstly described in 2003 and is now considered a pathogen of great importance in CIRD (Priestnall et al., 2014). The virus seemingly shows a strong association with disease and was not found in any control animals. In previous studies, CRCoV was also mostly found associated with disease. Joffe et al. (2016) looked at CIRD in pet populations attending Canadian clinics and found that 9.4% of cases were positive for CRCoV and no healthy animals tested positive for the virus. Schulz et al. (2014) reported a similar finding with 9.8% of diseased animals testing positive for the virus but with no positive results in healthy groups. Together these studies suggest that these respiratory coronaviruses may play a major role in CIRD and could represent a target for future vaccine development. Members of the *Coronaviridae* include both established and recently emerged respiratory pathogens in humans (Yin and Wunderink, 2018) and other animal species (Cavanagh, 2007; Saif, 2010), so it is not surprising that CRCoV has been found to be associated with CIRD (Erles and Brownlie, 2008).

CnPnV was found in three samples (2.27% of all samples) with two of these being cases (4.4% of cases). These numbers are significantly lower than findings previously reported in European mixed-origin samples (pet and kennelled), where this pathogen was detected in 23.4% of animals tested (Mitchell et al., 2017). It would be interesting to further explore these differences and understand whether they are related to the population being tested, in particular, pet dogs compared to kennelled animals.

Bordetella bronchiseptica, similarly to what was found in cats in the previous chapter, was found more often in controls than in cases, findings seemingly counter to the known role of *B. bronchiseptica* as a primary CIRD pathogen. These findings highlight the potential carrier role of healthy animals in the pet population (Bemis et al., 1977a) as well as the frequent role of *B. bronchiseptica* as an opportunistic pathogen

often acting synergistically after a virus firstly infects the host. As before, PCR was able to identify significantly more positives than bacterial culture. It is possible that the increased sensitivity of PCR meant we were able to detect lower levels of bacteria associated with a carrier state, below a threshold associated with disease. Being mostly live vaccines, *B. bordetella* can also be shed for some time after vaccination at high levels 3-10 days post vaccination, and at lower levels up to 28 days post-vaccination (Ruch-Gallie et al., 2016). Although previous studies have not found shedding of vaccine bordetella to be a common occurrence in the field (Binns et al., 1998), it would be interesting to genotype some of the isolates obtained in this study to see to what extent vaccine antigens as opposed to field isolates were responsible for the high rates of carriage in cases.

M. cynos was detected in two cases and two controls and pathogen detection was not found to be significantly associated with case status. The degree of importance of the role of *M. cynos* in the CIRD complex is still controversial mostly due to a paucity of studies focusing on this pathogen. However, some studies do indicate that *M. cynos* is associated with CIRD, its presence correlated with increased severity of disease (Chalker et al., 2004).

Several of the organisms tested for in this study were not detected. This is in part likely to reflect the underpowered nature of this study due to low number of dogs recruited. However, the absence of these pathogens still warrants some discussion. The absence of influenza A is in line with previous studies that suggest CIV has not been able to establish itself outside of the USA and Asia to become a major cause of CIRD. Similar findings have been previously recorded in Europe, Canada and Japan (Decaro et al., 2016; Joffe et al., 2016; Mitchell et al., 2017; Mochizuki et al., 2008). It will be important to continue to survey for canine influenza viruses in the UK, since it seems only a matter of time before human and canine movement will import this virus into what is likely to be a completely immunologically naïve population. If that does happen, it is likely that vaccines licensed for the control of these viruses would become available in the UK.

The absence of classical CIRD pathogens CAV-2, CDV or CHV mirrors that seen in pet and kennelled populations in a recent study by Decaro, et al. (2016). Given the fact vaccination against these pathogens is part of the widely recognised core vaccination schedule and that recent studies indicate that around 91.5% of UK vet-visiting dogs are vaccinated against these, these findings are not surprising and, if anything, are reassuring in regards to UK vaccination practices (Day et al., 2016; Sánchez-Vizcaíno et al., 2018). Similarly, CPiV was not found in this study's population. Most recent studies have reported CPiV as a major pathogen in CIRD with detection rates of 7.4 – 42% and a strong association with clinical signs (Decaro et al., 2016; Joffe et al., 2016; Mochizuki et al., 2008; Schulz et al., 2014). This is a curious finding and perhaps mirrors the fact that over 70% of the UK canine population seems to be vaccinated against CPiV (Sánchez-Vizcaíno et al., 2018).

S. equi subs. zooepidemicus was also not cultured from any of the samples submitted through this study. This pathogen is generally responsible for extremely severe and sporadic disease, with high mortality generally in larger groups of dogs with haemorrhage and pneumonia (Byun et al., 2009; Chalker et al., 2003a; Priestnall et al., 2014, 2010). This contrasts the generally mild nature of the disease seen in the cases recruited here.

As with the previous chapter, the broad case selection criteria applied in this study could lead to the inclusion of aetiologies of non-infectious origin. Other previously mentioned limitation (chapter 4) also apply here.

In conclusion, this chapter highlights the importance of newly emerged pathogens in CIRD, particularly CRCoV, as well as the importance of vaccination. More so, it highlights the need to re- evaluate the relevance of current CIRD vaccine antigens available with CRCoV seeming to be an important pathogen in which prevention through vaccination would be an interesting area to explore. Finally, with over 75% of cases not testing positive for any of the pathogens in this panel, it is important to question whether this is due to sampling collection, handling and testing issues,

whether there are still unidentified significant pathogens in circulation leading to CIRD clinical signs remaining to be discovered.

5.6 Appendix to Chapter 5

Appendix 5A: Questionnaires administered to participating owners via telephone.

Pet Health Study
Understanding infectious causes of respiratory disease in dogs and cats

PHONE ADMINISTERED OWNER QUESTIONNAIRE - DOG

1. General information:

Pet name	
Owner surname	
Owner postcode (to AA00 level eg.: CH60)	
Veterinary surgery	

2. Dog details:

a) Age: ☐ years ☐ months (known / estimated – circle correct)

☐ Don't know

b) Gender: Male ☐ Female ☐ Don't know ☐

c) Is your dog neutered (castrated/spayed)? Yes ☐ No ☐ Don't know ☐

d) Is your dog a: Purebreed ☐ Crossbreed ☐ Don't know ☐

e) If you answered purebreed, which breed is it? ☐

3. Lifestyle questions:

a) How many dogs are there in the house including this dog? ☐
b) Does your dog have contact with other animals?
Yes ☐ No ☐ Don't know ☐

c) If your answer to 3.b) was yes, which species does your dog have contact with?

☐ Other dogs
☐ Cats
☐ Cattle/sheep

☐ Pigs
☐ Poultry
☐ Horses
☐ Pet birds
☐ Wild animals (please specify) ☐
☐ Others (please specify) ☐

d) Does your dog have access to any of the following?

☐ Garden
☐ Woods
☐ Water streams
☐ Public spaces

e) During the last month has your dog stayed at or attended any of the following?

☐ Boarding kennel
☐ Vet surgery (excluding the one during which he/she was recruited for study)
☐ Rescue shelter
☐ Training/puppy classes
☐ Competitions/dog shows
☐ Groomers
☐ Other. Please specify: ☐

g) Your dog eats (choose all that apply):

☐ Commercial food (e.g: biscuits)
☐ Raw meat
☐ Home cooked meals
☐ Solely vegetarian
☐ Other. Please specify: ☐

h) Is your dog any of the following?

☐ Herding dog
☐ Hunting dog
☐ Assistance/service dog. Please specify: ☐
☐ None.

g) How long have you had your dog for? ☐

4. Medical information:

a) Is your dog vaccinated? When was he/she last vaccinated?

☐ Yes, within the last year.
☐ Yes, within the last three years.

☐ Yes, more than three years ago.
☐ Yes, he has had his first vaccination but hasn't received his booster yet.
☐ No, he is not old enough to receive vaccination.
☐ No, he was never vaccinated.
☐ Don't know.

b) Do you know the name of the last vaccine used? ☐

c) When was your dog last de-wormed?

☐ In the last 3 months.
☐ In the last year.
☐ Over a year ago.
☐ Don't know.
☐ Never.

d) When was your dog last treated for fleas and ticks?

☐ In the last 3 months.
☐ In the last year.
☐ Over a year ago.
☐ Don't know.
☐ Never.

e) What was the reason for your visit to the vet?

f) Before the vet visit you have just described, has your dog suffered from respiratory disease (example: coughing, sneezing, runny nose or eyes)? If yes, what was it? Did he/she receive any treatment?

g) Does your dog suffer from any other medical condition? If yes, explain.

h) Is your dog currently on antibiotics?

☐ Yes. Please specify: ☐
☐ No

i) Is your dog currently receiving any medical treatment?

☐ Yes
☐ No

j) If your answer to 4.c) was yes, what is the name of the product(s):

k) Is there any other information about your dog's health that you feel might be relevant?

l) Has your pet showed any of the following signs within the past 14 days?

☐ Coughing
☐ Sneezing
☐ Runny nose
☐ Runny eyes
☐ Nose bleed
☐ Red eye
☐ Trouble breathing
☐ Mouth ulcers
☐ Face/lip ulcers
☐ Fever

m) If you answered yes for any of these signs, when did they start?

☐ Last 48 hours
☐ Last 3 – 7 days
☐ Last 8 – 14 days
☐ or more days ago
☐ Don't know

n) Has your dog had any other signs of respiratory disease within the past 14 days?

☐ Yes. Please specify: ☐
☐ No
☐ Don't know

o) Do you have any thoughts on the reason your dog is suffering from respiratory disease?

p) Have you suffered from any signs of respiratory disease or conjunctivitis (red eye) yourself within the past 14 days?

Thank you for taking part in this study!

Appendix 5B Univariable logistic regression analysis of factors associated with case or control status in CIRD (questionnaires from 11 UK based veterinary practices; n=65, 22 cases and 43 controls)

Variable	*Case*		*Control*		*Odds*	*P-value*	*Lower*	*Upper*
	n	*%*	*n*	*%*	*ratio*		*95%CI*	*95%CI*
Gender								
Male	9	40.9%	21	48.8%	0.73	0.54	0.25	2.04
Female	13	59.1%	22	51.2%	Ref.			
Neutered status								
Yes	12	54.5%	37	86.1%	Ref.			
No	10	45.5%	6	13.9%	5.14	**0.008**	1.58	18.10
Breed								
Pedigree	17	13.9%	30	69.8%	Ref.			
Crossbreed	5	22.7%	13	30.2%	0.67	0.52	0.19	2.15
#dogs/household								
1	11	50%	22	51.2%	Ref.			
2	7	31.8%	13	30.2%	1.08	0.9	0.33	3.46
3 or more	4	18.2%	8	18.6%	1	1	0.23	3.96
Contact with other animals								
Yes	21	95.5%	42	97.7%	Ref.			
No	1	4.5%	1	2.3%	2	0.63	0.08	52.28
Contact with dogs								
Yes	21	95.5%	40	93.1%	Ref.			
No	1	4.5%	3	6.99%	0.63	0.70	0.03	5.31
Contact with cats								
Yes	7	31.8%	18	41.9%	0.65	0.42	0.21	1.88
No	15	68.2%	25	51.1%				

Variable	*Case*		*Control*		*Odds*	*P-value*	*Lower*	*Higher*
	n	*%*	*n*	*%*	*ratio*		*95%CI*	*95%CI*
Stay in multi-animal environment								
Yes	12	54.5%	21	48.8%	Ref.			
No	10	45.5%	22	51.2%	0.79	0.66	0.27	2.23
Access to private garden								
Yes	21	95.5%	39	90.7%	Ref.			
No	1	4.5%	4	9.3%	0.44	0.48	0.02	3.23
Access to public spaces								
Yes	21	95.5%	39	90.7%	Ref.			
No	1	4.5%	4	9.3%	0.46	0.50	0.02	3.39
Access to woods								
Yes	15	68.2%	35	81.4%	Ref.			
No	7	31.8%	8	18.6%	2.04	**0.24**	0.62	6.73
Access to water streams								
Yes	9	40.9%	26	60.5%	Ref.			
No	13	59.1%	17	39.5%	2.21	**0.14**	0.78	6.47
Food								
Commercial	16	72.7%	22	51.2%	Ref.			
Home cooked/raw	1	4.5%	2	4.7%	0.69	0.77	0.03	7.79
Both	6	27.3%	19	44.2%	0.43	**0.15**	0.13	1.29
Concurrent disease								
Yes	5	22.7%	13	30.2%	0.68	0.52	0.19	2.15
No	17	77.3%	30	69.8%	Ref.			

Variable	*Case*		*Control*		*OR*	*P-value*	*Lower 95%CI*	*Upper 95%CI*
	n		n					
Worming								
Within last 3m	14	63.6%	39	90.7%	Ref.			
Within last year	5	22.7%	2	4.7%	6.96	**0.03**	1.34	52.67
Over 1y ago/never	1	4.5%	2	4.7%	1.39	0.79	0.06	15.67
Flea/tick treatment								
Within last 3m	15	68.1%	38	88.4%	Ref.			
>3m ago	4	18.2%	2	4.7%	5.07	**0.08**	0.89	39.41
Never	1	4.5%	3	6.9%	0.84	0.89	0.04	7.21
Vaccination status								
Within last year	17	77.3%	40	93.0%	Ref.			
Over 1y/never	4	18.2%	2	4.7%	4.70	**0.09**	0.84	36.33
CIRD vaccination								
Yes	2	9.1%	8	18.6%	0.50	0.46	0.06	2.84
No	6	27.3%	12	27.9%	Ref.			
Prior respiratory disease								
Yes	6	27.3%	6	13.9%	2.25	**0.21**	0.62	8.27
No	16	72.7%	36	83.7%	Ref.			
Currently medicated								
Yes	8	36.4%	9	20.9%	2.16	**0.18**	0.68	6.8
No	14	63.6%	34	79.1%	Ref.			
Age (month)					1.01	**0.07**	0.99	1.02

Ref.= reference category

6 Cross-sectional and longitudinal surveys of canine and feline infectious respiratory disease-associated pathogens in four British animal rescue centres – a descriptive study

6.1 Abstract

Infectious respiratory disease in both dogs and cats is an important issue in rescue shelters, leading to increased running costs and decreased animal welfare, and is a frequent cause of failure to rehome animals. In this study, cats and dogs from four rescue shelters were sampled cross-sectionally in order to assess carriage status of FIRD- and CIRD-associated and potentially associated pathogens. One the participating shelters was also sampled longitudinally for a total period of 10 weeks.

During the cross-sectional sampling visits, oropharyngeal (OP) swabs were collected from 42 dogs and 108 cats. Using polymerase chain reaction (PCR), feline calicivirus feline herpesvirus and *Mycoplasma felis* were detected in 7.4%, 4.6% and 16.8% of feline samples. No feline samples were positive for *Chlamydophila felis*, influenza A or *Bordetella bronchiseptica*. Canine samples tested negative for all pathogens screened for including canine adenovirus type 2, canine parainfluenza virus, canine distemper virus, canine herpesvirus, canine respiratory coronavirus, canine pneumovirus, influenza A, *Bordetella bronchiseptica, Mycoplasma cynos and Streptococcus equi subps. zooepidemicus* (all tested for using PCR with the exception of the latter for which bacterial culture was used).

During the longitudinal study, OP swabs were collected from 10 dogs and 10 cats each week. *M. felis* was the microorganism identified the most from feline samples and through most of the sampling period. FCV was also detected in 6 of the 10 weeks of sampling. For both pathogens, most positive animals did not present any clinical signs at the time of sampling. In dogs, CnPnV was identified during weeks 9 and 10, which coincided with shelter staff reporting CIRD like clinical signs (especially cough); however, CnPnV-positive dogs did not present any clinical signs when sampled.

This study highlights the importance of clinically silent carriers of infection acting as disease reservoirs; such animals pose an infection risk for naïve animals entering rescue shelters. Further work is also needed to understand the potential for CnPnV to cause disease in these shelters.

6.2 Introduction

Rescue centres, often referred to interchangeably as animal shelters, rehoming centres or rescue kennels/catteries, have been estimated to handle approximately 90,000 dogs and 160,000 cats per year in the UK (Stavisky et al., 2012a). These institutions have multiple important roles, which can include not only providing shelter, rehoming animals, and controlling stray and unwanted animal populations, but also preventing animal cruelty, educating members of the public and improving animal welfare standards (Pesavento and Murphy, 2014).

Despite the positive role played by animal shelters in combatting overpopulation and improving animal welfare, these environments prove to be very challenging. The risk of exposure, susceptibility and transmission of infectious diseases in the shelter environment, where high numbers of animals are housed, is considered to be amplified due to several factors such as increased fomite contact, high levels of animal stress, high animal turnover and constant introduction of new, often immunologically naïve, animals (Pesavento and Murphy, 2014).

While upper respiratory tract infections in household pets are common and usually relatively easy to manage, in the shelter context of "herd health management" where disease is quickly transmitted between animals, this becomes a major issue (Bannasch and Foley, 2005; Pedersen, 1991; Pesavento and Murphy, 2014). Additionally, shelters have often been found to provide the perfect context for the emergence of new pathogens (Pesavento and Murphy, 2014; Priestnall et al., 2014).

In dogs, CIRD is a disease complex of multifactorial origin and a major issue in animals housed in shelters (Erles et al., 2004; Erles and Brownlie, 2005; Mitchell et al., 2017; Priestnall et al., 2014). The disease complex can become enzootic in these environments due to the constant introduction of new infectious agents to the canine population (Mochizuki et al., 2008). The disease is usually characterised by a dry hacking cough from which most animals recover in a short timeframe. However, in certain cases, more severe disease may develop leading to bronchopneumonia and can be a reason for euthanasia or result in fatalities (Priestnall, 2017). In addition to

causing discomfort to the affected animals, CIRD can also lead to increased costs to the shelter (i.e. treatment) as well as delays to training and rehoming of dogs. Additionally, sometimes it can result in the temporary closure of shelters (Erles et al., 2003; Erles and Brownlie, 2005).

While classic pathogens such as *B. bronchiseptica,* CAV-2, CDV, CPiV and CHV-1 are still considered important CIRD pathogens, the routine use of vaccination as well as antibiotics is thought to have exerted selective pressure on pathogens in the UK leading to a decrease in prevalence of these (Priestnall et al., 2014). Recently emerged pathogens have often been originally detected in the shelter environment and are commonly encountered in these settings. Examples are CRCoV, first isolated during an investigation into outbreaks of CIRD-like disease in a UK shelter (Erles et al., 2003), and CnPnV, described following a retrospective study of respiratory disease in USA shelters (Renshaw et al., 2010). *M. cynos* was also found to be associated with respiratory disease during a study undertaken at a large rehoming shelter (Chalker et al., 2004) and *S. equi subsp. zooepidemicus* has been often described in association with outbreaks of severe disease with high mortality in kennels (Britton and Davies, 2010; Byun et al., 2009; Priestnall et al., 2010). In respect to CIV, the pathogen is also most prevalent in multi-animal environments such as kennels, where it has been observed to rapidly spread especially among immunologically naïve dogs of all ages (Dubovi, 2010).

In the feline sheltered population, FIRD also comprises a major issue. Cats are characterised as solitary territorial predators and thus, the shelter environment leads to a significant loss of life quality and decreased wellbeing in this species. This occurs not only due to the high levels of stress cats are subject to, but also due to the constant introduction of new pathogens (Möstl et al., 2013). FIRD pathogens including FCV, FeHV-1, *M.felis, C.felis and B.bronchiseptica* are frequently introduced to shelters due to high animal turnover, with the shelter environment amplifying the spread of disease and leading to more severe clinical presentations (Pedersen et al., 2004). Molecular studies with FCV have shown high numbers and variability of viruses introduced into rescue shelters, as well as high potential for transmission

(Coyne et al., 2007a; Radford et al., 2001). More recently influenza viruses have been isolated associated with FIRD, with additional zoonotic spread to people working in the shelter (Belser et al., 2017). As in dogs, upper respiratory tract disease in pet cats is often relatively easy to manage. However, in shelters, it leads to serious problems as infected animals pose a high risk to other cats, decrease in adoption rates and increased costs due to treatment and disease management (Bannasch and Foley, 2005). The specific case of FeHV-1 infection is particularly problematic since the stress of being in a shelter environment often leads to reactivation of latent virus which can lead to disease outbreaks and severe chronic sequelae in affected animals (Pedersen et al., 2004). Furthermore, once adopted, these animals may again recrudesce in the first few days at a new home which can lead to frustration by adopters and have a negative effect on the public perception of shelters (Bannasch and Foley, 2005).

As highlighted above, it is challenging for shelters to manage infectious respiratory disease in housed animals. Additionally, each shelter faces different problems when faced with disease outbreaks or endemic CIRD or FIRD due to factors unique to each organisation (such as staffing, infrastructures, budget, biosecurity measures and management strategies). Thus, the goal of this study was to gain a basic understanding of the FIRD and CIRD pathogens currently circulating in UK based shelters with a focus on both classical and more recently emerged respiratory disease associated pathogens. In order to achieve this, a cross-sectional study was conducted across four UK based animal shelters housing both cats and dogs, with varied population dynamics and management philosophies. Additionally, a longitudinal study was conducted in one of the shelters looking at FIRD and CIRD pathogen shedding for a period of 10 consecutive weeks.

6.3 Materials and Methods

Sampling

Four rescue centres were selected to participate in this study based on convenience (ease of access, previous participation in similar projects as well as through professional contacts). These were designated as R1 (Merseyside), R2 (North Wales), R3 (North Wales) and R4 (West Midlands) (Table 6.1).

All centres were visited once with the exception of R1 which was visited once a week for 10 consecutive weeks. Oropharyngeal samples were collected by the author and University of Liverpool staff members from both cats and dogs as described in previous chapters. Gloves, single use overalls and shoe covers were changed between sampled animals when housed separately. Samples were transported back to the Leahurst Campus, University of Liverpool on the day of collection and handled and stored as previously described (Chapter 4 and 5).

Table 6.1 Details of the four rescue centres sampled within this study. All capacities are approximate as some animals are sometimes housed in groups.

Shelter	***Location***	***Capacity***	***Species***	***Contact with other animals***	***Isolation facilities***
R1	Merseyside	180	Dogs and cats	No	No
R2	North Wales	220	Dogs and cats	Yes (dogs only)	Yes
R3	North Wales	500	Dogs and cats	Yes (cats only)	No
R4	West Midlands	na	Dogs and cats	Yes (cats only)	Yes

Further information about participating shelters

Shelter R1 is part of a larger, nation-wide organisation and has in-house veterinary surgeons and veterinary facilities where consultations, treatment and small

procedures are undertaken. No isolation or quarantine facilities were present at the time of sampling. Cats and dogs are housed in separate buildings with an approximate capacity of up to 60 cats and 120 dogs. All animals were vaccinated upon entry to the shelter and, in a significant number of cases, even before this, as most were triaged in a larger branch of the organisation. Three weeks prior to sampling, a suspected outbreak of FIRD had occurred and the cat wing of the shelter was closed to the public. The clinical signs shown by affected animals during the outbreak comprised mouth ulcers, nasal discharge and sporadic coughing; During this suspected outbreak, nine animals were euthanised.

Shelter R2 is a modern, purpose built, independently run shelter with in-house veterinary surgeons and a fully equipped veterinary clinic. Isolation facilities are available and used for newly arrived, non-vaccinated animals, as well as animals showing clinical disease. At the time of sampling, approximately 85 dogs and 100 cats were housed in the shelter. Cats and dogs were housed in separate buildings and all animals were vaccinated upon admission.

Shelter R3 is an independent shelter based at the shelter manager's own residence set in farmland. Some animals (both cats and dogs, unknown number) lived with the shelter manager and were allowed to roam the premises. Cats and dogs were housed in separate buildings, but cats were often housed in groups ranging from 3 to 15 animals. Vaccination policies were unclear, biosecurity measures were found to be poor and records on animal history very unreliable.

Shelter R4 is comprised of modern built housing facilities and a fully equipped veterinary hospital and is part of a larger, nation-wide organisation. All animals were vaccinated upon entry and isolation facilities were available for unvaccinated or ill animals.

Cross-sectional study

In each shelter, sample collection was performed on a group of animals selected based on convenience by staff members at each of the centres. This was based on

ability to handle each animal, behavioural issues as well as concomitant non-respiratory disease health issues in which animals were deemed to be painful or uncomfortable in being handled; such animals were excluded. Table 6.2 shows number of animals sampled in each centre. Whenever available, details on age, gender, breed, age, vaccination and neutering status, length of stay in the centre and history of respiratory disease were collected.

Longitudinal study – R1

Rescue centre R1 was selected for a longitudinal study due to its convenient location. Following on from the cross-sectional study, R1 was visited weekly for 9 consecutive weeks (10 weeks in total). All animals that were sampled on the previous week were then re-sampled if still present at the centre. Otherwise, new animals were selected by convenience as previously described. Details on signalment and history of respiratory disease were collected as for the cross-sectional study. Throughout the 10 weeks of study, a total of 100 samples were collected from cats (corresponding to a total of 30 animals) and a total of 100 samples were collected from dogs (corresponding to a total of 31 animals).

Nucleic acid extraction, reverse transcription and diagnostic testing

Viral nucleic acid (RNA and DNA) was extracted from oropharyngeal swabs using the same method as described in Chapter 2 (Viral RNA mini-kit; Qiagen). Reverse transcription was performed using 200 ng random hexamers (Superscript III, Life Technologies). Diagnostic tests using PCR and bacterial culture are previously described in chapter 4 (feline FIRD pathogens) and chapter 5 (canine CIRD pathogens). Canine samples were tested for *B. bronchiseptica, S. equi subsp. zooepidemicus, M. cynos,* CHV, CnPnV, CRCoV, CPiV, CAV-2, CDV and influenza A. Feline samples were tested for *B. bronchiseptica, M. felis, C. felis,* FCV, FeHV-1 and influenza A.

Data analysis and result description

Pathogen prevalence and confidence intervals were calculated for the cross-sectional study using EpiTools (Ausvet). Data collection and recording by shelters was found to be unreliable and factors such as age and kennel length of stay found to often be estimations, therefore further structured epidemiological analysis was not performed.

6.4 Results

6.4.1 Cross-sectional kennel sampling

A total of 163 samples were collected (42 canine samples and 108 feline samples). Table 6.2 shows number of animals sampled in each centre. Overall, 7.4% of feline samples tested positive for FCV (8/108; 95% CI 3.8, 13.9), 4.6% of feline samples tested positive for FeHV-1 (5/108; 95% CI 2.0, 10.4) and 16.8% (18/108; 95% CI 10.8, 24.8) of samples tested positive for *M.felis.* Table 6.3 shows the prevalence breakdown of these pathogens per rescue centre.

Of the eight animals testing positive for FCV, five were from rescue centre R4: three had no recorded history of FIRD, one had unilateral eye discharge at the time of sampling and one had history of chronic gingivostomatitis. All of the above were fully vaccinated with the exception of one young animal who had only received his first vaccinations (primary vaccination; this animal was the one showing unilateral eye discharge at the time of sampling; figure 6.1A). Two FCV positive animals were from rescue centre R3. These were housed in the same pen with one of them having nasal discharge and sneezing at the time of sampling and the other showing no signs of FIRD. The remaining FCV positive sample was collected from an animal in R2 who had no recorded history of FIRD.

All the animals testing positive for FeHV-1 were housed in R3. Two of these animals shared the same pen and both had no history of FIRD, while one was housed with four other cats and had signs of FIRD at the time of sampling (sneezing and nasal discharge while its co-habitants had no clinical signs, figure 6.1B). The remaining animal was housed with a different group of cats and did not show any clinical signs of FIRD at the time of sampling. No reliable history was available for these animals.

A total of twenty samples tested positive for *M. felis* (n=2 in R1, n=12 in R3, n=6 in R4). The two positive samples from R1 corresponded to cats with recent (less than weeks) recorded history of bilateral eye discharge and conjunctivitis, respectively. In R4, one of the animals testing positive for this pathogen had a recent (less than 2 weeks) history of ocular discharge and one animal suffered from chronic

gengivostomatitis. There was no reliable history recorded for any of cats testing positive for *M. felis* from R3.

One cat tested positive for both *M.felis* and FCV (R4). No cats tested positive for Influenza A, *B. bronchiseptica* or *C. felis.* All canine samples were negative for all pathogens tested.

Figure 6.1 A – cat sampled from R1 showing unilateral ocular discharge, tested positive for FCV. B – cat from R3 showing signs of purulent nasal discharge, tested positive for FeHV-1.

Table 6.2 Number of animals sampled per rescue centre during cross-sectional sampling (four rescue centres, n=163 animals sampled in total, 42 dogs and 108 cats).

Shelter	***Number of dogs sampled***	***Number of cats sampled***	***Total of animals sampled***
R1	10	16	26
R2	21	21	42
R3	14	29	43
R4	10	42	52

Table 6.3 Breakdown of positive results for feline samples per shelter during cross-sectional sampling.

Shelter	***# cats sampled***	***FCV Positive***	***FeHV-1 positive***	**M. felis *positive***
R1	16	0 (0%)	0 (0%)	2 (12.5%)
R2	21	1(4.8%)	0(0%)	0(0%)
R3	29	2(6.9%)	5(17.2%)	12(41.4%)
R4	42	5(11.9%)	0 (0%)	6 (14.3%)

6.4.2 Longitudinal kennel sampling

Results of longitudinal sampling are shown in Table 6.6 (dogs) and 6.7 (cats). Cats and dogs were followed for 1 to 10 weeks, being lost to follow-up when rehomed or euthanased. Of the 31 dogs sampled in R1, three dogs were followed and sampled during the 10 weeks of study and the remainder were sampled between one and seven times for the duration of the study. Of the 30 cats sampled, only one was sampled 10 times (one per week of the study) while the others were sampled between one and eight times.

Over the ten-week period (Table 6.6), three dog samples tested positive for *B. bronchiseptica* (corresponding to two animals: one animal with a positive status on week 2 and another on weeks 6 and 7; neither had CIRD related clinical signs at the time of sampling or a recorded history of CIRD like disease).

A total of eight samples tested positive for CnPnV (corresponding to seven animals with a positive status with a duration of one to two weeks between weeks 9 and 10; during this period volunteer shelter staff reported coughing in some sheltered dogs but these observations could not be corroborated as these clinical signs were not observed during sampling by University of Liverpool staff). No co-infections were detected in sampled dogs.

For feline samples (Table 6.7), nine tested positive for FCV (corresponding to six animals with a positive status with a duration of one to three weeks; none of these animals had a recorded history of FIRD or signs at time of sampling with the exception of C17 who showed signs of bilateral conjunctivitis and lingual mouth ulcers, Figure 6.2). Interestingly, C21 and C22 which tested positive for FCV in week 8 were housed in the same pen. Two samples tested positive for FeHV-1 (corresponding to two animals, identified as positive once each, in different weeks; neither of these animals had CIRD signs or a recorded history of CIRD like disease and had been in the shelter for over one month each). A total of 19 samples tested positive for *M. felis* (corresponding to ten animals and a duration of positive status of one to four weeks). Of these, C1 and C2 had a recorded history of chronic gingivostomatitis, C5 and C20

had signs of mild conjunctivitis and eye discharge on the day of sampling and C3, C6, C9, C12, C13 and C26 had no recorded history of CIRD or signs at time of sampling. Finally, one feline sample tested positive for *B. bronchiseptica* on one single week (this animal had no history of respiratory disease). Cat C9 was positive for both FeHV-1 and *M.felis* on week 5 and C12 tested positive for both FCV and *M. felis* in weeks 5 and 6 (no history of FIRD had been recorded for either animal, nor did they present with any signs of respiratory disease at the time of sampling).

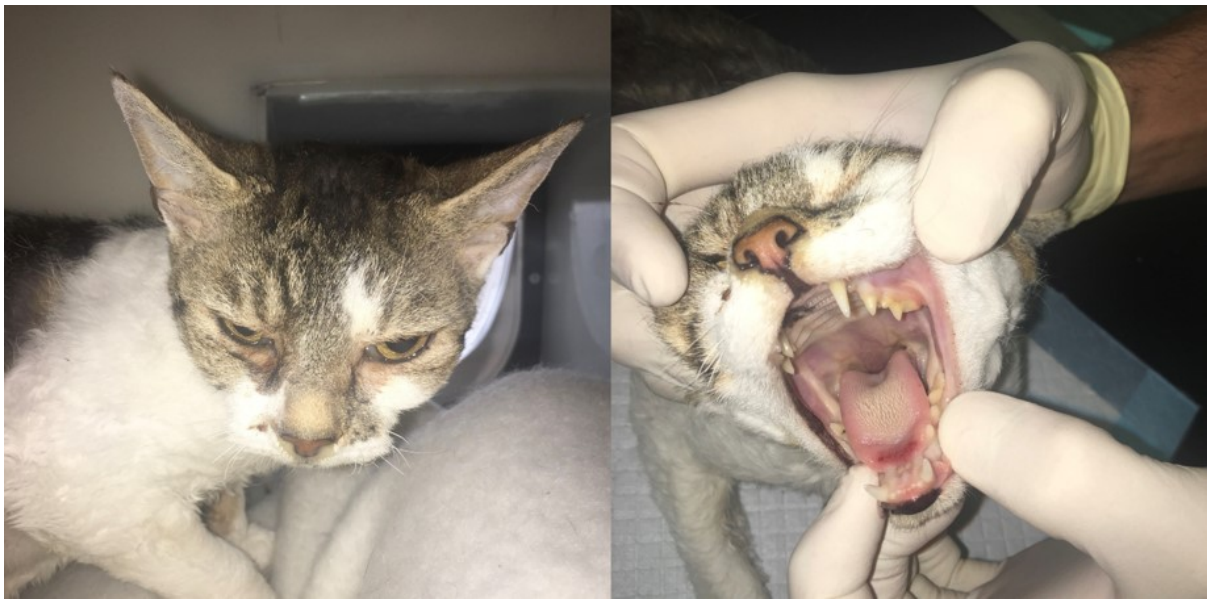

Figure 6.2 C17 from R1 showing signs of conjunctivitis and bilateral purulent ocular discharge (left) as well as a large lingual ulcer in the most cranial portion of the tongue (right).

Table 6.6 Results of cross-sectional (week 1) and longitudinal (week 2 - 9) sampling of dogs at R1. Each column represents the week of sampling (all animals sampled on the same day each week), and each row represents an individual dog. Cells are colour-coded: grey – animal was sampled, white – animal was not sampled.

	Week of sampling									
Dog n	1	2	3	4	5	6	7	8	9	10
D1										
D2										
D3										
D4									CnPnV	CnPnV
D5										
D6										
D7										
D8										CnPnV
D9										
D10										
D11										
D12		Bb								
D13										
D14										
D15										
D16										
D17						Bb	Bb			CnPnV
D18										
D19										
D20										
D21										
D22									CnPnV	
D23										
D24									CnPnV	
D25										
D26										
D27										CnPnV
D28										CnPnV
D29										
D30										
D31										
% (n)		Bb: 10% (1/10)				Bb: 10% (1/10)	Bb: 10% (1/10)		CnPnV: 30% (3/10)	CnPnV: 50% (5/10)

Table 6.7 Results of cross-sectional (week 1) and longitudinal (week 2 - 9) sampling of cats at R1. Each column represents the week of sampling (all animals sampled on the same day each week), and each row represents an individual cat. Cells are colour-coded: grey – animal was sampled, white – animal was not sampled.

	Week of sampling									
Cat n	1	2	3	4	5	6	7	8	9	10
C1	M.felis	M.felis	M.felis	M.felis						
C2	M.felis									
C3	M.felis	M.felis	M.felis							
C4										
C5	M.felis									
C6	M.felis									
C7										
C8										
C9			M.felis		FeHV-1 M.felis					
C10										
C11										
C12					FCV M.felis	FCV M.felis	M.felis	M.felis		
C13					M.felis					
C14										
C15										
C16										
C17						FCV				
C18						FeHV-1				
C19										
C20							M.felis			
C21								FCV		
C22							FCV	FCV	FCV	
C23								FCV		
C24										FCV
C25										
C26										M.felis
C27										
C28										Bb
C29										
C30										
% (n)	M.felis: 50% (5/10)	M.felis: 20% (2/10)	M.felis: 30% (3/10)	M.felis: 10% (1/10)	M.felis: 30% (3/10) FeHV-1: 10% (1/10) FCV: 10% (1/10)	M.felis: 10% (1/10) FCV: 20% (2/10)	M.felis: 20% (2/10) FCV: 10% (1/10)	FCV: 30% (3/10)	FCV: 10% (1/10)	M.felis: 10% (1/10) FCV: 10% (1/10) Bb: 10% (1/10)

6.5 Discussion and conclusions

Infectious upper respiratory tract disease in dogs and cats (i.e. FIRD and CIRD) is common in shelter environments worldwide and comprises a serious burden to these organisations (Belser et al., 2017; Byun et al., 2009; Möstl et al., 2013; Pereira et al., 2018; Pesavento and Murphy, 2014; Tanaka et al., 2017; Willi et al., 2016). Despite usually leading to low mortality rates, these multifactorial disease complexes cause various other issues such as increased shelter running costs and delays in animal adoption as well as having a significant impact on animal welfare (Chalker et al., 2004; Opperman and Brownlie, 2018; Schulz et al., 2014; Veir et al., 2008). In this study, we sought to investigate the presence of known and suspected CIRD and FIRD pathogens in UK animal shelters. Since some respiratory pathogens are potentially transmissible between dogs and cats, we for the first time focussed these studies on shelters housing both species.

The sampling strategy utilised in this study was very selective (convenience based). Therefore, generalisation of results is not possible. Aditionally, the lack of data such as length of stay and total number of animals present at the shelter (unavailable at the time of sampling) led to a limited analysis of results.

In the first and cross-sectional portion of this study, 18.4% (30/163) of the samples were positive for at least one of the tested pathogens. All these samples were from cats, with all dogs testing negative for the panel of pathogens tested for in this study.

Out of the 30 cats positive for any tested pathogen, only a small proportion of animals had a recent history of respiratory disease. Similar observations have been made in prior studies, where up to 80% tested animals were positive for FIRD-associated pathogens but clinical disease was uncommon (Coyne et al., 2007a; Tanaka et al., 2017). This partly could be due to the fact that, in the participating shelters, most animals were vaccinated at the time of admission, but also highlights the importance of asymptomatic carriers, especially for FCV, in the spread of infection in high-turnover environments such as these (Coyne et al., 2007a; Radford et al., 2009). Unfortunately, our conclusions here are limited due to the unreliable

record keeping at some of these shelters as well as the unknown medical history of most of these animals prior to arrival at the rescue centre.

The percentage of FCV and FeHV-1 positive samples during the cross-sectional study was 7.4% (8/108; ranging between 0 and 11.9% among participating shelters) and 4.6% (5/108; all samples from same shelter), respectively. These numbers are lower than those found in previous studies (Bannasch and Foley, 2005; Helps et al., 2005; Radford et al., 2001) and may mirror the good biosecurity and disease prevention measures applied in most of the participating shelters. With the exception of R3, all participating shelters had well trained members of staff and volunteers. Consistent biosecurity measures were seen to be applied, including, but not limited to, hand washing or change of gloves between handling different animals, thorough cleaning and disinfection of kennels, use of different facilities for vaccinated and unvaccinated animals and refraining from keeping animals in groups (apart from where mom and litters were present). This was not the case for R3 where often cats would be kept in large groups and new animals from unknown provenance were introduced to the group.

Previous studies have used the variability of FCV to look at the epidemiology of transmission of infection within shelters, suggesting, at least in the populations they studied, that infection was maintained by frequent import of new pathogens into the shelter with new animals, complemented by rarer examples of transmission within the shelter (Coyne et al, 2006). The pattern of infection we observe here, with some animals testing positive when first sampled, whilst others becoming positive after being in the shelter for some time, perhaps suggests a similar epidemiology here. Unfortunately time prevented the sequence analyses of the identified pathogens in this study,

M.felis was found in 16.8% of samples (20/108; ranging from 12.5% to 41.4% in participating shelters). These numbers are also lower than previous studies where the prevalence of *M.felis* were found to range from 20% to 47% depending on the population (Berger et al.; Fernandez et al., 2017). Interestingly, *M. felis* was, overall,

the microorganism most often found in these shelters. A recent systematic review and meta-analysis of the association between *Mycoplasma spp.* and FIRD suggested that *M. felis* may be a primary pathogen of the upper respiratory tract in cats but cautioned that this may not happen in all environments, especially not in shelters (Le Boedec, 2017). These observations are in line with the results found in this study as *M. felis* was found in a number of animals, but most of these did not present with any signs of respiratory disease at the time of sampling and no history of recent respiratory disease had been recorded for them (although, it is worth reiterating that records were often found to be unreliable).

The fact that, in the cross-sectional portion of the study, no dog tested positive for any of the pathogens is curious, especially given the fact that the same methods were used in chapter 5 where several samples were positive for the tested pathogens. This is particularly interesting given the fact that in this study we were looking at sheltered animals where population turnover was considered high with frequent opportunities for the introduction of new pathogens (Mochizuki et al., 2008; Schulz et al., 2014). It is likely this reflects the sporadic nature of respiratory disease in rescue shelters coupled with the relatively short period over which we were able to sample each population. In future studies it may be more fruitful to strategically sample shelters at the time of ongoing outbreaks of disease.

In the longitudinal portion of this study, pathogens were found sparingly, especially in the case of dogs where *B. bronchiseptica* was only found in two animals (weeks 2, 6 and 7). The staff in this shelter was well trained and biosecurity measures were constantly being improved under veterinary advice, which could in part explain low pathogen numbers. Additionally, animals sampled in the longitudinal study were often vaccinated before entry into the shelter (at a different branch of the charity) and therefore, live vaccine shedding was potentially no longer occurring. The same is possible for introduction of new pathogens into R1 since the animals had been at a different branch for a short period of time and could have introduced new pathogens at this branch and no longer be shedding them during their stay at R1. It

would be, therefore, interesting to compare pathogen and clinical disease prevalence between these two branches.

On the other hand, CnPnV was found in seven animals during weeks 9 and 10 (3 positive samples in week 9, 5 positive samples in week 10 with only one animal out of seven testing positive for CnPnV in both weeks 9 and 10). This coincided with staff and volunteers reporting coughing in a small number of dogs (data not presented as information passed on to author informally). Interestingly, none of the dogs with positive samples for CnPnV had clinical signs of respiratory disease at the time of sampling. The presence of a growing number of positives from weeks 9 and 10 could potentially represent the start of an outbreak. It is interesting to note that an high attack rate of 23.3% (7/30) was observed for the population sampled across the duration of the study. Although Koch's postulates are yet to be fulfilled for CnPnV, previous studies have found strong associations between presence of the virus and CIRD (Mitchell et al., 2013b; Renshaw et al., 2010).

In conclusion, this chapter re-emphasises the importance of kennel environments in maintaining infections through silent carriers that may act as reservoirs of disease to potentially immunologically naïve animals newly introduced into these environments. Similar findings have been reported in previous studies looking at pathogen shedding in clinically healthy animals including shedding canine parvovirus in cats housed in shelters (Clegg et al., 2012) and canine enteric coronavirus in kennelled dogs (Stavisky et al., 2012b).

7 Concluding discussion

7.1 General discussion

Despite widespread vaccination, CIRD and FIRD continue to be important issues affecting the health and general wellbeing of different feline and canine populations worldwide.

For many years, this field received little attention. However, within the last decade, a resurgence in interest in infectious respiratory disease in the feline and canine species has occurred. This has probably been fueled by the emergence of pathogens (such as CIV and CRCoV in dogs and VS-FCV in cats), as well as the perceived lack of efficacy of vaccination in the control of outbreaks especially within multi-animal, high-turnover populations such as rescue shelters (Priestnall et al., 2014; Radford et al., 2009). This project aimed to further the understanding of canine and feline infectious respiratory disease with a major focus on pet and unowned shelter populations in the UK. This was attempted through re-evaluation of the roles of known and suspected pathogens in this syndrome, and identification of risk-factors for both pathogen carriage and clinical disease. Despite some frustrations around sample sizes, it is hoped the results presented here will contribute to the field in a way that will inform the development of future disease reduction strategies.

Initially, a cross-sectional study involving the participation of veterinary practices in six European countries (United Kingdom, France, Germany, the Netherlands, Italy and Sweden) was used in order to evaluate the diversity and epidemiology of FCV (including the identification of risk factors for pathogen carriage) and to assess the efficacy of a widely used vaccine strain in neutralising recent field isolates of this virus (Chapter 2). To assess seroreactivity of UK cats and dogs to influenza viruses, a retrospective serosurvey study was undertaken (Chapter 3). A case-control study design was then used in order to further understand the role of known and potential FIRD and CIRD pathogens in dogs and cats attending British veterinary practices and to attempt the identification of risk factors for clinical disease in veterinary attending populations (Chapter 4 and 5). Finally, a cross-sectional and longitudinal study was undertaken in UK shelters in order to identify currently circulating CIRD and FIRD

associated pathogens (Chapter 6). Despite the variation in objectives and methodologies used in these chapters, several re-occurring themes were identified.

In CIRD, it becomes clearer through this body of work that pathogens traditionally considered the main culprits of disease (Priestnall, 2017; Wright et al., 1974) are perhaps becoming a much lesser threat to upper respiratory health in UK dogs. Throughout the studies described in this dissertation, both in pet and shelter populations, PCR failed to identify any samples positive to CAV-2, CDV and CHV. These findings are further strengthened by previous studies which also failed to identify these viruses in healthy CIRD-exposed animals as well as in animals showing CIRD clinical signs (Decaro et al., 2016; Erles and Brownlie, 2005) or that found these at much lower rates than older studies (Mochizuki et al., 2008; Schulz et al., 2014). The absence of these pathogens from collected samples, as well as of CPiV, is potentially related to the fact that a high percentage of animals are vaccinated against these pathogens in the UK (Sánchez-Vizcaíno et al., 2018). In contrast, emerging pathogens such as CnPnV and CRCoV were observed. Even though Koch's postulates are yet to be fulfilled for these viruses, it seems that they consistently appear in association with CIRD like disease both in this body of work as well as in other studies (Erles and Brownlie, 2008; Mitchell et al., 2013b; Priestnall et al., 2014). Proving (or otherwise) Kochs postulates for these viruses seems a sensible way forward.

In the work described in this dissertation, FCV is strongly reaffirmed as a common infectious agent encountered in feline populations in the UK and other European countries. Interestingly the FCV prevalence found in veterinary practice attending cats across six European countries (9.2%; chapter 2) was not far from the overall rates of infection found in the UK in the case-control study (10.8%; chapter 4). These findings are very similar to those of previous studies (Coyne et al., 2007b; Radford et al., 2009). While this virus is often identified in clinically healthy animals we have found, in the case-control study described in chapter 4, that it was also significantly associated with FIRD. This likely reflects the lower rates of cat vaccination in the UK (Sánchez-Vizcaíno et al., 2018), as well as the challenges of vaccinating against a

variable pathogen like FCV (and a herpesvirus like FeHV-1). For both these pathogens, vaccination does not prevent infection and vaccinated animals can be infected and become carriers. Perhaps the easiest way to impact on this in the future will be to understand the lower rates of vaccination by cat owners in an attempt to increase vaccine uptake.

In chapter 3, only 1.5% of canine serum samples were positive for equine H3N8 and 0.56% of feline samples were positive to human pandemic H1N1 suggesting that UK cats and dogs are exposed albeit rarely to influenza viruses. Additionally, the absence of Influenza A positives in canine and feline samples tested using PCR (chapters 4, 5 and 6) further confirms the currently low risk of infection where these viruses are concerned. However, there are an increasing number of reports of influenza A viruses in dogs and cats in different parts of the world as well as the emergence of several new strains that can establish infection and cause disease in these species, such as the case of the recent emergence of canine influenza H3N2 in the United States of America (Abente et al., 2016; Jeoung et al., 2013; Song et al., 2008). In addition, these viruses also pose an additional zoonotic risk (Belser et al., 2017). Together, this cautions the veterinary community to remain alert and continue investing their efforts in surveillance for these viruses.

It is also important to note that in a high number of animals showing signs of respiratory disease and suspected to suffer from upper respiratory infections no known or suspected CIRD or FIRD pathogens were detected. This has been previously observed and, while it could certainly be due to several human related factors (e.g. inappropriate sampling technique, inadequate sample handing or storage, misclassification of non-infectious disease as CIRD/FIRD) one must take into account the possibility that perhaps, in these animals, disease was caused by currently unknown pathogens. This would not be surprising since, for example, CRCoV (Erles et al., 2003) was originally described in animals suffering from CIRD like disease where classic pathogen testing was negative.

7.2 Study challenges and limitations

Challenges with comparing this study's results with prior studies were clear especially during the cross-sectional study of FCV diversity and vaccine cross-reactivity (chapter 2). Direct comparisons of this study with previous studies looking at FCV vaccine cross-reactivity were particularly challenging due to the differences in applied methodologies. For the work presented here, an effort was made to apply basic epidemiological principles in order to ensure, as far as possible, that the results were generalisable to the wider European feline population. Practices were recruited based on random selection and all samples were collected prospectively. Although it is understood that veterinary practice-attending animals are an inherently biased population (Murray and Gruffydd-Jones, 2012), in order to decrease selection bias as much as possible, participating vets had clear instructions to sample the next 30 cats presented for a consultation once sampling had started. It was postulated that, in order to collect field isolates representative of a feline population which is likely to be vaccinated, one should not select for diseased animals. FCV is an extremely variable virus with numerous circulating strains with different degrees of pathogenicity (Coutts et al., 1994; Monné Rodriguez et al., 2014; Radford et al., 2009; Rong et al., 2014). Therefore, in order to truly understand the capacity of antisera raised to the common FCV-F9 vaccine to neutralise recently circulating field isolates, these isolates must also mirror the range of pathogenicity of which FCV is capable and the range of isolates to which naïve cats might be exposed – including potentially apathogenic strains, isolates collected from carrier animals, isolates collected from animals with different FIRD clinical signs, etc. Additionally, since vaccination against FCV is known to decrease severity of disease but not infection itself, it was important to include isolates collected from both vaccinated (which can still be shedding the virus without clinical signs) and unvaccinated animals. In contrast to the methods presented here, it seems limiting to try to assess the efficacy of a vaccine strain using unstructured population sampling where only isolates collected from animals with FIRD clinical signs or retrospective samples from diseased animals submitted for diagnostic testing are used. This is the case with various previously published studies looking at FCV vaccine *in vitro* efficacy (Addie et al., 2008; Poulet et al., 2008; Wensman et al., 2015). In order to increase comparability of studies in the future it

will be necessary to agree an international study protocol as is available for other rapidly evolving pathogens such as influenza (e.g. https://sites.google.com/site/epiflu/Home). Whilst protocol issues are likely to be harmonised through such an approach, it is likely that future veterinary studies would largely still be funded by vaccine companies, as opposed to more independent funding that is available for human influenza vaccine surveillance. Any agreed protocol would need to take this into account and include open publication of all results. Although all the work in this thesis was funded by a commercial company, publication rights remained with the researchers.

Despite a recent shift towards more evidence-based approaches (Dean et al., 2017; Huntley et al., 2017), veterinary medicine still has a strong tradition of using anedoctal experiences and word of mouth in order to inform clinical decision-making (Stavisky, 2009). As previously mentioned, efforts were made throughout this body of work, to apply basic epidemiological principles whenever possible. However, these were frustrated on occasion due to various factors. Convenience based recruitment of veterinary practices and shelters was used in order to attempt to increase the numbers of collected samples. For example, in the case-control studies described in chapters 4 and 5, a small number of practices that had been enthusiastic participants in the FCV focused study described in chapter 2 were contacted again. This worked to an extent, and some of these practices did accept to participate in the case-control studies and performed well where sample collection was concerned. However, maintaining compliance throughout these studies was extremely challenging and, despite a significant number of practices accepting to participate, the number of samples collected fell very short of the original sample size calculation. This happened even though all practices confirmed that they would be able to provide the requested number of cases and controls in a period of three months from the start of sampling. Although practices were regularly contacted by phone and/or email, this did not seem to make a difference in terms of increased sample collection. In fact, it was occasionally mentioned by participating veterinary surgeons that they had “forgotten to collect samples” when a FIRD or CIRD suspected case presented to the practice. It is likely that the time constraints and pressures in the veterinary

profession may have had an impact on the compliance rates observed. In these studies, no incentives were offered to participating vets and this would perhaps be a way to increase sample numbers in the future.

Reliance on veterinary surgeons to submit samples can also introduce different biases. Firstly, we must take into account the fact that respiratory disease or respiratory signs may be misclassified as being of infectious origin. Additionally, even though practitioners were asked not to select cases or controls, it is not possible to discard this possibility and, in fact, it was noticed that in some instances, samples from animals belonging to staff members were selected as controls. However, time limitations means it is likely that future studies would also have to rely on practitioners, and not researchers, choosing cases and controls.

While for the FCV study (chapter 2), questionnaires were filled out by the participating veterinary surgeons with the help from cat owners, due to the longer nature of the case-control questionnaires, we postulated it would be more likely to be able to recruit veterinary surgeries if veterinary surgeons did not have to spend a large amount of time involved with the study (since OP swabs are very quick to collect) and therefore we decided to administer telephone questionnaires to the animal owners. Telephone questionnaires administered to owners in the case-control studies also proved to be very difficult to complete. Despite several attempts to telephone owners (at least three times for each participating animal, during a period of time selected by the owner upon agreement to take place in the study), a significant proportion of participants did not respond. It is possible that a significant proportion of participants did not answer calls from unknown numbers possibly given the recent increase in predatory and scam phone calls in the UK. In future studies this should be taken into account, and perhaps a shorter, simpler questionnaire should be used so that it can be filled out by the owner while waiting for their veterinary appointment or to be filled out directly by the participating vet with or without the owner.

In chapter 6, shelters were also recruited based on convenience. Personal connections, prior participation in University of Liverpool research studies and geographical location were some of the factors that influenced choice of sampling sites. This would have potentially introduced a bias especially since three of the shelters were in a 26 miles radius of the Leahurst campus of the University of Liverpool with the fourth shelter being about 100 miles away. Ideally, we would have preferred to sample shelters spread throughout the whole of the United Kingdom but this was not possible due to time constraints.

The results and conclusions taken from the cross-sectional and longitudinal shelter studies (chapter 6) have also been affected by several factors. For once, the unreliable record keeping in some of these organisations made it impossible to conduct more complex epidemiological analysis. Also, the fact that we were often only allowed to sample certain animals or were not allowed full access of the facilities proved challenging and, again, led to a biased sampling of the shelter populations. In future studies, it would be interesting to be embedded into the shelter environment for longer periods of time so that data collection could be made directly by the researcher (rather than relying on shelter records) and animals could be followed closely for longer periods of time (instead of one weekly sampling, spending a consecutive number of days or weeks with daily visits to the shelter would provide stronger information and better quality data).

7.3 Further work

The high proportions of feline and canine cases of suspected infectious respiratory disease where no pathogen was identified provides a great opportunity for further investigation. Next generation sequencing (NGS) techniques, allowing the analysis of viral genomes directly from samples without requiring any previous culture or isolation or even knowledge of potentially present microorganisms, has been applied to pathogen discovery in veterinary research with great success in recent years (Radford et al., 2012). As previously discussed, it is possible that disease in these cases where we were unable to identify any pathogen using PCR methods was caused by currently unknown pathogens. The application of NGS to these samples would

potentially provide further information and answers to this question. During this PhD project, a pilot study was undertaken (data not presented), which allowed us to attempt NGS in a small subset of samples collected during the FCV study described in chapter 2. These samples (n=13) comprised FCV isolates for which, using NGS methods, full or nearly full genomes were obtained (corresponding to the already sequenced isolates from chapter 2). Other viruses were also identified in these samples and further research is currently being undertaken in newly collected feline samples. This same approach could be applied to canine samples in future research in order to further understand whether currently unknown pathogens may be involved in causing disease in these animals where no known viruses or bacteria have been detected. Using NGS could also provide useful insight into the role of *B. bronchiseptica* vaccines in the observed high carriage of this organism in controls in this study.

In chapter 2, we observed that phylogenetic analysis on collected field strains of FCV, which also included the FCV-F9 vaccine strain capsid sequence, produced a radial phylogeny where FCV-F9 did not diverge from field strains any more than these did from one another. This is an extremely curious finding given that FCV is an RNA virus where high rates of evolution are observed (Radford et al., 1999). When compared with other RNA viruses such as influenza viruses, FCV seems to show a very different evolutionary behaviour. Influenza viruses are under continual immune and vaccine selection pressure leading to viral strains being replaced during seasonal outbreaks by new or adapted variants which evolve through antigenic drift and shift over time (Grenfell et al., 2004). As observed in Figure 2.2, this does not seem to occur with FCV where a strain which is approximately 50 years old (FCV-F9) remains clustered with the contemporary sequences obtained in this thesis. These findings could suggest that FCV is under a strong purifying selection which constrains its evolution. It would be therefore interesting to conduct further, more advanced phylogenetic analysis (using, for example, Bayesian coalescence frameworks) in order to further delve into this mystery. Such an analysis would also benefit from using full genome sequences more readily obtainable by NGS, rather than limiting the analyses to the more smaller and more immunogenic regions used in this thesis.

Finally, due to time constraints, sequencing and phylogenetic analysis of positive samples collected from the case-control and shelter studies were not undertaken. Doing so would provide additional information that would potentially allow us to infer on the transmission of infection within the studied populations. Such an approach would be particularly useful for the more variable pathogens identified including FCV.

7.4 Final conclusion

In conclusion, this thesis took a variety of approaches to review this important syndrome in cats and dogs. Although limitations around sampling sometimes restricted our interpretation of the results, taken together, this body of work provides new and interesting information. In cats, we observed that vaccine preventable infections are still common. These findings remind us that vaccination against FCV does not prevent infection and highlight the importance of vaccination in order to achieve good herd immunity in cats, which are generally less vaccinated than their canine cousins. In dogs, findings suggest that classical pathogens involved in CIRD seem to be fading away in UK populations, possibly due to success of vaccination schedules, although clearly larger sample sizes are needed to confirm this. However, other pathogens seem to be arising in the CIRD complex, shifting this old paradigm and raising the question of whether new vaccines against pathogens such as CRCoV should be developed.

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