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## Feline infectious diseases: genetic characterization of emerging and zoonotic pathogens

VET/05 – Infectious Diseases of Domestic Animals


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# RIASSUNTO

I gatti sono tra i più comuni animali da compagnia diffusi a livello mondiale. Negli ultimi decenni sono stati scoperti numerosi virus emergenti nei gatti. I virus emergenti felini, incluso il domestic cat hepadnavirus (DCH), potrebbero avere un importante ruolo patogeno nella specie felina. Si rende pertanto necessaria una valutazione della loro presenza; unitamente a studi di caratterizzazione genetica e di patogenicità al fine di indagare il loro potenziale ruolo patogeno. Inoltre, considerando il contatto stretto e diretto tra gatti ed esseri umani, è importante anche comprendere il ruolo dei gatti nell'epidemiologia dei virus emergenti che rappresentano una minaccia per l'uomo, come nel caso di severe acute respiratory coronavirus 2 (SARS-CoV-2). Oltre ai virus, anche i batteri antibiotico-resistenti sono diventati una delle maggiori minacce per la salute pubblica e, in accordo con il concetto One Health, la presenza di batteri multiresistenti dovrebbe essere monitorata anche negli animali da compagnia, inclusi i gatti. Pertanto, gli obiettivi di questa tesi di dottorato sono stati i) valutare la presenza, le caratteristiche genetiche e l'impatto dell'emergente DCH sulla salute felina (studi I-II) ii) indagare il ruolo dei gatti nell'epidemiologia della SARS-CoV-2 (studi III-V) e iii) studiare il *carriage* fecale di *Escherichia coli* antibiotico-resistenti nei gatti (studi VI-VII).

Per quanto riguarda il DCH, abbiamo indagato la presenza di DCH in gatti con e senza alterazioni biochimiche suggestive di malattia epatica (studio I) e gatti con e senza versamento cavitario (studio II); al fine di valutare il suo ruolo patogeno. Le analisi fillogenetiche hanno mostrato le sequenze DCH riscontrate nello studio II clusterizzavano con altre sequence di DCH australiane ma non erano incluse nel clade con altre sequenze DCH italiane, a supporto della presenza di due *clade* di DCH circolanti in Italia. Inoltre, DCH è stato rilevato sia in presenza che in assenza di alterazioni emato-biochimiche associate a danno epatico, e nei gatti con versamenti cavitari, suggerendo che DCH potrebbe non essere un patogeno felino significativo ma saranno necessari ulteriori studi per confermare questi risultati.

Al fine di indagare il ruolo dei gatti nell'attuale pandemia coronavirus diesease 2019 (COVID-19), nello studio III, abbiamo valutato la presenza di RNA di SARS-CoV-2 e anticorpi anti-SARS-CoV-2 in gatti randagi provenienti da colonie situate in un'area italiana fortemente colpita dalla pandemia. I risultati hanno suggerito che i gatti randagi potrebbero non avere un ruolo significativo nella diffusione di SARS-CoV-2 in aree con un'alta prevalenza di casi umani di COVID-19. Nello studio IV, abbiamo studiato se gatti e cani a stretto contatto con pazienti positivi al COVID-19 possano agire

come portatori meccanici passivi di SARS-CoV-2 attraverso la contaminazione di peli o zampe. I risultati hanno mostrato che gli animali domestici probabilmente non svolgono un ruolo epidemiologico nella trasmissione indiretta di SARS-CoV-2 all'uomo, anche se in contatto con proprietari positivi al COVID-19. Nello studio V abbiamo studiato le *performance* diagnostiche di tre diversi test immunologici disponibili in commercio per la rilevazione degli anticorpi anti-SARS-CoV-2 negli animali domestici, rispetto al gold standard *virus neutralization test*. I nostri risultati hanno mostrato che la validazione del test è un passo fondamentale per la sorveglianza epidemiologica negli animali domestici e che il *surrogate virus neutralization test* può essere un metodo efficace che non richiede un laboratorio di livello 3 di biosicurezza per prevedere la presenza di anticorpi neutralizzanti nel siero di gatti e cani.

Negli studi VI e VII abbiamo studiato la presenza a livello fecale di *E. coli* produttori di ESBL/AmpC e carbapenemasi nei gatti. *E. coli* produttori di ESBL/AmpC o carbapenemasi sono stati rilevati sia nei gatti randagi che domestici, solitamente in relazione allo stato di salute, alla predente terapia antibiotica ed al ricovero ospedaliero. I nostri risultati suggeriscono che i gatti potrebbero avere un ruolo come potenziali *carriers* di *E. coli* produttori di ESBL/AmpC/carbapenemasi, evidenziando la necessità di un uso prudente degli antibiotici in medicina veterinaria.

In conclusione, i risultati della presente tesi hanno consentito una valutazione preliminare del ruolo patogeno di DCH nel gatto, tuttavia saranno necessari ulteriori studi con una maggiore numerosità campionaria per confermare questi risultati.

Nonostante i gatti non abbiano mostrato un ruolo epidemiologico importante nella diffusione di SARS-CoV-2, il continuo monirotaggio dell'infezione da SARS-CoV-2 nei gatti è consigliabile data l'emergenza di nuove varianti virali. In fine, il ruolo dei gatti come *carriers* di *E. coli* produttori di ESBL/AmpC/carbapenemasi necessita di essere ulteriormente approfondito con un approccio One Health al fine di verificare il rischio di trasmissione all'uomo.

# ABSTRACT

Cats are one of the most common companion animals worldwide. Emerging viruses have been reported in cats worldwide in the last decades. Feline emerging viruses, including domestic cat hepadnavirus (DCH), may play an important role in cat diseases and evaluation of their presence, genetic characterization and pathogenicity is required to investigate the impact on feline health. Owing to their close and direct contact with humans, it is also important to understand the role of cats in the epidemiology of emerging viruses that represent a threat to humans, such as the pandemic severe acute respiratory coronavirus 2 (SARS-CoV-2). Besides viruses, antimicrobial resistant bacteria have become one of the major threats to public health and, according to the One Health concept, the presence of multidrug-resistant bacteria should also be monitored in cats. Therefore, the aims of this PhD thesis were i) to evaluate the presence, genetic characterization and impact of the novel DCH on feline health (studies I-II) ii) to investigate the role of cats in SARS-CoV-2 epidemiology (studies III-V), and iii) to study the faecal carriage of antimicrobial-resistant (AMR) *Escherichia coli* in cats (studies VI-VII).

Starting with DCH, we investigated the presence of the virus in cats and its potential pathogenicity by analysis of cats with and without biochemical alterations suggestive of liver disease (study I) and cats with and without cavitary effusion (study II). Phylogeny showed that DCH sequences from study II clustered with the prototypic Australian strain but were not included in the clade with other Italian DCH sequences, showing the circulation of two different groups of DCH in Italy. Moreover, DCH was detect in both animals with and without biochemical changes associated with liver disease and in cats with cavitary effusions suggesting that DCH may not be a significant feline pathogen but further studies are needed to confirm these findings.

To investigate the role of cats in the current coronavirus disease 2019 (COVID-19) pandemic, in study III we evaluated the presence of SARS-CoV-2 RNA and anti-SARS-CoV-2 antibodies in stray cats from colonies located in an Italian area highly affected by the COVID-19 pandemic. Results suggested that stray cats may not have a significant role in the spreading of SARS-CoV-2 in an area highly affected by the COVID-19 pandemic. In study IV we investigated whether cats and dogs living in close contact with COVID-19-positive patients may act as passive mechanical carriers of SARS-CoV-2 via contamination of hair or paws. The results showed that pets do not likely play an epidemiological role in the indirect transmission of SARS-CoV-2 to humans, even if in contact with COVID-19 positive

owners. In study V we studied the diagnostic performances of three different commercially available immunoassays for the detection of SARS-CoV-2 antibodies in pets, in comparison with the gold standard virus neutralization test. Our results showed that assay validation is a fundamental step for epidemiological surveillance in pets and surrogate virus neutralization test may be an effective method that does not require a biosafety level-3 laboratory for predicting serum neutralization antibodies in cats and dogs.

In studies VI and VII we investigated the faecal carriage of ESBL-/AmpC- and carbapenemase-producing *E. coli* in cats. ESBL-/AmpC- or carbapenemase-producing *E. coli* were detected in both stray and domestic cats, and their presence was associated with unhealthy status, previous antimicrobial therapy and hospitalization. Our results suggested that cats may have a role as potential carriers of ESBL/AmpC/carbapenemase-producing *E. coli* highlighting the need of antimicrobial stewardship in veterinary medicine.

In conclusion, the results of this thesis allowed the preliminary evaluation of the pathogenic role of DCH in cats and further studies with a larger sample size will be necessary to confirm our results.

Despite cats may not play an important epidemiological role in the spread of SARS-CoV-2, continuous monitoring of SARS-CoV-2 infection in cats may be suggested given the emergence of new viral variants. Ultimately, the role of cats as carriers of ESBL/AmpC/carbapenemase-producing *E. coli* needs to be further explored with a One Health approach in order to understand the risk of transmission to humans.

# SCIENTIFIC BACKGROUND

## Domestic cat hepdnavirus (DCH)

Viruses of the genus *Orthohepadnavirus*, family *Hepadnaviridae*, are circular, partially double-stranded DNA viruses reported in different mammal species, including bats, primates and rodents (Seeger & Mason, 2013). The prototype species of the genus, Hepatitis B virus (HBV), is a major global health problem causing potentially life-threatening liver diseases in humans. In 2019, HBV infections resulted in an estimated 820,000 deaths, mostly from cirrhosis and hepatocellular carcinoma (HCC) (Seeger & Mason, 2013; WHO, 2023a). The virus is transmitted from mother to child during birth and delivery or through contact with blood or other body fluids (WHO, 2023a). Moreover, HBV and human immunodeficiency virus (HIV) co-infections have been commonly reported (Petty et al., 2014).

The genome of HBV is approximately 3.2 kb and contains four overlapping open reading frames (ORFs), encoding for the polymerase (P), core (C), surface (S), and X proteins, similarly to other members of the genus *Orthohepadnavirus* (Aghazadeh et al., 2018; Seeger & Mason, 2013). The P protein plays a major role in viral genomic replication, having both RNA- and DNA-dependent DNA synthesis function, and ribonuclease H activity (Datta et al., 2012). The C protein is the major component of the viral nucleocapsid and a truncated form of the core protein circulates as the hepatitis B “e” antigen (HBeAg) in the early stages of infection (Seeger & Mason, 2013). The S proteins include three different domains: a large (LHBs), medium (MHBs), and small (SHBs) proteins. They provide the protein components of the virus envelope and have a major role in the host receptor recognition and binding (Churin et al., 2015). The sodium/bile acid cotransporter (NTCP) is the functional receptor for HBV, and a recent study show that it also likely for DCH (Seeger & Mason, 2013; Stone et al., 2022). The X protein is an enigmatic protein of the orthohepadnaviruses. It is required for efficient infection and can also bind to the tumor suppressor gene p53, increasing the risk of liver cancer (Seeger & Mason, 2013).

In 2018, a novel divergent member of the genus *Orthohepadnavirus*, named domestic cat hepadnavirus (DCH), was identified during transcriptomics studies in an Australian cat affected by a multicentric large B-cell lymphoma and feline immunodeficiency virus (FIV) infection. Whole

genome sequencing revealed a DCH genome of approximately 3.2 kb containing the four overlapping open reading frames (ORFs), encoding for the P, C, S and X proteins, similarly to other members of the genus *Orthohepadnavirus* (figure 1) (Aghazadeh et al., 2018). To date, function of DCH viral proteins have been suggested to be similar to HBV proteins but remains to be elucidated (Shofa et al., 2022).

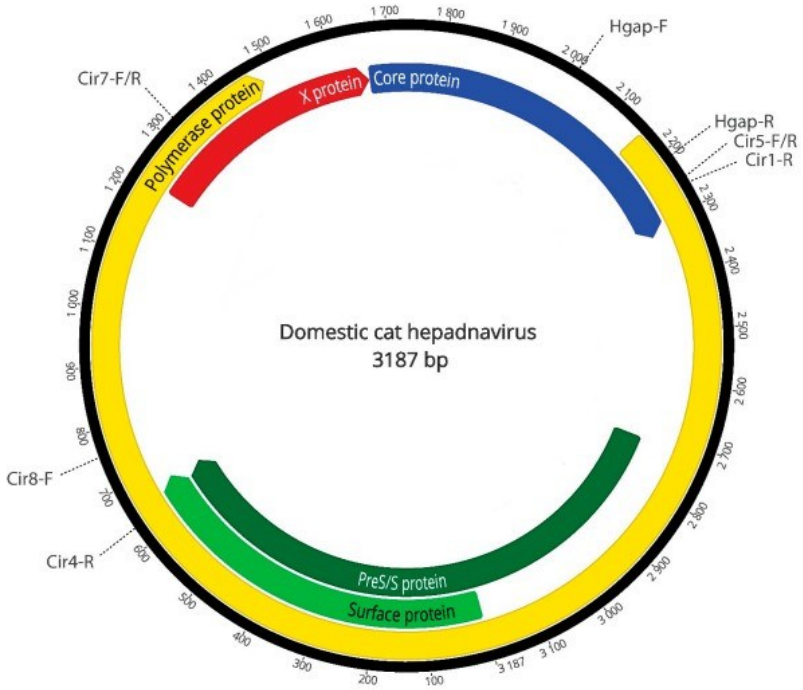


Figure 1. Genome organization of the domestic cat hepadnavirus (Aghazadeh et al., 2018).

Based on whole genome phylogenetic analysis and nucleotide differences (>8%) (Sunbul et al., 2014), DCH can be separated in two different genotypes. The genotype A, which is the most frequently reported, is divided into two clades: clade A1 which includes viral sequences from Asia and Australia and clade A2 including viral sequences from USA, Europe, Russia and Turkey. Currently, the genotype B is represented by one viral sequence only from Japan (Capozza et al., 2023). Based on the current data regarding phylogenetic analysis of DCH, viral genomes clustered in different clades according to the country of origin (Shofa et al., 2022). In addition, a putative DCH recombinant strain was found in Thailand. The recombination event was detected in 1,980–2,178 nucleotide region in the C gene, that were most closely related to DCH strains identified in Australia compared to the Thai strain, indicating Australian strain as the likely origin (Piewbang et al., 2020). These results may indicate the role of recombination in the evolution of DCH as suggested for HBV (Piewbang et al., 2020; Simmonds & Midgley, 2005). Indeed, recombination resulting in a deletion of 157 bp was

identified in the N-terminus of the core open reading frame of an American DCH strain, likely leading to a replication-incompetent virus (Stone et al., 2022). However, the confirmation of this findings requires viral replication systems (e.g., cell culture models), which are challenging to establish for hepadnaviruses due their narrow cellular specificity and the complex virus-host interaction (Witt-Kehati et al., 2016; Xu et al., 2021). Beside DCH, the presence of a closely related *Orthohepadnavirus,* tentatively named domestic dog hepadnavirus (DDH) has been reported in domestic dogs. However, DDH show high nucleotide identity (about 98%) and similar genomic organization to DCH suggesting the possibility of Hepadnaviruses circulation among domestic carnivores and a wider host range of DCH (Diakoudi et al., 2022).

Since its discovery in Australia, the presence of DCH in blood or serum samples of both healthy and unhealthy cats has been reported also in other countries including Thailand, Taiwan, Malaysia, China, Japan, Italy, Turkey, the United Kingdom and the USA, with prevalence ranging from 0.2% to 12.4% (Adıgüzel et al., 2023; Aghazadeh et al., 2018; Anpuanandam et al., 2021; Capozza et al., 2023; Jeanes et al., 2022; Lanave et al., 2019; Piewbang et al., 2020; Silva et al., 2023; Stone et al., 2022; Takahashi et al., 2022). Moreover, a 25% seroprevalence has been reported in cats in Italy using an antibody detection enzyme-linked immunosorbent assay based on the recombinant core antigen of DCH (Fruci et al., 2022).

DCH presence has been associated with feline retroviruses (FIV and/or feline leukemia virus, FeLV) infections, adult age and type of ownership, with higher detection of DCH among pet cats compared to shelter cats (Aghazadeh et al., 2018; Anpuanandam et al., 2021; Lanave et al., 2019; Piewbang et al., 2020; Piewbang et al., 2022). To date little is known about the infection progression and the potential transmission routes of DCH. Follow-up studies on DCH-positive cats have demonstrated that some cats can remain viremic for over 11 months, while others may be able to clear the infection (Capozza et al., 2021a; Wardhani et al., 2023). Moreover, in viremic cats the presence of DCH may be detected in auricular swab, nasal swab, oral swab and rectal swab suggesting horizontal transmission (Wardhani et al., 2023).

To date, the diagnosis of DCH infection in cats rely mainly on molecular methods. Several PCR protocols have been developed and used since the discovery of DCH (Adıgüzel et al., 2023; Aghazadeh et al., 2018; Lanave et al., 2019; Shofa et al., 2023). In contrast, only one study reported a serological test based on recombinant DCH core particles generated in insect cells using baculovirus expression system and their usage to develop an antibody detection ELISA to study DCH

seroprevalence (Fruci et al., 2022). This is a major limitation for DCH related research. Indeed, the detection of HBV in human is based mainly on the detection of antibodies and circulating viral antigens, such as the hepatitis B surface antigen (HBsAg) which is considered the definite marker of HBV infection (Lee & Kim, 2021). Viral DNA is considered a marker of viral active replication, however DNA levels in blood of chronically HBV-infected patients can fluctuate to the point of being undetectable (Chu, 2002). This may result in a likely underestimation of DCH prevalence, as circulating DCH DNA may not identify all infected cats within a population (Capozza et al., 2023). Supporting this hypothesis, Fruci and colleagues detected only 23 (35.9%) cats with positive results for DCH DNA out of 64 DCH-seropositive cats supporting previous DCH exposure (Fruci et al., 2022). Others diagnostic tools used by different studies to investigate the potential pathogenic role of DCH in formalin-fixed, paraffin-embedded tissue are in situ hybridization (ISH) and immunohistochemistry (IHC) which allow the analysis of viral distribution and localization among the affected tissue (Pesavento et al., 2019; Piewbang et al., 2020).

The potential pathogenetic role of DCH in feline liver diseases has been investigated. In an international multicenter investigation, DCH infection has been associated with HCC and chronic hepatitis in cats, with histopathological features of inflammation, neoplasia and viral distribution highly similar to that seen with HBV-associated liver disease in humans (Pesavento et al., 2019). Moreover, DCH has recently been associated with chronic lymphoplasmacytic hepatitis in cats (Piewbang et al., 2022). Cats with DCH viraemia are likely to have an increase in liver structural or functional damage markers, such as alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT) and total bilirubin, suggestive of liver injury (Anpuanandam et al., 2021; Capozza et al., 2021a; Lanave et al., 2019; Piewbang et al., 2022). However, not all the studies reported a correlation between ALT activity and DCH infection (Capozza et al., 2023). Besides liver diseases, a study has described DCH systemic infection in three cats, associated with inflammatory lesions in various organs including the liver, kidney, intestine, heart and lungs (Piewbang et al., 2020). Moreover, a recent study has detected a higher presence of DCH in cats with lymphoma compared to cats without lymphoma, supporting some pathophysiological mechanisms similar to HBV infection in human non-Hodgkin lymphoma (Piewbang et al., 2023). Despite hepadnaviruses are generally considered predominantly hepatotropic, the presence of DCH in others tissue may support extra-hepatic infections as previously described for HBV (Joshi & Coffin, 2018; Korba et al., 1989; Mason et al., 1993). Nevertheless, pathobiology of DCH infection in cats still need to be elucidated (Shofa et al., 2022).

## Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in cats

Coronavirus disease 2019 (COVID-19) is a human disease, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The first cases of COVID-19 in humans were identified in December 2019 in Wuhan city, China (Huang et al., 2020). SARS-CoV-2 most likely emerged from an animal source and after its emergence the disease was sustained by human-to-human transmission, quickly becoming the cause of the recent pandemic (Holmes et al., 2021). To date, the COVID-19 pandemic has led nearly 7 million of deaths worldwide, mostly in European counties, causing devastating effects on global health, society and economy (WHO, 2023b). According to the World Health Organization (WHO), this pandemic represents the most significant health challenge that the world has confronted in the last century (WHO, 2023c).

SARS-CoV-2 is a member of the genus *Betacoronavirus* within the family *Coronaviridae* (Woo et al., 2023). The genome of SARS-CoV-2 is composed by of a single-stranded positive-sense RNA with a length of about 30 kb (figure 2) (Wu et al., 2020a).

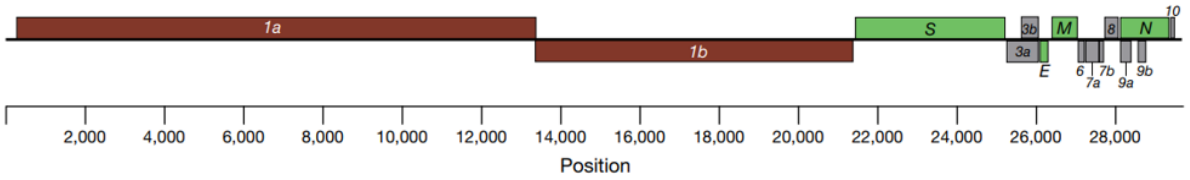


Figure 2. Genome organization of SARS-CoV-2. Brown: non-structural proteins; grey: accessory proteins; green: structural proteins (Wu et al., 2020a).

Coronaviruses possess the largest viral genome documented among viruses RNA viruses (Gorbalenya et al., 2006). The viral genome encodes for 29 proteins, including 16 non-structural, 9 accessory and 4 structural proteins (Zhou et al., 2020). Non-structural proteins play crucial roles in viral RNA replication, while accessory proteins are involved in different pathogenesis mechanisms during viral infection (Redondo et al., 2021; Wu et al., 2022). Particularly, two-thirds of the viral genome consists of two large open reading frames (ORFs 1a and 1b) that encode for two large non-structural polypeptides (pp1a and pp1b). These polyproteins are cleaved by the viral proteases present in the polypeptide itself, leading to the formation of 16 non-structural proteins (nsps), which include the RNA-dependent RNA polymerase, essential for the viral replication (Rohaim et al., 2021). Despite having a proofreading activity of its exonuclease during RNA synthesis, the viral RNA

polymerases are inherently error-prone, leading to a high rate of mutation (Bradley et al., 2019; Moeller et al., 2022). Indeed, since the beginning of the COVID-19 pandemic, several new SARS-CoV-2 variants, such as the 'variants of concern', designated as Alpha, Beta, Gamma, Delta and Omicron emerged independently and rapidly became dominant, regionally or globally, outcompeting previous variants (Carabelli et al., 2023).

Structural proteins, including spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins, are responsible for viral assembly and constitute the mature viral particles (Satarker & Nampoothiri, 2020). Particularly, the S protein plays a key role in host range determination through the recognition, binding, fusion and entry in the target host cell (Mahdy et al., 2020; Satarker & Nampoothiri, 2020). Species susceptibility to SARS-CoV-2 is predominantly determined by the interaction between the viral S protein and the angiotensin converting enzyme 2 (ACE2) that serves as the receptor for SARS-CoV-2 (Wu et al., 2020b).

Several viruses closely related to SARS-CoV-2 have been reported in bats and pangolin from different Asian countries, highlighting that SARS-CoV-2 may have zoonotic origin (Holmes et al., 2021). Indeed, the high rate of mutation, coupled with the high frequency of homologous RNA recombination and the large genomes of coronaviruses, makes coronaviruses capable of interspecies jumping which may lead to the emerging of zoonotic diseases (Woo et al., 2009). In addition to humans, SARS-CoV-2 infections have been reported in different animal species (WOAH, 2023). According to the last report of the World Organization for Animal Health, SARS-CoV-2 outbreaks in animals have been reported globally, affecting 29 mammal species in several countries (WOAH, 2023). Several cases of SARS-CoV-2 infection were detected among different zoo animal species (mainly belonging to the family Felidae), farmed mink, Syrian hamsters, wild ungulates, particularly in white-tailed deer and pets (Qiu et al., 2023).

Natural SARS-CoV-2 infection has been reported worldwide in cats (Goryoka et al., 2021; Hamer et al., 2021; Patterson et al., 2020). The highly susceptibility of cats to SARS-CoV-2 infection was previously reported under experimental conditions also showing that experimentally infected cats were capable to infect other cats (Bosco-Lauth et al., 2020; Shi et al., 2020). Particularly, intranasally infected cats showed oral and nasal viral shedding, lasting for up to 5 days post-infection (Bosco-Lauth et al., 2020). However, following re-infection with SARS-CoV-2, cats were unable to transmit the virus to other co-housed naïve cats (Gaudreault et al., 2021). These findings may be explained by the fact that the cat ACE2 (cACE2) is closely related to human ACE2 (hACE2), and that the SARS-

CoV-2 can interact with the cACE2, albeit with a lower binding affinity than with hACE2 (Wu et al., 2020b). Moreover, experimentally infected cats develop a strong antiviral immune response, leading to a rapid viral clearance, with only mild histopathological changes in the respiratory tract and with the absence of clinical signs (Vreman et al., 2023).

Given their close and direct contact with humans, the potential role of cats in SARS-CoV-2 transmission dynamics has been explored, according to the One Health approach (Dróżdż et al., 2021). The first identification of an infected domestic cat in Hong Kong and the finding of one stray cat harboring viral RNA in the proximity of SARS-CoV-2 infected mink farm in Netherlands has raised interest in the potential role of domestic and stray cats as reservoir of SARS-CoV-2, especially during the early stages of the COVID-19 pandemic (Barrs et al., 2020; Oreshkova et al., 2020), highlighting the need of monitoring SARS-CoV-2 in cats. Different SARS-CoV-2 variants have been identified in infected cats, including Alpha, Gamma, Delta and Omicron variants (Carneiro et al., 2022; Jairak et al., 2022; Krafft et al., 2021; Sánchez-Morales et al., 2022). Infection in cats has been usually associated with the presence of SARS-CoV-2-positive owners within the household, supporting human-to-cat transmission (Goryoka et al., 2021; Hamer et al., 2021; Kannekens-Jager et al., 2022; Patterson et al., 2020). Indeed, in almost all the case studies, human-to-cat transmission was hypothesized, based on epidemiological data and for the homology of viral sequence between owner and cat (Bessière et al., 2021; Chaintoutis et al., 2022; Curukoglu et al., 2021; Pagani et al., 2021). Despite these data may suggest that SARS-CoV-2 can achieve cross species transmission several studies have demonstrated that cats have a limited role on virus transmission (Bessière et al., 2021; Dróżdż et al., 2021). However, a case of cat-to-human transmission has been reported in Thailand, which represent the first suspected case of cat-to-human transmission. This case involved a veterinarian that tested positive after the clinical examination of an infected cat belonging to two SARS-CoV-2 positive patients. Whole genome sequencing (WGS) revealed that the viral genomes of the owners, the cat and the veterinarian were identical. The veterinarian had no contact with the cat's owner or other positive humans, suggesting that the veterinarian was infected by the cat (Sila et al., 2022). In addition to humans, the natural infection of cats with SARS-CoV-2 may also originate from other animals (Qiu et al., 2023). Indeed, animal to cat transmission of SARS-CoV-2 was suspected only in stray cats found positive by RT-PCR and serology near infected mink farms. Because these cats were not in close contact with humans, infected minks were considered as a likely source of the infection (Oreshkova et al., 2020; van Aart et al., 2022). Surveillance of infection in cats is

challenging when using molecular methods because cats usually shed virus only for few days, due their ability to clear the infection in a short time (Bosco-Lauth et al., 2020; Hamer et al., 2021). Moreover, the role of pets in the transmission pattern as passive mechanical carriers of SARS-CoV-2 was also taken into consideration by the Italian Ministry of Health during the first months of the pandemic, when contamination via surfaces was suggested as another potential route of transmission of SARS-CoV-2 due to the ability of SARS-CoV-2 to survive in the environment for up to 72h under laboratory conditions (Italian Ministry of Health, 2020; Riddell et al., 2020; van Doremalen et al., 2020).

In order to investigate the potential role of cats during the COVID-19 pandemic, several field studies have investigated the presence of SARS-CoV-2 infection in domestic cats have used serological and molecular methods (Murphy & Ly, 2021). SARS-CoV-2 RNA has been sporadically detected in domestic cats, mainly from oropharyngeal, nasal, and/or rectal swabs (Bessière et al., 2021; Chaintoutis et al., 2022; Curukoglu et al., 2021; Goryoka et al., 2021; Hamer et al., 2021; Hosie et al., 2021; Klaus et al., 2021a; Neira et al., 2021; Pagani et al., 2021). On the other hand, seropositive cats have been more frequently detected compared to molecular methods. Moreover, antibody levels in naturally infected cats have been shown to persist for up to 10 months (Decaro et al., 2022), making serological assays a useful tool to investigate SARS-CoV-2 infections in pets. Seroprevalence in cats has been reported to range from 0% to 31.7% (Deng et al., 2020; Jara et al., 2021; Klaus et al., 2021b; Michelitsch et al., 2020, Michelitsch et al., 2021; Oliveira et al., 2022; Patterson et al., 2020; Ramasamy et al., 2023; Spada et al., 2021; Udom et al., 2022; van der Leij et al., 2021; Villanueva-Saz et al., 2022; Wang et al., 2022; Zhang et al., 2020). Most of the studies reported a seroprevalence of less than 5% (Deng et al., 2020; Klaus et al., 2021b; Michelitsch et al., 2020, Michelitsch et al., 2021; Oliveira et al., 2022; Spada et al., 2021; Udom et al., 2022; van der Leij et al., 2021; Villanueva-Saz et al., 2022; Wang et al., 2022). It should be noted that the study with the highest prevalence (Jara et al., 2021) only included cats with COVID-19-positive owners, which may have led to a higher prevalence compared to others. Prevalences between studies may be hard to compare for different reasons, such as the characteristics of sampled population and the different methods used to detect SARS-CoV-2 exposure (Doliff & Martens, 2022). Moreveor, discordant results have been often reported also between different serological assays in cats and other animal species (Decaro et al., 2022; Jemeršić et al., 2021; Klaus et al., 2021b; Michelitsch et al., 2020; Udom et al., 2022; Zhang et al., 2020). Reliable methods with high diagnostic accuracy for antibody detection are essential not

only for epidemiological surveillance, but also to understand susceptibility and immune-response to SARS-CoV-2 in animals (Yamamoto et al., 2022).

The gold standard for the detection of serum neutralizing antibodies against SARS-CoV-2 in humans is the virus neutralization test (VNT). Serum neutralizing antibodies have shown their activity primarily against the SARS-CoV-2 S protein (Brouwer et al., 2020), representing only a small subset of the total polyclonal immune response (Girl et al., 2022). Serum neutralizing antibodies have been fundamental for the evaluation of convalescent plasma and efficacy of vaccination (Yamamoto et al., 2022). However, a limitation of VNT is the requirement of a biosafety level 3 (BSL-3) laboratory (WHO, 2021a), which is not always available in diagnostic laboratories. VNT is considered the gold standard also for SARS-CoV-2 antibody detection in pets (Embregts et al., 2021; Perera et al., 2021). VNT has been used as standalone or confirmation method for SARS-CoV-2 antibody detection by different studies (Calvet et al., 2021; Goryoka et al., 2021; Hamer et al., 2021; Krafft et al., 2021; Patterson et al., 2020; Zhang et al., 2020). To date, several serological tests are commercially available for the detection of SARS-CoV-2 antibodies against the spike or nucleocapsid protein in animals, including pets. The use of a surrogate VNT test (sVNT) detecting neutralizing antibodies, which can be performed in BSL-2 laboratories (Tan et al., 2020), has been reported in pets. The sVNT has shown high sensitivity and specificity in comparison to the VNT assay, without cross-reactivity to other animal coronaviruses, such as feline coronavirus (FCoV) and canine coronavirus (CCoV) (Embregts et al., 2021; Perera et al., 2021). A commercially available enzyme-linked immunosorbent assay (ELISA) for the detection of specific antibodies against the nucleocapsid antigen of SARS-CoV-2 has been used for antibody detection in animals (Decaro et al., 2022; Jemeršić et al., 2021; Udom et al., 2022). Despite the nucleoprotein does not elicit neutralizing antibodies, a good correlation between antibody responses to this protein and the neutralizing antibody titer has been described in humans (To et al., 2020). The discrepancy in results reported between ELISA and VNT has been reported to be due to the lack of antibodies with neutralizing activity (Michelitsch et al., 2020; Udom et al., 2022; Zhang et al., 2020) or to the different kinetics between the antibody responses against different viral antigens (Decaro et al., 2022). Therefore, an evaluation of different commercial serological assays is needed to define reliable methodologies for SARS-CoV-2 antibody detection in pets that may be used for the surveillance of the infection.

Continuous monitoring of SARS-CoV-2 presence in cats and other animals using reliable methods has been considered crucial considering the emergence of new viral variants that may adapt to new hosts (Doliff & Martens, 2022; Meekins et al., 2021; Pratelli et al., 2022).

# Extended-spectrum beta-lactamase-, AmpC-, and carbapenemase-producing *Escherichia coli* in cats

Antimicrobial resistance (AMR) has become one of the major threats to public health (WHO, 2022). Multisectoral surveillance systems focusing on resistance to antibiotics are key points to define and evaluate the effectiveness of measures against AMR. Due to their rapid emergence over the last few years, concerns have been raised about the spread of extended-spectrum beta-lactamase (ESBL)-, AmpC-, and carbapenemase-producing *Escherichia coli* (*E. coli)* in both humans and animals (Carvalho et al., 2021; Madec et al., 2017; Pomba et al., 2016). Indeed, the importance of ESBL-, AmpC-, and carbapenemase-producing *E. coli* strains has been emphasized by their association with increasing treatment failure, hospitalization, and mortality in humans and animals (Rubin & Pitout, 2014).

ESBLs have been defined as enzymes conferring resistance to penicillins, cephalosporins ($1^{st}$,$2^{nd}$ and $3^{rd}$ generations), and aztreonam by hydrolysis of these antibiotics. These enzymes are inhibited by β-lactamase inhibitors, such as clavulanic acid, and do not confer resistance to carbapenems or cephamycins (Paterson & Bonomo, 2005). The presence of ESBL has been reported in different members within the family *Enterobacteriaceae*, with *E. coli* being the most common species associated with global ESBLs (Peirano & Pitout, 2019). The most common ESBL enzyme reported worldwide is CTX-M, followed by TEM and SHV (Bush & Bradford, 2020). Particularly, over 220 CTX-M-β-lactamases have been reported to date, which can be divided into five subfamilies based on their amino acid identities, identified as: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25 subfamilies. Among CTX-M-β-lactamases, CTX-M-1 and CTX-M-9 subfamilies are widely distributed and commonly reported (Peirano & Pitout, 2019). The numbers of TEM and SHV type beta-lactamases currently exceed 200 and all of them, with the exception of TEM-1, TEM-2 and SHV-1, are ESBL (Ghafourian et al., 2015). Despite TEM- and SHV-type ESBLs are still being identified, it seems that the impact of their presence among clinical isolates is limited (Castanheira et al., 2021). The encoding genes for these enzymes are mostly located on plasmid, allowing efficient dissemination among bacteria (Dolejska & Papagiannitsis, 2018). Indeed, ESBL genes may be transferred between environment, animals and humans, highlighting the need of a One Health approach to reduce the spread of this resistance mechanism (Chong et al., 2018; Ramatla et al., 2023).

Another resistance mechanism to β-lactam in *Enterobacteriaceae* is the AmpC β-lactamase. This β-lactamase can hydrolyze cephalosporins (including cephamycins) and penicillins and in contrast to ESBLs are not inhibited by β-lactamase inhibitors (Meini et al., 2019). Another important difference between ESBLs and AmpC β-lactamase is that the latter can often be found as over-expressed chromosomal β-lactamases (Bush & Bradford, 2020; Tamma et al., 2019). As reported for ESBL, AmpC β-lactamases have been mobilized and can be found in plasmids as well (Bush & Bradford, 2020). The presence of plasmid-encoded AmpC β-lactamase, originated from chromosomal *ampC* genes have been reported in several Gram-negative species and falling into at least five phylogenetic groups, namely the *Enterobacter* group (MIR, ACT), the *Citrobacter freundii* group (CMY-2-like, LAT, CFE), the *Morganella morganii* group (DHA), the *Hafnia alvei* group (ACC), and the *Aeromonas* group (CMY-1-like, FOX, MOX) (Meini et al., 2019). Plasmid-encoded AmpC β-lactamase are mostly encoded by $bla_{\text{CMY-2}}$ worldwide, due its ability to be transmitted between bacterial species and between food-producing animals and humans (Pires et al., 2015; Winokur et al., 2001).

Compared to other classes of β-lactamases, carbapenemases have a broader spectrum, being able to hydrolyze penicillins, cephalosporins, monobactams, and carbapenems. Moreover, most of carbapenemase are resistant to all commercially available β-lactamase inhibitors (Queenan & Bush, 2007). The most frequently reported carbapenemase in human and animals belong to three different Amber classes: (i) class A, including the *Klebsiella pneumoniae* carbapenemase (KPC); (ii) class B, including Verona integron-encoded metallo-β-lactamase (VIM), imipenemase metallo-β-lactamase (IMP) and New Delhi metallo-β-lactamase (NDM); and (iii) class D, including oxacillinase-48-like (OXA-48) type enzymes (Bonomo et al., 2018; Silva et al., 2022). Carbapenemase genes are often located on mobile genetic elements, enhancing their ability to spread within the *Enterobacteriaceae* family (Bonomo et al., 2018). Carbapenems are important antibiotics that can be used in human medicine to threat serious infections caused by ESBL/AmpC-producing *E. coli* (Jacoby, 2009; Thomson, 2010). Given the importance of carbapenems in human health and the risk of resistance transmission, the administration of carbapenems has been prohibited in all animal species in Europe (EU Commission-Implementing Regulation 2022/1255/EU; Ramírez-Castillo et al., 2023). Despite this limitation in their use, carbapenemase-producing bacteria have been increasingly reported in companion animals, probably due to selective pressure by other classes of β-lactam commonly used in veterinary practice (Pulss et al., 2018; Ramírez-Castillo et al., 2023; Silva et al.,

2022), highlighting the need of surveillance program in companion animals and antimicrobial stewardship in veterinary medicine.

The need of a multisectoral surveillance system with a One Health approach to fight the spread of AMR has been highlighted (WHO, 2021b), and ESBL/AmpC-producing *E. coli* have been proposed as commensal indicator microorganisms for AMR surveillance systems (EU Commission-Decision 2020/1729/EU; WHO, 2021b). The detection of ESBL-, AmpC-, or carbapenemase-producing *Enterobacteriaceae* is usually based on samples screening, using selective media, and confirmation tests using both disc diffusion phenotypic assay and molecular methods (Noster et al., 2021). Particularly, the recommended strategy for the detection of ESBL in *Enterobacteriaceae* is based on the screening of resistance to an indicator oxyimino-cephalosporins (such as cefotaxime, ceftriaxone, ceftazidime and cefpodoxime) followed by phenotypic (and in some cases genotypic) confirmation tests on resistant strains (figure 3) (EUCAST, 2017).

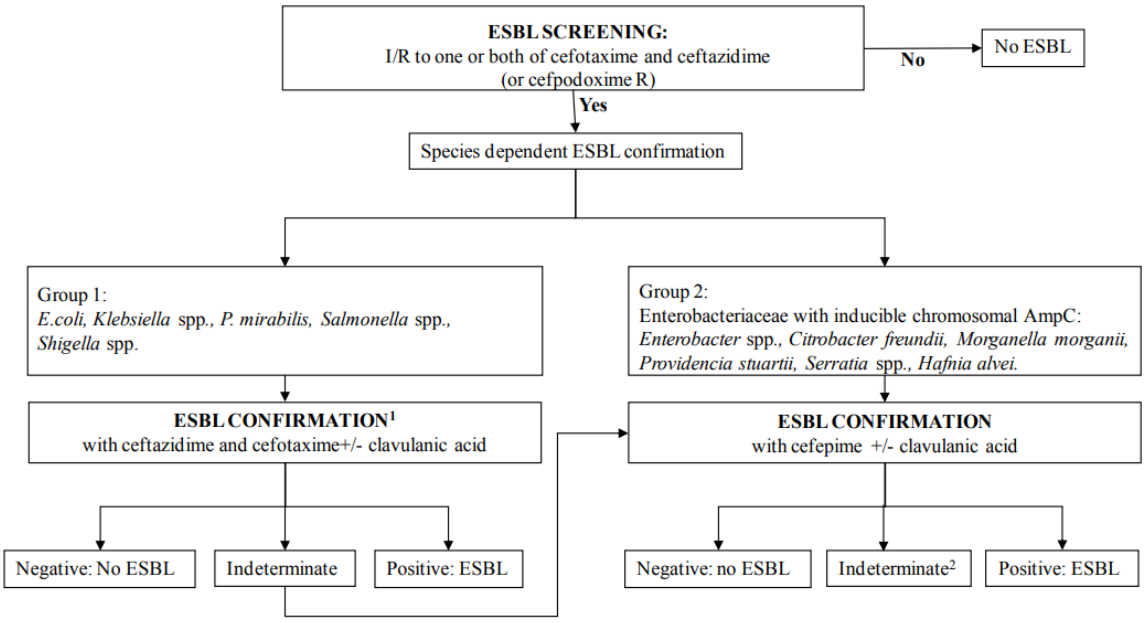


Figure 3. Algorithm for phenotypic detection of ESBL. [1] If cefoxitin has been tested and has an MIC >8 mg/L, perform cefepime+/- clavulanic acid confirmation test. [2] Cannot be determined as either positive or negative (e.g. if a gradient diffusion strip cannot be read due to growth beyond the MIC range of the strip or there is no clear synergy in combination-disk and double-disk synergy tests). In confirmation with cefepime +/- clavulanic acid is still indeterminate, genotypic testing is required (EUCAST, 2017).

Most of the phenotypic methods for ESBL confirmation are based on the *in vitro* inhibition of ESBL activity by clavulanic acid (EUCAST, 2017). These tests include: the combination disk test (CDT), the double-disk synergy test (DDST), the ESBL gradient test, and the broth microdilution test. Despite all

the aforementioned methods have high sensitivity and specificity values (Linscott & Brown, 2005), it should be noted that the presence of ESBL enzymes may be masked by the presence of others resistance mechanisms such as AmpC- and carbapenemase-production and in such cases, molecular methods should be used for ESBL detection (EUCAST, 2017). AmpC production may be suggested by cefoxitin resistance coupled with resistance to ceftazidime and/or cefotaxime. Phenotypic confirmation tests for AmpC production are generally based on inhibition of AmpC by either cloxacillin or boronic acid derivatives (figure 4) (EUCAST, 2017).

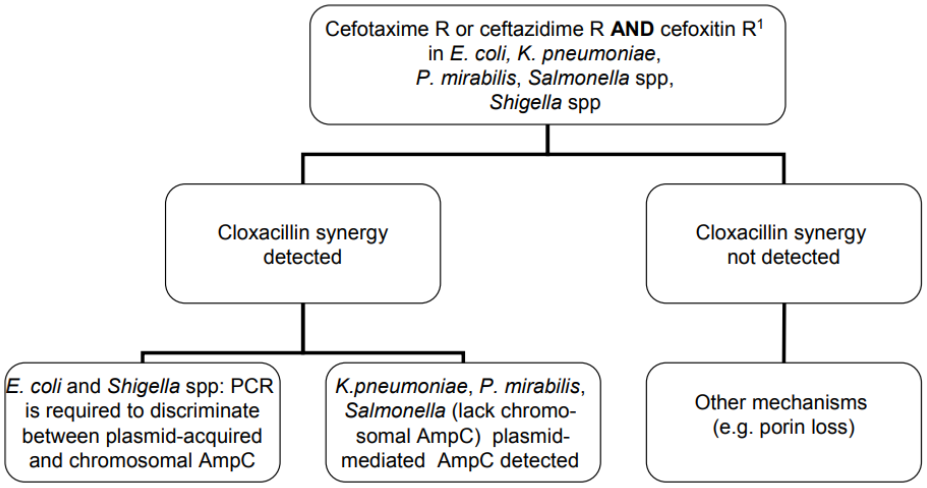


Figure 4. Algorithm for AmpC detection. [1] Cefoxitin 'R' is here defined as MIC >8 mg/L or zone diameter <19mm (EUCAST, 2017).

Several commercially available tests have been used as confirmatory tests for AmpC production, such as the Mast "AmpC Detection Disc Set" which show high sensitivity and specificity values (sensitivity 96-100%, specificity 98%-100%) compared to other in-house or commercially available test (Halstead et al., 2012; Ingram et al., 2011). However, AmpC confirmation tests cannot discriminate between plasmid encoded AmpC and over-expressed chromosomal AmpC. The presence of AmpC-production may be confirmed using PCR-based methods (EUCAST, 2017).

The screening for the presence of carbapenemase-producing bacteria has been suggested to be based on the resistance profile to carbapenems, such as imipenem, meropenem and ertapenem following MIC analysis of strains. However, the screening for the presence of carbapenemase-producing bacteria is more challenging as it cannot solely be based on the resistance profile to carbapenems, since isolates carrying OXA-48 can appear susceptible not only to cephalosporins but also to carbapenems (Silva et al., 2022). Therefore, the lower carbapenems epidemiological cut-off (ECOFF) instead of the clinical breakpoints have been used to screen for the presence of carbapenemase-producing *Enterobacteriaceae* (EUCAST, 2017) as OXA-48 strains may be resistant

to carbapenems only when using ECOFF. However, the resistance profile to temocillin has been reported as the best detection method for OXA-48-producing *Enterobacteriaceae* because high-level temocillin resistance is considered the only reliable phenotypic marker to detect the presence of OXA-48 (EUCAST, 2017). To confirm the screening results, several phenotypic methods for the detection of carbapenemases have been reported including: biochemical (colorimetric) tests, carbapenem inactivation method, lateral flow assays, detection of carbapenem hydrolysis with MALDI-TOF and combination disk test. These methods are based on the hydrolysis of carbapenem (biochemical tests, carbapenem inactivation method, detection of carbapenem hydrolysis with MALDI-TOF), immunological capture of epitopes of carbapenemase enzymes or in vitro inhibition of carbapenemase activity by addition of an inhibitor specific for a class of carbapenemases (disk test) coupled with temocillin resistance for OXA-48 since no inhibitors for OXA-48-like enzymes are available (Cohen Stuart & Leverstein-Van Hall, 2010; EUCAST, 2017; Silva et al., 2022). The Inhibition tests has been recommended by a group of experts from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) study group for antibiotic resistance surveillance (Cohen Stuart & Leverstein-Van Hall, 2010; Miriagou et al., 2010). Particularly, for detection of class A carbapenemases (e.g. KPC), the inhibitor used is boronic acid while for detection of class B metallo-carbapenemases (e.g. NDM), ethylene diamine tetra-acetic acid (EDTA) or dipicolinic acid can be used as an inhibitor (Cohen Stuart & Leverstein-Van Hall, 2010; EUCAST, 2017). Given that boronic acid can also inhibit class C enzymes (AmpC), comparison between the zone diameters of meropenem and meropenem plus cloxacillin should be included in the test (figure 5) (Miriagou et al., 2010).

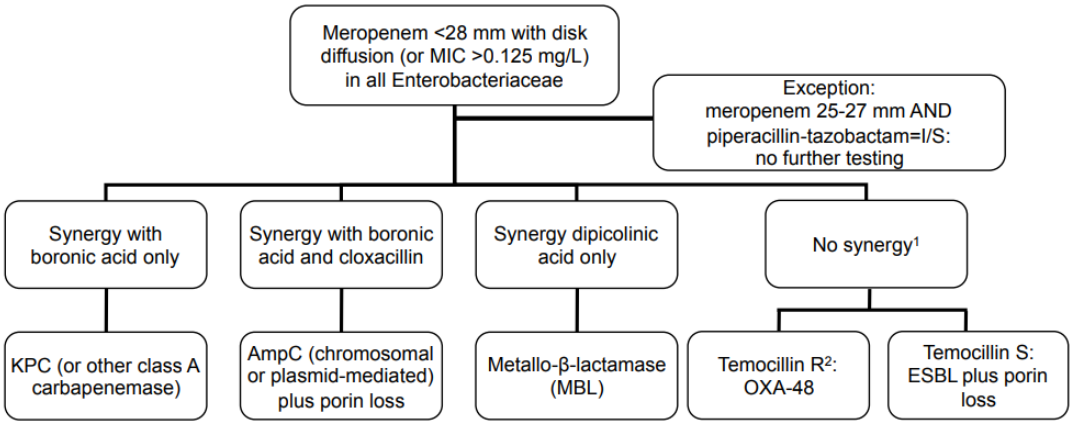


Figure 5. Algorithm for carbapenemase detection. [1] Combination of several carbapenemases can also contribute to no synergy – e.g. MBL and KPC in combination. Molecular testing is usually necessary in such cases. [2] High-level temocillin resistance (>128 mg/L, zone diameter <11 mm) is a phenotypic marker of OXA-48 (EUCAST, 2017).

Harmonized methods have been established by the European Union for the surveillance of AMR in food-producing animals (EU Commission-Decision 2020/1729/EU) and most of the studies and national surveillance programs related to AMR have focused on food-producing animals (Guardabassi, 2004). However standard protocols for AMR surveillance in companion animals have not been included (Timofte et al., 2021). The close contact and sharing of environments between humans and companion animals as well as the wide spread of dogs and cats in European households has raised concerns about the role of companion animals in AMR dissemination and the potential risk of the transmission of resistant bacteria to humans or vice versa (Overgaauw et al., 2020; Pomba et al., 2016).

The presence of ESBL-producing *E. coli* has been reported among dogs worldwide, with an estimated global prevalence of 6.87% [95% CI: 4.46-10.45%]. Different prevalences among countries have been observed likely reflecting differences in the diagnostic methods used; the levels of antibiotic use among veterinarians and owners; and other factors influencing bacterial transmission, including socioeconomic and behavioral components (Salgado-Caxito et al., 2021). Similarly to humans and food-producing animals, the most frequent genes associated with ESBL resistance in dogs encode for CTX-M enzymes, followed by TEM and SHV (Ewers et al., 2012; Formenti et al., 2021). Moreover, the presence of AmpC-producing *E. coli* in dogs, usually carrying the *bla*$_{\text{CMY-2}}$ gene, has been reported with a lower prevalence compared to ESBL-producing *E. coli* (Carvalho et al., 2021; Formenti et al., 2021; Melo et al., 2018). In contrast to ESBL-/AmpC- producing *E. coli*, the presence of carbapenemase-producing *E. coli* has been rarely reported in companion animals (Hong et al., 2020). Indeed, the prevalence of carbapenemase-producing *Enterobacteriaceae* in fecal samples from companion animals is usually low ranging from 0 to 2.5% (González-Torralba et al., 2016; Ortiz-Díez et al., 2023; Yousfi et al., 2016). NDM-5 and OXA-48 are the most frequently reported enzymes in carbapenemase-producing *E. coli* isolated from companion animals (Haenni et al., 2022; Hong et al., 2020; Pulss et al., 2018; Silva et al., 2022; Yousfi et al., 2016). However, it should be noted that the detection of carbapenemase-producing bacteria may be difficult and screening for this resistance mechanism is not usually performed in veterinary medicine, which may lead to underestimating their presence in companion animals (Silva et al., 2022).

Despite being the most popular companion animals in Europe, few studies have investigated the presence of ESBL/AmpC-producing *E. coli* in cats (FEDIAF, 2022; Salgado-Caxito et al., 2021). Particularly, there are twice as many studies reporting prevalence on dogs than cats, with only few

studies focused exclusively on cats (Salgado-Caxito et al., 2021). The presence of ESBL/AmpC-producing *E. coli* in cats has been reported in clinical samples from diseased cats (with a variety of common clinical conditions, including gastrointestinal disease, upper respiratory tract disease, otitis, conjunctivitis, stomatitis, skin abscess, and urinary tract infections) and in fecal samples from healthy cats (Carvalho et al., 2021; Melo et al., 2018; Piccolo et al., 2020; Zogg et al., 2018). In addition to owned cats, stray cats have also been reported as reservoirs of ESBL-producing *E. coli* (Gargano et al., 2022). Cats have been reported as potential reservoir of ESBL/AmpC-producing *E. coli*, with an estimate global prevalence of 5.04% (Salgado-Caxito et al., 2021) and there are increasing reports of carbapenemase-producing bacteria in cats (Silva et al., 2022). Therefore the role of cats in AMR spreading and the host factors associated with the presence of ESBL-, AmpC-, and carbapenemase-producing *E. coli* in cats still needs to be elucidated (Dazio et al., 2021; Pomba et al., 2016).

# AIMS

Cats are the most common companion animals in Europe (FEDIAF, 2022). Emerging viruses have been reported in cats worldwide in the last decades. Emerging pathogens may contribute to severe clinical diseases, whereas the pathogenicity of novel feline viruses, including DCH, is still under investigation (Shofa et al., 2022). Moreover, given their close and direct contact with humans, the potential role of cats as reservoirs and/or sentinels of zoonotic pathogens needs to be investigated (Guardabassi, 2004; Overgaauw et al., 2020). Several zoonotic pathogens that may be transmitted to humans have been reported in cats including the novel SARS-CoV-2 (Qiu et al., 2023). Besides viruses, multidrug-resistant microorganisms represent a major human threat and AMR in cats should be monitored, according to the One Health concept (WHO, 2021b). Therefore, during my PhD I covered three subtopics falling within this general subject:

## Subtopic 1: Emerging viruses in cats: DCH

After the discovery in 2018 of the novel Domestic Cat Hepadnavirus (DCH) in an Australian cat (Aghazadeh et al., 2018), the presence of DCH in cat blood and serum samples has been reported in different countries, including Italy (Lanave et al., 2019). Moreover, the pathogenetic role of DCH in feline liver diseases has been suggested by different studies. Indeed, DCH infection has been associated with hepatocellular carcinoma and chronic hepatitis (Pesavento et al., 2019) and DCH-positive cats have been often reported to have elevated alanine transaminase (ALT) values suggestive of liver injury (Anpuanandam et al., 2021; Capozza et al., 2021a; Lanave et al., 2019; Piewbang et al., 2022). Despite these findings, the pathogenetic role of DCH infection in cats still need to be elucidated (Shofa et al., 2022).

Therefore, the aims were to investigate the presence of DCH in two different scenarios and to genetically characterize the detected strains, particularly:

1. To evaluate the presence of DCH viraemia in cats with biochemical alterations suggestive of liver disease.
2. To evaluate the presence of DCH in cats with and without cavitary effusion.

## Subtopic 2: SARS-CoV-2 in cats

During the early stages of the COVID-19 pandemic the first identification of infected domestic cats in Hong Kong (Barrs et al., 2020) and the detection of infected stray cats nearby SARS-CoV-2 positive

mink farms (Oreshkova et al., 2020; van Aart et al., 2022) have raised interest in the potential role of cats in the SARS-CoV-2 epidemiology. This interest was also based on results of experimental infections showing that cats were highly susceptible to SARS-CoV-2 infection and capable to infect other cats (Bosco-Lauth et al., 2020; Shi et al., 2020). Moreover, serological tests using reliable methods are crucial for infection surveillance in cats, but discordant results among different serological assays were often reported in early pandemic stages (Decaro et al., 2022; Jemeršić et al., 2021; Klaus et al., 2021b; Michelitsch et al., 2020; Udom et al., 2022; Zhang et al., 2020).

Therefore, the aims of for this subtopic were:

1. To evaluate the presence of SARS-CoV-2 RNA and anti-SARSCoV-2 antibodies in free roaming cats belonging to colonies located in an area highly affected by the COVID-19 pandemic.
2. To verify whether cats and dogs may act as passive mechanical carriers of SARS-CoV-2 when they live in close contact with COVID-19 patients.
3. To evaluate the diagnostic performance of three different commercially available immunoassays for the detection of SARS-CoV-2 antibodies in pets, in comparison with conventional virus neutralization test.

## Subtopic 3: ESBL-/AmpC- and carbapenemase-producing *E. coli* in cats.

The WHO has recently listed AMR as one of the biggest threats to global health (WHO, 2022). ESBL-producing and carbapenem-resistant *Enterobacteriaceae* has been classified as priority 1 critical pathogens (WHO, 2017) and *E. coli* have been proposed as commensal indicator microorganisms for AMR surveillance systems (EU Commission-Decision 2020/1729/EU; WHO, 2021a). In veterinary medicine the presence of AMR has been studied mainly in food-producing animals. In pets, prevalence, molecular characterization and/or risk factors associated with carriage of ESBL-producing *E. coli* has been recently investigated mainly in dogs and less in cats, also in Italy (Dazio et al., 2021; Formenti et al., 2021; Salgado-Caxito et al., 2021). Carriage of ESBL-/AmpC- and carbapenemase-producing *E. coli* in domestic and stray cats remains poorly investigated and monitoring of carriage of AMR bacteria in cats is necessary to better understand the epidemiology and the risk of transmission for both animals and owners, according to the One Health approach.

Therefore, the aim of this subtopic was to investigate the presence of ESBL-/AmpC- and carbapenemase-producing *E. coli* in both domestic and stray cats, to perform bacteriological analysis for the isolation of ESBL-, AmpC- and carbapenemase-producing *E. coli*, followed by phenotypic, genetic characterization of AMR determinants.

ORIGINAL RESEARCH

Vet Record

# Evaluating the presence of domestic cat hepadnavirus viraemia in cats with biochemical alterations suggestive of liver disease

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**Foreword**: in this study, we evaluated the association between DCH viremia and clinicopathological changes consistent with liver disease in cats, to explore the potential pathogenetic role of DCH. My contribution to this work was related to laboratory analysis and manuscript review. The results of this study were published under the reference:

Scavone D, Lauzi S, Stranieri A, Tramontano G, Ratti G, Paltrinieri S. Evaluating the presence of domestic cat hepadnavirus viraemia in cats with biochemical alterations suggestive of liver disease. *Vet Rec*. 2022 Aug;191(4):e1626. doi: 10.1002/vetr.1626. Epub 2022 Apr 8. PMID: 35393638.

**Abstract**

Background: The association between domestic cat hepadnavirus (DCH) infection and feline chronic hepatitis and hepatocellular carcinoma has been suggested. However, studies focused on the association between DCH infection and clinicopathological changes consistent with liver disease in cats are not available.

Methods: This retrospective investigation included sera obtained from 96 cats that had the serum activity of at least alanine aminotransferase or alkaline phosphatase measured during initial diagnostic work-up. Based on these haematobiochemical results, cats were categorised according to their likelihood of having liver disease (absent, low, intermediate or high). DCH DNA was detected using real-time PCR, nested PCR and sequencing.

Results: Overall, potential liver damage was observed in 44 cats, including cats with low (n = 14), intermediate (n = 10) and high (n = 20) likelihood of liver disease. Four cats (4.2%) were DCH-positive, with three positive cats belonging to the liver disease group (two with low and one with intermediate likelihood of liver disease).

Conclusions: Although the pathogenic potential of DCH in cats still has to be clarified, these results suggest that DCH testing should not be based only on the presence of biochemical changes potentially consistent with liver disease.

**Aims**

The aim of this work was to define, by a retrospective investigation, if the prevalence of DCH infection is higher in cats with biochemical alterations suggestive of liver disease compared with cats without abnormalities consistent with structural or functional liver damage.

**Material and methods**

*Animals*

The study was performed on cats admitted to the Veterinary Teaching Hospital (VTH) of Lodi between January and December 2019. Serum specimens stored at −20°C were retrospectively selected from the routine diagnostic archive of samples previously sent to the laboratory of clinical pathology for diagnostic purposes. Blood samples collected in tubes with clot activator and submitted to the laboratory had been immediately centrifuged and the biochemical analyses required had been carried out on serum. Residual volumes of serum had been stored at −20°C within the day. According to the guidelines of our institution, a formal approval was not required because leftover serum used in this study was harvested from blood samples collected for diagnostic purposes or as part of health checks and with informed consent of the owners (decision no. 2/2016 of the Ethical Committee of the University of Milan). Cats and respective samples were included if at least the activity of ALT and ALP had been measured. When other parameters such as AST, GGT, creatine kinase (CK) activity and total bilirubin concentration had been measured, results were considered and recorded as well. Biochemistry analyses had been performed with an automated chemistry analyser (BT 3500, Biotecnica Instruments, Rome, Italy) and reagents provided by the instrument's manufacturer. Data regarding signalment and, when available, clinical history were recorded for each cat. The cats were then categorised into two groups according to their age: ≤2 and >2 years. Cats were also categorised according to clinical information and biochemical analyses

results for liver disease status. Given that the availability of clinical information was not exhaustive, a definite diagnosis of hepatopathy could not always be confirmed or ruled out for all cats. Therefore, cats were classified based on their probability of having liver disease according to biochemical analyses reported above (ALT, ALP and, if present, AST, GGT, CK activity and total bilirubin concentration). In particular, we selected three categories of liver disease probability based on signalment (particularly age) and biochemical results, considering the specificity of each biochemical change for primary liver disease and the magnitude of increase with respect to the relevant upper reference range. The three categories were the following:

- Low: less than two-fold increase in ALT activity or more than two-fold increase in ALT activity paired with simultaneous increase in CK activity and normal ALP, GGT activity and bilirubin concentration, suggestive of muscle damage; increase in ALP activity presumably due to isoenzymes of non-hepatic origin, such as increase in bone ALP activity related to growth or concurrent diseases (e.g. hyperthyroidism) (Archer & Taylor, 1996; Hall & German, 2016)
- Intermediate: more than two-fold and less than five-fold increase in ALT activity; less than two-fold simultaneous increase in ALT and ALP activity presumably not due to extrahepatic causes or not associated with abnormalities in other parameters (e.g. GGT), when available
- High: more than two-fold simultaneous increase in ALT and ALP activity or more than five-fold increase in ALT activity alone.

The algorithm used to select and classify the samples is reported in figure 6.

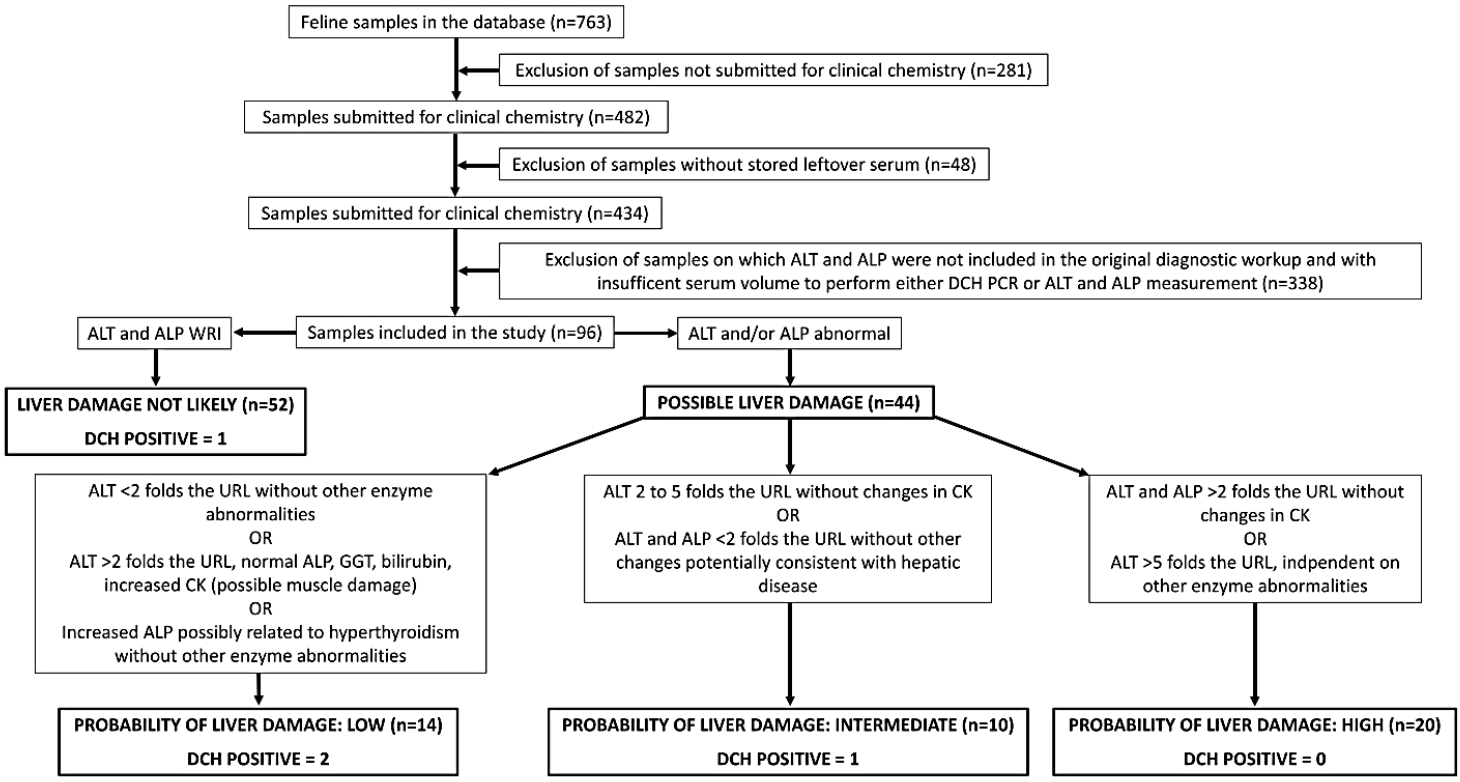


Figure 6. Flowchart summarising the criteria and procedure for sample selection. ALP, alkaline phosphatase; ALT, alanine transaminase; CK, creatine kinase; DCH, domestic cat hepadnavirus; GGT, gamma-glutamyl transferase; URL, upper reference interval; WRI, within reference interval.

*PCR and phylogenetic analysis*

Time of storage (ie, time from collection of sample to PCR analysis) was first calculated. DNA was extracted from frozen-thawed serum samples using a commercial DNA extraction kit (NucleoSpin Blood kit, Macherey-Nagle, Germany), following the manufacturer's instructions. DNA pre-analytical quality control targeting vertebrate 12S rRNA locus was performed on randomly selected samples (results not shown) (Kitano et al., 2007). All samples were subjected to quantitative PCR (qPCR) for specific DCH amplification (Lanave et al., 2019). Threshold cycle (Ct) number was used as the measure of viral load (the lower the Ct level the greater the amount of target nucleic acid is present in the sample). A pan-hepadnaviral nested PCR was also performed on all samples based on published methods using degenerate primers targeting a 258 bp fragment of the conserved domain of the polymerase gene of viruses in the family Hepadnaviridae in the second reaction (Wang et al., 2017). Nested PCR products were visualised under a UV transilluminator on a 1.5% agarose gel stained with ethidium bromide. DCH DNA extracted from a naturally infected cat was used as

positive control. A blank control (DNAse-free water sample) was also included in the qPCR and nested PCR reactions every 10 reactions, both at primary and secondary amplification in the nested PCR reaction. The amplicon of the expected size obtained from the second reaction of the pan-hepadnaviral PCR was purified from positive samples and sequenced using the outer forward and reverse primers used for DNA amplification. Sanger sequencing was performed by a commercial sequencing facility (Microsynth Seqlab, Germany). The sequences were then compared with those available in GenBank using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For phylogenetic analysis, the sequences were aligned with DCH reference sequences and with representative sequences of other mammalian orthohepdnaviruses retrieved from GenBank using Clustal X in BioEdit software v.7.0. Phylogeny was estimated by a neighbourjoining algorithm (NJ) and by maximum likelihood methods with 1000 bootstrap replicates using MEGA v.7 (Felsenstein, 1981; Kimura, 1980; Kumar et al., 2016). For sequences comparison, the percentage of nucleotide similarity of pairwise evolutionary distances was calculated using MEGA v.7. Animals showing positive qPCR and nested PCR results confirmed by sequencing and phylogeny were considered DCH positive. The DCH sequences obtained in this study were deposited in GenBank under accession numbers OM785180-OM785183.

*Data analyses*

The 95% confidence limits were calculated for DCH-positive cats. Pearson's chi-square test was used to evaluate the differences between proportions of DCH-infected cats and sex, age, type of ownership and likelihood of liver disease status. Cats with missing data on sex or age were not included in the sex and age categories, respectively, and were not analysed. Statistical comparisons were carried out using Epitools, taking $p < 0.05$ as significant (http://epitools.ausvet.com.au).

**Results**

*Caseload and occurrence of biochemical abnormalities*

The results of the selection and classification procedures are summarised in Figure 6. Overall, 598 cats were admitted to the VTH in 2019 for diagnostic purposes, corresponding to 763 feline samples submitted to the laboratory of clinical pathology. Out of the 482 feline serum samples, 434 residual samples were stored at −20°C, corresponding to samples collected from 301 cats. Among the stored samples corresponding to single sampling from cats with adequate aliquot for PCR analysis, a total of 96 serum samples had ALT and ALP measurements and were included and analysed in this study. Sex and age were unknown for eight and 20 cats, respectively. Among cats with recorded sex and

age, 39 were male and 49 were female, with median age of 8 years, ranging from 2 months to 20 years. Fifty-two cats (54.2%) had no biochemical changes potentially consistent with liver disease, while 44 (45.8%) showed abnormalities in at least ALT or ALP activity and possibly also in other parameters like AST, GGT, CK and bilirubin. Based on the subdivision into categories of likelihood to have liver disease, 14 cats were categorised as low probability, 10 as intermediate probability and 20 as high probability of liver disease. Data regarding signalment and biochemical analyses of these cats are summarised in Table 1 and in extended form in Table S1.

Table 1. Cats' characteristics according to the likelihood of liver disease group of animals analyzed in this study (number of cats and, between parentheses, percentages).

| Variable | Category | Total | Biochemical changes | | Likelihood of liver disease | | |
|---|---|---|---|---|---|---|---|
| | | | No | Yes | Low | Intermediate | High |
| Age | ≤ 2 years | 16 | 4 (25) | 12 (75) | 6 (37.5) | 1 (6.3) | 5 (31.3) |
| | > 2 years | 60 | 38 (63.3) | 22 (36.7) | 5 (8.3) | 5 (8.3) | 12 (20) |
| | nd | 20 | 10 (50) | 10 (50) | 3 (30) | 4 (40) | 3 (30) |
| Gender | male | 39 | 21 (53.8) | 18 (46.2) | 6 (15.4) | 3 (7.7) | 9 (23.1) |
| | female | 49 | 26 (53.1) | 23 (46.9) | 8 (16.3) | 7 (14.3) | 8 (16.3) |
| | nd | 8 | 5 (62.5) | 3 (37.5) | - | - | 3 (37.5) |
| Purebred cat | no | 84 | 46 (54.8) | 38 (45.2) | 12 (14.3) | 9 (10.7) | 17 (20.2) |
| | yes | 9 | 4 (44.4) | 5 (55.5) | 2 (22.2) | 1 (11.1) | 2 (22.2) |
| | nd | 3 | 2 (66.7) | 1 (33.3) | - | - | 1 (33.3) |
| Type of ownership | owned | 56 | 35 (62.5) | 21 (37.5) | 6 (10.7) | 4 (7.1) | 11 (19.6) |
| | stray | 40 | 17 (42.5) | 23 (57.5) | 8 (20) | 6 (15) | 9 (22.5) |
| Total | | 96 | 52 (54.2) | 44 (45.8) | 14 (14.6) | 10 (10.4) | 20 (20.8) |

nd= not determined

*Occurrence of DCH and phylogeny*

Serum samples were stored at −20°C for a median of 255 days (ranging from 14 to 349 days) before performing DCH analysis (Table S1). Overall, four cats tested positive using both the nested PCR and the qPCR reactions, with one positive sample showing a Ct value of 32.1 and the other three positive samples showing very high Ct values (Ct > 38) (Tables 2 and S1).

Table 2. Number and percentage of cats with DCH according to characteristics of cats and likelihood of liver disease group.

| Variable | Category | No. tested | DCH (%) |
|---|---|---|---|
| Age* | ≤ 2 years | 16 | 2 (12.5)[a] |
| | > 2 years | 60 | 1 (1.7)[a] |
| Gender | Male | 39 | 2 (5.1)[a,b] |
| | Female | 49 | 2 (4.1)[a] |
| Purebred cat | No | 84 | 4 (4.8) [a,b] |
| | Yes | 9 | 0 (0) |
| Type of ownership | Owned | 56 | 1 (1.8)[a] |
| | Stray | 40 | 3 (7.5) [a,b] |
| Biochemical changes potentially consistent with liver disease | No | 52 | 1 (1.9) [a] |
| | Yes | 44 | 3 (6.8) [a,b] |
| Likelihood of liver disease | Low | 14 | 2 (14.3%)[a] |
| | Intermediate | 10 | 1 (10.0%) [b] |
| | High | 20 | 0 (0.0) |

* Age was unknown for one DCH-positive cat; [a] DCH-positive cats with Ct value > 38; [b] DCH-positive cat with Ct value of 32.1.

Storage of the four positive samples ranged from 140 to 267 days (Table S1). The four sequences that were amplified using the pan-hepadnaviral PCR were further confirmed as DCH by BLAST analysis that showed the highest nucleotide similarity ranging from 96.1% to 99.2% with DCH sequences. The nucleotide percentages of similarity of pairwise evolutionary distances among the sequences detected in this study was 97.6%–99.2%. The phylogenetic tree based on maximum likelihood analyses showed that all the four sequences of this study clustered with DCH sequences and not with other orthohepadnaviruses (Figure 7). Results of the phylogenetic analysis were similar using the NJ method (data not shown). Our results showed a 4.2% (95% confidence interval: 0.2–8.2) DCH prevalence in the whole population of tested cats.

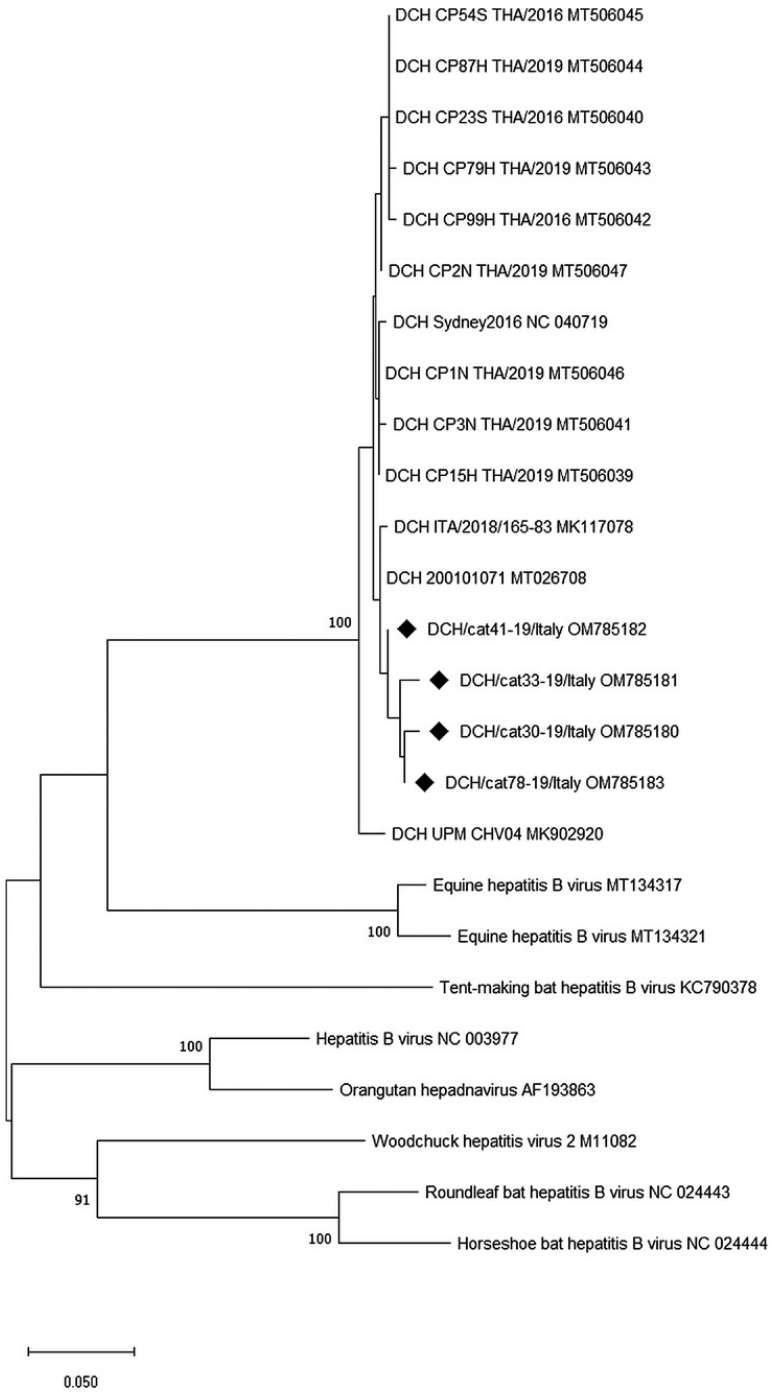


Figure 7. Phylogenetic tree generated with maximum likelihood analysis. A 258 bp region of the polymerase gene obtained from mammalian *Orthohepadnavirus* sequences retrieved from GenBank databases and the domestic cat hepadnavirus (DCH) representative sequence obtained in this study was used. Sequences are indicated by GenBank accession number (available at www.ncbi.nlm.nih.gov/pubmed/) and name of the strain. Distances were computed using the General Time Reversible model using MEGA v.7. Bootstrap values above 70% are given. The black diamonds indicate the sequences obtained in this study.

*Factors associated with DCH positivity*

Three of the four DCH PCR-positive cats showed abnormalities in at least ALT and ALP and, according to the criteria used in our study, the prevalence of positive cats among patients with increased ALT and/or ALP accounts for 6.8%. However, none of the positive cats had a high probability of liver disease based on the results of serum enzymes, since these three DCH-positive cats were categorised as having low (n = 2) and intermediate (n = 1) likelihood of liver disease. The statistical analyses showed no significant association of DCH positivity with sex, age, type of ownership or liver disease status (Table 2).

**Discussion**

We evaluated the frequency of DCH infection in the blood of cats with and without alterations in serum biomarkers suggestive of liver disease. The overall prevalence of DCH viraemia obtained in this study (4.2%) is in accordance with previous studies showing prevalence ranging from 1.9% to 12.4%, with differences in the sampled populations possibly due to geographical variances and control measures (Aghazadeh et al., 2018; Anpuanandam et al., 2021; Jeanes et al., 2022; Lanave et al., 2019; Piewbang et al., 2020). Further investigations are needed to assess if the geographical areas with different endemicity and different transmission dynamics previously reported for HBV may also account for DCH infection in cats (Alter, 2006). Differences in the efficiency of DNA extraction based on the use of different commercial kits and storage of samples may have accounted for the prevalence of DCH infection recorded in this study. DCH DNA in serum samples stored at −20°C may be damaged, and therefore low/intermediate viral loads may go undetected. However, positive samples were also found among those collected at the beginning of the study period, suggesting that long storage may not be the only issue accounting for the low prevalence of infection. In order to confirm DCH prevalence, diagnostic procedures in cats should include a panel of antibodies and antigens and viral DNA to profile the stage of infection, as reported in humans since there are some forms of HBV (i.e. occult infections) in which viral DNA is present at low titer, or it is not detected at all, in serum samples (Terrault et al., 2018).

The kinetic of DCH in serum as well as DCH transmission dynamics in cats are still unknown. Long-term infection, with DCH detectable in serum, has been recently reported in a proportion of DCH-infected cats (Capozza et al., 2021a). Transmission occurring through blood and body fluids, sexual intercourse and transplacental route, as reported for HBV in humans and for FIV and feline leukaemia virus (FeLV) in cats, could be hypothesised for DCH as well (Al-Sadeq et al., 2019; Hartmann, 2012;

Lanave et al., 2019). In this regard, close contact with other cats may pose a risk for DCH infection. Therefore, stray and free-roaming cats, and also client-owned cats with an outdoor lifestyle, may have a higher risk of infection from DCH, as happens for retroviral infections (Stavisky et al., 2017; Studer et al., 2019). It is interesting to observe that, among the DCH-positive cats found in our study, the majority of cats were housed in shelters or colonies, whereas only one was a client-owned cat. However, further investigations are needed to define type of ownership of cats as a risk factor for DCH infection because significant differences between stray cats and privately owned cats were not detected in our study. Moreover, the only other study that investigated this aspect detected a significantly higher prevalence of DCH in pet cats compared to shelter cats (Anpuanandam et al., 2021). Our results showing that sex and age are not associated with DCH infection are confirmed by recent reports (Piewbang et al., 2020). However, definition of age as a risk factor probably needs further investigations because both a significant association of DCH infection in cats older than 2 years as well as higher odds of being DCH positive in cats aged 4–7 months, albeit not significantly, have been previously reported (Anpuanandam et al., 2021; Lanave et al., 2019).

The association between hepadnaviral and retroviral infection previously reported in cats, mirroring HBV and HIV infections in humans, was not investigated in our study due to the lack of information regarding FIV and FeLV status of cats (Aghazadeh et al., 2018; Anpuanandam et al., 2021; Koziel & Peters, 2007; Lanave et al., 2019). These data were available only for two DCH-positive cats and one of them was FIV infected. Unfortunately, residual volume of other samples was not enough to perform viral antigen and antibody detection.

Regarding the pathogenic role of DCH, abnormalities in ALT or ALP activity and often in other possible markers of liver damage or dysfunction were detected in three out of four DCH-positive cats. This may suggest an association between DCH infection and liver disease. However, it should be considered that statistical analysis, despite potentially affected by the low number of observations, did not reveal a significant difference between the 3/44 (6.8%) DCH-positive cats included in this study with biochemical abnormalities potentially consistent with liver diseases (increased ALT or ALP, and in most cases other possible biomarker of hepatic dysfunction of damage) and the 1/52 (1.9%) positive cat belonging to the group without abnormalities, suggesting that DCH viraemia does not predict liver disease. The proportion of positive cats with enzyme abnormalities is lower than that reported in a recent study, on which, however, the threshold to classify as ‘high’ the ALT activity was so close to the upper reference limit to likely include mostly cats without hepatic diseases (Anpuanandam et al., 2021). As a matter of fact, in the absence of information on the actual

presence of liver disease based on clinical findings, diagnostic imaging or histopathology, that is a limitation of this and of many other retrospective studies, we estimated the likelihood of liver diseases rather than presence/absence of liver damage. The categorization of likelihood of liver disease was based on the cross-matching of the presence of biochemical changes in serum, with special emphasis on liver enzymes and paying attention either on the magnitude of the increase of single enzymes, or on the simultaneous change of more than one enzyme and the information about signalment of the cats. From this standpoint, only very high values of ALT activity alone (>5-fold increase) were considered as highly suggestive of liver damage, in accordance with recent recommendations in cats, although in people the magnitude of increases potentially consistent with liver damage is even higher (Center, 2007; Kim et al., 2008). Moreover, if ALT and/or ALP increase were moderate, we suspected liver disease in the presence of simultaneous increase of other enzymes potentially related with liver damage, since slight or mild increase of ALT and/or ALP alone may depend on extrahepatic causes (Center, 2007; Lidbury & Steiner, 2013). Unfortunately, the lack of additional information did not allow us to definitely conclude which type of liver disease, if any, was actually present and therefore it was not possible to compare the results of DCH testing with some specific type of liver disease observed in cats that usually induces relevant changes in liver enzymes such as lymphocytic cholangitis (Boland & Beatty, 2017). Conversely, we were aware that the lack of changes in ALT and/or ALP may have not excluded the presence of all hepatopathies, since their activity may remain within reference intervals if the functional hepatic mass is reduced (e.g. hepatic cirrhosis) or when dysfunctional hepatocytes are not structurally damaged (liver failure or insufficiency) (Lidbury & Steiner, 2013). Our analysis revealed that despite the increase of ALT and ALP, more than half of the cats with biochemical signs potentially consistent with hepatopathies (i.e. the cats with low or intermediate probability of hepatopathy), were likely not affected by hepatobiliary diseases and a high probability of liver disease was detected in only a quarter of the total cats analysed. Interestingly, none of the cats with high probability of liver disease was DCH positive suggesting that the possible association between DCH infection and liver disease hypothesised only on the basis of increased ALT or ALP is not supported by the individual analysis of biochemical and signalment data of the cats included in the study. In humans, high viral load in HBV infection has been correlated with virus replication and hepatocyte damage, and a similar correlation has been suggested for DCH in cats (Lanave et al., 2019). In this respect, our results showing Ct values indicative of higher viral load in the sample from the cat with intermediate probability of liver disease compared to the lower viral loads in the samples of cats without

biochemical abnormalities and with low probability of liver disease support this hypothesis but further investigations are needed. The presence of a correlation between DCH infection and liver disease still needs to be established, as recently suggested (Capozza et al., 2021a).

However, it should be considered that the prevalence of DCH in blood samples is lower than in liver tissue samples, since in some stages of the infection DCH DNA is only detectable in liver tissue (Anpuanandam et al., 2021). Consequently, it is important to outline that the prevalence of viraemia does not reflect, but rather underestimates, the true prevalence of DCH infection. Therefore, a more detailed information about the possible association between a clinical diagnosis of liver disease and DCH infection should be based on PCR and histopathological analysis of liver biopsies that, however, are not routinely collected in routine practice.

Overall, the limitations of our study include possible storage artefacts, poor knowledge of the biology of the virus and kinetic of DCH infection that would help to understand the impact of DCH on the feline population, absence of liver biopsies that does not allow to definitely conclude that cats with increased enzyme activity actually have liver disease and/or to define which type of liver disease is present and absence of whole genome sequences of the viruses.

In summary, our study showed that only a small proportion of cats with biochemical alterations suggestive of liver disease were DCH positive, and that DCH was not detected in cats that, based on clinical chemistry, had a high probability of liver disease. This suggests that further testing for DCH (i.e. liver biopsies, PCR on blood or hepatic tissues) should not be based only on the presence of biochemical changes potentially consistent with liver disease.

## Supplementary materials

Table S1. Age, sex, breed, type of ownership, storage, biochemical parameters, likelihood of liver disease and DCH status of cats analyzed in this study

| No. | Sex | Age (months) | Breed | Type of ownership | Storage days (collection-PCR analysis) | Total protein | Albumin | ALT | AST | ALP | GGT | Total Bilirubin | CK | Likelihood of liver disease | DCH qPCR (Ct) | DCH nested PCR | Accession no. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | MN | 96 | DSH | owned | 343 | 8,3 | 4,4 | 1157 | 208 | 138 | 1 | 1,98 | | H | neg | neg | |
| 2 | FN | 36 | DSH | owned | 270 | 6,7 | 2,8 | 104 | | 170 | 1 | | | H | neg | neg | |
| 3 | MN | | DSH | owned | 210 | 8,1 | 3,1 | 254 | 107 | 562 | 1 | | | H | neg | neg | |
| 4 | FN | 12 | Persian | owned | 209 | 7,2 | 3 | 442 | 11 | 46 | 3 | 0,19 | 74 | H | neg | neg | |
| 5 | F | 48 | DSH | stray/colony | 209 | 5,6 | 2,5 | 1260 | | 232 | 5 | 0,37 | | H | neg | neg | |
| 6 | MN | 132 | DSH | stray/colony | 196 | 7,5 | 3,5 | 267 | 139 | 325 | 6 | 10,98 | | H | neg | neg | |
| 7 | | 108 | DSH | owned | 119 | 5,8 | 3 | 315 | 212 | 61 | 1 | 3,26 | | H | neg | neg | |
| 8 | FN | 84 | DSH | stray/colony | 322 | 5,9 | 2,9 | 239 | | 172 | | | | H | neg | neg | |
| 9 | F | | DSH | stray/colony | 281 | 8,5 | 3,1 | 427 | 272 | 47 | 1 | 2,43 | 3451 | H | neg | neg | |
| 10 | MN | 156 | DSH | owned | 280 | 7,6 | 3,3 | 100 | 95 | 105 | 7 | 5,94 | | H | neg | neg | |
| 11 | M | 2 | DSH | stray/colony | 278 | 7,2 | 3,1 | 178 | | 125 | | | | H | neg | neg | |
| 12 | MN | 132 | DSH | owned | 270 | 7,4 | 5,45 | 274 | 30 | 98 | 1 | 0,43 | 67 | H | neg | neg | |
| 13 | M | 3 | DSH | stray/colony | 264 | 6,8 | 3,4 | 479 | 258 | 125 | 3 | 0,32 | 17572 | H | neg | neg | |
| 14 | MN | 132 | | owned | 259 | 7,4 | 3,1 | 357 | 258 | 147 | 0 | 4,45 | | H | neg | neg | |
| 15 | M | 4 | DSH | stray/colony | 250 | 8,5 | 3,5 | 539 | | 451 | | | | H | neg | neg | |
| 16 | | | DSH | stray/colony | 243 | 6,6 | 3,2 | 386 | 32 | 19 | 11 | 0,98 | 435 | H | neg | neg | |
| 17 | FN | 72 | DSH | owned | 14 | 7 | 2,8 | 487 | 510 | 507 | 8 | 6,9 | | H | neg | neg | |
| 18 | | 156 | Maine Coon | owned | 14 | 7,9 | 2,9 | 359 | 86 | 233 | 13 | 1 | | H | neg | neg | |
| 19 | FN | 240 | DSH | owned | 14 | 6,1 | 2,3 | 206 | 54 | 264 | 4 | 1,4 | | H | neg | neg | |
| 20 | F | 12 | DSH | stray/colony | 222 | 10,6 | 4 | 411 | 136 | 22 | 2 | 0,41 | 4580 | H | neg | neg | |
| 21 | F | | DSH | stray/colony | 346 | 4,7 | 2,1 | 223 | 140 | 23 | 4 | 0,58 | 9383 | L | neg | neg | |
| 22 | M | | Persian | stray/colony | 321 | 7 | 2,6 | 106 | 20 | 37 | 3 | 0,5 | 98 | L | neg | neg | |
| 23 | M | 7 | DSH | owned | 305 | 7,1 | 2,4 | 60 | 15 | 116 | 5 | 0,43 | | L | neg | neg | |
| 24 | F | | DSH | stray/colony | 294 | 4,8 | 2 | 231 | 56 | 30 | 3 | 0,29 | 761 | L | neg | neg | |
| 25 | M | 36 | DSH | stray/colony | 287 | 7 | 2,4 | 97 | 23 | 22 | 2 | 0,31 | 408 | L | neg | neg | |
| 26 | FN | 120 | DSH | owned | 255 | 7 | 1,9 | 145 | | 22 | | | | L | neg | neg | |
| 27 | MN | 48 | DSH | stray/colony | 215 | 6,6 | 2,8 | 117 | | 35 | | | | L | neg | neg | |
| 28 | F | 60 | DSH | stray/colony | 277 | 6,3 | 2,6 | 315 | 326 | 62 | 3 | 0,28 | 4982 | L | neg | neg | |

| No. | Sex | Age (months) | Breed | Type of ownership | Storage days (collection-PCR analysis) | Total protein | Albumin | ALT | AST | ALP | GGT | Total Bilirubin | CK | Likelihood of liver disease | DCH qPCR (Ct) | DCH nested PCR | Accession no. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 30 | F | 5 | DSH | owned | 266 | 8,1 | 3,7 | 34 | | 393 | | | | L | pos (39.3) | pos | OM785180 |
| 31 | M | 7 | DSH | owned | 256 | 7,4 | 3,3 | 41 | 40 | 179 | 2 | 0,45 | | L | neg | neg | |
| 32 | FN | 144 | DSH | owned | 238 | 8,3 | 3,5 | 40 | 15 | 122 | 1 | 0,28 | | L | neg | neg | |
| 33 | M | 3 | DSH | stray/colony | 223 | 7,5 | 3,8 | 400 | 88 | 245 | 2 | 0,35 | 3813 | L | pos (38.9) | pos | OM785181 |
| 34 | F | 4 | Norwegian | owned | 211 | 6,9 | 3,7 | 59 | 31 | 98 | 2 | 0,5 | | L | neg | neg | |
| 35 | FN | 132 | DSH | owned | 326 | 7,5 | 3,1 | 29 | 18 | 253 | 1 | 0,94 | | I | neg | neg | |
| 36 | FN | 156 | DSH | owned | 349 | 6 | 3,2 | 95 | | 104 | | | | I | neg | neg | |
| 37 | M | | DSH | stray/colony | 320 | 8,2 | 2,6 | 158 | 0 | 29 | 8 | 0,17 | 408 | I | neg | neg | |
| 38 | FN | | DSH | stray/colony | 300 | 6,8 | 2,5 | 85 | 28 | 275 | 1 | 0,37 | 275 | I | neg | neg | |
| 39 | FN | 156 | DSH | owned | 217 | 9,5 | 3,3 | 216 | 30 | 62 | 4 | 0,55 | 239 | I | neg | neg | |
| 40 | F | 24 | DSH | stray/colony | 206 | 8,4 | 3,4 | 99 | | 94 | | | | I | neg | neg | |
| 41 | M | | DSH | stray/colony | 140 | 7 | 3,7 | 109 | 4 | 105 | 3 | 0,65 | 136 | I | pos (32.1) | pos | OM785182 |
| 42 | F | | DSH | stray/colony | 243 | 7,3 | 2,9 | 344 | 170 | 0 | 0 | | | I | neg | neg | |
| 43 | F | 60 | DSH | stray/colony | 240 | 6,7 | 3,2 | 287 | 55 | 22 | 3 | 0,64 | 517 | I | neg | neg | |
| 44 | MN | 84 | Birman | owned | 233 | 7,3 | 2,3 | 45 | 387 | 1 | 0 | 1,59 | 7521 | I | neg | neg | |
| 45 | MN | 156 | DSH | owned | 329 | | | 39 | | 23 | | | | no | neg | neg | |
| 46 | F | | DSH | stray/colony | 324 | | | 30 | | 22 | | | | no | neg | neg | |
| 47 | MN | | DSH | stray/colony | 324 | | | 65 | | 28 | | | | no | neg | neg | |
| 48 | FN | 156 | DSH | owned | 323 | | | 58 | | 44 | | | | no | neg | neg | |
| 49 | | 144 | DSH | stray/colony | 323 | | | 43 | | 51 | 3 | | | no | neg | neg | |
| 50 | M | 156 | DSH | owned | 321 | | | 79 | | 25 | | | | no | neg | neg | |
| 51 | FN | 180 | DSH | owned | 320 | | | 47 | | 50 | 3 | | | no | neg | neg | |
| 52 | MN | 84 | DSH | owned | 317 | | | 32 | | 60 | | | | no | neg | neg | |
| 53 | F | 132 | DSH | owned | 310 | | | 78 | | 34 | 1 | | | no | neg | neg | |
| 54 | | 36 | DSH | owned | 306 | | | 54 | | 35 | | | | no | neg | neg | |
| 55 | MN | 144 | DSH | owned | 306 | | | 37 | | 61 | | | | no | neg | neg | |
| 56 | FN | 96 | DSH | owned | 300 | | | 53 | | 45 | | | | no | neg | neg | |
| 57 | M | 8 | Persian | owned | 285 | | | 36 | | 93 | 1 | | | no | neg | neg | |
| 58 | F | | DSH | stray/colony | 252 | | | 60 | | 19 | | | | no | neg | neg | |
| 59 | FN | 120 | DSH | owned | 282 | | | 70 | | 64 | | | | no | neg | neg | |
| 60 | F | | DSH | stray/colony | 282 | | | 46 | | 89 | | | | no | neg | neg | |
| 61 | MN | 108 | British shorthair | owned | 279 | | | 28 | | 50 | | | | no | neg | neg | |

| No. | Sex | Age (months) | Breed | Type of ownership | Storage days (collection-PCR analysis) | Total protein | Albumin | ALT | AST | ALP | GGT | Total Bilirubin | CK | Likelihood of liver disease | DCH qPCR (Ct) | DCH nested PCR | Accession no. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 63 | MN | 156 | DSH | owned | 275 | | | 39 | 58 | 3 | | | | no | neg | neg | |
| 64 | FN | 168 | DSH | owned | 274 | | | 52 | 38 | 1 | | | | no | neg | neg | |
| 65 | FN | 204 | DSH | owned | 278 | | | 78 | 44 | 4 | | | | no | neg | neg | |
| 66 | | 156 | DSH | stray/colony | 271 | | | 41 | 47 | | | | | no | neg | neg | |
| 67 | MN | 144 | DSH | owned | 272 | | | 41 | 44 | | | | | no | neg | neg | |
| 68 | FN | 144 | DSH | owned | 266 | | | 63 | 40 | | | | | no | neg | neg | |
| 69 | F | 96 | DSH | owned | 266 | | | 47 | 53 | | | | | no | neg | neg | |
| 70 | F | | DSH | stray/colony | 266 | | | 33 | 79 | | | | | no | neg | neg | |
| 71 | M | 36 | DSH | stray/colony | 262 | | | 21 | 36 | | | | | no | neg | neg | |
| 72 | MN | 144 | DSH | owned | 259 | | | 68 | 50 | 1 | | | | no | neg | neg | |
| 73 | FN | 144 | DSH | owned | 259 | | | 40 | 81 | | | | | no | neg | neg | |
| 74 | MN | 72 | DSH | owned | 259 | | | 30 | 14 | | | | | no | neg | neg | |
| 75 | FN | 108 | DSH | owned | 259 | | | 53 | 46 | | | | | no | neg | neg | |
| 76 | FN | 216 | DSH | owned | 258 | | | 80 | 43 | 1 | | | | no | neg | neg | |
| 77 | MN | 216 | DSH | owned | 257 | | | 56 | 40 | 2 | | | | no | neg | neg | |
| 78 | F | 96 | DSH | stray/colony | 267 | | | 34 | 37 | 5 | | | | no | pos (39.1) | pos | OM785183 |
| 79 | M | | DSH | stray/colony | 254 | | | 15 | 13 | | | | | no | neg | neg | |
| 80 | MN | 60 | DSH | owned | 251 | | | 54 | 10 | 1 | | | | no | neg | neg | |
| 81 | M | | DSH | stray/colony | 251 | | | 79 | 8 | | | | | no | neg | neg | |
| 82 | MN | 84 | Persian | owned | 251 | | | 29 | 46 | 1 | | | | no | neg | neg | |
| 83 | | 120 | DSH | stray/colony | 250 | | | 54 | 43 | | | | | no | neg | neg | |
| 84 | FN | 180 | DSH | owned | 244 | | | 35 | 33 | | | | | no | neg | neg | |
| 85 | FN | 96 | DSH | owned | 244 | | | 34 | 13 | | | | | no | neg | neg | |
| 86 | MN | 180 | DSH | owned | 238 | | | 74 | 81 | 1 | | | | no | neg | neg | |
| 87 | F | 84 | DSH | owned | 231 | | | 54 | 49 | | | | | no | neg | neg | |
| 88 | MN | | DSH | stray/colony | 230 | | | 36 | 15 | | | | | no | neg | neg | |
| 89 | FN | 60 | DSH | stray/colony | 226 | | | 39 | 37 | | | | | no | neg | neg | |
| 90 | M | 3 | DSH | stray/colony | 226 | | | 60 | 85 | | | | | no | neg | neg | |
| 91 | F | 36 | Norwegian | owned | 226 | | | 36 | 27 | | | | | no | neg | neg | |
| 92 | FN | 96 | DSH | owned | 226 | | | 59 | 61 | | | | | no | neg | neg | |
| 93 | FN | 48 | DSH | owned | 225 | | | 30 | 16 | 1 | | | | no | neg | neg | |
| 94 | F | 9 | DSH | stray/colony | 223 | | | 74 | 62 | | | | | no | neg | neg | |
| 95 | F | 12 | DSH | stray/colony | 218 | | | 51 | 36 | | | | | no | neg | neg | |
| 96 | MN | | | owned | 219 | | | 49 | 47 | | | | | no | neg | neg | |

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# Detection and genetic characterization of domestic cat hepadnavirus in cats with cavitary effusions

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**Foreword**: in this study, we investigated the presence of DCH in cats with and without cavitary effusions, to explore the potential pathogenetic role of DCH. My contribution to this work was related to conceptualization, sample collection, laboratory analysis, data analysis and interpretation and manuscript writing. The results of this study were published under the reference:

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**Abstract**

After the identification of the novel domestic cat hepadnavirus (DCH) in 2018, its potential pathogenetic role in feline hepatic diseases has been suggested. Following the detection of DCH in a cat's serum and peritoneal effusion, the aim of this study was to retrospectively investigate the presence of DCH in cats with and without cavitary effusions along with DCH presence in effusions.

Stored serum and effusion samples from cats with and without effusions admitted to the Veterinary Teaching Hospital of Lodi (Italy) in 2020–2022 were included based on results of hematobiochemical parameters. Effusions were classified based on cytological and physicochemical findings. The likelihood of liver damage was estimated based on clinical and laboratory findings. Samples were tested for DCH presence by quantitative PCR (qPCR). Positive samples were subjected to whole genome sequencing and phylogenetic analysis. DCH was detected in both serum and peritoneal effusion samples of 2/72 (2.8%) enrolled cats, included in the group with effusions (2/33; 6.1%), with one cat showing inflammatory and the other non-inflammatory effusion. Both DCH-positive cats belonged to the group with a likelihood of liver damage (2/22, 9.1%). Phylogeny showed that the DCH sequences from this study clustered with the prototypic Australian strain but were not included in the clade with other Italian DCH sequences. Results suggest the circulation of different DCH variants in Italy and show the presence of DCH in effusion samples from DCH-positive cats, mirroring the presence of HBV in body fluids from HBV-infected humans. Further studies are still recommended to define the pathogenic role of DCH in cats.

**Aims**

In November 2021, in the framework of a screening of pathogens carried out at the Veterinary Teaching Hospital (VTH) of Lodi (Italy), DCH was detected by quantitative real-time PCR (qPCR) (Lanave et al., 2019) in serum and peritoneal effusion samples of a cat with a history of hyperthermia, diffuse oedema, suspected liver disease and cavitary effusions of unknown etiology. Following this finding, this retrospective study aimed to investigate the presence of DCH infection in serum samples from cats with and without effusion and the presence of DCH in cavitary effusions to provide additional information about DCH infection and viral variants in cats.

**Materials and methods**

*Animals and samples selection*

Cats with or without cavity effusions from the Lombardy region, Northern Italy, admitted to the VTH of Lodi between January 2020 and March 2022 were randomly and retrospectively selected for this study. Criteria of inclusion were the availability of at least one residual leftover serum sample for molecular analysis, the availability of the results of ALT and alkaline phosphatase (ALP) activity measured at admission during the initial diagnostic work-up and the analysis of effusion (if present). Archive samples were stored at – 20°C. According to the Institution guidelines, formal approval of the

Ethical committee was not required because samples used in this study were obtained from leftover samples collected for diagnostic purposes or as part of health checks and with the informed consent of the owners (decision no. 2/2016 of the Ethical Committee of the University of Milan). Data regarding signalment, clinical history (with special emphasis on the presence or absence of cavitary effusion) and laboratory findings on serum samples and cavitary effusions recorded for each cat were retrieved from the laboratory database. The cats were categorized into two age groups: < 2 and ≥ 2 years, as previously reported (Anpuanandam et al., 2021). Regarding laboratory findings, results of biochemistry analyses performed on serum samples with an automated chemistry analyzer (BT 3500, Biotecnica Instruments, Rome, Italy) and reagents provided by the instrument's manufacturer were retrieved. Results of FeLV p27 antigen and FIV antibody presence detected using a commercially available kit (SNAP FIV/FeLV Combo, IDEXX Laboratories, USA) were recorded. When available, results of post-mortem examination of cats were also retrieved. Moreover, the analysis of effusions, if present, included:

1. The physicochemical analysis of the fluid, which in turn included the visual evaluation of the gross appearance of the fluid, the measurement of the specific gravity and total solids using a portable refractometer (Sper Scientific, Scottsdale AZ, USA), the measurement of total proteins and of additional analytes such as creatinine, urea, potassium, cholesterol, triglycerides, when the clinical presentation raised the suspicion of uroperitoneum or of chylous effusion, using the chemistry analyzer mentioned above;
2. The total cell count measured using a laser-based analyzer (Sysmex XN-V, Sysmex Europe, Norderstedt, Germany);
3. Cytology performed on smears and/or on cytocentrifuged fluids stained with a rapid stain (Hemacolor, Merck, Darmstadt, Germany);
4. Results of bacteriology and/or PCR diagnostic analysis.

The enrolled cats were further divided into two groups based on the presence or absence of cavitary effusions. Cats with cavitary effusions were then categorized based on the localization of effusion and the type of effusion. To achieve this latter classification, samples were first classified based on physicochemical and cytological features, according to the main classification schemes (Dempsey & Ewing, 2011; Stockham & Scott, 2008) and then integrated with clinical history and results of bacteriology, PCR and, if present, necropsy report. For this study, the types of effusion were clustered into two main categories: inflammatory effusions (septic effusions, non-specific exudates and FIP effusions) and non-inflammatory effusions (neoplastic, hemorrhagic or chylous effusions, transudates, uroperitoneum). Cats were also categorized according to biochemical analysis results for the likelihood

of liver damage based on the blood activity of ALT and ALP as previously reported (Scavone et al., 2022) and/or total bilirubin concentration. The three categories of the likelihood of liver damage were the following:

- Low: less than a two-fold increase in ALT activity without other enzyme abnormalities or more than two-fold increase in ALT activity paired with the simultaneous increase in CK activity and normal ALP, GGT activity and bilirubin concentration, suggestive of muscle damage or increase in ALP activity presumably due to isoenzymes of non-hepatic origin, possibly related to hyperthyroidism, without other enzyme abnormalities;
- Intermediate: more than two-fold and less than five-fold increase in ALT activity without changes in CK or less than two-fold simultaneous increase in ALT and ALP activity without other changes potentially consistent with hepatic disease and with high bilirubin (not associated with hematological changes consistent with hemolysis or with changes suggestive of cholestasis) or hyperbilirubinemia and other abnormalities consistent with liver failure (e.g. hypoalbuminemia or low urea and glucose level) even in absence of increased enzyme activity;
- High: more than two-fold simultaneous increase in ALT and ALP activity without changes in CK or more than five-fold increase in ALT activity alone, independent of other enzyme abnormalities.

*DNA extraction, PCR protocols and sequencing*

DNA was extracted from serum and effusion samples using a commercial DNA extraction kit (NucleoSpin Blood kit, Macherey-Nagel, Germany), following the manufacturer's instruction. DNA was extracted from liver biopsies, if available, following routine diagnostic procedures, using a commercial DNA extraction kit (NucleoSpin Tissue kit, Macherey-Nagel, Germany), following the manufacturer's instruction. DNA pre-analytical quality control targeting vertebrate 12 S rRNA gene was performed on randomly selected samples (Kitano et al., 2007). All samples were subjected to a qPCR specific for DCH (Lanave et al., 2019) using a QuantStudio™ 3 instrument (Applied Biosystems, MA, USA). For DCH quantification, a 105 bp fragment of the Australian DCH reference strain AUS/2016/Sydney genome (GenBank accession no. MH307930) was cloned into a pGEM-T Easy plasmid vector (Promega, WI, USA) to produce a standard for the qPCR. The DCH copy number in each sample was calculated based on the standard curve generated from 10-fold dilutions of the plasmid prepared between 109 and 1 copies/µL. DCH DNA extracted from a naturally infected cat was used as positive control and a blank control (DNase-free water sample) was also included in all the qPCR reactions.

*Whole genome sequencing of DCH positive samples*

To characterize the genome of DCH stains detected in this study, a six primers-set PCR (table 3) was designed based on the available DCH sequences deposited in GenBank. PCRs were performed using Phire Green Hot Start II PCR Master Mix (Thermo Scientific™, Vilnius, Lithuania) previously used for viral genome sequencing (Lin et al., 2016) in a final volume of 30 µL containing 1 µM of each primer and 5 µL (100–500 ng) of DNA template. Amplifications were carried out as follows: initial denaturation at 98°C for 30 s, 40 cycles of 98°C for 5 s, 54°C for 5 s and 72°C for 15 s; and a final elongation step at 72°C for 1 min. The amplicons were purified using a commercial kit (NucleoSpin Gel and PCR Clean-up, Macherey-Nagel, Germany) following the manufacturer's instruction and Sanger sequenced (Microsynth Seqlab, Germany) using the forward and reverse primers used for DNA amplification. The overlapping PCR products were used to generate consensus sequence of the full-length genome using Clustal X in BioEdit software v.7.0. Sequences were submitted to GenBank with accession numbers OQ859619- OQ859622. The sequences were compared with those available in GenBank using BLAST (http://blast.ncbi.nlm.nih.gov/Blast. cgi; accessed January 6th, 2023) and were used for phylogenetic analyses.

Table 3. PCR primers used to amplify DCH whole genome sequence.

| Fragment | Oligonucleotides | Sequence (5'-3') | Position* | Reference |
|---|---|---|---|---|
| **No.1** | HBV-pol-F1<br>HBV-pol-R1 | TAGACTSGTGGTGGACTTCTC<br>CATATAASTRAAAGCCAYACAG | 95-587 | (Wang et al., 2017) |
| **No.2** | FHBV-for<br>Cir7-R | CGTCATCATGGGCTTTAGGAA<br>CGTAGACGAAGGACACGTC | 458-1281 | (Aghazadeh et al., 2018; Lanave et al., 2019) |
| **No.3** | DCH 1127F<br>DCH 1885R | GGAACTCATTGCTGCCTGCGTAG<br>TCCCTACCTGTAAGTTCCTCTTCAT | 1127-1885 | This study |
| **No.4** | DCH 1733F<br>DCH 2433R | TAGATGGCTTTGGGGTATGGACAT<br>ATCAGCAGGCACATGGATATT | 1733-2433 | This study |
| **No.5** | DCH 2282F<br>DCH 3101R | TCGCAGACGTTCTCAATCTCCA<br>GTCCAGGTGCAGCAGTTGTCTTG | 2282-3101 | This study |
| **No.6** | DCH 2910F<br>DCH 290R | GCTCTCTCAGTTTCGCAACCCA<br>AGAACCAACGAGAAGATGAGCA | 2910-290 | This study |

*nucleotide position according to Australian reference strain AUS/2016/Sydney (GenBank accession nr. NC_040719).

*Phylogenetic analysis*

Phylogeny was performed on DCH genome sequences from this study that were aligned with whole genome DCH sequences and whole genomes of two mammalian orthohepadnaviruses retrieved from GenBank (accessed January 6th, 2023). All sequences were aligned using Clustal X in BioEdit software v.7. Phylogeny was estimated by a neighbourjoining algorithm (NJ) and by maximum likelihood (mL) methods with 1000 bootstrap replicates using MEGA v.11 (Felsenstein, 1981; Kimura, 1980; Kumar et al., 2016). For sequences comparison, the percentage of nucleotide similarity of pairwise evolutionary distances of DCH whole genome sequences of this study and all DCH whole genome sequences available from GenBank was calculated using MEGA v.11.

**Results**

*Caseload*

In total, 72 cats were included in this study, 33 and 39 cases with and without cavitary effusion respectively. Characteristics of analyzed cats are summarized in Table 4. All serum samples (72/72)

were subjected to molecular analysis. For the 33 cats with cavitary effusions, at least one residual effusion sample for PCR analysis was present for 28 (28/33; 84.8%) cats. Information regarding samples of cavitary effusions analyzed by DCH qPCR based on their classification are summarized in Supplementary material (Table S2). The liver biopsy was available for one cat. Follow-up samples collected at the moment of discharge (after 4 months from admission) were present for one cat.

*DCH detection*

Overall, DCH was detected in serum samples by qPCR in 2/72 (2.8%; 95% confidence interval: 0–6.6%) of the tested cats. Both DCH-positive cats belonged to the group with cavitary effusions, while all serum samples of the cats without effusion were DCH-negative (Table 4). DCH was also detected in peritoneal effusions of both DCH-positive cats, whereas DCH was not detected in the pleural and pericardial effusions of the DCH-positive cat with multiple effusions (identified as cat no. 1). According to the effusion type, DCH was detected in one inflammatory (cat no. 1) and one non-inflammatory effusion (cat no. 2) (Table 4). The liver biopsy obtained from cat no. 1 was DCH-negative based on immunohistochemical results. Results of qPCR, including viral quantification of the two DCH-positive cats, are reported in Table 5.

Table 4. Characteristics of cats analyzed in this study and DCH positivity in serum samples.

| **Population characteristics** | | **Cats with effusions (no. 33)** | **Cats without effusions (no. 39)** | **Total cats (no. 72)** | |
|---|---|---|---|---|---|
| | | **No. (%)** | **No. (%)** | **No (%)** | **Serum DCH positive no. (%)** |
| Sex | Male | 22 (51.2) | 21 (48.8) | 43 (59.7) | 1 (2.3) |
| | Female | 11 (37.9) | 18 (62.1) | 29 (40.3) | 1 (3.4) |
| Age[a] | <2 years | 5 (41.7) | 7 (58.3) | 12 (17.6) | 1 (8.3) |
| | ≥2 years | 26 (46.4) | 30 (53.6) | 56 (82.4) | 1 (1.8) |
| Breed | Domestic shorthair | 30 (46.2) | 35 (53.8) | 65 (90.3) | 2 (3.1) |
| | Purebreed | 3 (42.9) | 4 (57.1) | 7 (9.7) | - |
| Ownership | Owned | 17 (36.2) | 30 (63.8) | 47 (65.3) | 2 (4.2) |
| | Stray | 11 (64.7) | 6 (35.7) | 17 (23.6) | - |
| | Shelter | 5 (62.5) | 3 (37.5) | 8 (11.1) | - |
| FIV status[b] | FIV positive | 5 (55.6) | 4 (44.4) | 9 (22)[c] | - |
| | FIV negative | 18 (56.3) | 14 (43.7) | 32 (78) | 2 (6.2) |
| FeLV status[b] | FeLV positive | 5 (71.4) | 2 (28.6) | 7 (17.1)[c] | - |
| | FeLV negative | 18 (52.9) | 16 (47.1) | 34 (82.9) | 2 (5.9) |
| Biochemical changes potentially consistent with liver disease | No | 16 (32) | 34 (68) | 50 (69.4) | 0 (0) |
| | Yes | 17 (77.3) | 5 (22.7) | 22 (30.6) | 2 (9.1) |
| Likelihood of liver disease | Low | 4 (57.1) | 3 (42.9) | 7 (9.7) | - |
| | Intermediate | 9 (100) | - | 9 (12.5) | 2 (22.2) |
| | High | 4 (66.7) | 2 (33.3) | 6 (8.3) | - |
| Effusion | Present | 33 (100) | - | 33 (45.8) | 2 (6.1) |
| | Absent | - | 39 (100) | 39 (54.2) | - |

Table 4 continued

| Population characteristics | Cats with effusions (no. 33) | Cats without effusions (no. 39) | | Total cats (no. 72) | |
|---|---|---|---|---|---|
| | No. (%) | No. (%) | | No (%) | Serum DCH positive no. (%) |
| Localization of effusion | Peritoneal only | 20 (60.6) | - | 20 (27.8) | 1 (5) |
| | Pleural only | 7 (21.2) | - | 7 (9.7) | - |
| | Pericardial only | 1 (3) | - | 1 (1.4) | - |
| | Peritoneal and pleural | 4 (12.1) | - | 4 (5.6) | - |
| | Peritoneal, pleural and pericardial | 1 (3) | - | 1 (1.4) | 1 (100) |
| Inflammatory vs non inflammatory effusion[d] | Inflammatory | 18 (54.5) | - | 18 (25) | 1 (5.6) |
| | Non inflammatory | 15 (45.5) | - | 15 (20.8) | 1 (6.7) |
| Type of effusion[d] | septic | 4 (12.1) | - | 4 (5.6) | - |
| | FIP | 3 (9.1) | - | 3 (4.2) | - |
| | non specific exudate | 11 (33.3) | - | 11 (15.3) | 1 (9.1) |
| | neoplastic | 6 (18.2) | - | 6 (8.3) | - |
| | hemorrhagic | 3 (9.1) | - | 3 (4.2) | - |
| | chylous | 1 (3) | - | 1 (1.4) | - |
| | uroperitoneum | 1 (3) | - | 1 (1.4) | - |
| | transudate | 4 (12.1) | - | 4 (5.6) | 1 (25) |

[a] Age was unknown for four cats.

[b] FIV and FeLV status was unknown for thirty-one cats.

[c] Two cats were positive for both FIV and FeLV.

[d] Cats with more than one effusion were classified according to peritoneal effusion

- negative results (0)

Table 5. Results of DCH quantification in positive samples.

| | Serum DCH DNA copies/mL | Peritoneal effusion DCH DNA copies/mL | Pleural effusion DCH DNA copies/mL | Pericardial effusion DCH DNA copies/mL |
|---|---|---|---|---|
| **Cat no. 1** | $3.3 \times 10^4$ | $2.7 \times 10^4$ | Negative | Negative |
| **Cat no. 2** | $1.6 \times 10^9$ | $7.8 \times 10^6$ | Effusion not present | Effusion not present |

*Characteristics of DCH-positive cats*

Both DCH-positive cats were domestic shorthair privately owned cats admitted to the VTH with a history of hyperthermia, diffuse edema, suspected liver disease and cavitary effusions. In both cases cats were FIV-FeLV negative and bacterial culture and reverse transcription qPCR for FCoV RNA in the effusion were negative. Results of the biochemical, hematological and effusion analysis of the two cats are summarized in Table 6. In cat no. 1 (female, 6 years old), diagnostic imaging revealed the presence of pericardial, pleural and peritoneal effusions along with liver enlargement, with the presence of multiple hyperechoic lesions diffused to all the liver parenchyma. Hematology and clinical chemistry were unremarkable, except for a mild increase of AST and ALT. Therefore, the cat has been included in the intermediate likelihood of liver disease group. Based on the criteria listed in the material and methods section, the peritoneal and pleural effusions were classified as non-specific exudate, whereas pericardial effusion was hemorrhagic. The patient was initially treated with nonsteroidal anti-inflammatory and antibiotic therapy, without any clinical improvement. Consequently, regarding pericardial effusion, an effusive-constrictive pericarditis was supposed and subtotal pericardiectomy was performed by median sternotomy. During the same surgery, a core biopsy of the liver was collected. Intraoperative impression cytology revealed the presence of reactive lymphocytes and plasma cells in the liver and a few inflammatory cells in the pericardium. The cytological features were consistent with lymphoplasmacytic hepatitis, confirmed by histopathology. The histology of the pericardium was consistent with the diagnosis of fibrous pericarditis. The cat clinically improved, including the progressive disappearance of the effusion, over a 3 months period, and was discharged. ALT activity, that was mildly elevated at admission, strongly decreased at discharge, although values were still outside the reference intervals. No other significant clinical, hematological and biochemical findings were reported on follow-up clinical examination after four months after the hospitalization. Moreover, serum qPCR for DCH was negative during the follow-up of the cat. In cat no. 2 (male, 1 year old), which had a history of elevated liver enzymes, abdominal ultrasonography revealed the presence of diffuse peritonitis and lymph node enlargement, along with the presence of peritoneal effusion. Hematology was unremarkable, while clinical chemistry revealed some changes potentially consistent

with liver failure (hypoalbuminemia, decreased enzyme activity, hyperbilirubinemia), and therefore, also based on the classification of the effusion as a pure transudate, the cat was included in the intermediate likelihood of liver disease group. The cat was treated with nonsteroidal anti-inflammatory drugs, anti-hypertensive drugs and hepatoprotective agents. The cat was discharged upon request of the owners. Seven months after this episode, the owner reported good health conditions for the cat.

Table 6. Hematological and biochemical characteristics of the two DCH-positive cats

| **Sample** | **Hematological and biochemical characteristics (range values)** | **Cat no.1** | **Cat no.2** |
|---|---|---|---|
| **Blood and serum** | Ht (24-45 %) | 33.0 | 27.7 |
| | RBC (5.7-10 x $10^6$ /µL) | 8.43 | 6.12 |
| | WBC (6.0-17 x $10^3$ /µL) | 10.68 | 6.48 |
| | Total Proteins (5.4-8.5 g/dL) | 7.1 | 4.2 |
| | Albumin (2.1-3.3 g/dL) | 3.2 | 1.3 |
| | AST (26-43 IU/L) | 54 | ND |
| | ALT (<83 IU/L) | 267 | 21* |
| | ALP (25-93 IU/L) | 69 | 9* |
| | GGT (<5 IU/L) | 3 | ND |
| | Total Bilirubin (<0,5 mg/dL) | 0.45 | 1.74 |

Table 6 continued

| Sample | Hematological and biochemical characteristics (range values) | Cat no.1 | Cat no.2 |
|---|---|---|---|
| **Peritoneal effusion** | Total Proteins (g/dL) | 3.5 | 0.3 |
| | Cellularity ($10^3$ /µL) | 6.57 | 0.48 |
| | Main cell populations detected at cytology | Lymphocytes and non-degenerated neutrophils, more rarely foamy macrophages, plasma cells eosinophils, reactive mesothelial cells and mott cells | Foamy macrophages, non-degenerated neutrophils, lymphocytes, more rarely eosinophils and mast cells |
| | Macroscopic characteristics | Mildly sero-hematic | Transparent, colorless |
| **Pleural effusion** | Total Proteins (g/dL) | 2.4 | Effusion not present |
| | Total nucleated cell count ($10^3$ /µL) | 2.38 | |
| | Main cell populations detected at cytology | Lymphocytes and non-degenerated neutrophils, foamy macrophages displaying cytophagy, plasma cells more rarely eosinophils and metarubricyte | |
| | Macroscopic characteristics | Mildly sero-hematic | |
| **Pericardial effusion** | Total Proteins (g/dL) | 4.0 | Effusion not present |
| | Total nucleated cell count ($10^3$ /µL) | 13.5 | |
| | Main cell populations detected at cytology | Non-degenerated neutrophils, foamy macrophages foamy macrophages displaying cytophagy, more rarely lymphocytes, plasma cells eosinophils and reactive mesothelial cells | |
| | Macroscopic characteristics | Hemorrhagic | |

Abbreviations: Ht, hematocrit; RBC, red blood cells; WBC, white blood cells; AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase; ND, not determined. * elevated liver enzymes before admission to the VTH.

*DCH whole genome sequencing and phylogenetic analysis*

All six genome fragments were efficiently amplified only from samples with Ct ≤ 32 and whole genome sequences of 3184 bp were obtained for serum and peritoneal effusion of cat no. 2. In the presence of Ct > 32, five fragments (no. 1–2 and no. 4–6, corresponding to the partial C, P and X genes and the complete S gene) and two fragments (no. 1 and 6, corresponding to partial P and S genes) were obtained from the serum and the effusion sample of cat no. 1, respectively. Therefore, partial whole genome sequence of about 85.9% (2736 bp) of the genome was obtained from serum sample, whereas a partial sequence of 789 bp was obtained from the effusion sample of cat no.1. Both serum and effusion sample of cat no. 2 showed identical whole genome viral sequences (100% nucleotide similarity). Similarly, serum and effusion sequences of cat no. 1 showed the same partial viral sequence. The DCH 789 bp sequence from effusion of cat no. 1 (GenBank accession number OQ859622) was not included in further nucleotide comparison and phylogenetic analyses. Nucleotide pairwise identity between the whole genome sequences of cat no. 2 and the nearly complete DCH genome sequence from serum of cat no. 1 was 99.9% (Table S3). BLAST analysis and nucleotide comparison (Table S3) showed that the closest nucleotide similarity (98.7% and 98.6% for cat no. 2 and cat no. 1, respectively) was to the prototypic Australian sequence (GenBank Accession number NC_040719). The DCH whole and nearly complete genome sequences from the two cats of this study showed nucleotide similarity ranging from 96.8% to 97.4% with other DCH sequences from Italy (GenBank Accession numbers MK117078, OK574325, OK574326 and OM785182). The whole and nearly complete genome DCH sequences from this study showed > 96.0% nucleotide similarity with all the other DCH sequences except for the DCH sequence from isolate Rara, Japan (GenBank accession number LC685967), which showed nucleotide similarity < 92% (Table S3). Moreover, after DNA sequence identity comparison, results showed nucleotide differences > 8% of the DCH sequence from Japan, isolate Rara with all other DCH sequences available in GenBank, indicative of another DCH genotype (putative genotype B) compared to the rest of the sequences (putative genotype A). The phylogenetic tree based on mL analysis of whole genome sequences (figure 8) confirmed that the DCH sequences of this study clustered with the putative genotype A, within the prototype Australian DCH sequence (clade A1) but were not included in the clade A2 with other DCH sequences from Italy (Capozza et al., 2023). NJ analysis showed similar results (data not shown).

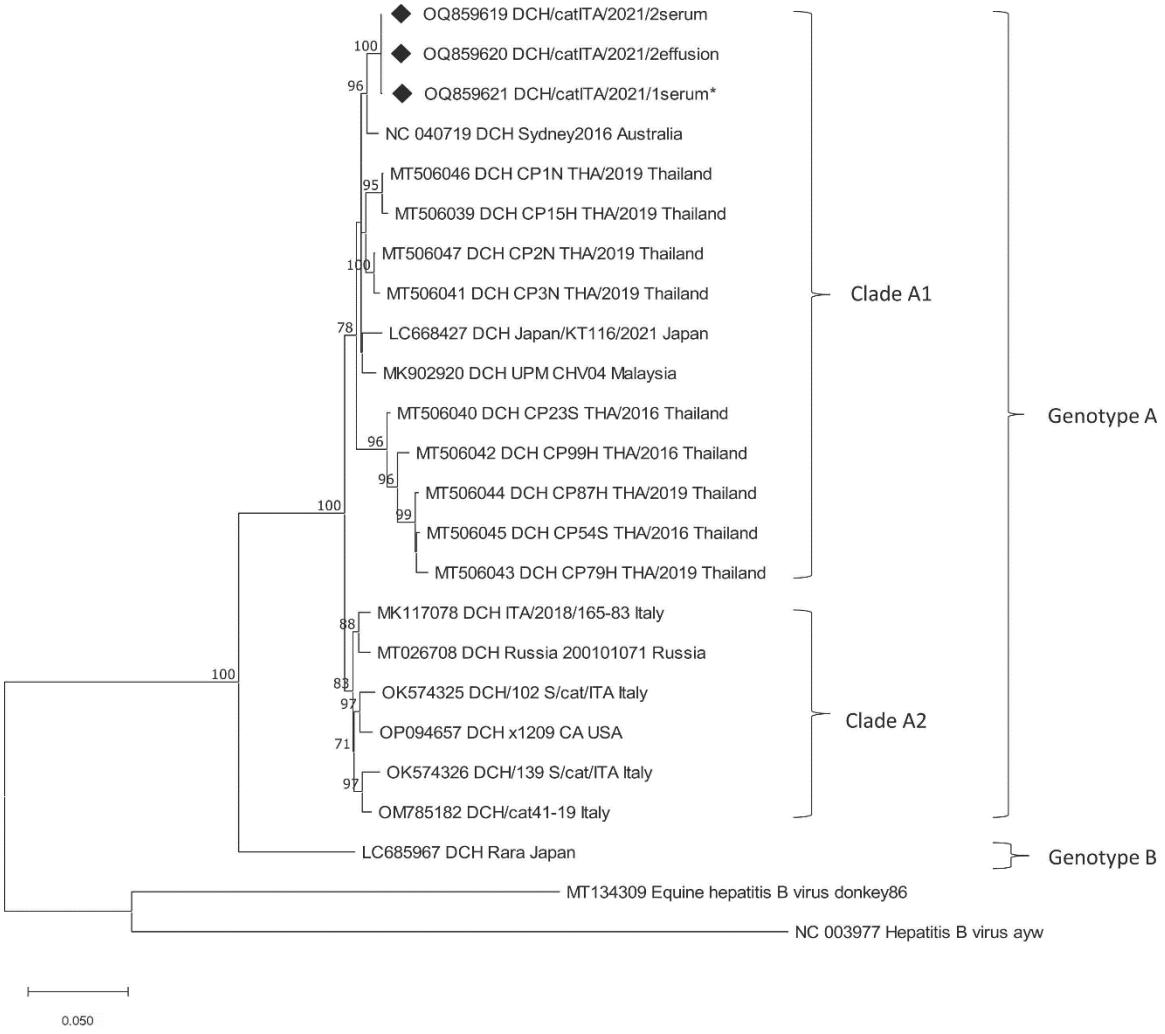


Figure 8. Phylogenetic tree generated with maximum likelihood analysis. Whole genome sequences from domestic cat hepadnavirus (DCH) retrieved from GenBank databases and the DCH obtained in this study were used. Mammalian orthohepadnaviruses whole genome sequences were used as outgroup. Sequences are indicated by GenBank accession number (available at www.ncbi.nlm.nih.gov/pubmed/) name of the strain and geographical origin. Distances were computed using the General Time Reversible model using MEGA v.11. Bootstrap values above 70% are shown. The black diamonds indicate the sequences obtained in this study; * indicates partial whole genome sequence.

**Discussion**

Since the discovery of the novel DCH, different studies have detected the presence of DCH in cats in Asia, Oceania, Europe and America (Aghazadeh et al., 2018; Anpuanandam et al., 2021; Lanave et al., 2019; Piewbang et al., 2020; Stone et al., 2022). Results of our study showing a 2.8% DCH positivity confirm that DCH is circulating in cats in Italy, where DCH infection has been previously reported

ranging between 4.2% and 10.8% (Lanave et al., 2019; Scavone et al., 2022). Nevertheless, it should be considered that the DCH infection detected by molecular investigations (Aghazadeh et al., 2018; Anpuanandam et al., 2021; Capozza et al., 2023; Jeanes et al., 2022; Lanave et al., 2019; Piewbang et al., 2020; Scavone et al., 2022; Stone et al., 2022; Takahashi et al., 2022) may be underestimated. Indeed, a high percentage of DCH infected cats has been identified using an optimized serology protocol able to identify anti-DCHc antibodies both with and without DCH DNA detectable at the blood/serum level (Fruci et al., 2022). In this respect, the absence of serological testing in this study has likely limited the identification of DCH-infected cats. Trying to make a parallelism with HBV patterns of infection in humans, DCH infection may also mirror HBV chronic infection that may be characterized by low viremia, normalization of the ALT levels and presence of antibodies against HBV core protein (anti-HBc Abs) whereas the presence of antibodies against HBV surface protein (anti-HBs Abs) is variable (Fruci et al., 2022; Terrault et al., 2018). Therefore, to assess DCH prevalence, diagnostic procedures in cats should profile the stage of infection by detecting antibodies, antigens and viral DNA, as reported for HBV infections in humans. Indeed, some forms of HBV (i.e. occult infections) may be undetected by PCR alone because of low viral loads, in serum samples (Terrault et al., 2018).

DCH whole genome analysis has been used for DCH genetic diversity assessment and molecular epidemiology. Analysis of DCH whole genome may help to clarify pathobiology and define if some viral variants may be associated with different clinical outcomes, as previously reported in humans infected with HBV (Gao, 2015). However, it should be considered that whole genome sequencing may be challenging in the presence of low viral loads. Indeed, in this study DCH complete genomes were obtained only for the samples of cat no. 2 showing Ct < 32, corresponding to > $10^4$ copies/mL of viral DNA that is usually required for whole genome sequencing (Margeridon et al., 2008). In the two samples from cat no.1, which showed lower viral loads compared to cat no.2, the discontinuous structure of relaxed circular (RC)-DNA of hepadnaviruses may have also accounted for the problematical amplification of at least one of the fragments (Margeridon et al., 2008). In this regard, rolling circle amplification or next-generation sequencing technique may be suggested to perform DCH whole genome sequencing analysis in samples with low viral load, as previously reported for other hepadnaviruses (Diakoudi et al., 2022; Margeridon et al., 2008). Moreover, cell culture systems for DCH isolation are also warranted.

Phylogenetic analysis based on whole genome sequences showed that the DCH sequences detected in this study were not included in the clade with other Italian DCH strains, one of which was recently

reported from the same study area, namely the Lombardy region (Scavone et al., 2022). The presence of two different DCH clades (A1 and A2), clustering Italian sequences and belonging to one DCH genotype in Italy, was highlighted. This result further confirms genetic diversity of DCH within the same geographic area (Piewbang et al., 2020; Stone et al., 2022). The potential pathogenetic role of DCH in liver disease is currently under investigation in cats (Anpuanandam et al., 2021; Capozza et al., 2021a; Lanave et al., 2019; Pesavento et al., 2019; Piewbang et al., 2022). Our study showed that the two cats with ≥ $10^4$ DCH DNA copies/mL in serum were likely affected by liver damage, confirming the positive correlation of DCH viral copy numbers with ALT values and with the degree of hepatitis in cats (Piewbang et al., 2022). The same results have been reported in HBV-infected humans for which $10^4$ HBV DNA copies/mL in serum samples have been considered as the lower threshold for hepatitis and liver damage (Seeger & Mason, 2013). Despite the increased liver enzymes in biochemical analyses prior to VTH admission, in our study the likelihood of liver disease of cat no. 2 was based on the increase of total bilirubin (not associated with hematological changes consistent with hemolysis or with changes suggestive of cholestasis) coupled with hypoalbuminemia. Indeed, it may be postulated that also this cat experienced some type of transient liver damage and that low ALT activity observed during admission could be due to the short half-life of this liver enzyme in cats, coupled with the possible occurrence of an ongoing liver failure supported also by hypoalbuminemia and a pure transudate (Hoffmann et al., 1977; Stockham & Scott, 2008). Therefore, we suggest that the likelihood of liver disease should rely not only on biochemical markers of liver damage, such as ALT and ALP that may be within reference interval and cannot rule out chronic hepatitis in cats (mirroring normal biochemistry in chronic hepatitis which accompany HBV infection in humans) (Seeger & Mason, 2013), but also on liver functionality markers, such as high total bilirubin, hypoalbuminemia or, better, bile acids (Stockham & Scott, 2008), that unfortunately have not been tested in the current case. The presence of DCH in cats showing an intermediate but not high likelihood of liver disease confirms previous findings (Scavone et al., 2022), suggesting that DCH infection is likely not correlated with liver disease severity. The ALT decreases during discharge in cat no.1 may indicate that, assuming that a hepatocellular damage was present at admission, a progressive recovery occurred over time. The presence of lymphoplasmacytic hepatitis has already been associated with DCH infection (Pesavento et al., 2019; Piewbang et al., 2022) but unfortunately the lack of DCH DNA in the liver biopsy of cat no. 1 did not allow to define the association of DCH with histopathological features of the liver. Further investigations are needed, considering that the negative PCR results may have been due to the patchy distribution of DCH in the liver, as previously reported (Pesavento et al., 2019). The results from our

study showing that DCH may be present in the peritoneal effusion of DCH-positive cats are not surprising and are in accordance with previous reports on HBV in humans showing the presence of HBV surface antigen (HBsAg) in human ascitic fluids of HBsAg carriers (Komatsu et al., 2016; Salo et al., 1980). The presence of DCH in the effusions of two cats suggests that the hepadnaviral infection may be linked to a cavitary effusion in individual cases. The presence of DCH in the inflammatory peritoneal effusion of cat no. 1 suggests that blood contamination of the peritoneal effusion may be accounted as a possible cause of DCH presence within the effusion although gross and cytological evaluation revealed only minimal blood contamination. On the other hand, the presence of DCH in the non-inflammatory peritoneal effusion of cat no. 2 may be due to altered oncotic pressure resulting from hypoalbuminemia, which is the primary cause of protein-poor transudative effusions in veterinary patients (Dempsey & Ewing, 2011). Results suggests that DCH DNA may be detected in an effusion from a cat that was viraemic at the time of sampling. The presence of DCH only in the peritoneal effusion of cat no.1, while pericardial and pleural effusions were negative, is in accordance with a previous study showing a higher DCH viral load in abdominal organs, compared to lungs or heart (Piewbang et al., 2020). Further investigations are needed to understand the mechanisms that lead to DCH presence in feline cavitary effusions and to define the presence of DCH in other body fluids, as HBV has been previously reported in a range of body fluids from HBV-infected humans (Komatsu et al., 2016). Overall, results of our study do not suggest that hepadnaviral infections are a differential diagnosis for effusions. Further investigations on DCH pathogenic role are recommended given that a previous study has detected DCH presence in various organs with inflammatory lesions, suggesting a broad cell tropism of DCH and a potential pathogenetic role of DCH in systemic disease (Piewbang et al., 2020; Shofa et al., 2022). Regarding risk factors, despite the low number of DCH-positive cats did not allow statistical analysis, the detection of DCH in two owned cats is in accordance with previous results showing a significantly higher prevalence in pets compared to shelter cats (Anpuanandam et al., 2021). The reasons for this difference are unclear (Shofa et al., 2022) and could rely on DCH transmission routes which are still unknown (Capozza et al., 2021b). Our results also confirm that DCH-positive cats may be FIV- and FeLV-negative, as previously reported (Stone et al., 2022). To date, clinical signs of DCH-positive cats have not been clearly defined and healthy DCH-positive cats have been reported (Anpuanandam et al., 2021). Non-specific signs, such as lethargy and anorexia, as well as acute hepatopathy (Anpuanandam et al., 2021; Capozza et al., 2021a), have been observed in DCH-positive cats, mirroring HBV-infected humans that may be asymptomatic or unhealthy according to the progression of the disease (Liang, 2009; WHO, 2023a). Unfortunately, it was not possible to confirm if

the clinical status observed in the two DCH-positive cats was due to DCH infection because other pathological conditions that may have caused the clinical status were not investigated, except for FIV, FeLV, FIP and sepsis that were ruled out based on laboratory findings and the clinical outcome (Fischer et al., 2011).

This study has some limitations, starting from the sample size and the identification of infected cats by detection of DCH DNA only and not by inclusion of serology, given that the absence of DNA does not indicate that a cat is uninfected. Therefore, the study design likely underestimated DCH positive cats and did not allow to perform statistical analysis of any association between DCH infection and a clinical outcome. Other limitations are due to the retrospective analysis of samples collected from cats admitted to a VTH, not allowing the availability of all the required samples. In this respect, the absence of follow-up and liver biopsy in cat no. 2 precluded crucial information about the presence of lesions in the liver and its functionality and neither allowed us to confirm the likely ongoing liver insufficiency in cat no. 2 based on clinical chemistry, possibly caused, among other pathophysiological conditions, by chronic hepatitis.

## Supplementary materials

Table S2. No. of tested cats and classification of cavitary effusions

| Effusions localization | No. of tested cats (%) | Effusions type: Inflammatory (No. analyzed/total cats) | | | Non inflammatory (No. analyzed/total cats) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Septic | FIP | Non-specific exudate | Neoplastic | Hemorrhagic | Chylous | Uroperitoneum | Transudate |
| **Peritoneal only** | 16/20 (80) | 2/4 | 2/2 | 5/7 | 1/1 | 3/3 | 0 | 1/1 | 2/2 |
| **Pleural only** | 6/7 (87.5) | 0 | 1/1 | 1/1 | 3/4 | 0 | 1/1 | 0 | 0 |
| **Pericardial only** | 1/1 (100) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1/1 |
| **Peritoneal and pleural** | 4/4 (100)[a] | 0 | 0 | 2/2 | 1/1 | 0 | 0 | 0 | 1/1 |
| **Peritoneal, pleural and pericardial** | 1/1 (100)[b] | 0 | 0 | 1/1 | 0 | 0 | 0 | 0 | 0 |
| **Total** | 28/33 (84.8) | 2/4 | 3/3 | 9/11 | 5/6 | 3/3 | 1/1 | 1/1 | 4/4 |

[a]only pleural effusions were available and were tested with qPCR.

[b]classification of the cat was based on peritoneal effusion.

Table S3. Nucleotide pairwise identity between the whole genome sequences

| | Strains | MT506043 | MT506045 | MT506039 | MT506046 | MT506041 | MT506047 | MK902920 | LC6684271 | NC_040719 | OQ859621* | OQ859620 | OQ859619 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Percentages of similarity | OQ859619 | 96,06 | 96,22 | 97,76 | 97,96 | 98,19 | 98,31 | 98,32 | 97,96 | 98,67 | 99,93 | 100,00 | |
| | OQ859620 | 96,06 | 96,22 | 97,76 | 97,96 | 98,19 | 98,31 | 98,32 | 97,96 | 98,67 | 99,93 | | |
| | OQ859621 | 96,01 | 96,20 | 98,00 | 98,22 | 98,30 | 98,45 | 98,41 | 98,11 | 98,63 | | | |
| | NC_040710 | 96,05 | 96,22 | 97,83 | 98,06 | 98,38 | 98,51 | 98,41 | 98,02 | | | | |
| | LC668427 | 95,92 | 96,22 | 97,96 | 98,06 | 98,09 | 98,38 | 98,31 | | | | | |
| | MK902920 | 96,05 | 96,25 | 97,99 | 98,22 | 98,48 | 98,57 | | | | | | |
| | MT506047 | 96,49 | 96,58 | 98,57 | 98,67 | 99,62 | | | | | | | |
| | MT506041 | 96,09 | 96,19 | 98,41 | 98,44 | | | | | | | | |
| | MT506046 | 96,88 | 97,11 | 99,69 | | | | | | | | | |
| | MT506039 | 96,62 | 96,85 | | | | | | | | | | |
| | MT506040 | 97,89 | 98,31 | | | | | | | | | | |
| | MT506045 | 99,21 | | | | | | | | | | | |
| | MT506043 | | | | | | | | | | | | |
| | MT506044 | | | | | | | | | | | | |
| | MT506042 | | | | | | | | | | | | |
| | MK117078 | | | | | | | | | | | | |
| | OK574325 | | | | | | | | | | | | |
| | OK574326 | | | | | | | | | | | | |
| | OM785182 | | | | | | | | | | | | |
| | MT026708 | | | | | | | | | | | | |
| | OP094657 | | | | | | | | | | | | |

Table S3 continued

| | Strains | OP094657 | MT026708 0 | OM785182 | OK574326 | OK574325 | MK117078 |
|---|---|---|---|---|---|---|---|
| Percentages of similarity | OQ859619 | 97,23 | 97,40 | 97,38 | 96,82 | 97,18 | 97,41 |
| | OQ859620 | 97,23 | 97,40 | 97,38 | 96,82 | 97,18 | 97,41 |
| | OQ859621* | 97,32 | 97,39 | 97,43 | 97,01 | 97,17 | 97,35 |
| | NC_040719 | 97,13 | 97,27 | 97,28 | 96,82 | 97,18 | 97,37 |
| | LC668427 | 97,06 | 97,10 | 97,11 | 96,71 | 96,95 | 97,24 |
| | MK902920 | 97,17 | 97,30 | 97,28 | 96,85 | 97,11 | 97,41 |
| | MT506047 | 97,16 | 97,46 | 97,37 | 96,95 | 97,21 | 97,47 |
| | MT506041 | 96,99 | 97,17 | 97,14 | 96,72 | 97,05 | 97,17 |
| | MT506046 | 96,85 | 97,07 | 96,98 | 96,69 | 96,88 | 97,08 |
| | MT506039 | 96,61 | 96,84 | 96,75 | 96,45 | 96,72 | 96,85 |
| | MT506040 | 96,36 | 96,61 | 96,59 | 96,25 | 96,55 | 96,65 |
| | MT506045 | 96,12 | 96,51 | 96,32 | 95,95 | 96,12 | 96,35 |
| | MT506043 | 95,66 | 96,24 | 95,92 | 95,68 | 95,82 | 96,02 |
| | MT506044 | 96,05 | 96,47 | 96,15 | 95,85 | 96,09 | 96,38 |
| | MT506042 | 96,08 | 96,27 | 96,25 | 95,95 | 96,25 | 96,15 |
| | MK117078 | 98,16 | 98,86 | 98,31 | 97,80 | 98,12 | |
| | OK574325 | 98,63 | 98,11 | 98,19 | 97,77 | | |
| | OK574326 | 97,82 | 97,89 | 98,64 | | | |
| | OM785182 | 98,23 | 98,28 | | | | |
| | MT026708 | 98,25 | | | | | |
| | OP094657 | | | | | | |

*Nearly complete

Transboundary and Emerging Diseases WILEY

*Original article*

# Absence of SARS-CoV-2 RNA and anti-SARS-CoV-2 antibodies in stray cats

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**Foreword**: in this study, we investigated the presence of SARS-CoV-2 RNA and antibodies in stray cats from an area highly affected by the COVID-19 pandemic, to verify the potential circulation of SARS-CoV-2 in stray cats. This study was carried out during the early stage of the COVID-19 pandemic. My contribution to this work was related to sample collection, laboratory analysis, data curation and manuscript review. The results of this study were published under the reference:

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**Abstract**

SARS-CoV-2 positive or seropositive owned cats have been reported worldwide. The detection of seropositive stray cats in the proximity of farms of infected minks, coupled with the demonstration of

cat-to-cat transmission in experimental settings, raise the question whether stray cats may have an epidemiological role in the COVID-19 pandemic and may act as sentinel for the circulation of SARS-CoV-2. The aim of this study was to evaluate the presence of SARS-CoV-2 RNA and anti-SARSCoV-2 antibodies in free roaming cats belonging to colonies located in an area highly affected by the COVID-19 pandemic and to correlate the results with the positivity rate in people sharing the same area. Interdigital, cutaneous, oropharyngeal, nasal and rectal swabs, as well as blood samples, were collected from 99 cats living in colonies and admitted to our hospital for neutering. This caseload corresponds to the 24.2% of the feline population living in the 25 sampled colonies and to the 5.6% of all the free-roaming registered cats. The presence of SARS-CoV-2 RNA in swabs was assessed using real time RT-PCR. Anti-SARS-CoV-2 serum antibodies were assessed using commercially available ELISA kits and confirmed by serum virus neutralization. In people, the SARS-CoV-2 positivity rate ranged from 3.0% to 5.1% (mean rate: 4.1%) and the seropositive rate from 12.1% to 16.3% (mean rate: 14.2%). Most of the colonies were in urban areas and resident cats had frequent contacts with external cats or people. A COVID-19 positive caretaker was found, whereas all the cats were negative for SARS-CoV-2 RNA and seronegative. Although the negative results cannot exclude previous infections followed by decrease of antibodies, this study suggests that colony cats do not have an important epidemiological role in SARS-CoV-2 transmission dynamics. Further studies on larger caseloads are warranted, also in the light of the emerging new viral variants, on a One Health perspective.

**Aims**

The aim of this study was to evaluate the presence of SARSCoV-2 RNA and anti-SARS-CoV-2 antibodies in free roaming cats belonging to several feline colonies located in one Veterinary District of the Regional Health Protection Agency (Agenzia di Tutela della Salute, ATS Città metropolitana di Milano–Distretto Alto Lodigiano) of Lodi province (ATS-AL), which was firstly and highly affected at the beginning of the COVID-19 pandemic and is still recording a high number of human cases (Dashboard-COVID-19, 2020), and to correlate the possible presence of SARS-CoV-2-positive or of seropositive cats with the rate of infection in people sharing the same area.

**Materials and Methods**

*Selection of feline colonies*

Feline colonies were included in this study if the following information were available: (a) data regarding the geographical localization (name of colony, municipality, urban vs rural location, and

distant from the city center vs close to the city center); (b) contact between stray cats of the colonies and people other than the caretakers; (c) contact between stray cats of the colony and other stray cats not belonging to the colonies or indoor/outdoor privately-owned cats; (d) number of caretakers per colony; I information regarding the occurrence of COVID-19 cases in colony caretakers (e.g., COVID-19 positive swabs). In order to compare the possible positivity rate in cats and people, we included in the study also the information about the human population of the whole veterinary district and of the municipalities that hosted the colonies, which were collected from public databases (number of inhabitants, area of each municipality, population density) as well as the information reported on the dashboard of Public Health Authorities (Dashboard-COVID-19, 2020) regarding the number and rate of people infected by SARS-CoV-2 in each municipality from the beginning of the pandemic to the end of the sampling period (22$^{nd}$ December, 2020). Based on these data, the possible correlation between the number of positive people and numbers of inhabitants or population density was also calculated using a Spearman's correlation test run on the Analyseit statistical software (Analyse-it Ltd, Leeds, UK). Cats were admitted to the Veterinary Teaching Hospital of the University of Milan to undergo orchiectomy or ovariectomy, as part of an agreement between the University and the ATS-AL regarding the demographic control of stray animals. During admission, the information about the colony as well as signalment (sex, breed and presumptive age) and any information regarding the clinical status of the cats was recorded. The cats were administered intramuscular dexmedetomidine (10 µg kg$^{-1}$; Dexdomitor, Vetoquinol, Italy), ketamine (2 mg kg$^{-1}$; Lobotor, Acme srl, Italy) and methadone (0.2 mg kg$^{-1}$; Semfortan, Eurovet Animal Health B.V., Italy). After 15 min, a blood sample was taken from the jugular vein and placed in a plain tube, and the following swabs were taken: interdigital swabs, collected by rotating the swabs between the digits of one front and one hind paws; cutaneous swabs, collected by rubbing the swabs on the skin and hair of the back and abdomen; oropharyngeal swabs, collected by inserting and gently rotating the swab into the posterior pharynx and tonsillar areas; nasal swabs, inserted into each nostril and gently rotated in order to harvest the nasal fluid; rectal swabs, collected by inserting the swab through the anal sphincter and gently rotating it.

The study was approved by the Institutional Animal Care and Use Ie and by the Institutional Ethical Committee (approval numbers_31/20 and 43/20, respectively).

*Sample handling and pre-processing*

Shortly after the collection, blood samples were centrifuged, serum was separated and divided in two aliquots placed in Eppendorf tubes The aliquots were frozen at −20°C until shipping, in cold chain, to

the Virology Department of the University of Bari (UniBA, Bari, Italy) and to the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER, Brescia, Italy) for further analyses, as below described. Each swab was dipped in a 1 ml solution based on phosphate buffered saline (PBS pH 7.2) supplemented with 10% glycerol and antibiotics (1%) and frozen at −80°C until shipping in cold chain to the IZSLER upon further analyses, as described below.

*Real time reverse transcription polymerase chain reaction*

Sample preparation, RNA extraction and the SARS-CoV-2 real time RT-PCR were carried out by IZSLER. Briefly, after centrifugation of swab samples at 3750 rpm for 15 min, viral RNA was extracted from 250 µl of supernatant using the QIAsymphony SP Instrument (Qiagen, Hilden, Germany) according to the manufacturer's instructions. SARS-CoV-2 RNA was detected by real time RT-PCR targeting a specific region of the SARS-CoV-2 N gene, according to the protocol described by Corman et al., 2020 (Corman et al., 2020). Positive and negative SARS-CoV-2 respiratory specimens from human patients sampled for COVID-19 diagnostic purposes and routinely processed at the IZSLER were used as positive and negative controls, respectively.

*Serological tests*

All the 99 serum samples were screened with the multispecies enzyme linked immunosorbent assay (ELISA) test, ID Screen SARS-CoV-2 Double Antigen Multi-species ELISA (ID.vet, Grabels, France) detecting antibodies against protein N. Additionally, 93 out of these 99 samples were tested also with the GenScript cPass SARS-CoV-2 Neutralization Antibody Detection Kit (GenScript Piscataway, NJ), a SARS-CoV-2 surrogate virus neutralization test (sVNT) based on antibody-mediated blockage of ACE2-spike (RBD) protein–protein interaction that was performed according to the manufacturer's instructions, and 75 samples were tested also with the ID Screen SARS-CoV-2-N IgG Indirect ELISA detecting antibodies against N protein (ID.vet). In 90 cases, results from ELISA tests were verified by serum virus neutralization (SVN) assay, performed by IZSLER as previously described (Meyer et al., 2020). Positive controls for all the serological tests included canine and feline sera that had tested positive by plaque reduction neutralization assay (Decaro et al., 2021a; Patterson et al., 2020). Briefly, sera were tested in two-fold serial dilutions (starting from 1:10) of heat-inactivated sera (30 min, 56°C) and were then incubated with 100 TCID50 of the SARS-CoV-2 strain HcoV-19/Italy/310904/46/2020 at 37◦C and 5% CO2, for 1 h in 96-wells plates. Vero-E6 cells were added in a concentration of 2 × 104 cells per well and incubated for 72 h at 37◦C with 5% CO2. The serum virus neutralization titer (VNT50)

was defined as the reciprocal value of the sample dilution that showed a 50% protection of virus growth. Sera with titres ≥ 10 were considered as positive for SARS-CoV-2 antibodies. SVN was not performed on 9 samples due to low volume or sera, hemolysis or cytotoxicity.

*Data analysis*

In the presence of negative results, the maximum possible prevalence in the total cat population was calculated using WinEpi (http://www.winepi.net).

**Results**

*Caseload and demographic information about feline and human populations of the sampled area*

The ATS-AL includes 35 out of the 60 (58.3%) municipalities comprised by the province of Lodi. Feline colonies are present in 34 out of the 35 municipalities of the Veterinary District, for a total of 221 colonies and a total of approximately 1768 registered cats. This study involved 25/221 (11.3%) of the colonies belonging to the ATS-AL, which are distributed in 12 municipalities (Figure 9, Table 7).

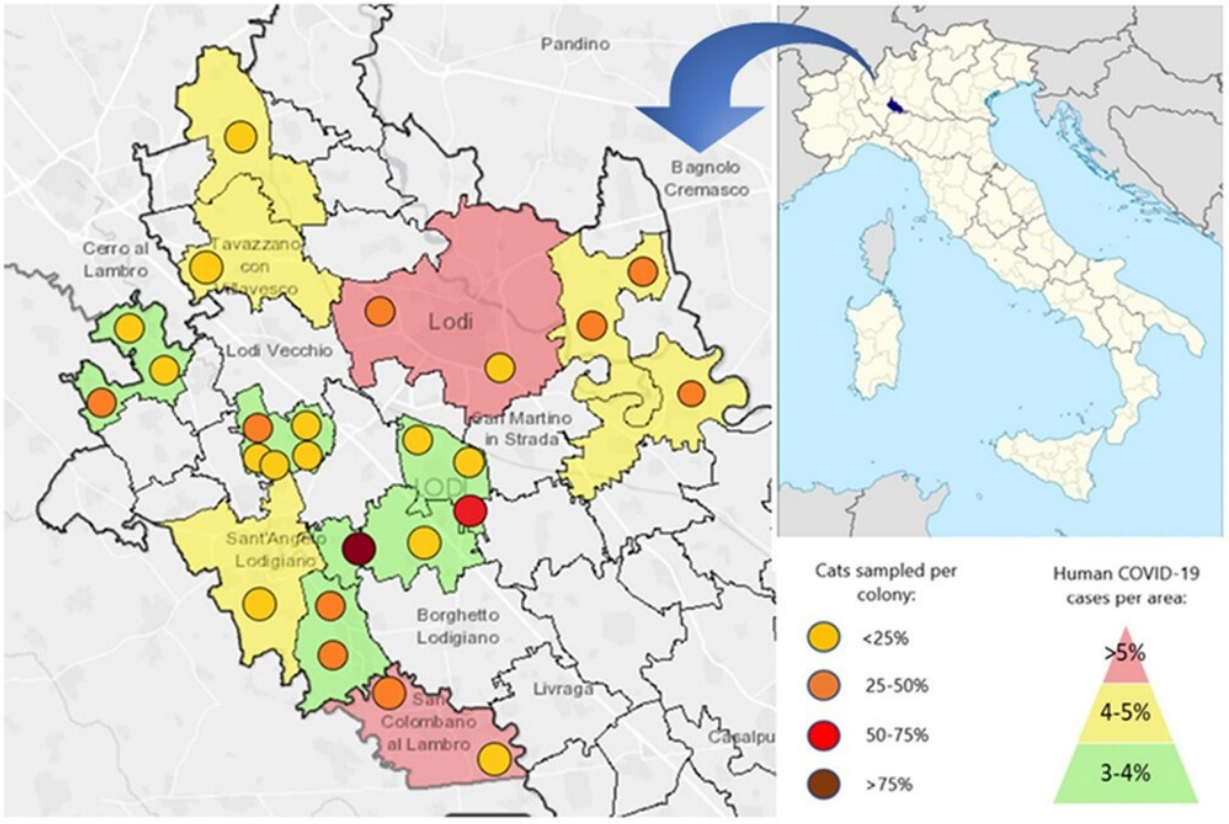


Figure 9. Spatial distribution of the feline colonies used in this study, with information regarding the number of cats enrolled from each.

Table 7. Data regarding the characteristics of the 25 feline colonies involved in this study, as reported in the main text.

| Municipality n° | Colony n° | Cats enrolled | Cats total | Enrolled cats % | Site | External cats | N° of caretakers | Other contacts | COVID-19-positive caretakers |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 5 | 10 | 60.0 | Urban | yes | 2 | yes | no |
| | 2 | 2 | 15 | 13.3 | Rural | no | 2 | no | no |
| | 3 | 3 | 12 | 25.0 | Urban | yes | 2 | yes | no |
| | 4 | 2 | 8 | 25.0 | Rural | yes | 2 | no | no |
| | 5 | 2 | 15 | 13.3 | Rural | no | 2 | no | no |
| 2 | 6 | 3 | 30 | 10.0 | Urban | yes | 2 | yes | no |
| | 7 | 2 | 9 | 22.2 | Urban | yes | 2 | no | no |
| | 8 | 1 | 2 | 50.0 | Urban | yes | 1 | no | no |
| 3 | 9 | 12 | 35 | 34.3 | Rural | yes | 3 | no | no |
| 4 | 10 | 8 | 29 | 27.6 | Urban | yes | 2 | yes | no |
| | 11 | 2 | 6 | 33.3 | Rural | no | 1 | yes | no |
| 5 | 13 | 8 | 20 | 40.0 | Rural | no | 2 | no | no |
| 6 | 14 | 6 | 18 | 33.3 | Urban | yes | 3 | yes | no |
| | 15 | 4 | 21 | 38.1 | Urban | yes | 3 | yes | no |
| 7 | 16 | 1 | 30 | 3.3 | Urban | yes | 3 | yes | no |
| | 17 | 3 | 26 | 11.5 | Urban | yes | 2 | yes | no |
| | 18 | 2 | 3 | 66.7 | Urban | no | 1 | no | no |
| 8 | 19 | 1 | 20 | 5.0 | Rural | no | 2 | no | no |
| 9 | 20 | 2 | 6 | 33.3 | Urban | yes | 3 | yes | yes |
| | 21 | 2 | 14 | 14.3 | Urban | yes | 2 | yes | no |
| 10 | 22 | 3 | 15 | 20.0 | Urban | yes | 3 | yes | no |
| 11 | 23 | 1 | 10 | 10.0 | Urban | yes | 2 | yes | no |
| 12 | 24 | 1 | 20 | 5.0 | Urban | yes | 2 | yes | no |
| | 25 | 19 | 25 | 76.0 | Rural | no | 4 | yes | no |

These colonies include 2–35 animals (mean and median: 16.4 and 15 cats per colony, respectively), for a total of 409 cats (23.1% of the colony cats registered at the ATS-AL). From each colony, one to 19 cats

were enrolled after being admitted for neutering (mean and median: four and two cats/colony, respectively) for a total of 99 cats, corresponding to the 24.2% of the 409 cats living in the 25 sampled colonies and the 5.6% of all the free-roaming registered cats of the Lodi province. Cats per colony ranged between two and 35 animals and the percentage of cats enrolled from each colony ranged from 3.3% to 76.0% of the total number of cats living in each colony. Of the 99 cats enrolled, 53 were female and 46 were male (53.5 and 46.4%, respectively). The presumptive age could be determined in 88/99 cats, and it ranged from 6 to 60 months (mean: 18.8 months, median: 12 months). The information on the site of the colonies and on the interactions with cats and people other than those of the colony are summarized in Table 8.

Table 8. Summary of the information regarding the localization of the colonies and the interactions between colony cats and external cats and people.

| | | Number of colonies | Total number of cats | Cats included in this study |
|---|---|---|---|---|
| Localization | Urban | 16 (64.0%) | 255 (62.3%) | 47 (47.5%) |
| | Rural | 9 (36.0%) | 154 (37.7 %) | 52 (52.5%) |
| Contacts with other cats | Yes | 17 (68.0%) | 295 (72.1%) | 59 (59.6%) |
| | No | 8 (32.0%) | 114 (27.9%) | 40 (40.4%) |
| Contacts with people other than caretaker | Yes | 15 (60.0%) | 272 (66.5%) | 63 (63.6%) |
| | No | 10 (40.0%) | 137 (37.5%) | 36 (36.4%) |

As seen in the table, most of the colonies were in urban areas and resident cats had frequent contacts with external cats (i.e., cats living in proximity of the colonies but not registered by the public Authorities, which may occasionally come into contact with colony cats) or people. The number of caretakers varied from 1–4 per colony (mean: 2.2 operators/colony; median: two operators/colony). COVID-19 cases were recorded among operators working in only one of the colonies used in this study. The municipalities where the feline colonies involved in this study are located, are inhabited by 94,955 citizens (41.4% of the citizens of the Lodi province, 60.5% of the citizens of the ATS-AL, with a population density ranging from 100.13/$Km^2$ and 1076/ $Km^2$ (mean density: 495.69/ $Km^2$). From the beginning of the COVID-19 pandemic to the end of sample collection, 4,335 COVID-19 confirmed cases were recorded in people in the 12 municipalities involved in the study (4.6% of the 94,955 citizens

inhabiting them). Specifically, the positive rate recorded in the 12 municipalities (supplementary table 4) ranged from 3.0% to 5.1% (mean positive rate: 4.1%), without a correlation either with the number of citizens per municipality (P = 0.131) or with the population density of each municipality (P = 0.417).

*Results of serological and molecular testing of sampled cats*

From the 99 examined cats, 98 nasal and 99 each of the other swabs were collected, for a total of 494 swabs. Serum was available for all the 99 cats. Despite an in-depth clinical examination was not performed, all non-domesticated animals during the pre-surgical physical examination did not show abnormal clinical findings. All the swabs tested negative for SARS-CoV-2 RNA. All sera tested negative using the ELISA tests and negative results were confirmed by SVN, except for one sample that tested positive using the indirect ELISA but resulted negative using the double antigen ELISA tests, the Surrogate VNT ELISA test and SVN and was then categorized as a false positive result. A 2.7% and a 2.9% maximum possible prevalence in the total cat population living in the 25 sampled colonies from the 12 municipalities or in the whole Lodi province was calculated for SARS-CoV-2 with negative RT-qPCR and serological results in all the samples tested, respectively.

**Discussion**

Investigation of SARS-CoV-2 infection in pet animals is of importance in the context of the current pandemic and the elucidation of the role of such animals, especially cats, in the epidemiology of the infection is needed (Hosie et al., 2021; Pagani et al., 2021; Patterson et al., 2020). Stray cats consist of a peculiar population of cats that can serve as a good indicator of pathogen propagation between cats and outside the domestic environment (Halfmann et al., 2020; Oreshkova et al., 2020; Shi et al., 2020). Results from the present study suggest that colony cats do not have an important epidemiological role in SARS-CoV-2 transmission dynamics, because all cats were RT-qPCR and serologically negative, despite possible contacts with other cats or with infected people either when colonies are located in urban area or when visitors or operators of the colony are infected. Contact with people or animals possibly eliminating the virus could have been possible, considering also that one caretaker was diagnosed as infected with SARS-CoV-2. However, the negative RT-qPCR results suggest that these short contacts did not induce infection in colony cats or that, whenever the cats were exposed to the virus (e.g., carried from asymptomatic caretakers or from other cats in 'open' colonies), they already cleared infection when subjected to RT-qPCR. This finding additionally supports what already reported regarding the need of a close contact between pets and their owners for SARS-CoV-2 transmission from

humans to pets (Decaro et al., 2021b). Moreover, seronegative results suggest the absence of previous SARS-CoV-2 infections in cats. On this regard it should be stressed that all the serological tests provided negative results, supporting the hypothesis of a good concordance of serological tests (to be confirmed by future studies specifically focused on the diagnostic performance of anti-SARS-CoV-2 serological tests in cats), except in one case. This was considered a false positive case because only one of the ELISA tests performed in this study showed a positive result that, however, was not confirmed by the other ELISA tests and by SVN. Experimentally infected cats with SARS-CoV-2 begin seroconversion about 7–13 days from infections, they maintain high titers 42 days after infection (Bosco-Lauth et al., 2020; Gaudreault et al., 2020; Shi et al., 2020), and may be found positive up to 54 days after owner diagnosis (Hamer et al., 2021). The few data on duration of seroconversion in natural settings suggest that antibodies in cats decrease below detection limits in 110 days (Pagani et al., 2021; Zhang et al., 2020). It may be possible that infection occurred months before sampling, followed by complete serological negativization, indicating that this possible infection was not followed by persistent cat-to-cat transmission and formation of SARS-CoV-2 endemic feline populations. In any case, also this scenario would support the hypothesis that colony cats do not play an epidemiological role in the COVID-19 pandemic. However, the fact that all our samples were negative for SARS-CoV2 may be indicative, in the total cat population of Lodi province, of the absence of infection or of a low (2.9%) maximum possible prevalence. This estimation in the stray cat population is in agreement with what reported by Villanueva-Saz et al., 2022 (Villanueva-Saz et al., 2022), which found 3.5% seroprevalence in stray cats from Spain, although analyzing 114 cats, a similar number of cats tested in our study. This discrepancy may depend on differences regarding factors not mentioned in the cited study, such as the actual rate of infection of people living in the sampled area or the site and structure of the colonies, including the type and frequency of contacts between cats and people and the total number of the stray cat population. Overall, our results show that stray cats are at lower risk of infection compared to privately owned cats from northern Italy (Patterson et al., 2020). This study has some limitations. First, even if the number of cats involved is considerable, they represent only a small percentage of all the registered cats in the analyzed district area. Therefore, it would be advisable to increase the sample size to confirm the findings of this study. Second, the cats involved were mostly young (mean age: 18.8 months), and this could have represented a bias, since among domestic pets, infected cats are generally adults (Patterson et al., 2020). However, in one study no association between age and seropositivity was found, but information on the mean age were not provided (Fritz et al., 2020). Third, the presence of coinfections, with special emphasis on feline immunodeficiency virus or feline

leukemia virus, has not been tested. Nevertheless, future studies should include more adult or aged cats, in order to clarify whether the negative results recorded in this study may depend on age, on a lower exposure to concurrent infections, or on a true absence of infection in colony cats. This study suggests that stray cats have no role in the spreading of SARS-CoV-2. Indeed, none of the examined cats was carrying the virus or has been infected, at least recently, suggesting that the short contacts with potentially infected people do not induce infection in colony cats or, if infection occurred before inclusion in this study, it is not followed by cat-to-cat transmission within the colony. This finding supports the knowledge that cats become infected if living in positive SARS-CoV-2 households, as already reported. Moreover, it can be assumed that free living cats might not be used as a sentinel species in a determined territory, even though some positive results might be found through future studies, as reported in cats living in specific environments (e.g., near mink farms with SARS-CoV-2 epidemics). Further studies increasing the sample size and including aged animals are warranted, also in the light of the emerging new viral variants that may change viral diffusion and tropism.

**Supplementary materials**

Supplementary Table 4. Data regarding the municipalities where the feline colonies were located. The area, number of citizens, density and COVID-19 cases are reported.

| | **Area ($Km^2$)** | **Citizens** | **Density of population (people/$Km^2$)** | **SARS-CoV-2 positive** | **SARS-CoV-2 positive rate** |
|---|---|---|---|---|---|
| 1 | 7.5 | 2420 | 322.67 | 87 | 3.6% |
| 2 | 9.75 | 2932 | 300.72 | 94 | 3.2% |
| 3 | 16.1 | 2135 | 132.61 | 93 | 4.4% |
| 4 | 15.68 | 1570 | 100.13 | 69 | 4.4% |
| 5 | 10.92 | 2611 | 239.1 | h102 | 3.9% |
| 6 | 41.38 | 44560 | 1076.85 | 2215 | 5.0% |
| 7 | 8.48 | 4426 | 521.93 | 150 | 3.4% |
| 8 | 15.58 | 5801 | 372.34 | 252 | 4.3% |
| 9 | 16.55 | 7452 | 450.27 | 383 | 5.1% |
| 10 | 20.05 | 13283 | 662.49 | 568 | 4.3% |
| 11 | 16.07 | 5890 | 366.52 | 266 | 4.5% |
| 12 | 13.5 | 1875 | 138.89 | 56 | 3.0% |

viruses

MDPI

*Communication*

# Do Dogs and Cats Passively Carry SARS-CoV-2 on Hair and Pads?

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**Foreword**: in this study, we investigated the presence of SARS-CoV-2 in hair and pads of domestic cats and dogs to verify if they may act as passive mechanical carriers of the virus. This study was carried out during the early stage of the COVID-19 pandemic. My contribution to this work was related to sample collection, data curation and manuscript review. The results of this study were published under the reference:

Lauzi S, Stranieri A, Giordano A, Lelli D, Elia G, Desario C, Ratti G, Decaro N, Paltrinieri S. Do Dogs and Cats Passively Carry SARS-CoV-2 on Hair and Pads? *Viruses*. 2021 Jul 13;13(7):1357. doi: 10.3390/v13071357. PMID: 34372563; PMCID: PMC8310179.

**Abstract**

The epidemiological role of domestic animals in the spread and transmission of SARS-CoV-2 to humans has been investigated in recent reports, but some aspects need to be further clarified. To date, only in rare cases have dogs and cats living with COVID-19 patients been found to harbour SARS-CoV-2, with no evidence of pet-to-human transmission. The aim of the present study was to verify whether dogs and cats act as passive mechanical carriers of SARS-CoV-2 when they live in close contact with COVID-19 patients. Cutaneous and interdigital swabs collected from 48 dogs and 15 cats owned by COVID-19 patients were tested for SARS-CoV-2 by qRT-PCR. The time elapsed between owner swab positivity and sample collection from pets ranged from 1 to 72 days, with a median time of 23 days for dogs and 39

days for cats. All samples tested negative, suggesting that pets do not passively carry SARS-CoV-2 on their hair and pads, and thus they likely do not play an important role in the virus transmission to humans. This data may contribute to confirming that the direct contact with the hair and pads of pets does not represent a route for the transmission of SARS-CoV-2.

**Aims**

Since the data on SARS-CoV-2 contamination of hair and pads of pets are limited and mostly refer to dogs or cats infected by SARS-CoV-2, as demonstrated by PCR positive oropharyngeal, nasal, and/or rectal swabs (Goryoka et al., 2021; Hamer et al., 2021; Klaus et al., 2021a). The aim of this study was to assess whether hair and pads of pets from COVID-19 positive households are contaminated by their owner(s) and/or indirectly by the environment in order to determine if pets, even if they are not actively infected, may passively carry the virus.

**Materials and Methods**

The study included 48 dogs and 15 cats from Italian COVID-19-positive owners that were diagnosed in April–May and October–November 2020 with COVID-19 on the basis of the presence of symptoms and signs consistent with the disease (Wong et al., 2020) and a positive nasopharyngeal swab for SARS-CoV-2, as officially defined by the National Public Health services. The inclusion criteria for dogs and cats were the absence of clinical signs and, in order to exclude self-contamination, the absence of SARS-CoV-2 RNA in nasal, oropharyngeal, and rectal swabs of the animals, determined as previously described (Patterson et al., 2020).

Samples from dogs and cats were collected during routine veterinary visits under the informed consent of the owners, who also filled out a questionnaire including information about the date of their clinical and molecular diagnosis. Dogs and cats were brought to the veterinary visit by their owners (after the owners had recovered and received negative results of nasopharyngeal swabs) or, if veterinary visits were requested before the recovery of the owners, by healthy dog sitters or relatives not living with the owners. In four cases, animals were owned by veterinarians, who directly performed the samplings following the authors' instructions. The study was approved by the Institutional Animal Care and Use Committee and by the Institutional Ethical Committee (approval numbers_31/20 and 43/20, respectively).

Cutaneous samples from dogs and cats were collected by brushing sterile swabs on the skin and hair of the dorsal part of the neck and moving the swab towards the lumbar region. To collect interdigital

samples, swabs were brushed on the surface of the footpads and then gently inserted into the interdigital spaces. These anatomical regions were selected based on the frequency of direct contact between pets and owners (dorsal part of animal bodies) and between pets and the environment (footpads and interdigital spaces). When delivered to the Veterinary Teaching Hospital (VTH) of the University of Milan, each swab was dipped in a solution based on phosphate-buffered saline (PBS pH 7.2) supplemented with 10% glycerol and antibiotics (1%) and frozen at −20 °C until shipping in cold chain to the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna for analyses, which are described below. Swabs taken by veterinarians were frozen and then delivered to the VTH after the veterinarians' recovery and negative testing, then treated as described above.

Swabs were thawed and centrifuged at 3750 rpm for 15 min. Viral RNA was extracted from 250 µL of supernatant using the QIAsymphony™ SP instrument (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Swabs from dogs and cats were analysed by real-time reverse transcription-PCR (qRT-PCR) tests targeting a specific region of the SARS-CoV-2 E gene to detect SARS-CoV-2 as previously described (Corman et al., 2020). Thermal cycling was performed at 52 °C for 15 min for reverse transcription, followed by 95 °C for 10 s and then 45 cycles of 95 °C for 5 s and 60 °C for 30 s.

In our study, the analytical sensitivity (LOD) of the assay was 11–15 copies of SARS-COV-2 RNA per reaction at a 99% detection probability in human respiratory specimens. Multiple negative controls were included in each run to rule out contamination, while an internal positive control (IC) was added to each sample in order to reveal the presence of RT-PCR inhibitors. ICs were simultaneously extracted and/or amplified in the same tube with the pathogen target; combined with the amplification positive control, they proved the functionality of the reaction mixture for correct amplification of the SARS-COV-2 E gene target. This combination ruled out inhibition and other malfunctions and confirmed that negative results were truly negative. The amplification positive control consisted of SARS-CoV-2 RNA extracts from swabs of infected human patients, diluted until a Ct value (cycle threshold) of 26–30 was obtained.

**Results**

The time elapsed from the positive testing of owners and the collection of swabs from pets ranged from 1 to 72 days (median 23 days) in dogs and from 5 to 68 days (median 39 days) in cats (Figure 10). Specifically, of the 63 samples, 42.9% (n = 27) were collected within 21 days of the owner's positive test and comprised 22 swabs from dogs and 5 from cats, whereas 57.1% of samples (n = 36) were

collected between 22 and 72 days after the owner's positive test and comprised 26 swabs from dogs and 10 from cats. All cutaneous and interdigital swabs tested negative for SARS-CoV 2 RNA.

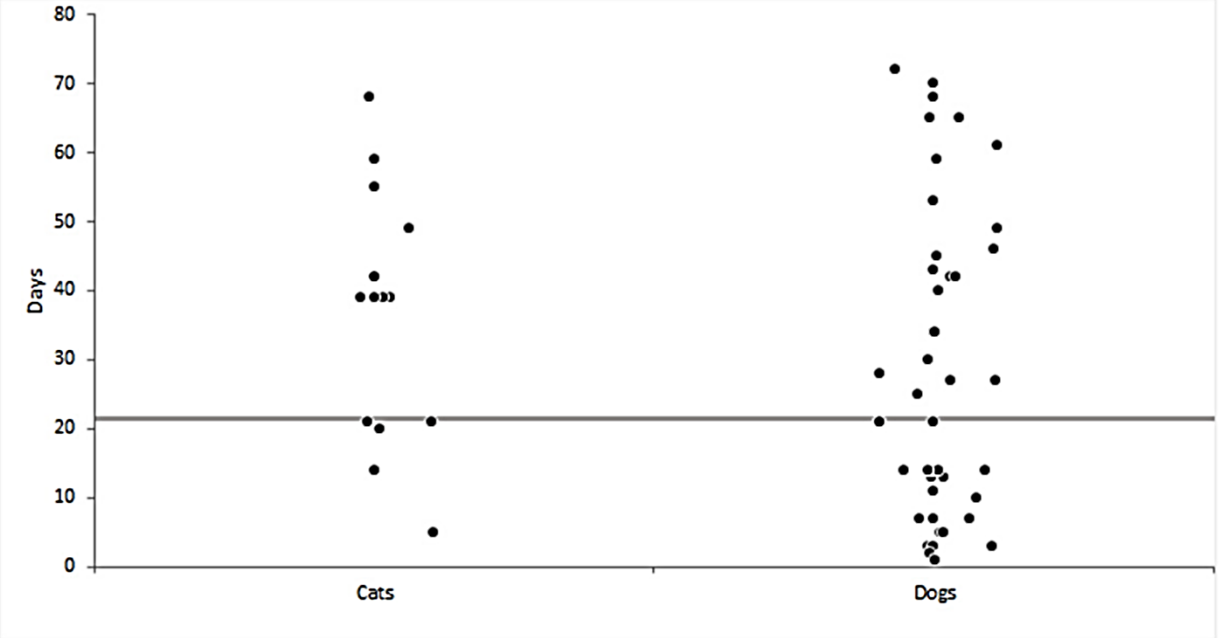


Figure 10. Time elapsed from the detection of SARS-CoV-2 in owners by oropharyngeal swab and the collection of swabs of hair and footpads from dogs and cats. The grey horizontal line indicates 21 days from the owner's positive test.

**Discussion**

The current guidelines of the World Organization for Animal Health (WOAH) recommend that owners infected with SARS-CoV-2 avoid close contact with their companion animals (WOAH, 2020). However, the complete absence of contact or indirect contamination of pets through environmental surfaces/fomites (e.g., through the owners' coughing or sneezing) is unlikely in pets in COVID-19-positive households. The results of the present study suggest that pets are not contaminated by their owners or the environment or that routine hygiene practices are adequate to avoid persistent contamination of pets' hair and pads with SARS-CoV-2. The time elapsed from the owner's COVID-19 diagnosis to pet sample collection is considered a potential source of bias for some of our negative results (Ruiz-Arrondo et al., 2021). It was reported that SARS-CoV-2 in humans is contagious for several days after onset of symptoms, and some patients test positive for SARS-CoV-2 RNA for weeks if not months (WOAH, 2020). Contagiousness in patients rapidly decreases after about 10–15 days from the onset of COVID-19, depending on the severity of the clinical presentation and whether the patients are immunocompromised. The longest duration of viral viability that has been reported thus far is 20 days

from the onset of clinical signs (Rhee et al., 2021). Moreover, the viral load and temperature were shown to influence the presence of infectious virus on common surfaces for up to 28 days (Riddell et al., 2020). Although it has been demonstrated that the probability of pets being seropositive increases with the time of exposure to the infected owner (Colitti et al., 2021), it may be possible that the virus was present on the hair or pads of pets before they were sampled. This is in line with a recent report showing that of 56 dogs with negative oropharyngeal and rectal swabs who lived with SARS-CoV-2 positive owners, 1 tested positive in a fur sample but tested negative soon after (Goryoka et al., 2021). Nevertheless, negative results were also observed in our study in samples from pets that were collected within 21 days after the diagnosis of COVID-19 in their owners, when it was likely that symptomatic people or housemates still harbored the virus. These results suggest that pets do not play an epidemiological role in the spread and indirect transmission of SARS-CoV-2 to humans via contamination of hair or paws. The limitations of this study include the low number of enrolled animals (due to the challenges of collecting samples from pets belonging to COVID-19 patients) and the possible influence of preanalytical or analytical factors. Among these, the specificity and sensitivity of the method seem not to be an issue based on the results of our own evaluation of the analytical performances of the method and on the use of positive and negative controls. This evaluation did not include the assessment of possible storage artefacts. Although storage at −20 °C may have theoretically affected sensitivity especially if the virus load in skin or pad swabs was low, previous reports have demonstrated limited pre-analytical artefacts in samples stored at room temperature, +4 °C, or −20 °C, and it is therefore unlikely that freezing conditions led to false-negative results (Basso et al., 2020; Li et al., 2020). Despite these limitations, our results suggest that pets do not passively carry the virus or play an important role in the transmission of SARS-CoV-2 to humans through direct contact with the hair or skin of pets. Considering the data obtained in this study, future investigations using a One Health approach are needed to update the knowledge of the possible risk of pet-to-human transmission in the early phase of infection. Moreover, future studies are recommended to evaluate whether owners' behaviors and interactions with their pets influence the likelihood that their pet will test positive or have positive hair or paw swabs in order to design effective strategies for COVID-19 prevention and control and to avoid unnecessary discrimination against animals.

Transboundary and Emerging Diseases WILEY

*Original article*

# Comparison of diagnostic performances of different serological tests for SARS-CoV-2 antibody detection in cats and dogs

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**Foreword**: in this study, we evaluated the diagnostic performance of three different commercially available immunoassays for the detection of SARS-CoV-2 antibodies in pets with conventional virus neutralization test as reference method. My contribution to this work was related to sample collection, data analysis, data interpretation and manuscript writing. The results of this study were published under the reference:

Ratti G, Lelli D, Moreno A, Stranieri A, Trogu T, Giordano A, Grassi A, Luzzago C, Decaro N, Paltrinieri S, Lauzi S. Comparison of diagnostic performances of different serological tests for SARS-CoV-2 antibody detection in cats and dogs. *Transbound Emerg Dis*. 2022 Nov;69(6):3530-3539. doi: 10.1111/tbed.14716. Epub 2022 Oct 13. PMID: 36183165; PMCID: PMC9538080.

**Abstract**

Serosurveillance among animals, including pets, plays an important role in the current coronavirus disease 2019 (COVID-19) pandemic, because severe acute respiratory coronavirus 2 (SARS-CoV-2) infections in animal populations could result in the establishment of new virus reservoirs. Serological assays that offer the required sensitivity and specificity are essential. In this study, we evaluated the

diagnostic performance of three different commercially available immunoassays for the detection of SARS-CoV-2 antibodies in pets, namely two ELISA tests for the detection of antibodies against SARS-CoV-2 nucleocapsid [ID Screen SARS CoV-2 double antigen multispecies (Double antigen) and ID Screen® SARS-CoV-2-N IgG indirect ELISA (Indirect)] and one test for the detection of neutralizing antibodies against SARS-CoV-2 receptor-binding-domain [surrogate virus neutralization test (sVNT)]. The obtained results were compared with those of conventional virus neutralization test (VNT), which was regarded as reference method. A total of 191 serum samples were analysed. Thirteen (6.8%) samples showed VNT-positive results. The overall sensitivity was higher for sVNT (100%) compared to nucleocapsid-based ELISA assays (23% for Double antigen and 60% for Indirect). The specificity was 100% for Indirect ELISA and sVNT, when a higher cut-off (>30%) was used compared to the one previously defined by the manufacturer (>20%), whereas the other test showed lower value (99%). The sVNT test showed the highest accuracy and agreement with VNT, with a perfect agreement when the higher cut-off was applied. The agreement between each nucleocapsid-based ELISA test and VNT was 96% for Indirect and 94% for Double antigen. Our findings showed that some commercially available serological tests may lead to a high rate of false-negative results, highlighting the importance of assays validation for the detection of SARS-CoV-2 antibodies in domestic animals.

## Materials and Methods

### *Sample collection*

Cats and dogs from Italy were sampled between 2 April 2020 and 12 September 2021. Samples were collected for the purpose of this study (approval n. 31/20 of the Institutional Animal Care and Use Committee and n. 43/20 of the Institutional Ethical Committee of the University of Milan) or were collected following diagnostic procedures performed within the Veterinary Teaching Hospital (VTH) of Lodi after obtaining written consent from the pet owner. According to the Ethical Committee of the University of Milan decision 29 October 2012, renewed with the protocol no. 02–2016, the use for research purposes of residual aliquots of samples collected for diagnostic purposes at the VTH under informed consent of the owners is allowed without any additional formal request of authorization. Complete information regarding animal signalment, including breed, sex, age, localization and timing of exposure to COVID-19 infected humans was collected when available. Blood samples were collected by jugular or cephalic venipuncture and placed immediately in serum-separating tubes. After collection, blood samples were centrifuged at 2500 × *g* for 10 min and serum was stored at –20°C until serological analysis.

In cats, rectal swabs were also collected and stored at –80°C until RNA extraction for feline coronavirus (FCoV) detection.

*Serological tests*

Serum samples were tested by VNT and three commercial serological tests.

*Virus neutralization test*

The virus neutralization assay was performed as described by Rijkers et al. (2020) (Rijkers et al., 2020) with few modifications. Briefly, sera were previously heat-inactivated (30 min, 56°C) and tested in duplicate. Twofold serial dilutions (starting at 1:5) of the sera were incubated with 100 TCID50 of the SARS-CoV-2 HCoV-19/Italy/310904/46/2020 strain (EPI_ISL_9011947) at 37°C and 5% $CO_2$, for 1 h at 37°C in 96-well plates. Vero-E6 cells were added at a concentration of 2 × $10^4$ cells per well and incubated for 72 h at 37°C with 5% $CO_2$. Serum virus neutralization titre (VNT50) was defined as the reciprocal value of the sample dilution that showed 50% protection of virus growth. Sera with titres ≥1/10 were considered positive for SARS-CoV-2 antibodies. The analysis is considered valid when there is a difference of less than 1 log2 between the two replicates. For each serum, the mean between the titres of the two replicates is reported. Samples reactive in VNT with a titre of 5 were further classified as positive in case of positive results obtained using the commercial serological assays.

*Surrogate virus neutralization assay (sVNT)*

sVNT kits were obtained from GenScript, Inc., NJ, USA, and performed following the manufacturer's instructions. This assay is based on the binding inhibition between SARS-CoV-2 receptor binding domain (RBD) and the human angiotensin-converting enzyme 2 (hACE2) by the neutralizing antibodies present in the sera. Briefly, serum samples were diluted 1:10 and mixed with an equal volume of horseradish peroxidase (HRP) +conjugated to SARS-CoV-2 RBD and then incubated for 30 min at 37°C. One hundred microlitres were transferred to each well coated with hACE2 receptor and incubated for 15 min at 37°C. Mixture was removed, and plates were washed with wash solution. One hundred microlitres of tetramethylbenzidine (TMB) substrate were added to each well and incubated in dark at room temperature for 15 min. Reaction was stopped by adding 50 µl of stop solution to each well. The optical densities (OD) of each sample were read at 450 nm in an ELISA microplate reader (Biosan SIA, Latvia). As reported in the manufacturer's instructions, percentage of inhibition was calculated with the following formula: (1 – OD sample value/OD negative control) × 100. Samples with a percentage of

inhibition value >20% (low cut-off, as previously established by the manufacturer) and samples with >30% inhibition (cut-off value defined recently by the manufacturer) were considered positive for SARS-CoV-2 antibody. Positive and negative sera supplied by the manufacturer were used as positive and negative controls.

*Double antigen ELISA*

A commercial double antigen multispecies ELISA (ID Screen SARS CoV-2 double antigen multispecies; ID.Vet, France) was used for the detection of specific antibodies against SARS-CoV-2 N antigen, following the manufacturer's instructions. Briefly, 25 µl of dilution buffer and 25 µl of each sample were added to each well and incubated for 45 min at 37°C. Wells were then washed five times with wash solution. One hundred microlitres of HRP conjugate N protein recombinant antigen was added to each well and incubated for 30 min at 21°C. Wells were washed five times and 100 µl of substrate solution (TMB) was added, subsequently plates were incubated for 20 min at 21°C in dark. Reaction was stopped by adding 100 µl of stop solution to each well. The OD values of each sample were read at 450 nm in an ELISA microplate reader. Sample to positive ratio (*S*/*P*) was calculated with the following formula: (OD sample value – OD negative control)/(OD positive control – OD negative control). Samples with *S*/*P* >0.60 were considered positive. Positive and negative sera supplied by the manufacturer were used as positive and negative controls.

*Indirect ELISA*

A commercially available indirect ELISA (ID Screen® SARS-CoV-2-N IgG indirect ELISA; ID, Vet, France) was used to detect specific antibodies against SARS-CoV-2 N antigen with protocol modification for the detection of dog and cat antibodies. Briefly, 10 µl controls and samples were diluted in 200 µl of dilution buffer, 100 µl of diluted samples and controls were added to each well and incubated for 45 min at room temperature. Wells were then washed three times with wash solution. One hundred microlitres of anti-multispecies IgG HRP-conjugate were added to each well and incubated for 30 min at room temperature. Wells were then washed three times with wash solution. One hundred microlitres of substrate solution (TMB) were added to each well and incubated for 20 min kept in a dark place at room temperature. Reaction was stopped by adding 100 µl of stop solution to each well. The OD of each sample was read at 450 nm in an ELISA microplate reader. *S*/*P* was calculated with the following formula: (OD sample value – OD negative control)/(OD positive control – OD negative

control). Samples with $S/P \geq 0.40$ were considered positive. Positive and negative sera supplied by the manufacturer were used as positive and negative controls.

*FCoV real-time reverse transcriptase PCR (real-time RT-PCR)*

RNA extraction from rectal swabs was performed using commercial NucleoSpin viral RNA isolation kit (Macherey-Nagel, Bethlehem, PA) following manufacturer's instructions. RNA quality control targeting vertebrate 12S rRNA locus (Kitano et al., 2007) was performed on randomly selected samples (results not shown). Real-time RT-PCR based on the amplification of the 7b gene of FCoV was performed on extracted RNA, according to a previously described protocol (Gut et al., 1999) with minor modifications. The real-time RT-PCR reaction was performed using a commercial kit (TaqMan Fast Virus 1step master mix, Applied Biosystems) in a final volume of 25 µl: 5 µl master mix, 600 nM of primers FCoV1128f (GATTTGATTTGGCAATGCTAGATTT) and FCoV1229r (AACAATCACTAGATCCAGACGTTAGCT), 200 nM of probe FCoV1200p (FAM-TCCATTGTTGGCTCGTCATAGCGG-TAMRA) and 5 µl of template RNA. Reactions were performed using a QS3 instrument (Applied Biosystems). As a positive control, an FCoV-positive cat sample was used, while the negative control consisted of an FCoV-negative sample from a domestic cat. A sample was considered positive in the presence of an amplification curve and a value of threshold cycle (Ct) <40, as previously reported (Felten et al., 2020). For absolute quantitation, a pCR4 plasmid (Invitrogen, Carlsbad, California, USA), containing the FCoV 7b target sequence produced according to previously published protocols (Balboni et al., 2012) and kindly provided by Professor Mara Battilani, was used. Serial $\log_{10}$ dilutions of the recombinant plasmid with a known copy number ($10^1$–$10^7$ copies/µl) were amplified with the samples in order to obtain a standard curve.

*Data analysis*

For each of the three commercial serological assays, sensitivity and specificity were calculated using VNT as the reference method (Embregts et al., 2021; Perera et al., 2021). Concordance among the assays was calculated using the Cohen's Kappa coefficient. Kappa value <0.00 indicates a poor concordance, 0.00 to 0.20 a slight concordance, 0.21 to 0.40 a fair concordance, 0.41 to 0.60 a moderate concordance, 0.61 to 0.80 a substantial concordance, and ≥.81 represents almost perfect concordance (Landis & Koch, 1977). Spearman's correlation coefficient was used to evaluate the correlation between VNT titres and the results obtained using the commercial tests (OD and percentage of inhibition). A Spearman's rho value between 0.81 and 1 indicated a very strong correlation, $r$ = 0.61 to 0.80 strong, 0.41 to 0.60 moderate, 0.21 to 0.40 weak and 0 to 0.20 negligible

correlation (Prion & Haerling, 2014). All statistical analyses were performed using Epitools (https://epitools.ausvet.com.au) and Analyse-it v5.66 software (Analyse-it software, Ltd, Leeds, United Kingdom). The significance was set at $p$ value < 0.05.

**Results**

In total, 191 serum samples from dogs ($n$ = 66) and cats ($n$ = 125) were included in this study. Thirty-nine animals belonged to COVID-19-positive owners, 42 belonged to COVID-19-negative owners. Information on owner's disease status was not available for 11 privately owned animals, whereas the other 99 animals were stray cats. Regarding time of sampling from owners' positivity, samples collection ranged from 16 to 251 days, with a median of 93 days (Table 9).

Table 9. Serum samples positive for at least one assay: characteristics of samples and comparison of results of three commercially available tests and gold standard VNT.

| Species | No. of days after owner tested positive for COVID-19 that animal was sampled | VNT titers | sVNT (Percentage of inhibition) | Double antigen ELISA (S/P) | Indirect ELISA (S/P) |
|---|---|---|---|---|---|
| Dog | Positive (251 days) | 5 | Positive† (71%) | ND‡ | Negative |
| Dog | ND | Negative | Positive (23%) | Negative | ND |
| Dog | Positive (78 days) | 20 | Positive (42%) | Negative | Positive (0.77) |
| Dog | Positive (16 days) | Negative | Positive (24%) | ND | Negative |
| Dog | Positive (47 days) | 20 | Positive (71%) | Negative | Positive (0.75) |
| Dog | Positive (17 days) | 40 | Positive (66%) | Negative | Positive (0.74) |
| Dog | Positive (ND) | 10 | Positive (71%) | Negative | ND |
| Dog | Positive (ND) | 20 | Positive (73%) | Positive (0.83) | ND |
| Dog | Positive (ND) | 20 | Positive (82%) | Positive (3.2) | ND |
| Dog | Positive (ND) | Negative | Negative | Positive (0.78) | ND |
| Dog | ND | 5 | Positive (75%) | Negative | Positive (1.22) |
| Dog | ND | 40 | Positive (67%) | Negative | Positive (1.30) |
| Dog | ND | 20 | Positive (74%) | Negative | Negative |
| Dog | ND | 20 | Positive (68%) | Positive (0.62) | Positive (1.17) |
| Cat | 251 | 160 | Positive (94%) | ND | Negative |
| Cat | ND (stray) | Negative | Negative | Positive (1.37) | ND |
| Cat | 59 | 80 | Positive (89%) | Negative | Negative |

† Positive sample using the low cut-off of percentage of inhibition value >20% (as previously established by the manufacturer); ‡ ND = not determined

All serum samples were analysed using VNT and sVNT assays. Out of these samples, 189 (65 dogs and 124 cats) and 123 (18 dogs and 105 cats) sera were analysed with Double antigen and Indirect ELISA, respectively. Overall results showed that 17 (8.9%) serum samples were positive to at least one test (Table 9), while all the others serum samples tested negative for all the applied tests. Thirteen (6.8%) samples tested seropositive with the VNT assay, including 11 (16.7%) sera from dogs and 2 (1.6%) sera from cats. Nine VNT-positive animals belonged to COVID-19 owners, whereas information on owners'

COVID-19 status was not available for 4 animals. Available information on timing of samples from owner's positivity showed that samples from positive animals belonging to COVID-19-positive owners were collected between 17 and 251 days from owner's diagnosis (Table 9). Results of commercial assays showed that false-negative results were observed using Double antigen ELISA and Indirect ELISA. The false-negative results obtained in our study using the N-based ELISA tests were observed regardless of the VNT titre, since the absence of antibodies against the N protein was observed in animals showing both high and low neutralization antibody titres (Table 9). The 8 false-negative results using Double antigen ELISA were obtained in four samples collected less than 2 months after owner's COVID-19-positivity, whereas this information was not available for the remaining four animals. The four false-negative results obtained using the Indirect ELISA were from 3 samples collected more than 2 months after owners' diagnosis of SARS-CoV-2 infection, whereas for one sample this information was not available. Two false-positive results were obtained using sVNT with cut off of >20% and Double antigen ELISA.

Sensitivity and specificity of each of the assays for overall samples and samples collected from dogs and from cats are reported in Table 10. Due to the low number of positive cat samples, sensitivity for the two ELISA assays was not calculated for feline samples.

Table 10. Tests performance results.

| | Ab Positive samples/total | Sensitivity % (95% C.I.) | Specificity % (95% C.I.) |
|---|---|---|---|
| **Overall** | | | |
| sVNT (cut-off >20%) | 15/191 | 100 (75-100) | 99 (96-100) |
| sVNT (cut-off >30%) | 13/191 | 100 (78-100) | 100 (98-100) |
| Double antigen ELISA | 5/189 | 23 (5-54) | 99 (96-100) |
| Indirect ELISA | 6/123 | 60 (26-89) | 100 (97-100) |
| **Dog** | | | |
| sVNT (cut-off >20%) | 13/66 | 100 (71-100) | 96 (87-100) |
| sVNT (cut-off >30%) | 11/66 | 100 (75-100) | 100 (93-100) |
| Double antigen ELISA | 4/65 | 30 (6-65) | 98 (90-100) |
| Indirect ELISA | 6/18 | 75 (35-97) | 100 (69-100) |
| **Cat** | | | |
| sVNT (cut-off >20%) | 2/125 | 100 (15-100) | 100 (97-100) |
| sVNT (cut-off >30%) | 2/125 | 100 (15-100) | 100 (97-100) |
| Double antigen ELISA | 1/124 | NC† | 99 (95-100) |
| Indirect ELISA | 0/105 | NC | 100 (96-100) |

†NC= not calculated

The overall concordance between each of the assays and VNT is reported in Table 11.

Table 11. Overall proportion of concordance between immunoassays. A color gradient illustrates the Cohen Kappa measure (orange=fair; blue=substantial; green= perfect)

| | VNT % (Kappa) | sVNT cut-off >20% % (Kappa) | sVNT cut-off >30% % (Kappa) | Double antigen ELISA % (Kappa) | Indirect ELISA % (Kappa) |
|---|---|---|---|---|---|
| VNT % (Kappa) | 100% (1) | | | | |
| sVNT cut-off >20% % (Kappa) | 99% (0.92) | 100% (1) | | | |
| sVNT cut-off >30% % (Kappa) | 100% (1) | 99% (0.92) | 100% (1) | | |
| Double antigen ELISA | 94% (0.31) | 94% (0.30) | 95% (0.35) | 100% (1) | |
| Indirect ELISA | 97% (0.73) | 96% (0.69) | 97% (0.79) | 95% (0.24) | 100% (1) |

Comparison between VNT titres and results of the different assays is reported in Figure 11. Spearman's rho value showed a very strong correlation ($r = 0.935$) between the VNT titre and the sVNT percentage of inhibition value, a strong correlation ($r = 0.753$) between the VNT titre and Indirect ELISA S/P value and a weak correlation ($r = 0.379$) between the VNT titre and Double antigen ELISA S/P value, always with a statistical significance level of $p < 0.001$. The best correlation was observed between VNT and sVN with the use of cut-off value of >30%.

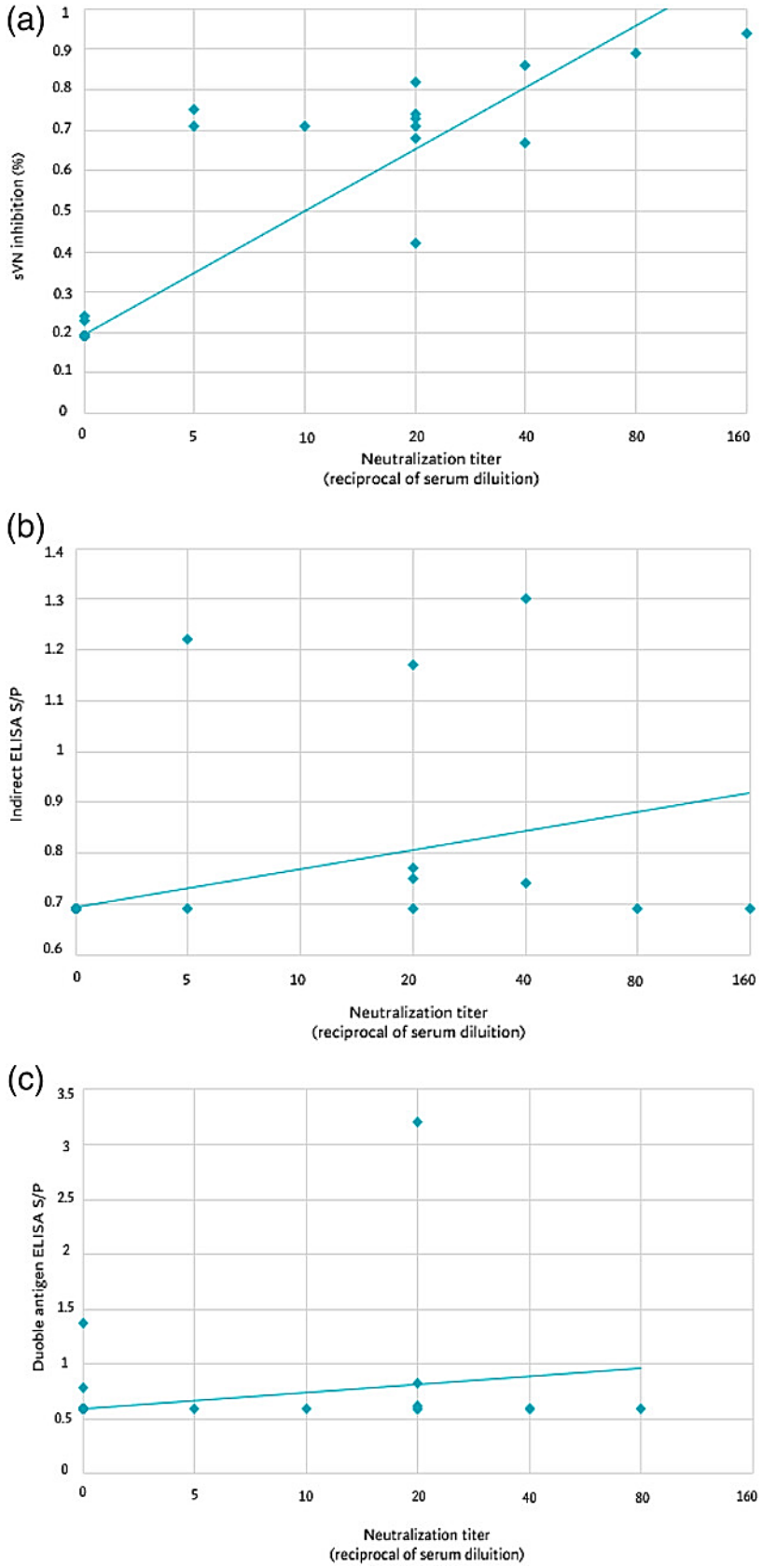


Figure 11. Correlation between per cent inhibition in the sVNT and VNT titres (reciprocal of serum dilution) with r = 0.935 (a), correlation between S/P values in the indirect ELISA and the VNT titres with r = 0.753 (b), correlation between S/P values in the double antigen ELISA and VNT titres with r = 0.379 (c). Spearman's test was used for correlation analysis. For clarity, the negative VNT sera that were negative in all three assays were not included in the figure.

Regarding samples from cats, real-time RT-PCR for FCoV detection was performed on a subset of 106 available feline rectal swabs. FCoV RNA was detected in 57 (53.8%) of the tested cats, with Ct values ranging from 15.6 to 39.8, corresponding to $5.6\times10^7$ and 1.8×10° copy numbers/µl, respectively. Two (1.9%) out of the 106 FCoV tested cats showed VNT-positive results. More precisely, among the 57 FCoV-positive cats, one (1.8%) cat was VNT-positive (VNT titre 80). This VNT-positive sample, collected 59 days after owner's positivity, was correctly identified by sVNT but resulted negative by the N-antigen based ELISA tests. The Double antigen ELISA identified as seropositive an FCoV-positive stray cat with VNT-negative result. Among the 49 FCoV-negative cats, one (2%) cat showed neutralization antibodies. This VNT-positive sample, collected 251 days after owner's positivity, was correctly identified by sVNT but not by the Indirect ELISA test, whereas it was not tested using the Double Antigen test.

**Discussion**

Reliable methods for antibody detection are essential to understand susceptibility and immune-response to SARS-CoV-2 in animals and assays with high sensitivity should be used for epidemiological surveillance (Yamamoto et al., 2022). However, the gold standard VNT execution requires BSL-3 laboratories and trained personnel, making it inaccessible for a wider community of diagnostic and research laboratories. Therefore, in this study we investigated the diagnostic accuracy of widely accessible and easy-to-perform assays. More precisely, we evaluated sensitivity, specificity and correlation with neutralizing antibodies, considered as the gold standard for antibody detection, of three different commercially available immunoassays for the detection of SARS-CoV-2 antibody in pets that can be performed in BSL-2 laboratories.

The VNT cut-off for positive samples was set to antibody titre 10; however, considering that low antibody titres have been observed in SARS-CoV-2-infected domestic dogs following experimental infection (Bosco-Lauth et al., 2020), samples reactive in VNT with a titre of 5 were subsequently classified as positive in cases where positive results were obtained using the commercial serological assays. Neutralizing antibodies were identified in animals enrolled in this study at different timing of sample collection from the owner's COVID-19 positivity. Indeed, exposure to COVID-19-positive owners was considered as the likely source of infection in the animals (Patterson et al., 2020). As previously mentioned, neutralizing antibodies in pets can persist for up to 10 months (Decaro et al., 2022). This finding is in accordance with our results that showed VNT positivity in animals after more than 8 months from owner's COVID-19-positive status.

Our results confirmed the best performances of the sVNT when using the higher cut-off value recommended by other authors (Embregts et al., 2021; Tan et al., 2020) and recently also by the manufacturer, compared with the lower cut-off and with the other two ELISA commercial tests. Indeed, the higher cut-off value (>30%) allowed the correct negative identification of two samples from dogs that showed false-positive results when using the lower cut-off value (>20%). Moreover, even if the sVNT is not meant to be quantitative, the strong correlation between sVNT percentage of inhibition and VNT titres confirms previous results (Perera et al., 2021) and further confirms the high performance of this test.

Regarding the two N-antigen-based ELISA assays, the discrepancies between these ELISA tests and the neutralization assay for both positive and negative results observed in our study are consistent with previous reports that have frequently performed SARS-CoV-2 serological investigations on animal samples based on a screening test using commercial assays and subsequent confirmation of results with neutralization assays (Adler et al., 2022; Barua et al., 2021; Decaro et al., 2022; Jemeršić et al., 2021; Klaus et al., 2021b; Michelitsch et al., 2020; Udom et al., 2022). Concerning the low sensitivity values of the two N-antigen-based ELISA assays from our study, it should be reminded that the false-negative results detected in samples from pets were based on the confirmation by VNT of all serum samples regardless of their positive ELISA results. Previous reports may have underestimated false-negative results of N-antigen-based ELISA because only ELISA-positive samples or randomly selected ELISA-negative samples were confirmed by VNT (Jemeršić et al., 2021; Udom et al., 2022). False-negative results using the N-based-antigen ELISA tests evaluated in this study may be due to the absence or lower presence of antibodies against the viral nucleoprotein compared to the gold standard assay detecting neutralizing antibodies. Indeed, a lower persistence of anti-nucleocapsid compared to anti-spike antibodies has been reported in humans (Van Elslande et al., 2022), and this may explain why ELISA tests based on the spike (S) antigen have shown a higher sensitivity and a better correlation with the presence of neutralizing antibody in humans compared to the N antigen-based ELISA tests (Kontou et al., 2020; Mohit et al., 2021; Ni et al., 2020; Rathe et al., 2021). The different kinetics between the antibody responses raised against the viral nucleoprotein and the one directed against the spike protein has also been suggested as a possible cause of the lower sensitivity of ELISA N-based assays compared to VNT in domestic animals (Decaro et al., 2022). In this respect, it is intriguing that two samples with neutralizing antibodies collected from animals after 251 days from owner's COVID-19 positivity were both negative in the Indirect ELISA. Further investigations are needed to define the kinetics between the antibody responses against different SARS-CoV-2 antigens in pets as well as the

possible explanations of the lower sensitivity of N-based ELISA assays also considering that other studies have shown similar diagnostic performances between S- and N-based commercially available assays (Folegatti et al., 2020; Ni et al., 2020; Okba et al., 2020) or higher specificity and sensitivity for in-house N antigen-based ELISA in comparison with RBD antigen-based ELISA for the detection of SARS-CoV-2 antibody in pets (Dileepan et al., 2021).

Given that both the ELISA assays investigated in the present study are N-antigen-based, the higher sensitivity and the better correlation of the indirect ELISA with the presence of neutralizing antibodies compared with the double antigen ELISA could also be ascribed to the different type of N protein used and assay procedure for antibody detection that may have influenced the assay performance (Adler et al., 2022; Rikhtegaran Tehrani et al., 2020).

The conserved structure of the N protein has raised concerns on a possible cross-reactivity with antibodies against other animal coronaviruses when using N-based ELISA (Udom et al., 2022). This aspect was apparently not observed in our study and serological cross-reactivity between SARS-CoV-2 and other animals coronaviruses was likely ruled out by our results, confirming previous reports (Decaro et al., 2022; Dileepan et al., 2021; Embregts et al., 2021; Michelitsch et al., 2020; Perera et al., 2021; Zhang et al., 2020). Indeed, our results did not show different prevalences of SARS-CoV-2 seropositive cats among cats with and without FCoV. Furthermore our results showed very high specificity values for the N-based-antigen immunoassays, especially considering the high prevalence of FCoV-positive cats, thus confirming the widespread presence of FCoV in cat population (Addie et al., 2009) and the consideration that canine coronaviruses are known to be widespread in dog populations (Priestnall et al., 2007) . However, recent reports have observed a significantly higher number of SARS-CoV-2 seropositive cats in FCoV-infected groups (Adler et al., 2022). For the only sample showing N antigen-based ELISA positivity in the absence of neutralizing activity, we cannot definitively rule out that cross-reactivity may have caused the false-positive result, also considering that the cat was shedding FCoV RNA in the faeces, but recognition of non-neutralizing epitopes or different antibody kinetics could also explain this result, as previously reported (Decaro et al., 2022; Michelitsch et al., 2020; Udom et al., 2022; Zhang et al., 2020). Therefore, further studies are needed to definitively rule out cross-reactivity with antibodies against endemic carnivore coronaviruses when using N-based ELISA.

This study has some limitations, which should be considered. First, the low number of domestic animals with neutralizing antibodies, due to the sporadic frequency of infection among pets, may have impacted the accuracy of the diagnostic tests (Leeflang et al., 2013), especially for cats in this study.

Second, the results of our study are related to SARS-CoV-2 variants that circulated from 2020 to 2021 and diagnostic accuracy is unknown for SARS-CoV-2 variants that have circulated after 2021. Therefore, further studies, with a higher number of SARS-CoV-2-positive pet samples and with samples collected during 2022, are needed to confirm tests accuracy. Finally, the antibody kinetics in pets was not evaluated and further studies are needed to investigate the development of antibody responses against different SARS-CoV-2 antigens in cat and dog.

In summary, several studies have performed SARS-CoV-2 serological investigations on animal samples based on a screening test using commercial assays and confirmation of results with neutralization assays. However, assays with high sensitivity should be used for epidemiological surveillance and therefore the diagnostic performances of commercial test for SARS-CoV-2 should be taken into account for surveillance in pets as some methods can incorrectly identified the presence of SARS-CoV-2 specific antibody. Overall, our results confirm that assay validation is a fundamental step for serologic studies in cats and dogs and suggest that the sVNT used with a cut-off value of 30% may be an effective method that does not require a BSL-3 laboratory for predicting serum neutralization antibodies in dogs and cats.

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*Article*

# Fecal Carriage of Extended-Spectrum β-Lactamase-/AmpC-Producing *Escherichia coli* in Pet and Stray Cats

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**Foreword**: in this study, we investigate the fecal carriage of ESBL-/AmpC-producing *E. coli* in pet and stray cats, to estimate carriage, the antimicrobial resistance phenotypes and genotypes of the isolates, and identify risk factors associated with the carriage. My contribution to this work was related to conceptualization, sample collection, laboratory analysis, data analysis and manuscript writing. The results of this study were published under the reference:

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**Abstract**

Dogs have been reported as potential carriers of antimicrobial-resistant bacteria, but the role of cats has been poorly studied. The aim of this study was to investigate the presence and the risk factors associated with the fecal carriage of extended-spectrum β-lactamase and AmpC (ESBL/AmpC)-producing *Escherichia coli* (*E. coli*) in pet and stray cats. Fecal samples were collected between 2020 and 2022 from healthy and unhealthy cats and screened for ESBL/AmpC-producing *E. coli* using selective media. The presence of ESBL/AmpC-producing *E. coli* was confirmed by phenotypic and

molecular methods. The evaluation of minimum inhibitory concentrations (MICs) was performed on positive isolates. Host and hospitalization data were analyzed to identify risk factors. A total of 97 cats' samples were collected, and ESBL/AmpC-producing *E. coli* were detected in 6/97 (6.2%), supported by the detection of $bla_{CTX\text{-}M}$ (100%), $bla_{TEM}$ (83.3%), and $bla_{SHV}$ (16.7%) genes and the overexpression of chromosomal *ampC* (1%). All *E. coli* isolates were categorized as multidrug-resistant. Unhealthy status and previous antibiotic therapy were significantly associated with ESBL/AmpC-producing *E. coli* fecal carriage. Our results suggest that cats may be carriers of ESBL/AmpC-producing *E. coli*, highlighting the need for antimicrobial stewardship in veterinary medicine and an antimicrobial-resistance surveillance program focusing on companion animals, including stray cats.

**Aims**

Given the importance of cats as a potential ESBL/AmpC-producing *E. coli* reservoir because of their wide diffusion as pet animals and their close contact and sharing of environments with humans, the aims of this study were to estimate the presence of ESBL/AmpC-producing *E. coli* fecal carriage in cats, characterize the antimicrobial-resistance phenotypes and genotypes of the isolates, and identify risk factors associated with the fecal carriage of ESBL/AmpC-producing *E. coli*.

**Materials and Methods**

*Sample Collection*

Fecal samples were collected from both owned and stray cats admitted to the Veterinary Teaching Hospital (VTH) of Lodi, University of Milan, Italy from 2020 to 2022. Fecal samples from owned cats were collected from leftover material submitted to the VTH laboratory for diagnostic purposes or collected for research purposes in collaboration with local veterinarians. Stray cats were admitted to undergo neutering programs for the demographic control of stray population. Samples were stored at 4 °C until arrival at the laboratory (within 24 h from collection). The study was approved by the Institutional Animal Care and Use Committee and the Institutional Ethical Committee (approval no. OPBA_40_2020). Furthermore, residual fecal samples from cats collected for diagnostic purposes at the VTH with the informed consent of the owners were used for this study without any additional formal request for authorization, according to the decision of the Ethical Committee of the University of Milan (EC decision 29 October 2012, renewed with protocol no. 02-2016).

*Identification of ESBL/AmpC-Producing E. coli*

Samples were screened for the presence of ESBL/AmpC-producing *E. coli* according to the standard protocol of the DTU National Food Institute, the reference laboratory for antimicrobial resistance in Europe, with minor modifications (DTU, 2019). One gram of each fecal sample was pre-enriched in buffered peptone water (BPW) and incubated at 37 ± 1°C for 18–22 h. For ESBL/AmpC-producing *Enterobacteriaceae*, a loopful of BPW was inoculated on MacConkey agar supplemented with 1 mg/L cefotaxime and incubated overnight at 37 ± 1°C. Up to four bacterial colonies from positive growths of each sample were submitted to species identification regardless of colony morphology (lactose +/−). Species identification was accomplished in duplicate via matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (MBT Microflex ® LT/SH MALDI-TOF mass spectrometer, Bruker Daltonik, GmbH, Bremen, Germany) using the direct transfer method (Singhal et al., 2015). Following species identification, all four colonies/samples recovered from MacConkey agar supplemented with 1 mg/L cefotaxime were stored in brain heart infusion (BHI) broth with 15% glycerol at −80°C. One confirmed *E. coli* isolate for each sample was further thawed and used for ESBL and AmpC phenotypes confirmation using a combination disk test (CDT) and AmpC detection set D69C (MAST Group Ltd., Bootle, UK), respectively, according to EUCAST guidelines (EUCAST, 2017). Briefly, a pure fresh culture of the tested isolate was suspended in physiological saline to obtain a 0.5 McFarland standard density equivalent suspension. The suspension was spread uniformly across the surface of a Mueller Hinton agar plate. Disks used for ESBL phenotyping contained ceftazidime or cefotaxime (30 µg) with and without clavulanic acid (10 µg). Disks used for AmpC phenotyping were disks A, B, and C. Disks were placed on the inoculated medium and incubated at 35 ± 1 °C for 18 ± 2 h. Results were interpreted following the manufacture's instructions. For ESBL phenotyping, the isolate was considered positive in the presence of a ≥5 mm increase in the inhibition zone diameter in the disk containing ceftazidime or cefotaxime and clavulanic acid compared to the one without clavulanic acid. For AmpC phenotyping, the isolate was considered positive if the difference in the inhibition zone diameter between disks C and A and between disks C and B was ≥5 mm. Isolates were also subjected to PCR analysis and the evaluation of minimum inhibitory concentrations (MICs).

*PCR Analysis for Resistance Genes and E. coli Phylogroup*

Single thawed *E. coli* colonies recovered from MacConkey agar supplemented with 1 mg/L cefotaxime were resuspended in 100 µL of sterile distilled water, and DNA was extracted by the boiling method at 95°C for 10 min and subjected to PCR analysis to determine ESBL/AmpC genes and *E. coli* phylogroup. For the detection of ESBL-producing *E. coli*, a multiplex PCR targeting $bla_{\text{CTX-M}}$, $bla_{\text{TEM}}$, and $bla_{\text{SHV}}$ was

performed on the DNA of all isolates, as previously reported (Monstein et al., 2007). Isolates positive for *bla*$_{CTX-M}$ genes were further analyzed with a specific PCR for the *bla*$_{CTX-M-1}$ group, *bla*$_{CTX-M-2}$ group, *bla*$_{CTX-M-8}$ group, *bla*$_{CTX-M-9}$ group, and *bla*$_{CTX-M-25}$ group, as previously reported (Bertrand et al., 2006; Chmelnitsky et al., 2005; Jiang et al., 2005; Jouini et al., 2007; Saladin et al., 2002). Since the ESBL phenotype could be masked by the presence of AmpC β-lactamases and/or carbapenemases, cats were considered positive for the presence of ESBL-producing *E. coli* in the presence of at least one ESBL-encoding gene, regardless of phenotypic confirmation, as previously reported (EUCAST, 2017; Garrec et al., 2011). The DNA of *E. coli* isolates showing the AmpC phenotype were subjected to PCR for the detection of major plasmid-mediated ampC β-lactamase (*pamp*C)-encoding genes and chromosomal-mediated ampC β-lactamases (*camp*C). The presence of *pamp*C was determined using a multiplex PCR, as previously reported (Dallenne et al., 2010). The presence of *camp*C hyperproduction was investigated by the amplification of a 271 bp fragment of the promoter/attenuator region, as previously reported (Corvec et al., 2007). Amplicons were purified and Sanger sequenced by a commercial sequencing facility (Microsynth Seqlab, Göttingen, Germany). The sequences were aligned against the promoter/attenuator region of the *camp*C gene of *E. coli* strain ATCC 25922 using Clustal X in BioEdit software v.7.0. Strains were labeled *camp*C hyperproducers when promoter mutations were found, according to previous reports (Coolen et al., 2021; Peter-Getzlaff et al., 2011). *E. coli* isolates showing the AmpC phenotype supported by the overexpression of *camp*C or by the presence of *pamp*C genes were considered as AmpC-producing *E. coli*.

The *E. coli* phylogenetic group was determined following previously published protocols (Clermont et al., 2013). The different PCR assays were performed using positive control strains, and a blank control (DNAse-free water sample) was also included in all the PCR reactions. The primers used in this study are shown in Table S5.

*Antimicrobial Susceptibility Testing*

MICs were determined on single ESBL- and/or AmpC-producing *E. coli* isolates by the broth microdilution method using a commercially available plate (COMPGN1F Sensititre plates, Thermo Fisher Scientific®, Waltham, MA, USA). The list of antimicrobials used, the related cut-off values, and the MIC (µg/mL) range are reported in Table S6: *E. coli* ATCC 25922 was used as the control strain for susceptibility testing. Results were defined manually using a Sensititre Manual Viewbox (Sensititre™, Thermo Fisher Scientific®, Waltham, MA, USA). The MIC results were interpreted according to Clinical and Laboratory Standard Institute breakpoints CLSI VET01S ED6:2023 (CLSI, 2023) following the

manufacturer's instructions. Multidrug resistance (MDR) was defined as non-susceptibility to at least one antimicrobial agent in three or more antimicrobial categories, as previously reported (Magiorakos et al., 2012).

*Data Analysis*

For each animal, information regarding sex; age; type of ownership; and clinical history (clinical status, history of hospitalization, and previous antibiotic treatment performed within three months) were collected, as well as the results of biochemical analyses on blood and serum samples, if performed as part of the VTH diagnostic procedures. The stray cats belonged to 11 cat colonies from Lodi province, and the geographic location of the feline colony of origin was recorded. For the age variable, two categories were considered: <2 years old and ≥2 years old, as previously reported for the identification of age as a risk factor for infectious diseases (Anpuanandam et al., 2021). The clinical status variable was classified into two categories: healthy and unhealthy, according to the presence/absence of a clinical manifestation and the results of routine laboratory analyses, if available. The clinical status of unhealthy cats was further classified, according to the main clinical presentation on admission, into gastrointestinal, respiratory, urogenital, dermatological, neurologic, traumatic, or systemic. Previous antibiotic treatment was further classified according to the antibiotic class used to treat the cats.

Pearson's chi-square test and Fisher's exact probability test were used to evaluate the differences between the proportions of ESBL/AmpC-producing *E. coli*-positive cats and sex, age, type of ownership, clinical status, hospitalization, and previous antibiotic therapy. Statistical comparisons were carried out using Epitools (https://epitools.ausvet.com.au/ (accessed on: 16 February 2023)), taking $p < 0.05$ as significant.

**Results**

In total, 97 fecal samples from cats were included in this study. Characteristics of analyzed cats are summarized in Table 12.

Table 12. Characteristics of cats analyzed in this study.

| Population characteristics | | n. (%) | ESBL/AmpC positive n. (%) | *P* value[a] |
|---|---|---|---|---|
| Sex | Male | 54 (55.7) | 5 (9.3) | 0.22 |
| | Female | 43 (44.3) | 1 (2.3) | |
| Age[b] | <2 years | 55 (57.9) | 3 (5.5) | 1 |
| | ≥2 years | 40 (42.1) | 3 (7.5) | |
| Type of ownership | Stray | 50 (51.5) | 2 (4.0) | 0.43 |
| | Owned | 47 (48.5) | 4 (8.5) | |
| Clinical Status | Healthy | 70 (72.2) | 2 (2.9) | **0.049** |
| | Unhealthy | 27 (27.8) | 4 (14.8) | |
| Clinical syndrome at the admission | Gastrointestinal | 12 (44.4) | 2 (16.7) | nd |
| | Respiratory | 8 (29.6) | 1 (12.5) | |
| | Urogenital | 2 (7.4) | 0 (0) | |
| | Systemic | 2(7.4) | 1 (50) | |
| | Dermatological | 1 (3.7) | 0 (0) | |
| | Neurologic | 1 (3.7) | 0 (0) | |
| | Traumatic | 1 (3.7) | 0 (0) | |
| Hospitalization | Yes | 10 (10.3) | 2 (20.0) | 0.11 |
| | No | 87 (89.7) | 4 (4.6) | |
| Previous antibiotic therapy | Yes | 25 (25.8) | 4 (16) | **0.037** |
| | No | 72 (74.2) | 2 (2.8) | |
| Antibiotic class used in treated cats | Fluoroquinolones | 12 (48.0) | 0 (0) | nd |
| | β-Lactams and β-Lactamase Inhibitors | 10 (40.0) | 1 (10.0) | |
| | Cephalosporins | 7 (28.0) | 2 (28.6) | |
| | Macrolide-nitroimidazole | 2 (8.0) | 1 (50.0) | |
| | Tetracyclines | 2 (8.0) | 0 (0) | |

[a] Numbers in bold indicate $p<0.05$; [b] Age was unknown for two cats; [c] Cats treated with single and combination therapy are included; nd = not determined. Statistical analysis was not performed due to low numbers of cats included in the different groups.

Statistical analysis showed that the unhealthy status of animals (OR = 5.91; 95% CI: 1.01–34.44; p = 0.049) and previous antibiotic therapy (OR = 6.67; 95% CI: 1.14–38.99; p = 0.037) were significantly

associated with the fecal carriage of ESBL/AmpC-producing *E. coli*, whereas the other variables considered in the analysis were not risk factors (Table 12). The two positive stray cats detected in this study originated from two different feline colonies. The characteristics of the unhealthy cats compared to healthy cats analyzed in this study are given in Table S7.

Overall, the presence of ESBL/AmpC-producing *E. coli* were detected in 6/97 (6.2%; 95% confidence interval: 1.4-11%) cats and characteristics of positive cats are reported in Table 12. More precisely, ESBL-producing *E. coli* only were observed in five (83.3%) isolates whereas ESBL and AmpC-producing *E. coli* was observed in one (16.7%) strain only. Results of phenotypic and genetic characterization of *E. coli* isolates are summarized in table 13. Among $bla_{\text{CTX-M}}$ positive isolates, all *E. coli* isolates carried $bla_{\text{CTX-M-1}}$ group gene.

Table 13. Phenotypic and genetic characterization of ESBL/AmpC-producing *E. coli* isolates.

| Isolate ID | Animal characteristics | | | | | | Phenotype | Resistance genetic determinants | Phylogenetic group | Resistance pattern |
|---|---|---|---|---|---|---|---|---|---|---|
| | Sex | Age | Ownership | Clinical status | Hospitalization | Previous antibiotic therapy (antibiotic) | | | | |
| 46/1 | Female | 6 months | Stray | Healthy | No | No | ESBL/AmpC | *bla*$_{CTX-M-1}$ group, *campC* hyperproducer | B2 | AMP, FAZ, FOV, POD, LEX, AUG2, DOX, TET |
| 51/1 | Male | 3 years | Stray | Healthy | No | No | ESBL | *bla*$_{CTX-M-1}$ group, *bla*$_{TEM}$ | A | AMP, FAZ, FOV, POD, LEX, DOX, TET |
| 77/1 | Male | 13 years | Owned | Unhealthy | No | Yes (cephalosporin) | ESBL | *bla*$_{CTX-M-1}$ group, *bla*$_{TEM}$ | B2 | AMP, FAZ, FOV, POD, LEX, DOX, TET, SXT |
| 137/1 | Male | 1 year | Owned | Unhealthy | Yes | Yes (cephalosporin) | ESBL | *bla*$_{CTX-M-1}$ group, *bla*$_{TEM}$, *bla*$_{SHV}$ | B2 | AMP, FAZ, FOV, POD, LEX, DOX, TET, SXT |
| 161/1 | Male | 7 years | Owned | Unhealthy | Yes | Yes (amoxicillin +clavulanic acid) | ESBL | *bla*$_{CTX-M-1}$ group, *bla*$_{TEM}$ | B2 | AMP, FAZ, FOV, POD, LEX, AUG2, DOX, CHL, GEN, TET, SXT |
| 195/1 | Male | 10 months | Owned | Unhealthy | No | Yes (metronidazole and spiramycin) | Negative | *bla*$_{CTX-M-1}$ group, *bla*$_{TEM}$ | F | AMP, FAZ, FOV, POD, LEX, DOX, TAZ, AUG2, P/T4, IMI, GEN, ENRO, MAR, ORB, PRA |

AMP: ampicillin; AUG2: amoxicillin/clavulanic acid 2:1 (value refers to amoxicillin concentration); CHL: chloramphenicol; DOX: doxycycline; ENRO: enrofloxacin; FAZ: cefazolin; FOV: cefovecin; GEN: gentamicin; IMI: imipenem; LEX: cephalexin; MAR: marbofloxacin; ORB: orbifloxacin; PRA: pradofloxacin; POD: cefpodoxime; P/T4: piperacillin/tazobactam contant 4; SXT: trimethoprim/sulfamethoxazole 1:19 (value refers to trimethoprim concentration); TAZ: ceftazidime; TET: tetracycline.

The analysis of the promoter/attenuator region of the AmpC-producing *E. coli* detected in this study showed the presence of -32 T>A mutation in the -35 promoter box and -28 G>A mutation in the spacer region (Table S8), previously associated with the upregulation of AmpC production (Coolen et al., 2021).

Based on MIC results (Table 14), the highest level of resistance was observed for β-lactam with all isolates resistant to ampicillin, cefazolin, cephalexin, cefovecin and cefpodoxime, and for doxycycline. Resistance to fluoroquinolones (16.7%), phenicol (16.7%), β-lactam in combination with β-lactamase inhibitor agent (16.7% and 50% for amoxicillin/clavulanic acid 2:1 and piperacillin/tazobactam content 4, respectively), tetracyclines (83.3%) and carbapenems (16.7%) was less frequent, with at least one isolate resistant to all the agents in each category. All isolates were susceptible to amikacin, accounting for the lowest resistance rate. All ESBL/AmpC-producing *E. coli* isolates detected in this study were MDR.

Table 14. Distribution of MICs among ESBL/AmpC-producing *E. coli* isolates.

| **Antimicrobial class** | **Antimicrobial agent** | **No. resistant** | **No. of isolates at the indicated MIC µg/ml** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | **0.12** | **0.25** | **0.5** | **1** | **2** | **4** | **8** | **16** | **32** | **64** | **128** |
| β-Lactam (penicillins) | AMP | 6/6 | | | | | | | | 6 | | | |
| β-Lactam (Cephalosporin I) | FAZ | 6/6 | | | | | | | | | | 6 | |
| | LEX | 6/6 | | | | | | | | | 6 | | |
| β-Lactam (Cephalosporin III) | FOV | 6/6 | | | | | | | | 6 | | | |
| | POD | 6/6 | | | | | | | | 6 | | | |
| | TAZ | 1/6 | | | | | | 2 | 3 | | 1 | | |
| β-Lactam combination agent | AUG2 | 3/6 | | | | | | 1 | 2 | 3 | | | |
| | P/T4 | 1/6 | | | | | | | 4 | 1 | | | 1 |
| Aminoglycosides | AMI | 0/6 | | | | | | 5 | 1 | | | | |
| | GEN | 2/6 | | 2 | 1 | | 1 | | | 2 | | | |
| Phenicol | CHL | 1/6 | | | | | 1 | 3 | 1 | | | 1 | |
| Fluoroquinolones | ENRO | 1/6 | 3 | 2 | | | | | 1 | | | | |
| | MAR | 1/6 | 3 | 2 | | | | | 1 | | | | |
| | ORB | 1/6 | | | | 3 | 2 | | | 1 | | | |
| | PRA | 1/6 | | 5 | | | | 1 | | | | | |
| Tetracyclines | TET | 5/6 | | | | | | 1 | | | 5 | | |
| | DOX | 6/6 | | | | 2 | 1 | | 1 | 2 | | | |
| Carbapenems | IMI | 1/6 | | | | 5 | | | | 1 | | | |
| Folate pathway antagonists | SXT | 3/6 | | | 3 | | | | 3 | | | | |

White fields denote range of dilutions tested for each antimicrobial agent. Vertical lines indicate CLSI VET01S ED6:2023 cutoff values (Intermediate results were considered as susceptible). AMP: ampicillin; FAZ: cefazolin; LEX: cephalexin; FOV: cefovecin; POD: cefpodoxime; TAZ: ceftazidime; AUG2: amoxicillin/clavulanic acid 2:1 (value refers to amoxicillin concentration); P/T4: piperacillin/tazobactam

contant 4; AMI: amikacin; GEN: gentamicin; CHL: chloramphenicol; ENRO: enrofloxacin; MAR: marbofloxacin; ORB: orbifloxacin; PRA: pradofloxacin; TET: tetracycline; DOX: doxycycline; IMI: imipenem; SXT: trimethoprim/sulfamethoxazole 1:19 (value refers to trimethoprim concentration).

**Discussion**

Cats are the most common companion animals worldwide, but only a few studies have investigated their role as potential carriers of AMR bacteria (FEDIAF, 2022; Guardabassi, 2004; Salgado-Caxito et al., 2021). In the present study, fecal samples were analyzed from stray and pet cats to estimate the presence of ESBL/AmpC-producing *E. coli* fecal carriage in cats from Italy and the associated risk factors. The overall presence of ESBL/AmpC-producing *E. coli* in 6.2% of cats detected in this study was consistent with previous reports that have investigated the fecal carriage of ESBL-producing *E. coli* in healthy and sick cats from Portugal (Carvalho et al., 2021) and in sheltered cats (Weese et al., 2022). The recent meta-analysis performed by Salgado-Caxito and colleagues (Salgado-Caxito et al., 2021) showing that the global estimated prevalence of ESBL-producing *E. coli* in cats is 5.04% (95% CI: 2.42–10.22%) also confirms our results. However, other studies have detected a prevalence of up to 20% for ESBL-producing *E. coli* among cat fecal samples (Hordijk et al., 2013; Sfaciotte et al., 2021), probably due to the different backgrounds of the tested cats or the different diagnostic methods used. Indeed, a comparison between studies may be challenging due to the lack of standardized diagnostic methods for AMR surveillance in pets (Formenti et al., 2021; Salgado-Caxito et al., 2021). Therefore, according to standardized protocols for food-producing animals (EU Commission-Decision 2020/1729/EU), the implementation of a specific standardized diagnostics methodology for AMR surveillance programs in companion animals may be suggested.

The presence of the ESBL genotype supported by the detection of $bla_{\text{CTX-M}}$ as the main resistance gene in all isolates in this study, followed by $bla_{\text{TEM}}$ and $bla_{\text{SHV}}$, confirmed previous studies reporting these as the most common genes in ESBL-producing bacteria from both humans and animals (Liebana et al., 2013), though it should be noted that we did not determine the TEM and SHV groups of the isolates, which do not always encode for ESBL (Bush & Bradford, 2020). Our results showing that the majority of *E. coli* isolates carried more than one resistance gene confirmed the genetic diversity of ESBL-producing *E. coli* in companion animals (Ewers et al., 2012; Salgado-Caxito et al., 2021). Moreover, the high detection rate of $bla_{\text{CTX-M-1}}$ group genes was expected, as this group is the most common worldwide (Peirano & Pitout, 2019).

The presence of mutations of the AmpC-producing *E. coli* isolates detected in this study associated with the upregulation of AmpC production (Coolen et al., 2021) was consistent with previous reports highlighting the presence of *camp*C resistance in companion animals (Bogaerts et al., 2015; Rocha-Gracia et al., 2015). However, it must be noted that AmpC hyperproduction is less frequently investigated compared to plasmid-mediated AmpC resistance, since the latter mechanism is capable of being horizontally transferred to other bacteria, posing a greater threat to AMR control (Coolen et al., 2019, Coolen et al., 2021). Indeed, the absence of pampC among the AmpC-producing *E. coli* detected in this study could have been due to the limited sample size and the lower presence of pAmpC-producing *E. coli* compared to ESBL-producing *E. coli* in companion animals (Carattoli et al., 2005; Carvalho et al., 2021; Formenti et al., 2021; Melo et al., 2018; Piccolo et al., 2020). Therefore, further studies with a wider sample size are necessary to assess the role of cats in the dissemination of pAmpC-producing *E. coli*. Moreover, our results confirmed that commercially available kits for AmpC detection failed to differentiate between pAmpC and cAmpC production in *E. coli*, as previously reported (Coolen et al., 2019).

Interestingly, one isolate detected in this study that carried $bla_{\text{CTX-M}}$ and $bla_{\text{TEM}}$ genes was phenotypically negative for ESBL production. The ESBL phenotype could have been masked by the presence of AmpC β-lactamases and/or carbapenemases (EUCAST, 2017). However, the presence of AmpC β-lactamases was unlikely, as the isolate was negative for the AmpC phenotype. Interestingly, this isolate was the only one resistant to imipenem, with an MIC value ≥16 µg/mL. A high imipenem MIC value has been used as a screening method for carbapenemase production in *Enterobacteriaceae* (Miller & Humphries, 2016; Vlek et al., 2016), as isolates with an MIC value of 2 µg/mL or above are likely to be carbapenemase producers (Ong et al., 2020; Tamma et al., 2016). Unfortunately, the aim of our study did not include the detection of carbapenemase-producing bacteria. In this respect, further studies are needed to clarify the epidemiological role of cats in the carriage of carbapenemase-producing *E. coli*, as the presence of carbapenemase-producing *Enterobacteriaceae* in companion animals has been rarely investigated, despite recent studies having shown infection or colonization by carbapenemase-producing Enterobacterales in companion animals (Cole et al., 2020; Hong et al., 2020; Silva et al., 2022).

Regarding *E. coli* phylogroups, the detection of B2 as the most frequent phylogenetic group was consistent with previous reports showing a high presence of phylogroup B2 among the AMR *E. coli* in companion animals and humans but not in food-producing animals, suggesting the need for further investigations to understand the zoonotic potential of these isolates (Bortolami et al., 2019; Clermont

et al., 2013; Giufrè et al., 2021; Jakobsen et al., 2011; Piccolo et al., 2020; Zhou et al., 2022). Moreover, given that *E. coli* belonging to phylogroups B2 and F has been commonly associated with the presence of virulence factors responsible for extra- intestinal infections (Bortolami et al., 2019; Clermont et al., 2013), further investigations are suggested to assess the virulotyping of ESBL/AmpC-producing *E. coli* in cats.

The high presence of MDR E. coli was expected, as ESBL-producing *E. coli* usually not only exhibit resistance to β-lactam, but frequently carry other resistance genes conferring resistance to other antimicrobial drugs, leading to MDR (Walther et al., 2017). Moreover, the high detection rate of MDR *E. coli* highlighted the need for antimicrobial stewardship in veterinary practice to also reduce the emergence and spread of MDR bacteria in cats.

The unhealthy status of cats as a risk factor associated with the fecal carriage of ESBL/AmpC-producing *E. coli* observed in our study was likely influenced by the fact that a higher percentage of sick animals were treated with antibiotics compared to healthy cats. This suggests that antibiotic treatment alone may have increased the risk of the fecal carriage of AMR organisms in this study, as it is a well-recognized risk factor for the acquisition of drug-resistant bacteria in both humans and animals (Ortiz-Díez et al., 2023; Pomba et al., 2016; Shnaiderman-Torban et al., 2020). It was not possible to define if specific antibiotic classes were associated with ESBL/AmpC-producing *E. coli* due to the low number of cats treated with antibiotics in this study. The absence of an association between hospitalization and the fecal carriage of ESBL/AmpC-producing *E. coli* could have been due to the low number of hospitalized cats included in the present study or to the differences in management and hygiene protocols among hospitals (Dazio et al., 2021), since hospitalization has been associated with the carriage of drug-resistant bacteria in companion animals (Dazio et al., 2021; Ortiz-Díez et al., 2023; Shnaiderman-Torban et al., 2020). In this respect, further studies focusing on hospitalized cats are needed to evaluate the contribution of veterinary hospitals in the spread of drug-resistant bacteria.

The presence of ESBL/AmpC-producing *E. coli* among both stray and owned cats is of interest and confirms a recent report detecting AMR in stray cats (Gargano et al., 2022). Given the unknown backgrounds of the stray cats in this study, we could not conclude if the AMR acquisition was associated with environmental sources, antimicrobial administration, the presence of subclinical disease (cats were healthy at physical examination), or other factors. Moreover, the apparent non-statistically significant higher ESBL/AmpC-producing *E. coli* carriage in owned cats compared to stray cats may have been influenced by the high percentage of unhealthy animals among the owned cats, likely requiring antimicrobial therapy. Indeed, although this group of cats was raised in an enclosed environment, with

a clean water and food supply and a good level of care from their owners, more than half of the owned cats were unhealthy, whereas all stray cats were apparently healthy and were brought to the VTH only for neutering. Further investigations are needed to understand the potential route of transmission of AMR in stray cat populations as well as to clarify the role of stray cats, as previously reported for stray dogs in South America (Marchetti et al., 2021; Melo et al., 2018).

This study had some limitations, starting from the low number of analyzed cats, which was not sufficient for population estimation. Further studies with a greater sample size and an ad hoc sampling strategy are needed to assess the epidemiological role of stray and owned cats in the spread of ESBL/AmpC-producing *E. coli*. The whole-genome sequencing of the *E. coli* isolates detected in this study is suggested in order to define virulence factors and the presence of other resistance genes that were not investigated in this study. Moreover, it would be interesting to analyze more than one AMR isolate from each sample to investigate if the genetic variability of *E. coli* isolates can occur within the same fecal sample.

## Supplementary materials

**Table S5.** Primers sequences used in this study**.**

| Target | Primer | Sequence 5'-3' | Reference |
|---|---|---|---|
| CTX-M | *bla*$_{CTX-M}$ F | ATGTGCAGYACCAGTAARGTKATGGC | (Monstein et al., 2007) |
| | *bla*$_{CTX-M}$ R | TGGGTRAARTARGTSACCAGAAYCAGCGG | |
| TEM | *bla*$_{TEM}$ F | TCGCCGCATACACTATTCTCAGAATGA | (Monstein et al., 2007) |
| | *bla*$_{TEM}$ R | ACGCTCACCGGCTCCAGATTTAT | |
| SHV | *bla*$_{SHV}$ F | ATGCGTTATATTCGCCTGTG | (Monstein et al., 2007) |
| | *bla*$_{SHV}$ R | TGCTTTGTTATTCGGGCCAA | |
| CTX-M-1 group | *bla*$_{CTX-M-1}$ F | GGTTAAAAAATCACTGCGYC | (Saladin et al., 2002) |
| | *bla*$_{CTX-M-1}$ R | TYGGTGACGATTTTAGCCGC | |
| CTX-M-2 group | *bla*$_{CTX-M-2}$ F | ATGATGACTCAGAGCATTCG | (Bertrand et al., 2006) |
| | *bla*$_{CTX-M-2}$ R | TCAGAAACCGTGGGTTAC | |
| CTX-M-8 group | *bla*$_{CTX-M-8}$ F | TGATGAGACATCGCGTTAAG | (Jouini et al., 2007) |
| | *bla*$_{CTX-M-8}$ R | TAACCGTCGGTGACGATTTT | |
| CTX-M-9 group | *bla*$_{CTX-M-9}$ F | ATGGTGACAAAGAGAGTGCA | (Jiang et al., 2005) |
| | *bla*$_{CTX-M-9}$ R | CCCTTCGGCGATGATTCTC | |
| CTX-M-25 group | *bla*$_{CTX-M-25}$ F | CACACGAATTGAATGTTCAG | (Chmelnitsky et al., 2005) |
| | *bla*$_{CTX-M-25}$ R | TCACTCCACATGGTGAGT | |
| ACC-1 and ACC-2 | MultiCaseACC_F | CACCTCCAGCGACTTGTTAC | (Dallenne et al., 2010) |
| | MultiCaseACC_R | GTTAGCCAGCATCACGATCC | |
| FOX-1 to FOX-5 | MultiCaseFOX_F | CTACAGTGCGGGTGGTTT | (Dallenne et al., 2010) |
| | MultiCaseFOX_R | CTATTTGCGGCCAGGTGA | |
| MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11 and CMY-19 | MultiCaseMOX_F | GCAACAACGACAATCCATCCT | (Dallenne et al., 2010) |
| | MultiCaseMOX_R | GGGATAGGCGTAACTCTCCCAA | |
| DHA-1 and DHA-2 | MultiCaseDHA_F | TGATGGCACAGCAGGATATTC | (Dallenne et al., 2010) |
| | MultiCaseDHA_R | GCTTTGACTCTTTCGGTATTCG | |
| LAT-1 to LAT-3, BIL-1, CMY-2 to CMY-7, CMY-12 to CMY-18 and CMY-21 to CMY-23 | MultiCaseCIT_F | CGAAGAGGCAATGACCAGAC | (Dallenne et al., 2010) |
| | MultiCaseCIT_R | ACGGACAGGGTTAGGATAGY | |
| ACT-1 and MIR-1 | MultiCaseEBC_F | CGGTAAAGCCGATGTTGCG | (Dallenne et al., 2010) |
| | MultiCaseEBC_R | AGCCTAACCCCTGATACA | |
| *campC* promoter/attenuator region | AmpC AB1 | GATCGTTCTGCCGCTGTG | (Corvec et al., 2007) |
| | AmpC-2 | GGGCAGCAAATGTGGAGCAA | |
| *chuA* | chuA.1b | ATGGTACCGGACGAACCAAC | (Clermont et al., 2013) |
| | chuA.2 | TGCCGCCAGTACCAAAGACA | |
| *yjaA* | yjaA.1b | CAAACGTGAAGTGTCAGGAG | (Clermont et al., 2013) |
| | yjaA.2b | AATGCGTTCCTCAACCTGTG | |
| TspE4.C2 | TspE4C2.1b | CACTATTCGTAAGGTCATCC | (Clermont et al., 2013) |
| | TspE4C2.2b | AGTTTATCGCTGCGGGTCGC | |
| *arpA* | AceK.f | AACGCTATTCGCCAGCTTGC | (Clermont et al., 2013) |
| | ArpA1.r | TCTCCCCATACCGTACGCTA | |

**Table S6**. List of antimicrobials used, related cut-off values and the range of MIC (mg/L) used in this study.

| Antimicrobial class | Antimicrobial agent | Cut-off (µg/mL)* | range of MIC (µg/mL) |
|---|---|---|---|
| β-Lactam (penicillins) | Ampicillin (AMP) | >8 | 0.25-8 |
| β-Lactam (Cephalosporin I) | Cefazolin (FAZ) | ≥32 | 1-32 |
| | Cephalexin (LEX) | ≥32 | 0.5-16 |
| β-Lactam (Cephalosporin III) | Cefovecin (FOV) | ≥8 | 0.25-8 |
| | Cefpodoxime (POD) | ≥8 | 1-8 |
| | Ceftazidime (TAZ) | ≥16 | 4-16 |
| β-Lactams and β-Lactamase Inhibitors | Amoxicillin/clavulanic acid 2:1 (AUG2) | >8/4 | 0.25/0.12-8/4 |
| | Piperacillin/tazobactam contant 4 (P/T4) | ≥32/4 | 8/4-64/4 |
| Aminoglycosides | Amikacin (AMI) | ≥16 | 4-32 |
| | Gentamicin (GEN) | ≥8 | 0.25-8 |
| Phenicol | Chloramphenicol (CHL) | ≥32 | 2-32 |
| Fluoroquinolones | Enrofloxacin (ENRO) | ≥4 | 0.12-4 |
| | Marbofloxacin (MAR) | ≥4 | 0.12-4 |
| | Orbifloxacin (ORB) | ≥8 | 1-8 |
| | Pradofloxacin (PRA) | ≥2 | 0.25-2 |
| Tetracyclines | Tetracycline (TET) | ≥16 | 4-16 |
| | Doxycycline (DOX) | ≥0.5 | 0.25-8 |
| Carbapenems | Imipenem (IMI) | ≥4 | 1-8 |
| Folate pathway antagonists | Trimethoprim/sulfamethoxazole 1:19 (SXT) | ≥4/72 | 0.5/9.5-4/72 |

* CLSI VET01S ED6:2023 cutoff values (CLSI, 2023) .

**Table S7**. Characteristics of unhealthy cats compared to healthy cats analyzed in this study.

| Characteristics of cats | | Total cats (no. 97) | Unhealthy cats (no. 27) | Healthy cats (no. 70) |
|---|---|---|---|---|
| Sex | Male n. (%) | 54 (55.7) | 17 (63) | 37 (52.9) |
| | Female n. (%) | 43 (44.3) | 10 (37) | 33 (47.1) |
| Age | <2 years n. (%) | 55 (56.7) | 12 (44.4) | 43 (61.4) |
| | ≥2 years n. (%) | 40 (41.2) | 14 (51.9) | 26 (37.1) |
| | ND (%) | 2 (3.1) | 1 (3.7) | 1 (1.4) |
| Type of ownership | Stray n. (%) | 50 (51.5) | 0 (0) | 50 (71.4) |
| | Owned n. (%) | 47 (48.5) | 27 (100) | 20 (28.6) |
| Hospitalization | Yes n. (%) | 10 (10.3) | 10 (37) | 0 (0) |
| | No n. (%) | 87 (89.7) | 17 (63) | 70 (100) |
| Previous antibiotic therapy | Yes n. (%) | 25 (25.8) | 18 (66.7) | 7 (10) |
| | No n. (%) | 72 (74.2) | 9 (33.3) | 63 (90) |

**Table S8.** Results of the chromosomal ampC promoter/ attenuator regions analysis of the AmpC-producing *E. coli* detected in this study.

| Position within the *ampC* promoter/attenuator regions[1] | | ATCC 25922 (accession number: CP009072) | 46/1 (AmpC-producing *E. coli* detected in this study) |
|---|---|---|---|
| Displaced -35 box | -42 | C | |
| -35 box | -32 | T | A |
| | -28 | G | A |
| | -26.1 | - | |
| | -23 | T | |
| | -20.1 | - | |
| Displaced -10 box | -18 | G | |
| Spacer | -15.2 | - | |
| | -15.1 | - | |
| | -15 | G | |
| -10 box | -11 | C | |
| Attenuator | +17 | C | |
| | +21.1 | - | |
| | +22 | C | |
| | +23 | G | |
| | +24 | C | |
| | +26 | T | |
| | +27 | A | |
| | +28 | T | |
| | +30 | G | |
| | +32 | G | |
| | +34 | G | |
| | +35 | C | |
| | +36 | C | |
| | +37 | G | |

[1]Descriptions of functional elements are used as reported by Peter-Getzlaff et al., 2011 and Coolen et al., 2021 (Coolen et al., 2021; Peter-Getzlaff et al., 2011).

4th INTERNATIONAL CONFERENCE OF THE EUROPEAN COLLEGE OF VETERINARY MICROBIOLOGY

*Oral presentation*

# Prevalence and Risk Factors Associated with Faecal Carriage of Extended-Spectrum Beta-Lactamase-, AmpC- and Carbapenemase-Producing *Escherichia coli* in Stray Cats

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**Foreword:** in this study, we investigated the fecal carriage of ESBL-/AmpC- and carbapenemase-producing *E. coli* focusing on stray cats, in order to estimate the carriage, the antimicrobial resistance phenotypes and genotypes of the isolates, and identify risk factors associated with the carriage of ESBL-/AmpC- and carbapenemase-producing *E. coli*. My contribution to this work was related to conceptualization, sample collection, laboratory analysis, data interpretation and writing the first manuscript draft. I presented the preliminary results of this study during the 4th International Conference of the European College of Veterinary Microbiology that was accepted as an oral presentation. Moreover, the first manuscript draft is currently under internal revision by the authors and will be further submitted for publication.

**Abstract**

Antimicrobial resistance in pets is gaining attention worldwide. The aim of this study was to determine the presence and risk factors associated with faecal carriage of ESBL-, AmpC-, and carbapenemase-producing *E. coli* in stray cats from Northern Italy. Rectal swabs were collected between 2020 and 2021 from stray cats admitted to the Veterinary Teaching Hospital of Lodi. Samples were

microbiologically and molecularly analyzed to investigate the presence of ESBL-, AmpC-, and carbapenemase-producing *E. coli*. Anamnestic data were analyzed to identify risk factors. In total 20/121 (16.5%) animals were positive for the presence of ESBL-/AmpC-/carbapenemase-producing *E. coli*. Eleven (9.1%) ESBL-, 3 (2.4%) ESBL- /AmpC- and 2 (1.7%) AmpC- producing *E. coli*. ESBL were detectd by phenotypic methods, confirmed by genetic ESBL determinants $bla_{\text{CTX-M}}$ (14, 100%), $bla_{\text{TEM}}$ (12, 86.7%) and $bla_{\text{SHV}}$ (2, 14.3%) whereas $bla_{\text{CMY-2}}$ gene was the most frequently detected gene in AmpC-producing *E. coli* (4/5, 80%). Moreover, 4 (3.3%) isolates showed carbapenemase-producing phenotype supported by the detection of $bla_{\text{NDM}}$ gene in all these isolates. *E. coli* phylogroups F (6/20, 30%) and B2 (5/20, 25%) were the most frequently detected. Nineteen (95%) isolates showed multidrug resistance. Risk factors associated with ESBL-/AmpC-/ carbapenemase-producing *E.coli* faecal carriage were hospitalization ($P$ <0.0001), previous antibiotic treatment ($P$ <0.0001) and presence of clinical disease ($P$ <0.0001). Our findings highlight the need of surveillance programs and antimicrobial stewardship to reduce the emergence and spread of resistant bacteria. Further studies are needed to define the risk of transmission to humans and other animals.

**Aims**

The aims of this study were i) to estimate the presence of ESBL-/AmpC- and carbapenemase-producing *E. coli* fecal carriage in stray cats, ii) to phenotypically and genotypically characterize the antimicrobial-resistance the detected *E. coli* isolates, and iii) to identify risk factors associated with the fecal carriage.

**Material and methods**

*Sample collection*

Fecal samples were collected from stray cats admitted to the Veterinary Teaching Hospital (VTH) of Lodi, University of Milan, Italy from 2020 to 2022. Stray cats were were admitted to the VTH to undergo neutering programs for demographic control of stray cat population or for for receiving medical care for poor health conditions, prior being released to their respective feline colony or being adopted. For each animal, information regarding sex, age and clinical status were collected. For the age variable, two categories were considered: <2 years old and ≥2 years old, as previously reported for the identification of age as a risk factor for infectious diseases (Anpuanandam et al., 2021). The clinical status variable was classified into two categories: healthy and unhealthy, based on the clinical history and examination. Moreover, for hospitalized stray cats additional information regarding the

clinical history (antibiotic treatment performed during hospitalization, clinical status and hospitalization) were collected. Fecal samples from hospitalized stray cats were collected from residual materials submitted to the VTH laboratory for diagnostic purposes. Samples were stored at 4 °C until arrival at the laboratory (within 24 hours from the collection). The study was approved by the Institutional Animal Care and Use Committee and by the Institutional Ethical Committee (approval n. OPBA_40_2020).

*Sample screening for the presence of ESBL-/AmpC-producing E. coli*

Briefly, on gram of each fecal sample was pre-enriched in buffered peptone water (BPW) and incubated at 37 ± 1°C for 18–22 h. After the pre-enrichment, a loopful of BPW was inoculated on MacConkey agar supplemented with 1 mg/L cefotaxime and incubated overnight at 37 ± 1°C. Bacterial species identification was performed with MALDI-TOF MS) (MBT Microflex ® LT/SH MALDI-TOF mass spectrometer, Bruker Daltonik, GmbH, Bremen, Germany) using the direct transfer method (Singhal et al., 2015).

*Antimicrobial susceptibility testing*

MICs were determined on single *E. coli* isolates grown on MacConkey agar supplemented with 1 mg/L cefotaxime using broth microdilution method with a commercially available plate (COMPGN1F Sensititre plates, Thermo Fisher Scientific®, Waltham, MA, USA). Results were defined manually using a Sensititre Manual Viewbox (Sensititre™, Thermo Fisher Scientific®, Waltham, MA, USA). MIC results were interpreted according to Clinical and Laboratory Standard Institute breakpoints CLSI VET01S ED6:2023 (CLSI, 2023). Multidrug resistance (MDR) was defined as non-susceptibility to at least one antimicrobial agent in three or more antimicrobial categories, as previously reported (Magiorakos et al., 2012).

*Identification of ESBL-/AmpC-/carbapenemase-producing E. coli*

One confirmed *E. coli* isolate grown on MacConkey agar supplemented with 1 mg/L cefotaxime for each sample was subjected to ESBL and AmpC phenotypes confirmation using a combination disk test (CDT) and AmpC detection set D69C (MAST Group Ltd., Bootle, UK), respectively, according to EUCAST guidelines (EUCAST, 2017). Briefly, a pure fresh culture of the tested isolate was suspended in physiological saline to obtain a 0.5 McFarland standard density equivalent suspension. The suspension was spread uniformly across the surface of a Mueller Hinton agar plate. Disks used for

ESBL phenotyping contained ceftazidime or cefotaxime (30 µg) with and without clavulanic acid (10 µg). Disks used for AmpC phenotyping were disks A, B, and C. Disks were placed on the inoculated medium and incubated at 35 ± 1 °C for 18 ± 2 h. Results were interpreted following the manufacture's instructions. For ESBL phenotyping, the isolate was considered positive in the presence of a ≥5 mm increase in the inhibition zone diameter in the disk containing ceftazidime or cefotaxime and clavulanic acid compared to the one without clavulanic acid. For AmpC phenotyping, the isolate was considered positive if the difference in the inhibition zone diameter between disks C and A and between disks C and B was ≥5 mm.

For carbapenemase-production, a commercially available test (Carba plus D73C, MAST group Ltd, UK) based on carbapenemase inhibition disk test (EUCAST, 2017) was performed on imipenem-resistant isolates following MIC analysis. Briefly, a pure fresh culture was suspended in physiological saline to obtain a 0.5 McFarland standard density equivalent suspension. The suspension was spread uniformly across the surface of a single Mueller Hinton Agar plate. Disks were placed on the inoculated medium and incubated at 35 ± 1 °C for 18 ± 2 hours. Results were interpreted following the manufacture's instruction. More precisely, if disc B only showed a zone difference ≥5 mm than disc A (C - A and D – A should be <5 mm), the isolate was considered positive for MβL activity. If disc C only showed a zone difference ≥5mm than disc A (B – A and D – A should be <5 mm), the isolate was considered positive for KPC activity. If no synergy was obtained between discs A, B, C and D and disc E showed a zone of inhibition of <10 mm, the isolate was considered positive for OXA-48 activity.

*PCR Analysis for Resistance Genes and E. coli Phylogroup*

DNA of *E. coli* isolates grown on MacConkey agar supplemented with 1 mg/L cefotaxime was extracted by the boiling method at 95°C for 10 min and subjected to PCR analysis. For the detection of ESBL-producing *E. coli*, all *E. coli* isolates were subjected to a multiplex PCR targeting $bla_{\text{CTX-M}}$, $bla_{\text{TEM}}$, and $bla_{\text{SHV}}$ as previously reported (Monstein et al., 2007). Isolates positive for $bla_{\text{CTX-M}}$ genes were further analyzed with a specific PCR for the $bla_{\text{CTX-M-1}}$ group, $bla_{\text{CTX-M-2}}$ group, $bla_{\text{CTX-M-8}}$ group, $bla_{\text{CTX-M-9}}$ group, and $bla_{\text{CTX-M-25}}$ group, as previously reported (Bertrand et al., 2006; Chmelnitsky et al., 2005; Jiang et al., 2005; Jouini et al., 2007; Saladin et al., 2002). The DNA of *E. coli* isolates showing the AmpC phenotype were subjected to multiplex PCR for the detection of major plasmid-mediated ampC β-lactamase (*pamp*C)-encoding genes ($bla_{\text{ACC}}$, $bla_{\text{FOX}}$, $bla_{\text{MOX}}$, $bla_{\text{CMY}}$, $bla_{\text{LAT}}$, $bla_{\text{BIL}}$, $bla_{\text{ACT}}$, $bla_{\text{MIR}}$) as previously reported (Dallenne et al., 2010). Isolates positive for $bla_{\text{CMY}}$ genes were further analyzed with a specific PCR for the $bla_{\text{CMY-2}}$ gene as previously reported(Rehman et al., 2019). For the detection

of the major carbapenemase-encoding genes (*bla*$_{NDM}$, *bla*$_{KPC}$, *bla*$_{VIM}$ and *bla*$_{OXA-48}$), DNA of *E. coli* isolates showing carbapenemase phenotype were subjected to a specific PCR protocol based on the detected phenotype as previously reported (ECDC, 2019).

*Data analysis*

Pearson's chi-square test and Fisher's exact probability test were used to evaluate the differences between the proportions of ESBL-/AmpC-/carbapenemase-producing *E. coli*-positive cats and sex, age, clinical status, hospitalization, and antibiotic therapy administered during hospitalization. Statistical comparisons were carried out using Epitools (https://epitools.ausvet.com.au/ (accessed on: 14 May 2022)), taking $p < 0.05$ as significant.

**Results**

In total, 121 fecal samples from stray cats were included in this study. Characteristics of analyzed cats are summarized in Table 15.

Table 15. Results and characteristics of cats analyzed in this study.

| Population characteristics | | n. (%) | ESBL/AmpC/carbapenemase positive n. (%) | *P* value[a] |
|---|---|---|---|---|
| Sex | Male | 61 (50.4) | 11 (9.1) | 0.84 |
| | Female | 60 (49.6) | 9 (7.4) | |
| Age[b] | <2 years | 55 (46.6) | 6 (5.0) | 0.17 |
| | ≥2 years | 63 (53.4) | 14 (11.9) | |
| Clinical Status | Healthy | 79 (65.3) | 4 (3.3) | **<0.001** |
| | Unhealthy | 42 (34.7) | 16 (13.2) | |
| Hospitalization | Yes | 41 (33.9) | 16 (13.2) | **<0.001** |
| | No | 80 (66.1) | 4 (3.3) | |
| Antibiotic therapy during hospitalization | Yes | 36 (29.8) | 14 (11.6) | **<0.001** |
| | No | 85 (70.2) | 6 (5.0) | |

[a] Numbers in bold indicate $p<0.05$; [b] Age was unknown for three cats.

Overall, the presence of ESBL-/AmpC-/carbapenemase-producing *E. coli* were detected in 20/121 (16.5%; 95% confidence interval: 10.9-24.1%) cats. More precisely, ESBL-producing *E. coli* only were observed in 11 (9.1%) cats, ESBL and AmpC-producing *E. coli* was observed in 3 (2.4%) cats, AmpC-producing *E. coli* only were observed in 2 (1.7%) cats and carbapenemase-production, particularly metallo-beta-lactamase (MβL) was detected in 4 (3.3%) cats. *E. coli* phylogroups F (6/20, 30%) and B2 (5/20, 25%) were the most frequently detected, followed by B1 (4/20, 20%), A/C (2/20, 10%), A (1/20, 5%), while for one isolate the phylogroup was undetermined. ESBL genetic determinants were *bla*$_{CTX-}$

$_{M}$ (14, 100%), *bla*$_{TEM}$ (12, 86.7%) and *bla*$_{SHV}$ (2, 14.3%) whereas *bla*$_{CMY-2}$ gene was the most frequently detected gene in AmpC-producing *E. coli* (4, 80%). All carbapenemase-producing *E. coli* were positive for the presence of *bla*$_{NDM}$ gene. Results of phenotypic and genetic characterization of *E. coli* isolates are summarized in table 16.

Table 16. Combination of AMR genes and phenotypes detected in the 20 ESBL/AmpC/carbapenemase-producing *E. coli* isolates in this study.

| **Phenotypes** | **Genes combinations** | **n. (%)** |
|---|---|---|
| ESBL only | *bla*$_{CTX-M-1}$ group | 2 (10) |
| | *bla*$_{CTX-M-1}$ group + *bla*$_{TEM}$ | 5 (25) |
| | *bla*$_{CTX-M-9}$ group + *bla*$_{TEM}$ | 3 (15) |
| | *bla*$_{CTX-M-1}$ group + *bla*$_{TEM}$ + *bla*$_{SHV}$ | 1 (5) |
| ESBL + AmpC | *bla*$_{CTX-M-1}$ group | 1 (5) |
| | *bla*$_{CTX-M-1}$ group + *bla*$_{TEM}$ + *bla*$_{CMY-2}$ | 2 (10) |
| AmpC only | *bla*$_{TEM}$ + *bla*$_{SHV}$ + *bla*$_{CMY-2}$ | 1 (5) |
| | *bla*$_{CTX-M-1}$ group + *bla*$_{CMY-2}$ | 1 (5) |
| MβL only | *bla*$_{CTX-M-1}$ group + *bla*$_{TEM}$ + *bla*$_{NDM}$ | 4 (20) |

N.D.=not determined.

Based on MIC results (Table 17), the highest level of resistance was observed for β-lactam with all isolates resistant to ampicillin, cefazolin, cephalexin, cefovecin and cefpodoxime. Resistance to fluoroquinolones, phenicol, β-lactam in combination with β-lactamase inhibitor agent, tetracyclines was less frequent, with at least one isolate resistant to all the agents in each category. Imipenem resistant strains (4/20; 20%) were all confirmed as carbapenemase-producing *E. coli*. The lowest resistance rate was detected for amikacin with only one resistant isolate. Multidrug-resistance was detected in 19/20 (95%) *E. coli* isolates.

Table 17. Distribution of MICs among ESBL-/AmpC-/carbapenemase-producing *E. coli* isolates.

| Antimicrobial class | Antimicrobial agent | No. resistant | No. of isolates at the indicated MIC µg/ml | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0.12 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 |
| β-Lactam (penicillins) | AMP | 20/20 | | | | | | | | 20 | | | |
| β-Lactam (Cephalosporin I) | FAZ | 20/20 | | | | | | | | | | 20 | |
| | LEX | 20/20 | | | | | | | | | 20 | | |
| β-Lactam (Cephalosporin III) | FOV | 20/20 | | | | | | | | 20 | | | |
| | POD | 20/20 | | | | | | | | 20 | | | |
| | TAZ | 9/20 | | | | | | 6 | 5 | | 9 | | |
| β-Lactam combination agent | AUG2 | 10/20 | | | | | | 4 | 6 | 10 | | | |
| | P/T4 | 9/20 | | | | | | | 11 | | | | 9 |
| Aminoglycosides | AMI | 1/20 | | | | | | 16 | 3 | 1 | | | |
| | GEN | 8/20 | | 1 | 10 | 1 | | | | 8 | | | |
| Phenicol | CHL | 3/20 | | | | | 3 | 6 | 6 | 2 | | 3 | |
| Fluoroquinolones | ENRO | 9/20 | 2 | 5 | 3 | 1 | | | 9 | | | | |
| | MAR | 9/20 | 2 | 2 | 3 | 4 | | | 9 | | | | |
| | ORB | 10/20 | | | | 6 | 3 | 1 | 1 | 9 | | | |
| | PRA | 9/20 | | 11 | | | | 9 | | | | | |
| Tetracyclines | TET | 12/20 | | | | | | 6 | 2 | | 12 | | |
| | DOX | 20/20 | | | 1 | 2 | 3 | 4 | 5 | 5 | | | |
| Carbapenems | IMI | 4/20 | | | | 16 | | | 2 | 2 | | | |
| Folate pathway antagonists | SXT | 10/20 | | | 10 | | | | 10 | | | | |

White fields denote range of dilutions tested for each antimicrobial agent. Vertical lines indicate CLSI VET01S ED6:2023 cutoff values (Intermediate results were considered as susceptible). AMP: ampicillin; FAZ: cefazolin; LEX: cephalexin; FOV: cefovecin; POD: cefpodoxime; TAZ: ceftazidime; AUG2: amoxicillin/clavulanic acid 2:1 (value refers to amoxicillin concentration); P/T4: piperacillin/tazobactam contant 4; AMI: amikacin; GEN: gentamicin; CHL: chloramphenicol; ENRO: enrofloxacin; MAR: marbofloxacin; ORB: orbifloxacin; PRA: pradofloxacin; TET: tetracycline; DOX: doxycycline; IMI: imipenem; SXT: trimethoprim/sulfamethoxazole 1:19 (value refers to trimethoprim concentration).

Statistical analysis (Table 15) showed that unhealthy status of animals (OR = 11.5385; 95% CI: 3.535–37.6623; $p$<0.001), hospitalization (OR = 12.16; 95% CI: 3.7172–39.7788; $p$<0.001) and previous antibiotic therapy (OR = 8.3788; 95% CI: 2.8835–24.3471; $p$<0.001) were significantly associated with the fecal carriage of ESBL-/AmpC-/carbapenemase-producing *E. coli* whereas sex and age were not risk factors.

**Discussion**

The aims of the present study were to preliminary estimate the prevalence of ESBL-/AmpC-/carbapenemase-producing *E. coli* in stray cats, to phenotypically and genetically characterize the detected isolates and to investigate the risk factors associated with feacal carriage, whereas only few studies have explored the potential role of stray as carriers of AMR bacteria (Gargano et al., 2022;

Salgado-Caxito et al., 2021). The overall prevalence of ESBL-/AmpC-producing *E. coli* detected in this study was lower compared with a previous study performed on owned dogs and cats in Italy (Formenti et al., 2021; Piccolo et al., 2020). However, the prevalence was higher compared to the global estimated prevalence of ESBL-producing *E. coli* in cats of 5.04% reported by a recent meta-analysis (Salgado-Caxito et al., 2021) which may reflect some geographical variation or other differences in the sampled population. Indeed, considering a parallelism between cats and dogs, Marchetti and colleagues reported a higher prevalence of suspected ESBL-producing *E. coli* among stray dogs (22%) compared to owned dog (2%) in Argentina, likely reflecting antimicrobial-usage and legislation among different countries (Marchetti et al., 2021). Another possible explanation for the different prevalences among studies is the lack of standardized diagnostic methods and surveillance program in companion animals as previously reported (Salgado-Caxito et al., 2021; Timofte et al., 2021), highlighting the need of standardized diagnostic methodology for AMR surveillance in companion animals.

In this study, the most frequent genes associated with ESBL resistance encoded for CTX-M enzymes, followed by TEM and SHV, similarly to previous report in humans, food-producing animals and dogs (Ewers et al., 2012; Formenti et al., 2021). Moreover, the results of our study showed a lower presence of AmpC-producing *E. coli*, usually carrying the $bla_{\text{CMY-2}}$ gene, compared to ESBL-producing *E. coli* as previously reported in companion animals (Carvalho et al., 2021; Formenti et al., 2021; Melo et al., 2018). In this regard, one AmpC-producing *E. coli* detected in this study was negative for the presence of major plasmid-mediated AmpC genes (Dallenne et al., 2010). These results may be due to the presence of an overexpression of chromosomal ampC β-lactamases as previously reported in companion animals (Bogaerts et al., 2015; Rocha-Gracia et al., 2015). Unfortunatelly, the aims of this study did not include the investigation of this resistance mechanisms. AmpC hyperproduction has been less frequently investigated compared to plasmid-mediated AmpC resistance by others studies, since the latter mechanism is capable of being horizontally transferred to other bacteria, posing a greater threat to AMR control (Coolen et al., 2019, Coolen et al., 2021). However, future studies are needed to confirm the overexpression of chromosomal ampC β-lactamases and it will be interesting to use whole genome sequencing for the genetic characterization of the strains.

The detection of carbapenemase-producing *E. coli* among stray cats is of concern. Indeed, carbapenem are used in human medicine to treat serious infections caused by ESBL/AmpC-producing *E. coli* (Jacoby, 2009; Thomson, 2010), and the administration of carbapenems has been recently prohibited in all animal species, including companion animals (EU Commission-Implementing Regulation 2022/1255/EU; Ramírez-Castillo et al., 2023). In the present study, we detected

carbapenemase-producing *E. coli* carrying *bla*$_{NDM}$ gene using resistance to imipenen as a screening method previously suggested (Miller & Humphries, 2016; Vlek et al., 2016). The presence of *E. coli* carrying *bla*$_{NDM}$ gene has been rarely reported in dog and cats from different countries including Italy (Alba et al., 2021; Silva et al., 2022). Morevover, all *E. coli* isolates from our study carrying *bla*$_{NDM}$ gene were also positive for the presence of *bla*$_{CTX\text{-}M\text{-}1}$ group and *bla*$_{TEM}$ genes, but were negative for ESBL phenotype. This result was not surprising, since ESBL phenotype may be masked by the presence of carbapenemase production (EUCAST, 2017). Despite resistance to imipenem has been suggested as a screening method for the detection of carbapenemase-producing bacteria (Vlek et al., 2016), it may lead to an underestimation of *E. coli* carrying OXA-48, which may appear susceptible to cephalosporins and carbapenems (Silva et al., 2022). Indeed, it would be interesting to verify if our work based exclusively on the resistance profile observed by MIC analysis (including imipenem as the only carbapenem analyzed) may have underestimated the presence of *E. coli* carrying OXA-48 (Silva et al., 2022). Therefore, further studies employing specific screening methods for the detection of carbapenemase-producing *E. coli* are needed to investigate the potential role of cats in the carriage of these bacteria.

The detection of phylogoups F and B2 may be linked with the presence of virulence factors responsible for extra-intestinal infections (Bortolami et al., 2019; Clermont et al., 2013). Therefore, further investigations are needed to investigate the presence of virulence factor in AMR *E. coli* detected in this study.

MDR was frequently detected in this study, particularly against antimicrobials commonly used for empirical antimicrobial-therapy in Northern Italy, such as beta-lactams, fluorochinolones and tetracyclines (Barbarossa et al., 2017). In contrast, resistance to aminoglycosides was rare, reflecting it scarce used in veterinary medicine in Italy (Barbarossa et al., 2017), problaly due to the severe side effects of this antimicrobial class (Durante-Mangoni et al., 2009). These results, highlight the need of good antimicrobial stewardship in veterinary medicine.

The risks factor detected in this study, namely hospitalization, previous antibiotic therapy and presence of clinical disease are factors associated with antimicrobial resistance in humans and dogs (Ortiz-Díez et al., 2023; Pomba et al., 2016). However, our results may be biased because discrimination between risk factors may be challenging due to the severity of diseased stray cats that often require hospitalization and the use of antimicrobials during their hospitalization and given the unknown backgrounds of the free-roaming cats. Indeed, all unhealthy stray cats were hospitalized for their need of medical care and the majority of hospitalized stray cats required antimicrobial therapy.

Moreover, even if previous antimicrobial therapy was not reported for stray cats, other sources of exposure to antimicrobials (e.g. anitimicrobial administration by non-veterinary caretaker, antimicrobial residues in the garbage or environment) could not be completely ruled out, as previously reported for stray dogs (Marchetti et al., 2021). Therefore, further studies are needed to confirm if veterinaty hospitals may play an important role in the selection and acquisition of antibiotic resistant organisms as previously reported (Dazio et al., 2021). Moreover, the spread of antimicrobials resistance within the environment should be monitored according to the One Health approach.

This study had some limitations, starting from the lack of a specific selective media for the primary screening of carbapenemase-producing *E. coli* from feacal samples. Therefore, further studies, focusing on carbapenemase producing-*Enterobacteriaceae* are needed to better understand the role of companion animals in the dissemination of AMR. Moreover, another limitation of this study was the absence of whole genome sequencing analysis for complete genetic characterization of the isolates, which may be useful for the detection of other antimicrobial resistance and virulence genes of the *E. coli* detected in this study.

# GENERAL CONCLUSIONS

Emerging infectious diseases have occurred in cats worldwide in the last decades and emerging pathogens may contribute to severe clinical diseases, whereas the pathogenicity of novel feline viruses is still under investigation (Capozza et al., 2021b; Shofa et al., 2022). Companion animals are important for humans physical and mental health. Indeed, pet ownership can reduce anxiety, loneliness, depression and increases physical condition (Overgaauw et al., 2020). However, pets can also be carriers or reservoirs of zoonotic pathogens but their role in the transmission of pathogens to humans is often underestimated. Therefore, according to the One Health approach, the role of pets in the transmission of zoonotic pathogens to humans, as for the novel SARS-CoV-2, needs to be elucidated (Overgaauw et al., 2020; Qiu et al., 2023). ESBL-/AmpC- and carbapenease-producing *E. coli* have been recognized as human health threats that require surveillance programs but compared to others animal species the presence of these AMR bacteria in domestic and stray cats has been poorly investigated (Guardabassi, 2004; Overgaauw et al., 2020; Salgado-Caxito et al., 2021)
Therefore, the aims of this PhD thesis were to investigate the impact of the novel DCH on feline health, the role of cats in the spread of emerging zoonotic pathogens and AMR.

Starting from DCH, the results of this PhD thesis showed the circulation of different DCH variants in Italy, which need to be further investigated as some viral variants may be associated with different clinical outcomes, as previously reported in humans infected with HBV (Gao, 2015).
Results of our thesis added some information on the potential pathogenic role of DCH considering that:

- The results of our study showed that only a small proportion of cats with biochemical alterations suggestive of liver disease were DCH positive and that DCH was not detected in cats which had a high probability of liver disease. These results suggests that DCH may not be an important pathogen of cats and that DCH surveillance should not be based only on the presence of biochemical changes potentially consistent with liver disease.
- DCH DNA was detected in peritoneal effusions from DCH-positive cats, adding information on presence of the virus also in cavitary effusions likely mirroring the presence of HBV in body fluids of HBV-infected humans.

Based on our results, we cannot definitively conclude that DCH is a significant feline pathogen. However, considering that recent studies have suggested the correlation between DCH and chronic lymphoplasmacytic hepatitis in cats (Piewbang et al., 2022) further studies are still needed to confirm or dismiss the potential pathogenic role of DCH in cats.

Regarding SARS-CoV-2, the general conclusion that can be drawn is that up to now cats have played a limited role in SARS-CoV-2 epidemiology, particularly:

- Stray cats likely did not play a significant role in the spreading of SARS-CoV-2 in area with a high number of human COVID-19 cases.
- Pets may not play a significant epidemiological role in the spread and indirect transmission of SARS-CoV-2 to humans via contamination of hair or paws, even if in contact with COVID-19 positive owners.
- For SARS-CoV-2 epidemiological surveillance in pets, assay validation is a fundamental step and sVNT may be an effective method that does not require a BSL-3 laboratory for predicting serum neutralization antibodies in dogs and cats.

Continuous monitoring of SARS-CoV-2 in cats and other animals may be suggested to explore the emergence of new viral variants that may adapt to new hosts (Doliff & Martens, 2022; Meekins et al., 2021; Pratelli et al., 2022).

Lastly, our results showed that cats may play a role as potential carriers of ESBL/AmpC/carbapenemase-producing *E. coli* and highlight the need of antimicrobial stewardship in veterinary medicine, considering that:

- The fecal carriage of ESBL/AmpC-producing *E. coli* was detected in both owned and stray cats from Italy and was associated with unhealthy cats and antimicrobial usage.
- The fecal carriage of ESBL/AmpC/carbapenemase-producing *E. coli* in stray cats was associated with unhealthy status, hospitalization, and antimicrobial therapy.

Given that we detected the presence of carbapenemase-producing *E. coli* in cats and considering that companion animals may be carriers of carbapenemase-producing bacteria (Silva et al., 2022), further studies focusing on carbapenemase-producing *Enterobacteriaceae* are needed to better understand the role of companion animals in the epidemiology of carbapenem resistance, as well as the risk of transmission of AMR between companion animals, owner and environment.

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# PUBBLICATIONS, COMMUNICATIONS AND OTHER ACTIVITIES

## Publications

**Related to PhD topics**

- Lauzi, S., Stranieri, A., Giordano, A., Lelli, D., Elia, G., Desario, C., Ratti, G., Decaro, N., & Paltrinieri, S. (2021). Do Dogs and Cats Passively Carry SARS-CoV-2 on Hair and Pads?. *Viruses, 13*(7), 1357. https://doi.org/10.3390/v13071357

- Stranieri, A., Lauzi, S., Giordano, A., Galimberti, L., Ratti, G., Decaro, N., Brioschi, F., Lelli, D., Gabba, S., Amarachi, N. L., Lorusso, E., Moreno, A., Trogu, T., & Paltrinieri, S. (2022). Absence of SARS-CoV-2 RNA and anti-SARS-CoV-2 antibodies in stray cats. *Transboundary and emerging diseases*, *69*(4), 2089–2095. https://doi.org/10.1111/tbed.14200

- Scavone, D., Lauzi, S., Stranieri, A., Tramontano, G., Ratti, G., & Paltrinieri, S. (2022). Evaluating the presence of domestic cat hepadnavirus viraemia in cats with biochemical alterations suggestive of liver disease. *The Veterinary record*, *191*(4), e1626. https://doi.org/10.1002/vetr.1626

- Ratti, G., Lelli, D., Moreno, A., Stranieri, A., Trogu, T., Giordano, A., Grassi, A., Luzzago, C., Decaro, N., Paltrinieri, S., & Lauzi, S. (2022). Comparison of diagnostic performances of different serological tests for SARS-CoV-2 antibody detection in cats and dogs. *Transboundary and emerging diseases*, *69*(6), 3530–3539. https://doi.org/10.1111/tbed.14716

- Ratti, G., Stranieri, A., Scavone, D., Cafiso, A., Meazzi, S., Luzzago, C., Dall'Ara, P., Tagliasacchi, F., Cavicchioli, L., Ferrari, F., Giordano, A., Paltrinieri, S., & Lauzi, S. (2023). Detection and genetic characterization of domestic cat hepadnavirus in cats with cavitary effusions. *Veterinary microbiology*, *284*, 109828. https://doi.org/10.1016/j.vetmic.2023.109828

- Ratti, G., Facchin, A., Stranieri, A., Giordano, A., Paltrinieri, S., Scarpa, P., Maragno, D., Gazzonis, A., Penati, M., Luzzago, C., Dall'Ara, P., & Lauzi, S. (2023). Fecal Carriage of

Extended-Spectrum β-Lactamase-/AmpC-Producing *Escherichia coli* in Pet and Stray Cats. *Antibiotics (Basel, Switzerland), 12*(8), 1249. https://doi.org/10.3390/antibiotics12081249

**Unrelated to PhD topics**

- Ratti, G., Stranieri, A., Giordano, A., Oltolina, M., Bonacina, E., Magnone, W., Morici, M., Ravasio, G., Paltrinieri, S., & Lauzi, S. (2022). Molecular Detection of Feline Coronavirus in Captive Non-Domestic Felids from Zoological Facilities. *Animals: an open access journal from MDPI*, *12*(14), 1864. https://doi.org/10.3390/ani12141864
- Bardi, E., Manfredi, M., Capitelli, R., Lubian, E., Vetere, A., Montani, A., Bertoni, T., Talon, E., Ratti, G., & Romussi, S. (2021). Determination of Efficacy of Single and Double 4.7 mg Deslorelin Acetate Implant on the Reproductive Activity of Female Pond Sliders (*Trachemys scripta*). *Animals: an open access journal from MDPI*, *11*(3), 660. https://doi.org/10.3390/ani11030660

**Oral communications/posters related to PhD topics**

* Indicates speaker:

- Stranieri A., Lauzi S., Giordano A., Galimberti L., Ratti G., Decaro N., Brioschi F., Lelli D., Gabba S., Amarachi N.L., Lorusso E., Moreno Martin A., Trogu T., Paltrinieri S. (2021). Molecular and serological survey for SARS-CoV-2 in stray cats in Northern Italy. In: Proceedings of the 74th Convegno SiSVet, Società Italiana della Scienze Veterinarie, ISBN: 9788890909290, online, 23-26 June 2021, p. 145.
- Ratti G., Stranieri A., Penati M., Dall'Ara P., Luzzago C., Lauzi S. (2021) Extended-spectrum β-lactamase producing *Escherichia coli* in stray cats from northern Italy. In: virtual Eighth International Meeting on Emerging Diseases and Surveillance, IMED 2021 Conference, 4-6 November 2021 (online).
- Ratti G., Facchin A., Giordano A., Paltrinieri S., Scarpa P., Masiero G., Gazzonis A., Penati M., Lauzi S. (2022) Prevalence and risk factors associated with fecal carriage of extended-spectrum β-lactamase- and AmpC-producing *Escherichia coli* in cats. In: One Health European Joint Programme Annual Scientific Meeting, 11-13 April 2022 (Orvieto, Italy).

- Ratti G.*, Lelli D., Moreno A., Stranieri A., Giordano A., Decaro N., Grassi A., Paltrinieri S., Lauzi S. (2022) Comparison of different serological assays for SARS-CoV-2 antibody detection in cats and dogs. In: online event of the Association of Institutions for Tropical Veterinary Medicine (AITVM), the Society for Tropical Veterinary Medicine (STVM) and the French Agricultural Research Centre for International Development (CIRAD), 17 May 2022 (online).

- Ratti G.*, Stranieri A., Scavone D., Meazzi S., Giordano A., Paltrinieri S., Lauzi S. (2022) Detection of Domestic Cat Hepadnavirus in cats with cavitary effusions. In: Proceedings of the 75th Convegno SISVet, Società Italiana della Scienze Veterinarie, 15-18 June 2022 (Lodi, Italy), p. 176.

- Ratti G.*, Facchin A., Stranieri A., Giordano A., Paltrinieri S., Scarpa P., Maragno D., Villa L., Penati M., Lauzi S. (2022) Prevalence and risk factors associated with faecal carriage of extended-spectrum β-lactamase-, AmpC- and Carbapenemase-producing *Escherichia coli* in stray cats In 4rd International Conference of the European College of Veterinary Microbiology (4rd ICECVM), 15-17 September 2022 (Bari, Italy), AO12, p. 22-23.

**Oral communications/posters unrelated to PhD topics**

- Ratti G., Lubian E., Giussani E., Penati M., Luzzago C., Scavia G., Tozzoli R., Morabito S., Lauzi S. (2021) Spread of shiga toxin-producing *Escherichia coli* and extended-spectrum β-lactamase producing *Escherichia coli* from Mesola red deer In: Webinar 3rd International Conference of the European College of Veterinary Microbiology (3rd ICECVM), 16-17 October 2021, (online).

- Lauzi S., Tozzoli R., Chiani P., Michelacci V., Nava M., Pedrotti L., Ratti G., Crespi C., Scavia G., Morbito S., Luzzago C. (2022) Characterization of shiga toxin-producing *Escherichia coli* from environmental feces of free-ranging red deer and cattle sharing an alpine pasture. In: One Health European Joint Programme Annual Scientific Meeting, 11-13 April 2022. (Orvieto, Italy).

- Lauzi S., Ratti G., Chiani P., Michelacci V., Scavia G., Morabito S., Luzzago C., Tozzoli R. (2022) Whole genome characterization of shiga toxin-producing *Escherichia coli* from environmental feces of mesola red deer. In: International Conference on "Role of Veterinary Medicine in One Health Approach" Academy of Sciences, Albania, 27 May 2022 (online).

- Facchin A., Ratti G., Penati M., Gazzonis A., Masiero G., Scarpa P., Dall'Ara P., Luzzago C., Alborali L. G., Lauzi S. (2022), Prevalence and risk factors associated with extended-spectrum β-lactamase- and AmpC-producing *Escherichia coli* in dogs. In: Proceedings of the 75th Convegno SISVet, Società Italiana della Scienze Veterinarie, 15-18 June 2022 (Lodi, Italy), p. 178.

- Sala G. Ratti G., Ferrulli V., Scavone D., Giordano A., Boccardo A., Pravettoni D., Lauzi S. (2022) First detection of "*Candidatus Mycoplasma haemolamae*" in alpaca (*Vicugna pacos*) in Italy. In: 31th World Buiatrics Congress, 4-8 September 2022 (Madrid, Spain).

- Giordano A., Meazzi S., Martini V., Ratti G., Scavone D., Bettoni V., Sala G., Ferrulli V., Lauzi S., Stranieri A. (2022) Sysmex XN-V multispecies hematology analyzer setting for whole blood analysis of healthy adult alpacas reared in Italy (*Vicugna pacos*). European Society of Veterinary Clinical Pathology (ESVCP)/European College of Veterinary Clinical Pathology (ECVCP) 24th Annual Meeting, October 5-8th, 2022. (Belgrade, Serbia) Abstract n. 42.

- Ratti G., Lubian E., Semenza Esposito R., Lauzi S. (2023). Coronaviruses in Domestic Ferrets and Hedgehogs: Detection of Ferret Coronavirus and Hedgehog Coronavirus 1 in Italy. International Conference on Livestock, Companion Animals and Wildlife Coronaviruses, May 25th-26th, 2023 (Belfast, United Kingdom).

- Ratti G.*, Ravasio G., Roccabianca P., Giordano A., Oltolina M., Bertola L., Morici M., Magnone W., Lauzi S. (2023). Detection of *Helicobacter* spp. in captive non-domestic felids from Italy In: Proceedings of the 76th Convegno SiSVet, Società Italiana della Scienze Veterinarie, 21-23 June 2023. (Bari, Italy) p. 165.

- Dalla Costa E., Ratti G., Ferrulli V., Brioschi F., Sala G., Probo M., Gazzonis A., Zani D. (2023). VetScape: an escape room experience at the skill lab. The annual Veterinary Education Symposium, VetEd. 5th-7th July 2023. (Edinburgh, United Kingdom)

- Battiato P., Ratti G., Lapira L., Zani D., Longo M., Dalla Costa E. (2023). A novel ultrasound imaging model for training fine needle paracentesis to veterinary students. The annual Veterinary Education Symposium, VetEd. 5th-7th July 2023. (Edinburgh, United Kingdom)

- Ferrari F., Bassi J., Sala G., Brioschi F. A., Gariboldi E. M., Ratti G., Dalla Costa E., Zani D.D. (2023) Impact of 3D printed feline skulls as a learning tool on radiographic interpretation of

craniomaxillofacial traumatic injuries. The annual Veterinary Education Symposium, VetEd. 5th-7th July 2023. (Edinburgh, United Kingdom)

- Ratti G., Sala G., Ferrulli V., Scavone D., Stranieri A., Giordano A., Boccardo A., Pravettoni D., Lauzi S. (2023) Detection of *Candidatus* Mycoplasma Haemolamae In South American Camelids Farmed in Italy. 5th International Conference of the European College of Veterinary Microbiology (5th ICECVM), 21th-23th September 2023. (Bled, Slovenia)

## Other activities

**Teaching activity**

- Collaboration in practical teaching activity "Clinical rotation (microbiology/molecular diagnostics)" (32 hours) (years 2021-2022), Veterinary Medicine course, Supervisor Prof. Stefania Lauzi.
- Tutoring activity for the Veterinary Medicine "skill lab". (100 hours) (years 2022-2023), Art. 45, Supervisor Prof. Davide Zani.

**Thesis**

*Vetrinary Medicine degree thesis*

- Co-tutor in Veterinary Medicine degree thesis: *Escherichia coli* produttori di shiga tossine in campioni fecali di cervi della mesola (*Cervus elaphus italicus*) (tutor Dr. Lauzi S.), Candidate: Giussani E., graduation December 2021
- Co-tutor in Veterinary Medicine degree thesis: Diffusione di *Escherichia coli* produttori di beta-lattamasi a spettro esteso (ESBL) e AmpC in campioni fecali di gatti del nord Italia (tutor Dr. Lauzi S.), Candidate: Maragno D., graduation June 2022.
- Co-tutor in Veterinary Medicine degree thesis: Domestic cat hepadnavirus in gatti del nord Italia: indagine molecolare e analisi dei fattori di rischio (tutor Dr. Lauzi S.), Candidate: Sorresina M., graduation in October 2023.
- Co-tutor in Veterinary Medicine degree thesis: Indagine molecolare sulla presenza di *Helicobacter* spp. nei felidi non domestici in cattività del nord Italia (tutor Dr. Lauzi S.), Candidate: Mascheroni S.., graduation expected in February 2024.

*School of Specilization*

- Co-tutor in School of specialization in animal health, breeding and productions thesis: Indagine sulla presenza di *Escherichia coli* produttori di beta-lattamasi a spettro esteso e AmpC in campioni fecali di cane e di gatto (tutor Dr. Lauzi S.), Candidate: Masiero G., graduation July 2022.

**Attended courses**

- Genomics for ecological and evolutionary studies: from DNA sequencing to data analysis. Prof. Gentile Francesco Ficetola, November 2020, online course, 2 CFU
- Biochemistry and molecular biology techniques.Prof. Simona Laura Anna Polo, December 2020, online course, 4 CFU
- Surveillance and dynamics of the relevant infections in Public Health. Prof. Gianguglielmo Zehender, May 2021, online course, 2 CFU
- Statistical methods applied to epidemiology and animal sciences. Prof. Annamaria Costa and Prof. Valerio Bronzo, December 2022, Lodi, 2 CFU
- One Health approach for the control of emerging zoonoses. Prof. Alfonso Zecconi, January 2022, Milan, 2 CFU

**Abroad period**

- 1 October 2023-31 December 2023: Erasmus Traineeship Programme at the University of Nottingham, United Kingdom. Traineeship in bioinformatic analysis applied to molecular virology. Supervisor: Prof. Rachael Tarlinton

**Visiting to external laboratories**

- 2 May 2022-6 May 2022: Laboratory of Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Brescia, Italy
- 1 April 2023-4 September 2023: Department of Biomedical and Clinical Sciences, University of Milan, Milan, Italy (one day a week)
- 26 May 2023-07 May 2023: Department of Veterinary Medicine, University of Bari, Valenzano, Italy

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*"So take me, sweet release!*
*I've found it's naught but stone and silver"*
-Slice the cake