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Serological and Molecular Surveillance of Influenza A Virus in Dogs and Cats in Central Chile

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ABSTRACT

Introduction: Influenza A virus (IAV) is a zoonotic pathogen with pandemic potential that infects a wide range of species, including companion animals. Although surveillance efforts have primarily focused on North America, Europe and Asia, data from South America remain scarce. This study evaluated the molecular and serological evidence of IAV circulation in dogs and cats from shelters and multi-pet households in central Chile.

Methods: Between June and November 2020, oropharyngeal swabs and serum samples were collected from dogs and cats in shelters and multi-pet households in central Chile. Samples were analysed by RT-qPCR, NP-ELISA and hemagglutination inhibition assay.

Results: IAV was detected by RT-qPCR in 3.2% (11/342) of dogs (95% CI: 1.3%–5.1%) and 5.8% (4/69) of cats (95% CI: 0.3%–11.3%). Serological analysis revealed IAV seropositivity in 55.5% (96/173) of dogs (95% CI: 48.1%–62.9%) and 50.0% (10/20) of cats (95% CI: 28.1%–71.9%). Additionally, pdmH1N1 antibodies were detected in 26 animals (25.7%; 95% CI: 17.2%–34.2%) out of 101 NP-ELISA-positive serum samples, with higher median titres for cats (median = 160) compared to dogs (median = 20). Low-level antibody titres against Canine/H3N2 (range = 10–20) were identified in three dogs (3.0%; 95% CI: 0.0%–6.3%), whereas no antibodies were detected against Canine/H3N8 or Avian/H3N6. No significant associations were observed between seropositivity and animal age, sex, origin or area.

Conclusions: This study presents the first report of IAV detection in cats in South America and highlights a high level of IAV exposure among companion animals in central Chile. These findings underscore the importance of including pets in IAV surveillance efforts under a One Health approach and highlight the need for expanded monitoring and genetic characterization of circulating strains to assess zoonotic risk.

Cecilia Baumberger and Francisca Di Pillo should be considered joint first authors.

Impacts

- Influenza A virus is actively circulating in companion animals in central Chile, with high prevalence and seroprevalence levels in both dogs and cats from shelters and multi-pet households.
- Detection of specific influenza A virus antibodies indicates exposure to pdmH1N1 in both dogs and cats, and to canine H3N2 in dogs.
- The circulation of influenza A virus in a substantial proportion of companion animals raises concerns about the potential for reassortment events between canine and human influenza A virus, which could lead to the emergence of novel strains.

1 | Introduction

The emergence of new zoonotic pathogens represents one of the greatest challenges to global health security. Influenza A virus (IAV), due to its high mutation rate and ability to cross species barriers, poses a particular concern for both human and animal health (Borland et al. 2020; Short et al. 2015). Beyond wild birds (especially aquatic birds), which serve as the reservoir of the virus, IAV is capable of infecting a wide range of species, including equids, swine, canids, felines and humans, thereby facilitating cross-species transmission events (Abdelwhab and Mettenleiter 2023; Joseph et al. 2017). These events have led to four major influenza pandemics since 1918, each originating from avian or porcine hosts and resulting in significant global morbidity and mortality (Abdelwhab and Mettenleiter 2023). The frequent and close interactions between wild birds, domestic animals and humans increase the risk of spillover and genetic reassortment between different IAV strains, potentially leading to the emergence of novel viruses with pandemic potential (Escudero-Pérez et al. 2023; Jimenez-Bluhm et al. 2018).

The number of cat and dog owners has increased in recent decades, accompanied by growing pet-human contact rates, which may potentially elevate the risk of pathogen transmission (Chomel 2014). This is particularly concerning given that dogs and cats are susceptible to IAV infection (Jimenez-Bluhm et al. 2021; Ramírez-Martínez et al. 2013; Sekine et al. 2024), suggesting a potential role in the epidemiology of IAV. Since the early 2000s, the H3N8 equine-origin and the H3N2 avian-origin strains have shown significant adaptability in the canine population, being associated with several outbreaks. The H3N8 subtype was initially described in dogs in the United States in 2004 and has circulated in the canine population without significant genetic reassortment (Payungporn et al. 2008). Current evidence indicates canine H3N8 is no longer circulating in the United States (Wasik et al. 2023). Conversely, the H3N2 subtype was first reported in South Korea in 2007 associated with cases of respiratory disease in dogs (Song et al. 2011) and has undergone reassortment with other subtypes, including H5N1 (Zhu et al. 2015) and pdmH1N1 (Moon et al. 2015; Song et al. 2012). Regarding domestic cats, documented cases of IAV infection prior to 2021 were limited but included several subtypes, such as canine H3N2, low pathogenic avian influenza H7N2 and the pdmH1N1 virus (Hatta et al. 2018; Knight et al. 2016; Lee et al. 2017; Song et al. 2011). Notably, the recent emergence of highly pathogenic avian influenza H5N1 in domestic cats in France (Briand et al. 2023) and the deadly outbreak among cats in Poland in 2023 (Rabalski et al. 2023) illustrate the ongoing risks posed by these viruses to pet populations. These incidents, along with asymptomatic cases of H5N1 in dogs and cats in Italy (Moreno et al. 2023), emphasise the potential for silent circulation of IAV in companion animals, which could have implications for both animal and human health.

Given the increasing frequency of IAV detection in dogs and cats, it is crucial to enhance our understanding of their role in the ecology of influenza viruses. There is a limited number of documented cases in which mammalian species have acted as intermediate hosts between avian and mammalian species. The emergence of pdmH1N1 is one of these events where an avian, swine and human triple-reassortant virus was transmitted from pigs to humans (Garten et al. 2009). The equine origin of Canine H3N8, after decades of continuous circulation in equine populations, is another well-documented case of a mammalian intermediate host event. In both cases, the outcome was the establishment of epidemics in the secondary mammalian host (Parrish et al. 2015; Sekine et al. 2024). Moreover, over 40 human cases of highly pathogenic avian influenza H5N1 have been attributed to exposure to infected dairy cows by last year (Morel et al. 2026). Regarding companion animals, the only documented zoonotic case of IAV transmission from pets occurred during an outbreak of low pathogenic avian influenza H7N2 in shelter cats in New York (Lee et al. 2017). To date, no cases of dog-to-human IAV transmission have been reported. However, the increasing circulation of IAV in pets, together with the extensive interaction at the human-pet interphase, highlights the need for continued surveillance and research (Parrish et al. 2015; Sekine et al. 2024).

Most surveillance efforts of IAV in companion animals originate from North America, Europe and Asia; however, very few studies have evaluated the circulation of IAV in dogs and cats in Latin America. Therefore, sporadic or even epizootic events may go unnoticed in the region. Among the scarce literature, serological evidence of IAV infection was shown in dogs (pdmH1N1, H1N2, H3N2 and H3N8) in Mexico (Maya-Badillo et al. 2024; Ramírez-Martínez et al. 2013) and in dogs and cats (pdmH1N1) in Chile (Jimenez-Bluhm et al. 2021). However, molecular findings have been described in only one recent study performed in Chile, where IAV positivity by RT-qPCR was 1.1% in dogs, although no evidence was found in cats (Jimenez-Bluhm et al. 2021). Therefore, the present study aims to investigate the seroprevalence and prevalence of IAV in companion animals from shelters and multi-pet houses in central Chile.

2 | Material and Methods

2.1 | Study Area and Study Population

A cross-sectional study was conducted in central Chile between June and November 2020. The study populations consisted of domestic pets in central Chile from high-density animal

environments: (i) multi-pet households (defined as households with more than five animals, including both cats and dogs) and (ii) dog shelters. Within each study population, animals were selected through a convenience sampling method in eight municipalities belonging to Metropolitan and Valparaíso regions in central Chile (Figure 1). The sampling focused on two specific groups: the map was constructed using R (version 4.2.1) using the packages: 'chilemapas', 'ggplot2', 'ggspatial', 'sf' and 'cowplot' (R Core Team 2016).

2.2 | Sample Collection

The collected samples included blood and oropharyngeal swabs for serological and molecular analysis, respectively. Samples were collected by trained veterinarians. Physical restraint was used; however, chemical restraint was not employed at any time. Blood samples were collected from cephalic or jugular veins, obtaining up to 1 mL from cats and 5 mL from dogs. Blood samples were kept at 4°C using an insulated cooler containing frozen gel packs during transportation to the Faculty of Veterinary Science at the University of Chile. Serum was obtained by centrifuging the blood samples at 1300*g* for 15 min and stored at −20°C until analysis. Oropharyngeal swabs collected from cats and dogs were preserved in 1 mL Universal Transport Media (Copan group, Brescia, BS, Italy) and maintained at 4°C until arrival at the Faculty of Veterinary Medicine at the University of Chile, where samples were stored at −80°C until further analysis. All samples were collected by trained veterinarians.

2.3 | Sera Analysis

A competitive IAV nucleoprotein ELISA assay (NP-ELISA) was used to detect anti-NP antibodies in serum samples, following the manufacturer's guidelines (Virusys Corporation, USA). The ELISA plates were read at 450 nm using a Sunrise absorbance microplate reader (TECAN, Switzerland). ELISA-positive serum samples were further tested using hemagglutination inhibition (HAI) assays to detect antibodies against the following IAV strains: (i) A/California/7/09 (pdmH1N1; human-origin), (ii) A/canine/Indiana/1177-17-1/2017 (Canine/H3N2; avian-origin), (iii) A/canine/Florida/14/2006 (Canine/H3N8; equine-origin) and (iv) A/red-fronted coot/Chile/5/2013 (Avian/H3N6; avian-origin; Table 1). For HAI, 25 μL of each serum sample was first treated with receptor-destroying enzyme (Denka Seiken Co., Japan) and then subjected to 2-fold serial dilution in 25 μL of PBS, performed in duplicate in a 96-well v-bottom plate. Subsequently, 25 μL of a solution containing 4 hemagglutinin units of each virus was added and the plate was incubated at room temperature for 15 min. Following this, 50 μL of 0.5% chicken red blood cells, diluted in PBS, were added. The plate was then kept at 4°C for 30 min before analysis.

2.4 | Molecular Analysis

To evaluate the presence of IAV, RNA extraction was performed on 50 μL of oropharyngeal swab samples using the MagMax AI/ND-96 viral extraction kit (ThermoFisher Scientific, USA),

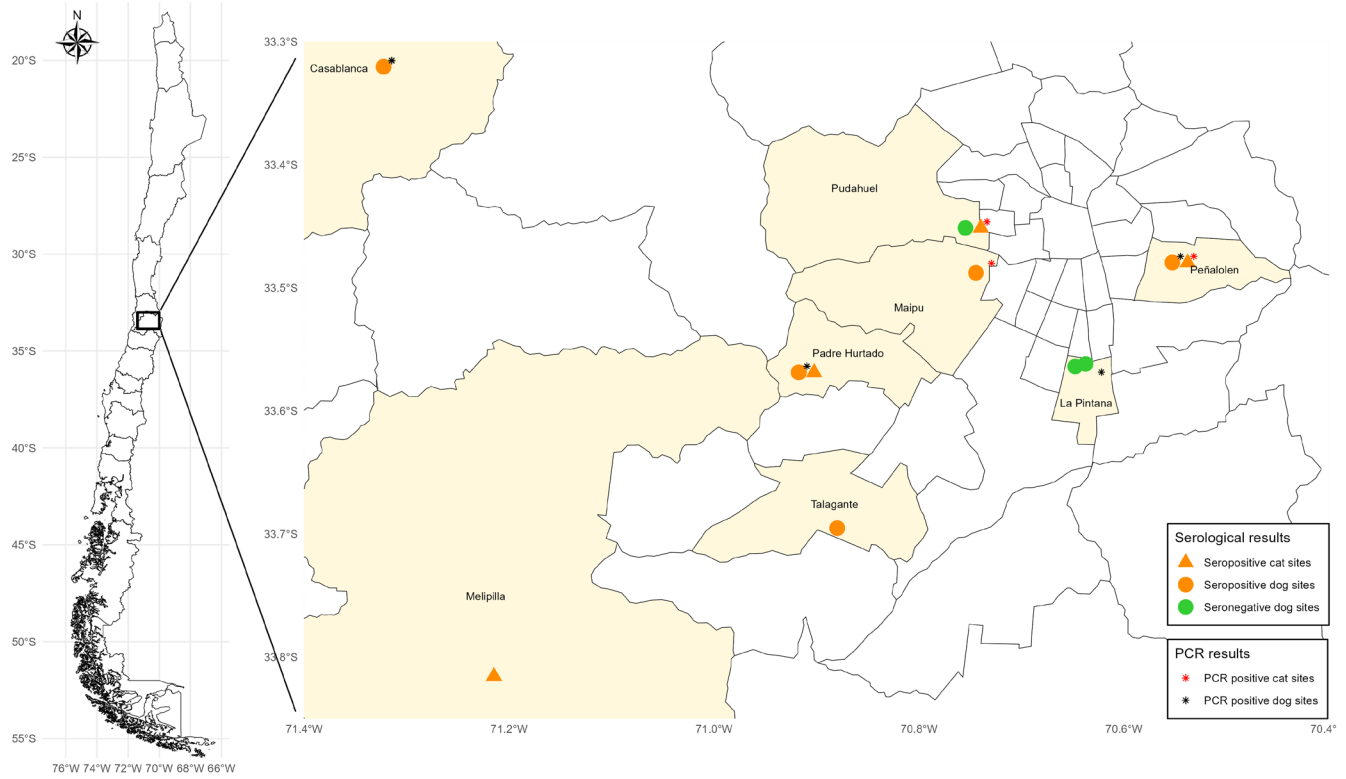

FIGURE 1 | Companion animals sample area. Sampled area including eight municipalities across two administrative regions of central Chile. The map shows the geographic distribution of sampling sites in the Metropolitan and Valparaíso regions. Each symbol represents a different result: Orange circles indicate sites with seropositive dogs; orange triangles represent sites with seropositive cats; green circles correspond to sites with seronegative dogs; red stars mark sites with RT-qPCR positive cats; black stars indicate sites with RT-qPCR positive dogs.

TABLE 1 | Influenza A virus strains used on the hemagglutination inhibition assay.

Subtype	ID	Virus origin	GenBank access	Abbreviation
pdmH1N1	A/California/7/09	Human	ACP44189	pdmH1N1
H3N2	A/canine/Indiana1177-17-1/2017	Avian	MF173230	Canine/H3N2
H3N8	A/canine/Florida/14/2006	Equine	GU473367	Canine/H3N8
H3N6	A/red-fronted coot/Chile/5/2013	Avian	KX101146	Avian/H3N6

following the manufacturer's instructions. For RT-qPCR, TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems, USA) with primers designed for the amplification of the Matrix protein gene was used (Spackman 2020). Samples with a cycle threshold (Ct) value below 38 were classified as positive (Shu et al. 2011). Extraction controls and positive controls consisting of BPL inactivated CA/09 pdmH1N1 were included, in accordance with the laboratory protocol.

2.5 | Data Analysis

The comparison of quantitative variables between groups was evaluated using the Student's *t*-test or the Mann–Whitney *U* test, according to the distribution of the variable, which was graphically assessed. Comparisons of the proportions of categorical variables were made using the Chi-square test. Logistic regression models were built separately for cats and dogs to assess the effect of independent variables on IAV seropositivity as the outcome variable. The independent variables evaluated included sex, animal origin (animal shelter or multi-pet house) and area (peri-urban or urban). Significance level was set at 5%, and the analyses were conducted using InfoStat statistical software (Di Rienzo 2020).

3 | Results

3.1 | Molecular Analysis

A total of 411 animals (342 dogs and 69 cats) were swabbed. All sampled cats originated from multi-pet households, while the majority of dogs (90%) originated from shelters. Of 411 oropharyngeal swab samples, 15 tested positive for IAV by RT-qPCR (Ct range = 34.9–37.4), with four positive cases observed out of 69 cats (5.8%; 95% CI: 0.3%–11.3%) and 11 positive cases out of 342 dogs (3.2%; 95% CI: 1.3%–5.1%) (Figure 1). Ninety percent of positive cases in dogs were from shelters. Regrettably, none of the RT-qPCR positive samples had a cycle threshold (Ct) value below 32, which is the established cutoff for successful sequencing in our laboratory. As a result, no molecular characterisation of the viruses could be obtained from these samples.

3.2 | ELISA and Hemagglutination Inhibition Assay

Additionally, a total of 193 serum samples were collected from dogs ($n = 173$) and cats ($n = 20$). NP-ELISA results showed a seropositivity rate of 50.0% (10/20; 95% CI: 28.1%–71.9%) among cats, with no significant difference between cats younger than 7 years (40%; 4/10) and those ≥ 7 years old (60%; 6/10; $\chi^2 = 0.80$; $p = 0.371$). Seropositivity among dogs was 55.5% (96/173; 95% CI: 48.1%–62.9%; Table 2; Table 3; Figure 1), also with no significant difference between dogs younger than 7 years (51.5%; 52/101) and those ≥ 7 years old (53.7%; 29/54; $\chi^2 = 0.07$; $p = 0.792$). The mean ages were 6.6 years (SD = 4.2) and 5.7 years (SD = 2.9) for

TABLE 2 | Cat characterisation by NP-ELISA result ($n = 20$ cats).

Variables	Cats ($n = 20$) NP-ELISA (+) ($n = 10$)	Cats ($n = 20$) NP-ELISA (−) ($n = 10$)	*p*
Age, mean ± SD	7.7 ± 4.1	5.6 ± 4.3	0.287
Sex			0.068
Female	8 (80%)	4 (40%)	
Male	2 (20%)	6 (60%)	
Area			0.149
Peri-urban	1 (12.5%)	4 (44%)	
Urban	7 (87.5%)	5 (56%)	

TABLE 3 | Dog characterisation by NP-ELISA result ($n = 173$ dogs).

Variables	Dogs ($n = 173$) NP-ELISA (+) ($n = 96$)	Dogs ($n = 173$) NP-ELISA (−) ($n = 77$)	*p*
Age, mean ± SD	5.8 ± 2.9	5.6 ± 3.0	0.618
Sex			0.128
Female	52 (57%)	34 (45%)	
Male	40 (43%)	42 (55%)	
Area			
Peri-urban	86 (93%)	67 (88%)	0.229
Urban	6 (7%)	9 (12%)	
Origin			0.352
Shelter	84 (91%)	66 (87%)	
Multi-pet house	8 (9%)	10 (13%)	

TABLE 4 | Logistic regression models for the association between NP-ELISA results and independent variables by animal species.

Predictor	Estimate	SE	OR	95% CI	*p*
Logistic model for dogs					
Intercept	−0.3	0.63			
Age	0.02	0.06	1.02	0.92–1.14	0.689
Sex (Ref: female)					
Male	−0.3	0.33	0.74	1.42–0.83	0.364
Animal origin (Ref: Multi-pet house)					
Shelter	0.43	0.52	1.53	0.55–4.25	0.414
Logistic model for cats					
Intercept	−1.51	1.28			
Age	0.35	0.2	1.42	0.96–2.10	0.082
Sex (Ref: female)					
Male	−3.27	1.71	0.04	0.0013–1.09	0.056

cats and dogs, respectively and did not differ by NP-ELISA result ($p = 0.287$; Tables 2 and 3).

There was no observed association between NP-ELISA positivity and sex, animal origin or area for both dogs and cats (Table 4). None of the cats tested positive by both NP-ELISA and RT-qPCR. Both serological and molecular data were available for 168 dogs, of which two exhibited positive NP-ELISA and RT-qPCR results.

Out of a total of 106 NP-ELISA positive animals, 101 ($n = 9$ cats; $n = 92$ dogs) had sufficient serum volume for HAI testing. Hemagglutination inhibition titre ranged between 10 and ≥160 for the pdmH1N1 strain, with 26 (five cats and 21 dogs) out of the 101 tested samples (25.7%; 95% CI: 17.2%–34.2%) showing detectable titers (≥10). The median HAI titre against pdmH1N1 among HAI-positive samples was 20 (Q1 = 20; Q3 = 75; Figure 2), suggesting moderate levels of prior exposure. Cats exhibited significantly higher median HAI titers against pdmH1N1 (median = 160) compared to dogs (median = 20; $p < 0.001$). Conversely, only three dogs out of 101 animals (3.0%; 95% CI: 0.0%–6.3%) showed low-level antibodies against the Canine/H3N2 strain (titers range = 10–20; Figure 2). Only one dog was positive to both pdmH1N1 and Canine/H3N2. Finally, no detectable antibodies were found against the Canine/H3N8 or Avian/H3N6 strains, suggesting limited or no recent circulation of these subtypes in the study population (Figure 2).

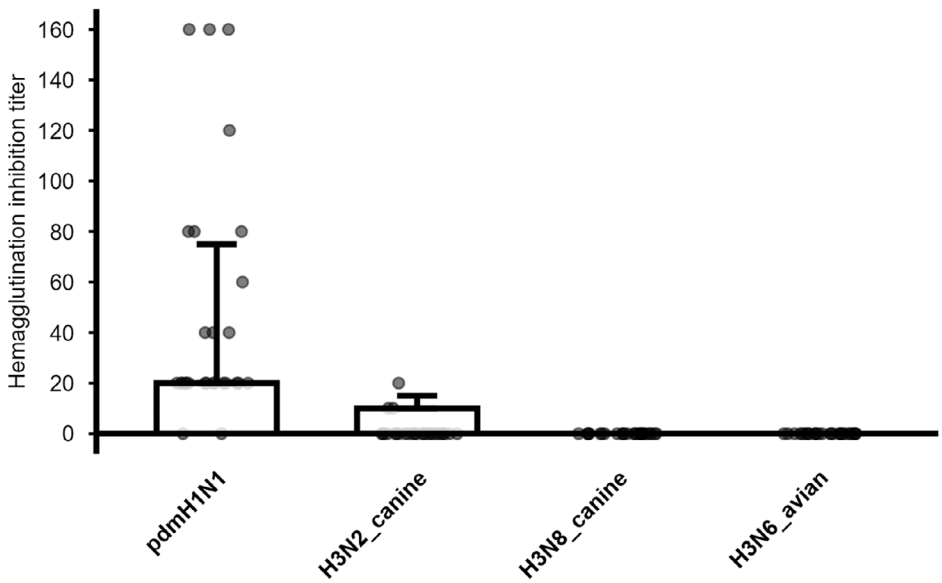

FIGURE 2 | Hemagglutination results of tested sera. Median and interquartile range of influenza hemagglutination inhibition (HAI) antibody titers (1:X) against pdmH1N1, Canine/H3N2, Canine/H3N8 and Avian/H3N6 strains in serum samples from 28 animals that were HAI-positive for at least one strain ($n = 5$ cats; $n = 23$ dogs).

4 | Discussion

This study represents a significant contribution to understanding the epidemiology of IAV in companion animals in central Chile, offering both serological and molecular evidence of IAV circulation in dogs and cats. Our results showed a RT-qPCR IAV prevalence of 3.2% in dogs and 5.8% in cats. This is particularly interesting as it constitutes the first report of IAV positivity by RT-qPCR in cats in South America. This finding expands on previous studies in the region, which primarily focused on dogs and reported an IAV prevalence of 1.1% in dogs using RT-qPCR (Jimenez-Bluhm et al. 2021).

The NP-ELISA IAV seroprevalence rates observed in this study, 55.5% in dogs and 50.0% in cats, are higher than those reported in previous studies conducted in the United States, China, the Netherlands and Chile (Jimenez-Bluhm et al. 2021; Su et al. 2013; Zhao et al. 2020). This greater seroprevalence could be attributed to the specific population sampled in our study, particularly animals from shelters and multi-pet households. These environments are characterised by higher animal density that increases direct and indirect animal contact rates, potentially facilitating viral transmission. The greater seropositivity in these settings suggests that densely populated environments may play a crucial role in the spread of IAV among companion animals. Notably, all but one of the eight sampled municipalities showed seropositivity in either cats or dogs, indicating widespread IAV circulation in companion animals across central Chile.

Individual factors, such as sex, age or animal origin, may influence exposure risk through behavioural patterns and susceptibility to infection through differences in immunity (Albery et al. 2023). However, our study did not identify significant associations between seropositivity and variables such as sex, age or animal origin. This finding is consistent with previous studies that have also identified these animal-level variables as non-significant risk factors for IAV seropositivity (Barrell et al. 2010; Jimenez-Bluhm et al. 2021; Ramírez-Martínez et al. 2013). The lack of association suggests that IAV exposure in companion animals may be more influenced by environmental factors and the specific conditions of the population rather than individual animal characteristics.

Hemagglutination inhibition results align with previous evidence from Chile and Mexico, showing seropositivity to pdmH1N1 in companion animals (Jimenez-Bluhm et al. 2021; Maya-Badillo et al. 2024; Ramírez-Martínez et al. 2013). Notably, the highest pdmH1N1 titers (≥ 160) were observed in three cats aged 7, 8 and 15 years, suggesting that older animals may have experienced earlier or repeated exposure to this virus subtype. In contrast to reports from Mexico where dogs showed HAI titers against both pdmH1N1 and equine H3N8 (Ramírez-Martínez et al. 2013), our study did not detect antibodies to canine H3N8 in any tested animal. Interestingly, three dogs (3.0%) showed low-level antibodies to the canine H3N2 strain. To our knowledge, this is the first report of canine-origin H3N2 seropositivity in companion animals in South America. While these findings may suggest sporadic exposure and warrant consideration of including canine H3N2 vaccines in regional prevention strategies, the low titers observed (10–20) argue against active viral circulation. It is also possible that these results may reflect cross-reactivity with human seasonal H3 viruses, which are known to circulate in Chile. However, none of the tested samples showed cross-reactivity to the Avian/H3N6 strain, which is enzootic in wild birds in Chile (Bravo-Vasquez et al. 2020), supporting the specificity of the observed responses.

These findings have important implications for public health, particularly in the context of the One Health framework. Notably, only one case of cat-to-human IAV transmission has been reported to date during an outbreak of low pathogenic avian influenza H7N2 in shelter cats in New York (Lee et al. 2017). In contrast, transmission of IAV from dogs to humans has not been documented. However, the circulation of IAV in a substantial proportion of companion animals raises concerns about the potential for reassortment events between canine and human IAV, which could lead to the emergence of novel IAV (Chomel 2014; Parrish et al. 2015). The potential of canine H3N8 to mutate into variants capable of infecting human respiratory cells further emphasises the public health risk posed by canine IAV (Sekine et al. 2024). The close bond between humans and pets, often characterised by high levels of physical interaction, may facilitate human exposure to IAV circulating in companion animals (Parrish et al. 2015). This underscores the need for enhanced surveillance and monitoring of IAV in companion animals, particularly in regions where human-animal interactions are frequent. Furthermore, the identification of IAV in both rural and peri-urban settings highlights the need for a broader geographic approach to surveillance. Public health strategies should consider the role of companion animals in the ecology of IAV, particularly given the increasing integration of pets into human households. This trend may increase the risk of cross-species transmission, highlighting the need for surveillance to prevent the emergence of novel IAV strains with zoonotic potential (Sun et al. 2017). Understanding contact rates at the pet-human interface is critical, as these rates can significantly influence the dynamics of viral transmission. The inability to genetically characterise positive samples in the present study highlights the need for improved strategies to recover viruses with lower Ct values or through virus isolation. Larger-scale studies are also fundamental to identify population level, environmental and individual risk factors associated with IAV infection in dogs and cats. In addition, longitudinal surveillance is desirable to better capture patterns of viral shedding during high-prevalence seasons and to detect possible seasonal dynamics of infection.

5 | Conclusions

This study represents the first report of IAV RT-qPCR-positive cases in cats in South America, expanding on previous research that has primarily focused on dogs. Our findings demonstrate the active circulation of IAV in dogs and cats in central Chile, underscoring the importance of including pets in IAV surveillance efforts within a One Health framework. Further studies are needed to isolate circulating IAV subtypes in companion animals, enabling full genomic sequencing and phylogenetic characterisation.

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Ethics Statement

All sampling activities and animal experiments were approved by the Institutional Animal Care and Use Committee (CICUA) of the University of Chile (19265-VET-UCH approved 24 March 2019).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data presented in this study are available on request from the corresponding author.

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