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Exposure of dogs to influenza A and B viruses

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ABSTRACT

Dogs have not typically been considered natural hosts for influenza viruses. However, two canine influenza subtypes, H3N8 and H3N2, have been associated with infection in this species, posing a potential zoonotic threat. In this study, a serological investigation was conducted to assess the seroprevalence of human seasonal influenza viruses (type A and B), as well as canine and avian influenza A viruses, and the H7N1 virus, in a population of adult household dogs. A total of 256 serum samples were analysed by the hemagglutination inhibition and virus neutralization assays. The seroprevalence rates were 0.78% and 77.34% for human seasonal H1N1 and H3N2 influenza viruses; 0.39% for both the B Yamagata and B Victoria lineages; and 1.17% for H7N1 virus. None of the samples tested positive for avian or canine viruses. These findings suggest that household dogs can be exposed to human seasonal influenza viruses, emphasizing the importance of continued surveillance of influenza viruses within canine populations.

Keywords: influenza A virus, influenza B virus, dog, prevalence

INTRODUCTION

Influenza viruses (IVs) represent a persistent threat to public health, with significant epidemiological, clinical, economic, and societal impacts. This threat stems from several factors, including the widespread and contagious nature of the disease, the antigenic variability of the viruses, the seasonal and occasionally pandemic circulation of certain subtypes, and the risk of serious complications, especially in vulnerable populations [1].

IVs are highly contagious pathogens affecting both animals and humans, belonging to the *Orthomyxoviridae* family. They are classified into four types: A (IAV), B (IBV), C (ICV), and D (IDV) [2]. IAVs infect avian and mammalian species, with swine and poultry considered key reservoirs. IBVs are almost exclusive to humans, with the rare exception of seals [3]. ICVs are primarily human pathogens but have also been detected in pigs, dogs, and cattle [4,5]. IDV, although first isolated in 2011 from a pig with respiratory disease, has its natural reservoir in cattle [6]. The presence of IDV has since been reported globally, either directly or indirectly, in livestock, feral pigs, wild boars, camelids, small ruminants, horses, and, more recently, dogs [7,8].

Dogs are not usually natural hosts for IVs, but two IA subtypes, H3N8 and H3N2, have infected dogs and are known as canine IVs (CIVs) [9]. In 2004, an equine-like H3N8 virus was identified in Florida greyhounds, USA, though serological evidence suggests it had already been circulating in dogs since 1999 [10]. The virus was identified across several North American states but was considered extinct by 2016 [11]. Independent spillover events of H3N8 from horses to dogs also occurred in UK (2002) and Australia (2007) [12,13].

In 2007, an avian-origin H3N2 CIV was reported in a pet dog in the Republic of Korea, while around the same time, an avian-origin H3N2 IAV was isolated from clinically ill dogs in southern China [14,15]. Between 2015 and 2020, the virus was identified repeatedly in dogs from the USA and Canada [16,17]. Genetic analyses have identified at least 6 clades, suggesting multiple introductions [18].

Dogs have occasionally been infected with other IAV subtypes, including a fatal H5N1 case in Thailand (2004) [19], suggesting they could serve as a new source for the transmission of novel IAVs.

Further studies have demonstrated that dogs can also be infected with human IVs, including the 2009 pandemic H1N1 virus (H1N1pdm09), seasonal H3N2, and B viruses [20,21].

Dogs and cats, as close human companions, could act as important intermediate hosts or even participate directly in the transmission of human IVs. Moreover, pre-existing CIVs could reassort with human seasonal viruses, potentially generating novel viruses that could lead to new pandemics [22]. During 2007-2010 canine surveillance in South Korea, a novel H3N1 virus was isolated, likely a natural reassortant of H3N2 CIV (avian-origin) and pandemic H1N1, with the HA gene from H3N2 and the NA gene from H1N1 [23].

Despite increasing evidence that dogs can be infected with IVs of both animal and human origin, important knowledge gaps remain regarding the extent of their natural exposure to different IV types and subtypes, particularly among household dogs. Although sporadic infections and outbreaks have been described, comprehensive serological data evaluating exposure to multiple human, canine, and avian IVs in healthy companion dogs are still limited. Such information is essential to better understand the epidemiological role of dogs in influenza ecology, to assess their potential involvement in interspecies transmission, and

to strengthen One Health surveillance strategies. Therefore, the aim of this study was to conduct a serological investigation to assess the seroprevalence of human seasonal IVs (IAV and IBV), canine and avian IAV, and H7N1 virus in a population of adult household dogs.

MATERIALS AND METHODS

Serum samples and influenza viruses

A total of 256 serum samples from adult household dogs were collected in 2023 in the Apulia region of Southern Italy. All samples were collected by veterinary offices either for presurgical evaluation or for routine analysis, with aliquots of the remaining serum used for this study. Ethical approval was waived by the Ethics Committee of the Department of Veterinary Medicine of the University of Bari Aldo Moro, as the samples were collected during routine veterinary procedures and no additional sampling procedures were performed for the purposes of this study. Information on sex, age and health status was not available. None of the dogs had received vaccination against CIVs H3N8 or H3N2. All serum samples were screened in duplicate by the hemagglutination inhibition (HI) assay using human seasonal IAVs (A/Wisconsin/588/2019 (H1N1) and A/Darwin/6/2021 (H3N2) and IBVs (B/Phuket/3073/2013 B/Yamagata lineage and B/Austria/1359417/2021 B/Victoria lineage); canine IAVs (A/canine/Korea/LBM412/08 (H3N2) and A/canine/Florida/43/04 (H3N8), avian IAV (A/turkey/Italy/23VIR1964-19/2023 (2.3.4.4b clade) (H5N1), and NIBRG-127 (H7N1), reassortant developed at NIBSC with the HA gene from A/Prague/56 (H7N7), the NA gene from A/California/7/2009 (H1N1) and the six internal genes from A/PR/8/34 (H1N1), available in house at the time of testing (Table 1). Samples were further tested for human H3N2 and avian H5N1 IVs by virus neutralization (VN) assay using Madin-Darby canine kidney (MDCK; ATCC: CCL-34) cells.

Origin	Influenza type/subtype Lineage	Strain
Human	A/H1N1	Wisconsin/588/2019
Human	A/H3N2	Darwin/6/2021
Human	B Yamagata	Phuket/3073/2013
Human	B Victoria	Austria/1359417/2021
Canine	A/H3N8	Florida/43/04
Canine	A/H3N2	Korea/LBM412/08
Avian	A/H5N1	Italy/23VIR1964-19/2023
Equine (reassortant)	A/H7N1	NIBRG-127

Table 1. Overview of all the IVs included in the study.

HI assay

All serum samples, including positive and negative controls, were pre-treated with receptor-destroying enzyme (RDE) from *Vibrio cholerae* (ratio 1:4) (Denka, Tokyo, Japan) for

18 hours at 37°C in a water bath, heat inactivation for 1 hour at 56°C, and then re-treated with 15% turkey red blood cells (ratio 1:1, final sample dilution 1:10). The antibody titre was expressed as the reciprocal of the highest sample dilution inhibiting agglutination, with titres below < 10 assigned a value of 5. In agreement with previous studies, an HI titre ≥ 40 was considered positive [24,25], with the median titres recorded.

Virus neutralization assay

MDCK cell cultures were grown at 37°C and 5% CO₂ in EMEM (Euroclone, Pero MI, Italy), supplemented with 10% v/v FBS (Euroclone, Pero MI, Italy), 1% v/v L-glutamine (Euroclone, Pero MI, Italy), 1% v/v MEM Non-Essential Amino Acids Solution 100X (NEAA) (Euroclone, Pero MI, Italy) and 1% v/v penicillin-streptomycin P/S (Euroclone, Pero MI, Italy). For the VN assay, cells were seeded in 96-well plates, and heat-inactivated samples were twofold diluted and mixed with an equal volume of virus (100 TCID₅₀/well). After 1 hour incubation, the mixtures were added to the cells and incubated for 4 days. Neutralizing antibody titres were expressed as the highest serum dilution protecting more than 50% of cells from cytopathic effect (CPE).

Statistical analysis

Data analysis was performed using GraphPad Prism Version 8.4.2 (GraphPad Software) and Microsoft Excel 2019. Data were log transformed. Lognormality of distribution was evaluated by Shapiro-Wilk test. Median and 25th and 75th percentile were assessed. Nonparametric Kruskal-Wallis method was performed to evaluate statistical significance among the IVs analysed in this study. Statistical significance was set at $*p < 0.05$.

RESULTS

To assess the seroprevalence of human IAVs and IBVs in dogs, we tested 256 samples collected in 2023 in the Apulia region, Southern Italy. Using the HI assay, H1N1 antibodies were detected in 2 samples (0.78%; 95%CI 0.09–2.79%) with titres of 40. H3N2 antibodies were found in 198 samples (77.34%; 95%CI 71.72–82.132%), with titres ranging 40–640 (Figure 1 and Table 2), and nearly all were confirmed by the VN assay. For IBV, 1 sample (0.39%; 95%CI 0.01–2.16%) tested positive for both Yamagata and Victoria lineages with a titre of 80 (Figure 1 and Table 2).

No samples tested positive for avian H5N1 or canine H3N2 and H3N8 by the HI assay, nor for H5N1 by the VN assay.

Three samples (1.17%; 95%CI 0.24–3.39%) tested HI-positive for H7N1 (titres 40 – 160) (Figure 1 and Table 2).

No sample was positive for all IAVs and IBVs, but some showed multiple positivity: 3 samples (1.17%; 95%CI 0.24-3.39%) to human seasonal H1N1 and H3N2, 1 sample (0.39%; 95%CI 0.01–2.16%) to human seasonal H3N2 and IBVs, and 3 samples (1.17%; 95%CI 0.24-3.39%) to human seasonal H3N2 and H7N1.

The comparison of HI results showed statistically significant differences between H3N2 and H1N1, H3N2 and B Yamagata, H3N2 and B Victoria ($p < 0.0001$) and H3N2 and H7N1 viruses ($p < 0.0001$). Comparisons between H1N1 and B Yamagata, H1N1 and B Victoria viruses, as well as between the two IBVs, were not statistically significant.

HI assay	IAV H1N1	IAV H3N2	IBV Yamagata lineage	IBV Victoria lineage	H7N1
Positive	2/256 (0.78%)	198/256 (77.34%)	1/256 (0.39%)	1/256 (0.39%)	3/256 (1.17%)
Negative	254/256 (99.22%)	58/256 (22.66%)	255/256 (99.61%)	255/256 (99.61%)	253/256 (93.83%)

Table 2 Prevalence of positive samples (HI titre ≥ 40) in dogs from Southern Italy in 2023 for seasonal human IAVs, A/Wisconsin/588/2019 (H1N1) and A/Darwin/6/2021 (H3N2), IBVs, Phuket/3073/2013 (B Yamagata lineage) and Austria/1359417/2021 (B Victoria lineage) and A H7N1 NIBRG-127.

DISCUSSION

Dogs and cats are susceptible to multiple IAV strains, raising concerns about zoonotic transmission due to their close contact with humans.

With roughly 900 million dogs worldwide and 8.8 million in Italy, dogs are the most common companion animals

In this study, we investigated the prevalence of antibodies against human seasonal IVs, canine and avian IAVs, and the H7N1 virus in household adult dogs, in Italy in 2023. Our findings suggest that dogs may be exposed to human seasonal IVs and H7N1, albeit at varying levels of prevalence. Avian H5N1 and canine H3N2/H3N8 antibodies were not detected.

Only a small proportion of dogs tested positive for antibodies against H1N1 IAV (0.78%) and IBVs of the Yamagata and Victoria lineages (0.39%). The H1N1-positive samples were negative for IBVs, while the IBV-positive sample showed reactivity to both B lineages. Given that circulation of the B/Yamagata lineage has not been confirmed since March 2020, and in the absence of age information for the surveyed dogs, it is plausible to hypothesize that the dog was infected with the B/Victoria lineage, and the reactivity to the B/Yamagata lineage may be due to cross-reactive antibodies[26]. Cross-reactive humoral responses between IBV lineages have been described in humans, suggesting that a large proportion of antibodies

elicited by primary infection or seasonal vaccination can recognize both B lineages [27]. Our findings are consistent with a study conducted in Japan, which reported a 1.6% seroprevalence of antibodies against human seasonal IBV (B Yamagata lineage) in dogs [20]. Although a possible natural IBV infection in a dog was reported in an earlier study, it was not confirmed by established diagnostic criteria [28]. Taken together, these findings add to the growing body of evidence that dogs can be infected with IBVs, despite these viruses being considered almost exclusively human-specific, with only rare cases reported in seals [27].

The observed 0.78% seropositivity to H1N1 IAV in dogs aligns with findings reported in other countries: 0.64% for H1N1pdm09 in Thailand, 0.9% for both H1N1pdm09 and H1N1 in Mexico, and 0.13% for H1N1pdm09 in Germany [25,29,30]. Notably, the study from Mexico City revealed that 22% and 20% of dog owners tested positive for H1N1pdm09 and H1N1 antibodies, respectively, providing evidence that these human influenza subtypes infect both dogs and their owners. This suggests the presence of factors that may be directly associated with the infection that warrant further investigation [25]. A serological study conducted in Italy during the 2009 pandemic found that 0.7% of canine samples tested positive for the pandemic virus, with no evidence of antibodies to other IVs, indicating exposure to the H1N1 pandemic strain [31]. The positive samples were collected during a period that closely coincided with the reported peak of human pandemic cases, suggesting that transmission likely occurred through aerosols or close contact between infected owners and their dogs. Given the low percentage of positivity observed in these and similar studies, it is unlikely that dogs are particularly susceptible to the H1N1 pandemic virus or H1N1 viruses in general [31]. However, considering the inherent plasticity of IVs and their capacity to adapt to new hosts such as dogs, coinfection with multiple influenza strains could facilitate reassortment events. This, in turn, may lead to the emergence of novel reassortant viruses with potential zoonotic pandemic risk [32]. However, other studies have reported higher seropositivity rates when tested for H1N1pdm09, including 1.2% in China, 1.61% in Poland, 3.8% in Japan, 4% in Ohio, 20.5% in Southern China, and 9.5% in Hong Kong [9,20,32–36]. Interestingly, the prevalence observed in household dogs differs from the serological profile of shelter dogs, which did not show reactivity to human H1N1 IVs. This suggests that the contact dynamics of dogs in urban settings may influence their exposure risk to human IVs [33].

Surprisingly, our study found that 77.34% of samples were HI-positive for human seasonal IAV H3N2, a result further confirmed by the VN assay. While this finding partially aligns with previous studies reporting H3N2 seropositivity in dogs, the prevalence observed in our study was markedly higher. Reported seropositivity rates in dogs in other regions include 1.1% in shelter dogs in Hong Kong, 4.7% in southern China, 2.2% and 2.1% in Japan, 1.2% in China, 0.9% in Mexico, and 2.4% in Ohio, USA [20,25,32–35,37]. A serological study conducted in Italy in 1981 on shelter dogs from Rome reported a 14% seropositivity rate to the human IAV H3N2 strain A/Bangkok/1/79, suggesting a likely unidirectional

transmission pathway from infected humans to dogs, rather than vice versa [38]. In Sao Paulo, Brazil, approximately 92% of urban dogs tested positive for human H3N2 [39]. Experimental and observational studies have demonstrated the susceptibility of dogs to human IVs, including the human H3N2 Hong Kong/68 virus [28,40]. Song et al. demonstrated that dogs can act as hosts for human seasonal IVs, including H3N2 and H1N1pdm09 [22]. Their study further revealed differences in the infectious efficacy of these viruses in dogs, with the seasonal H3N2 virus exhibiting higher infectivity compared to the others. These findings align with and support the high prevalence observed in our study. Notably, during the 2022–2023 influenza season, IAVs accounted for 80.2% of human cases, with the majority belonging to the H3N2 subtype [41]. The high prevalence of the H3N2 virus observed in our study may be attributed to its widespread circulation among humans. We can speculate, but cannot confirm, that transmission of H3N2 occurred through close contact or aerosol exposure between infected owners and their dogs. Other factors, such as the specific influenza strain, the level of contact between dogs and humans, and the health status of both, may also contribute to interspecies virus transmission and warrant further investigation. As our study was based on serologic analyses, no direct correlation between IAV/IBV infection and clinical signs in dogs could be established.

Dogs possess both avian-like α -2,3-linked sialic acid receptors and human-like α -2,6-linked sialic acid receptors in the endothelial cells of their respiratory tract and other organs [42]. The α -2,3-linked sialic acid receptors are the preferred receptor types for avian IVs, suggesting a molecular basis for the potential direct transmission of avian viruses to dogs [43,44]. Existing field and experimental data collectively suggest dogs are susceptible to infection by highly pathogenic avian influenza (HPAI) of the H5N1 subtype, although clinical outcomes vary [44–46]. Infection appears to be limited primarily to dogs with high levels of viral exposure. A recent study of bird-hunting dogs in Washington, USA, reported antibodies to H5 and N1 subtype IAVs in 2% of the sampled population [47]. By contrast, in our study population, no antibodies were detected to these antigens, likely because the dogs had not been exposed to any source of the H5N1 virus, as they were primarily domestic and not involved in activities with higher exposure risk.

Three dogs tested positive for H7N1 subtype with various HI titres. To the best of our knowledge, serological evidence of H7 virus exposure in dogs has not been previously reported. However, it is important to point out that the H7N1 included in this study is not highly pathogenic and of avian origin. Wild birds are the natural reservoir of H7 subtypes [48]. In Europe, H7 avian IVs have caused multiple poultry outbreaks that also resulted in occasional human infections, often following direct contact with infected birds. Between 1999 and 2003 both highly (HPAIV) and low pathogenicity (LPAIV) A(H7N1) viruses, as well as LPAIV A(H7N3), have caused outbreaks on poultry farms located mainly in North-Eastern Italy [48]. Between 1999 and 2001, LPAIV H7N1 was identified in 199 outbreaks, mostly in commercial poultry in north-eastern Italy and in December 1999 a mutated HPAIV H7N1 variant emerged, infecting over 413 poultry farms.

Although our data are preliminary, the detection of antibodies to an H7 virus in our canine population is a novel finding, and it may suggest potential spillover of an H7 virus similar to the strain used in this study. Indeed, we used a laboratory-generated reassortant H7N1 IAV strain, with the HA derived from an extinct equine H7N7 IAV strain, and the genome backbone of a human H1N1 IAV. Accordingly, the reactivity to the H7 antigen in the canine serum samples should be considered with caution. Additional investigations, using, for instance, a broad array of H7 antigens representative of the major H7 lineages, could help better contextualize these findings.

Our population also tested negative for canine H3N2 and H3N8 IVs. A previous seroprevalence study of H3N8 and H3N2 CIVs conducted primarily in the same area on samples collected from pet and kennel dogs between 1997 and 2011, reported a low ELISA positivity rate of 3.56%, with 2 H3N8 and 47 H3N2 positive samples confirmed by HI assay [49]. This low seropositivity was attributed to the nature of the study as a population-based prevalence survey, similar to ours, rather than one focused on animals showing clinical signs of respiratory disease. These considerations, along with the fact that H3N2 CIV has limited or no circulation in Europe, and that H3N8 CIV is considered extinct since 2016 may explain our negative results [18].

This study has some limitations. Serum samples were collected from a single geographic area, and data on the sex and age of the dogs were not available. Additionally, we did not have paired samples from dogs and their owners, which limited our ability to assess the seroprevalence of influenza virus within households.

Overall, our findings suggest that household dogs can be exposed to human seasonal IAVs and IBVs. The potential for dogs to be exposed to human IVs underscores the importance of monitoring interspecies transmission between humans and companion animals. As dogs are susceptible to multiple influenza virus subtypes, co-infection with different viruses may lead to viral reassortment and the emergence of novel viruses potentially able to infect humans. Ongoing surveillance of IVs in dog populations is therefore crucial to advancing the “One Health” approach in managing the risks associated with this zoonotic disease and should be considered as an important component of pandemic preparedness.

Author Contributions

Claudia Maria Trombetta: conceptualization, data curation, formal analysis, funding acquisition, project administration, resources, supervision, visualization, writing – original draft. Maria Giovanna Marotta: data curation, formal analysis, investigation, writing – review and editing, visualization. Serena Marchi: investigation, writing – review and editing. Francesco Bonfante: investigation, resources. Silvia Maniero: investigation. Alessandra Napolitano: investigation. Ana Moreno: writing – review and editing. Chiara Chiapponi: writing – review and editing. Nigel Temperton: conceptualization, writing – review and editing. Simon Scott: writing – review and editing. Janet M. Daly: writing – review and editing. Alessandro Manenti: resources, writing – review and editing. Emanuele Montomoli: resources. Gianvito Lanave: data curation, formal analysis, visualization, writing – review and editing. Michele Camero: resources, writing – review and editing. Vito Martella: writing – review and editing.

All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding author.

Conflicts of Interest

EM is founder and Chief Scientific Officer of VisMederi srl. AM is an employee of VisMederi Srl.

Ethics approval statement

Ethical approval was waived by the Ethics Committee of the Department of Veterinary Medicine of the University of Bari Aldo Moro, as the samples were collected during routine veterinary procedures and no additional sampling procedures were performed for the purposes of this study

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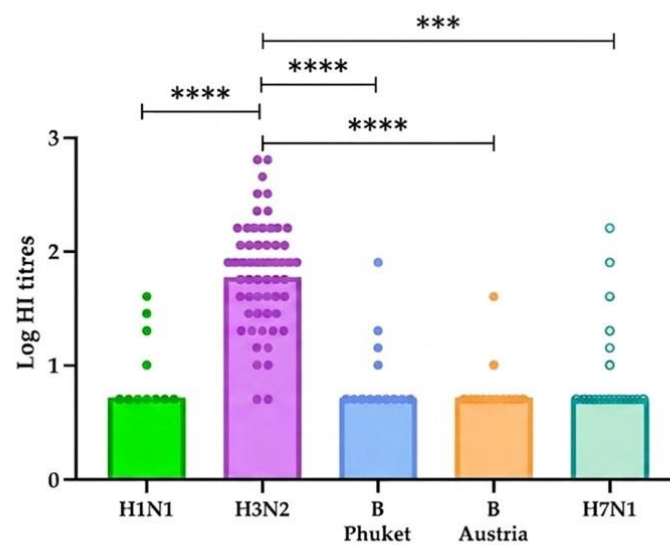
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Figure 1



Highlights

- Dogs have not typically been considered natural hosts for influenza viruses.
- A serological investigation was conducted to assess the seroprevalence of human seasonal influenza A and B viruses, canine and avian influenza A viruses, and the H7N1 virus in a population of adult household dogs.
- Household dogs can be exposed to human seasonal influenza A and B viruses.