Serological exposure to influenza A in cats from an area with wild birds positive for avian influenza

Short running title: Anti-influenza A antibodies in cats

Sergio Villanueva-Saz[1,2,3*], Mariví Martínez[2], Pablo Rueda[1], María Dolores Pérez[3,4], Delia Lacasta[2,3], Diana Marteles[2], Héctor Ruíz[2], Ana Gonzalez[2], Maite Verde[1,2,3], Julián Pardo[5,6,7], Maykel Arias[5,7], Natacha Peña-Fresneda[5], Antonio Fernández[1,2,3], Michele Trotta[1]

[1]Clinical Immunology Laboratory, Veterinary Faculty, University of Zaragoza, Spain

[2] Department of Animal Pathology, Veterinary Faculty, University of Zaragoza, Spain

[3]Instituto Agroalimentario de Aragón-IA2 (Universidad de Zaragoza-CITA), Spain

[4] Department of Animal Production and Sciences of the Food, Veterinary Faculty, University of Zaragoza, Spain

[5]Aragon Health Research Institute (IIS Aragón), Zaragoza, Spain

[6]Department of Microbiology, Pediatrics, Radiology and Public Health, Zaragoza University of Zaragoza, Spain

[7]CIBER de Enfermedades Infecciosas, Instituto de Salud Carlos III, Spain

Corresponding author*: Sergio Villanueva-Saz, Veterinary Faculty, University of Zaragoza, 50013 Zaragoza, Spain Tel. +34 679 727 285, E-mail: svs@unizar.es (SVS)

Summary

Influenza A is an emerging zoonotic virus with worldwide distribution. To our knowledge, no studies have been conducted to assess influenza A exposure in stray cats in regions with positive cases of wild birds. This study aimed to determine the seroprevalence of anti-influenza A antibodies in feral cats from a region in Spain with cases of positive wild birds. A cross-sectional study of stray cats (n=183) was conducted between March 2022 and March 2023. The presence of antibodies against the influenza A virus was tested using a commercial enzyme-linked immunosorbent assay kit adapted for this study and confirmed by competitive enzyme-linked immunosorbent assay for the detection of antibodies against the hemagglutinin H5. During sample collection, none of the cats exhibited clinical signs of illness. Four of the 183 animals tested showed anti-influenza A antibodies by ELISA, and the seroprevalence of influenza A was 2.19% (95% confidence interval 0.85-5.48%). Due to the low number of positive cases detected, it appears that cats did not have an important epidemiological role in influenza A transmission during this period.

Keywords: Cat; ELISA; Epidemiology; Influenza A; Serology

Impacts

- According to the literature, cats appear to be the dead-end host species for influenza viruses.
- Cats are more likely to be infected by humans because of seasonal influenza viruses (anthrophozoonosis) than cat-to-human transmission.
- In stray cats, the main risk could be associated with feeding or playing with dead, potentially infected wild birds.

1 INTRODUCTION

Influenza is a highly contagious viral infection that affects humans. The virus belongs to the Orthomyxoviridae family and is characterised by four known types: A, B, C, and D (Hause et al., 2014). Influenza type A (IAV) is the most common influenza virus in humans and causes seasonal epidemics (Taubenberger et al., 2010). However, IAV may also affect other mammals and birds (Frymus et al., 2021).

Traditionally, the reservoirs of IAV have been waterbirds of the orders Anseriformes and Charadriiformes (Latorre-Margalef et al., 2014). Birds, which are often subclinically infected, can shed IAV in their faeces. Therefore, the seasonal migration of these birds can help to circulate IAV around the world (Krammer et al., 2018). The strains of avian influenza are classified into two groups according to the severity of the clinical disease: low pathogenicity avian influenza (LPAIV, associated with subclinically infected birds) and highly pathogenicity avian influenza (HPAIV, associated with high mortality rates) (Spackman, 2020).

IAV has been isolated from numerous mammalian species, including humans, pigs, horses, cats, dogs, pinnipeds, cetaceans, and minks (Landolt & Olsen, 2007). Several studies have demonstrated the circulation of IAV strains adapted to domestic dogs and cats. In 2004 in Thailand, several domestic cats were found to be infected with an HPAI H5N1 strain (Tiensin et al., 2005). In subsequent years, single cases of cat infection with H5N1 HPAI have been reported in different parts of the world (Songserm et al. 2006; Yingst et al. 2006; Amonsin et al. 2007; Klopfleisch et al. 2007). After the 2014 outbreak of H5N6 in humans, a cat was reported to die following infection with H5N6 in China (Yu et al. 2015).

An outbreak of the H7N2 influenza virus occurred in late 2016 among cats housed in an animal shelter in New York (Lee et al., 2017). Furthermore, the H1N1 virus caused an outbreak of disease in 90 cats in Italy (Fiorentini et al., 2011). Recently, the H5N1 clade 2.3.4.4b virus was detected in a domestic cat that lived near a duck farm infected by a closely related virus in France during December 2022 (Briand et al., 2023). The presence of antibodies to IAV of both avian and human origin is common in cats living in shelters in Europe (Zhao et al., 2020).

Reports have confirmed the presence of a high number of positive for the HPAIV H5N1 virus, clade 2.3.4.4b, in Europe between 3 December 2022 and 1 March 2023 (EFSA et al., 2023). In Spain, an outbreak of HPAIV between May 2022 and March 2023 was reported, and the Spanish authorities also reported 100 cases in wild birds and one in poultry (WOAH, 2023).

To date, limited information is available on the degree of IAV exposure in stray cats. Therefore, this epidemiological survey aimed to investigate the seroprevalence of the influenza A virus in urban stray cats in an area with cases positive for HPAIV.

2 METHODS

2.1 Animals and sample collection

The study was conducted in the city of Zaragoza, Aragón, Spain (41°38′58.8948″N, 0°53′15.7632″W) on stray cats. The cats were captured from different urban and peri-urban areas of Zaragoza. Data on the breed, sex, date of blood collection, and colony of origin of each cat were recorded. A complete physical examination, including chest auscultation, was performed before sampling. Only cats older than one year, classified as apparently healthy based on general examination, were included. Blood was collected aseptically to obtain the serum. Separated serum was stored at −40 °C until processing and analysis. This survey was included under Project Licence PI75/20 approved by the Ethic Committee for Animal Experiments for the University of Zaragoza. The care and use of animals were performed according with the Spanish Policy for Animal Protection RD 53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

Samples were collected between April and May 2016 (n=80), March and July 2022 (n=87), and September 2022 and March 2023 (n=96). In the first period, we analysed 85 frozen (-40 °C) archived serum samples from feral cats from the region using enzyme-linked immunosorbent assay (ELISA) threshold determination.

The second group was collected during the official declaration of the presence of positive cases of H5N1 in wild birds in the Aragón region. The third group of samples was collected during a period of positive cases of HPAIV H5N1 in Zaragoza City, including different infected wild bird species (Boletin Epidemiológico Semanal de Aragón, 2023).

Finally, a control group consisting of serum samples from cats that were not allowed outdoor access was also included. These samples were collected before (n=15) and during (n=15) the official declaration of the presence of positive cases of H5N1.

2.2 Modified commercial ELISA for the detection of anti-influenza A antibodies

Serum samples were tested using a modified commercial indirect ELISA kit (IDVet, Montpellier, France). The modification involved the replacement of the commercial conjugate with another conjugate composed of protein A/G peroxidase. This reagent interacts with immunoglobulin G in several animal species (Ejercito et al., 1993; Inoshima et al., 1999).

First, a 70 µl aliquot of cat serum diluted 1:40 in dilution buffer 2 was added to each well. The plates were then incubated for 1 h at 37 °C in a moist chamber. The wells were washed with approximately 300 µl of wash solution five times. Then, 50 µl of protein A/G conjugated to horseradish peroxidase (Thermo Fisher Scientific) diluted 1:10,000 in PBS containing 0.05% Tween 20 and 1% dry skim milk were added to each well. The plate was incubated for 30 min at 25 °C in a moist chamber and was washed again with wash solution three times as described above. The substrate solution (ortho-phenylene-diamine) and stable peroxide substrate buffer (Thermo Fisher Scientific) were added to each well, and the reaction was allowed to develop for 10 ± 1 min at room temperature in the dark. The reaction was terminated by adding 2.5 M H_2SO_4 to each well. Absorbance was read at 492 nm using an automatic microplate reader.

As a positive control, each plate included a positive control provided by the kit and serum from a mouse that had been experimentally infected. C57BL6/j mice from Envigo were infected with 2×10^2 PFU of mouse-adapted PR8 influenza A H1N1 virus. This experimental infection was included under Project Licence PI78/20 approved by the Ethic Committee for Animal Experiments for the University of Zaragoza. Blood and serum samples were collected through cardiac puncture 21 days

after infection. Serum was collected 21 days after virus inoculation. Negative controls provided by the kit and serum from healthy, non-infected mice were used as negative controls. All samples and controls were analysed in duplicate.

2.3 ELISA cut-off determination

To determine the cut-off in the absence of serum from a positive status, we used the mean value from a known negative animal population reference and added three standard deviations (SD) to the mean optical density (OD) to determine the cut-off, which is the most common approaching used in veterinary diagnostics for detecting different pathogens (Saraswati et al., 2019; Villanueva-Saz et al., 2022a).

In our study, the cut-off of the modified commercial ELISA was calculated as the mean + 3 SD of the value of 80 stray cats, and the results above this value were considered positive. Serum samples obtained from stray cats in the same area in 2016 were included in the negative cat population. In 2016, no cases of H5N1 infection were reported in this region. These samples were collected from previously published epidemiological studies performed in the same region as this study, and the animals were classified as healthy (Alcover et al., 2021). The cut-off value was established at 0.23 OD.

2.4 Commercial competitive ELISA for the detection of antibodies against the hemagglutinin H5 of the Avian Influenza

Serum samples were tested using a modified commercial competitive ELISA kit (IDVet, Montpellier, France). The ELISA was performed following the instructions of the manufacturer. The wells of the plate were coated with the hemagglutinin H5. Specimens to be tested and controls were added to the microwells. Anti-H5 antibodies, if present, form an antibody-antigen complex which masked the H5 epitopes. An anti-H5-peroxidase (HRP) conjugate was added to the microwells. It fixed to the remaining free H5 epitopes, forming an antigen-conjugate-HRP complex. After washing in order to eliminate the excess conjugate, the substrate solution (TMB) was added. The resulting coloration depends on the quantity of specific antibodies present in the specimen to be tested. In the absence of antibodies, a blue coloration appeared which became yellow after addition of the Stop Solution. By contrast, no coloration was observed when the antibodies were present. For each sample, calculate

the competition percentage was obtained (S/N % $= \frac{\text{OD sample}}{\text{OD negative control}}$ x 100. Sera were classified as negative status, when having a competition percentage greater than or equal to 60%, doubtful status were classified as competition percentage between 50% and 60%. Finally, positive status were sera from those cats with a competition percentage less than or equal to 50%. This test was performed in serum samples that tested positive in the modified commercial ELISA for influenza A antibody detection as complementary technique.

2.5 *Detection of feline coronavirus (FCoV) antibodies using an immunochromatographic test and anti-SARS-CoV-2 antibodies using an in-house ELISA*

Two serological diagnostic techniques were employed to detect FCoV and anti-SARS-CoV-2 antibodies in cases that were positive for anti-influenza A antibodies in the serum. A rapid test was employed for FCoV antibodies (FASTest® FIP, MEGACOR Diagnostik), while, an in-house ELISA was performed to detect SARS-CoV-2 antibodies as previously described (Villanueva-Saz et al., 2022b).

2.6 *Statistical analysis*

The Statistical Package for the Social Sciences (SPSS Inc. IBM Corp., Chicago, IL, USA) was used for the statistical analysis. Associations between the presence of anti-influenza A antibodies and the recorded variables (period of sampling, sex, and type of environment [urban/peri-urban]) were analysed. The significance of these differences was assessed using Fisher's exact test and the chi-square test. A *P* value of <0.05 was considered statistically significant.

3 RESULTS

A total of 183 stray cats (77 males, 101 females, and five undetermined) were included in this study. All animals were of the shorthaired type and were classified as apparently healthy, with no evident systemic signs on general physical examination. No auscultation problems were observed in any cat. The modified commercial ELISA found that four cats (three males and one female) were positive, with a seroprevalence of influenza A of 2.19% (95% confidence interval [CI] 0.85-5.48%) and with OD units ranging from 0.26 (n=1), 0.29 (n=1), 0.35 (n=1), to 0.96 (n=1) (cut-off $\geq$0.23). Among the four tested positive cats, antibodies against the hemagglutinin H5 of the Avian Influenza virus were detected with a competition percentage ranging from 37% (n=1) 40% (n=1), 42% (n=1) to 45% (n=1).

All seropositive cats were of peri-urban origin, and no seropositive results emerged from cats living in urban areas. The seropositive samples were obtained at the following time points: September 2022 (n=1), November 2022 (n=2), and March 2023 (n=1). None of the cats that were seropositive for influenza A were also positive for SARS-CoV-2 or FCoV.

Considering the different time points of sample collection, the OD (mean±SD) detected by the modified commercial ELISA from March to July 2022 was 0.09±0.03, while the OD from September 2022 to March 2023 was 0.11±0.10. In the case of control group, the OD was 0.08±0.04.

Finally, no significant associations ($p>0.005$) were found between the positivity for anti-influenza A antibodies and the period of sample collection or sex and, seropositivity between samples from control group and samples obtained during study period. However, a significant association was observed between the type of environment (peri-urban) and seropositivity ($p=0.047$).

4 DISCUSSION

We have serologically demonstrated the exposure to IAV in stray cats in Spain for the first time. Since 2004, when the first infection occurred in a cat, different subtypes of influenza virus have been detected in cats, including different strains with different pathogenicities (H5N1 HPAI, H5N6 LPAI, H1N1, two novel H5N6, H7N2, and the last variant H9N2; Table 1).

Three mass mortality events related to HPAI A (H5N1) in mammals have been reported (the United States of America (USA) and Spain in 2022 and Peru in 2023). The first was an unusual mortality event of seals on the coast of Maine, USA, in the summer of 2022, which coincided with an outbreak of HPAI A (H5N1) virus in wild birds in the region (Puryear et al., 2022). The second outbreak occurred at a farm of 52,000 American minks housed in open barns in Galicia, northwest Spain (Aguero et al., 2023) and was associated with a mortality rate of 4.3%. The open housing system of mink farming possibly allowed direct infection through wild bird to mink contact (Sikkema et al., 2022). The third HPAI A (H5N1) outbreak in mammals involved two species of sea lions in Peru (Gamarra-Toledo et al., 2023).

Cat to cat transmission was reported in a shelter in New York and was attributed to close contact between the animals (Lee et al., 2017; Blachere et al., 2018; Hatta et al., 2018). Due to the high number of animals and high viral circulation, the infection was able to pass from cat to cat and later from cat to human shelter workers. Housed cats are more likely to be infected by humans with

seasonal influenza viruses via human to cat transmission than cat to human transmission. According to the literature, cats appear to be the dead-end host species for influenza viruses (Harder et al., 2010).

In conclusion, the serological results provide evidence of influenza A virus exposure in cats in Spain between September 2022 and March 2023. However, the classification of these animals as apparently healthy based on physical examinations suggests that the epidemiological role of cats is limited. Due to the low number of seropositive animals detected in our study, cat to cat transmission was considered highly unlikely, and predation or consumption of an infected source was considered the most plausible form of transmission.

Author Contributions

Conception and design of the study: SVS, DP and JP. Collection the serum samples: AG and MTV. Experimental infection for serum controls: MA and NPF. Assembly of the data set: MM, PR and DM. Statistical analysis: AF. Interpretation of results: SVS, AF and MT. Drafter the initial article: SVS, MM and MT. Reviewing and editing the article: DL and HR. Final approval of article: All authors.

Conflict of Interest Statement

The authors declare that they have no competing interests.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics Statement

The samples analysed in this study were part of a Project Licence PI75/20 (cat) and PI78/20 (mouse) approved by the Ethic Committee for Animal Experiments for the University of Zaragoza. The care and use of animals were performed according with the Spanish Policy for Animal Protection RD

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