



## Prevalence of influenza A H5N1 virus in cats from areas with occurrence of highly pathogenic avian influenza in birds

**Julia Marschall<sup>1</sup>, Bianka Schulz** Dr Med Vet, Dipl ECVIM-CA<sup>1</sup>,  
**Timm C Harder Priv-Doz** Dr Med Vet, PhD<sup>2</sup>,  
**Thomas W Vahlenkamp Priv-Doz** Dr Med Vet, PhD<sup>3</sup>,  
**Janine Huebner** Dr Med Vet<sup>4</sup>, **Elke Huisinga** Dr Med Vet<sup>5</sup>,  
**Katrin Hartmann** Dr Med Vet, Dr Habil, Dipl ECVIM-CA, Prof<sup>1\*</sup>

<sup>1</sup>*Clinic of Small Animal Medicine, Ludwig Maximilian University, Veterinärstrasse 13, 80539 Munich, Germany*

<sup>2</sup>*Office International des Epizooties and National Reference Laboratory for Avian Influenza, Friedrich-Loeffler-Institute, Greifswald-Insel Riems, Germany*

<sup>3</sup>*Institute of Molecular Biology, Friedrich-Loeffler-Institute, Greifswald-Insel Riems, Germany*

<sup>4</sup>*Laboklin GmbH & Co KG, Bad Kissingen, Germany*

<sup>5</sup>*Vet Med Laboratory GmbH, Ludwigsburg, Germany*

Natural and experimental infections have shown that cats are susceptible to highly pathogenic avian influenza A virus subtype H5N1 (HPAIV H5N1). Cats can be severely affected and die from the disease, but subclinical infections have also been reported. To learn more about the role of cats in the spread of the virus and about the risk posed to cats, the prevalence of H5N1 virus was examined in 171 cats from areas in Germany and Austria in which birds infected with HPAIV H5N1 had been found. Pharyngeal swabs were examined for H5N1 virus using real-time polymerase chain reaction, and serum samples were tested for antibodies to influenza virus. None of the cats showed evidence of infection with H5N1 virus. Prevalence of H5N1 virus was determined to be <1.8% (95% confidence interval (CI): 0.000000–0.017366); prevalence of antibodies was <2.6% (95% CI: 0.000000–0.025068).

Date accepted: 13 March 2008

© 2008 ESFM and AAEP. Published by Elsevier Ltd. All rights reserved.

Since its first appearance in 1996 (Xu et al 1999, Li et al 2004), highly pathogenic avian influenza virus of the subtype H5N1 (HPAIV H5N1) has spread nearly worldwide resulting in high mortality in poultry (World Health Organization 2007). Its ability to cross the species barrier without prior adaptation and to infect many mammal species including humans, has raised concerns about a new influenza pandemic (Subbarao et al 1998, Vahlenkamp and Harder 2006).

Among mammalian species, especially cats proved to be susceptible to natural and experimental infections with HPAIV H5N1

(Kuiken et al 2004, Songserm et al 2006). Large felids as well as domestic cats can be severely affected and die from the disease. Acute respiratory signs caused by severe pulmonary changes (interstitial pneumonia, diffuse alveolar damage, haemorrhage, and oedema) and pleural effusion are among the main features (Thanawongnuwech et al 2005, Rimmelzwaan et al 2006). Affected cats can also show pyrexia, depression, serosanguinous nasal discharge, conjunctivitis, protrusion of the nictitating membrane, and neurological signs (Thiry et al 2007). Subclinical infections, however, have also been reported (Leschnik et al 2007).

All natural infections described in cats so far, have been spatially and temporally associated with the occurrence of HPAIV H5N1 infections in poultry or wild birds in the surrounding area

\*Corresponding author. Tel: +49-89-2180-2651. E-mail: hartmann@uni-muenchen.de

(Keawcharoen et al 2004, Klopfleisch et al 2007, Leschnik et al 2007). Cats cannot only be infected by direct or indirect contact with infected birds (Songserm et al 2006, Leschnik et al 2007); the virus can also be transmitted horizontally from cat to cat (Kuiken et al 2004, Thanawongnuwech et al 2005). Experts, therefore, think that cats might play a role in the epidemiology of HPAIV H5N1 (Kuiken et al 2004, 2006, Songserm et al 2006).

It is unknown, how high the actual risk is for pet cats to become infected in areas with highly pathogenic avian influenza in birds. It is also unknown if cats play a role in the spread of the virus. The aim of this study, therefore, was to determine the prevalence of H5N1 virus and antibodies in cats with access to outdoors in areas with occurrence of highly pathogenic avian influenza in birds.

## Materials and methods

A total number of 171 cats were included in this prospective study. Inclusion criterion to enter the study was regular and current outdoor access. Additionally, cats had to meet at least one of the following criteria. Cats included either lived in a restriction zone, which is defined as an area within a 10 km radius around the location of a detected outbreak of avian influenza in birds, or they showed signs of acute respiratory disease in an area close to a restriction zone. Samples were taken between March and June 2006, in August and September 2006, and in July and August 2007. During these periods, cases of HPAIV H5N1 were observed in wild birds in Germany. Samples of cats from restriction zones were taken during the time of official declaration of the respective area as 'protection zone' or 'surveillance zone'. Cats with respiratory signs also came from areas relatively close to areas with prior or future outbreaks of avian influenza in birds (less than 100 km).

One hundred and thirty-two cats lived in restricted zones in Germany (131/132) and Austria (1/132), 28 cats were presented to a veterinarian because of acute respiratory signs, and 11 cats met both criteria. All cats underwent routine physical examination including auscultation of the chest. In 94 animals, complete blood count and serum biochemistry profiles were performed.

A real-time reverse transcriptase polymerase chain reaction (RRT-PCR) for H5N1 virus detection was performed in all cats from pharyngeal swabs. Either dry rayon swabs were used (Copan innovation, Brescia, Italy) (36/171 animals)

or swabs were placed into virus transport medium containing antibiotics (Virocult, Medical Wire and Equipment, Corsham, UK) (135/171 cats). Swabs were stored at  $-70^{\circ}\text{C}$  prior to further examination.

RNA was extracted with RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol in 154 cats. In 17 cats, a modified protocol of QIAamp DNA blood mini kit (Qiagen, Hilden, Germany) was used.

Three different RRT-PCR assays were used. Samples of 158 cats were examined using an RRT-PCR for the detection of influenza A matrix gene. Out of those, 141 samples were examined according to the method described by Spackman et al (2002) that was modified by inclusion of an internal control (Hoffmann et al 2006). In the other 17 cats, the commercially available kit 'Light Mix for the detection of Influenza Virus A M2' (TIB-Molbiol, Berlin, Germany) was used. H5- and N1-specific RRT-PCRs would have followed in positive samples for subtype confirmation. RRT-PCRs using primers for influenza A matrix gene and haemagglutinin gene (H5) were conducted in 13 cats with TaqMan Influenza A/H5 Detection Kit v1.0 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol.

Plasma samples were obtained from 118 cats and were stored at  $-20^{\circ}\text{C}$ . They were tested for antibodies to avian influenza viruses of the subtypes H5 and H7 (H5N1, H5N2, H7N1, and H7N7) using a haemagglutination inhibition test according to the procedures of the World Organization for Animal Health (Alexander 2004).

Statistical analysis was performed using an exact binomial test for determination of exact confidence intervals (CIs) (Clopper and Pearson 1934). The exact binomial test was one-tailed and was used to prove the alternative hypotheses that the prevalence of H5N1 virus and the prevalence of antibodies are within the 95% CI. A significance level of  $<0.05$  was chosen.

## Results

Samples from all cats were negative for influenza A H5 virus-specific nucleic acid by RRT-PCR. Likewise, all serum samples obtained were negative for antibodies to the tested influenza virus subtypes. On the basis of these data, prevalence of H5N1 virus was determined between 0 and 1.8% (95% CI: 0.000000–0.017366). Prevalence of antibodies to H5N1 virus was between 0 and 2.6% (95% CI: 0.000000–0.025068).

## Discussion

In this study, no evidence of infection with avian influenza virus H5N1 in cats was found. No symptomatic infections were detected; no antibodies to subclinical or past infections were present.

A limitation of the study is the relatively small number of animals. Nevertheless, it is important to publish these data, as very little is known about the epidemiological role of cats in avian influenza so far. When the first cases of avian influenza occurred in Germany, there was great uncertainty among cat owners and veterinarians, especially when the virus was detected in cats on the Isle of Rügen. Rectal swabs could have been examined instead of pharyngeal swabs, as it is now known that infected cats may excrete virus in their faeces (Rimmelzwaan et al 2006). In subclinically infected cats, however, virus has only been detected in pharyngeal swabs (Leschnik et al 2007). Despite the limited data, this study is able to show that in epidemiological situations, as they occurred in Germany and Austria, the risk of cats contracting influenza A H5N1 is low. In the cats examined in this study, the prevalence of H5N1 virus (171/171 cats negative) and antibodies (118/118 cats negative) was 0%. Hence, it can be concluded that, with a probability of 95%, the prevalence of H5N1 virus in cats in Germany and Austria is less than 1.8% and prevalence of antibodies less than 2.6%.

The majority of the samples (157/171) were collected in Bavaria, South-Eastern Germany. Bavaria was the German state exhibiting the largest number of birds (poultry and wild birds) that had died from HPAIV H5N1 infection. Until the time of writing, 92 infected wild birds have been found and two poultry farms have been affected. Sixty-two (62/171) samples were collected in the rural district of Landsberg am Lech, Bavaria, Germany. In this district, five dead wild aquatic birds had confirmed infections of HPAIV H5N1 between February and May 2006. Thirty-six (36/171) samples derived from an area around a lake north of Munich, Bavaria, Germany, where three dead diving ducks were found infected with HPAIV H5N1 in August 2007. Samples of 34 cats were collected in Nuremberg, Bavaria, Germany, where in June and July 2007 HPAIV H5N1 was found in 16 wild aquatic birds. According to EU legislation (Council of the European Union 2006), protection zones with a radius of 3 km were established for 21 days and surveillance zones with a 10 km radius were established

for 30 days around the places of discovery of the birds. Within these zones, cats were supposed to be kept indoors. All cats included in this study, however, were still allowed to roam outside despite this recommendation and thus certainly belonged to a high-risk group.

The fact that no evidence of subclinical or past infections was found in the cats included in this study is in contrast to the indication that in Asia a high percentage of cats may carry antibodies to H5N1 virus (Butler 2006, Mackenzie 2007). As the results of these Asian studies have not yet been scientifically evaluated, and there is no reference to the applied methods, it must be considered that the numbers are falsely high. On the other hand, experts think that the numbers could be even higher, as cats that were ill or had died had not been included (Mackenzie 2007). The virus strains circulating in Asia could possibly have a higher pathogenicity in cats than the European lineage. However, mainly isolates of the European–Middle Eastern–African (EMA) lineage, which circulates in Europe, show a mutation associated with high pathogenicity in mammals (Salzberg et al 2007), and EMA-type viruses were also responsible for fatal infections in previously healthy cats (Yingst et al 2006, Klopffleisch et al 2007). For a definite answer, however, experimental studies would be necessary. The difference may also be due to lower risk of exposure to the virus and lower infection pressure in Europe. Contrary to the situation in Asia, in Europe only single wild birds and just four poultry farms were affected. Cats included in this study were pet cats, which were fed by their owners and thus did not rely on hunting birds. A serological study in cats from Milan, Italy, also did not find any evidence of antibodies to influenza A viruses – neither to subtype H5N1 nor to other subtypes (Paltrinieri et al 2007). However, no outbreaks of avian influenza had occurred in this sampling area in Italy and thus, the likelihood of finding cats with antibodies there was extremely low.

Cats, at least in Europe, do not seem to play a major role in the transmission of the virus. This circumstance may change, however, as the virus can rapidly acquire new properties by genetic mutation and reassortment (Webster et al 1992).

This study may contribute to show cat owners and veterinarians that – in anticipation of further outbreaks of highly pathogenic avian influenza H5N1 virus – there is no reason to believe that cats pose a major risk to humans.

## Acknowledgements

We thank all persons involved in the sample collection for their support and Monia Mahling and Prof Dr Helmut Küchenhoff from the Institute of Statistics, University of Munich, for statistical analysis.

## References

- Alexander DJ (2004) Avian influenza. In: OIE International Committee (ed), *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Mammals, Birds and Bees)* (5th edn). Paris: Office International des Epizooties. Available at: <[http://www.oie.int/eng/normes/mmanual/A\\_00037.htm](http://www.oie.int/eng/normes/mmanual/A_00037.htm)>.
- Butler D (2006) Thai dogs carry bird-flu virus, but will they spread it? *Nature* **439**, 773.
- Clopper CJ, Pearson ES (1934) The use of confidence or fiducial limits illustrated in the case of the binomial. *Biometrika* **26**, 404–413.
- Council of the European Union. (2006) Council directive 2005/94/EC of 20 December 2005 on community measures for the control of avian influenza and repealing directive 92/40/EEC. *Official Journal of the European Union* **L 10**, 16–65 (14.1.2006).
- Hoffmann B, Depner K, Schirmer H, Beer M (2006) A universal heterologous internal control system for duplex real-time RT-PCR assays used in a detection system for pestiviruses. *Journal of Virological Methods* **136**, 200–209.
- Keawcharoen J, Oraveerakul K, Kuiken T, Fouchier RA, Amonsin A, Payungporn S, Noppornpanth S, Wattanodorn S, Theamboonlers A, Tantilertcharoen R, Pattanarangsarn R, Arya N, Ratanakorn P, Osterhaus AD, Poovorawan Y (2004) Avian influenza H5N1 in tigers and leopards. *Emerging Infectious Diseases* **10**, 2189–2191.
- Klopffleisch R, Wolf PU, Uhl W, Gerst S, Harder T, Starick E, Vahlenkamp TW, Mettenleiter TC, Teifke JP (2007) Distribution of lesions and antigen of highly pathogenic avian influenza virus A/Swan/Germany/R65/06 (H5N1) in domestic cats after presumptive infection by wild birds. *Veterinary Pathology* **44**, 261–268.
- Kuiken T, Rimmelzwaan GF, Van Riel D, Van Amerongen G, Baars M, Fouchier RA, Osterhaus AD (2004) Avian H5N1 influenza in cats. *Science* **306**, 241.
- Kuiken T, Fouchier RA, Rimmelzwaan GF, Osterhaus AD, Roeder P (2006) Feline friend or potential foe? *Nature* **440**, 741–742.
- Leschnik M, Weikel J, Möstl K, Revilla-Fernández S, Wodak E, Bagó Z, Vanek E, Benetka V, Hess M, Thalhammer JG (2007) Subclinical infection with avian influenza A (H5N1) virus in cats. *Emerging Infectious Diseases* **13**, 243–247.
- Li KS, Guan Y, Wang J, Smith GJ, Xu KM, Duan L, Rahardjo AP, Puthavathana P, Buranathai C, Nguyen TD, Estoepongastie AT, Chaisingh A, Auewarakul P, Long HT, Hanh NT, Webby RJ, Poon LL, Chen H, Shortridge KF, Yuen KY, Webster RG, Peiris JS (2004) Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. *Nature* **430**, 209–213.
- Mackenzie D (2007) Deadly H5N1 may be brewing in cats. *New Scientist* 6–7.
- Paltrinieri S, Spagnolo V, Giordano A, Martin AM, Luppi A (2007) Influenza virus type A serosurvey in cats. *Emerging Infectious Diseases* **13**, 662–664.
- Rimmelzwaan GF, Van Riel D, Baars M, Bestebroer TM, Van Amerongen G, Fouchier RA, Osterhaus AD, Kuiken T (2006) Influenza A virus (H5N1) infection in cats causes systemic disease with potential novel routes of virus spread within and between hosts. *American Journal of Pathology* **168**, 176–183.
- Salzberg SL, Kingsford C, Cattoli G, Spiro DJ, Janies DA, Mehrez Aly M, Brown IH, Couacy-Hymann E, De Mia DM, Dung DH, Guercio A, Joannis T, Maken Ali AS, Osmani A, Padalino I, Saad MD, Savić V, Sengamalay NA, Yingst SL, Zaborsky J, Zorman-Rojs O, Ghedin E, Capua I (2007) Genome analysis linking recent European and African influenza (H5N1) viruses. *Emerging Infectious Diseases* **13**, 713–718.
- Songserm T, Amonsin A, Jam-on R, Sae-Heng N, Meemak N, Pariyothorn N, Payungporn S, Theamboonlers A, Poovorawan Y (2006) Avian influenza H5N1 in naturally infected domestic cat. *Emerging Infectious Diseases* **12**, 681–683.
- Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, Perdue ML, Lohman K, Daum LT, Suarez DL (2002) Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. *Journal of Clinical Microbiology* **40**, 3256–3260.
- Subbarao K, Klimov A, Katz J, Regnery H, Lim W, Hall H, Perdue M, Swayne D, Bender C, Huang J, Hemphill M, Rowe T, Shaw M, Xu X, Fukuda K, Cox N (1998) Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. *Science* **279**, 393–396.
- Thanawongnuwech R, Amonsin A, Tantilertcharoen R, Damrongwatanapokin S, Theamboonlers A, Payungporn S, Nanthapornphiphat K, Ratanamungklanon S, Tunak E, Songserm T, Vivatthanavanich V, Lekdumrongsak T, Kesdangsakonwut S, Tunhikorn S, Poovorawan Y (2005) Probable tiger-to-tiger transmission of avian influenza H5N1. *Emerging Infectious Diseases* **11**, 699–701.
- Thiry E, Zicola A, Addie D, Egberink H, Hartmann K, Lutz H, Poulet H, Horzinek MC (2007) Highly pathogenic avian influenza H5N1 virus in cats and other carnivores. *Veterinary Microbiology* **122**, 25–31.
- Vahlenkamp TW, Harder TC (2006) Influenza virus infections in mammals. *Berliner und Muenchener tierärztliche Wochenschrift* **119**, 123–131.
- Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y (1992) Evolution and ecology of influenza A viruses. *Microbiological Reviews* **56**, 152–179.
- World Health Organization. (2007) Areas reporting confirmed occurrence of H5N1 avian influenza in poultry and wild birds since 2003. Available at: <[http://gamapserver.who.int/mapLibrary/Files/Maps/Global\\_SubNat\\_H5N1inAnimalConfirmedCUMULATIVE\\_20070717.png](http://gamapserver.who.int/mapLibrary/Files/Maps/Global_SubNat_H5N1inAnimalConfirmedCUMULATIVE_20070717.png)>.
- Xu X, Subbarao, Cox NJ, Guo Y (1999) Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. *Virology* **261**, 15–19.
- Yingst SL, Saad MD, Felt SA (2006) Qinghai-like H5N1 from domestic cats, northern Iraq. *Emerging Infectious Diseases* **12**, 1295–1297.