Protection of cats against lethal influenza H5N1 challenge infection

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Highly pathogenic avian influenza virus (HPAIV) H5N1 of Asian origin continues to circulate in poultry and wild birds, causing considerable concern for veterinary and public health in Asia, Europe and Africa. Natural transmission of HPAIV H5N1 from poultry to humans, resulting in infections associated with high mortality, and from poultry or wild birds to large felids and domestic cats has been reported. Experimental infection of cats with HPAIV H5N1 derived from a human patient resulted in lethal disease. The role of cats in the adaptation of HPAIV H5N1 to mammals and vaccination regimens for the eventual protection of cats, however, remain to be elucidated. Here, it was shown that cats can be protected against a lethal high-dose challenge infection by an inactivated, adjuvanted heterologous H5N6 avian influenza virus vaccine. The challenge HPAIV H5N1 was derived from a naturally infected cat. In non-vaccinated cats, low-dose exposure resulted in asymptomatic infections with minimal virus excretion. As diseased cats can transmit the infection to naïve contact animals, the epidemiological role of H5N1-infected cats in endemically infected areas as a link between wild birds, poultry and humans needs close inspection, and vaccination of cats should be considered to reduce possible human exposure.

INTRODUCTION

In 1996, highly pathogenic avian influenza virus (HPAIV) subtype H5N1 (A/Goose/Guangdong/1/96) was detected in southern China (Xu et al., 1999) and subsequently spread among poultry in Hong Kong. During the outbreak, direct transmission to humans occurred, clinically affecting 18 patients and resulting in six fatalities (Claas et al., 1998; Subbarao et al., 1998). Despite strict control measures, the virus continued to circulate in domestic waterfowl populations of south-east Asia, causing severe economic losses in more than 10 countries (Webster et al., 2005; Chen et al., 2006). The continuous evolution of HPAIV H5N1 gave rise to genotype Z, which has been dominant since 2003 (Li et al., 2004; Chen et al., 2006).

Analysis of the haemagglutinin (HA) gene revealed 10 different viral clades. Different geographically restricted co-circulating sublineages of H5N1 virus were responsible for the outbreaks in south-east Asia. The HPAIV H5N1 outbreak at Lake Qinghai among migratory birds in the spring of 2005, now assigned to sublineage 2.2 of HPAIV H5N1, marked the beginning of a westward spread into countries in central Asia, the Middle East, Europe and Africa. Viruses of this sublineage have formed at least three further clusters, all of which have been associated with recent outbreaks among wild birds and poultry in Europe (Chen et al., 2006; Salzberg, 2007; Starick et al., 2007).

To date, infection of 348 humans in 14 countries from Asia, the Middle East and Africa has resulted in more than 210 deaths as listed by the World Health Organization (http://www.who.int/csr/disease/avian_influenza/en/). Sporadic fatal disease due to natural HPAIV H5N1 infection has also been reported in various carnivores, including domestic cats, leopards and tigers, which were previously considered to be resistant to influenza A virus infection (Paniker & Nair, 1970, 1972; Hinshaw et al., 1981). Meanwhile, natural infection of felids has been reported from seven countries in Asia, the Middle East and Europe including China (http://www.promedmail.org; archive number 20041023.2873), Thailand (Keawcharoen et al., 2004; Thanawongnuwech et al., 2005; Songserm et al., 2006), Vietnam (http://www.promedmail.org; archive number 20050826.2527), Indonesia (http://www.promedmail.org; archive number 20070126.0347), Iraq (Yingst et al., 2006), Austria (Leschnik et al., 2007) and Germany (Klopfleisch et al., 2007; Weber et al., 2007).

After natural HPAIV H5N1 infection, fatal disease may ensue in felids (Keawcharoen et al., 2004; Thanawongnuwech et al., 2005).
Methods

Cats. Animals were obtained from Charles River Laboratories and housed at the high-containment animal facility (Biosafety Level 3+) at the Friedrich-Loeffler-Institut, Germany. The animal experiments were approved by the regional ethical committee. Animals used in the vaccination experiment were between 3.5 and 4 months at the time of challenge. The other animals were between 8 and 10 months at the time of infection.

Infection experiments. In order to determine the challenge dose and route of infection for the vaccination experiments, three specific-pathogen-free cats were infected via the oculo-nasopharyngeal route with 10⁵ 50 % egg infectious dose (EID₅₀) and two cats each with 10³, 10² and 1 EID₅₀ of HPAIV H5N1 A/cat/Germany/R606/2006 (Weber et al., 2007) in a volume of 1 ml each. The inoculation was performed with one drop (~100 µl) in the eyes and nostrils and the remaining volume (~600 µl) in the pharynx. The virus used for the infection experiments belongs to the clade 2.2.2 genotype (‘Qinghai-like’) and was derived from a naturally diseased domestic cat found during the outbreak among wild birds on the Isle of Rügen, Germany, in 2006 (Klopfleisch et al., 2007). The virus was propagated once in embryonated hen eggs. The titre of the inoculated virus was determined by serial dilutions in embryonated hen eggs. Two animals were housed in a different room of the containment facility and served as negative controls. All animals were monitored for 21 days by physical examination and virus excretion using pharyngeal and rectal swabs taken at days 2, 4, 7, 9, 14 and 21 post-infection (p.i.). Animals were euthanized and investigated by necropsy on day 21 p.i. unless they developed clinical symptoms and had to be euthanized prior to this.

Immunization and challenge experiments. For immunization, an inactivated, adjuvanted H5-specific whole-virus vaccine was produced based on low pathogenic avian influenza virus A/Duck/Potsdam/2243/84 (H5N6). The virus was grown in Madin–Darby canine kidney cells and harvested when cytopathic effects were apparent. The harvested virus was inactivated with 0.1 % formaldehyde at 37 °C for 18 h. The inactivated antigen was formulated in Diluvac Forte adjuvant (Intervet). Five cats were immunized subcutaneously twice with a 4-week interval with 1 ml of the vaccine preparation containing 80 haemagglutinating units (HU). Five non-vaccinated animals served as controls.

For the ocu-lo-nasopharyngeal challenge experiments, the influenza virus A/cat/Germany/R606/2006 (H5N1) was used after a single passage in embryonated hen eggs. The animals were monitored for the study period of 21 days by physical examination and virus excretion was tested using pharyngeal and rectal swabs taken on days 2, 4, 7, 9, 14 and 21 p.i.

Virus titration. The amount of infectious virus present in the organ and swab samples was determined by titration of homogenates on mink lung (MV1Lu) cells in a 96-well-plate format. After incubation for 48 h at 37 °C and 5 % CO₂, the cells were stained for influenza virus antigen by an immunoperoxidase-mediated method using the anti-nucleoprotein (NP)-specific antibody HB65 (ATCC) and 3-amino-9-ethylcarbazole as the chromogen.

Virus neutralization. Serial dilutions of sera were incubated with 100 50 % TCID₅₀ of influenza virus A/cat/Germany/R606/2006 (H5N1) for 1 h at 37 °C in microtitre plate wells, after which MV1Lu cells were added and cultured for 48 h at 37 °C and 5 % CO₂. After incubation, the cells were stained for influenza virus antigen as described above.

Real-time RT-PCR (rRT-PCR). RNA was isolated from swab samples using a QIAamp Viral RNA Mini kit (Qiagen). One-step rRT-PCR was performed on a Stratagene Mx3000 PCR machine using a Superscript III One-Step RT-PCR system and Platinum Taq DNA polymerase (Invitrogen). Cycling conditions were 30 min at 50 °C and 2 min at 94 °C, followed by 42 cycles for 30 s each at 94, 56 and 68 °C. Primer and probe sequences for amplification of part of the influenza M gene were 5′-AGATGAGTCTTCTAACCAGGCTTGCG-3′ (forward), 5′-TGAAAAACACTTCTAAGGTTAAGGCAGGAGCCGA-(BHQA1a-6FAM)-3′ (reverse) and 5′,-(6FAM)-TCAGGCCCTCTCAAGACCGA-BHQ1a-6FAM)-3′ as described previously (Spackman et al., 2002). An internal control based on RNA run-off transcripts of an enhanced green fluorescent gene fragment was added on a copy basis (2 × 10⁵).

ELISA. A competitive commercial ELISA (Pourquier) to detect antibodies against the NP of type A influenza viruses was used.

Haemagglutination inhibition (HI). HI antibodies were detected as described in the EU Diagnostic Manual for Avian Influenza. Briefly, sera were inactivated for 30 min at 56 °C and serial twofold dilutions were incubated at room temperature using 4 HU for 45 min, followed by incubation with erythrocytes for 30 min.

Immunohistochemistry (IHC). The influenza A virus NP was detected in paraffin-wax sections. Briefly, dewaxed sections were incubated with a polyclonal rabbit anti-NP serum (diluted 1 : 500). As secondary antibody, biotinylated goat anti-rabbit IgG1 (Vector) was applied. A bright red signal was produced using the avidin–biotin–peroxidase complex (ABC) method.

Protection against influenza virus H5N1
RESULTS AND DISCUSSION

Experimental infection using HPAIV H5N1 A/cat/Germany/R606/2006

The infection experiments using $10^6$, $10^4$, $10^2$ and 1 EID$_{50}$ of HPAIV H5N1 A/cat/Germany/R606/2006 revealed that all three cats inoculated oculo-nasopharyngeally with $10^6$ EID$_{50}$ developed fever (≤40.5 °C), anorexia and respiratory distress within 2 days p.i. Two cats were euthanized because of the severity of symptoms on days 2 and 4 p.i. Viral RNA was detected by rRT-PCR in pharyngeal and rectal swabs on days 2 and 4 p.i. Histopathologically, these cats showed acute multifocal hepatocellular coagulative necrosis (Fig. 1a and c) and bronchointerstitial and necrotizing pneumonia with intralesion detection of influenza virus antigen (Fig. 1b and d). Sequence analysis of the HA and neuraminidase (NA) genes derived from the virus in the lung and liver proved to be identical to the sequence of the virus used for infection. The third cat inoculated with $10^6$ EID$_{50}$ developed fever (40.0 °C) and the pharyngeal swab samples were positive by rRT-PCR on day 4 p.i., but generally its symptoms were milder. From day 5 p.i. onwards, this cat improved clinically and had no more fever. At necropsy on day 21 p.i., the cat was found to have developed specific antibodies (Table 1). Cats inoculated with $10^4$ EID$_{50}$ did not show any clinical symptoms. In these cats, influenza viral RNA was detected in the pharyngeal swabs up to day 7 p.i. and both animals developed specific antibodies (Table 1). Cats that had been inoculated oculo-nasopharyngeally with $10^2$ and 1 EID$_{50}$ of HPAIV H5N1 A/cat/Germany/R606/2006 did not show clinical symptoms. All swab samples remained negative for viral RNA and the animals did not develop specific antibodies. The uninfected control cats did not show any clinical symptoms and were also negative for viral RNA in swab samples. Thus, HPAIV A/cat/Germany/R606/2006 was pathogenic for cats under experimental conditions and caused systemic disease symptoms similar to those described previously (Kuiken et al., 2004). The minimal infectious dose for cats using influenza virus A/cat/Germany/R606/2006 by the

![Fig. 1. Gross lesions (a, b) and IHC (c, d) in cats after infection with HPAIV H5N1. (a) Disseminated pinpoint-sized foci of acute necrosis throughout the liver, a few of them with central areas of haemorrhage. (b) Almost 60% of the lung parenchyma is dark red, oedematosus, slightly consolidated and atelectatic. (c) Scattered throughout the parenchyma in the liver, mainly with periportal orientation, are sharply demarcated foci of acute coagulative hepatocellular necrosis, rimmed by numerous viable or degenerate hepatocytes with strong intracytoplasmic and intranuclear immunostaining for avian influenza virus antigen. (d) In these areas of the lung, avian influenza virus antigen was detected in degenerate and necrotic epithelial cells of the bronchi and within the luminal cellular debris. Cells in (c) and (d) were stained using the ABC method with a haematoxylin counterstain. Bars, 150 µm (c) and 50 µm (d).](image-url)
oculo-nasopharyngeal route was between 10^2 and 10^4 EID_{50}. The route of infection was shown to be efficient and seemed to us more natural than intratracheal application of the virus as used before in experimental infections (Kuiken et al., 2004). Therefore, in the subsequent challenge experiments, the oculo-nasopharyngeal route of infection was used.

**Immunization**

Domestic cats commonly live in close contact with humans. As diseased cats were shown to excrete virus, we aimed to protect cats from disease and/or virus excretion by vaccination. For immunization, an inactivated H5-specific whole-virus vaccine based on low pathogenic avian influenza virus A/Duck/Potsdam/2243/84 (H5N6) was prepared. Five cats were immunized subcutaneously twice as described in Methods. Control and vaccinated animals were investigated for the development of specific antibodies. In contrast to the control group, the immunized cats developed antibody titres between 8 and 16 against the homologous H5N6 antigen 2 weeks after the second immunization (Table 2). All immunized cats also developed antibody titres of between 8 and 16 against the H5N1 antigen 2 weeks after the second immunization (Table 2) and neutralizing antibodies against the H5N1 challenge virus with titres of between 20 and 40 (Table 3). No neutralizing antibodies could be measured in the unvaccinated control cats.

**Protection of cats against heterologous challenge infection**

Vaccinated cats were challenged oculo-nasopharyngeally with 10^6 EID_{50} HPAIV A/cat/Germany/R606/2006 (Weber et al., 2007) 4 weeks after the second immunization. All five inoculated control cats showed high fever (≤41.4 °C) within 2 days p.i. and developed severe respiratory distress by day 3 p.i. Three of the five cats had to be euthanized by day 6 p.i. Two of the infected control cats died, despite appearing to have an improving clinical condition the day before. At necropsy, the main lesions were confined to the liver and lungs. All control animals exhibited systemic infections in the organs as determined by IHC or rRT-PCR (Table 4). Pharyngeal swab samples were positive by rRT-PCR in all control cats from day 4 p.i. onwards and two cats had positive rectal swab samples on days 5 and 6 p.i. The diseased animals excreted substantial amounts of virus, with peak titres in pharyngeal swabs of up to 10^4.4 TCID_{50} ml^{-1} (Table 5).

In contrast, in the vaccinated animals, body temperatures after challenge infection never exceeded 39.3 °C and clinical

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**Table 1. Development of influenza H5N1 virus-specific antibodies**

Sera of animals infected via the oculo-nasopharyngeal route were examined by HI using the influenza virus A/cat/Germany/R606/2006 (H5N1) as antigen. Results are given as titres. Competitive NP ELISA results are given in parentheses as − (inhibition from 0 to <65 %), + (inhibition from 65 to <85 %) and ++ (inhibition >85 %). NS, No sample due to death of the animal.

**Table 2. Development of influenza virus-specific antibodies in vaccinated cats**

Results are given as titres and were measured by HI using the challenge influenza virus A/cat/Germany/R606/2006 (H5N1) and the vaccine influenza virus A/Duck/Potsdam/2243/84 (H5N6) as antigen. NS, No sample; p.v., post-vaccination.
signs were not observed. In three of the vaccinated cats, all pharyngeal and rectal swab samples remained negative for virus RNA. The other two vaccinated cats had positive pharyngeal and rectal swab samples by rRT-PCR up to day 8 p.i., and infectious virus could be isolated from individual swabs of one animal during that time (Table 5). At necropsy, this animal also harboured viral RNA, although at low copy numbers, in five of the nine organs and tissues (lung, trachea, colon, mesenteric lymph node and the central nervous system; Table 4). This cat also showed the lowest antibody titres against the homologous H5N6 antigen at the time of challenge and developed the lowest antibody titres against H5N1 antigen after the challenge infection (Table 2). Also, neutralizing antibody titres in this animal were the lowest among the group of vaccinated cats (Table 3). All other vaccinated cats developed anti-H5N1 antibody titres of \( \geq 64 \), neutralizing antibody titres of \( \geq 320 \) and, at necropsy, harboured viral RNA only in single organs. One cat efficiently cleared the infection, as viral RNA could not be detected in any organ or swab sample over the observation period. This animal showed neutralizing antibody titres of 640 at the time of necropsy (Table 3). The control animals showed infectious virus titres of \( >10^{5.5} \) TCID\(_{50}\) ml\(^{-1}\) in selected organs at necropsy on day 5 or 6 p.i. (Table 5). We were not able to isolate infectious virus from any of the RNA-positive samples of the vaccinated cats taken on day 21 p.i. We therefore do not believe that the animals became persistently infected and continued virus replication at low levels, although we cannot fully rule out this possibility. This indicated that vaccination of cats with an adjuvanted inactivated avian influenza H5N6 virus protected all animals from lethal challenge with HPAIV H5N1, prevented clinical signs and inhibited or greatly reduced challenge virus excretion. The cross-protecting potential of the vaccine virus used was supported by the increase in anti-H5N6 antibody titres in three cats after HPAIV H5N1 challenge (Table 2). Comparison of the HA sequence between the vaccine and challenge virus revealed amino acid homologies for HA1 and HA2 of 91 and 93 %, respectively. Similar amino acid sequence homologies have also been shown to induce cross-protective immune responses in mice against lethal H5N1 challenge in studies investigating different inactivated H5 vaccine preparations (Lu et al., 2006). These results suggest that adjuvanted

### Table 3. Development of H5N1 influenza virus-specific neutralizing antibodies in vaccinated animals

Influenza virus A/cat/Germany/R606/2006 (H5N1) antibody neutralization titres were measured as described in Methods. NS, No sample; p.v., post-vaccination.

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<th>Cat no.</th>
<th>Days p.v./p.i.</th>
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<td></td>
<td>0 days p.v.</td>
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<tr>
<td>324</td>
<td>&lt;10</td>
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<td>374</td>
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<td>520</td>
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### Table 4. Systemic spread in cats after experimental infection with HPAIV A/cat/Germany/R606/2006(H5N1)

Control animals were euthanized on days 5 and 6 p.i. Vaccinated cats were euthanized on day 21 p.i. rRT-PCR results are given as: +++ [cycle threshold (C\(_T\)) values of 10 to <17], ++ [C\(_T\) values of 17 to <24], + [C\(_T\) values of 24 to <31] and - (C\(_T\) values of 31 to <38). Ln mes, mesenteric lymph node; CNS, central nervous system; NT, not evaluated due to PCR inhibitory effects of the sample.

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<th>Cat no.</th>
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<td>Control</td>
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<td>114</td>
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<td>895</td>
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<td>324</td>
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<td>520</td>
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vaccines may also be useful in humans for inducing cross-reactive neutralizing antibody responses, particularly in situations where the available vaccine strain is not an optimal match with the circulating virus (Stephenson et al., 2005).

To date, there is no evidence that HPAIV H5N1-infected cats have transmitted the virus to humans. However, cats may play a considerable role in the evolution and adaptation of the virus to mammals, including humans, and thus may lead to an increased pandemic potential. In order to clarify their epidemiological role, cats and other feral carnivores should be monitored closely in areas of H5N1 infection. Vaccination of cats in areas where HPAIV H5N1 continues to circulate should also be considered. Although a sterile immunity was not induced in all of the vaccinees, a considerable reduction in virus excretion was achieved. Given the possible close contact between infected cats and humans, including children and immunosuppressed patients, this effect would markedly reduce the risk of virus exposure for humans.

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