AVIAN INFLUENZA H5N1 OUTBREAK IN A FLOCK OF MUTE SWANS IN THE CITY OF TORUŃ, POLAND, IN 2006

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Abstract

The outbreak of highly pathogenic avian influenza (HPAI) infection caused by H5N1 virus in a flock of mute swans wintering in the city of Toruń in western Poland in 2006 was described. Following the confirmation of H5N1 infection in 3 dead swans, 113 apparently healthy swans were placed in an aviary. After 5 d, another dead swan was found in the aviary and was later proved positive for HPAI/H5N1. A lack of regulations concerning the control of HPAI in wild birds, lead to the prolonged indecision regarding the measures that were to be applied to the flock. Finally, due to the quickly approaching flood wave on the Vistula River, and a danger of the aviary being flooded, samples of tracheal and cloacal swabs as well as blood samples were collected from 112 live swans and submitted for testing. Under the pressure of time, initially RT-PCR/H5 and haemagglutination inhibition (HI) test with H5N2 antigen were performed and based on the results, 32 PCR-positive swans were euthanised while the rest of the flock (regardless of serological status) was released. Thereafter, secondary tests were carried out comprising of real time RT-PCR/M, virus isolation, HI with antigens H1N1, H3N2, H5N1, H7N1 and H9N2, agar gel immunodiffusion test, and competitive ELISA. The additional serological tests confirmed that more than 70% of swans were infected with H5 virus, but a group of the swans were also positive for other subtypes, mainly H1. The low mortality rate in the flock might indicate the previous contact with the AIV virus and a partial protection against HPAI/H5N1, or that innate resistance to AIV infection is higher than commonly believed.

Key words: swans, avian influenza, H5N1.

Wild aquatic birds seem to be a natural reservoir for low pathogenic influenza A viruses (23). Until recently, highly pathogenic avian influenza (HPAI) outbreaks in wild birds were sporadically recorded (3). In the second half of 2002, a significant outbreak caused by HPAI H5N1 took place in Hong Kong SAR, China, with multiple species involved (8, 9). Since 2003, when the largest HPAI epidemic started in South-East Asia, the H5N1 virus has been isolated from wild birds in other countries (14), but a major outbreak occurred between April and June 2005, at the Qinghai Lake in North-Western China (6). The subsequent appearance of H5N1 of the “Qinghai-type” in Europe (2) raised a question about the possible role of migratory birds in the introduction of the virus into Europe. However, in 2006, European HPAI/H5N1 was only recovered from dead birds (2, 9) and the active surveillance of live birds did not prove the existence of H5N1 in the population of wild birds. Another vital issue is the role of mute swans in the epidemiology of HPAI H5N1 infections and the reason for the increased mortality of this species observed during the epidemic in Europe in 2006.

This paper reports the details of the first outbreak of HPAI/H5N1 in Poland, in a flock of mute swans in Toruń.

Material and Methods

History of HPAI outbreak. HPAI caused by the H5N1 subtype in wild birds was first diagnosed in three dead swans found on the Vistula riverbank in the city of Toruń on March 2-3, 2006 (16). The dead swans formed part of a larger flock of apparently healthy swans. On March 10, 113 swans were placed in an aviary built on the riverbank. On March 15, one swan in the aviary was found dead in the aviary and was positive for the highly pathogenic H5N1 virus (amino acid sequence at the HA0 cleavage site: PQGERRRKRGLF). On March 26, the swans were given identification rings by ornithologists and two days later tracheal and cloacal swabs as well as samples of blood were collected from all swans of the flock and then sent to the National Reference Laboratory for...
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Samples. The samples of tracheal swabs were placed in 2 ml of phosphate-buffered saline (PBS) containing penicillin (2,000 U/mL), streptomycin (2 mg/mL), gentamicin (50 µg/mL), and nystatin (1,000 U/mL). The cloacal swabs were treated in the same way, but the antibiotic concentration was fivefold higher. Following 30 min incubation at room temperature, 200 µl of each sample was used for RNA extraction, and the rest of the sample was stored at -20°C for further tests. The serum was obtained from blood by centrifugation and used for serological studies.

Virus isolation. The virus was cultured according to EU Commission Decision 2006/437/EC (1), by inoculation of 200 µl of the supernatant of tracheal/cloacal swabs into the allantoic cavity of 9-11-d-old specific pathogen free (SPF) embryonated eggs. Samples from up to five individuals were pooled. Two blind passages were performed.

RNA extraction. The RNA was extracted by using RNeasy Mini Kit (QIAGEN) according to the manufacturer’s instruction. The RNA was then eluted with approximately 50 µl of RNA-se free water.

RT-PCR for H5 gene. The One Step RT-PCR/H5 was performed in one tube with primers H5-kha-1: CCT CCA GAR TAT GCM TAY AAA ATT and H5-kha-3: TAC CAA CCG TCT ACC ATK CCY TG (21). The Qiagen One Step RT-PCR Kit was used in a total volume of 50 µl containing prefixed specific pathogen free (SPF) embryonated eggs. The RNA was then eluted using RNeasy Mini Kit (QIAGEN) according to the manufacturer’s instruction. The RNA was then eluted with approximately 50 µl of RNA-se free water.

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Results

RT-PCR/H5. Nineteen tracheal swabs and twenty-seven cloacal swabs collected from 32 swans were identified as positive in RT-PCR/H5, but the PCR bands were of a very small size (Fig. 1). Fourteen birds were found to be positive in both types of samples.

RT-PCR/M. Out of 32 birds identified as positive for RT-PCR/H5, 10 birds were also positive for RT-PCR/M (six tracheal and five cloacal swabs). The cycle threshold (Ct) values were very high and varied between 33 and 38, but the curves were of a characteristic sigmoidal shape (Fig. 2).

Virus isolation. None of the samples was positive on SPF embryos following two blind passages.

HI test. The HI results (Table 1) revealed that the most sera showed positive reaction (titres 16 and higher) with the H5 antigens of AIV. From 112 serum samples tested with H5N2 antigen, 81 (72.3%) were positive (titres: 16 to 64) while eighty (71.4%) sera showed positive results against H5N1 antigen (with the range of positive titres 16 to ≥512). However, more than half of the sera (58.75%) exhibited HI titres up to fourfold higher with H5N1 antigen. The seroconversion against H1N1 was demonstrated in 58 sera (110 tested, 58.75%), but only one serum showed positive result with this antigen exclusively, and titres of 12 sera (20.7%) were 2-3 times higher in comparison to the results with other antigens. Out of 119 sera tested with H9N2 antigen, 23 (21.1%) were positive but the titre of only one serum was higher than titres obtained with other antigens.

Haemagglutination inhibition test (HI). The HI test was performed on 96-well microplates according to EU Commission Decision 2006/437/EC (1), using four haemagglutinating units. The following antigens were used: H5N2 (initial test), H1N1, H3N2, H5N1, H7N1, and H9N2 (secondary tests). Sera exhibiting titres ≥16 were considered positive.

Agar gel immunodiffusion test (AGID). The AGID test was performed according to EU Commission Decision 2006/437/EC (1).

Competitive ELISA (C-ELISA). C-ELISA for the detection of AIV antibodies in different species of birds was prepared in-house with the use of commercial monoclonal antibody HB-65 (ATCC) (15).

![Fig. 1. RT-PCR/H5 amplifications from RNA samples of cloacal and tracheal swabs obtained from swans in Toruń. Lane M – molecular size marker; lane 1: negative control (water); lanes 2-5 and 7-13: samples from swans; lane 6: positive control.](image-url)
The number of seropositive sera with H7N1 and H3N2 was 11 (9.8%, 112 sera were tested) and eight (7.3%, 109 sera tested), respectively. However, the titres in both cases were low (range: 16-32) and in all cases lower when comparing to the results with other tested antigens.

**AGID.** Thirty-four samples of sera (30.4%) were found positive (Table 1). All AGID-positive samples were also positive for HI/H5N1, HI/H5N2.

**C-ELISA.** One hundred and one (90.4%) of the swans seroconverted in C-ELISA test (Table 1), among them 18 (16%) were positive only in this test.

### Table 1

Results of serological tests performed with samples collected from mute swans in Toruń

<table>
<thead>
<tr>
<th>Method</th>
<th>Positive/tested (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HI</td>
<td>80/112 (71.4)</td>
</tr>
<tr>
<td>H5N2</td>
<td>75/112 (67.0)</td>
</tr>
<tr>
<td>H7N1</td>
<td>11/112 (9.8)</td>
</tr>
<tr>
<td>H1N1</td>
<td>58/110 (52.7)</td>
</tr>
<tr>
<td>H3N2</td>
<td>8/109 (7.3)</td>
</tr>
<tr>
<td>H9N2</td>
<td>23/109 (21.1)</td>
</tr>
<tr>
<td>AGID²</td>
<td>34/112 (30.4%)</td>
</tr>
<tr>
<td>C-ELISA</td>
<td>101/112 (90.4%)</td>
</tr>
</tbody>
</table>

¹ haemagglutination inhibition test (due to small amounts of collected sera, all swans were only tested against H5 and H7 antigens), ² agar gel immunodiffusion test.

### Discussion

The first case of HPAI-H5N1 in Poland was discovered at the beginning of March 2006 in a flock of mute swans in the city of Toruń. The flock constituted a mixed population of native swans, spending summer in the nearby water bodies, as well as swans migrating along the Vistula River and Notec River valleys towards Germany and Baltic seashore. Their mean body weight, size and general condition, were similar to those of other flocks wintering in Poland (24). After a confirmation of HPAI/H5N1 in this flock, a problem that emerged was the type of measures that could be applied to the flock. Since the mute swan is a protected species, permission for euthanasia can only be granted by the Minister of the Environment. Thus, while waiting for the decision, the swans were placed in a makeshift aviary with food and water ad libitum and were kept isolated. The isolation of the HPAI H5N1 virus from a swan that died in the aviary 5 d after the flock had been isolated (16) proved that the virus was still circulating in the flock. In the meantime, the early spring flood-wave on the Vistula River was approaching Toruń, and posed a danger to the birds in the aviary. Therefore, samples of tracheal and cloacal swabs along with blood samples were collected from each bird and submitted for testing. Time pressure hindered scrutinised testing, so initially only RT-PCR/H5 and HI/H5 tests were carried out. Based on the results, 32 RT-PCR positive birds were euthanised, while the remaining swans (regardless of the serological status) were released. A lack of regulations concerning the control of HPAI in wild birds did not allow applying the “stamping-out” method to all of the birds. In that case, the applied measure was the result of a compromise between the General Veterinary Inspectorate and the Ministry of the Environment. This unprecedented case should facilitate the establishment of appropriate measures concerning the control of HPAI in wild birds.

All the released swans were ringed in order to follow their migration routes. The fate of one swan from Toruń flock is known: 7 d after the swans had been released, one dead swan from this flock was found dead in Lipno, approximately 50 km south–east from Toruń.
but it was found to be H5N1 negative. The cause of its death is unknown.

In the following weeks, secondary tests were performed on the samples of swabs and sera from the Toruń flock. A lower number of positive birds by RRT-PCR/M when comparing to RT-PCR/H5 can be explained by the fact that the former test was performed four months later on the samples that were previously frozen/thawed, and the virus present in the sample might have been degraded. Moreover, temporary storage of the samples at -20°C surely contributed to the partial decomposition of the RNA. Unsuccessful attempts to isolate the virus on SPF embryonated eggs can also be explained by a very low viral load in the sample. As shown in the study of Munster et al. (17), approximately 33% of M-based RRT-PCR positive samples could be recovered on embryonated eggs, which was explained by either a low viral load (expressed by a high Ct value, >35) and/or improper transport conditions of the samples. In our study, all RRT-PCR-positive samples had the Ct value above 33, which partially explains the failure of the isolation on embryos.

Little is known about the epidemiology of HPAI in wild birds. Few experimental studies show a variable degree of resistance of certain wild bird species to HPAI/H5N1 (4, 11, 18, 19). There have been rare recoveries of HPAI H5N1 from apparently healthy birds (5, 12). Nevertheless, the assumption that migratory birds may serve as asymptomatic carriers capable of transferring the virus over long distances has recently been questioned as lacking strong evidence (10). Low-level viral shedding by apparently healthy birds found in our study, cannot exclude the fact that mute swans can serve as virus carriers but their role in spreading the virus over long distances seems to be unlikely due to a limited migration of this species.

There are also few serological data on AIV infections in wild birds. Chen H.X. et al., (7) performed HI test with H2-H13 antigens on 493 sera samples collected from 15 migratory species in 2004 and 2005 in China, and found positive results with H2, H9, and H10 antigens. Our HI serological studies of the swans from Toruń revealed that most of them were infected with the subtype H5 of AIV (above 70% of swans were seropositive for H5N1 and H5N2 antigens) and most likely by H5N1 virus (HI titres of more than 50% of sera were higher when testing with H5N1 antigen). A high percentage (52.7%), as well as higher mean titres with H1 antigen in more than 20% of the swans could also indicate a previous exposure to AIV/H1. On the other hand, lower seroconversion and lower titres for H3N2, H7N1 and H9N2 subtypes, suggest the occurrence of cross-reactions caused by the interference of N1 or N2 antigens. As expected, AGID test showed a lower sensitivity with only 30.4 % of seropositive birds. It is worth noting that all AGID-positive sera were also positive with H5 antigen in HI test. The highest percentage (90.4%) of seroconverted swans was found in C-ELISA method. This test, by using the NP specific monoclonal antibody, can be utilised for the detection of antibodies for all influenza A viruses, including all subtypes of AIV, in multiple species. Our study confirms its higher sensitivity than that of AGID (20) but also indicates a possible infection of a part of the swans with AIV of other subtypes than tested.

Although in Europe, H5N1 was recovered mainly from mute swans, the overall mortality in this species seems not to be dramatically high. In most cases, when outbreaks occurred in larger flocks, usually single birds were being found dead. In Iran, 153 birds out of flock of around 3,000 swans were positive, i.e. circa 5% (9). In Toruń, out of 116 swans only four birds died (3.4%). During the incident of HPAI/H5N1 in the Abbotsbury swannery (Dorset, UK), out of 38 tested swans (in the flock of 766 birds), 10 were found H5N1-positive (J.Brown - oral presentation during the 14th Annual meeting of the national laboratories for avian influenza and Newcastle disease of the Member States, Brussels, 2008). The clinical observations and results of laboratory investigations clearly indicate that HPAI infection in mute swans can vary significantly from severe disease with nervous signs and quick death, through emaciation, subclinical infection with slight viral shedding, to complete resistance to infection (single birds from the Toruń flock were negative in all of the tests despite very close proximity of infected swans on a highly restricted area of the aviary).

However, the results of experimental infection of mute swans revealed that most of the immunologically naïve birds died (11). We therefore conclude that a number of factors such as dosage of the virus, immunological status, presence of concurrent infections, and previous contact with AIV may contribute to the overall clinical picture of the disease.

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