

Virological and serological investigation of avian influenza in black-headed gulls captured on a rubbish dump in Zagreb, Croatia

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ABSTRACT

A total of 142 black-headed gulls (BHG), *Chroicocephalus ridibundus*, were captured during February and March 2009 at the Zagreb city rubbish dump (45.45 N 16.01 E) in order to collect cloacal swabs and serum samples. Cloacal swabs were tested by virus isolation in embryonated chicken eggs, which resulted in isolation of one avian influenza virus (AIV) that was of the H16 subtype. The collected sera were tested by blocking ELISA for avian influenza antibodies, resulting in 28.2% positive samples, which were retested by haemagglutination inhibition (HI) using H5 and H7 subtype antigens. Only one serum sample was positive for H5 and none for H7 antibodies. Statistically, no significant difference was found between the ages of AIV seropositive birds ($\chi^2 = 2.08$, $df = 1$, $P = 0.15$). In contrast, regarding seroprevalence in different months of capture, a higher proportion of positive gulls was found during March than during February ($\chi^2 = 4.53$, $df = 1$, $P = 0.03$), especially in younger birds ($\chi^2 = 7.67$, $df = 1$, $P = 0.006$). This finding suggests that BHGs might contract AIV infection during their aggregations in large numbers on rubbish dumps or similar feeding sites during the winter. Although only one of the 142 tested birds was positive for H5 subtype antibodies, this finding cannot be neglected seeing that apparently healthy BHGs can carry highly pathogenic AIV of the H5N1 subtype. Nevertheless, the results of our study have shown that BHGs are more often infected with other AIV subtypes and therefore are most likely not the primary carriers of H5 AIV. To our knowledge this is the first detection of H16 AIV in Croatia.

Key words: avian influenza, H5 subtype, gulls, *Chroicocephalus ridibundus*

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Introduction

Highly pathogenic avian influenza (HPAI) is a viral disease that can cause high mortality of both poultry and wild birds. The main natural reservoirs of avian influenza (AI) are birds belonging to the orders *Anseriformes* (ducks, geese and swans) and *Charadriiformes* (gulls, terns and waders) (WEBSTER et al., 1992). AI of the H13 subtype is the most frequent one found in gulls (OLSEN et al., 2006). It is closely related to the H16 subtype and they are both genetically distant from the influenza viruses found in hosts other than gulls. During the study in Norway in 2006/07 it was found that H13 and H16 are the most frequently occurring AI subtypes found in gulls, but the authors also isolated H1, H4, H5 and H6 AI subtypes from these birds (GERMUNDSSON et al., 2010).

In May 2005 the HPAI H5N1 virus killed more than 6,000 wild birds at Lake Qinghai. Two out of six bird species that were affected were gulls: great black-headed gulls (*Ichthyaetus ichthyaetus*) and brown-headed gulls (*Chroicocephalus brunnicephalus*) (ZHOU et al., 2006).

During the outbreak of the HPAI H5N1 virus of Asian lineage in Croatia in the winter of 2005/06 seventeen H5N1 viruses were isolated from wild birds. Five of them were from black-headed gulls (BHG), *Chroicocephalus ridibundus*. All the gulls were apparently healthy in contrast to the infected mute swans (*Cygnus olor*) and a mallard (*Anas platyrhynchos*), which showed clinical signs or were found dead (SAVIĆ et al., 2010).

This virus can be also fatal to humans. In the period from 2003 until this paper was written there were 637 confirmed cases of people infected with HPAI H5N1 virus, 378 of which had fatal outcome (ANONYMOUS, 2013).

The Zagreb city rubbish dump, Jakuševac, is one of places with the highest aggregation of BHGs in Croatia. During winter months up to 13,500 BHGs feed on Jakuševac (JURINOVIĆ and KRALJ, 2012). This concentration of gulls creates a great opportunity for the spread of AI viruses (AIVs) among these birds (OLSEN et al., 2006).

The aim of this study was to determine whether the AI viruses of H5 and H7 subtypes are circulating in gulls feeding on the Zagreb city rubbish dump.

Materials and methods

Sampling. During February and March 2009 gulls were captured at the Zagreb city rubbish dump (45.45 N 16.01 E) with a clap net. Captured gulls were divided into three age groups according to their plumage: unknown age, second calendar year and third calendar year or older (OLSEN and LARSSON, 2004). Cloacal swabs and serum samples were collected from all the gulls.

Serology. All the sera were tested by ELISA for the presence of antibodies against the avian influenza virus using the FlockCheck AI MultiS-Screen Ab Test (IDEXX, Maine, USA) according to the manufacturer's instructions.

Positive and suspicious samples were tested by haemagglutination inhibition (HI) using H5 and H7 antigens (Istituto Zooprofilattico Sperimentale delle Venezie, Padova, Italy). For the first test H5N1 and H7N1 antigens were used respectively. Samples that were positive for H5 antibodies were retested with the H5N2 antigen to eliminate neuraminidase cross reactivity.

Virus isolation. AIV isolation was carried out in embryonated chicken eggs, according to the standard procedures (ANONYMOUS, 2009). A battery of monospecific antisera covering all 16 H subtypes of AIV and a monospecific serum against avian paramyxovirus type 1 (Istituto Zooprofilattico Sperimentale delle Venezie, Padova, Italy) were used for typing of haemagglutinating isolates.

Molecular methods. Viral RNA was extracted from all haemagglutinating allantoic fluids using a High Pure Viral Nucleic Acid Kit (Roche Applied Science, Mannheim, Germany). Extracted RNA samples were tested by RT-qPCR specific for type A influenza viruses (SPACKMAN et al., 2002), and positive samples were then tested by RT-qPCR specific for the H5 (SLOMKA et al., 2007) and H7 subtypes (SLOMKA et al., 2009). All RT-qPCR tests were carried out in a LightCycler 1.5 (Roche Applied Science, Mannheim, Germany) with slight modifications of the original protocols due to the different platform (available upon request).

Statistical analysis. Data were analyzed using Statistica software package (StatSoft, Inc. 8.0, Tulsa, USA). Possible differences between months of capture or age classes were tested using χ^2 tests. Differences at $P < 0.05$ were regarded statistically significant.

Results

A total of 142 BHGs were captured (Table 1).

Virus isolation yielded two haemagglutinating agents and the RNA of only one was positive for avian influenza type A virus, but negative for H5 and H7 subtypes. Serotyping of the isolate showed it to be of the H16 subtype. The other isolate was serotyped as avian paramyxovirus type 1. The bird harbouring the AIV was in its third calendar year or older.

The results of ELISA are given in Table 1. Briefly, 28.2% sera were positive for avian influenza antibodies, while only one of them was positive for H5 antibodies with a titer of 4 log₂ with both H5N1 and H5N2 antigens, and none of them was positive for H7 antibodies.

When testing for variations in proportions of gulls positive versus negative for avian influenza antibodies, no significant statistical differences were found between ages ($\chi^2 =$

2.08, $df = 1$, $P = 0.15$). A significant statistical difference was found when testing all gulls in different months of capture ($\chi^2 = 7.67$, $df = 1$, $P = 0.006$). Consequent analysis showed no significant difference between months of capture for gulls in their 3rd calendar year or older ($\chi^2 = 1.96$, $df = 1$, $P = 0.16$), but revealed a significantly higher proportion of positive gulls during March than during February for gulls in their 2nd calendar year ($\chi^2 = 4.53$, $df = 1$, $P = 0.03$).

Table 1. Results of the ELISA test for the presence of antibodies against avian influenza in Black-headed Gull sera

	Tested birds by months		Positive birds by months		Total tested birds	Total positive birds
	February	March	February	March		
Unknown age	1	0	0 (0%)	0 (0)	1	0 (0%)
2 nd calendar year	64	13	12 (18.75%) ^a	6 (46.16%) ^a	77	18 (23.38%)
3 rd calendar year or older	31	33	8 (25.80%)	14 (42.42%)	64	22 (34.38%)
Total for all ages	96	46	20 (20.83%) ^b	20 (43.48%) ^b	142	40 (28.17%)

^a - Statistically significant differences among months of sampling for BHGs in their second year ($P = 0.03$)

^b - Statistically significant differences among months of sampling for all BHGs sampled ($P = 0.006$).

Discussion

BHGs are one of the most common gull species in Europe. In cities with large water areas, such as lakes or rivers, they interact very closely with humans and may serve as vectors for many diseases. The prevalence of the influenza virus in BHGs varies greatly between different ages of birds, the population and the time of year when the birds were sampled. Percentages can be very misleading in small samples, such as 60% prevalence in juvenile birds caught in August 1999 in Sweden (FOUCHIER et al., 2005) or 21% in Norway in 2006 (GERMUNDSSON et al., 2010) with samples of 10 and 21 birds respectively. Other authors (GLOBIG, 2006, PEREZ-RAMIREZ et al., 2010) failed to detect AI viruses in small samples. In largerr samples such as 1,583 birds from Northern Europe and the Netherlands (MUNSTER et al., 2007) or 4,303 birds from the European Union (HESTERBERG et al., 2009) the prevalence was less than 1%, 0.9% and 0.5% respectively, and is probably closer to the true prevalence. In all these studies RT-qPCR was used, except in one study (GLOBIG, 2006) where virus isolation in embryonated chicken eggs was used. Virus isolation success from RT-qPCR positive samples varies from 83.3% (FOUCHIER et al., 2005) to 33.5% (MUNSTER et al., 2007). The results of our study are in accordance with the larger samples and show 0.7% (1/142) of birds positive for AI virus.

No AIV of the H5 subtype was isolated during this study. Most of the documented cases of isolation of the HPAI H5N1 virus from BHGs were from dead birds (SHARASHOV et al., 2010, ELLIS et al., 2004) but SAVIĆ et al. (2010) described isolation from apparently healthy BHGs. However, AIV of the H16 subtype was isolated from a BHG and to our knowledge this is the first detection of this AIV subtype in Croatia.

Overall AIV seroprevalence in BHGs at Jakuševac in February and March 2009 was 28.2%. It was higher in older birds (34.4%) than in birds in their second calendar year (23.4%), but this difference does not show statistical significance. The probable cause of this may be found in fact that older birds had had more time to become infected (GRAVES 1992; DE MARCO, 2004).

The percentage of seropositive birds was significantly higher in March (overall 43.48%) than in February (overall 20.83%) both for birds in their second calendar year and older. In the 2001-2005 period there was large difference between February and March in the average number of BHGs in a day. There were 5302.00 in February and 2742.38 in March (JURINOVIĆ, 2006). This change in numbers and subsequently the change in flocks feeding at Jakuševac is the probable reason for the change in seroprevalence.

Although only one of the 40 seropositive birds was positive for H5 antibodies, this finding cannot be neglected. The bird was in its second calendar year. Nevertheless, the results of our study indicate that BHGs are not the primary carriers of H5 AIV. Most probably they contracted the infection from other water birds during mass feeding or roosting on water surfaces, because waterfowls are very efficient in influenza virus transmission via fecal material in the water supply (WEBSTER et al., 1992). Gulls are not typical migratory birds with regular routes of movement. They are, especially before reaching sexual maturity, capable of crossing very long distances in small periods of time, and are therefore capable of transmitting the disease over a vast area before showing symptoms.

Therefore there is a need for systematic monitoring of gulls at mass feeding or roosting sites (such as rubbish tips or large water areas) for the timely prevention of the possible spread of the virus to domestic poultry, and to eliminate subsequent potential major economic losses.

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SAŽETAK

Tijekom veljače i ožujka 2009. na gradskom odlagalištu otpada u Zagrebu (45.45 N 16.01 E) uhvaćena su 142 riječna galeba (*Chroicocephalus ridibundus*) u cilju prikupljanja brisova nečisnica i uzoraka krvi. Brisovi nečisnica su pretraženi izdvajanjem virusa u embrioniranim kokošjim jajima što je rezultiralo izdvajanjem virusa influence ptica koji je bio podtipa H16. Prikupljeni uzorci seruma su pretraženi imunoenzimnim testom za protutijela virusa influence ptica od kojih je 28,2% bilo pozitivno. Pozitivni uzorci su dodatno pretraženi inhibicijom hemaglutinacije s antigenima podtipa H5 i H7 među kojima je samo jedan bio pozitivan na H5 i nijedan na H7 protutijela. Statistički nije ustanovljena značajna razlika među skupinama različite dobi seropozitivnih ptica ($\chi^2 = 2,08$, $df = 1$, $P = 0,15$). Nasuprot tome, seroprevalencija u različitim mjesecima

lova je imala veći udio tijekom ožujka nego tijekom veljače ($\chi^2 = 4,53$, $df = 1$, $P = 0,03$), naročito u mladim ptica ($\chi^2 = 7,67$, $df = 1$, $P = 0,006$). Ovakav nalaz ukazuje na moguću infekciju riječnih galebova tijekom njihovoga brojnog okupljanja na odlagalištima otpada ili sličnim mjestima za hranjenje tijekom zime. Iako je samo jedna od 142 ptice bila pozitivna na protutijela za H5 podtip, nalaz se ne smije zanemariti znajući da naizgled zdravi riječni galebovi mogu nositi virus jako patogene influence podtipa H5N1. Unatoč tome, rezultati našeg istraživanja pokazuju da riječni galebovi češće bivaju inficirani drugim podtipovima virusa influence ptica te stoga najvjerojatnije nisu primarni nosioci virusa influence podtipa H5. Sukladno našim spoznajama, ovo je prvi dokaz virusa influence podtipa H16 u Hrvatskoj.

Ključne riječi: influenza ptica, podtip H5, galebovi, *Chroicocephalus ridibundus*
