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Antibodies against human influenza viruses in sentinel duck flocks in the ornithological reserve Kopački rit in Croatia

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ABSTRACT

Direct transmission of avian influenza viruses to humans has been confirmed and ever since it has been the main topic of influenza virus research. The opposite form of virus transmission is still unclear. In our study we used duck flocks as sentinels for surveillance of wild birds for influenza viruses and the possibility of bird infections with human influenza A viruses. Tested sera were collected from ducks in a free breeding system on a fish pond in the ornithological reserve Kopački rit (Croatia). Ducks were bred in an isolated unit for the first three weeks, and this was followed by four weeks breeding on the fish pond without contact with humans. Sera were tested by haemagglutination inhibition (HI) test using human influenza viruses A/New Caledonia/20/99/ VR-116 (H1N1), A/Panama/2007/99 (RESVIR - 17) (H3N2), B/Hong Kong/330/01 and B/Sichuana/379/99 as antigens. To determine the time of infection, sera were collected twice during the period of isolation (day 0 and day 21) and at the end of breeding (day 49). Sera collected during the period of isolation were negative to influenza A and influenza B viruses. The high titer of influenza A specific antibodies in the serum samples at the end of breeding confirmed infection during exposure on the fish pond. As ducks on the fish pond had no contact with humans and were only in close contact with wild migratory birds these results confirmed that wild migratory birds were a source of infection and present a reservoir of influenza viruses. Very high seroprevalence in sentinel ducks with a high HI titers in some animals was determined at the end of breeding. The results of this study indicate that sentinel ducks were infected with influenza A virus strains closely related to the human

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strains used as an antigen. Also this study confirmed that sentinel ducks could be successfully used in influenza monitoring in wild birds.

Key words: influenza, surveillance, ducks, sentinels, wild birds

Introduction

Influenza A is a viral disease of global threat with high morbidity and mortality in annual epidemics, and in pandemics which have very high attack rates. Influenza A viruses have been isolated from pigs, horses, seals and a variety of birds, as well as humans (WRIGHT and WEBSTER, 2000). Recently, equine influenza virus has been transmited to dogs (CRAWFORD et al., 2005). Phylogenetic studies have revealed host species-specific lineages of viral genes with occasional examples of virus transmission among different host species. The epidemiological role of animal species in the maintenance and spread of human influenza is a subject of interest throughout the world. Nowadays, the possibility of reassortment of human, avian and swine type of influenza A virus in animals or humans is a matter of high scientific interest. It is proved that triple reassortant swine virus reassorted with an Eurasian avian-like swine virus, resulting in the swine-origin H1N1 that are now circulating in humans (NEUMANN et al., 2009).

Avian species, particularly wild waterfowl, have been recognized as the main reservoir of influenza A viruses in nature (HINSHAW and WEBSTER, 1982). All 16 subtypes of influenza A virus are able to replicate in intestinal tract causing no signs of disease, and are excreted in the faeces in high concentration (WEBSTER et al., 1978). They have been isolated from freshly deposited faecal material and from lake water, indicating that the waterfowl environment is an important source of influenza virus infection (WEBSTER, 2002).

Surveillance studies of the dynamics of influenza infection in the wildlife population, especially in wild waterfowl, are important to understand the spread of influenza in humans. All influenza viruses isolated in mammals, including those that cause epidemics and pandemics in humans, have been originated, either directly or indirectly, from avian influenza viruses. Transmission of an avian and human type of virus to pigs has been reported many times, supporting the theory that pigs serve as the mixing vessels. In May 1997, the outbreak of direct transmission of avian virus H5N1 to humans was reported in Hong Kong (CLASS et al., 1998). However, receptor specificity for avian viruses, as defined by oligosaccharides that contain the Sia (α2,3) Gal-linkage (ROGERS and PAULSON, 1983), does not necessarily restrict transmission between species (MATROSOVICH et al., 1999), indicating that even transmission of human viruses to birds is a real possibility. Sentinel domestic birds as a model to obtain information about the circulation of influenza viruses in wild birds has been used before (SINNECKER et al., 1982) but the new epidemiological situation and problems with surveillance of influenza have again highlighted sentinel animals as a possibly effective system for influenza control (GLOBIG et al., 2009).

The object of this paper was to study the use of ducks as sentinel animals in influenza surveillance and to determine circulation of influenza viruses in wild birds in the ornithological reserve Kopački rit in Croatia.

Materials and methods

Sera. In the preliminary investigation 133 duck (Cherry Valley) blood samples were collected following seven weeks of breeding. The ducks were reared in the experimental free breeding system on a fish pond in the area of the Natural Park and ornithological reserve Kopački rit in Croatia where they were in close contact with wild migratory birds. Before rearing on the fish pond, the ducks were placed in an isolated unit for the first three weeks of their life, without any contact with other animals.

The following year, a total of 187 blood samples were collected from ducks (Cherry Valley) that were placed for the period of seven weeks in similar conditions as those a year before. The samples were collected from 10% of the population on the first day (day 0) and the last day (day 21) of breeding in the isolated unit prior to the ducks being moved into the external free breeding system, and at the last day (day 49) of breeding.

Blood samples were collected from v. ulnaris in sterile test tubes and transported to the laboratory on ice. The serum samples were separated from blood clots and stored at -20 °C until analysis.

Viruses. Two reference influenza A viruses of human origin -A/New Caledonia/20/99/VR-116 (H1N1) and A/Panama/2007/99 (RESVIR - 17) (H3N2) were used, as well as the reference influenza B viruses of human origin - B/Hong Kong/330/01 in the preliminary investigation and B/Sichuana/379/99 in a 2004 experiment. The viruses were supplied from the World Health Organization Collaborating Centre for Influenza, Centre for Disease Control, Atlanta, Georgia, USA.

Hemagglutination inhibition test. Hemagglutination inhibition (HI) test was performed as described by ALEXANDER (1989). HI titers ≥1:20 detected against four hemagglutinating units were considered positive. In order to inactivate nonspecific inhibitors of hemagglutination, serum samples were treated both with receptor destroying enzyme, as described (ANONYM., 1982), and at 37 °C for 1 h with 10% (v/v) chicken red blood cells.

Results

Out of 133 serum samples from the preliminary investigation collected on the last day of breeding, i.e. after three weeks in an isolated unit and four weeks of exposure on the fish pond, HI antibody titers ≥1:20 to A/New Caledonia/20/99/VR-116 (H1N1) were detected in 54 (40.6%) samples, and to strain A/Panama/2007/99 (RESVIR - 17) (H3N2)

in 120 (90.2%) samples (Table 1). HI antibodies titers to A/New Caledonia/20/99/VR-116 (H1N1) ranged from 1:20 to 1:160 and to A/Panama/2007/99 (RESVIR - 17) (H3N2) from 1:20 to 1:320 (Table 2).

Table 1. Number and percentage of duck sera positive for the presence of hemagglutination inhibition antibodies to human influenza virus strains tested in 2003

	A/New Caledonia/20/99 VR-116 (H1N1)	A/Panama/2007/99 (RESVIR - 17) (H3N2)
Total number of tested sera	n (%)	n (%)
133	54 (40.6)	120 (90.2)

Table 2. Number of positive duck sera and titer of hemagglutination inhibition antibodies to human influenza virus strains tested in 2003

	A/New Caledonia/20/99 VR-116 (H1N1)	A/Panama/2007/99 (H3N2)
Antibody titer	n	n
<1:10	58	4
1:10	21	9
1:20	34	12
1:40	17	38
1:80	2	44
1:160	1	24
1:320	0	2

Table 3. Number and percentage of duck sera tested positive for the presence of hemagglutination inhibition antibodies to human A influenza virus strain A/Panama/2007/99 (H3N2) in 2004

Day of samples collection	Total number of tested sera	Positive (%)	Negative (%)
0	20	0 (0.0)	20 (100.0)
21	20	0 (0.0)	20 (100.0)
49	187	92 (49.1)	95 (50.9)

Table 4. Number of positive duck sera and titer of hemagglutination inhibition antibodies to human influenza virus A/Panama/2007/99 (H3N2) in 2004

Antibody titer	A/Panama/2007/99 (H3N2)
<1:10	40
1:10	35
1:20	42
1:40	34
1:80	13
1:160	3

In our study performed in 2004 we again detected a high percentage of positive serum samples among the duck population tested on the last day of rearing in the free breeding system on the fish pond, and negative sera before exposure.

In 20 duck sera, collected on day 0, with HI assay we did not find any antibodies for any of the tested virus strains. The same result was obtained with sera collected at day 21.

HI antibody titers ≥1:20 were detected to strain A/Panama/2007/99 (RESVIR - 17) (H3N2) in 92 (49.10%) out of 187 tested duck serum samples after exposure for four weeks in the free breeding system on the fish pond (day 49) (Table 3). The titers of HI antibodies to strain A/Panama/2007/99 (RESVIR - 17) (H3N2) in positive sera ranged from 1:20 to 1:160 (Table 4).

Antibodies against the other tested virus strains (A/New Caledonia/20/99/VR-116 (H1N1), B/Hong Kong/330/01 and B/Sichuana/379/99) were not detected.

These negative results also confirmed the absence of nonspecific hemagglutination inhibitors in the tested sera.

Discussion

The role of mammals and birds in the maintenance and spread of human influenza is a matter of interest in a many related epidemiological studies. It is generally accepted that the human influenza pandemics from the last centuries and numerous outbreaks in domestic and wild animals, were correlated with interspecies transmission of avian influenza A virus among reservoirs in nature, from which viruses can be transmitted to poultry, pigs, horses, marine mammals and humans. Influenza A viruses can also be transmitted from poultry and pigs to humans. Surveillance of wild migratory bird reserves for influenza should be performed systematically worldwide because migratory birds play a key role of virus evolution (WEBSTER, 2002). In most cases surveillance of influenza in wild birds includes investigation of dead or sick wild birds (HAPPOLD et al.,

2008). As surveillance of influenza in the ecosystem gives just a partial insight through the investigation of dead or sick wild birds only, in our study we evaluated a sentinel approach to monitor the prevalence of influenza in the ecosystem. Very high prevalence of influenza A antibodies in the investigated duck flocks confirmed the effectiveness of sentinel birds as a monitory tool for influenza surveillance in an ecology system. Our results are corroborated by a recent study where ducks were used as sentinels for avian influenza in wild birds (GLOBIG et al., 2009).

In this study we used as antigens the human influenza A virus, subtypes H1N1 and H3N2, which have been circulating for some twenty years in most European countries to investigate the possibility of bird infections with influenza A viruses related to human strains. Sera with antibody titer of ≥1:20 were considered positive, whereas SCHRÖDER and FISCHER (1992) set the level of antibody titer as positive at ≥1:8. If the level of antibody titer was considered positive at ≥1:20, 40.6% of sentinel duck sera were positive for H1N1 subtype and 90.2% for H3N2 subtype in our investigation in 2003, and 49.1% were positive for H3N2 subtype in the experiment in 2004. Tested sera were negative to human influenza B virus.

These results are in accordance with earlier world-wide serological findings in domestic and wild birds. Thus, AYOUB et al. (1974) report the finding of HI antibodies in hens and turkeys for certain human influenza type A strains. Testing two different age groups of separated colonies of hens and one group of turkeys, they found a positive HI titer in 46.3% of hens aged about 18 months to A/Hong Kong/1/68 (H3N2) virus, in 43% to A/Hong Kong/107/71 (H3N2), and in 64.7% to A/England/42/72 (H3N2), while in hens aged about 20 months they found HI antibodies to A/Hong Kong/107/71 in 46.6% and to A/England/42/72 in 50.0%. In turkeys aged 10-13 weeks they recorded a positive HI antibody titer to A/Hong Kong/107/71 (H3N2) virus in 70.7% and for A/England/42/72 (H3N2) virus in 78.0% of the sera examined. Studying HI antibodies to the Victoria/3/75 (H3N2) variant of human influenza virus type A in wild birds, ROMVARY et al. (1976) reported on having found these antibodies in 29.0% of black-headed gull sera, in 40.0% of turtle-dove sera, and in 29.0% of mallard duck sera originating from the Zoological Gardens in Budapest. They also recorded specific antibodies for the same virus in 37.1% of turtle-dove sera, 17.7% of crow sera, and 24.4% of sparrow sera. All these birds were caught along the river Tisza and on some dairy farms with milking cattle and calves. AGRIMI et al. (1978) detected HI antibodies to human influenza type A virus strains A/Victoria/3/75 and A/New Jersey/76 in the sera of hens and pheasants; antibodies to A/New Jersey/76 virus strain in hens were detected in 6.85% and to A/Victoria/3/75 even in 49.2% of the tested sera, while in pheasants specific antibodies were detected to A/New Jersey/76 strain in 9.6% and to A/Victoria/3/75 in 35.2% of tested sera. In Hong Kong, SHORTRIDGE et al. (1979) detected HI antibodies to A/HKU/10/77 (H1N1) human

influenza type virus strain in 19.4% of the sera of hens out of a total of 237 examined samples. In Japan, ITAGAKI et al. (1983) found HI antibodies to human influenza A virus in seven out of 17 tested ducks, in four out of 11 tested pheasants, in two out of nine tested crows, and in three out of five tested pigeons.

The results of our study showed a high percentage of sentinel animals positive to H1N1 and H3N2 subtypes. That was expected because the sentinel animals were in close contact on a fenced fish pond, and with possible contact with wild birds from the whole ornithological reserve. To clarify the time of infection, in our experiment 2004 sera were tested on day 0, day 21 and on day 49. The negative serum samples tested on day 0 and day 21 and the high percentage of positive tested after exposure on the fish pond (day 49) suggest that the ducks were infected after exposure on the fish pond without any contact with humans. These results confirmed that wild migratory birds in the ornithological reserve were the source of infection for the sentinel ducks. The hemagglutination inhibition test results are limited by the possibility of cross-reaction to give a general conclusion, but the high level of seroprevalence and high antibodies titer in some animals suggested infection with an influenza strain closely related to strains used as antigen. Recently hemagglutination inhibition antibodies were detected only rarely and at low titers using homosubtypic but not autologous antigen (GLOBIG et al., 2009).

Evidence of the circulating of influenza virus related to the human strain in wild birds in the ornithological reserve is very important for influenza epidemiology because the ecology system of the ornithological reserve has great potential for the reassortment of different influenza virus strains and the emergence of a potential pandemic of influenza viruses.

Comparing data of human influenza viruses isolated from humans in Croatia with our results in sentinel ducks, we found the same subtype in both populations for each year (DRAŽENOVIĆ and BARIŠIN, 2005). Further investigations will be performed to clarify if this is just a coincidence or if wild migratory bird could be a reservoir of human influenza virus strains.

Now when infection with influenza virus H5N1 is spreading worldwide and has been confirmed in many countries, the main question is the possibility of the reassortment of human and avian types of the influenza A virus. The first confirmation of infection with the H5N1 influenza virus in Croatia was in October 2005 (ANONYM., 2005). Positive swans were found on two locations in the eastern part of Croatia (Našice and Zdenci), on locations near the ornithological reserve Kopački rit. In both cases the infected animals were found on fish ponds. Finally, the recent pandemic caused by the H1N1 influenza virus containing genes originating from avian, human, and swine influenza viruses, with a still unknown reassortment host (GARTEN et al., 2009) have confirmed the importance

of global influenza surveillance, part of which may be surveillance by sentinel animals in different ecology systems.

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SAŽETAK

Mogućnost prijenosa ljudskih sojeva virusa influence izravno s ljudi na ptice još uvijek nije dovoljno istražena. U ovom radu prikazana je uporaba domaćih pataka kao modela prokazivačkih (engl. sentinel) životinja za promatranje širenja influence u divljih ptica, te je pomoću njih istražena infekcije ptica virusima influence antigenski bliskim humanim sojevima. Pretraživani su uzorci seruma pataka slobodno držanih na ribnjaku u ornitološkom rezervatu Kopački Rit. Patke su prva tri tjedna bile uzgajane u zatvorenom objektu, a potom su sljedeća četiri tjedna bile uzgajane na ribnjaku bez dodira s ljudima. Serumi su bili uzorkovani nultoga i 21. dana u zatvorenom objektu te 49. dana, tj. nakon završetka uzgoja na ribnjaku. Svi uzorci seruma bili su pretraženi inhibicijom hemaglutinacije, a kao antigeni bili su rabljeni humani sojevi virusa influence A/New Caledonia/20/99/VR-116 (H1N1) i A/Panama/2007/99 (RESVIR - 17) (H3N2), te humani sojevi virusa influence B/Hong Kong/330/01 i B/Sichuana/379/99. Svi pretraženi uzorci seruma uzeti u zatvorenom objektu nultog i 21. dana uzgajanja bili su negativni, dok su u uzorcima seruma uzetima na kraju uzgoja na ribnjaku (49. dana) ustanovljena specifična protutijela za viruse influence tipa A. Ovi rezultati potvrđuju infekciju pataka tijekom boravka na ribnjaku bez kontakta s ljudima što dokazuje da su divlje ptice bile izvor zaraze. Visoka seroprevalencija, te poglavito visoki titri specifičnih protutijela u pojedinih životinja dokaz su infekcije virusima influence antigenski srodnima humanim sojevima rabljenima u testu inhibicije hemaglutinacije. Postignuti rezultati potvrđuju mogućnost uporabe jata domaćih pataka kao prokazivačkih životinja za promatranje širenja influence u divljih ptica, te upućuju na nužnost daljnjega istraživanja mogućnosti infekcije ptica humanim sojevima virusa influence.

Ključne riječi: influenca, nadzor, prokazivačke patke, divlje ptice