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Influenza A virus surveillance in wild birds

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Abstract

Surveillance studies in wild animals provide information on the prevalence of avian influenza viruses in the environment, and enables banking of reference reagents and putative vaccine strains to be used in times of outbreaks in humans and animals. In the past five years we have performed surveillance studies in wild birds primarily in The Netherlands and Sweden. In ducks, geese and gulls the prevalence of influenza A virus ranged from 0 to 60 percent, depending on bird species, location and season. The detection of avian influenza A viruses in other bird species was rare. Most of the fifteen haemagglutinin subtypes and all nine neuraminidase subtypes described to date were found in birds in Northern Europe. In addition, we have identified a novel haemagglutinin subtype(H16) in black-headed gulls. Viruses of subtypes H5 and H7 were found less frequently than other subtypes, and were closely related to the H5 and H7 highly pathogenic avian influenza viruses that have caused outbreaks in poultry in Italy and The Netherlands between 1997 and 2003.
Keywords: bird; poultry; avian influenza; *Orthomyxovirus*; surveillance; ecology; prevalence; PCR; subtype

Introduction

Influenza virus types A, B and C all belong to the family of *Orthomyxoviridae* and have therefore many biological properties in common (Murphy and Webster 1996). A key difference between them is their in-vivo host range; whereas influenza viruses of types B and C are predominantly human pathogens that have also been isolated from seals and pigs, respectively (Osterhaus et al. 2000; Guo et al. 1983), influenza A viruses have been isolated from many species including humans, pigs, horses, marine mammals and a wide range of domestic and wild birds (Webster et al. 1992). It is generally accepted that in the human influenza pandemics from the last centuries and numerous outbreaks in domestic and wild animals, interspecies transmission of avian influenza viruses has played a crucial role (Webster et al. 1992).

Predominantly water-associated wild birds such as ducks, geese, gulls and shorebirds form the reservoir of influenza A viruses in nature (Figure 1). All fifteen antigenic subtypes of the virus surface glycoprotein haemagglutinin and all nine

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subtypes of neuraminidase that have been identified to date have been isolated from these bird species (Webster et al. 1992). Avian influenza viruses preferentially infect cells lining the intestinal tract of birds and are excreted in high concentrations in their faeces. The transmission of influenza viruses between birds is thought to occur primarily via the faecal-oral route. Whereas avian influenza viruses are generally non-pathogenic in their natural hosts, they may cause significant morbidity and mortality upon transmission to other species.

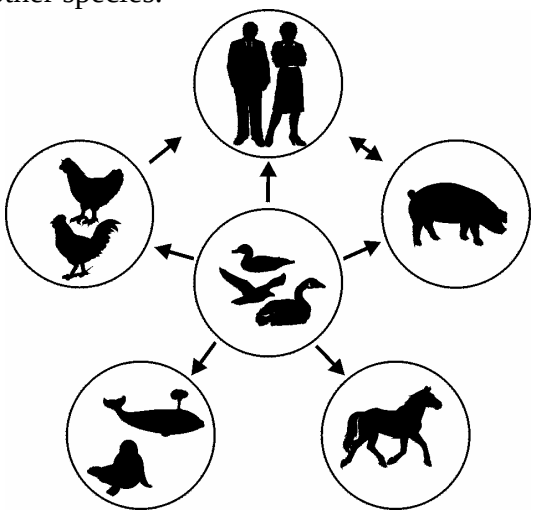

Figure 1. The natural hosts of influenza A virus. Wild aquatic birds are thought to form the influenza A virus reservoir in nature, from which viruses can be transmitted to poultry, pigs, horses, marine mammals and humans. Influenza A viruses can also be transmitted from poultry and pigs to humans

It is virtually impossible to prevent outbreaks of influenza A virus infection in domestic animals and to prevent novel influenza pandemics in man. Through the continuous surveillance of wild birds around the world the prevalence of influenza A viruses in the environment can be monitored and the pathogenic and antigenic properties of the circulating viruses can be determined. In addition, such studies will ensure that panels of reference reagents required for testing of animals and man can be updated continuously and that virus isolates with the appropriate antigenic properties will be available for the generation of potential influenza vaccines (Fouchier et al. 2003).

Previously, we have described a RT-PCR-based screening procedure for influenza A virus that is suitable for the detection of all influenza A virus strains identified to date (Fouchier et al. 2000). This screening method is more sensitive than classical virus propagation in eggs and cell cultures and provides a good alternative for rapid high-throughput screening for influenza A viruses in wild birds. Using this method and with the help of a large network of ornithologists, we have screened a wide spectrum of wild birds of different taxa in Northern Europe since 1998 for the presence of influenza A virus. Although our primary focus was on ducks, geese and gulls because these species are known to be susceptible to influenza A virus, we have also tested thousands of samples from passerines, waders, birds of prey, etc.

Materials and methods

Specimens

Birds were trapped using duck traps, wader funnel traps, mist nets, clap nets or Helgoland funnel traps. For bird species that could not be trapped, fresh dropping

samples were collected from the ground at locations where large numbers of birds congregate. Cloacal swabs and fresh dropping samples were collected using cotton swabs and subsequently stored in transport media at -70ºC. From small birds, mainly passerines and small shorebirds, we collected fresh dropping samples rather than cloacal swabs after capture because the cotton swabs were too large. Transport media consisted of Hanks balanced salt solution supplemented with 10 % glycerol, 200 U/ml penicillin, 200 µg/ml streptomycin, 100 U/ml polymyxin B sulphate, 250 µg/ml gentamicin and 50 U/ml nystatin (all from ICN, Zoetermeer, The Netherlands). Most duck and goose samples were collected in the autumn and winter whereas for most passerine birds and shorebirds samples were collected in spring, summer and early autumn.

RNA isolation

RNA was isolated using a high-pure RNA isolation kit (Roche Molecular Biochemicals) according to the instructions from the manufacturer, with minor modifications. A 0.2 ml sample was homogenized by vortexing and subsequently lysed with 0.4 ml lysis/binding buffer. After binding to the column, DNase-I digestion and washing, the RNA was eluted in 50 µl nuclease-free double-distilled water. Initially, pools of 5 samples are tested (without significant loss of sensitivity). Upon identification of positive pools, the individual positive samples were identified.

RT-PCR

Samples were amplified in a one-step RT-PCR in 25 µl final volume, containing 50 mM Tris.HCl pH 8.5, 50 mM NaCl, 7 mM $MgCl_2$, 2 mM DTT, 1 mM each dNTP, 0.4 µM each oligonucleotide, 2.5 U recombinant RNAsin, 10 U AMV reverse transcriptase, 2.5 U Ampli-Taq DNA polymerase (all enzymes from Promega Benelux BV, Leiden, The Netherlands) and 5 µl RNA. Primers M52C (5'- CTT CTA ACC GAG GTC GAA ACG -3') and M253R M253R (5'- AGG GCA TTT TGG ACA AAG/T CGT CTA -3') were used. Thermo-cycling was performed in an MJ PTC-200 apparatus using the following cycling conditions: 30 min. at 42ºC, 4 min. at 95ºC once; and 1 min. at 95ºC, 1 min. at 45ºC, 3 min. at 72ºC repeated 40 times. Each reaction (5 µl/sample) was analysed by dot-blot hybridization.

Dot-blot hybridization

Five µl of the RT-PCR products was incubated for 5 min. at room temperature with 45 µl 10 mM Tris.HCl pH 8.0, 1 mM EDTA and 50 µl 1 M NaOH for denaturation. Samples were transferred to prewetted Hybond N+ membranes (Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands) using a dot-blot apparatus while applying vacuum. Samples were then treated for 3 min. with 0.1 ml 1 M Tris.HCl pH8.0, after which vacuum was applied again for 10 sec, and the membrane removed from the apparatus. Blots were washed three times for 10 min. with 0.3 M NaCl, 30 mM Na-citrate pH 7, dried, and stored at 4ºC. Blots were prehybridized for 5 min. at 55ºC in 2 x SSPE (0.3 M NaCl, 20 mM NaH_2PO_4, 2 mM EDTA, pH 7.4) and 0.1 % SDS, after which biotinylated oligonucleotide probe Bio-M93C (5'- CCG TCA GGC CCC CTC AAA GCC GA –3') was added to 2 pmol/ml and hybridization was continued for 45 min. at 55ºC. Blots were washed twice for 10 min. at 55ºC with hybridization buffer, transferred to 2 x SSPE with 0.5 % SDS after which streptavidin peroxidase (Roche Molecular Biochemicals) was added to 0.125 U/ml and incubated for 45 min. at 42ºC. Blots were washed 10 min. at 42ºC in 2 x SSPE, 0.5 %SDS, 10 min. at 42ºC in 2 x SSPE, 0.1 % SDS and 10 min. at room temperature in 2 x SSPE,

after which the samples were visualized using ECL detection reagents and exposure to hyperfilm (Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands) for 5 - 60 seconds.

Virus isolation and characterization

For all RT-PCR-positive samples, 200 µl of the original specimen was inoculated in the allantoic cavities of 11-day-old embryonated chicken eggs (Rimmelzwaan et al. 1998). When necessary, the allantoic fluid was harvested and passaged again in embryonated eggs. HA titers in virus stocks were determined with turkey erythrocytes using standard procedures. Virus isolates were characterized by haemagglutination and neuraminidase-inhibition assays with subtype-specific hyper-immune rabbit antisera raised against HA/NA preparations of virus isolates representing all 15 HA subtypes (Rohm et al. 1996). Sequencing was also conducted of HA and NA genes.

Results

PCR-based detection of influenza A virus

From 1998 to 2003 we have collected cloacal swabs and fresh dropping samples from more than 15,000 birds that were tested for the presence of influenza A virus RNA by RT-PCR. The majority of these samples were collected at different locations in The Netherlands (Fouchier et al. 2004) and Sweden (Wallensten et al. 2003). Samples were collected from 252 different bird species with the majority of samples originating from ducks, geese, gulls and shorebirds. RT-PCR positive samples were obtained only from geese (white-fronted, greylag and brent geese), ducks (mallard, wigeon, shoveler and teal), black-headed gulls and guillemots (Table 1). For ducks, gulls and guillemots almost exclusively cloacal swabs were tested, whereas geese samples included both cloacal swabs and fresh droppings, both of which included influenza A virus-positive samples. With 345 RT-PCR-positive samples, the overall detection of influenza A virus was approximately 2.3 percent of all samples.

Table 1. RT-PCR-based screening of wild birds for the presence of influenza A virus

Order (No. species tested)	No. tested	No. PCR-positive (%)	PCR-positive bird species
Anseriformes (22)			
Geese (8)	1627	20 (1.2)	White-fronted, Greylag, Brent goose
Ducks (12)	5223	301 (5.8)	Wigeon, Mallard, Shoveler, Teal
Others (2)	93	0	
Charadriiformes (39)			
Gulls (5)	1606	10 (0.6)	Black-headed gull
Guillemots (2)	30	3 (10.0)	Guillemot
Others (32)	2130	0	
Others (181)	4348	0	

However, at selected locations the prevalence in specific species was much higher at some occasions. For instance, in October 1999 14 of 132 (10.6 %) of all mallard ducks in duck trap ‘Bakkerswaal’ in Lekkerkerk, The Netherlands were positive for influenza A virus and in the second week of August 1999 6 out of 10 (60 %) black-headed gulls in Ottenby, Sweden were positive.

Virus isolation and characterization

Virus could be isolated in embryonated chicken eggs for about fifty percent of the 345 RT-PCR-positive cloacal swabs and dropping samples. Most of the samples that remained negative even upon repeated attempts to isolate the virus contained relatively low copy numbers of viral RNA as judged by the low intensity of the bands upon hybridization of dot-blots. A low proportion of RT-PCR-positive samples that appeared to contain relatively high copy numbers of viral RNA may have been stored improperly in the field, or may contain virus that cannot be isolated efficiently in embryonated chicken eggs.

Most of the fifteen haemagglutinin subtypes (except subtypes 9, 14, 15) and all nine neuraminidase subtypes described throughout the world to date were found in birds in Northern Europe in the past five years. The haemagglutinin gene of 4 virus isolates obtained from black-headed gulls could not be identified using our panels of reference reagents; these HA genes represent a novel subtype, H16.

Viruses of subtypes H5 and H7 were found less frequently than other subtypes. Sequence analyses of haemagglutinin and neuraminidase genes of influenza A viruses obtained from mallard ducks indicate that these viruses are very closely related to the H5 and H7 highly pathogenic avian influenza viruses that have caused outbreaks in poultry in Italy and The Netherlands between 1997 and 2003.

Discussion

This study demonstrates that influenza A viruses are highly prevalent in wild birds in Northern Europe. For ducks (~5200 tested), geese (~1600 tested) and guillemots, the prevalence varied from 0 to 11 %, and for gulls (~1600 tested) from 0 to 60 % dependent on the birds' age, time, location and species. Using a highly sensitive RT-PCR procedure, we only found evidence for influenza A virus infection of aquatic birds (ducks, geese, gulls, guillemots), which is in agreement with previous studies performed by many laboratories using virus isolation in embryonated chicken eggs (Webster et al. 1992; Ito and Kawaoka 1998), but not in more than 6500 samples from other bird species (252 bird species were included in our dataset). A clear difference with other studies (Ito and Kawaoka 1998) is the apparent absence of influenza A virus in our collection of shorebirds. In Northern Europe, the prevalence of avian influenza in ducks and geese is at its peak between late summer and early winter when the birds leave their breeding grounds and start migrating, and up to 30 % of a flock or colony may be excreting virus

Many different influenza A virus subtypes were found to circulate at the same time, in the same bird species at a single location in The Netherlands. For instance, in duck trap 'Bakkerswaal' in Lekkerkerk, The Netherlands, 14 out of 132 mallard ducks caught in October 1999 were positive for influenza A virus, and HA subtypes 1, 2, 4, 5 and 11 were identified. The genetic and antigenic heterogeneity observed for some of our virus isolates indicates that continuous influenza A virus surveillance is required to keep panels of reference reagents and potential future vaccine strains updated.

Viruses of subtypes H5 and H7 were found less frequently than other subtypes. Sequence analyses of haemagglutinin and neuraminidase genes of influenza A viruses obtained from mallard ducks indicate that these viruses are very closely related to the H5 and H7 highly pathogenic avian influenza viruses that have caused outbreaks in poultry in Italy and The Netherlands between 1997 and 2003 (Capua and Marangon 2000; Banks et al. 2000; Fouchier et al. 2004).

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