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Determination of hemagglutinin and neuraminidase subtypes of avian influenza A viruses in urban pigeons by a new nested RT-PCR

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Summary. – The prevalence of avian influenza viruses (AIVs) together with determination of hemagglutinin (HA) and neuraminidase (NA) subtypes were studied in urban pigeons using a new nested RT-PCR. Both oropharyngeal and cloacal swabs from birds were collected. Altogether, screening of all samples revealed that 12% of oropharyngeal and 20% of cloacal samples were positive for AIVs. However, samples from both the oropharynx and cloaca coming from one animal were positive in only 8% of pigeons. Four different HA and NA combinations H7N3, H7N6, H9N5, and H14N8 respectively, were identified using a new nested RT-PCR.

Keywords: avian influenza virus; hemagglutinin; neuraminidase

Introduction

Avian influenza is caused by the Influenza A virus that belongs to the family *Orthomyxoviridae*. While all birds are thought to be susceptible to the infection with avian influenza viruses (AIVs), many wild bird species carry these viruses with no apparent signs of infection (Webster *et al*., 1992). In poultry, AIV causes two different forms of disease – one common and mild, the other rare and highly lethal. All 16 hemagglutinin and 9 neuraminidase subtypes of AIV are known to infect wild waterfowl providing an extensive reservoir of influenza viruses circulating in bird populations. Transmission of AIVs among susceptible birds occurs mainly through direct contact with infectious excretions and secretions, particularly feces. The pigeons may be infected by AIVs directly by contact with infective birds or indirectly through contact with a contaminated environment.

Subtyping is essential for the identification of circulating strains, investigation of viral reassortment, and epidemiological characterization of emerging influenza viruses. Serological testing is the most common technique used for the identification of subtypes. There are also several assays detecting nucleic acid as RT-PCR, real-time RT-PCR, and microarray (Dawson *et al.,* 2006, 2007; Cattoli *et al.,* 2007; Runstadler *et al*., 2007), but none of them is easily technically available as the nested RT-PCR described here.

The aim of the present study was to evaluate a method, which will be able to differentiate the known HA and NA subtypes and identify AIV in the sample without previous isolation of the virus.

Materials and Methods

Collection of samples. 50 urban pigeons were caught into the traps located in the centre of Košice (48º43'N; 21º15'E). Cloacal and oropharyngeal samples were collected and the birds were released. The samples were immediately frozen in liquid nitrogen and transferred to the laboratory.

Nested RT-PCR. The swabs were extracted with 2.5 ml of PBS each and 100 µl aliquots were used for purification of RNA with RNeasy MiniKit (Qiagen). cDNA was synthesized from purified RNA by reverse transcription using random oligonucleotide primers. Nested RT-PCR was done using the primers for the conserved

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Abbreviations: AIV(s) = avian influenza virus(es); HA = hemagglutinin; NA = neuraminidase

Table 1. Primers used in the nested RT-PCR for subtyping of HA of AIVs

Subtype Acc. No.	Primers[a]	Product size (bp)	Subtype Acc. No.	Primers[a]	Product size (bp)
H1 CY035250.1	F1 (31-52) R1 (731-713) F2 (117-139) R2 (542-520)	700bp 425bp	H9 FJ231866.1	F1 (185-207) R1 (844-822) F2 (346-358) R2 (557-537)	659bp 211bp
H2 AY422017.1	F1 (419-440) R1 (908-887) F2 (505-778) R2 (800-778)	489bp 295bp	H10 CY006001.1	F1 (141-161) R1 (893-869) F2 (380-400) R2 (682-662)	752bp 302bp
H3 EU557492.1	F1 (46-69) R1 (829-808) F2 (232-254) R2 (709-690	783bp 477bp	H11 CY006005.1	F1 (667-687) R1 (1288-1268) F2 (796-819) R2 (991-971)	621bp 195bp
H4 AY633156.1	F1 (63-84) R1 (1083-1064) F2 (575-593) R2 (732-713)	1020bp 157bp	H12 CY021293.1	F1 (71-94) R1 (748-727) F2 (201-223) R2 (432-408)	677bp 231bp
H5 FJ602868.1	F1 (610-632) R1 (1109-1087) F2 (697-718) R2 (930-906)	499bp 233bp	H13 CY014603.1	F1 (441-460) R1 (1127-1107) F2 (759-780) R2 (991-971)	686b 232bp
H6 DQ376653.1	F1 (63-81) R1 (1039-1018) F2 (277-297) R2 (614-592)	976bp 337bp	H14 CY014604.1	F1 (371-394) R1 (1285-1263) F2 (699-720) R2 (1174-1150)	914bp 475bp
H7 EF675618.1	F1 (364-384) R1 (788-765) F2 (450-471) R2 (644-614)	424bp 194bp	H15 CY006032.1	F1 (29-49) R1 (758-739) F2 (266-289) R2 (565-544)	710bp 299bp
H8 AF310989.1	F1 (103-126) R1 (996-973) F2 (391-411) R2 (727-706)	893bp 336bp	H16 AY684890.1	F1 (88-110) R1 (1005-985) F2 (293-316) R2 (887-865)	917bp 594bp

[a]nt position of forward (F) and reverse (R) primers.

region of M gene as described previously (Betáková *et al.,* 2005*;* Gronesová *et al.,* 2007).

Nested RT-PCR for subtyping AIVs. AIV-positive samples were used in nested RT-PCR with primers specific for each HA and NA subtype. The primers were designed to be specific to the conserved regions of each subtype of HA or NA (Table 1 and 2).

The RT-PCR for each HA and NA subtype was run in separate tube containing 2 µl of cDNA, 10 µl of 2x MasterMix (Fermentas), 1 µl of each F1 and R1 primer and 6 µl of H_2O. Reaction mixtures were placed in a thermal cycler at 94°C for 5 mins; cycled 35 times through 94°C for 30 secs, 55°C for 30 secs, and 72°C for 2 mins; and finally kept at 4°C. The obtained PCR product was mixed with 10 µl of BioMix (Bioline), 1 µl of second set of primers (F2 and R2), and 8 µl of H_2O. Cycling condition were as follows: 95°C for 15 mins followed by 36 cycles of 95°C for 30 secs, 55°C for 30 secs, and 72°C for 45 secs and a final extension of 72°C for 10 mins before cooling to 4°C. PCR products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Results and Discussion

AIVs were detected in both oropharyngeal and cloacal samples. Altogether, 10 cloacal (20%) and 6 oropharyngeal (12%) samples were AIV-positive. From that amount 6 cloacal samples were positive in the pigeons with negative oropharyngeal samples, and two oropharyngeal samples were positive in the pigeons with negative cloacal samples. Samples from both the oropharynx and cloaca were positive in only 4 pigeons (8%). In total, samples of 12 pigeons from 50 pigeons tested (24%) were found positive for AIVs in one or both of the collected samples from cloaca or oropharynx (Table 3). Prevalence of AIVs in passerines has previously been reported as being particularly low (Fouchier *et al.*, 2003; Schnebel *et al*., 2005; Lebarbenchon *et al*., 2007), although the application of the nested RT-PCR may have increased the sensitivity of virus detection (Gronesová *et*

Table 2. Primers used in the nested RT-PCR for subtyping of NA of AIVs

Subtype Acc. No.	Primers[a]	Product size (bp)	Subtype Acc. No.	Primers[a]	Product size (bp)
N1 AY646425.1	F1 (612-634) R1 (1370-1345) F2 (900-919) R2 (1175-1149)	758bp 275bp	N6 AB270600.1	F1 (13-34) R1 (1413-1391) F2 (868-887) R2 (1226-1204)	1400bp 358bp
N2 CY021063.1	F1 (557-578) R1 (828-808) F2 (604-625) R2 (734-711)	271bp 130bp	N7 CY005251.1	F1 (15-35) R1 (1440-1420) F2 (333-353) R2 (1164-1145)	1425bp 831bp
N3 AY207510.1	F1 (241-267) R1 (1396-1373) F2 (371-393) R2 (647-625)	1155bp 276bp	N8 DQ885998.1	F 1(544-565) R1 (1376-1356) F2 (865-887) R2 (1271-1253)	832bp 406bp
N4 CY005359.1	F1 (344-365) R1 (1382-1361) F2 (685-704) R2 (1226-1207)	1038bp 541bp	N9 CY014859.1	F1 (177-196) R1 (1429-1409) F2 (454-476) R2 (1370-1351)	1252bp 916bp
N5 AB270600.1	F1 (117-139) R1 (936-915) F2 (241-259) R2 (536-514)	819bp 295bp			

[a]nt position of forward (F) and reverse (R) primers.

al., 2007; Mižáková *et al.,* 2008; Keawcharoen *et al*., 2008, Gronesova *et al.,* 2008a). We have eliminated the false-positive assessment of our samples, since each sample proved positive in three different nested RT-PCRs and moreover, the different subtypes of HA and NA were identified.

Relatively high percentage of AIV-positive samples from urban pigeons can be also affected by the method used for sampling of the birds. The traditional method of sampling wild birds is through the cloacal swabbing only. However, we found 2 birds positive only in the oropharynx and they would have been missed when tested only with cloacal sampling. Thus, using oropharyngeal and cloacal sampling should be routinely used in surveillance of AIV in wild birds (Mižáková *et al.,* 2008; Gronesová *et al*., 2008b; Ellstrom *et al.,* 2008).

AIVs with 3 different subtypes of hemagglutinin and 4 different subtypes of neuraminidase were detected in the collected samples. Five birds were infected with the H7N3 viruses, 2 birds were infected with H7N6 viruses, and 4 birds were infected with H9N5 viruses (Table 3). Surprisingly, the H14N8 virus was detected in 1 sample, since this virus seems to be particularly adapted to the replication in ducks and pelagic birds (Kawaoka *et al.,* 1990; Rohm *et al.,* 1996). However, H5 viruses were not detected.

In Europe, low-pathogenic H5 and H7 viruses were isolated from poultry in Italy (H7N3 in years 2002–2003 and H5N2 in year 2005), Netherlands (H7N3 in year 2002), France (H5N2 in year 2003), and Denmark (H5N7 in year 2003) (Alexander 2007). Infections with H9N2 subtype viruses were widespread in Asia during the years 2002–2006 (Alexander, 2007). These subtypes of viruses also circulated in population of wild birds living in this area (Gronesova *et al.,* 2008c). Slovakia is crossed by two dominant north-south and east-west birds' migratory routes and urban pigeons may mix with these wild birds and come into contact with a contaminated environment (Panigrahy *et al.,* 1996; Perkins *et al.,* 2002). Thus, it is not surprising that we found two prevalent AIVs subtypes of H7 and H9 that are predominant in Europe and Asia, respectively.

The presented subtyping nested RT-PCR was about 100 times more sensitive than diagnostic PCR (data not shown).

Table 3. HA and NA subtypes of AIVs in pigeons

No. of pigeons	HN subtype present in	
	oropharynx	cloaca
2	H7N3	H7N3
3	–	H7N3
2	–	H7N6
2	H9N5	H9N5
2	H9N5	–
1	–	H14N8
*Total 12 (24%)	6 (12%)	10 (20%)

*The number of tested pigeons was 50.

In addition, all subtypes of HA and NA can be detected in the diluted samples without previous virus isolation, while majority of subtyping assays require virus isolation. The other advantage of this assay is its availability. Equipments for real-time PCR or microarray are not usually accessible in regular laboratories. Subtyping of AIVs by a nested RT-PCR can be accomplished in a comparably short and straightforward process that has the potential to be used as a diagnostic tool for the detection of AIVs strains and does not require the special equipment.

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