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

Abbreviations: AIV(s) = avian influenza virus(es); HA = hemagglutinin; NA = neuraminidase
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 H2O. 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 H2O. 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 al., 2007).
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 2. Primers used in the nested RT-PCR for subtyping of NA of AIVs

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Acc. No.</th>
<th>Primers&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Product size (bp)</th>
<th>Subtype</th>
<th>Acc. No.</th>
<th>Primers&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>AY646425.1</td>
<td>F1 (612-634) R1 (1370-1345) F2 (900-919) R2 (1175-1199)</td>
<td>758bp 275bp</td>
<td>N6</td>
<td>AB270600.1</td>
<td>F1 (13-34) R1 (1413-1391) F2 (868-887) R2 (1226-1204)</td>
<td>1400bp 358bp</td>
</tr>
<tr>
<td>N2</td>
<td>CY021063.1</td>
<td>F1 (557-578) R1 (828-808) F2 (604-625) R2 (734-711)</td>
<td>271bp 130bp</td>
<td>N7</td>
<td>CY005251.1</td>
<td>F1 (15-35) R1 (1440-1420) F2 (333-353) R2 (1164-1145)</td>
<td>1425bp 831bp</td>
</tr>
<tr>
<td>N3</td>
<td>AY207510.1</td>
<td>F1 (241-267) R1 (1396-1373) F2 (371-393) R2 (647-625)</td>
<td>1155bp 276bp</td>
<td>N8</td>
<td>DQ885998.1</td>
<td>F1 (544-565) R1 (1376-1356) F2 (865-887) R2 (1271-1253)</td>
<td>832bp 406bp</td>
</tr>
<tr>
<td>N4</td>
<td>CY005359.1</td>
<td>F1 (344-365) R1 (1382-1361) F2 (685-704) R2 (1226-1207)</td>
<td>1038bp 541bp</td>
<td>N9</td>
<td>CY014859.1</td>
<td>F1 (177-196) R1 (1429-1409) F2 (454-476) R2 (1370-1351)</td>
<td>1252bp 916bp</td>
</tr>
<tr>
<td>N5</td>
<td>AB270600.1</td>
<td>F1 (117-139) R1 (936-915) F2 (241-259) R2 (536-514)</td>
<td>819bp 295bp</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</table>

<sup>a</sup>nt position of forward (F) and reverse (R) primers.

### Table 3. HA and NA subtypes of AIVs in pigeons

<table>
<thead>
<tr>
<th>No. of pigeons</th>
<th>HN subtype present in oropharynx</th>
<th>HN subtype present in cloaca</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>H7N3</td>
<td>H7N3</td>
</tr>
<tr>
<td>3</td>
<td>–</td>
<td>H7N3</td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>H7N6</td>
</tr>
<tr>
<td>2</td>
<td>H9N5</td>
<td>H9N5</td>
</tr>
<tr>
<td>2</td>
<td>H9N5</td>
<td>–</td>
</tr>
<tr>
<td>1</td>
<td>–</td>
<td>H4N8</td>
</tr>
</tbody>
</table>

<sup>*Total 12 (24%)</sup> 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 comparatively 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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References


