LETTER TO THE EDITOR

DETECTION OF INFLUENZA A VIRUS IN WILD BIRDS IN WEST SLOVAKIA BY NESTED PCR

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Avian influenza, caused by Influenza A virus (IAV) (the genus *Influenzavirus A*, the family *Orthomyxoviridae*) is a highly contagious disease affecting respiratory and digestive tracts and/or nervous system of many species of birds, domestic and wild as well. All 16 hemaglutinin and 9 neuraminidase subtypes of IAV are know to infect wild waterfowl, thus providing an extensive reservoir of viruses circulating in birds populations. A routine testing of samples obtained from wild birds nearly always detects some IAVs. A vast majority of these viruses cause no harm to their hosts. Only after the transmission from wild birds to domestic poultry and subsequent circulation in the poultry populations, lowly pathogenic viruses may mutate into highly pathogenic ones, like those causing major disease problems in parts of Asia and affecting also humans (*1*). There is some evidence that the excretion of IAVs by domestic ducks increased by early 2004 and that they can be transmitted by wild birds. The migratory birds from which IAVs had been isolated were usually sick or dead, suggesting that they would have a limited potential for carrying the viruses over long distances unless subclinical infections were prevalent (*2*). However, there is strong circumstantial evidence that wild birds can become infected from domestic poultry and potentially can exchange viruses while sharing the same environment (*2, 3*). The minor genetic and antigenic diversity between the viruses recovered from wild birds and those causing highly pathogenic avian influenza outbreaks indicate that IAV surveillance studies in wild birds are indeed useful in the design and evaluation of diagnostic tests and in the generation of prototype vaccine candidates prior to the occurrence of outbreaks in animals and humans (*3–5*).

One of the dominant European north-south wild birds' migratory routes passes across Slovakia and the surveillance of IAVs in wild birds in Slovakia began in 2004. The first study of this kind using a simple RT-PCR proved the presence of the virus in two species of wild waterbirds (*6*). Therefore, in this study, an attempt was made to investigate the positivity of wild birds in West Slovakia for IAVs using a more efficient technique, a nested PCR.

In June 2006, cloacal and tracheal swabs were collected from 42 captured wild birds of 11 free living species living in National Park Parížske Močiare, one of the largest wetlands in West Slovakia, located near the villages of Gbelce and Nová Vieska. All captured birds belonged to the order *Passeriformes.* Species as *Acrocephalus* spp., *Emberiza schoeniclus*, *Locustella luscinioides*, and *Panurus biarmicus* are water or near water living birds. The other

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Abbreviations: IAV = Influenza A virus; p.i. = post infection

species represent forest-dwelling birds or birds living in other terrestrial habitats. The samples were immediately frozen in liquid nitrogen and transferred to the laboratory. The swabs were extracted each with 3 ml of PBS and 100 µl aliquots of the extracts were used for purification of RNA with RNeasy Mini Kit (Qiagen). cDNAs were synthesized from purified RNAs by reverse transcription using random oligonucleotide primers. The first PCR was done using the primers for the conserved region of M gene as described previously (*6*). The second PCR was done with the primers M2F (5'-GCTAGGCAGATGGTGCAGGCAATG-3') and M2R (5'-GTAGAAGGCCCTCTTTTCAAAC-3'). The PCR was performed in 35 cycles of 94°C/30 secs (denaturation), 55°C/30 secs (annealing), and 72°C/1min (extension) using 2xPCR Master mix (Fermentas). The PCR products of 258 bp were identified by agarose gel (1.5%) electrophoresis and ethidium bromide staining.

The results of this study (the table) showed that the virus was detected in both tracheal and cloacal samples in 3 birds only. Five cloacal samples were positive in the birds with negative tracheal samples, while 3 tracheal samples were positive in the birds with negative cloacal samples. Altogether 11 birds (26%) were found positive for the virus.

These findings show how important is the kind of samples, which are collected and analyzed. The majority (*8*) of positive samples were obtained from cloaca, while a little less (*6*) from trachea. In comparing water birds with forest-dwelling species we found the positivity of 22% and 30%, respectively**.** Because of small number of tested samples, this finding should be considered preliminary.

The results of this study do not correspond to those of previous reports, which showed maximum positivity for tracheal swabs from the chicken infected intranasally (*7, 8*). The virus in the chickens infected intranasally could be detected in tracheal swabs already on day 1 post infection (p.i.) (*8*). In the chickens infected orally and by contact, the virus could be detected only on days 2 and 3 p.i., respectively (*8*). There is also evidence on the virus detection in tracheal swabs for at least 6 days p.i. (*9*). However, there are no data on the virus in cloacal swabs. Moreover, only a little is known about the propagation of IAV in wild birds.

Use of a nested PCR increased the sensitivity of the virus detection by simple PCR reported previously (*6*). Namely, in our conditions, the positivity of samples increased from 2% to 26%. Previous studies of other authors have reported 9.9–10.5% of positive samples collected from wild birds (*10, 11*) and considerable variation of positivity among different species (*11*). It is obvious that the positivity of samples depends on the sensitivity of the used methods.

Whereas our previous findings indicated that IAV was present in the birds migrating through Slovakia (*6*), this study proved its presence also in the wild birds living in this area. The species barriers between the birds are much more permeable to IAV than previously anticipated. The diversity of genotype, gene composition, and host receptor specificity provides this virus with multiple options of hosts (*12–14*). There is a possibility that virtually all IAVs presenting as low- or even non-pathogenic for their natural hosts may have the capacity to become more pathogenic upon transmission to "non-natural" hosts (*3*). Thus it is very important to continue the surveillance and characterization of IAV in wild birds.

Species	No. of tested birds	No. of positive samples from		No of positive birds
		Trachea	Cloaca	
Hirundo rustica (swallow)	3	0	1	1
Locustella luscinioides (Savi's warbler)	3	0	0	0
Acrocephalus melanopogon (moustached warbler)	2	1	1	1
Acrocephalus schoenobaenus (sedge warbler)	2	0	0	0
Acrocephalus scirpaceus (reed warbler)	10	1	1	3
		1	0	
		0	1	
		0	0	
Acrocephalus arundinaceus (great reed warbler)	2	1	0	1
Phylloscopus collybita (chiffchaff)	1	0	0	0
Panurus biarmicus (bearded tit)	7	1	0	1
Parus caeruleus (blue tit)	3	0	1	1
Sturnus vulgaris (starling)	3	0	0	0
Emberiza schoeniclus (reed bunting)	6	0	2	3
		1	1	
Total	42	6	8	11

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