www.czasopisma.pan.pl PAN www.journals.pan.pl
POLSKA AKADEMIA NAUK

Polish Journal of Veterinary Sciences Vol. 15, No. 2 (2012), 323-328

DOI 10.2478/v10181-011-0150-4

Original article

Avian influenza viruses in wild birds at the Jeziorsko reservoir in Poland in 2008-2010

K. Śmietanka[1], Z. Minta[1], R. Włodarczyk[2], K. Wyrostek[1], M. Jóźwiak[1], M. Olszewska[1], P. Minias[2], K. Kaczmarek[3], T. Janiszewski[2], A. Kleszcz[4]

[1] Department of Poultry Diseases, National Veterinary Research Institute, Al. Partyzantów 57, 24-100 Puławy, Poland
[2] Department of Teacher Training and Biodiversity Studies, University of Łódź, ul. Banacha 1/3, 90-237 Łódź, Poland
[3] Medical University of Łódź, Al. Kościuszki 4, 90-419 Łódź, Poland
[4] Ornithological Section of the Student's Scientific Club of Biologists, ul. Banacha 1/3, 90-237 Łódź, Poland

Abstract

During a 3-year surveillance study for avian influenza virus (AIV) infections at the Jeziorsko reservoir in central Poland, 549 oropharyngeal or cloacal swabs from 366 birds of 14 species belonging to 3 orders (*Anseriformes*, *Charadriiformes* and *Gruiformes*) were tested. AIV was detected in 14 birds (3.8%): Common Teals (12x), Mallard (1x) and Garganey (1x). Three potentially dangerous H5 AIV were detected in Common Teals (2x) and Garganey (1x) but all of them revealed a low pathogenic pathotype. A unique cleavage site amino acid motif PQREIR*GLF was found in one H5 isolate from a Garganey.

Key words: avian influenza, wild birds, Jeziorsko

Introduction

Low pathogenic avian influenza viruses (LPAIV) are widely distributed in wild birds, mostly of the orders *Anseriformes* and *Charadriiformes*, and to date the presence of AIV has been confirmed in at least 105 species (Olsen et al. 2006). Based on the surface antigens haemagglutinin (HA) and neuraminidase (NA), 16 HA subtypes and 9 NA subtypes of AIV have been described that form different combinations, for example H5N1, H7N7, H3N8 etc. (Alexander 2007). LPAIV infections in wild birds are asymptomatic but virus transmission to domestic poultry can lead to a series of adaptation processes and in some cases poultry-adapted viruses can evolve into highly pathogenic (HP) AIV. The unprecedented outbreaks and spread of H5N1 HPAI in wild birds in recent years is believed to be a result of a spill-over from infected poultry (Alexander 2007, Spackman 2009). So far only AIV of H5 and H7 subtype have had the potential to become highly pathogenic. Despite the recent efforts on the expansion of AIV surveillance in wild bird population all over the world (Gaidet et al. 2007, Munster et al. 2007, Breed et al. 2010, Hansbro et al. 2010, Bui et al. 2011, Goekjian et al. 2011), numerous aspects of the ecology and evolutionary dynamics of AIV still remain unsolved. For example, the mechanisms of virus maintenance in breeding and wintering populations, duration and patterns of virus shedding and long-distance transmission, virus persist-

Correspondence to: K. Śmietanka, e-mail: ksmiet@piwet.pulawy.pl

ence in the environment, frequency of virus introduction into domestic poultry or major mechanisms of genetic diversity of AIV (reassortment, genetic drift) have only been partially recognized (Dugan et al. 2008, Latorre-Margalef et al. 2009, Costa et al. 2010, 2011, Henaux and Samuel 2011, Lebarbenchon and Stallknecht 2011, Smietanka et al. 2011). A better understanding of these mechanisms can be achieved through the continuous, high quality and risk-based surveillance as well as experimental and simulation studies.

The present study reports on the occurrence of AIV carried by migratory birds captured at the Jeziorsko (a lake in central Poland) reservoir over a 3-year period and molecular characterization of isolates potentially dangerous for poultry.

Materials and Methods

Sample collection. Samples were collected at the Jeziorsko reservoir in central Poland in three consecutive years (2008-2010) in late summer/early autumn, with major sampling activity in August-September. Cloacal and/or oropharyngeal samples were collected from wild birds captured by funnel traps using commercial swabs (COPAN UTM, Italy) or "dry" cotton swabs, placed in portable freezers and transported chilled to our laboratory. In the laboratory, "dry" swabs were placed in 2 ml phosphate-buffered saline (PBS) containing penicillin (10 000 IU/mL), streptomycin (10 mg/mL), gentamicin (0,25 mg/mL), and nystatin (5000 U/mL). A total of 549 samples from 366 birds belonging to 3 orders (*Anseriformes*, *Charadriiformes* and *Gruiformes*) and 14 species were tested (Table 1).

AIV detection, isolation and subtyping. Viral RNA was extracted from viral transport medium or PBS according to manufacturers' instructions by using RNeasy Mini kit (QIAGEN, Germany). AIVs were detected by real-time reverse transcription-PCR (rRT-PCR) with primers and probe targeting the conserved matrix (M) gene (Spackman et al. 2002) using the Quantitect Probe PCR kit (Qiagen Hilden, Germany) in a 25-µl reaction volume on an ABI 7500 (Applied Biosystems, USA). The RT and PCR conditions were as follows: RT step: 30 min at 50°C and 15 min at 95°C, PCR: 40 cycles of 95°C for 10 s and 60°C for 20 s. rRT-PCR/M-positive samples were subsequently tested in rRT-PCR and conventional RT-PCR targeting H5 and H7 genes (Slomka et al. 2007a, 2007, 2009) as well as by virus isolation in 9-11 day-old SPF embryonated hens' eggs (OIE 2008). Haemagglutinating isolates were identified by means of the haemagglutination inhibition (HI) assay according to standard procedure (OIE 2008) using specific hyperimmune chicken antisera to all 16 HA subtypes (x-oVo, UK).

Another RT-PCR (primer sequences and PCR conditions available on request) was performed to amplify selected regions of all internal genes of two H4N6 AIV found in Common Teals: PB2 (465 bp), PB1 (660 bp), PA (610 bp), NP (460 bp), M (450 bp), NS (780 bp).

Sequencing, molecular characterization and phylogenetic analysis. PCR products were purified and sequences were generated using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA) and sequenced in the 3730xl DNA analyzer (Applied Biosystems, USA) in Genomed, Warsaw. Sequences were assembled and edited with SeqMan, Lasergene (DNASTAR, USA), aligned and compared with 21 sequences of H5 available on GenBank and from our own collection. Translation of DNA sequence into amino acid sequence and phylogenetic analysis of the 280 bp fragment of H5 gene (from nucleotide 813 to 1092) using neighbor-joining method were performed in MEGA5 software (Tamura et al. 2011).

Sequences obtained in this study have been submitted to the EpiFlu databank (accessible at www.gisaid.org) with the following isolate IDs: EPI_ISL_81323, EPI_ISL_93618, EPI_ISL_93619, EPI_ISL_93620.

Results

Fourteen AIVs were detected by rRT-PCR, representing a total PCR-positive detection rate of 3.8%. Three species of birds were found positive: Common Teal (12 birds), Garganey (1 bird) and Mallard (1 bird). Three viruses belonging to subtype H5 (Table 1) were only detected by RT-PCR (virus isolation negative). Two viruses from Common Teals, detected by PCR and virus isolation, were classified into subtype H4N6. Nine rRT-PCR/M positive samples were negative in rRT-PCR/H5&H7 and failed to grow in embryonated chicken eggs and therefore their subtype could not be determined. Only cloacal swabs were found positive. Two different cleavage site amino acid sequences were found in H5 viruses: PQRETR*GLF (Common Teals) and PQREIR*GLF (Garganey) and both were consistent with a low pathogenicity. Phylogenetic analysis (Fig. 1) showed that two H5 AIV from Common Teals were located closely to each other and were substantially different from H5 virus detected from a Garganey. The internal genes of two H4N6 AIV found in Teals shared identical sequences over the regions examined.

Table 1. Species of birds captured at the Jeziorsko reservoir in 2008-2010 and detected avian influenza viruses.

Order	Species	Number of birds tested	Number of swabs tested	AIV detected
Anseriformes	Common teal *Anas crecca*	208	371	2xH5 LPAI 2xH4N6 8xAIV (subtype unidentified)
	Garganey *Anas querquedula*	15	21	1x H5LPAI
	Mallard *Anas platyrhynchos*	12	14	1xAIV (subtype unidentified)
	Mute Swan *Cygnus olor*	12	17	–
	Eurasian Wigeon *Anas penelope*	1	1	–
Charadriiformes	Common Snipe *Gallinago gallinago*	92	98	–
	Wood Sandpiper *Tringa glareola*	17	17	–
	Dunlin *Calidris alpina*	1	1	–
	Spotted Redshank *Tringa erythropus*	1	1	–
	Ringed Plover *Charadrius hiaticula*	1	1	–
	Black Tern *Chlidonias niger*	1	1	–
	Whiskered Tern *Chlidonias hybridus*	1	1	–
Gruiformes	Spotted Crake *Porzana porzana*	3	4	–
	Water Rail *Rallus aquaticus*	1	1	–
Total		366	549	14

Discussion

The Jeziorsko reservoir is one of the largest man-made reservoirs in Poland, located in central part of the country with a maximum surface area of 43 km^2. Jeziorsko is home to more than 250 species of birds, including 150 breeding in this area. It is also an important stop-over site for waterfowl and waders, and during the autumn migrations the total population size exceeds 10 000 individuals (Janiszewski et al. 1998). Before 2008, avian influenza surveillance was not performed in this reservoir on a large scale. However, H5N1 HPAIV was found in a Mute swan found in this area during the epidemic in 2006 (Minta et al. 2007).

In the present study, the vast majority of tested birds (67.7%) belonged to *Anseriformes*, order and Common Teals *Anas crecca* constituted almost 57% of all birds sampled. An overall detection rate in wild birds during the 3-year period was 3.8% and in relation to Teals 5.7%. In other studies, the average prevalence in this species was 3.6% (Lebarbenchon et al. 2007), 6.4% (Munster et al. 2007) and 14.8% (Terregino et al. 2007). With such a relatively high prevalence it seems likely that Common Teals may play an important role in the AIV dispersal. Lebarbenchon et al. (2009) investigated the role of Common Teals in the spread of AIV by building a simulation model based on bird-ring recoveries, population size, average prevalence of AIV infections, duration of viral shed-

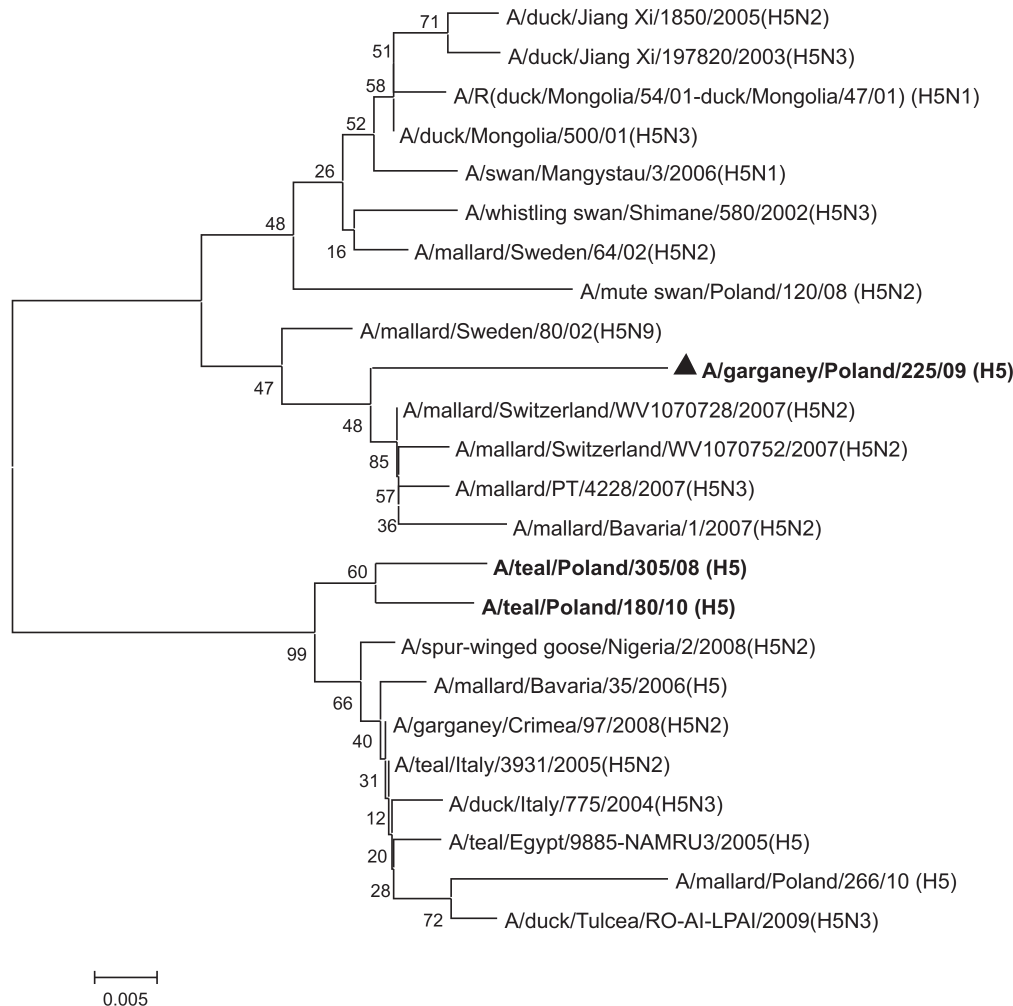

Fig. 1. Phylogenetic characterization based on the neighbor-joining analysis of 280 bp fragment of the HA gene of 3 viruses isolated from Common Teals and Garganey.

ding and persistence of AIV in the environment and concluded that efficient AIV dispersal by Teals is only possible for birds excreting virus for more than 7 days. The median infectious period for LPAI-infected wild ducks was found to be 10-11.5 days as opposed to HPAI-infected ducks in which the mean shedding period was only 5 days (Henaux and Samuel 2011). Therefore, it seems plausible to conclude that Common Teals may play a role in the long-distance dispersal of LPAIV but highly pathogenic viruses are unlikely to be efficiently spread by birds of this species.

Out of 14 positive results in rRT-PCR specific for influenza A type, only 5 could be subtyped. The reason for failures in virus isolation attempts can be either a very high sensitivity of rRT-PCR method (higher than in case of virus isolation method) or insufficient storage and transport conditions (high temperature, duration of transport etc.) that led to the virus decline. In other studies an overall virus isolation rate was also low and ranged from 15% to 33.5% (Munster et al. 2007, Terregino et al. 2007). Three isolates belonged to potentially dangerous subtype H5 but all of them exhibited low pathogenic amino acid profile. Interestingly, the isolate from a Garganey had a unique amino acid sequence at the cleavage site of HA: "PQREIR*GLF" that differed slightly from a much more prevalent motif "PQRETR*GLF" found in numerous H5 LPAIV, including two H5 found in Common Teals. The database search (Influenza virus resource, NCBI, available at http://www.ncbi.nlm.nih.gov/ genomes/ FLU/FLU.html) did not reveal isolates with a similar sequence so we decided to perform additional sequencing which also confirmed our initial finding. The cause of the substitution was a nucleotide point mutation that changed the triplet codon to "ATA" and contributed to the replacement of Threonione (T) with Isoleucine (I) (Fig. 2).

www.czasopisma.pan.pl PAN POLSKA AKADEMIA NAUK www.journals.pan.pl

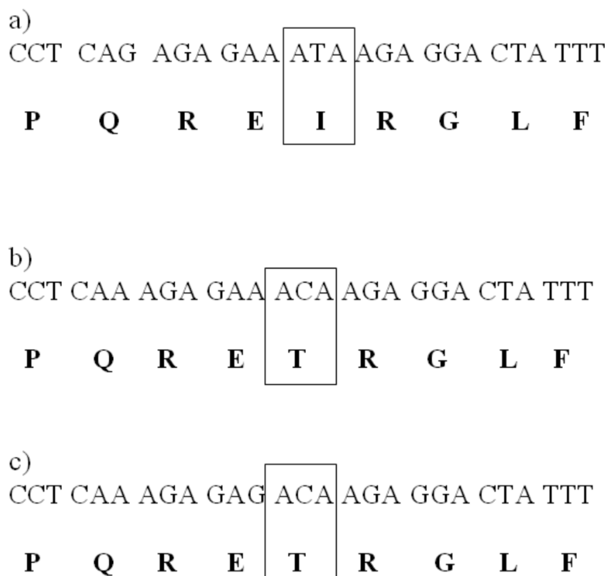

Fig. 2. Cleavage site of Polish H5 LPAIV: a) A/garganey/Poland/225/09; b) A/teal/Poland/305/08; c) A/teal/Poland/180/10. The unique cleavage site motif: "PQREIRGLF" (a) was caused by a nucleotide point mutation in the triplet codon "ATA" leading to the amino acid substitution (T --> I)

Although not as dangerous as H5N1, low pathogenic H5 AIV pose a threat to poultry industry and in 2006-2009 the outbreaks occurred in at least 10 countries, with significant spread of H5N3 LPAI in Germany, where 35 premises containing turkeys were affected (Brown 2010). However, none of the H5 viruses detected at Jeziorsko could grow in embryonated chicken eggs and subtyping was only possible by means of H5-specific real time RT-PCR with confirmation in conventional RT-PCR. The virus isolation failure made a thorough characterization of these viruses impossible. Therefore, the phylogenetic analysis could only be performed over a short region (280 bp) and not the whole gene. Thus, the results are approximate and only provide a rough insight into genetic relationship between H5 LP viruses. The H5 AIV from Common Teals, detected 2 years apart, belonged to the same major group, possibly sharing a recent common ancestor, while the isolate from a Garganey was clearly different. All 3 isolates revealed a genetic similarity to H5 LPAIV isolated from wild birds in Europe and Africa (Fig. 1).

Another interesting finding was the detection of 4 viruses from 3 females of Common Teals foraging together and captured at the same time (on 26 August 2010 at 6.00 a.m.). The birds with ring numbers PA 12906 and PA 12907 were infected with H4N6 AIV while the Teal with the ring number PA 12905 was positive for H5 AIV and additionally for avian paramyxovirus type 4. Due to the fact that the presence of different AI viruses in birds found at the same place creates a perfect condition for reassortment event (mixing of the genetic material of viruses with segmented genome into new combinations), an additional study was undertaken to compare sequences of internal gene fragments from H4N6 isolates. We found a 100% homology between partial sequences of 6 internal genes which suggests that in that case reassortment did not take place. Unfortunately, the same study was not possible for H5 virus due to the paucity of original specimen to perform enough sequencing reactions. On the other hand, Dugan et al. (2008) found five H4N6 AIV in Mallards sampled at the same location and time in Ohio, USA, and sequencing of internal genes revealed the existence of 4 different genome constellations with only 2 viruses sharing identical gene segments.

The results confirm that avian influenza viruses, including potentially dangerous subtypes for domestic birds, are circulating in the population of wild birds, mainly waterfowl. The Jeziorsko reservoir should be taken into consideration when planning AI surveillance activity in the future.

Acknowledgments

We thank Elżbieta Juszczuk and Zofia Kopińska for excellent technical assistance and Dr. Andrzej Kęsy for organizing shipment of the samples from Jeziorsko to Puławy. We would also like to thank all the people that helped during sample collection at the ringing site especially Anna Piasecka, Tomasz Iciek and Łukasz Krajewski.

This work was supported by the EU 6th framework programmes NEW-FLUBIRD (044490).

References

Alexander DJ (**2007**) An overview of the epidemiology of avian influenza. Vaccine 25: 5637-5644.

Breed AC, Harris K, Hesterberg U, Gould G, Londt BZ, Brown IH, Cook AJ (**2010**) Surveillance for avian influenza in wild birds in the European Union in 2007. Avian Dis 54: 399-404.

Brown IH (**2010**) Summary of avian influenza activity in Europe, Asia, and Africa, 2006-2009. Avian Dis 54: 187-193.

Bui VN, Ogawa H, Karibe K, Matsuo K, Nguyen TH, Awad SS, Minoungou GL, Xininigen, Saito K, Watanabe Y, Runstadler JA, Happ GM, Imai K (**2011**) Surveillance of avian influenza virus in migratory water birds in eastern Hokkaido, Japan. J Vet Med Sci 73: 209-215.

Costa TP, Brown JD, Howerth EW, Stallknecht DE (**2010**) The effect of age on avian influenza viral shedding in mallards (Anas platyrhynchos). Avian Dis 54: 581-585.

Costa TP, Brown JD, Howerth EW, Stallknecht DE (**2011**) Variation in viral shedding patterns between different

wild bird species infected experimentally with low-pathogenicity avian influenza viruses that originated from wild birds. Avian Pathol 40: 119-124.

Dugan VG, Chen R, Spiro DJ, Sengamalay N, Zaborsky J, Ghedin E, Nolting J, Swayne DE, Runstadler JA, Happ GM, Senne DA, Wang R, Slemons RD, Holmes EC, Taubenberger JK (**2008**) The evolutionary genetics and emergence of avian influenza viruses in wild birds. PLoS Pathog 4: e1000076.

Gaidet N, Dodman T, Caron A, Balanca G, Desvaux S, Goutard F, Cattoli G, Lamarque F, Hagemeijer W, Monicat F (**2007**) Avian influenza viruses in water birds, Africa. Emerg Infect Dis 13: 626-629.

Goekjian VH, Smith JT, Howell DL, Senne DA, Swayne DE, Stallknecht DE (**2011**) Avian influenza viruses and avian paramyxoviruses in wintering and breeding waterfowl populations in North Carolina, USA. J Wildl Dis 47: 240-245.

Hansbro PM, Warner S, Tracey JP, Arzey KE, Selleck P, O'Riley K, Beckett EL, Bunn C, Kirkland PD, Vijaykrishna D, Olsen B, Hurt AC (**2010**) Surveillance and analysis of avian influenza viruses, Australia. Emerg Infect Dis 16: 1896-1904.

Henaux V, Samuel MD (**2011**) Avian influenza shedding patterns in waterfowl: implications for surveillance, environmental transmission, and disease spread. J Wildl Dis 47: 566-578.

Janiszewski T, Włodarczyk R, Bargiel R, Grzybek J, Kaliński A, Lesner B, Mielczarek S (**1998**) Awifauna zbiornika Jeziorsko w latach 1986-1996. Notatki Ornitologiczne 39: 121-150.

Latorre-Margalef N, Gunnarsson G, Munster VJ, Fouchier RA, Osterhaus AD, Elmberg J, Olsen B, Wallensten A, Haemig PD, Fransson T, Brudin L, Waldenstrom J (**2009**) Effects of influenza A virus infection on migrating mallard ducks. Proc Biol Sci 276: 1029-1036.

Lebarbenchon C, Albespy F, Brochet AL, Grandhomme V, Renaud F, Fritz H, Green AJ, Thomas F, van der Werf S, Aubry P, Guillemain M, Gauthier-Clerc M (**2009**) Spread of avian influenza viruses by common teal (Anas crecca) in Europe. PLoS One 4: e7289.

Lebarbenchon C, Stallknecht DE (**2011**) Host shifts and molecular evolution of H7 avian influenza virus hemagglutinin. Virol J 8: 328.

Lebarbenchon C, van der Werf S, Thomas F, Aubin JT, Azebi S, Cuvelier F, Jeannin P, Roca V, Chang CM, Kayser Y, Roche B, Guegan JF, Renaud F, Gauthier-Clerc M (**2007**) Absence of detection of highly pathogenic H5N1 in migratory waterfowl in southern France in 2005-2006. Infect Genet Evol 7: 604-608.

Minta Z, Śmietanka K, Domańska-Blicharz K, Tomczyk G, Wijaszka T (**2007**) Highly pathogenic avian influenza H5N1 in wild birds in Poland – analysis of first cases. Med Weter 63: 1349-1352.

Munster VJ, Baas C, Lexmond P, Waldenstrom J, Wallensten A, Fransson T, Rimmelzwaan GF, Beyer WE, Schutten M, Olsen B, Osterhaus AD, Fouchier RA (**2007**) Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. PLoS Pathog 3: e61.

OIE (**2008**) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Chapter 2.3.4 Avian influenza. 6th ed., available at www.oie.int

Olsen B, Munster VJ, Wallensten A, Waldenstrom J, Osterhaus AD, Fouchier RA (**2006**) Global patterns of influenza a virus in wild birds. Science 312: 384-388.

Slomka MJ, Coward VJ, Banks J, Londt BZ, Brown IH, Voermans J, Koch G, Handberg KJ, Jorgensen PH, Cherbonnel-Pansart M, Jestin V, Cattoli G, Capua I, Ejdersund A, Thoren P, Czifra G (**2007**) Identification of sensitive and specific avian influenza polymerase chain reaction methods through blind ring trials organized in the European Union. Avian Dis 51: 227-234.

Slomka MJ, Pavlidis T, Banks J, Shell W, McNally A, Essen S, Brown IH (**2007a**) Validated H5 Eurasian real-time reverse transcriptase-polymerase chain reaction and its application in H5N1 outbreaks in 2005-2006. Avian Dis 51: 373-377.

Slomka MJ, Pavlidis T, Coward VJ, Voermans J, Koch G, Hanna A, Banks J, Brown IH (**2009**) Validated Real-Time reverse transcriptase PCR methods for the diagnosis and pathotyping of Eurasian H7 avian influenza viruses. Influenza Other Respi Viruses 3: 151-164.

Smietanka K, Pikula A, Minta Z, Meissner W (**2011**) Evidence of persistence and multiple genetic modifications of H7N7 low-pathogenic avian influenza virus in wild mallards in Poland provided by phylogenetic studies. Avian Pathol 40: 131-138.

Spackman E (**2009**) The ecology of avian influenza virus in wild birds: what does this mean for poultry? Poult Sci 88: 847-850.

Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, Perdue ML, Lohman K, Daum LT, Suarez DL (**2002**) Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J Clin Microbiol 40: 3256-3260.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (**2011**) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28: 2731-2739.

Terregino C, De Nardi R, Guberti V, Scremin M, Raffini E, Martin AM, Cattoli G, Bonfanti L, Capua I (**2007**) Active surveillance for avian influenza viruses in wild birds and backyard flocks in Northern Italy during 2004 to 2006. Avian Pathol 36: 337-344.