Molecular epidemiology of avian influenza virus and incidence of H5 and H9 virus subtypes among poultry in Egypt in 2009–2011

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Summary. – Egypt has experienced outbreaks of avian influenza (AI) since 2006. A total of 3583 cloacal swabs were collected from chickens, ducks, geese and turkeys from commercial farms, backyards and local bird markets in Qena and Luxor governorates in South Egypt during 2009–2011. These samples were examined for the presence of AI virus (AIV) and positive samples were further subtyped for the H5 and H9 by real time RT-PCR. In this way, 202 (5.64%) samples were found to be AIV-positive of which 186 (92.08%) and 7 (3.46%) belonged to H5 and H9 subtypes, respectively. Higher infection rates were observed in backyard birds and birds from local bird markets in comparison to birds from commercial farms. In conclusion, the predominance of H5 infection indicates a need for continuous monitoring of AIV among avian species and the awareness against public health risk.

Introduction

Avian influenza viruses (AIVs) contain segmented, single-stranded, negative sense RNA and belong to the genus *Influenza virus* A in the family *Orthomyxoviridae*. Its genome consists of 8 segments that code for 10 proteins; HA (hemagglutinin), NA (neuramindase), NP (nucleoprotein), matrix proteins (M1 and M2), polymerase subunits (PB2, PB1, PA) and two non-structural proteins (NS1 and NS2) (Cox *et al.*, 2000; Swayne and Halvorson, 2003).

AIVs have been isolated sporadically from domestic poultry, most frequently from chickens, turkeys and ducks. However, all combinations of the 16 HA and 9 NA subtypes have been reported in free-flying birds, especially from

orders *Anseriformes* (ducks and geese) and *Charadriformes* (shorebirds, gulls, terns and auks), which are the ultimate source of influenza A viruses. The incidence and distribution varies based on geographic region, species, age of birds, time of the year and the environmental system (Webster *et al.*, 1992; Alexander, 1993; Perdue *et al.*, 1999; Suarez and Schultz, 2000).

Several waves of AI have been recorded during 1924–1925 and 1929 in USA. It re-emerged again between 1953 and 1971. The most destructive wave has occurred in 1997 in Hong Kong and spread in Asia, Europe and Africa with interspecies transmission and human deaths threaten the world with a global AI pandemic (Swayne and Halvorson, 2003; Elbers *et al.*, 2004; Capua and Alexander; 2006).

AI became the most important disaster threat to poultry industry all over the world after the reported occurrence of highly pathogenic (HP) AIV outbreaks in many countries (Alexander, 2000) such as; H5Nl in Hong Kong, H5N2 in Italy, Mexico and USA, H7Nl in Italy, H7N4 in Australia,

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H7N3 in Canada and Pakistan, H7N7 in the Netherlands (Capua *et al.*, 1999; Capua and Alexander, 2004). However, outbreaks of low pathogenic (LP) AIV and HPAIV have occurred in China, Taiwan, Thailand, Singapore, Australia, Italy, Ireland, Belgium, Russia and Ukraine, and also the disease spread to Germany, France and Great Britain (Henzier *et al.*, 2003; Werner *et al.*, 2003; Capua and Alexander, 2004).

Since 2006, Egypt has been severely affected by continuous outbreaks of HPAIV H5, resulting in sever losses in the poultry industry, with more than 100 human cases, and 34 human deaths (WHO, 2010a). Outbreaks in 9 governorates in Egypt (Gharbiyah, Minufiyah, Kafr El-Shaykh, Daqahliyh, Sharqiyah, Minya, Giza, Sohag, and Luxor) in commercial and live market poultry and backyard birds from February to June 2008 have been reported (WHO, 2010a,b). In 2010, 36 H5 HPAIV outbreaks have been reported in poultry (chickens, ducks, geese and turkeys) from 12 governorates including Qena and Luxor (FAO, 2010).

HPAIV H5 and H7subtypes, usually in chickens or closely related gallinaceous birds, are associated with a wide range of clinical signs (Capua and Alexander, 2004), morbidity and mortality up to 100%. LPAIVs H5 and H7 subtypes are the progenitors of HPAIV, so it is essential for veterinary authorities to detect and manage LPAIV endemics caused by H5 and H7 (Capua and Alexander, 2006).

LPAIV H9N2 infections have been reported in the Middle East since 1998 and caused widespread outbreaks in commercial chickens in Iran (Nili and Asasi, 2003). The H9 AIV has emerged in different parts of the world among poultry and wild birds (Banks *et al.*, 2000). Also, the human infection with H9N2, which is closely related to that isolated from a quail, has been reported in Hong Kong in 1999 (Peiris *et al.*, 1999).

This study was carried out to evaluate the epidemiological status and elucidate the prevalence of H5 and H9 subtypes of AIV in poultry species that have been reared in endemic governorates (Qena and Luxor) in the South Egypt during 2009–2011.

Materials and Methods

Sample collection. The study comprised of a total of 3583 cloacal swab samples collected from poultry (chicken, duck, geese and turkey) of different ages, 2–12 months, from commercial farms, local bird markets and backyards during 2009–2011. The samples were collected from birds with or without clinical signs of AIV infection in two endemic governorates; Qena and Luxor. Information about species, age, farm system, vaccination and clinical signs were collected. Samples were placed in phosphate buffer saline (PBS, pH 7.4) containing antibiotics and antimycotics (WHO, 2002) and stored at -80°C for further analysis. Birds from local bird markets were apparently healthy, while backyard birds and commercial market birds suffered from AI characteristic clinical signs (cyanosis of comb and wattles, hemorrhages in shank, greenish diarrhea and high mortality rate). H5N1 and H5N2 vaccines were administrated to commercial chicken farms but not to backyard birds. On the other hand, birds from local bird markets have unknown vaccination history.

RNA extraction. RNA was extracted from the cloacal swab suspensions using QIAamp® viral RNA mini kit (Qiagen) according to manufacturer's instructions.

Primers and probes. The real-time reverse-transcription polymerase chain reaction (real-time RT-PCR) primers and probes used to detect M segment of AI type A virus and HA gene subtype (H5 and H9) of the AIV M segment positive samples were synthesized according to previously described reports. The primers and probes details are shown in Table 1.

One-step real-time RT-PCR for M gene. Samples were examined by One-Step real-time RT-PCR to amplify the M segment using Quanti Tect probe-PCR Kit (Qiagen) according to manufacturer's instructions. Master Mix contained 2× Quanti Tect probe PCR mix, 10 μmol/l primers (Com F, Com R), 5 μmol/l probe SeProb, 6.25 μl Quanti Tect RT Mix and 2.5 μl template RNA. The PCR plate was loaded into real-time cycler (Stratagene, MX3005P, qRNA system) and the one-step real-time RT-PCR was performed under the following conditions; 1 cycle at 50°C/30 min, 1 cycle at 95°C/15 min followed by 40 cycles of denaturation at 95°C/30 sec and annealing/extension at 60°C/1 min (data were collected at this step).

Table 1. Primers and probes for M, H5 and H9 genes of AIV

ID	Target	Sequence 5'-3'	Note	Reference
Com F	M	5' - AGATGAGTCTTCTAACCGAGGTCG-3'	Primer	VLA, 2007a
Com R	M	5' - TGCAAAAACATCTTCAAGTCTCTG-3'	Primer	VLA, 2007a
SEPRO	M	FAM-5'-TCAGGCCCCCTCAAAGCCGA-3'-TAMRA	Probe	VLA, 2007a
H5LH1	H5	5' -ACATATGACTACCCACARTATTCAG-3'	Primer	VLA, 2007b
H5RH1	H5	5' -AGACCAGCTAYCATGATTGC-3'	Primer	VLA, 2007b
H5PRO	H5	FAM-5'-TCW ACA GTG GCG AGT TCC CTA GCA-3'- TAMRA	Probe	VLA, 2007b
H9F	H9	5'-GGAAGAATTAATTATTATTGGTCGGTAC-3'	Primer	Ben Shabat *et al.*, 2010
H9R	H9	5'- GCCACCTTTTTCAGTCTGACATT-3'	Primer	Ben Shabat *et al.*, 2010
H9PRO	H9	FAM-5'-AACCAGGCCAGACATTGCGAGTAAG ATCC-3'-TAMRA	Probe	Ben Shabat *et al.*, 2010

Table 2. Prevalence of AIV among poultry samples from 2009–2011

Location	Year	No. of isolates				Total
		Chickens	Ducks	Geese	Turkeys	
Qena	2009	5	2	2	1	10
	2010	36	15	20	9	80
	2011	50	5	3	4	62
Luxor	2009	4	2	3	1	10
	2010	5	3	1	1	10
	2011	20	4	3	3	30
Total		120	31	32	19	202

One-step real-time RT-PCR for H5 and H9 genes. HA gene subtyping was performed for M segment positive samples using H5 or H9 primers and probes (Table 1) and Quanti Tect probe-PCR Kit (Qiagen) according to manufacturer's instructions. The one-step real-time RT-PCR for H5 was conducted under the following conditions; 1 cycle at 50°C/30 min, 1 cycle at 95°C/15 min followed by 40 cycles of denaturation at 95°C/15 sec, annealing 54°C/30 sec (data were collected at this step) and extension at 72°C/10 sec. The same conditions were conducted for H9 with the annealing temperature of 50°C.

Results

Detection of AIV in collected samples

AIV was detected in 202 (5.6%) out of the 3583 samples analyzed by one-step real-time RT-PCR using primers and probe targeting the M segment of AI type A virus with cycle threshold (Ct) values ranged from 19–27. The majority of AIV positive samples (152/202) were from poultry in the Qena province, while 50/202 AIV positive samples were from Luxor province (Table 2).

Out of the 202 AIV positive samples 120 (59.4 %) were from chickens, 31(15.4 %) from ducks, 32 (15.8 %) from geese and 19 (9.4 %) from turkeys (Table 2). Characteristic clinical signs of AI; high mortality up to 100% within 3–4 days after onset of infection, cyanosed comb, hemorrhages on shanks and nervous signs in survived birds, were observed in commercial broiler chickens and backyard birds but not in birds from local bird markets.

Identification of H5 and H9 subtypes in AIVs

To elucidate the prevalence of AIV H5 and H9 subtypes among examined avian species, the positive AI type A virus samples were subjected to one step real-time RT-PCR using specific primers and probes for H5 and H9 (Table 1). A total of 186/202 (92.08%) were H5 subtype while 7/202 (3.46%) were H9 subtype (Table 3). Although, H5 subtype was predominant among avian species (chickens, ducks, geese and turkeys) in both endemic areas during 2009–2011 especially in the backyard birds and birds from local bird markets. It is interesting that the H9 subtype was only detected in chicken samples from backyard birds and birds from local bird markets in both regions and in the same year, 2011 (Table 3).

Epidemiological features of AIVs from endemic areas

The overall results revealed that the prevalence of AIV in Qena 152/202 (75.25%) was higher than that in Luxor 50/202 (24.75%), and also the infection among backyard birds and birds from local bird markets (86.84% and 76%) was higher than in birds from commercial farms (13.16% and 24%) in Qena and Luxor, respectively. The AIV infections among the poultry was congruent in both endemic areas with the highest rate in 2011 (10%) of the total examined samples (62/598 and 30/300) in Qena and Luxor, respectively. However, the peak of epidemic rate among poultry in Qena was 52.5 % (80/152) and 40.7% (62/152) while in Luxor it was 20% (10/50) and 60% (30/50) in 2010 and 2011, respectively (Table 2).

The HA subtyping revealed that H5 is predominant among various species in both endemic localities, 141/152 (92.8%)

Table 3. The incidence of H5 and H9 subtypes in the AIV-positive samples

Location	Qena			Luxor		
Year	AIV positive	H5	H9	AIV positive	H5	H9
2009	10	10	N[a]	10	10	N
2010	80	72	N	10	10	N
2011	62	59	3	30	25	4
Total	152	141	3	50	45	4

[a]No AIV-positive samples were recognized as H9 subtype.

in Qena and 45/52 (90%) in Luxor, during the surveillance period. The peak of H5 infections was in 2011. H9 subtype 7/202 (3.5%) could be only detected in both localities in 2011 and all isolates were recovered from chickens. The remaining 9 isolates were neither H5 nor H9 subtype (Table 3).

Discussion

Recent records from WHO in Dec. 2013 showed 648 confirmed human cases of AIV H5N1 with 384 deaths. Egypt was the 2nd country that had highest number of infected cases (173) and 63 deaths (WHO, 2013).

AIVs have been isolated most frequently from chickens, turkeys, ducks and captive wild birds held as caged pets, or in quarantine stations, private collections/reserves and zoological parks (Alexander, 1993). However, incidence and distribution varies greatly with geographic region, species, age, time of year, and the environmental or agricultural system occupied. Turkeys and other gallinaceous birds (including chickens) are not natural reservoirs of AI viruses (Perdue *et al.*, 1999; Suarez and Schultz, 2000).

The present study revealed that the prevalence of AIV was 5.6 % and occurred among poultry species (Table 2) during the surveillance period especially the local bird markets and backyard birds. This attributed to the well-known risk factors for AIV as bird movement, rearing of mixed populations and contact with migratory waterfowl (Capua *et al.*, 1999). In addition to these, local bird markets are recognized as important places for the maintenance and exchange of AIVs (Kung *et al.*, 2003). The real-time RT-PCR targeting the M segment is widely used to estimate the prevalence of AIV and sometimes used to screen samples for virus isolation and characterization (Runstadler *et al.*, 2007; Ferro *et al.*, 2008, 2010). The amount of viral RNA in the examined samples correlated inversely to the Ct values; samples with low Ct values have more viral RNA. All positive samples that were detected by real-time RT-PCR had low Ct values (19–27) which indicates that they contain higher amount of viral RNA and these birds were shedding virus.

Although the vaccination strategy was conducted in the commercial farms the infection has occurred, and symptoms of AI appeared 2 months after H5N1vaccination in birds in the Luxor province. Two H5 AIVs oil-emulsion formulation imported vaccines; one derived from killed LPAIV H5N2 virus and the other form a reassortant H5N1, are used for control HPAIV in Egypt.

Hoffmann *et al.* (2001) and Bahgat *et al.* (2009) have reported that for high efficiency vaccines, the strains used to produce the vaccine must be sufficiently closely related to the circulating strains to ensure the induction of the effective protective immunity against infection. Thus the infection among vaccinated chickens may be due to the differences in the immunogenicity between the field isolates and vaccine derived ones.

Waterfowl represents the natural reservoir of all subtypes of influenza A viruses, including H5N1. Ducks are considered major contributors to the spread of HPAIV because they exhibit diversity in morbidity and mortality. Therefore, as a preventive strategy against endemic as well as pandemic influenza, it is important to reduce the spread of H5N1 influenza A viruses in duck populations (Chen *et al.*, 2004; Kim *et al.*, 2008). The incidence of infection among waterfowl was 9.7% (31/320) and 11.1% (32/288) of the examined samples from ducks and geese, respectively, as well as in turkeys where it was 18.8% (19/101) (Table 2). The infected ducks, geese and turkeys may cause environmental contamination and be a source of infection for chickens and other birds. Hulse-Post *et al.* (2005) has reported that mallard ducks experimentally infected with H5N1 excreted the virus asymptomatically for 17 days.

The spread of HPAIV H5 or H7, LPAIV of H5, H7 or H9 subtypes among poultry could have caused emerged pandemic infection through adaptive mutations or reassortments (Butt *et al.*, 2005; Capua and Alexander, 2004). The H9 considered as LPAIV continuously circulates in poultry flocks causing enormous economic losses to poultry industry in Pakistan (Naeem *et al.*, 1999). H9N2 viruses are double or even triple reassortants that have amino acids signatures in their HA indicating their potential to directly infect human (Li *et al.*, 2003).

In this study the prevalence of H5 and H9 subtypes was 92.08% (186/202) and 3.46% (7/202), respectively. Although H5 was prevalent among avian species, H9 was detected only in chickens. The H9 appeared to be newly introduced into chickens in these two endemic provinces in the same year, 2011, and disseminated among the farm chickens only. Some reports indicated that H9 has not been detected in Egypt until the end of 2010 (Abdel-Moneim *et al.*, 2012; Afifi *et al.*, 2013). One possibility is that the infected chickens came from other provinces with H9 infections, because the main commercial chicken breeders are established in the North Egypt and they distribute broiler chicks to farms all over the country.

In conclusion, the risk of infection with AIV is high among poultry especially from local bird markets and backyard birds. The H5 subtype is predominant among all avian species even in the vaccinated birds. This indicates the necessity for applying strict regulations for bird trafficking and developing vaccine from the local isolates. The continuous monitoring of the H9 subtype should be warranted especially amongst the commercial farms.

References

Abdel-Moneim AS, Afifi MA, El-Kady MF (2012): Isolation and mutation trend analysis of influenza A virus subtype H9N2 in Egypt. Virol. J. 9, 173. http://dx.doi.org/10.1186/1743-422X-9-173

Afifi MA, El-Kady MF, Zoelfakar SA, Abdel-Moneim AS (2013): Serological surveillance reveals widespread influenza A H7 and H9 subtypes among chicken flocks in Egypt. Trop. Anim. Health Prod. 45, 687–690. http://dx.doi.org/10.1007/s11250-012-0243-9

Alexander DJ (1993): Orthomyxovirus infections. In Mc Ferran JB, McNutly MS (Eds): Virus Infections of Birds. Elseiver Science : London, pp. 287–316.

Alexander DJ (2000): A review of avian influenza in different bird species. Vet. Microbiol. 74, 3–13. http://dx.doi.org/10.1016/S0378-1135(00)00160-7

Bahgat MM, Kutkat MA, Nasraa MH, Mostafa A, Webby R, Bahgat IM, Ali MA (2009): Characterization of an avian influenza virus H5N1 Egyptian isolate. J. Virol. Methods 159, 244–250. http://dx.doi.org/10.1016/j.jviromet.2009.04.008

Banks J, Speidel EC, Harris PA, Alexander DJ (2000): Phylogenetic analysis of influenza A viruses of H9 haemagglutinin subtype. Avian Pathol. 29, 353–360. http://dx.doi.org/10.1080/03079450050118485

Ben Shabat M, Meir R, Haddas R, Lapin E, Shkoda, I, Raibstein S, Perk S, Davidson I (2010): Development of a real-time TaqMan RT-PCR assay for the detection of H9N2 avian influenza viruses. J. Virol. Methods 168, 72–77. http://dx.doi.org/10.1016/j.jviromet.2010.04.019

Butt KM, Smith GJ, Chen H, Zhang LJ, Leung YH, Xu KM, Lim W, Webster RG, Yuen KY, Peiris JS, Guan Y (2005): Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. J. Clin. Microbiol. 43, 5760–5767. http://dx.doi.org/10.1128/JCM.43.11.5760-5767.2005

Capua I, Alexander DJ (2004): Avian influenza: recent developments. Avian Pathol. 33, 393–404. http://dx.doi.org/10.1080/03079450410001724085

Capua I, Alexander DJ (2006): The challenge of avian influenza to the veterinary community. Avian Pathol. 35, 189–205. http://dx.doi.org/10.1080/03079450600717174

Capua I, Marangon S, Selli L, Alexander DJ, Swayne DE, Pozza MD, Parenti E, Cancellotti FM (1999): Outbreaks of highly pathogenic avian influenza (H5N2) in Italy during October 1997–January 1998. Avian Pathol. 28, 455–460. http://dx.doi.org/10.1080/03079459994470

Chen H, Deng G, Li Z, Tian G, Li Y, Jiao P, Zhang L, Liu Z, Webster RG, Yu K (2004): The evolution of H5N1 influenza viruses in ducks in southern China. Proc. Natl. Acad. Sci. USA 101, 10452–10457. http://dx.doi.org/10.1073/pnas.0403212101

Cox NJ, Fuller F, Kaverin N, Klenk HD, Lamb RA, Mahy BW, McCauley JW, Nakamura K, Palese P, Webster RG (2000): Orthomyxoviridae. In Van Regenmortel MH, Fauquet CM, Bishop DHL, Carstens EB, Estes MK, Lemon SM, Maniloff J, Mayo MA, McGeoch DJ, Pringle CR, R. Wickner B (Eds): Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses. Academic Press : San Deigo, pp. 585–597.

Elbers AR, Fabri TH, de Vries TS, de Wit JJ, Pijpers A, Koch G (2004): The Highly pathogenic avian influenza A (H7N7) virus epidemic in the Netherlands in 2003-lessons learned from the first five outbreaks. Avian Dis. 48, 691–705. http://dx.doi.org/10.1637/7149

FAO (2010): H5N1 HPAI Global Overview- July and August 2010. Available on: http//www.fao.org/docerp/013/ak784e00.pdf

Ferro PJ, Peterson MJ, Merendino T, Nelson M, Lupiani B (2010): Comparison of real-time reverse transcription-PCR and virus isolation for estimating prevalence of avian influenza virus in hunter-harvested wild birds at waterfowl wintering grounds along the Texas mid-Gulf Coast (2005-2006 through 2008-2009). Avian Dis. 54, 655–659. http://dx.doi.org/10.1637/8810-040109-ResNote.1

Ferro PJ, El-Attrache J, Fang X, Rollo SN, Jester A, Merendino T, Peterson MJ, Lupiani B (2008): Avian influenza surveillance in hunter-harvested waterfowl from the Gulf Coast of Texas (November 2005-January 2006). J. Wildl. Dis. 44, 434–439. http://dx.doi.org/10.7589/0090-3558-44.2.434

Henzier DJ, Kradel DC, Davison S, Ziegler AF, Singletary D, De-Bok P, Castro AE, Lu H, Eckroade R, Swayne D, Lagoda W, Schmucker B, Nesselrodt A (2003): Epidemiology, production losses, and control measures associated with an outbreak of avian influenza subtype H7N2 in Pennsylvania (1996-1998). Avian Dis.47, 1022–1036. http://dx.doi.org/10.1637/0005-2086-47.s3.1022

Hoffmann E, Stech J, Guen Y, Webster RG, Perez DR (2001): Universal primer set for the full-length amplification of all influenza A viruses. Arch. Virol. 146, 2275–2289. http://dx.doi.org/10.1007/s007050170002

Hulse-Post DJ, Sturm-Ramirez KM, Humberd J, Seiler P, Govorkova EA, Ktauss S, Scholtissek C, Puthavathana P, Buranathai C, Nguyen TD, Long HT, Naipospos TS, Chen H, Ellis TM, Guan Y, Peiris JS, Webster RG (2005): Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia. Proc. Natl. Acad. Sci. USA 102, 10682–10687. http://dx.doi.org/10.1073/pnas.0504662102

Kim JK, Seiler P, Forrest HI, Khalenkov AM, Franks J, Kumar M, Karesh WB, Gilbert M, Sodnomdarjaa R, Douangngeun B, Govorkova EA, Webster RG (2008): Pathogenicity and vaccine efficacy of different clades of Asian H5N1 avian influenza A viruses in domestic ducks. J. Virol. 82, 11374–11382. http://dx.doi.org/10.1128/JVI.01176-08

Kung NY, Guan Y, Perkins NR, Bissett L, Sims L, Morris RS, Shortridge KF, Peiris JSM (2003): The impact of a monthly rest day on avian influenza virus isolation rates in retail markets in Hong Kong. Avian Dis. 47, 1037–1041 http://dx.doi.org/10.1637/0005-2086-47.s3.1037

Li KS, Xu KM, Peiris JS, Poon, LL, Yu KZ, Yuen KY, Shortridge KF, Webster RG, Guan Y (2003): Characterization of H9 subtype influenza viruses from ducks of southern China: a candidate for the next influenza pandemic in humans? J. Virol. 77, 6988–6994. http://dx.doi.org/10.1128/JVI.77.12.6988-6994.2003

Naeem K, Ullah A, Manvell RJ, Alexander DJ (1999): Avian influenza A subtype H9N2 in poultry in Pakistan. Vet. Record 145–560.

Nili H, Asasi K (2003): Avian Influenza (H9N2) outbreak in Iran. In: Proceedings of the fifth international symposium on avian influenza, Athens, Georgia, April 14 – 17 2002. Avian Dis. 47, 828–831. http://dx.doi.org/10.1637/0005-2086-47.s3.828

Peiris M, Yuen KY, Leung CW, Chan KH, Ip PLS, Lai TWM, Orr WK, Shortridger KF (1999): Human infection with influenza H9N2. Lancet 354, 916–917 http://dx.doi.org/10.1016/S0140-6736(99)03311-5

Perdue ML, Suarez DL, Swayne DE (1999): Avian influenza in the 1990s. Poult. Avian Biol. Rev. 11, 1–20.

Runstadler JA, Happ GM, Slemons RD, Sheng ZM, Gundlach N, Petrula M, Senne D, Nolting J, Evers DL, Modrell A, Huson H, Hills S, Rothe T, Marr T,Taubenberger JK (2007): Using RRT-PCR analysis and virus isolation to determine the prevalence of avian influenza virus infections in ducks at Minto Flats State Game Refuge, Alaska, during August 2005. Arch. Virol. 152, 1901–1910. http://dx.doi.org/10.1007/s00705-007-0994-1

Swayne DE, Halvorson DA (2003): Avian influenza. In Saif YM, Barnes HJ, Gilson JR, Fadly AM, MC-Dougald IR, Swainy DE (Eds): Avian Influenza in Disease of Poultry. 11 ed. Iowa State University Press, Iowa, USA, pp. 135–160.

Suarez DL, Schultz CS (2000): Immunology of avian influenza virus A review. Dev. Comp. Immunol. 24, 269–283. http://dx.doi.org/10.1016/S0145-305X(99)00078-6

VLA (Veterinary Laboratories Agency) (2007a): Detection of influenza A matrix gene by real-time Taqman® RT-PCR. SOP VI 493 edition 2. http://www.defra.gov.uk/ahvla-en/files/pub-protocol-ai-vi493.pdf

VLA (Veterinary Laboratories Agency) (2007b): Eurasian H5 avian influenza Real-Time PCR. SOP VI.492 edition 4. http://www.defra.gov.uk/ahvla-en/files/pub-protocol-ai-vi492.pdf

Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y (1992): Evolution and ecology of influenza A viruses. Microbiol. Rev. 56, 152–79.

Werner O, Starick E, Grund CH (2003): Isolation and characterization of a low-pathogenicity H7N7 influenza virus from a Turkey in a small mixed free- range poultry flock in Germany. Avian Dis. 47, 1104–1106. http://dx.doi.org/10.1637/0005-2086-47.s3.1104

WHO (2002): WHO manual on animal influenza diagnosis and surveillance. http://www.who.int/csr/resources/publications/influenza/en/whocdscsrncs20025rev.pdf

WHO (2010a): H5N1 Avian Influenza: timeline of major events reported to WHO available at: www.who.int/csr/disease/Avian_Influenza/timeline_10_01_04.pdf Accessed April 9/2010.

WHO (2010b): Situation updates – Avian Influenza. http//www.who.int/csr/disease/avian_Influenza/updates/en.

WHO (2013): Cumulative number of confirmed human cases for avian influenza A (H5N1) reported to WHO, 2003–2013. http://www.who.int/influenza/human_animal_interface/EN_GIP_20131210CumulativeNumberH5N1cases.pdf