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.

Keywords: avian influenza; H5; H9; subtyping; real-time RT-PCR; poultry; Egypt

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 Charadriiformes (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; H5N1 in Hong Kong, H5N2 in Italy, Mexico and USA, H7N1 in Italy, H7N4 in Australia,
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 severe 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, Daqahliyah, Sharqiyyah, 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 H7 subtypes, 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 enemics 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, goose 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 antimiocytics (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 A1 characteristic clinical signs (cyanosis of comb and wattles, hemorrhages in shank, greenish diarrhea and high mortality rate). H5N1 and H5N2 vaccines were administered 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 QuantiTect probe-PCR Kit (Qiagen) according to manufacturer’s instructions. Master Mix contained 2× QuantiTect 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) according to manufacturer’s instructions.

Polymerase chain reaction (real-time RT-PCR) primers and probes details are shown in Table 1.

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

<table>
<thead>
<tr>
<th>ID</th>
<th>Target</th>
<th>Sequence 5’-3’</th>
<th>Note</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Com F</td>
<td>M</td>
<td>5’- AGATGAGTCTTCTAACCGAGGTCG-3’</td>
<td>Primer</td>
<td>VLA, 2007a</td>
</tr>
<tr>
<td>Com R</td>
<td>M</td>
<td>5’- GCATAAAACATCTCAGATCTGTG-3’</td>
<td>Primer</td>
<td>VLA, 2007a</td>
</tr>
<tr>
<td>SEPRO</td>
<td>M</td>
<td>FAM-5’-TCAGGCCCCCTCAACGAGCA-3’-TAMRA</td>
<td>Probe</td>
<td>VLA, 2007a</td>
</tr>
<tr>
<td>H5LH1</td>
<td>H5</td>
<td>5’-ACATATGACTACCCACATATTTCAG-3’</td>
<td>Primer</td>
<td>VLA, 2007b</td>
</tr>
<tr>
<td>H5RH1</td>
<td>H5</td>
<td>5’- AAGCCGACTYCATGATTGTC-3’</td>
<td>Primer</td>
<td>VLA, 2007b</td>
</tr>
<tr>
<td>H5PRo</td>
<td>H5</td>
<td>FAM-5’-TCW ACA GTG CGC AGT TCC CTA GCA-3’-TAMRA</td>
<td>Probe</td>
<td>VLA, 2007b</td>
</tr>
<tr>
<td>H9F</td>
<td>H9</td>
<td>5’- GGAAGAATATTATTTATTTGCACGTAAC-3’</td>
<td>Primer</td>
<td>Ben Shabat et al., 2010</td>
</tr>
<tr>
<td>H9R</td>
<td>H9</td>
<td>5’- GCCACCTTTTTCAGTCTGCAGTAT-3’</td>
<td>Primer</td>
<td>Ben Shabat et al., 2010</td>
</tr>
<tr>
<td>H9PRO</td>
<td>H9</td>
<td>FAM-5’-AACCAGGCCAGACATTGCGAAG ATCC-3’-TAMRA</td>
<td>Probe</td>
<td>Ben Shabat et al., 2010</td>
</tr>
</tbody>
</table>
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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 QuantiTect 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%)
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 H5N1 vaccination 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


