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REGULAR ARTICLES

Widespread of H5N1 infections in apparently healthy backyard poultry

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Abstract Highly pathogenic avian influenza subtype H5N1 represents a threat to the poultry industry and human health worldwide. Inapparently infected birds are suspected to play an essential role in the spread of avian influenza virus. In the current study, a total of 25,646 samples (16,185 chicken, 4696 ducks, 1633 geese and 3132 turkeys) from apparently healthy birds were screened for the presence of positive samples for H5N1 during 2009–2014. The samples were examined by reverse transcriptase real-time polymerase chain reaction (rRT-PCR) for M, H5 and N1 genes of avian influenza viruses. The results revealed that the HPAI H5N1 existed in an inapparent manner in ducks (4.68 %), geese (4.10 %), chickens (2.48 %) and turkeys (2.29 %). The current finding highlights the serious impact of such type on birds in the epidemiology of H5N1 in birds, animals and humans. It also highlights the existence of another reason other than vaccination that contributes to the widespread of inapparent infection of H5N1 in Egypt.

Keywords Highly pathogenic avian influenza (HPAI) · H5N1 · Inapparent infection · Domestic birds · rRT-PCR · Egypt

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Introduction

Egypt is considered one of the major epicentres for influenza H5N1 and constitutes a greater pandemic potential to other countries. The long-term endemicity of H5N1 and the co-circulation of other avian influenza subtypes in poultry complicate the situation in Egypt. H5N1 was first recorded in Egypt by the end of 2005 in wild birds, and later in different poultry species in 2006, as reviewed in (Abdelwhab et al. 2016; Abdelwhab and Abdel-Moneim 2015). H5N1 exists in an endemic manner and has resulted in many fatal human infections.

To date, 350 confirmed human infections and 116 deaths from H5N1 in Egypt have been confirmed, and more than 90 % of the cases in Egypt were linked to close contact with backyard birds (WHO 2016). H5N1 infected other mammals in Egypt but in an inapparent manner (Abdel-Moneim et al. 2010).

Four sectors of poultry production in Egypt: the first and second sectors include the grandparent and parent commercial production with considerably fair to good hygienic and biosafety measures. The third sector includes non-regulated, non-registered small to medium-scale commercial activities while the fourth sector includes backyard, rural, in-house and rooftop-raised poultry (Peyre et al. 2009). Both backyard-bird rearing and live bird market are two main obstacles that hinder the eradication of H5N1 from Egypt. Rearing backyard birds and livestock in the countryside is a complex phenomenon with cultural, social and ecological background. In addition, raising multiple and different species of birds and mammals promotes inter- and intraspecies transmission (Abdel-Moneim et al. 2010, 2011). The existence of different gene pools of the influenza viruses constitutes a potential risk for evolution of new strains with enhanced virulence to birds, animals and humans.

In a trial evaluated, from July 2007 to April 2009, the authorities have adopted different strategies to control H5N1 spread in Egypt including (i) massive vaccination strategy with different H5 vaccines, (ii) banning of live birds trading and (iii) providing vaccination free-of-charge twice a year (El Masry et al. 2014). H5N1-positive birds detected during the routine surveillance were culled but with very low, if any, compensation (Abdelwhab and Hafez 2011). This strategy failed to eradicate the disease in the backyard. It is known that the long-term endemic influenza virus infections in poultry increases the exposure risk to humans and in turn, creates opportunities for the emergence of human-adapted strains with subsequent pandemic potential (Webster et al. 1992). To the end of this point, the current study intended to screen the H5N1 incidence in apparently healthy backyard poultry system in the Upper Egypt.

Materials and methods

Samples

The samples were collected under the umbrella of the national strategy adopted in passive, active and targeted types of AIV surveillance in backyard poultry. A total of 25,646 samples (16,185 chickens, 4696 ducks, 1633 geese and 3132 turkeys) were collected from three governorates in the Upper Egypt (Beni-Suef, Fayoum and Minia Governorates) during the period between 2009 and 2014. All samples were collected from apparently healthy birds. Tracheal and cloacal swab samples were collected in 1–2 mL sterile phosphate buffered saline (PBS) containing antimicrobials, pH 7.0–7.4 according to OIE manual (2008). The samples were placed on ice-packs and transported promptly to the laboratory using well-closed ice boxes. Tracheal and cloacal swab samples from five birds from each species within the same house were pooled together. If any pooled samples were found positive, then screening of individual bird samples was conducted to detect individual positive birds among the pooled bird samples. The processed samples were kept at −80 °C until tested.

Detection of influenza A, M, H5 and N1 genes in the clinical samples

The extraction procedures were performed on 140 μl of pooled swab samples using QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Extracted RNA was subjected to reverse transcriptase real-time polymerase chain reaction (rRT-PCR) for influenza A, M gene using One-Step-Quantitect probe rRT-PCR kit (Qiagen, Valencia, CA, USA) as described in Spackman et al. (2002). Positive samples were then subjected to rRT-PCR for both H5 and N1 genes as described according to Veterinary Laboratories Agency, UK (VLA-UK 2009) and Aguero et al. (2007), respectively. The primers and probe used for M, H5 and N1 genes are listed in (Table 1).

Results and discussion

During the collection of samples, we found that different species of backyard birds are reared together and they were roaming freely within and in the vicinity of houses or through streets and fields. Aquatic birds were found in water canals and ponds during the day and are kept inside primitive cages on rooftops at night. Domestic ducks, that are in contact with wild waterfowl, wild birds and other poultry species, can act as key intermediaries in the transmission of avian influenza among birds (Li et al. 2004). Interestingly, from 25,646 tested samples, only 1126 (4.39 %) were found positive to influenza A virus using rRT-PCR for AIV M gene (Table 2). H5N1 strains were found in 762/1126 (67.67 %) of the AIV positive samples while 364/1126 (32.33 %) of the positive AIV samples were found negative to H5 and N1 rRT-PCR (Table 3). The finding that about one third of AIV samples was negative to H5 and N1 rRT-PCR, coincided with the detection of other influenza subtypes, H7 and H9, in Egypt during that period of time (Afifi et al. 2013; Abdel-Moneim et al. 2012). The percentage of H5N1 total positive samples related to the total samples of each species was the highest in ducks (220, 4.68 %) followed by geese (105, 4.10 %), then chicken (403, 2.48 %) and turkeys (72, 2.29 %) (Table 3). The percentage of AIV positive samples rather than H5N1 (non-H5N1 AIV) was the highest in turkeys (64, 2.04 %) and geese (29, 1.78 %), followed by chickens (215, 1.33 %) and ducks (56, 1.19 %) (Table 3). The percentages of H5N1 positive samples in all the examined birds in different years did not show considerable differences with a range of 2.77 to 3.58 % of the total of samples tested (Table 2). The risk of HPAI H5N1 virus present in countries like Egypt, Thailand and Vietnam has been associated with free-ranging duck numbers and the local abundance of both duck and geese (Gilbert et al. 2006). The overall H5N1 percentage is highly reduced in comparison to previous studies in Egypt that showed very variable ranges of incidence. In one study, the overall results of positive H5N1 cases along Egypt were 26.8 % (20.1 % in chicken and 13.2 % in aquatic birds), 13.5 % (23 % in chicken and 4.6 % in aquatic birds), 20 % (24.3 % in chicken and 8.3 % in aquatic birds) and 26 % (29.5 % in chicken and 15.7 % in aquatic birds) during 2007, 2008, 2009 and 2010, respectively (Safwat 2012). In another study, H5N1 positive cases were detected in 0.97 and 30 % of commercial farms and backyard birds, respectively, in 2007, while in 2008, the percentages were 0.31 and 5.2 %, respectively (Hafez et al. 2010). In 2009, the H5N1 positive cases were

Table 1 Primer/probe sequences used for the detection of influenza A virus M, H5 and N1 genes

Gene	Primer/probe name	Sequence (5′–3′)	Position[a]	Reference
M	M-25F	AGATGAGTCTTCTAACCGAGGTCG	9–32	(Spackman et al. 2002)
	M-124	TGCAAAAACATCTTCAAGTCTCTG	84–107	
	Probe M+64	6-FAM-TCAGGCCCCCTCAAAGCCGA-TAMRA	59–78	
H5	H5LH1	ACATATGACTACCCACARTATTCAG	1507–1531	(VLA-UK 2009)
	H5RH1	AGACCAGCTAYCATGATTGC	1639–1658	
	Probe FAM	FAM-TCWACAGTGGCGAGTTCCCTAGCA-TAMRA	1612–1635	
N1	AIV-N1-F1	GGCATAATAACAGACACTATCAA	588–610	(Aguero et al. 2007)
	AIV-N1-R1	CACATGCACATTCAGACTCT	641–660	
	AIV-N1-S1Probe	FAM-TCAGTATGTTGTTCCTCCA-MGB	615–633	

[a] Oligonucleotide positions were matched according to A/chicken/Egypt/2253-1/2006(H5N1)

recorded in 0.1, 10.5 and 11.4 % in commercial farms, backyard birds and LBM poultry, respectively (El-Zoghby et al. 2013). This conflicting finding could be explained by the fact that the surveillance in the current study was conducted only in apparently healthy birds; however, the other studies screened the presence of H5N1 in diseased birds or birds showed typical signs of H5N1. Silently infected free-ranging ducks and geese as well as mixed species and lack of biosafety measures in backyard birds in Egypt make the susceptibility of backyard birds to AIV infection very high. This highlights the epidemiological role of backyard birds as a constant reservoir of H5N1 virus in Egypt and consequently maintains the threat to commercial poultry industry and indeed to public health (Cristalli and Capua 2007). Few studies reported the presence of HPAI in an inapparent form in domestic birds including duck and geese (Shortridge et al. 1998; Li et al. 2004; Chen et al. 2006; Ma et al. 2014) and to less extent in chickens in live poultry markets (Shortridge

Table 2 Number of positive samples for H5N1 and non H5N1 influenza A viruses from three governorates in Upper Egypt

Year	Real-time assay	Positive numbers (%)							
		Beni-Suef		Fayoum		Minia		Total	
		Number	Positive	Number	Positive	Number	Positive	Number	Positive
2009	M gene	1516	59 (3.89)	1884	82 (4.35)	1976	78 (3.95)	5376	219 (4.07)
	H5/N1 genes		41 (2.70)		55 (2.92)		53 (2.68)		149 (2.77)
	Non-H5N1		18 (1.19)		27 (1.43)		25 (1.27)		70 (1.30)
2010	M gene	1437	52 (3.61)	1613	78 (4.84)	1569	67 (4.27)	4619	197 (4.26)
	H5/N1 genes		43 (2.99)		49 (3.04)		48 (3.06)		140 (3.03)
	Non-H5N1		9 (0.62)		29 (1.80)		19 (1.21)		57 (1.23)
2011	M gene	1043	48 (4.60)	1189	62 (5.21)	1072	51 (4.76)	3304	161 (4.87)
	H5/N1 genes		30 (2.87)		43 (3.61)		39 (3.64)		112 (3.39)
	Non-H5N1		18 (1.73)		19 (1.60)		12 (1.12)		49 (1.48)
2012	M gene	1398	56 (4.00)	1590	71 (4.47)	1514	73 (4.82)	4502	200 (4.44)
	H5/N1 genes		39 (2.79)		54 (3.40)		50 (3.30)		143 (3.17)
	Non-H5N1		17 (1.21)		17 (1.07)		23 (1.52)		57 (1.27)
2013	M gene	1484	48 (3.23)	1673	56 (3.34)	1563	62 (3.96)	4720	166 (3.51)
	H5/N1 genes		32 (2.15)		38 (2.27)		36 (2.30)		106 (2.24)
	Non-H5N1		16 (1.07)		18 (1.02)		26 (1.66)		60 (1.27)
2014	M gene	785	48 (6.11)	1193	68 (5.69)	1147	67 (5.84)	3125	183 (5.86)
	H5/N1 genes		30 (3.82)		42 (3.52)		40 (3.49)		112 (3.58)
	Non-H5N1		18 (2.29)		26 (2.17)		27 (2.35)		71 (2.27)
Cumulative	M gene	7663	311 (4.05)	9142	417 (4.56)	8841	398 (4.50)	25,646	1126 (4.39)
	H5/N1 genes		215 (2.8)		281 (3.1)		266 (3.0)		762 (2.97)
	Non-H5N1		96 (1.3)		136 (1.5)		132 (1.5)		364 (1.42)

Table 3 Numbers and percentages of H5N1 and non-H5N1 positive samples in different species of backyard poultry in Egypt based on rRT-PCR of M, H5 and N1 genes

Species	AIV-M-gene		H5N1 positive		Non-H5N1 positive	
	Tested	Positive	Number	Percentage	Number	Percentage
Chickens	16,185	618 (3.81 %)	403	2.48	215	1.33
Turkeys	3132	136 (4.34 %)	72	2.29	64	2.04
Ducks	4696	276 (5.88 %)	220	4.68	56	1.19
Geese	1633	96 (5.88 %)	67	4.10	29	1.78
Cumulative	25,646	1126 (4.39 %)	762	67.67	364	32.33

et al. 1998; Guan et al. 2002; Ma et al. 2014). Interestingly, we recorded considerable inapparent infection of H5N1 in Egypt not only in ducks and geese but also in chicken and turkeys.

Webster et al. (2006) suggested that inappropriate agricultural vaccines could be able to prevent influenza disease signs but unable to prevent virus shedding, promote discreet spread of the virus on farms and to live poultry markets, thus promoting antigenic drift. Egypt, China and Indonesia have adopted poultry vaccination to control H5N1. However, the resurgence of H5N1 in Egypt (Abdelwhab and Abdel-Moneim 2015) and the detection of H5N1 in apparently healthy birds in live poultry markets in China (Chen et al. 2005; Ma et al. 2014) suggest that some vaccines are of suboptimal quality (Shany et al. 2011) or that co-infection masks disease. The early vaccines used in Egypt (H5N1 and H5N2) and H5N2 vaccine in Mexico were proved to be not protective (Lee et al. 2004; Shany et al. 2011).

In the current study, some of the positive backyard birds were vaccinated with H5 vaccines under the umbrella of the national vaccination strategy. Unsound behaviour of the owners hindered the efficiency of the H5 vaccination: some owners vaccinate only some birds and hide the rest while others may not be available at the time of visit and did not notify that their birds are not vaccinated. Meanwhile, obligatory free-of-charge vaccination was discontinued by the end of 2009. Accordingly, inapparent infection could be explained by the application of the obligatory H5 vaccination till the end of 2009 to the beginning of 2010 (Kayali et al. 2014). Most of the backyard owners do not have sufficient awareness, and by the termination of the free of charge vaccination against H5, many owners did not vaccinate their birds, a fact that reduces and calls into question the role of the vaccine as an only factor that masks the clinical signs.

On the other hand, it was clear that there was higher incidence of AIV-H5N1 and non-H5N1 viruses in aquatic birds in comparison to chicken and turkeys. Although outbreaks of fatal HPAI H5N1, in ducks from farms, backyards, live bird markets, rooftops, and also in wild ducks and even vaccinated ducks were previously reported (OIE 2008), inapparent infection of ducks and geese was recorded in the current study and also in a previous recent study (Kayali et al. 2014). Domestic ducks have been implicated in the dissemination and evolution of H5N1 HPAI viruses, and their inclusion in disease control programs is therefore important (Li et al. 2004; Chen et al. 2004). The role of backyard aquatic birds in evolutions of viral mutants was evidenced in Egypt (Abdel-Moneim et al. 2009). The prevalence of H5N1 in the current study showed that ducks and geese, as well as chicken and turkeys, are of great importance as a source of infection to humans that agrees with other previous findings (Chen et al. 2004; Cristalli and Capua 2007). In addition, the high level of relatedness of an avian virus from LBM market to human infection (El-Zoghby et al. 2012) confirms the critical role of transmission of H5N1 from birds with inapparent infection to humans. Interestingly, subclinical infections of humans with H5N1 virus have been reported in China, Cambodia, Vietnam, Thailand and Turkey (Vong et al. 2009; Ceyhan et al. 2010; Khuntirat et al. 2011; Huo et al. 2012; Powell et al. 2012). In Egypt, transmission and widespread occurrence of the disease among different poultry backyard species has been shown to be due to raising chicken, turkeys, ducks and geese together. Interspecies transmission usually occurs especially between closely related host species in the same taxonomic family (Swayne 2000).

In conclusion, the current study reported the existence of HPAI in an inapparent manner in ducks, geese, chicken and turkeys in the backyard birds without considerable differences in different years. This finding highlights the serious impact of such type of birds in the epidemiology of H5N1 in birds, animals and humans. It also highlights the existence of other factors including the evolution of less virulent strains, the possibility of co-infections with different influenza subtypes, in addition to vaccination that may contribute to the widespread of inapparent infection of H5N1 in Egypt.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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