ORIGINAL ARTICLE

Distribution of avian influenza H5N1 viral RNA in tissues of AI-vaccinated and unvaccinated contact chickens after experimental infection

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Abstract Avian influenza due to highly pathogenic avian influenza (HPAIV) H5N1 virus is not a food-borne illness but a serious panzootic disease with the potential to be pandemic. In this study, broiler chickens were vaccinated with commercial H5N1 or H5N2 inactivated vaccines prior to being challenged with an HPAIV H5N1 (clade 2.2.1 classic) virus. Challenged and non-challenged vaccinated chickens were kept together, and unvaccinated chickens served as contact groups. Post-challenge samples from skin and edible internal organs were collected from dead and sacrificed (after a 14-day observation period) birds and tested using qRT-PCR for virus detection and quantification. H5N1 vaccine protected chickens against morbidity, mortality and transmission. Virus RNA was not detected in the meat or edible organs of chickens vaccinated with H5N1 vaccine. Conversely, H5N2 vaccine did not confer clinical protection, and a significant virus load was detected in the meat and internal organs. Phylogenetic analysis showed that the H5N1 virus vaccine and challenge virus strains are closely related. The results of the present study strongly suggest a need for proper selection of vaccines and their routine evaluation against newly emergent field viruses. These actions will help to reduce human exposure to HPAIV H5N1 virus from both infected live birds and slaughtered poultry. In addition, rigorous preventive measures should be put in place in order to minimize the public-health risks of avian influenza at the human-animal interface.

Abbreviations

AIV	Avian influenza virus
Dpc	Days post-challenge
HI	Hemagglutination inhibition
HPAIV	Highly pathogenic avian influenza virus
NLQP	National Laboratory for Quality Control on Poultry Production
NVNC	Non-vaccinated non-challenged
OIE	World Organization for Animal Health
qRT-PCR	Quantitative real-time reverse transcription polymerase chain reaction
SPF	Specific-pathogen-free
VC	Vaccinated challenged
VNC	Vaccinated non-challenged
WHO	World Health Organization

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Introduction

The unprecedented spread of bird flu since 2003 due to highly pathogenic avian influenza virus (HPAIV) of the H5N1 subtype has not only devastated the poultry industry in many countries but also posed a pandemic threat [21]. For control of H5N1 infection in chickens, commercial vaccines, mainly from the H5N1 and H5N2 subtypes, have

been developed using traditional and reverse genetics technologies. These vaccines are able, with variable efficacy, to reduce morbidity, mortality, and losses in egg production and to decrease virus excretion through the respiratory and alimentary tracts of infected birds [33]. However, silent spread of the virus in vaccinated birds with or without clinical disease was not uncommon [24]. It is well documented that AIVs undergo antigenic drift, especially within the hemagglutinin (HA) gene, enabling the virus to circumvent the neutralizing antibodies elicited by the vaccine [29]. In fact, immune pressure induced by the vaccine could accelerate the antigenic drift of field viruses away from vaccine strains [11, 18, 19]. Therefore, a regular update of the vaccines used with regard to the genetic and antigenic variations of field viruses has been recommended recently in countries endemic with HPAIV H5N1 [11, 19, 30].

Since 2006, infection with HPAIV subtype H5N1 of clade 2.2.1 has resulted in the culling of hundreds of millions of birds and has disrupted food security in Egypt [6]. The poultry industry has embarked on a mass vaccination policy to reduce circulation of the H5N1 virus, but with limited success [4].

Recent phylogenetic analysis of the HA genes of Egyptian H5N1 viruses indicated the circulation of two main sublineages among poultry. The first group (classic strain) is related to the viruses originally introduced into Egypt in 2006. This group was detected mainly in household and farm birds and linked to all human cases reported during 2010. The other group (variant strain) emerged in late 2007 and is mostly linked to cases of vaccination failure. This group is genetically and antigenically different from the commonly used vaccine strains [8, 9, 11, 12].

Currently, HPAIV H5N1 is endemic in Egypt, is deeply entrenched in poultry, and poses a significant public-health threat. By August 09, 2011, it had caused 52 fatalities out of 150 confirmed human cases [36]. All human infections but three were linked to direct contact with poultry, i.e., keeping, handling, and slaughtering of apparently healthy or sick birds [15]. In this regard, backyard birds, live-bird markets and rooftops represent a major source of H5N1 virus exposure to humans due to the lack of adoption of biosecurity measures, hygienic practices and veterinary inspection [1, 3, 4, 8]. The virus can easily contaminate personnel handling and/or processing chicken meat. To date, it is recognized that bird flu due to H5N1 virus infection is not a food-borne disease [35]. However, transmission of the Asian HPAIV H5N1 to tigers and leopards due to consumption of raw infected meat or consumption of experimentally infected chickens and ducks has been reported [14, 16]. Therefore, there is a legitimate concern that H5N1 virus could be transmitted to humans via contact with contaminated poultry products

[35]. Little information is available regarding the dissemination of HPAIV H5N1 in skin and edible organs from vaccinated and unvaccinated chickens. Recently, we isolated HPAIV human-like H5N1 of the classic 2.2.1 sublineage from the internal egg contents of vaccinated layer chickens [17].

The objectives of the present study were to assess the efficacy of two commercial H5N1 and H5N2 vaccines after experimental challenge with a recently isolated HPAIV H5N1 strain, to monitor contact transmission to non-challenged vaccinated and unvaccinated chickens, and to detect and quantify H5N1 virus residues in chicken meat and edible organs in experimentally infected and contact birds.

Materials and methods

H5N1 vaccines and vaccination scheme

Two commercial oil-adjuvant, whole inactivated vaccines that are commonly used in Egypt for the control of HPAIV H5N1 in poultry were used in this study. The first vaccine is a reverse-genetics-modified H5N1-based vaccine (referred to as EGY18H/H5N1), which contains the HA and NA genes of H5N1 virus A/chicken/Egypt/18-H/2009 (GenBank accession number CY062601.1) and other internal genes from the high-growth A/Puerto Rico/8/1934 (H1N1) virus. The second vaccine is the H5N2 vaccine prepared from the low-pathogenic H5N2 A/Chicken/Mexico/232/94/CPA virus (referred to as Mex/H5N2) (GenBank accession number AY497096.1). The vaccination scheme consisted of a single-dose vaccination of 0.5 ml administered subcutaneously in the distal part of the neck at 7 days of age as recommended by the manufacturers.

H5N1 challenge virus

Birds were challenged with classical A/chicken/Egypt-102d/2010 (H5N1) virus (referred to as EGY102d/H5N1, GenBank accession number HQ198270.1), obtained from the influenza virus repository of the National Laboratory for Quality Control on Poultry Production (NLQP), Giza, Egypt. The virus (EGY102d/H5N1) was isolated in 2010 from a vaccinated chicken on a commercial farm where birds were infected. The intravenous pathogenicity index was determined to be 1.8, as described [5]. A tenfold serial dilution of the challenge virus was titrated using 9-day-old specific-pathogen-free (SPF) embryonated chicken eggs via allantoic sac inoculation of five eggs per dilution. The median egg infectious dose (EID_{50}) was calculated based on the formula described by Reed and Muench [23]. Experimental infection consisted of intranasal administration of 0.1 ml containing 10^6 EID_{50} of EGY102d/H5N1 virus per bird.

Vaccination of chickens

Sixty one-day-old commercial broiler chickens were obtained from a broiler breeder farm in Egypt where the birds were not vaccinated. The chicks were leg-banded for identification, received feed and water *ad-libitum*, and were kept in cages under good hygienic conditions. Experimental birds all tested negative for maternal antibody. At the age of 7 days, 15 birds were vaccinated with H5N2 vaccine, and another 15 birds were vaccinated with H5N1 vaccine. The remaining 30 birds were not vaccinated and served as unvaccinated controls.

Challenge test

At the age of 35 days (four weeks post-vaccination), birds were transferred to BSL3 animal isolators and were separated into four groups as described in Table 1. Group 1 consisted of 15 birds that were vaccinated with H5N2 vaccine and five unvaccinated birds, group 2 consisted of 15 birds that were vaccinated with H5N1 vaccine and five unvaccinated birds, and groups 3 and 4 had 10 unvaccinated birds each. Five vaccinated birds from groups 1 and 2 as well as 10 unvaccinated birds from group 3 received intranasal inoculations of 0.1 ml containing 10^6 EID_{50} of the challenge virus. The remaining birds in groups 1, 2, and 3 remained unchallenged. Groups 1 and 2 were thus vaccinated and challenged (VC), while those in group 3 were unvaccinated and challenged (NVC). Birds in group 4 were

Table 1 Experimental design of the vaccination and challenge study

Group	Treatment*	No. of chickens	Vaccine used	Challenge**
Group 1	VC	5	H5N2	Yes
	VNC	10	H5N2	No
	NVNC	5	None	No
Group 2	VC	5	H5N1	Yes
	VNC	10	H5N1	No
	NVNC	5	None	No
Group 3	Control positive	10	None	Yes
Group 4	Control negative	10	None	No

VC = Vaccinated challenged birds at 35 days of age

VNC = Vaccinated non-challenged birds served as vaccinated contacts and were placed in contact with the VC birds on day 2 post-challenge

NVNC = Non-vaccinated non-challenged birds served as sham contact birds and were placed in contact with the VC birds on day 2 post-challenge

* Birds were vaccinated with H5N1- or H5N2-based vaccines at 7 days of age

** Birds were inoculated via the intranasal route with 0.1 ml containing $10^{6.0}$ EID_{50} of the Egyptian classic virus (A/chicken/Egypt-102d/2010(H5N1))

unvaccinated and non-challenged (NVNC). At day 2 post-infection, 10 vaccinated non-challenged (VNC) birds and 5 NVNC birds were introduced into the first and second group as contact birds. At 5 and 10 dpc, five chickens from VC and VNC in groups 1 and 2 were sacrificed as shown in Tables 2 and 3. All birds were observed daily for signs and mortality for 14 days post-challenge (dpc), after which all surviving chickens were sacrificed. The dead (or sacrificed) chickens were necropsied, and organ samples from skin, mixed breast and leg muscles, heart, brain, gizzard, trachea, kidney and liver were packed separately in plastic bags and prepared for virus detection (using qRT-PCR as described below). The amount of excreted virus in each organ tested was calculated as EID_{50} using embryonated chicken eggs [5, 23].

RNA extraction

Automated extraction of viral RNA from supernatant fluid of each organ collected separately from birds was done using a MagNA Pure LC Total Nucleic Acid Extraction Kit (Roche, Mannheim, Germany), following manufacturer's recommendations, in a MagNA Pure LC instrument (Roche, Mannheim, Germany). RNA was eluted in a final volume of 60 μl diethylpyrocarbonate-treated water and was stored at −70 °C until tested.

qRT-PCR

Partial amplification of the matrix gene of avian influenza type A viruses (AIV) was done using a OneStep Real-Time PCR Kit (QIAGEN, Valencia, CA). Primers and probes published by Spackman et al. [25] targeting the M gene of AIV were used; namely, forward primer M+25: 5′-AGA TGA GTC TTC TAA CCG AGG TCG-3′, reverse primer M-124: 5′-TGC AAA AAC ATC TTC AAG TCT CTG -3′and probe M+64: 5′-FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA-3′. The QRT-PCR reaction was performed in a Stratagene MX3005P real-time PCR machine following the protocol described by Spackman et al. [25].

Hemagglutination inhibition (HI) test

Serum samples were collected from all vaccinated birds immediately before challenge. Titration of hemagglutinating antibody was carried out by HI test. Two serial twofold dilutions of collected sera were tested against four hemagglutinating units of both the variant H5N1 and H5N2 antigens of the vaccines used as homologous antigens and the challenge virus as heterologous antigen, using a 1% suspension of chicken erythrocytes in V-bottom, 96-well microtiter plates as recommended previously [5]. The titer was defined as the reciprocal of the serum dilution in the

Table 2 Recorded daily morbidity and mortality of H5N2-vaccinated chickens, contact non-challenged vaccinated and sham chickens (group 1)

	Chicken no.	HI titer			Bird status (dpc)													
		A	B	C	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Vaccinated challenged (VC)	1	6	<2	<2		+	++	D										
	2	10	3	<2			+		S									
	3	8	3	<2			+	+	++	D								
	4	9	2	<2				+	S									
	5	7	2	<2			+	D										
Vaccinated non-challenged (VNC)	6	6	<2	<2			+	D										
	7	10	4	<2						+	D							
	8	7	<2	<2					S									
	9	10	4	<2							+	+	+	D				
	10	7	2	<2					S									
	11	7	3	<2							+		D					
	12	7	3	<2								+	++	D				
	13	8	3	<2					S									
	14	6	<2	<2									+	S				
	15	8	3	<2							+	++	D					
Sham (NVNC)	16	<2	<2	<2										+	+	D		
	17	<2	<2	<2							+	++	D					
	18	<2	<2	<2									+	+	+	D		
	19	<2	<2	<2													+	S
	20	<2	<2	<2											+	+	+	D

+ Sick birds showed one of the following clinical signs: ruffled feathers, depression or respiratory disorders

++ Severely sick birds showed more than one clinical sign

dpc = Days post-infection D = dead S = sacrificed

A = A/Chicken/Mexico/232/94/CPA (H5N2) vaccine antigen

B = A/chicken/Egypt-102d/2010 (H5N1) challenge virus antigen belonging to classic 2.2.1 clade

C = A/chicken/Egypt/18-H/2009 (H5N1) vaccine antigen belonging to variant 2.2.1 clade

last well that gave complete inhibition of the hemagglutination activity of the H5 antigen.

Sequence and phylogenetic analysis

The HA_1 gene sequences of challenge and vaccine viruses used in this study as well as other relevant H5N1 viruses were retrieved from the GenBank public database. Nucleotides and deduced amino acid sequences were aligned using BioEdit software version 7.0.0 [13]. Multiple and pairwise sequence alignments of the HA1 gene segment were constructed using the ClustalW algorithm, and phylogenetic trees were constructed using the neighbor-joining method in the MegAlign program from the LaserGene Biocomputing Software Package (DNASTAR, Madison, WI), as shown in Fig. 1.

Statistics

The HI antibody titers were compared by the Kruskal-Wallis test, while the Mann-Whitney U-Test was used to assess significance of means for different groups ($P < 0.05$) using SPSS for Windows 15 (SPSS Inc., Chicago, IL). For statistical purposes, all negative serum samples were given a numeric value of 0.9.

Results

Challenge

The results of the challenge experiment are summarized in Tables 2 and 3. Chickens vaccinated with H5N2 vaccine (group 1) were not protected when challenged with H5N1 virus, which spread to infect contact birds. As shown in Table 2, marked morbidity and mortality were observed in all groups (VC, VNC and NVNC). The onset of clinical illness (ruffled feathers, depression or respiratory symptoms) in group 1 was observed at 2 dpc in VC birds and 3 dpc in VNC birds. A total of 13 birds in this group died, during the period from 4 dpc to the end of observation period (14 dpc). In contrast, birds in the H5N1-vaccinated

Table 3 Recorded daily morbidity and mortality of H5N1-vaccinated chickens, contact non-challenged vaccinated and sham chickens (group 2)

	Chicken no.	HI titer			Bird status (dpc)														
		A	B	C	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Vaccinated challenged (VC)	1	2	8	10				+	S									
	2	<2	7	7			+	+	+	+	+			S				
	3	<2	6	7					S									
	4	<2	7	9										S				
	5	3	8	10										S				
Vaccinated non-challenged (VNC)	6	<2	7	8					S									
	7	1	8	8					S									
	8	2	8	9										S				
	9	<2	8	8					S									
	10	1	7	8														S
	11	<2	6	7								+	+	S				
	12	2	6	8														S
	13	1	6	8														S
	14	<2	6	7														S
	15	<2	7	8														S
Sham (NVNC)	16	<2	<2	<2														S
	17	<2	<2	<2											+	+	+	D
	18	<2	<2	<2												+	+	S
	19	<2	<2	<2														S
	20	<2	<2	<2														S

HI titer against the challenge virus

+ Sick birds showed one of the following clinical signs: ruffled feathers, depression or respiratory disorders

dpc = Days post-infection D = dead S = sacrificed

A = A/Chicken/Mexico/232/94/CPA (H5N2) vaccine antigen

B = A/chicken/Egypt-102d/2010 (H5N1) challenge virus antigen belonging to classic 2.2.1 clade

C = A/chicken/Egypt/18-H/2009 (H5N1) vaccine antigen belonging to variant 2.2.1 clade

group 2 had no clinical signs, and none of the VC or VNC birds died (Table 3). Only one out of five NVNC birds in this group displayed ruffled feathers and died at 14 dpc. All birds in group 3 died within four days after challenge, while no signs of illness or deaths were recorded in the negative control group 4 throughout the observation period (data not shown).

H5N1 virus detection and quantification in chicken carcasses

The challenge H5N1 virus was detected at 8 dpc by qRT-PCR in muscles of only one NVNC bird in the H5N1-vaccinated group 2, with a very low titer equal to $10^{1.2}$ EID_{50}. Also, no virus was detected in the 10 sacrificed chickens slaughtered at 5 and 10 dpc (data not shown). Although H5N1 vaccine efficiently protected birds from morbidity and mortality, transmission of the challenge virus to one NVNC contact bird indicates existing but limited shedding of the challenge virus. In contrast, in the

H5N2-vaccinated group 1, the H5N2 vaccine failed to protect vaccinated chickens against challenge with H5N1 virus or virus transmission due to contact with VNC or NVNC birds.

We tested 17 out of 20 birds for virus detection and quantification (Table 4). The results showed that the highest rate of virus detection was from the trachea of (9 out of 17), and the lowest rate of detection was from the brain (1 out of 17). No viral RNA was detected in five slaughtered, clinically normal birds (1 VC and 4 VNC birds), while one slaughtered VC bird had the virus in three organs. A significant virus load was recorded in the organs of four dead NVNC contact birds, in which the kidneys had the highest virus load ($10^{5.7}$ EID_{50}).

Serum samples

Serum samples collected from birds of group 1, vaccinated with H5N2 vaccine, had a significantly higher HI mean titer (7.7 ± 1.4) against the homologous H5N2 antigen

Fig. 1 Phylogenetic analysis of the HA_1 segment of Egyptian H5N1 and other relevant viruses using the MegAlign program from the LaserGene Biocomputing Software Package (DNASTAR, Madison, WI). Shown are the vaccine H5N1 and H5N2 strains (white arrows) and the challenge virus (black arrow)
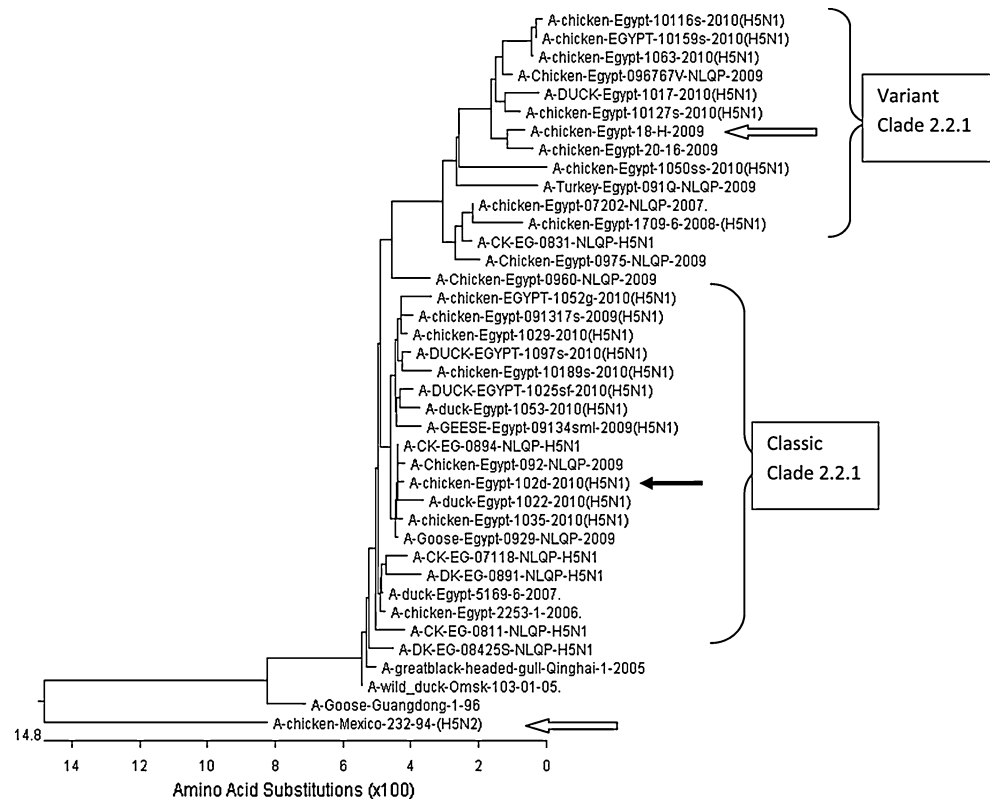

Table 4 Results of H5N1 virus quantification (EID_{50}) from H5N2-vaccinated chickens, contact non-challenged vaccinated and sham chickens

	Chicken no.	Bird status (dpc)	Skin	Muscles	Liver	Heart	Gizzard	Kidneys	Brain	Trachea
Vaccinated challenged (VC)	1	Died (4 dpc)	-	-	-	-	-	-	-	$10^{5.7}$
	2	Slaughtered (5 dpc)	-	-	-	-	$10^{2.9}$	$10^{4.5}$	$10^{1.5}$	-
	3	Died (12 dpc)	-	$10^{3.1}$	-	-	$10^{5.3}$	-	-	-
	4	Slaughtered (5 dpc)	-	-	-	-	-	-	-	-
Vaccinated non-challenged (VNC)	5	Died (7 dpc)	-	-	-	-	-	-	-	$10^{4.5}$
	6	Slaughtered (5 dpc)	-	-	-	-	-	-	-	-
	7	Died (10 dpc)	-	-	-	-	-	-	-	$10^{5.82}$
	8	Slaughtered (5 dpc)	-	-	-	-	-	-	-	-
	9	Died (9 dpc)	$10^{3.5}$	$10^{3.56}$	-	$10^{2.8}$	-	$10^{3.4}$	-	$10^{3.2}$
	10	Died (10 dpc)	-	-	-	-	-	-	-	$10^{4.9}$
	11	Slaughtered (5 dpc)	-	-	-	-	-	-	-	-
	12	Slaughtered (10 dpc)	-	-	-	-	-	-	-	-
	13	Died (9 dpc)	-	-	-	-	-	-	-	$10^{3.1}$
Sham	14	Died (4 dpc)	-	$10^{2.5}$	-	-	-	-	-	$10^{4.45}$
Non-vaccinated non-challenged (NVNC)	15	Died (6 dpc)	$10^{5.2}$	$10^{3.6}$	-	$10^{3.6}$	$10^{3.1}$	$10^{4.5}$	-	$10^{3.8}$
	16	Died (7 dpc)	$10^{3.2}$	-	$10^{3.5}$	$10^{3.9}$	$10^{3.4}$	$10^{4.3}$	-	$10^{5.4}$
	17	Died (9 dpc)	-	$10^{3.5}$	$10^{5.5}$	$10^{5.1}$	$10^{5.1}$	$10^{5.7}$	-	-

The birds at 7 days of age received 0.5 ml of H5N2 vaccine administered by the subcutaneous route in the distal part of the neck and were challenged via the intranasal route with 0.1 ml 10^6 EID_{50} at 35 days after challenge with H5N1 virus

dpc = Days post-challenge . - = negative PCR

than that produced using the heterologous antigen of the challenge virus (2.1 ± 1.1). Meanwhile, no cross-reaction (HI titer <2) was observed against the variant H5N1 antigen. Serum samples collected from birds of group 2, vaccinated with H5N1 variant vaccine had a mean HI titer of 8.1 ± 0.9 using the variant H5N1 vaccine antigen and

7.0 ± 0.8 using challenge virus antigen. Meanwhile, no cross reaction (HI titer <2) against H5N2 vaccine antigen was observed.

Sequence comparison

The HA1 part of EGY102d/H5N1 (the challenge virus) hemagglutinin had 94.2% and 78% nucleotide and 91.9% and 84.1% amino acid identity to that of the H5N1 and H5N2 vaccine virus, respectively. The two vaccine viruses (EGY18H/H5N1and /MexH5N2) shared 77.5% nucleotide identity and 80.9% amino acid identity. Furthermore, the Egyptian challenge virus was located on the tree within the classic group and was found to be more closely related to the original viruses introduced into Egypt in 2006 and more distant from the H5N1 vaccine seed virus related to the Egyptian variant group. Furthermore, the challenge H5N1 virus had undergone several substitutions in the HA_1 protein, particularly a deletion of serine at position 129 in the receptor-binding domain (data not shown). Both the challenge and H5N1 vaccine viruses are completely distinct from the Mexican H5N2 vaccine seed strain (Fig. 1).

Discussion

Control of enzootic H5N1 avian influenza in Egypt is based on risk-reduction practices such as effective vaccination, biosecurity and hygienic procedures to reduce the risk of bird infection and, subsequently, to minimize human exposure. In March 2006, mass vaccination was implemented, and several types of inactivated H5N1 and H5N2 AIV vaccines with different seed viruses were used. In late 2007, vaccinal breaks were observed in some flocks vaccinated with H5N1. This was accompanied by high mortality due to infection with the new immune-escape variant HPAI of the H5N1 subtype [1, 8]. Because of the continuous mutation of H5N1 viruses in animals and humans, vaccination failure in vaccinated birds, recorded human cases, and possible reassortment with other endemic viruses such as H1N1 virus, the likelihood of the evolution of pandemic virus cannot be neglected.

In this study, the efficacy of two commercial inactivated vaccines based on H5N1 and H5N2 strains was investigated in commercial chickens challenged with the classic 2.2.1 Egyptian virus, which showed dramatic differences in morbidity and mortality. Despite the satisfactory HI titer evoked by both vaccines against the homologous HI antigens, the H5N1 vaccine induced significantly high titers against the challenge virus, while very low or undetectable titers were produced by the H5N2 vaccine. Birds vaccinated with H5N1 vaccine were clinically protected against challenge with classic 2.2.1 virus infection, although there was limited virus transmission to one unvaccinated control bird. In contrast, birds vaccinated with heterologous H5N2 vaccine did not withstand the infection, and the virus transmitted to and killed all but one vaccinated non-challenged contact bird. These results are in accordance with those of Grund et al. [11], who showed that H5N1-, but not H5N2-, vaccinated SPF chickens were protected against Egyptian classic 2.2.1 virus infection four weeks post-vaccination.

There is a paucity of information on the efficacy of H5 vaccines to prevent or limit distribution of HPAIV H5N1 in different organs of vaccinated chickens. Nevertheless, the use of inactivated AIV vaccines to prevent the presence of HPAIV, other than H5N1, in the meat of vaccinated poultry was reported previously [28, 32]. In the current study, the RNA of the challenge virus was detected in skin, muscles and tissues of edible organs of most of the H5N2-vaccinated birds (group 1), and in only one H5N1-vaccinated NVNC bird (group 2). Other studies have shown that HPAIV of H5N1, H5N2, and H7N1 subtypes are able to disseminate in meat of infected unvaccinated chickens [7, 10, 20, 22, 28, 32].

In this study, the deduced amino acid sequences of the HA_1 segment showed that the challenge H5N1 virus was closely related to the H5N1 vaccine strain (91.9%) rather than the H5N2 vaccine strain (84.1%). Close genetic identity between field virus and vaccine strains has been reported to be a decisive factor for successful vaccination against HPAIV [31, 34]; however, earlier studies showed that it had no effect on the efficacy of the vaccine [27].

Reports have indicated that HPAIV H5N1 can be transmitted to mammals such as cats, dogs and leopards due to ingestion of raw poultry meat [14], and two cases have been reported in which humans were infected with H5N1 due to the ingestion of uncooked duck blood [26]. The silent spread of HPAIV H5N1 in vaccinated chickens and its dissemination in raw poultry products raise the possibility of zoonotic infection with unrecognized viruses. In such cases, large-scale exposure to infected birds would not be limited to poultry workers but would also include consumers who otherwise have no contact with live poultry and could be exposed to contaminated poultry products. Subsequently, the virus could be transferred to mucous membranes via contaminated hands and fomites [26].

In Egypt, at the animal-human interface, chickens are woven in the fabric of the society, and backyard and household birds constitute a large sector of rural poultry, in addition to commercial production. Approximately 4-5 million families live with about 250 million backyard birds in the same vicinity. Native chickens, waterfowl and turkeys are usually kept together in the same house [12]. Furthermore, it has been observed that the Egyptian H5N1 viruses isolated from ducks and humans are closely related

to each other, suggesting an important epidemiological role of ducks as a reservoir and/or mixing vessel for H5N1 viruses with zoonotic potential [2].

In conclusion, the results of this study indicate that recently evolved H5N1 viruses can adversely affect vaccine efficacy, with increased risk of transmission to humans due to virus residue in infected chicken meat and edible organs. In order to reduce human exposure to infected live and slaughtered poultry, efficacious vaccines should be selected and routinely evaluated against newly emergent HPAIV H5N1. Nevertheless, enforcement of biosecurity measures, elimination of infected poultry and awareness campaign remain the first line of defense against influenza virus infections.

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