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Development of broadly reactive H5N1 vaccine against different Egyptian H5N1 viruses

Mahmoud Ibrahim[a,b], Hesham A. Sultan[b], Alaa G. Abdel Razik[b], Kyung-il Kang[a], Abdel Satar Arafa[d], Awad A. Shehata[b], Yehia M. Saif[a,c], Chang-Won Lee[a,c,*]

[a] Food Animal Health Research Program, Ohio Agricultural Research and Development Center; The Ohio State University, 1680 Madison Avenue, Wooster, OH 44691, United States
[b] Department of Poultry Diseases, Faculty of Veterinary Medicine, University of Sadat City, Menoufia 22857, Egypt
[c] Department of Veterinary Preventive Medicine, College of Veterinary Medicine, The Ohio State University, Columbus, OH 43210, United States
[d] National Laboratory for Veterinary Quality Control on Poultry Production, PO Box 246—Dokki, Giza 12618, Egypt

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ABSTRACT

The H5N1 highly pathogenic avian influenza (HPAI) virus was isolated for the first time in Egypt in 2006, since then, the virus has become endemic causing a significant threat to the poultry industry and humans. H5N1 HPAI outbreaks continue to occur despite extensive vaccination programs that have been implemented nationwide in different poultry species. Several studies showed that the co-circulating H5N1 viruses in Egypt are genetically and antigenically distant raising a question on the cross protective efficacy of commercial vaccines. In this study, we introduced mutations at the antigenic sites of the hemagglutinin (HA) to broaden reactivity of the Egyptian H5N1 virus. A reverse genetically created variant H5N1 virus (A/chicken/Egypt/1063/2010) with five amino acid mutations (G140R, Y144F, I190L, K192Q, D43N) in the HA gene showed enhanced cross reactivity. This virus showed up to 16 fold increase in reactivity to the classic-lineageH5N1viruses measured by hemagglutination inhibition (HI) assay while maintaining similar level of reactivity with the variant-lineage viruses compared to wild-type virus. In addition, a single amino acid substitution (N165H), which removes potential glycosylation site at the HA globular head of two classic strains (A/chicken/Egypt/527/2012 and A/chicken/Egypt/102d/2010) broadened the reactivity to antisera generated against H5N1 viruses from different clusters. The broadened reactivity of the mutant viruses were also confirmed by testing reactivity of antisera prepared from the mutant viruses against reference viruses from both classic and variant clades. The virus neutralization test using selected antisera and viruses further confirmed the cross HI results. This study highlights that targeted mutation in the HA may be effectively used as a tool to develop broadly reactive influenza vaccines to cope with the continuous antigenic evolution of viruses.

1. Introduction

The H5N1 highly pathogenic avian influenza (HPAI) virus isolated for the first time from geese in Guangdong Province, China in 1996 [1]. The first isolation of the HPAI H5N1 virus from poultry in Egypt was in mid February 2006 [2]. Since then the virus has spread rapidly throughout the country causing severe economic losses with frequent human infections [3]. Stamping out, vaccination, movement control, biosecurity, and monitoring were implemented to control the disease [4]. However, continued circulation of the virus resulted in the evolution into multiple clades with emergence of antigenic variants [5–9], with two main clades of H5N1 co-circulating in Egypt; the classic 2.2.1 and the variant 2.2.1.1 [10]. The antigenic analysis showed that the classic 2.2.1 strains are antigenically different from the co-circulating variant 2.2.1.1 strains [11–13].

Experimental challenge studies reported the inconsistent efficacy of the commercial inactivated H5N1 and H5N2 vaccines used in Egypt [14–17]. Moreover, field evidence showed an increased virulence of recent field isolates in poultry causing drop in egg production and mortality up to 27% even with three vaccinations with commercial H5N1 vaccine [18]. This vaccine failure appears to be primarily due to antigenic difference between the vaccine seed

* Corresponding author at: Food Animal Health Research Program, Ohio Agricultural Research and Development Center, The Ohio State University, 1680 Madison Avenue, Wooster, OH 44691, United States. Tel.: +1 330 263 3750; fax: +1 330 263 3677.
E-mail address: lee.2854@osu.edu (C.-W. Lee).

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and circulating strains although vaccine quality, improper storage, handling and administration of the vaccine should be considered as well [19].

The antigenic diversity of different influenza A virus subtypes presents a challenge to influenza vaccine development. This directed researchers toward development of universal influenza vaccines that induce immunity to conserved epitopes expressed in multiple subtypes which could protect against newly emerging influenza strains. Several experimental studies tested the protective efficacy of the conserved epitopes like M2e and HA2 against different influenza subtypes [20–22]. However, M2e-mediated and HA2-mediated protection are much weaker than HA-mediated protection [23,24].

Multiple-clade influenza H5N1 vaccines using strains from different H5N1 virus clades as inactivated whole virus vaccine with adjuvants were tested in different animals and revealed broad cross protection against viruses from distant clades [25,26]. However, the inclusion of more than one strain from each influenza subtype has been limited by the need to include large doses of antigen [27]. As an alternative approach, the consensus-based H5N1 DNA vaccines have been created and tested for several influenza proteins including HA, NA, NP and M1 [28–30]. A new method of antigen design using multiple rounds of consensus generation termed computationally optimized broadly reactive antigen (COBRA) successfully elicited broadly reactive antibodies against different strains of clade 2 HPAI H5N1 viruses [31]. However, the consensus-based antigen design is inherently influenced by the input sequences used to generate the synthetic molecule and therefore being a subject to sampling bias [31]. Another method has been described to design H5N1 vaccine that cross-react and broadly protect against viruses from different clades by reconstructing ancestral protein sequences at several nodes of the HA and NA gene phylogenies that represent ancestors to diverse H5N1 virus clades [32].

In our previous study, sequence analysis revealed about 28 conserved mutations in the antigenic sites of HA1 between co-circulating classic and variant H5N1 viruses [12]. Among these 28 amino acids, it was previously demonstrated that substitutions at 74, 140, 141, 144, and 162 residues in the HA gene of a variant H5N1 strain were mainly involved in the antigenic drift from a classic H5N1 virus [33]. It was also shown that the D43N mutation resulted in antigenic drift between strains from different Egyptian H5N1 virus clusters [13]. In addition, influenza A viruses have been shown to use glycosylations to mask the antigenic epitopes [34–41]. So the removal of glycosylations at the globular head may expose the antigenic epitopes for the immune system.

In this study we tried to develop a broadly reactive vaccine that cross protects against different H5N1 strains circulating in Egypt using *in vitro* mutagenesis coupled with reverse genetics. Two H5N1 strains were used as templates; A/chicken/Egypt/1063/2010 (63/10) representing the variant 2.2.1.1 cluster and A/chicken/Egypt/527/2012 (527/12) from the classic 2.2.1 cluster. Two additional classic strains; A/chicken/Egypt/102d/2010 (2d/10) and A/chicken/Egypt/1112/2011 (12/11), were also used to validate some of the findings. We targeted two potential glycosylation sites (PGSs) at the globular head of the HA protein in addition to mutations at the antigenic epitopes based on previous antigenic studies, especially sites with conserved mutations observed among different H5N1 clusters.

2. Materials and methods

2.1. Viruses and antisera

The list of H5N1 viruses and antisera used in this study are shown in Table 1. All the inactivated viruses and the antisera were obtained from our previous study [12] except the following:12/11 virus was provided by the Animal Health Research Institute (AHRI-NLQP, Giza, Egypt) while A/chicken/Egypt/116/2009 (116/09) was obtained from the depository maintained at Food Animal Health Research Program (FAHRP), The Ohio State University, Wooster, Ohio. Viruses used for generation of the reassortant viruses were inactivated using phenol (Biotechnology grade PH 4.3, GmbH, Germany) before shipping to FAHRP (USDA shipping permit #110710). The hyperimmune sera against three viruses (116/09, 12/11, and 527/12) and reverse genetically created viruses were prepared in chickens as previously described [12].

Table 1
The antisera against reference H5N1 isolates used in this study.

No.	Isolate name	Sublineage	Abbreviation
1	A/chicken/Egypt/06459-3-NLQP/2006	Classic 2.2.1, C1	459-3/06
2	A/chicken/Dakahlia/116/2009*	Classic 2.2.1, C1	116/09
3	A/duck/Egypt/0918-NLQP/2009	Classic 2.2.1, C2	18/09
4	A/chicken/Egypt/102d/2010	Classic 2.2.1, C2	2d/10
5	A/turkey/Egypt/112694V/2011	Classic 2.2.1, C2	2694V/11
6	A/duck/Egypt/11117S/2011	Classic 2.2.1, C2	117S/11
7	A/chicken/Egypt/111AF/2011	Classic 2.2.1, C2	1AF/11
8	A/chicken/Egypt/112AF/2011	Classic 2.2.1, C2	2AF/11
9	A/chicken/Egypt/1112/2011*	Classic 2.2.1, C2	12/11
10	A/chicken/Egypt/527/2012*	Classic 2.2.1, C2	527/12
11	A/chicken/Egypt/1709-6/2008	Variant 2.2.1.1, V1	1709-6/08
12	A/chicken/Dakahlia/106/2008	Variant 2.2.1.1, V2	106/08
13	A/chicken/Egypt/09519S-NLQP/2009	Variant 2.2.1.1, V2	519S/09
14	A/chicken/Egypt/1063/2010	Variant 2.2.1.1, V2	63/10
15	A/turkey/Egypt/101474v/2010	Variant 2.2.1.1, V2	1474v/10
16	A/chicken/Egypt/10135ss/2010	Variant 2.2.1.1, V2	135ss/10
17	A/chicken/Egypt/116AD/2011	Variant 2.2.1.1, V2	6AD/11

* indicate antisera prepared in this study. Other antisera were prepared previously [12].

2.2. Generation of the reassortant viruses

The polybasic amino acid at the HA cleavage site was removed and sequence of the wild-type virus (RERRRKKR) changed to the avirulent-type sequence (RETR) as described previously [42]. The avirulent form of HA gene was cloned into pHH21 plasmid as previously described [43,44]. Targeted *in vitro* mutations in the HA gene were conducted using the QuickChange Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA). Twenty one reassortant viruses containing the avirulent form of HA of the H5N1 strains in a genetic background of A/WSN/33 (H1N1) were generated by reverse genetics (RG) as described [43,44] and were listed in Table 2. The viruses were propagated up to 4 passages in MDCK cells to prepare the stocks. The incorporated changes in the HA gene of the RG reassortants were confirmed by sequencing.

2.3. Serologic assays

Sera were heat inactivated at 56 °C for 30 min and tested by hemagglutination inhibition (HI) and virus neutralization (VN) assays. HI test was carried out according to the OIE manual [45] with minor modifications. Briefly, 2-fold serial dilutions of sera were mixed with 8 hemagglutination units of each virus. The HI reactivity was determined using a 1% suspension of turkey red blood cells. The HI antibody titer was determined as the reciprocal of the highest serum dilution that had complete inhibition of hemagglutination. The VN test was carried out with selected mutants as previously described [12,46]. Two-fold serial dilutions of serum starting at a 1:16 dilution were incubated with an equal volume of virus containing 100 $TCID_{50}$/25 μl in a 96-well plate for 30 min at 37 °C. The virus-serum mixture was transferred to an MDCK cell monolayer and incubated at 37 °C with 5% CO_2. Plates were observed for cytopathic effect for 4 days post-inoculation to determine the

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