Genetic and antigenic analysis of H5N1 viruses for selection of HA-donor virus for vaccine strains

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Received: 14 May 2013 / Accepted: 24 July 2013 / Published online: 8 August 2013
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Abstract Genetic and antigenic analysis of H5N1 viruses, isolated in India during a period from year 2006 to 2010, was carried out for selection of the potential H5-HA (haemagglutinin) gene donor virus for developing a reverse genetics based DIVA marker H5 vaccine for poultry in India. Out of the 47 H5N1 viruses (clade 2.2), 14 representative viruses were selected on the basis of amino acid sequence analysis of HA1 gene for further antigenic characterization. Using antigenic cartography, an antigenic map was constructed based on the data of cross-HI (haemagglutinin inhibition) titration of 14 sera versus 14 viruses to visualize the relatedness among the antigens and antigenic coverage of the sera. Sera against five H5N1 viruses (A/crow/Assam/142119/2008, A/chicken/West Bengal/100879/2008, A/chicken/West Bengal/155505/2009, A/chicken/West Bengal/80995/2008 and A/chicken/West Bengal/81760/2008) exhibited maximum (100 %) antigenic coverage, hence, were selected as the potential HA donor viruses. However, the virus strain A/chicken/West Bengal/80995/2008 matched completely with the consensus amino acid sequence of the 47 viruses, therefore, was considered the best HA donor candidate out of the five showing 100 % antigenic coverage. The present study demonstrates a step-wise methodology for logical selection of vaccine strain or HA gene donor strain for developing H5 vaccines using genetic and antigenic data.

Keywords Avian influenza · Antigenic cartography · H5N1 · Vaccine

Introduction

Avian influenza (AI) has emerged as a highly contagious and economically important ailment of poultry caused by type A influenza viruses of the family Orthomyxoviridae [5, 7]. AI in domestic chickens and turkeys is classified according to disease severity, with two recognized forms: highly pathogenic avian influenza (HPAI) and low-pathogenic avian influenza (LPAI). Since the first outbreak of HPAI H5N1 in India in February 2006 [12], several outbreaks of HPAI H5N1 in poultry have occurred in India [9, 11, 21]. All these outbreaks resulted repeatedly in severe economic losses due to high mortality, culling operations, reduced price of chicken and eggs and ban on exports [3]. As part of preparedness against HPAI in poultry, development of new generation vaccines for HPAI has become essential for possible use in emergency situations. Vaccination has been used in Pakistan to reduce numbers of chickens culled during AI outbreaks and has helped Pakistan in controlling H7N3 infection in poultry [10]. On the contrary, Bangladesh and eastern India have not used AI vaccine and have recurring H5N1 HPAI outbreaks in poultry with possibility of endemic status of H5N1 infection in certain areas [20]. Vaccination of poultry against HPAI is an option when other measures are not able to control the infection but should be exercised with utmost caution and proper planning since improper vaccination may result in silent spread of the virus and emergence of antigenic variants. Office International des Epizooties (OIE) recommends use of only LPAI based H5 vaccine which could be manufactured safely in vaccine manufacturing units with a

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provision of a marker for differentiation of infected from vaccinated animals (DIVA). With the availability of reverse genetic systems for AI virus, inactivated AI vaccines with heterologous neuraminidase (NA) marker can be developed for use in poultry as DIVA marker vaccine.

Since HA (haemagglutinin) is the primary site for neutralizing antibody, it is considered the prime target for the development of AI vaccines mandating the need for its antigenic characterization for vaccine seed strain selection. In case of human influenza vaccines, the high sequence similarity between the HA protein amino acid sequence of the vaccine and challenge strain is known to be critical for optimal efficacy [14, 16]. Though, in case of AI vaccines for poultry, the protection is much broader than human influenza vaccines, but a correlation in sequence similarity of vaccine virus with challenge strain and virus shedding after challenge has been observed [19]. The present study reports genetic and antigenic analysis of HA of Indian isolates of H5N1 for selection of potential HA donor viruses for developing reverse genetics based vaccine strains.

Materials and methods

SPF chicken and embryos

Specific pathogen free embryonated chicken embryos (SPF-ECE) and SPF chicken (4 weeks age), produced at the SPF unit of the institute, were used in the study. The SPF chickens were kept under standard management practices in the bio-containment (BSL 3+) animal facility and were provided autoclaved feed and water ad libitum before and during the experiments. The approval of Institute Animal Ethics Committee was obtained before starting the experiment on SPF chickens.

Virus strain

The H5N1 virus isolates were obtained from the virus repository of the institute and were propagated in SPF-ECE for preparation of antigen for raising hyper-immune sera.

Genetic analysis of Indian H5N1 virus isolates

The AA sequence data of HA gene was retrieved from NCBI GenBank for 47 H5N1 viruses isolated from field samples in India between 2006 and 2010 by High Security Animal Disease Laboratory, Bhopal, India as well as National Institute of Virology, Pune, India. The AA sequences of HA1 part of HA gene segments were analyzed by clustal W alignment algorithm in MEGA 5.1

version. Pair wise sequence alignment was also performed with the MEGA 5.1 program to determine AA sequence similarity and heterogeneity among 47 H5N1 viruses. Phylogenetic analysis of the HA1 sequences was performed using MEGA 5.1 version. The evolutionary history was inferred using the Neighbor-Joining method [15]. Out of these 47 viruses, only 23 viruses were available at the Institute's virus repository. Out of the 23 viruses, representative viruses were selected on the basis of similar AA substitutions at the antigenic sites of HA1 and were characterized further by cross-HI assay and subsequent antigenic cartography.

Preparation of inactivated viral antigens and anti-sera production in SPF chickens

The selected AI virus isolates (14) were grown in 10-day old SPF-ECE and the allantoic fluid was harvested after incubation at 37 °C for 48–72 h. The viruses were inactivated by beta-propiolactone (BPL) at a final concentration of 0.05 % for 4 h at 37 °C with intermittent stirring and then keeping at 4 °C overnight for hydrolysis of the BPL followed by adjustment of pH to 7.2 using sterile 7.5 % sodium bicarbonate solution (w/v). The inactivation was confirmed by inoculation in chorio-allantoic sac of 9-day-old SPF-ECE till three passages without any evidence of virus growth as detected by HA test. Inactivated viral antigen (0.25 ml) prepared from each of the 14 H5N1 viruses and Freund's complete adjuvant (FCA; 0.25 ml) were inoculated in SPF chickens by s/c route. Sera were collected on 28th day and were stored at −40 °C in small aliquots till further use.

Cross HI titration and antigenic cartography

Cross haemagglutination inhibition (HI) was performed with 14 H5N1 viruses against 14 anti-sera. Briefly, 14 sera were tested by HI assay with each of the 14 viral antigens. The HI assays were performed in accordance with standard procedures [13]. Titers were calculated as the reciprocal of the last HI positive serum dilution. The cross-HI titrations were repeated on three consecutive days and the mean HI values of 3 days were used in the antigenic cartography for mapping the viruses and anti-sera using the open access software viz. Analysis Tool for Influenza Virus Surveillance (ATIVS) from Division of Biostatistics and Bioinformatics, National Health Research Institutes, Zhunan, Taiwan available at web-address <http://influenza.nhri.org.tw/ATIVS/index.jsp> [8]. The bar chart was also generated by the same software which determined the range of antigenic coverage.

Results

Genetic analysis of Indian H5N1 virus isolates

The virus isolate A/chicken/West Bengal/80995/2008 matched with the consensus sequence derived from the alignment of AA sequence data of HA gene of 47 H5N1 viruses. The AA substitutions were observed at variable frequency with most prominent differences at positions 145 (antigenic site A), 171 and 205 (B) and 223 (C) (Fig. 1). In the phylogenetic analysis of the 47 H5N1 viruses based on HA1 AA sequence, the viruses isolated in 2006, 2009 and 2010 grouped distinctly and originated from a single branch while the viruses isolated in 2008 were distributed in different branches (Fig. 2). Out of these 47 viruses, only 23 viruses were available to us from HSADL's repository. Out of 23, 14 were selected as representative viruses based on differences in AA substitution from the consensus sequence at or near the antigenic sites A, B, C and D excluding the ones with similar substitutions. The 14 selected viruses were subjected to further characterization by antigenic cartography.

Antigenic analysis of Indian H5N1 virus isolates

The mean HI titres induced by different viruses varied from 1:32 to 1:3072 (homologous titre) (Table 1). In the antigenic cartography (Fig. 3), sera against five H5N1 viruses (A/crow/Assam/142119/2008, A/chicken/West Bengal/100879/2008, A/chicken/West Bengal/155505/2009, A/chicken/West Bengal/80995/2008 and A/chicken/West Bengal/81760/2008) were found to be in a central position of map indicating a reasonably good reactivity with most of the viruses. On the other hand, sera which had peripheral position in the map e.g. A/chicken/West Bengal/239022/2010, would not cover all the viruses within two HI distance and hence would not be

sufficiently reactive to all the viruses. The antigenic coverage of these five viruses was estimated to be 100 % by the ATIVS software. A/chicken/West Bengal/82613/2008 also had high antigenic coverage (93 %) followed by A/chicken/West Bengal/148713/2009 (86 %), A/chicken/West Bengal/142121/2008 and A/chicken/West Bengal/170564/2009 (71 %), A/chicken/Navapur/India/7972/2006 and A/chicken/Jalgaon/India/8824/2006 (57 %), A/chicken/West Bengal/106181/2008 (50 %), A/chicken/Assam/140187/2008 (36 %) and A/chicken/West Bengal/239022/2010 (29 %). The five viruses showing 100 % antigenic coverage were selected as potential H5-HA gene donor viruses for developing DIVA marker vaccine for H5N1 infection in the poultry. The clustering of these five viruses in the antigenic map (Fig. 3) did not correspond to the phylogenetic grouping (Fig. 2).

Discussion

The goal of the present study was to select the potential HA donor H5N1 viruses for reverse genetics based vaccine for possible use in poultry in India. The selection was performed based on cross-HI profile of the available H5 isolates along with the AA sequence analysis of HA1 gene. Previous studies with fowlpox vectored H5 vaccine [2] and reverse genetics based inactivated vaccines [6] have shown that viral shedding in vaccinated and infected birds depends on antigenic match between vaccine strain and challenge strain. These studies have demonstrated that a closer AA sequence similarity of the HA1 protein between vaccine strain and challenge strain results in comparatively lesser viral shedding. On the other hand, it was demonstrated that a challenge strain antigenically distinct (12 % AA differences) from vaccine strain would result in significantly higher virus shedding [6]. In the present study, differences in AA sequences in the HA1 protein were

Fig. 1 Frequency distribution of variation of amino acid at different key positions of HA1 gene among 47 studied H5N1 viruses isolated in India

Fig. 2 Amino acid sequence based phylogenetic analysis of the HA1 protein of 47 Indian H5N1 strains included in the study (clade 2.2). The viruses selected as HA gene donor strains are shown in *bold font*. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 63 amino acid sequences

found among various isolates and were spread randomly throughout the HA1 protein (Fig. 1). Some AA substitutions were present within the predicted antigenic sites known to be important for virus neutralization. The overall maximum similarity of H5N1 virus isolate A/chicken/West Bengal/80995/2008 to the consensus sequence was correlated significantly with its antigenic coverage (100 %). However, AA sequence based phylogeny of HA1 gene (Fig. 2) did not correlate with the placement of antigen and sera in the antigenic map (Fig. 3) except that A/chicken/Navapur/India/7972/2006 and A/chicken/Jalgaon/India/8824/2006 were grouped together in the phylogeny as well as in the antigenic map. These findings lead to the

interesting inference that antigenic clustering based on phenotypic data (HI values) may not match always with the phylogenetic grouping which is based purely on genetic data. A change in single AA due to point mutation in the antigenic site of HA1 may lead to altered antigenicity of the virus while such a point mutation will not alter the phylogenetic position of the virus. A comprehensive antigenic profiling of Asian H5N1 viruses has shown that virus phylogeny does not correlate with antigenic groups A, B, C and D and one antigenic group may include viruses from even five different genetic clades [23]. In 2008, the H5N1 outbreaks in India covered a large geographical area (15 states) and continued for longer time extending till 2009

Table 1 Mean HI values of three independent cross-HI titrations of 14 H5N1 viruses versus 14 anti-H5N1 sera

Antigen	Antiserum													
	8824/06 [#]	7972/06	239022/10	170564/09	142119/08	100879/08	155505/09	106181/08	142121/08	81760/08	148713/09	140187/08	80995/08	82613/08
8824/06	853	384	597	768	853	299	341	12	24	1195	6	19	192	1536
7972/06	768	683	853	683	1024	256	341	21	59	1707	13	21	256	1707
239022/10	469	683	3072	853	1365	517	597	128	149	1109	32	128	256	1195
170564/09	213	256	597	853	555	384	299	171	107	1195	32	53	85	1877
142119/08	341	149	683	277	1365	256	341	27	27	1365	8	53	171	1365
100879/08	128	32	768	53	853	427	427	43	37	1195	19	256	149	597
155505/09	149	64	597	75	768	341	512	75	21	853	13	192	192	853
106181/08	597	683	1195	853	1195	213	597	213	107	1024	16	64	171	1707
142121/08	597	597	853	683	853	427	597	277	128	1536	27	48	213	1707
81760/08	597	101	469	64	597	427	469	27	21	1707	9	277	85	853
148713/09	171	149	299	597	352	213	213	149	85	939	32	59	85	1024
140187/08	171	85	512	64	1024	256	427	53	43	1195	16	341	171	853
80995/08	768	299	427	427	768	192	213	13	37	853	9	35	256	1365
82613/08	171	341	683	683	683	299	341	27	85	1024	19	75	171	2731

Values shown in bold are homologous HI titres

[#] Virus names are shown in short format (isolate number/year of isolation). See Fig. 2 for full names of the viruses

with possibility of more than one introduction [22], hence, a more divergent population of H5N1 viruses is available from isolations made during this year than other years. Due to this reason, the viruses isolated in 2008 were distributed in different branches in the phylogenetic tree (Fig. 2).

The use of antigenic cartography has become an important tool for study of antigenic relatedness and vaccine strain selection for AI vaccine in poultry. Recently, Abbas et al. [1] characterized the H7 isolates through

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focus of antibody response without considering the height of the HI titre. This was done with an aim to select vaccine strains with best coverage of circulating clade 2.2 viruses and to develop the vaccine candidates from all the five strains through reverse genetics. The final selection of the best suitable strain can be based on comprehensive evaluation which would also include the height of HI titre apart from protection and virus shedding in vaccinated and challenged chickens. This stepwise approach for selection of vaccine candidate virus may be much better than previously reported since it is based on genotypic as well as phenotypic (antigenic) data and avoids antigenic characterization of too many viruses. This approach should be able to include all the desirable characters of a vaccine in the selected strain.

In conclusion, we report here a systemic approach for vaccine strain selection for H5 viruses based on genetic and antigenic analysis. In the present study, the five viruses showing 100 % antigenic coverage (A/crow/Assam/142119/2008, A/chicken/West Bengal/100879/2008, A/chicken/West Bengal/155505/2009, A/chicken/West Bengal/80995/2008 and A/chicken/West Bengal/81760/2008) were selected as potential H5-HA gene donor viruses for developing DIVA marker vaccine against clade 2.2 H5N1 viruses in poultry in India. However, since the virus strain A/chicken/West Bengal/80995/2008 matched completely with the consensus AA sequence, it could be the best HA donor candidate out of the five showing 100 % antigenic coverage. Nevertheless, vaccine candidate viruses should be developed through reverse genetics using the HA gene of all the five viruses selected in this study. The vaccine candidates should then be finally evaluated in challenge studies in SPF chicken against currently circulating H5 viruses of clade 2.2.

Acknowledgments We are thankful to the Deputy Director General, Education Division, Indian Council of Agricultural Research for providing the necessary funds under National Fellow project and to the Director, Indian Veterinary Research Institute, Indian Council of Agricultural Research for providing necessary infrastructure and other facilities to undertake the work at HSADL, Bhopal.

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