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Chemical Biology
LETTERS

Identification of immunodominant epitopes in the HA2 subunit of H5N1 haemagglutinin by immunoassay using synthetic peptides as antigens

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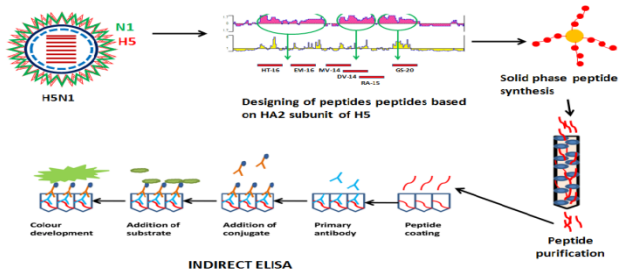
ABSTRACT



Schematic diagram representing the workflow of peptide designing, synthesis, purification and use for detection of anti-AIV antibody by indirect ELISA

H5N1 causes high mortality in domestic poultry. It is the causative agent of H5N1 flu and is the world's largest current pandemic threat. Haemagglutinin (HA) is a surface glycoprotein of the virus which facilitates viral attachment to target cell and its entry. HA frequently accumulates mutation to escape immune response of host. In this study, conserved amino acid sequences in HA2 subunit of H5N1 HA protein of both clade 2.2 and 2.3 were identified and the conserved sequence was further analyzed in silico for its antigenic index, hydrophilicity and surface probability. Six peptides showing potential antigenicity were selected and synthesized. Reactivity of the peptides were analysed by indirect ELISA using antisera against different subtypes of avian influenza. Five out of the six peptides showed reactivity. Thus, five epitopes in the conserved region of HA2 subunit of H5 could be identified which can detect positive serum against avian influenza.

Keywords: H5N1, haemagglutinin, synthetic peptides, ELISA, immunodominant epitopes

INTRODUCTION

Highly pathogenic avian influenza virus (HPAIV) subtype H5N1 has been isolated from avian species in different countries. In India, the first H5N1 outbreak was reported in 2006 in chickens at Maharashtra.[1] Since then, many outbreaks of H5N1 in poultry population have been reported from different states of India and the most recent outbreak occurred in the state of Karnataka in May 2016. H5N1 outbreaks in 2016 have also been reported from our neighbouring countries like, Bangladesh, Myanmar and Bhutan.[2]

HPAI H5N1 virus infection can lead to severe disease in chicken causing upto 90-100% mortality often within 48 hr though aquatic birds can be infected without provoking significant clinical disease.[3] H5N1 was previously thought to infect only birds but it has been confirmed that the virus can infect human, cat, pig and other mammals.[4] As per WHO, 2016 humans infected with avian influenza A (H5N1) have a history of exposure to poultry or poultry related environment prior to illness.[5] Establishment of HPAIV H5N1 as endemic in poultry populations and its ability to cross species barrier makes this virus a potential pandemic threat.[6]

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H5N1 viruses belong to species Influenza A virus, genus Influenza virus A and family, Orthomyxoviridae.[7] Influenza A viruses are enveloped viruses with segmented genome of eight negative sense, single strands of RNA which code for 11 proteins. The two major surface glycoproteins on the viral envelope haemagglutinin (HA) and neuraminidase (NA) are coded by segment 4 and 6 respectively.[8] HA mediates attachment of the virus particle to susceptible cells and initiates the process of virus entry while NA is involved in the release of viral progeny from infected cell surface.[9] Till date, 18 HA subtypes (H1 to H18) and 11 NA subtypes (N1 to N11) have been identified.[10,11] These viruses are classified on the basis of the subtypes of HA and NA present on its envelope such as H5N1 has H5 HA and N1 NA subtypes on its surface.

HA protein being a major surface protein is the primary target of neutralizing antibodies and hence frequently accumulates mutation and phylogenetically evolves into different clades.[12] HA is a homotrimeric protein and the precursor monomers are cleaved into two fragments to produce HA1 and HA2 subunits. HA1 forms the globular head of the HA protein and plays a central role in virus-host interactions.[13] Since HA1 contains all the antigenic sites of HA protein, many current influenza vaccines target the globular head domain. It had also been reported that antibody targeting conserved epitope in HA1 subunit of H5N1 are protective both prophylactically and therapeutically against numerous H5N1 viruses.[14] However, continuous immune driven mutations at head domain causes HA1 targeted vaccines to produce a low broad spectrum activity. [15-17] HA2 forms the stem of HA protein and plays a major role in membrane fusion.[18,19] Hence, any antibody targeting HA2 subunit may prevent membrane fusion by blocking the important structural changes required in the membrane fusion process.[20] Moreover, sequence analysis revealed that HA2 subunit is relatively well conserved which means that antibodies targeting HA2 region may provide cross protection against different clades. HA2 had been targeted for subtype identification in TaqMan real-time RT-PCRs,[21,22] to suppress H5N1 infection by ScFv (Single chain variable fragment),[23] to construct HA-based universal vaccine,[24] generation of antibody specific toward a broad collection of H5N1 viruses.[25] In order to produce antibodies against HA2 subunit, either recombinant HA2 protein or synthetic peptides based on selected epitopes can be used as antigen. However, recombinant proteins are not necessarily correctly folded and may have incorrect disulfide bonding. On the other hand, synthetic peptides consist of only few amino acids and cannot fold like longer polypeptides.[26] Synthetic peptides can be used as an alternative to the whole virus antigen and also has the advantages of easier standardization and safe to handle with no risk of infection.[27,28] Considering the advantages of synthetic peptides in developing a safer diagnostic against virulent virus like H5N1 and the role of HA2 subuint in viral entry, the present work was formulated to identify the immunodominant epitopes in the HA2 subunit of H5N1 using synthetic peptides as antigens.

EXPERIMENTAL PROCEDURES

Designing and synthesis of peptides:

All the H5 HA sequences of avian influenza clade 2.2 and 2.3 reported from India since 2006 till the end of 2014 were aligned by Clustal W of MEGA version 6.[29] These include HA sequences of 92 isolates of both the clade 2.2 and 2.3 reported since 2006. Whole HA sequence was scanned for conserved region for designing antigenic peptides. HA sequence of an isolate of clade 2.3.2.1 was then randomly selected for analysis of antigenic index, hydrophilicity and surface probability by PROTEAN of DNAstar. Six peptides were then designed based on the amino acid sequence of HA2 subunit. The peptides were coded as HT-16, EM-16, MV-14, DV-14, RA-15 and GS-20. The peptides were custom synthesized in linear form by Genscript, USA.

Characterization of peptides:

Properties of the peptides including charge, isoelectric point (PI) and molecular weight (M.W.) were predicted using PROTEAN of DNAstar. Peptides were purified by RP-HPLC using C18 column (Alltima TM C18, 4.6 X 250 mm) with a flow rate of 1 ml/min at wavelength 220 nm. These purified peptides were characterized by ESI-MS (electrospray ionization – mass spectra) and mass was confirmed.

Structural prediction of peptides:

Secondary structures of the peptides were predicted using Chou and Fasman method of DNAstar and online tool, Pep2D.[30,31] Three dimensional (3D) structures of the peptides were predicted by PEPFOLD and visualized in Jmol.[32-34]As the peptides were intended to be analysed by indirect ELISA for their reactivity where a coating buffer of pH>9.0 is to be used, difference in 3D structures of the peptides at pH 7.0 and 9.0 were also determined using PEPLOOK.[35]

Analysis of reactivity of the peptides:

Indirect ELISA of the synthetic peptides using sera available at the repository of ICAR-NIHSAD, Bhopal (OIE reference laboratory of avian influenza), was carried out to determine their reactivity. Briefly, 10 µg of peptides in 50 µl of coating buffer (pH 9.6) per well was coated in duplicate on a high binding polystyrene ELISA plate (Nunc Maxisorp) and incubated overnight at 4ºC. The plate was washed once with PBS and the unoccupied sites were blocked with 250 µl of 5% skimmed milk powder at 37ºC for 2 hrs. The plate was washed once and then 50 µl of identified positive and negative sera (1:100 in PBST containing 2% SMP) was dispensed in the designated wells and incubated for 1 hr at 37ºC. Three washes with PBST (PBS + 0.05% Tween 20 v/v) followed by one wash with PBS was done to remove the unbound antibodies. 50 µl of anti-chicken HRP conjugate (Sigma) diluted 1:10,000 in PBST containing 2% SMP was then added to the wells to detect the bound antibodies and incubated for 1 hr. Plate was again washed four times and substrate (TMB, Amresco) was added and incubated in dark at room temperature for 15 min. The reaction was stopped by adding 1M H_2SO_4 and OD was recorded at 450 nm on ELISA reader (EIAQuant Microplate Reader). The experiment was repeated thrice each with H1, H3, H4, H5, H6 and H9 antisera.

RESULTS AND DISCUSSION

Early and accurate diagnosis of H5N1 is the need of the hour considering the pathogenic potential of these viruses. Currently, conventional virus isolation and characterisation are the methods of choice for diagnosis of avian influenza. However, these methods tend to be expensive, labour intensive and time consuming. Moreover, there are biosafety issues as these agents can be a serious zoonotic threat causing over 50% mortality in infected humans and handling of suspected samples requires specialized biocontainment facilities.[36] There are several commercial kits based on enzyme immunoassays or immuno-chromatography for detection of influenza A viruses in poultry.[37] These kits have the advantages of ability to detect influenza A virus within minutes but are expensive and may not be suitable for application in field condition. Diagnostic tests like RT-PCR, single step real time RT-PCR have been developed for detecting viral RNA and successfully employed in many laboratories.[38,39] These molecular detection methods though allow rapid direct detection of viral RNA may lack sensitivity in samples like faecal swabs, faeces due to the presence of PCR inhibitors unless improved RNA extraction methods are employed.[40] Another drawback of such methods is the requirement of highly sophisticated equipments which are not available in all diagnostic laboratories. This instigates the idea of developing a novel safer and rapid diagnostic which can be used at field level away from biocontainment.

In the present study, HA sequences of 92 H5N1 Indian isolates were aligned and it was found that HA2 subunit consists of few stretches of amino acids showing 100% identity in all the H5 sequences. The conserved amino acids sequence in HA2 are at position 347 to 370, 372 to 388, 415 to 461 and 513 to 528. Since amino acids at C-terminal of HA2 beyond 528 are hydrophobic, possibility of them to be antigenic are minimum and therefore not considered for designing of peptides.[41] After analyzing all the sequences for conserved region, HA sequence of an isolate, (A/duck/02CA10/2011(H5N1)) of 2.3.2.1 clade was randomly picked up to feed into PROTEAN of DNastar to find out antigenic index, hydrophilicity and surface probability of the conserved amino acid stretches. It is an approach adopted successfully to predict antigenic determinants using software, chemical synthesis of the selected sequences and analysis of their reactivity by enzyme immunoassays.[42,43] Virus of clade 2.3.2.1 was selected because the present circulating H5N1 viruses in India belong to this clade.[44]

Amino acid sequence in the region 347 to 370 though conserved, showed less antigenic index as per Jameson and Wolf prediction.[45] In hydrophilicity plot by Kyte and Doolittle and surface probability plot by Emini, this region showed no hydrophilicity and surface probability.[46,47] Therefore, there is least probability of the region to be a suitable epitope. Sequence 372 to 388 showed positive antigenic index, surface probability and consists of hydrophilic amino acids. Sequence 415 to 461 consists of two hydrophilic regions and one hydrophobic region in between. The hydrophobic region in between is of about 14 amino acids where no antigenic index and surface probability could be observed. Sequence 513 to 528 showed positive antigenic index, surface probability and consists of hydrophilic amino acids. Based on these analysis six peptides, HT-16, EM-16, MV-14, DV-14, RA-15 and GS-20 were designed. HT-16, EM-16 and GS-20 were designed based on different stretch of amino acid sequence while MV-14, DV-14 and RA-15 are overlapping peptides in which two peptides are overlapped by 7 amino acids (Figure 1). Strategies of overlapping peptides are followed in epitope mapping of a protein by constructing peptide libraries in which two consecutive peptides are overlapped by few amino acids and testing all the peptides by enzyme immunoassays.[48,27]All peptides listed in Table 1 were synthesized in linear format and purified. More than 80% purity was observed for all the peptides (Figure 2). Molecular weight of the synthesized peptides was confirmed by ESI-MS (Figure 3).

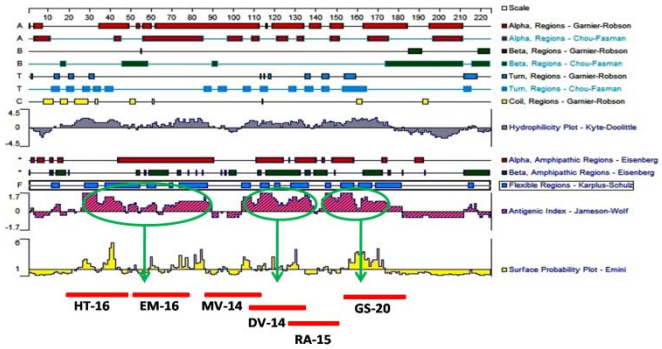

Figure 1. Designing of peptides based on the amino acids sequence on HA2 subunit showing positive antigenic index, hydrophilicity and surface probability

Table 1 Peptides designed based on the conserved region of HA2 subunit of H5N1 haemagglutinin

Sl. No.	Peptide code	Peptide sequences	Number of amino acids
1	HT-16	HSNEQGSGYAADKEST	16
2	EM-16	EFNNLERRIENLNKKM	16
3	MV-14	MENERTLDFHDSNV	14
4	DV-14	DFHDSNVRNLYDKV	14
5	RA-15	RNLYDKVRLQLKDNA	15
6	GS-20	GTYDYPQYSEEARLKREEIS	20

ESI-MS data are presented in Table 2 along with predicted charge, PI and molecular weight. The knowledge of peptide's pI value and charge at different pH is of great importance in downstream experiments including purification and medical applications.[49] Similarly, prediction of peptide secondary structures can be helpful in understanding their biological activity.[27] In Chou and Fasman method, HT-16 and DV-14 showed only turn as secondary structure while EM-16 and GS-20 showed only alpha-helical structure.[30] MV-14 and RA-15 showed both alpha helix and turn but none of the peptides showed beta structure. Pep2D prediction also revealed the presence of only helix and coil with no beta strand in all the peptides.

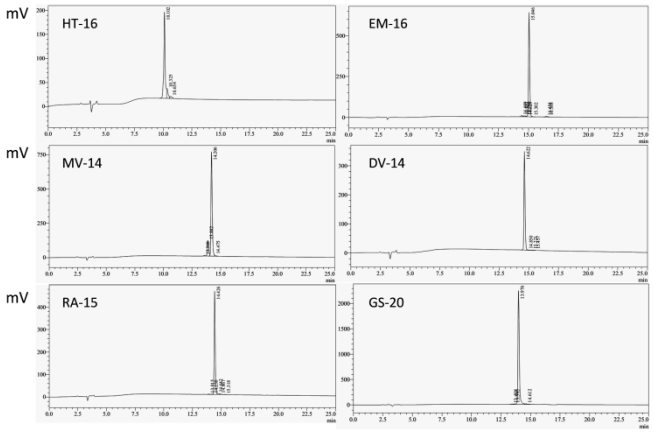

Figure 2. RP-HPLC chromatogram of the peptides, HT-16, EM-16, MV-14, DV-14, RA-15 and GS-20.

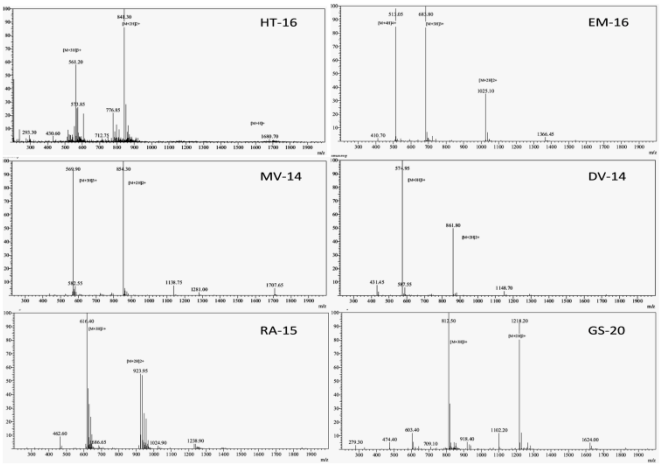

Figure 3 Mass confirmation of the peptides, HT-16, EM-16, DV-14, MV-14, RA-15 and GS-20 by ESI-MS.

Table 2 Properties of peptide predicted by PROTEAN of DNAstar and Molecular weight (M.W.) estimated by ESI-MS

Sl. No.	Peptide code	pI	Charge at pH 7.0	M.W. (Predicted)	M.W. (ESI-MS)
1	HT-16	4.55	-1.92	1679.64	1682.6
2	EM-16	8.83	0.91	2047.34	2051.4
3	MV-14	4.14	-2.92	1705.80	1708.6
4	DV-14	5.31	-0.93	1720.83	1724.85
5	RA-15	9.74	1.91	1845.1	1849.2
6	GS-20	4.49	-2.08	2433.59	2437.5

The observations in both secondary structure predictions were found to be reflected in their 3D structures predicted by PEPFOLD (Figure 4). There is a good correlation between the helicity of peptide antigen and relative affinities of the derived antibody against the native protein antigen and even enhanced affinity has been observed when the epitope is presented on helical scaffolding.[50] Differences in the conformation of the 3D structure of peptides at pH 7.0 and 9.0 could be observed in the predictions by PEPLOOK (Figure 5). pH dependent changes in conformation could also be visualized in 3D structures of all the peptides which may be due to the alteration in electrostatic interactions between charged amino acids with change of pH.[51]

The peptides were tested for their reactivity with anti-AIV sera. Detection of anti-AIV antibodies may not be very useful in highly susceptible species but there are species like aquatic birds which serve as reservoir of avian influenza. Moreover, most viruses of the H5 subtype isolated from birds have been of low pathogenicity for poultry which causes either a mild or no clinical disease. However, presence of low pathogenic H5 strain cannot be overlooked as there is always a risk of mutation and can turn into highly pathogenic.[12] In such scenario, sero-surveillance with highly sensitive and specific, low cost and easy to handle diagnostic can contribute to preparedness against H5N1.

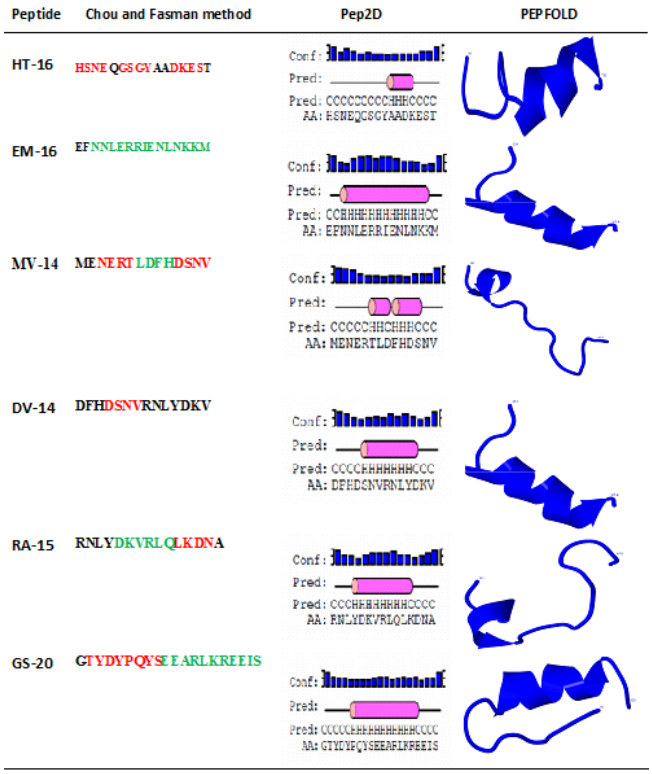

Figure 4. Structural predictions of the peptides by Chou and Fasman method, Pep2D, PPEPFOLD. Red coloured amino acids in the sequence indicate turn and green indicates alpha in Chou and Fasman method. C and H represents coil and helix respectively in Pep2D.

Synthetic peptides have been used in the development of diagnostics against various viral infections like infectious bursal disease virus, peste des petits ruminants (PPR) virus, rotaviruses.[42,52,53] Reactivity of the synthetic peptides were analysed by indirect ELISA using titrated sera. Titre of each serum was determined before use by haemagglutination inhibition (HI) assay. Serum showing a HI titre of more than 2^7 was selected for ELISA. Serum from SPF (specific pathogen free) birds was used as negative control. All the peptides except RA-15 showed reactivity with positive H5 sera (Figure 6). It may be because that RA-15 carries no net charge when

dissolved in coating buffer as its pI value and pH of coating buffer are very close. Passive adsorption of peptides on ELISA plates are primarily through hydrophobic interactions or ionic interactions between the peptides and the surface.[54] When tested with H1, H3, H4, H6 and H9 sera, the reactive peptides were also found to show good reactivity (Figure 7). It indicates that these peptides may serve as template for development of synthetic antigens for diagnosis of avian influenza or for generation of anti-peptide antibodies. Antibodies generated against these peptides may react with HA2 region of H5N1 and prevent virus entry. Moderation of virus infection by antibodies against HA2 in mice has been reported.[55]

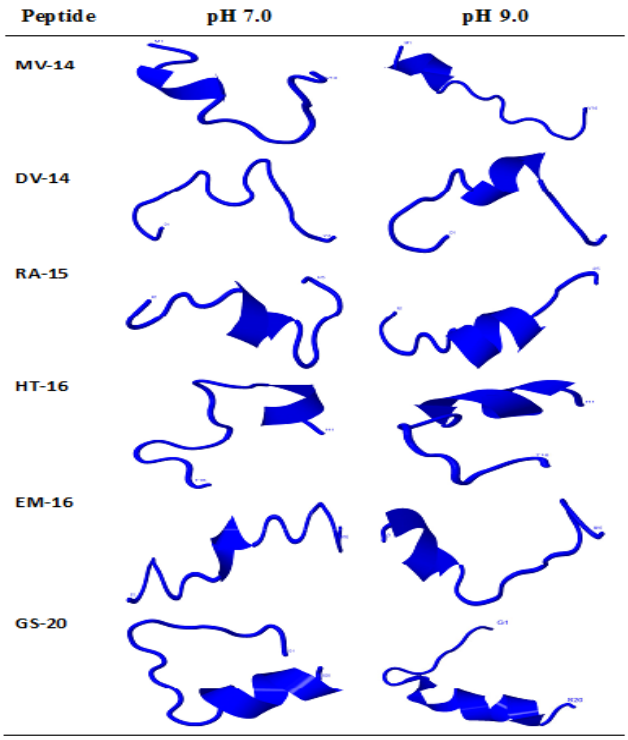

Figure 5. Three dimensional structure of the peptides at pH 7.0 and 9.0 predicted by PEPLOOK

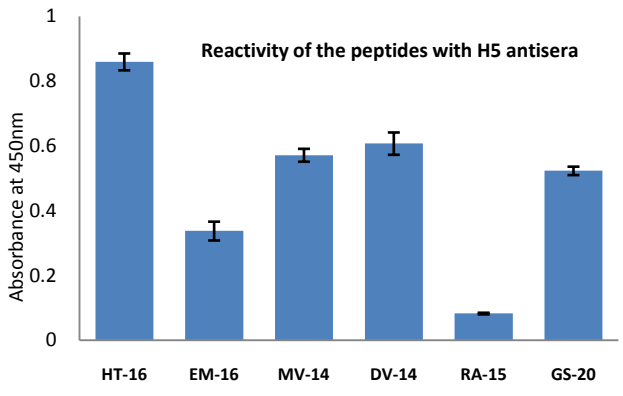

Figure 6. Reactivity of the peptides with H5 antisera in indirect ELISA. All results are presented as Mean of six values ± SE.

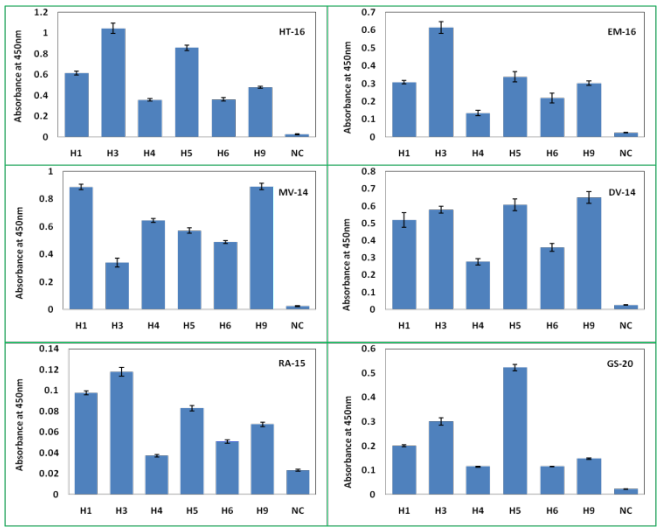

Figure 7 Reactivity of the peptides with serum of different subtypes of avian influenza, H1, H3, H4, H5, H6, H9 and serum of specific pathogen free (SPF) birds as negative control (NC). All results are presented as Mean of six values ± SE.

Out of the reactive peptides, reactivity of GS-20 is comparatively lower but the specificity towards serum of H5 was higher than other HA subtypes. H5 specific peptides in HA2 region have been identified by antibody repertoire analyses of H5N1 influenza survivors in Vietnam using whole-genome-fragment phage display libraries.[56] GS-20 has lesser amino acids compared to the reported H5 specific peptide. Lesser number of amino acids in a peptide has the advantage of lower production cost. This requires further confirmation of the specificity of GS-20 with more number of samples and protocol optimization to develop an user friendly H5 specific diagnostic agent.

CONCLUSIONS

Six potential antigenic sites on the conserved region of HA2 subunit of H5 were predicted using bioinformatics tools and chemically synthesized. Out of six, five peptides showed reactivity with anti-AIV antibodies in indirect ELISA. Among the reactive peptides, one peptide, GS-20 showed higher specificity towards H5 sera. Specificity of GS-20 towards H5 antisera needs to be analysed with further optimization of the reaction protocol.

FUTURE PROSPECTS

In future, overlapping peptides can be designed based on HA2 sequence starting from position 347 to 528 which includes all the conserved region. Screening of the peptides by immunoassay will reveal those peptides which has best coating efficiency as well as reactivity with antisera. This will also reveal the evidence of conformational epitopes in HA2 subunit, if any. Further the peptides showing best result may be synthesized in multiple antigenic peptide (MAP) format which may be used as antigen both for detection of antibodies and generation of antibodies without using a carrier protein.

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SUPPLEMENTARY INFORMATION

The enlarged and high resolution figures are provided as supplementary file and can be downloaded from journal site.

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