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Immunoinformatics Study of Promiscuous HLA Binding Antigenic Nanomers of Surface Glycoproteins of Influenza A (H1N1) pdm09 of Asian Origin

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Abstract.- The swine flu virus [(A(H1N1)pdm09)] is the current health problem over the globe and declared to be pandemic at phase 6. WHO report illustrates swine flu cases over 214 countries and overseas territories including 19000 deaths. Several human cases of A(H1N1)pdm09 infection have been recorded in different geographical regions. Immunoinformatics analysis was applied for the prediction of epitopes from surface glycoproteins of A(H1N1)pdm09 of Asian origin against HLAs that cover 99% Asian population. Alleles of MHC class I supertypes A2, A3, B15, B57 and ABX were well respondents for promiscuous predicted epitopes. DRB1-0101 was the highest binding allele of MHC II supertype DRB1. Epitope VSFNQNLEY of NA protein was excellent MHC I promiscuous binder at average binding score 111.64 covering 15 MHCs. YSTVASSLV was predicted as MHC II promiscuous binding epitope of HA protein covering 6 MHC alleles at average score 224.49. Considerable number of predicted epitopes were found true positive as analyzed by IEDB conservancy tool. Hence, application of systemic *in silico* method provided good vaccine candidates for all Asian countries.

Key words: H1N1, glycoprotein, epitope, immunoinformatics, major histocompatability complex, human leukocyte antigen.

INTRODUCTION

The pandemic swine flu virus (also known as pig influenza and hog flu) of influenza A [(A(H1N1)pdm09)] belongs to *Orthomyxoviridae* family (Kedwaii *et al.*, 2011), is the current public health problem over the globe (Adhikari *et al.*, 2011; Aydin et al., 2012 ; Gunson *et al.*, 2010). It is a negative sense RNA virus declared to be pandemic at phase 6. World Health Organization (WHO) report of 1st August 2010 illustrates A(H1N1)pdm09 cases over 214 countries and overseas territories/communities including 19,000 deaths). The dynamics and impact of this A(H1N1)pdm09 is expected to be different in different geographical regions. Control of viral infection become low in densely populated low-income countries where public health facilities and drug availabilities are low, resulting increase in infection rate and death (Mullick *et al.*, 2011). Although the infection rate in Pakistan remained to be low in contrast to other countries of the World specially the Asian, the number of infections in Pakistan is at steady increase. About 1242 suspected swine flu cases were identified of which 262 cases were confirmed by laboratory tests (Nishtar, 2010). The first index case of A(H1N1)pdm09 infection in human was identified in March 2009 in Mexico and the laboratory confirmation was on 17 April 2009 (Liu *et al.*, 2011; MMWR, 2009). Several human cases of A(H1N1)pdm09 infection have been recorded since 2009 exhibiting influenza like symptoms *i.e.* pneumonia, respiratory complications, fever, headache, vomiting, cough, fatigue, diarrhea, loss of appetite, aching muscles etc. Unlike the seasonal influenza, majority of A(H1N1)pdm09 infection and death was accounted in children and young adults (Hancock *et al.*, 2009; Sun *et al.*, 2011; Chua and Chai , 2012). The genome of Influenza A viruses is composed of 8 RNA strands of about 13,500 bases that encode 11 viral proteins: PB2, PB1, PB1-F2, PA, HA, NP, NA, M1, M2, NS1, and NEP/NS2 (Furuse *et al.*, 2010; Li *et al.*, 2011). Influenza A viruses are categorized into various classes based on

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2 envelope proteins *i.e.* HA (hemagglutinin) and NA (neuraminidase) (Furuse *et al.*, 2010). Up till now, seventeen subtypes of HA and ten subtypes of NA have been identified (Peirong Jiao *et al*., 2012). The emergence of A(H1N1)pdm09 virus is because of differences in hemagglutinin sequence (HA) of the swine origin influenza A virus (S-OIV) and conventional influenza vaccine (CIV) strains resulting in failure of vaccinated individuals against A(H1N1)pdm09 by CIV vaccine to produce cross-reactive antibodies to the A(H1N1)pdm09 viral strain. Because of this fact the novel A(H1N1)pdm09 strain resisted to drugs and antivirals such as amantadine and rimantadine, increasing the potential for the spread of disease at the global scale (De Groot *et al.*, 2009).

Vaccine development is an effective way to control the viral spread. However, variations in A(H1N1)pdm09 genomic sequences and distribution with regard to geographical area, the target of Asian population could provide more effective results. Epitopic vaccine involves the use of small segments of viral proteome that has more potential to cause viral infections (Shehzadi *et al.*, 2011). The use of such segments provides effective and controlled immune response reducing the lethal effects of the use of whole viral proteome. However, a major problem of epitopic vaccine is the HLA polymorphism. HLA is a highly dense genomic region comprising about 200 genes in human genome. A large number of these genes play an important role for immune response and some exhibit high genetic polymorphisms (Yao *et al.*, 2009).

Hence, study was designed for prediction of antigenic nanomers (also known as epitopes) of A(H1N1)pdm09 from surface glycoproteins proteins i.e. HA and NA of Asian origin against HLA that are more frequently populated in Asian population. Since most of the Asian countries are developing, having nearly similar climatic, hygienic conditions and the mode of viral infection, it was hypothized that promiscuous epitope prediction of A(H1N1)pdm09 virus of Asian origin against Asian HLA can provide more sophisticated results. The use of such predicted promiscuous epitopes can serve as potential vaccine candidates for all the Asian countries covering a wide geographical region and eliminating past and future mutations (Hoof *et al.*, 2009).

MATERIALS AND METHODS

Sequence retrieval and alignment

Full length protein sequences of HA and NA of A(H1N1)pdm09 of 17 Asian countries Asian countries were collected from NCBI (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) (Bao *et al.*, 2008). These Asian countries are namely China, Hong Kong, India, Japan, Jordan, Kazakhstan, Kyrgyzstan, Malaysia, Mongolia, Myanmar, Russia, Singapore, South Korea, Taiwan, Thailand, Turkey and Turkmenistan. Since public databases contain errors, duplications and discrepancies (Khan *et al.*, 2006), so the collected sequences were edited manually using BioEdit tool and sequences such as precursor polyproteins were removed prior to analysis.

All the selected sequences were aligned and compared by using multiple sequence alignment software ClustalW2 (Larkin *et al.*, 2007). Consensus sequence based approach was applied to sequences of each country separately. From the all aligned sequences of one origin, a consensus sequence was drawn. Finally all the consensus sequences were aligned to find the regions of sequence conservancy. Only the conserved segments comprising minimum amino acid length of 9 were marked for further analysis. This typical length of amino acids comprises significance for many immunological applications for their ability to bind to many HLA molecules.

Entropy-based analysis of A(H1N1)pdm09 sequence variability

Prediction of short vaccine targets typically 9-mers, from conserved regions are important for the mutational escape. One of the used dynamic method for the measure of degree of variability of peptides and their evolutionary stability is the determination of entropy (Shannon, 2001). Entropy (H) representing the site variability is computed from the probability, p_a of each residue in the protein sequence. $H = -\Sigma_a p_a \log_2 (p_a)$. Low entropy of each amino acid residue or peptide characterizes the site stability. A change in entropy value from 0

to upwards indicates the respective decrease in conservancy from 100% to lower value effected by the number of site variants and their respective frequencies (Khan *et al*., 2006). Hence, the entropy of protein sequences aligned by ClustalW2 was calculated prior to epitope prediction. Any predicted epitope that fall in variable region was rejected.

HLA selection and epitope prediction of A(H1N1)pdm09 surface glycoproteins

Epitopes were predicted against selected alleles of 10 MHC I Supertypes (A2, A3, B7, B15, A24, B44, B57, ABX, B27 and BX) and 8 MHC II alleles of Supertype DRB1 that covers 99% Asian population (De Groot *et al*., 2002; Reche and Reinherz, 2005).

A large number of web servers are available freely on net for the prediction of linear epitopes of proteins from infecting organisms. However, NetMHCpan (http://www.cbs.dtu.dk/services/NetMHCpan) and NetMHCpanII (http://www.cbs.dtu.dk/services/NetMHCIIpan-2.0/) were used for the prediction of MHC I and MHC II binding epitopes of A(H1N1)pdm09 of Asian origin against MHC alleles that were predominantly found in Asian countries (De Groot, Sbai, Saint Aubin, McMurry and Martin, 2002, Reche and Reinherz, 2005). It uses artificial neural networks (ANNs) for epitope (9-15 mer) prediction for human as well as non human primates including chimpanzee, rhesus macaque, gorilla and mouse and pig (Hoof *et al*., 2009). In the only benchmark performed so far and was trained on more than 110,000 quantitative binding data covering more than 100 different MHC molecules. It was validated as the best of the methods compared, and a recent update of this method is able to accurately predict peptide binding even for MHCs from several non-human mammals (Lundegaard *et al*., 2010). A good illustration for the accuracy of predicted epitopes is for the pig peptides against SLA-1* 0401 molecule. 93% predicted peptides using NetMHCpan were demonstrated to bind stronger than 500 nM (Hoof *et al*., 2009). It also facilitates the user to make MHC restricted peptide predictions using full length MHC protein sequences and given protein of interest. The results are provided in nM IC500 values as well as %-Rank. Peptides having prediction value lower than the set threshold are declared as binders and the rest are non-binders. One drawback of the server is that it can use up to 5,000 sequences per submission, average length of the sequences not higher than 20,000 amino acids and also should not be less than 8 amino acids and predictions against maximum of 20 MHC alleles (Hoof *et al*., 2009).

All the MHC I and MHC II binding epitopes of A(H1N1)pdm09 surface glycoproteins, were predicted via NetMHCpan-I and II server. Using the protein sequences in FASTA format, epitopes were predicted at default threshold where sensitivity and specificity is nearly same. All the epitopes were predicted as nanomers because large number of HLA molecules bind more strongly with nanomers. Irrespective to the fact that MHC-II molecules usually present peptides longer than 9mers, the server predicts the peptide binding core as nonomer that varies for each subjected MHC allele. Moreover, to find out the common epitopes for MHC I and MHC II alleles, only nanomers were predicted. The use of common epitopes can save considerable future experimentation and sources for their first step validation. All the promiscuous epitopes that fall in the conserved regions of A(H1N1)pdm09 sequences were selected and are summarized in Tables I, II; along with starting position in the protein sequence, average score and number of MHC bindings (frequently distributed in Asian countries).

Validation of predicted epitopes

The IEDB database contains experimentally determined epitopes (http://www.immuneepitope.org/). All the predicted epitopes were confirmed for binding to MHC alleles and T cells. We found considerable number of predicted epitopes experimentally confirmed as found in IEDB database and are highlighted by (*) in the predicted epitope tables. It also highlights that NetMHCpan server predicts true positive results at default thresholds.

RESULTS

A large number of epitopes were predicted against 10 selected supertypes at various binding scores. Any epitope that falls in hotspot or warm

Table I.- Predicted promiscuous MHC I and MHC II epitopes of HA proteins of A(H1N1)pdm09 virus (Asian origin) with sequence, start position, binding score and number of MHC binding alleles.

HA MHC I					MHC II			
No.	**Position**	**Peptide**	**MHC binders**	**Average Score**	**Position**	**Peptide**	**MHC binders**	**Average Score**
1	20-Dec	FATANADTL	8	320.71	35-43	TVLEKNVTV *	3	297.05
2	87-95	LSTASSWSY *	9	127.04	43-51	VTHSVNLLE *	4	317.59
3	88-96	STASSWSYI *	6	120.08	309-317	FQNIHPITI	3	156.56
4	120-128	EQLSSVSSF *	4	101.33	323-331	YVKSTKLRL	4	146.14
5	123-131	SSVSSFERF *	5	168.26	324-332	VKSTKLRLA	3	259.73
6	126-134	SSFERFEIF *	5	113.67	329-337	LRLATGLRN *	5	205.16
7	153-161	CPHAGAKSF *	4	58.64	443-451	LVLLENERT *	3	77.89
8	167-175	WLVKKGNSY	5	124.76	534-542	YSTVASSLV *	6	224.49
9	188-196	KEVLVLWGI	4	257.61	541-549	LVLVVSLGA *	5	90.58
10	207-215	SLYQNADAY	5	126.38	543-551	LVVSLGAIS *	3	294.42
11	209-217	YQNADAYVF	6	139.28	544-552	VVSLGAISF *	3	202.88
12	242-250	GRMNYYWTL *	11	104.43				
13	243-251	RMNYYWTLV *	9	57.95				
14	257-265	ITFEATGNL *	4	339.99				
15	259-267	FEATGNLVV	6	218.79				
16	265-273	LVVPRYAFA *	5	151.26				
17	281-289	GIIISDTPV	4	279.68				
18	301-309	GAINTSLPF	6	104.8				
19	307-315	LPFQNIHPI *	8	144.22				
20	309-317	FQNIHPITI *	10	160.83				
21	330-338	RLATGLRNV	6	124.83				
22	341-349	IQSRGLFGA *	5	184.16				
23	345-353	GLFGAIAGF *	7	131				
24	360-368	GMVDGWYGY *	4	43.84				
25	370-378	HQNEQGSGY	3	70.23				
26	419-427	KRIENLNKK	4	185.61				
27	436-444	WTYNAELLV *	4	206.19				
28	451-459	TLDYHDSNV	5	124.54				
29	495-503	SVKNGTYDY *	4	208.1				
30	525-533	TRIYQILAI *	4	310.93				
31	526-534	RIYQILAIY *	4	126.54				
32	528-536	YQILAIYST *	6	188.31				
33	529-537	QILAIYSTV *	7	183.49				
34	534-542	YSTVASSLV *	5	274.19				
35	536-544	TVASSLVLV *	7	46.05				
36	544-552	VVSLGAISF *	4	226.26				
37	546-554	SLGAISFWM *	6	193.31				

(*) in 2nd column indicates that these predicted epitopes are experimentally confirmed in past as studied by IEDB

spot (as determined by entropy) was rejected. Conserve promiscuous binders covering minimum of 3 MHC I alleles were selected as epitopes. Out of 10 MHC I supertypes, 37 epitopes were predicted as promiscuous binders for all the HA proteins of H1N1 influenza A virus of Asian origin. Epitope GRMNYYWTL (242-250) was predicted to bind 11 MHC I alleles and FQNIHPITI (309-317) binds 10 MHC I alleles at average binding score 104.43 and 160.83, respectively. About 24.32 % predicted epitopes (FATANADTL, LSTASSWSY, RMNYYWTLV, LPFQNIHPI, GLFGAIAGF, QILAIYSTV and TVASSLVLV) have binding specificity to 7-9 MHC I alleles of Asian origin at average binding score 320.71, 127.04, 57.95, 144.22, 131.0, 183.49 and 46.05 respectively. 75.67% predicted epitopes covers less than 7 MHC I alleles of Asian origin (Table I).

Table II.- Predicted promiscuous MHC I and MHC II epitopes of NA proteins of A(H1N1)pdm09 virus (Asian origin) with sequence, start position, binding score and respective MHC binding alleles.

NA MHC I					MHC II			
No.	**Position**	**Peptide**	**MHC binders**	**Average Score**	**Position**	**Peptide**	**MHC binders**	**Average Score**
1	10-Feb	NPNQKIITI	5	253.89	15-Jul	IITIGSVCM *	4	197.52
2	15-23	MTIGMANLI	5	266	32-40	IWISHSIQL *	4	217.15
3	66-74	YVNISNTNF	4	144.98	67-75	VNISNTNFA	3	381.28
4	147-155	GTIKDRSPY	5	166.5	83-91	VKLAGNSSL *	3	184.12
5	166-174	VPSPYNSRF *	4	137.3	91-99	LCPVSGWAI	3	226.63
6	212-220	ITDTIKSWR	4	109.83	216-224	IKSWRNNIL *	3	137.07
7	304-312	VSFNQNLEY *	15	111.64	349-357	FSFKYGNGV *	3	194.65
8	345-353	GVKGFSFKY *	4	171.52	359-367	IGRTKSISS *	5	313.92
9	363-371	KSISSRNGF	5	136.02	436-444	IWTSGSSIS *	3	242.31
10	437-445	WTSGSSISF	5	85.25				
11	457-465	WPDGAELPF *	4	113.45				

(*) in 2nd column indicates that these predicted epitopes are experimentally confirmed in past as studied by IEDB

Among the subjected MHC I alleles, HLA-B*1503, HLA-A*0206, HLA-A*6802, HLA-A*0203 and HLA-B*1501 were found to be the highest binders for the predicted epitopes of HA proteins of A(H1N1)pdm09 virus of Asian origin. In contrast, HLA-B*3801, HLA-B*5702, HLA-A*0207, HLA-B*5101, HLA-B*5401, HLA-B*4402, HLA-B*2701, HLA-B*2702, HLA-B*2706, HLA-B*1509 and HLA-B*1510 responded zero or litter to predicted HA epitopes. Other subjected HLAs have intermediate binding efficiency (Fig. 1).

For the MHC II, 11 epitopes were selected as conserve promiscuous binders for HA proteins of Asian origin. Epitope YSTVASSLV (534-542) was predicted to bind maximum of 6 MHC II alleles at average score 224.49 and was also proved by Sidney and Sette., 2006. Epitope LRLATGLRN (329-337) and LVLVVSLGA (541-549) are the promiscuous binders of 5 MHC II alleles at average score 205.16 and 90.58 respectively. 72.72% predicted promiscuous epitopes covers only 3-4 selected MHC II alleles of Asian origin (Table I). DRB1-0101 and DRB1-0701 were found to be the highest binders for a large number of promiscuous epitopes. The DRB1-1301 did not respond to any epitope while DRB1-0301 was the binder of only 2 epitopes (Fig.2).

Considerable number of predicted epitopes of HA proteins, were found to be common epitopes for

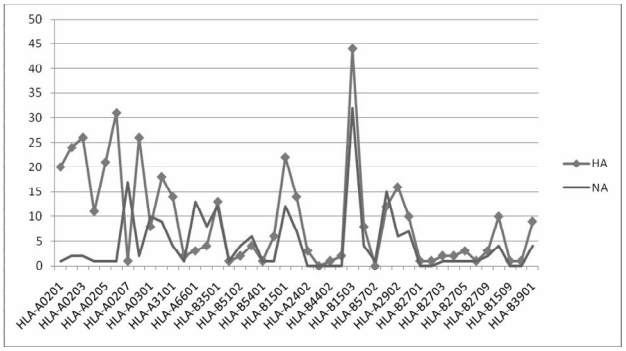

Fig.1. Binding specificity of different MHC I alleles to originally predicted epitopes of HA and NA proteins A(H1N1)pdm09 virus (Asian origin).

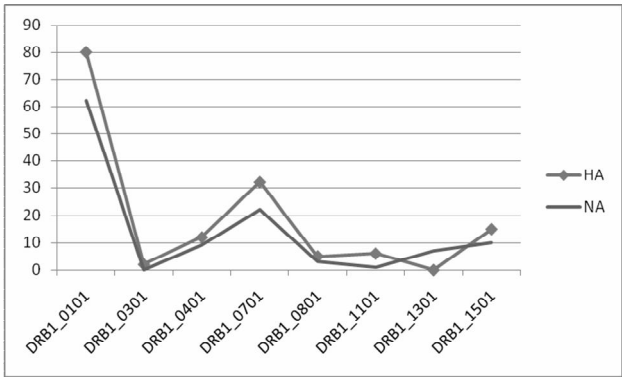

Fig.2. Binding specificity of different MHC II alleles to originally predicted epitopes of HA and NA proteins of A(H1N1)pdm09 virus (Asian origin).

both MHC I and MHC II alleles. However few epitopes were also found having variation of 1, 2 or 3 amino acid residues. Predicted FQNIHPITI (309-317) was found to be exactly same epitope against MHC I and MHC II alleles having good antigenicity against the large number of alleles. RLATGLRNV (330-338), IQSRGLFGA (341-349), YSTVASSLV (534-542) and VVSLGAISF (544-552) were also common epitopes.

NA protein of A(H1N1)pdm09 comprises 469 amino acids, 51549.49 KDa molecular weight and 5.93 pI values. Amino acid Serine (S/Ser) is the highly repeated amino acid (11.5%) in the NA protein sequence while Histidine (H/His) is the least repeated amino acid residue by 1.3% in the protein. Entropy of NA proteins of Asian origin depicted the highest mutation rate at about 250 and 390 amino acid positions. A large number of MHC binding epitopes were predicted against 10 selected supertypes at various binding scores. 11 MHC I promiscuous epitopes were predicted against 10 selected supertypes of Asian population. Epitope VSFNQNLEY positioned 304-312 is the conserve promiscuous binder for 15 alleles at average score 111.64 and has also been proved experimentally by Zhang *et al.* (2011). Other predicted epitopes of NA protein are the promiscuous binders of 5 and 4 MHC I alleles (Table II). Among the subjected MHC I alleles for epitope prediction of NA viral proteins of Asian origin, HLA-B*1503, HLA-A*0207, HLA-A*6601 and HLA-B*1501 were found to be the highest binders. However, HLA-A*2402, HLA-B*3801, HLA-B*4402, HLA-B*4403, HLA-B*2701, HLA-B*2702, HLA-B*1509 and HLA-B*1510 never responded to any predicted promiscuous epitope (Fig. 1). Other subjected HLAs were respondent to some extent.

9 MHC II conserve promiscuous epitopes were predicted for NA proteins of Asian origin. Epitope IGRTKSISS (359-367) binds to 5 MHC II alleles at score 313.92. Other Epitopes are the promiscuous binders of 4 or 3 MHC II alleles at varying binding score are also listed in (Table II). DRB1-0101 and DRB1-0701 were the highest binders to a large number of predicted epitopes. However, DRB1-0301 and DRB1-1101 were found to be the zero binders for predicted epitopes of NA proteins. Other HLA and their binding efficiency is also depicted (Fig. 2).

2 epitopes YVNISNTNF (66-74) and WTSGSSISF (437-445) were found to be common among the predicted MHC I and MHC II epitopes for NA proteins. Epitope GVKGFSFKY (345-353) was partly similar.

Validity of predicted epitopes by IEDB analysis tool showed that 67.56% MHC I and 72.73% MHC II predicted epitopes of HA protein are true positive. For NA proteins, 36.36% MHC I and 77.77% MHC II are also found true positive. These results highlight that server predicts true positive results and thus have remarkable consideration in *in silico* studies to save money and time.

DISCUSSION

In vitro epitope determination and their utilization as vaccine candidates is a very complex phenomenon requiring considerable money and time. However, Bioinformaticians have developed a large number of tools for epitope prediction. These tools are based on various algorithms such as quantitative matrix (QM), Support Vector Machine (SVM), matrix based method, artificial neural network, etc (Bhasin and Raghava, 2004). Such tools are being used largely for epitope prediction of various viruses and pathogens.

The prediction of T cell epitopes is important because these are linear and hence easy to synthesize and proven successful in case of HIV, malaria and tuberculosis (Khan *et al.*, 2006; Shehzadi *et al.*, 2011). It is largely based on phenomenon of immune response associated with Major Histocompatabiliy complex protein (MHC) and T lymphocytes/T cell. Viral antigens/epitopes are initially recognized by surface glycoprotein *i.e.* MHCs that present them to T lymphocytes for their degradation (Ordaz *et al.*, 2010). Two broad categories of MHCs are MHC I and MHC II. MHC I associated epitopes are presented to CD8 T cells. CD8 T cells or cytotoxic T cells (CTL or Tc) recognizes and subsequently kills the infecting cells and secretes cytokines (Yao *et al.*, 2007). MHC II presents the antigenic epitopes to CD4 T cells or helper cells (Th cells) that ultimately provide growth factors and signals for generation of naive

CD8 T cells and maintenance of existing cells.

Because of the fact that pre-existing antibodies offered only limited protection against the pandemic S-OIV of 2009 and resisted to previously used drugs, the utilization of T cell epitopes can postulate T cell mediated immunity and control the viral spread (Jameson *et al.*, 2010). Hence, we used the strategy for epitope prediction against pandemic swine flu virus that has successfully being used for other viruses. The prediction of epitope using A(H1N1)pdm09 surface glycoproteins of a specific geographical area against the HLA that are mostly distributed over that area can provide more sophisticated results. Moreover, the epitopes having litter or more mutation can disturb the binding efficiency and hence were eliminated based on entropy of that peptide in protein sequences.

The majority of the predicted epitopes of A(H1N1)pdm09 virus listed here are promiscuous having intermediate to high HLA binding affinity. These epitopes can effectively be used singly or fused together as polytopes and eventually become vaccines and represents the antigenicity of the whole surface proteins. Another advantage is that their number is significantly smaller then the use of full length proteins and thus provides a control over the immune response and eliminates the side effects then the use of whole viral proteome (De Groot *et al*., 2002).

We predicted the potential T cell epitopes from surface glycoproteins of A(H1N1)pdm09 virus of Asian origin. A considerable number of predicted epitopes have already been confirmed experimentally that also indicate the true positive predictions. This study can serve as a reference for subsequent design and validation via in vitro and in vivo analysis facilitating their usage as epitopic vaccine against pandemic swine flu virus over a wide geographical range.

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