Molecular and Bioinformatic Strategies in Reducing the Burden of Influenza Infections in Slum Areas of Dhaka

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Dedicated

To

My Beloved Family

Acknowledgement

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Abbreviations and Acronyms

cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
CD4	Cluster of differentiation 4
M	Matrix
mRNA	Messenger RNA
NA	Neuraminidase
NCBI	National Center for Biotechnology Information
NEP	Nuclear export protein
NIC	National Influenza Center
NP	Nucleoprotein
NS	Nonstructural protein
PCR	Polymerase chain reaction
PB1	Polymerase basic 1
PB2	Polymerase basic 2
RT-PCR	Reverse transcriptase polymerase chain reaction
rtRT-PCR	Real time reverse transcriptase PCR
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
vRNA	Viral RNA
AA	Amino Acid
vRNP	Viral ribonucleoprotein
Icddr,b	International Center for Diarrhoeal Disease Research, Bangladesh
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
HA	Hemagglutinin
M	Matrix
mRNA	Messenger RNA
NA	Neuraminidase
NCBI	National Center for Biotechnology Information
NEP	Nuclear export protein
NP	Nucleoprotein
NS	Nonstructural protein
ER	Endoplasmic reticulum
FcR	Fc receptor
HA	Haemagglutinin
HAI	Haemagglutination inhibition
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL	Influenzailike illness

kDa	Kilo dalton
L	Liter
M.W	Molecular weight
M1	Matrix protein 1
M2	Matrix protein 2
MHC	Major histocompability complex
Ml	Milliliter
Mm	Milimole
mRNA	Messenger RNA
NA	Neuraminidase
NEP	Nuclear export protein
RNP	Ribonucleoprotien
SAα-(2,3)Gal	Sialic acid α2,3 Galactose
SAα-(2,6)Gal	Sialic acid α2,6 Galactose
SC	Secretory component
S-IgA	Secretory IgA
ssRNA	Single stranded ribonucleic acid
TBE	Tris-borate EDTA
Tc	Cytotoxic T cell
TcR	T-cell receptor
Th	T helper cell
TLR	Toll like receptor
TNF	Tumor necrosis factor
VN	Virus neutralisation
vRNA	Viral ribonucleic acid
WHO	World health organization

Amino Acid Abbreviations

G	Glycine
V	Valine
L	Leucine
I	Isoleucine
F	Phenylalanine
P	Proline
Y	Tyrosine
W	Tryptophan
S	Serine
T	Threonine
A	Alanine
M	Methionine
N	Asparagine
Q	Glutamine
A	Aspartate
G	Glutamate
K	Lysine
R	Arginine
C	Cysteine
H	Histidine

Abstract

Influenza epidemics are caused by rapid evolution of the viral genotypes and play a significant role in the annual mortality and morbidity due to respiratory tract infections in tropical countries like Bangladesh. In low-income countries, influenza associated hospitalization is more prevalent in impoverished population such as slums. In Bangladesh, data on influenza infections in slums of densely populated Dhaka city is limited. Aims of this study were to detect circulatory influenza strains in slums, genetic characterization by molecular and serological methods, assessment of antiviral drug susceptibility of the virus and prediction of putative candidate epitope by in silico approach for alternate vaccine design. In this study course, 993 nasal and throat swabs were collected from patients presented influenza-like illness (ILI) from Rayerbazar, Hazaraibagh, Mohammadpur slums in Southeast Dhaka, Bangladesh between June 2012 and August 2013. Influenza viruses were detected using real time RT-PCR. One hundred one (10%) samples were identified as influenza positive, 47 influenza A (19 A/H1N1pdm09 and 26 A/H3N2) and 54 influenza B viruses including both B/Yamagata and B/Victoria lineages. Influenza positive samples were antigenically characterized by Hemagglutination Inhibition Assay (HAI) which revealed that Bangladeshi strains were antigenically similar to the WHO recommended vaccine strain for the Northern Hemisphere. Complete genome sequencing of two representative influenza A strains were conducted by sanger method and analyzed by BLAST, BioEdit, and Mega tools. Based on complete genome sequence, A/H1N1pdm and A/H3N2 strains were almost identical to other contemporary Bangladeshi strains as well as the globally circulating strains. In the phylogenetic analysis, HA gene of A/H1N1pdm and A/H3N2 were clustered in clade 6B and 3C.3, respectively. Compared to the vaccine strain, A/H1N1pdm showed at least three mutations, K163Q, S185T, and S203T, respectively in the HA antigenic sites (Sa, Sb, and Ca) while receptor binding sites remained conserved. Mutations were also observed at residue E374K of HA which is essential for membrane fusion. N-linked glycosylation sites were conserved in HA gene but one alteration at position 42 in NA gene was identified. The A/H3N2 showed two mutations at the antigenic site A with substitutions T144A and B, R158G in HA while receptor binding sites remained conserved. No strains had mutations that have been reported as responsible for enhanced virulence. Antiviral assay was performed in confluent monolayer of MDCK cells in 96-well plate in duplicate by MTT assay. Both strains were susceptible to the antiviral drugs routinely

prescribed. Candidate epitope prediction was performed through in silico approach and putative peptide vaccine was determined.

This study demonstrates several intriguing findings towards the understanding of genetic diversity of influenza viruses in Bangladesh and prediction of alternate vaccine approaches to reduce the burden of the disease. First, at least four types of influenza viruses were circulating during the study period which justifies that a quadrivalent influenza vaccine formulation that includes influenza A, influenza B, and both lineages of influenza B viruses could be more effective to reduce influenza disease burden in the country. Second, although Bangladeshi strains and WHO recommended vaccine strains are antigenically similar, several mutations were identified. It is interesting to see whether these mutations have any impact on vaccine efficacy. Third, all slum strains were found to be sensitive to the drugs routinely used for influenza treatment. The strains were analyzed based on known molecular markers for the drug resistance which could be useful for the clinical management of the patients particularly during pandemic situation. Fourth, prediction of HA and NA based candidate epitopes on the study strains could facilitate alternate vaccine approach. Notably, in Bangladesh the influenza vaccination has not been implemented in the national vaccination schedule and the vaccine effectiveness among Bangladeshi population is remained unknown. Therefore, assessment of current vaccine effectiveness as well as efforts to assess alternate vaccine approaches are required. In summary, the findings of this study contribute to understanding the characterization of slum influenza viruses that will be useful for routine surveillance, potential drug recommendation. Moreover, candidate epitope prediction will guide for alternate and improved vaccine for the control of influenza in future.

Chapter 1

Introduction & Literature Review

1 Introduction and Literature Review

1.1 General Introduction

Influenza is the paradigm of a viral respiratory disease in which continued evolution of the virus is of paramount importance for annual epidemics and occasional pandemics in humans (Webster, Bean et al. 1992). During the past century, three such pandemics occurred. The first of these is the so called 'Spanish Flu' which occurred in 1918 and which is considered as the greatest natural disaster of the 20th century. This pandemic occurred as a result of the introduction of an influenza A virus of the H1N1 subtype probably derived from an unidentified avian-like precursor virus which became adapted to mammals (Morens, Taubenberger et al. 2009). An estimated 50 million people died as a result (Patterson and Pyle 1991). The second such pandemic 'Asian Flu' occurred in 1957 caused by H2N2 which was a reassortment between the viruses in humans-avian reservoir killing an estimated 2 million people worldwide (Webster 1993). The last pandemic of the 20th century occurred in 1968 and was due to an influenza A/H3N2 virus subtype derived from both human and avian viruses (de Wit and Fouchier 2008). In 2009, a new swine-origin influenza A (H1N1) virus emerged which is the first pandemic of the 21st century (WHO 2009). In this situation, a global preparedness is necessary to combat with upcoming pandemics. One of the key aspect in pandemic preparedness is a rapid response and containment of a novel virus with pandemic potential. If Bangladesh, a country with basic public health infrastructure and currently facing a real pandemic threat due to H5N1, have the capacity in country to determine early the presence of a novel influenza virus, this would have a significant impact on the timeliness of their response. In addition, vaccination strategies in the country including vaccine strains should be monitored and compared with the circulating strains to evaluate the effectiveness of the current vaccines. This would benefit not only the country of Bangladesh but have a great global health impact due to the universal threat of an influenza pandemic.

1.2 Literature Review

1.2.1 Classification and Nomenclature

The influenza viruses belong to the family Orthomyxoviridae (Cox and M.K Estes 2000) and infects cells of the mucous membranes in the respiratory tract (Metselaar 1982). The family Orthomyxoviridae is divided into five genera; Influenza virus A, Influenza virus B, Influenza virus C, Isavirus and Thogotovirus, based on antigenic differences in two of the major structural proteins of the virus, the nucleoprotein (NP) and the matrix protein (M). Influenza A viruses are further classified into 16 HA subtypes and 9 NA subtypes based on the antigenic differences between these two glycoproteins on the surface of the virus particle, the neuraminidase (NA) and the haemagglutinin or HA (Luke and Subbarao 2006) though several new subtypes have recently been proposed (Tong, Zhu et al. 2013). Historically however, influenza A virus subtypes infecting humans have been limited to H1, H2, and H3 and N1 and N2. In recent years, H5N1, H7N7 and H9N2 have sporadically infected humans and continue to pose a pandemic threat due to their possible interspecies transmission into humans. On the other hand, only one HA and one NA subtype has been identified among influenza B influenza viruses which are classified as two lineages, Yamagata and Victoria (Nicholson, Wood et al. 2003).

Nomenclature of influenza viruses includes the host of origin, geographical origin, strain number and year of isolation; then follows in parentheses the antigenic description of the hemagglutinin and the neuraminidase. For example, A/Swine/Iowa/3/70(H1N1) was isolated from swine in Iowa in 1970. If human viruses host of origin is omitted; A/Scotland/42/89(H3N2) for example was isolated from a human host in 1989 in Scotland (Nguyen-Van-Tam and Hampson 2003).

1.2.2 Influenza Virion Structure

Influenza virions are spherical, 80-120 nm in diameter, but may be filamentous, sometimes up to several micrometers in length as illustrated in Figure 1.1. Two distinct types of spikes (approximately 16 nm in length), corresponding to the HA and NA molecules, reside on the surface of the virions. The HA spike appears rod shaped and protrudes from the envelope as a trimer (Wilson, Skehel et al. 1981); the NA spike is a mushroom shaped tetramer (Colman, Laver et al.

1987). These two glycoproteins are anchored to the lipid enveloped derived from the plasma membrane of host cells by short sequences of hydrophobic amino acids (the transmembrane region).

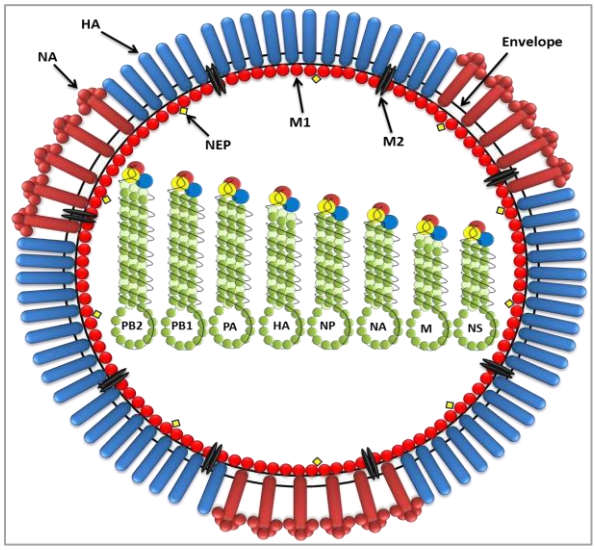

Figure 1.1. Schematic diagram of influenza virus A virus (adapted from Flint 2004)

In addition to HA and NA, a limited number of M2 proteins are integrated into influenza A virions. The mRNA for M2 is transcribed from RNA segment 7 and is an integral membrane protein whose membrane-spanning domain also serves as a signal for transport to the cell surface (Webster, Bean et al. 1992). M2 proteins form tetramers and have H+ ion channel activity. When activated by the low pH in endosomes, they acidify the inside of the virion, facilitating its uncoating (Pinto, Holsinger et al. 1992). M1 protein that lies within the envelope is also derived from the splicing of RNA segment 7. It is the most abundant protein in the influenza virion (Webster, Bean et al. 1992). M1 is thought to play an important role in assembly and budding.

Eight segments of single stranded RNA molecules (negative sense, or complementary to mRNA) are contained within the viral envelope, in association with NP and three subunits of viral polymerase (PB1, PB2, and PA), which together form a ribonucleoprotein (RNP) complex (Figure 1.2) that participates in RNA replication and transcription.

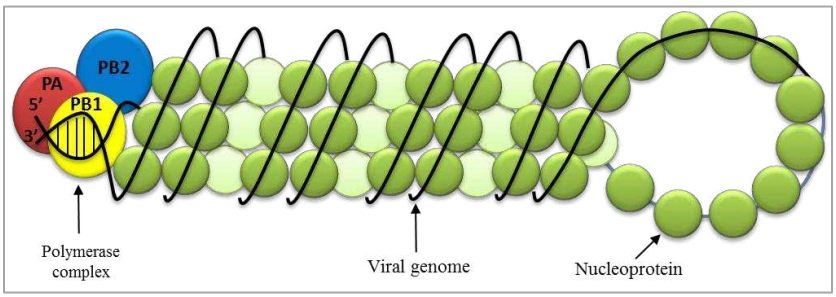

Figure 1.2. NP and PA/PB1/PB2 complex

1.2.3 Genomic Organization of Influenza Virus

The influenza A virus genome contains eight negative sense RNA segments (Palese and Schulman 1976). The viral mRNAs from segments 1 to 6 are monocistronic while viral mRNAs derived from segments 7 or 8 are spliced to form mRNAs coding for two proteins (Lamb 2001,). The sizes of the viral RNA segments and the proteins encoded are summarized in Table 1.1. Of these proteins, only the NS1 protein from segment 8 (NS segment) is a non-structural protein.

Table 1.1: Influenza A virus genome RNA segments*

vRNA Segment	Encoded polypeptide	Abbreviation	vRNA length (bp)	mRNA length (bp)
1	Polymerase Basic 2	PB2	2341	2320
2	Polymerase Basic 1	PB1	2341	2320
3	Polymerase Acid	PA	2233	2211
4	Hemagglutinin	HA	1778	1757
5	Nucleoprotein	NP	1565	1540
6	Neuraminidase	NA	1413	1392
7	Matrix	MP	1027	1005 (M1) 315 (M2)
8	Nonstructural Protein	NS	890	868 (NS1) 395 (NS2/NEP)

*Adapted from (Lamb and Krug, 2001)

1.2.3.1 Segment 1- Basic Polymerase Protein 2 (PB2)

The first segment of influenza A viruses encodes a 2.3Kb protein that forms part of the influenza viral polymerase subunit named PB2. Studies have shown that PB2, PB1, PA and NP form the minimum set of proteins required for viral transcription and replication (Perales and Ortin 1997, Honda, Mizumoto et al. 2002). Like the other polymerase proteins, PB2 contains a nuclear

localization signal (Mukaigawa and Nayak 1991, Perales and Ortin 1997) and is transported into the nucleus of infected cells for viral transcription and replication (Jones, Reay et al. 1986). PB2 is an important protein for generating the cap structure for viral mRNAs (Braam, Ulmanen et al. 1983). The PB2 protein specifically plays a role in generating 5′-capped RNA fragments from cellular pre-mRNA molecules that are used as primers for viral transcription (Guilligay, Tarendeau et al. 2008). Specifically, analysis of deletion mutants of PB2 indicate that the amino-terminus of this protein is a binding site for PB1 (Toyoda, Adyshev et al. 1996). Recently, functional analysis of PB2 protein has shown that this polymerase subunit contains a novel binding site for PB1 subunit and two regions for binding nucleoprotein (NP) with regulatory interactions potential (Poole, Elton et al. 2004).

The PB2 gene is reported to play a key role in the adaptation of a virus that infects birds to one that infects humans. In particular, it has been reported that the amino acid at position 627 is critical for this adaptation. Viruses that infect birds typically have a glutamic acid (E) in this position. On the other hand, influenza A viruses that are fully adapted to humans usually have a lysine (K) at this position. This same position in PB2 appears to be key in determining the lethality of flu viruses (Hatta, Gao et al. 2001). In the 1918 pandemic and in H5N1 viruses, a lysine in this position was associated with a higher level of lethality. The presence of a lysine at position 627 appears to permit flu viruses to replicate in both the lungs and nose and thus spread more easily from person to person.

1.2.3.2 Segment 2- Basic Polymerase Protein 1 (PB1)

The PB1 RNA polymerase subunit of influenza viruse is encoded by segment 2. Several lines of evidence have indicated that PB1, itself, is an RNA polymerase. The central location of the polymerase domain is predicted from observing the presence of conserved motifs characteristic of segmented negative-strand RNA-dependent polymerases (Poch, Sauvaget et al. 1989) and that mutations in these motifs abolished the polymerase activity. Several studies have described the functional domains of PB1 involved in interaction with the other polymerase subunits. Immunoprecipitation studies of the influenza virus RNA polymerase indicate that PB1 contains independent binding sites for PB2 and PA (Digard, Blok et al. 1989) while deletion mutant analyses of PB1 suggest that the amino- and carboxyl-termini of PB1 are binding sites for the PA

and PB2 polymerase subunits, respectively (Gonzalez, Zurcher et al. 1996, Toyoda, Adyshev et al. 1996). The nuclear localization signal of PB1 has been mapped to a region near the amino-terminus (Nath and Nayak 1990). The PB1 subunit plays a key role in both the assembly of the three polymerase protein subunits and serves the catalytic function of RNA polymerization. It has been proposed that the catalytic specificity of PB1 subunit is modulated to the transcriptase by binding PB2 or the replicase by interaction with PA (Honda, Mizumoto et al. 2002). The presence of Serine at the position 66 associated with increased virulence (Medina and Garcia-Sastre 2011).

1.2.3.3 Segment 3- Acidic Polymerase Protein (PA)

The PA protein is encoded by segment 3 and is the smallest subunit of the influenza RNA polymerase complex. Like the other influenza viral polymerase subunits, it contains nuclear localization signals (Nieto, de la Luna et al. 1994) required for transport into the nucleus (Jones, Reay et al. 1986). The PA subunit has no significant homology to other proteins, and for a long time, its function was unclear (Webster, Bean et al. 1992, Perales and Ortin 1997). Various functions were proposed including helicase and ATP binding activities (de la Luna, Martinez et al. 1989). It has also been suggested to be a protease (Sanz-Ezquerro, Zurcher et al. 1996) but this property is not related to any known viral function.

Recent studies however have shown that PA is separable by trypsinization into a large carboxy-terminal domain-the crystal structure of which has recently been reported (Obayashi, Yoshida et al. 2008) and a small N-terminal domain, which contains residues important for protein stability, promoter binding, cap-binding and endonuclease activity of the polymerase complex (Hara, Schmidt et al. 2006). The viral endonuclease activity which is critical for synthesizing viral messenger mRNA's have previously been thought to reside in the PB2 (Shi, Summers et al. 1995) or PB1 (Li, Rao et al. 2001) subunits. However, recent biochemical and structural studies have shown that the amino-terminal 209 residues of the PA subunit contain the endonuclease active site and not PB2 (Dias, Bouvier et al. 2009). As such, during transcription, the PB2 subunit binds the 5′,7-methylguanosine cap of a host pre-mRNA molecule, which is subsequently cleaved 10–15 nucleotides downstream by the PA endonuclease. The resulting short capped RNA primer is used to initiate polymerization by the RNA-dependent RNA polymerase of the PB1 subunit using 5′-

and 3′-bound vRNA as template, resulting in capped, polyadenylated, chimeric mRNA molecules that are exported to the cytoplasm for translation into viral proteins (Boivin, Cusack et al. 2010).

1.2.3.4 Segment 4- Hemagglutinin (HA)

HA plays an essential role in the early stages of infection and is responsible for the virus binding to its receptors, sialic acid, which is present on the host cell surface and promotes fusion of viral and endosomal membrane and eventually facilitates viral entry into the host cell. During infection and vaccination, HA elicits neutralizing antibodies. It is therefore considered the most important target of antibody-mediated protection (Steinhauer 1998,). The HA is synthesized as a precursor polypeptide, HA0 (Webster, Bean et al. 1992, Lamb 2001,). This precursor polypeptide is post-translationally cleaved into two disulphide-linked subunits, HA1 and HA2. The cleavage of the HA0 is a prerequisite for viral infectivity. This process liberates the "fusion peptide" at the amino-terminus of HA2 required for membrane fusion. In addition, this cleavage also allows the native HA molecule to undergo a conformational change, a process which is triggered by an acidic environment and is essential for membrane fusion (Skehel, Bayley et al. 1982). In general, the HA0 is believed to be cleaved by trypsin-like proteases extracellularly. However, the presence of multiple basic amino acid residues within the cleavage site allow the protein to be cleaved by intracellular proteases, e.g. furin (Horimoto, Nakayama et al. 1994), which are ubiquitously expressed in most tissues. Hence, influenza viruses containing HA with multiple basic amino acids near the cleavage site are often highly infectious and can infect a wide range of cells. The generated HA1 surface subunit mediates the binding to cell surface sialic acid receptors and the HA2 transmembrane subunit that mediates membrane fusion between viral and endosomal membranes after endocytosis (Skehel and Wiley 2000).

The specificity of the interaction of HA with sialic acid (SIA), the cellular receptor, largely explains the host range of influenza A virus (Taubenberger and Kash 2010). Thus, viruses that infect humans bind preferentially to SIA linked to the penultimate galactose via an α 2–6 configuration, whereas avian viruses prefer binding to SIA with α 2–3 linkages (Connor, Kawaoka et al. 1994). The HA receptor binding site (RBS) is formed by three structural elements at the tip of the HA molecule, an α-helix composed of residues 190–198 (the 190-helix) and two loop structures formed by residues 133–138 (the 130-loop) and 220– 229 (the 220-loop). Four

conserved residues, comprising Tyr98, Trp153, His183 and Tyr195, form the base of the RBS (Skehel and Wiley 2000). The amino acid residues in the RBS that are critical for the recognition of either avian or human receptors have been well characterized (Connor, Kawaoka et al. 1994, Matrosovich, Tuzikov et al. 2000). For H1, glutamic acid and glycine residues at positions 190 and 225, respectively, result in binding to avian SIA receptors, whereas H1 proteins that carry aspartic acid residues at these (Matrosovich, Tuzikov et al. 2000) positions interact with human SIA receptors. For H2 and H3, mutations of glutamine and glycine residues at positions 226 and 228 to leucine and serine, respectively, correlate with a shift from avian to human receptor specificity (Matrosovich, Tuzikov et al. 2000). The same mutations also allow binding of H5 to human SIA receptors (Stevens, Blixt et al. 2006).

For H1 strain antigenic determinant sites are as follows: site Sa include 125,128-129,157-158, 160-165, Sb include: 156, 160, 189, 192-194, 196-198, Ca site include: 140-43-145,169,171-174207-208, 224-225, Cb include :73-79, 119 and for H3 strain antigenic determinant sites are as follows A includes 139147, B includes: 154-161, 188-198, C includes: 276-282, D includes: 204-221 and E includes: 169-174, 259-266. (Medina and Garcia-Sastre 2011).

1.2.3.5 Segment 5- Nucleoprotein (NP)

NP protein which is an essential component for transcription and replication is encoded by segment 5 of influenza A viruses. NP is distinct among influenza A, B, and C viruses and is recognized as one of the type-specific viral antigens. It encodes a protein with approximately 500 amino acids; it plays an important role in assembly and budding of influenza virus and has a putative role in host range (Snyder, Buckler-White et al. 1987, Ruigrok, Crepin et al. 2010). The primary function of NP is to form oligomers and bind with the viral RNA segments to form the nucleocapsid of a virus particle playing a pivotal role in the viral genome transcription machinery (Perales and Ortin 1997). Like the other influenza viral polymerase subunits, it contains nuclear localization signals and important for vRNA nuclear transport (Whittaker, Bui et al. 1996). During the early stage of viral infection, the transport of incoming vRNPs from the viral particle into the nucleus is believed to be mediated by NP whereas, in the late infection stage, progeny vRNAs associated with NP, M1 and NS2 are exported to the cytoplasm for viral packaging (Whittaker, Bui et al. 1996).

1.2.3.6 Segment 6- Neuraminidase (NA)

The three-dimensional structure of the NA has revealed that the NA monomer is a homotetramer (Hausmann, Kretzschmar et al. 1997). It consists of a box-shaped globular head, a thin stalk, a transmembrane domain and a cytoplasmic domain (Varghese and Colman 1991). The NA is a surface glycoprotein and the glycosylation of the NA might be an important determinant (but not the sole determinant) of the neurovirulence of influenza viruses (Li, Schulman et al. 1993). It has receptor-destroying activity to cleave the α–ketosidic linkage between a terminal sialic acid and an adjacent D-galactose or D-galactosamine residue (Colman and 1998). The NA is a type II integral membrane protein. It is a sialidase that possesses an enzymatically active domain which cleaves sialic acids from host cells, leading to the release of progeny virions and preventing the aggregation of virion during the budding process (Palese and Schulman 1976). Strong binding to receptor analogs on mucins, cilia, and cellular epithelia would inhibit virus access to functional receptors on surface membrane of target cells. Therefore, NA is important for releasing viruses from the decoy receptor and plays an essential role of virus entry in the early stage of infection (Couceiro, Paulson et al. 1993, Matrosovich, Matrosovich et al. 2004). It is for this reason that NA is a favorable target for antiviral drugs aiming at reducing its sialidase enzymatic activity and thus release of progeny virions (Suzuki, Takahashi et al. 2005). In the NA catalytic sites position that directly interact with positon 118, 151, 152, 224, 276, 292, 371 and 406 and supporting framework (Matrosovich, Matrosovich et al. 2004) sites position that directly interact with the substrate are 156, 179, 222, 227, 247, 277, 294 and 425. The presence of a tyrosine at position 275 of NA appears to resistant against oseltamivir.

1.2.3.7 Segment 7- Matrix Protein (M1 and M2)

The segment 7 of influenza virus encodes two proteins, M1 and M2, by differential splicing of mRNA. The M1 Protein is the product of collinear transcript of the mRNA while differential splicing of the M been RNA yields the M2 protein (Lamb and Choppin 1981). M1 is recognized as another type specific antigen and constitutes the most abundant polypeptide in the virion which provides rigidity to the viral membrane and functions in the viral assembly and budding during viral cycle (Lamb and Choppin 1981). It is found to interact with the viral surface glycoproteins and the RNP complex (Taubenberger and Kash 2010). The segment 7 of influenza A virus encodes two proteins, M1 and M2 by differentials splicing of mRNA. The M1 protein is the product of

collinear transcript of the mRNA while differential splicing of the M gene RNA yields the M2 protein (Lamb and Choppin 1981). M1 is recognized as another type specific antigen and constitutes the most abundant polypeptide in the virion which provides rigidity antigen and constitutes the most abundant polypeptide in the virion which provides rigidity to the viral membraned and functions in the viral assembly and budding during viral life cycle (Lamb and Choppin 1981). It is found to interact with the viral surface glycoprotein and the RNP complex (Taubenberger and Kash 2010). The presence of an asparagine at position of M2 in 31 appears to resistance to adamantine (Medina and Garcia-Sastre 2011).

1.2.3.8 Segment 8- Nonstructural Proteins (NS1 and NS2)

The eighth vRNA segment of the influenza A virus directs the synthesis of two mRNAs. The first of these encodes the non-structural (NS) protein, NS1, while the other is derived from splicing of the NS1 mRNA is translated into a protein that localizes to the cell nucleus and which was originally named NS2 (Nemeroff, Utans et al. 1992) but which has now been renamed the nuclear export protein (NEP) (O'Neill, Talon et al. 1998). NS1 is the only non-structural protein of influenza virus and is found mainly in the host cell nucleus. It is a multifunctional protein involved in nuclear exportation of mRNA, posttranscriptional regulation, and inhibition of cellular interferon response (Hale, Randall et al. 2008). Recently, NS1 protein was found to be responsible for the unusual severity of H5N1 diseases by inducing exaggerated proinflammatory cytokine responses (Cheung, Poon et al. 2002). NS2 protein exists in low amounts and binds to M1 protein in virion. The protein may promote the formation of a stable export complex of new viral RNP (Neumann, Hughes et al. 2000). In association with the matrix protein 1 (M1), it interacts with cellular export factor (CEF1) and mediates the nuclear export of viral ribonucleoprotein (vRNP) complexes by connecting the cellular export machinery with vRNPs (Neumann, Hughes et al. 2000).

1.2.4 Glycosylation

The genome of the influenza A virus encodes 11 proteins. However, only hemagglutinin (HA) and neuraminidase (NA) undergo N-linked glycosylation, and no observation of O-linked glycosylation has been reported (Blake, Williams et al. 2009, Zhirnov, Vorobjeva et al. 2009). Glycosylation of HA and NA can affect the host specificity, virulence and infectivity of an

influenza strain either directly, by changing the biological properties of HA and NA (Schulze 1997, Mishin, Novikov et al. 2005) or indirectly, by attenuating receptor binding (Ohuchi, Ohuchi et al. 1997, Gambaryan, Marinina et al. 1998, Gao, Zhang et al. 2009), masking antigenic regions of the protein (Munk, Pritzer et al. 1992, Schulze 1997, Abe, Takashita et al. 2004, Wang, Chen et al. 2009, Das, Puigbo et al. 2010) impeding the activation of the protein precursor HA0 via its cleavage into the disulfide-linked subunits. HA1 and HA2 (Bosch, Garten et al. 1981, Deshpande, Fried et al. 1987, Ohuchi, Orlich et al. 1989), regulating catalytic activity or preventing proteolytic cleavage of the stalk of NA (Matsuoka, Swayne et al. 2009, Wu, Ethen et al. 2009). N-linked glycosylation sites generally fall into the N-X-S/T sequence motif (sequon) in which X denotes any amino acid except proline. (Bause 1983). The number and distribution of the N-glycosylation sites over the viral proteome can therefore be computationally determined by scanning the sequences for these sequons (Zhang, Gaschen et al. 2004, Zhang, Loriaux et al. 2006).

1.2.5 Influenza Viral Cycle

The influenza viral cycle can be divided into five separate stages and these include viral attachment and entry into a host cell; entry of vRNP into the host muscles, transcription and replication of the viral genome, export of the vRNPs from the nucleus, and assembly and budding at the host plasma membrane (Figure 1.3).

Viral attachment to the host cell membrane begins with HA which, binds to the host cell sialic acid receptor. Two major classifications of this receptor are either α (2,3) or α (2,6). (White et al., 2008). The α (2,3) and α (2,6) linkages are also important in determining the specificity of HA in binding in different species, for example influenza virus which effectively infect humans recognize the α (2,6) linkage whereas those from avian or equine preferentially bind to the α (2,3) linkage and those from swine recognize both. Binding the host cell sialic acid receptor triggers both clatherin dependent and independent pathways resulting in the endocytosis of the virus (Lakadamyali, Rust et al. 2003). The low pH of the endosome initiates an irreversible conformational change exposing the HA2 portion of the HA protein allowing fusion of the viral and endosomal membranes (White, Delos et al. 2008). The acidic environment of the endosome also opens the M2 ion channel allowing protons to be pumped into the viral core; it is this internal

acidification of the virion which releases the vRNP from M1 allowing it to be released into the host cell cytoplasm (Shimbo, Brassard et al. 1996).

Negative sense strand RNA, requires a conversion into positive strand RNA before it can be translated into viral protein, the 5' and 3' ends of the vRNA contain partially inverted sequences, when these ends bind to each other they create a 'cork-screw' confirmation to which the RNA polymerase (composed of PB1, PB2 and PA) binds to initiate transcription (Samji 2009). In order for mRNA to be translated by host cell machinery it requires both a 5' methylated cap and a 3' polyadenylated tail. The poly(A) tail is produced by a 'stuttering' step where the viral polymerase continuously replicates a short run of 5-7 uradines at the end of the vRNA (Digard, Blok et al. 1989). The 5'cap is 'snatched' from host mRNAs. The endonuclease activity of the PB2 protein is essential to this process and as it cleaves host methylated cap from host mRNA and uses it to prime host transcription of the viral mRNA.

In the early stages of infection, the newly transcribe mRNA is then exported from the nucleus to the host cytoplasm where the translation of early a protein, PA, PB1, PB2 and NP occur. The viral polymerase complex and the NP are then transported back into the nucleus to allow for replication of uncapped RNA, which serves as the template for new vRNA (Wise, Foeglein et al. 2009; Wise et al., 2009). Later in the viral infection, newly synthesized vRNA are encapsidated with NP and these vRNPs are translocated to the host cytoplasm where they associate with NS2/NEP for transportation to the apical surface and eventually with M1 for viral packaging. Also, later in the viral cycle, the mRNA transcripts for the viral surface proteins M2, HA and NA are transported to the host cytosol, translated and transported through to the Golgi apparatus to the apical membrane (Johansson and Cox 2011).

Once vRNPs and viral proteins accumulate at the apical cell membrane, viral packaging and budding occur (Barman, Ali et al. 2001). All three viral surface proteins associate with M1 and once this protein begins to accumulate, it allows protrusion from the host membrane, thus initiating budding of the virion. After budding, it is released through the cleavage of host sialic acid, by the NA protein (Air and Laver 1989).

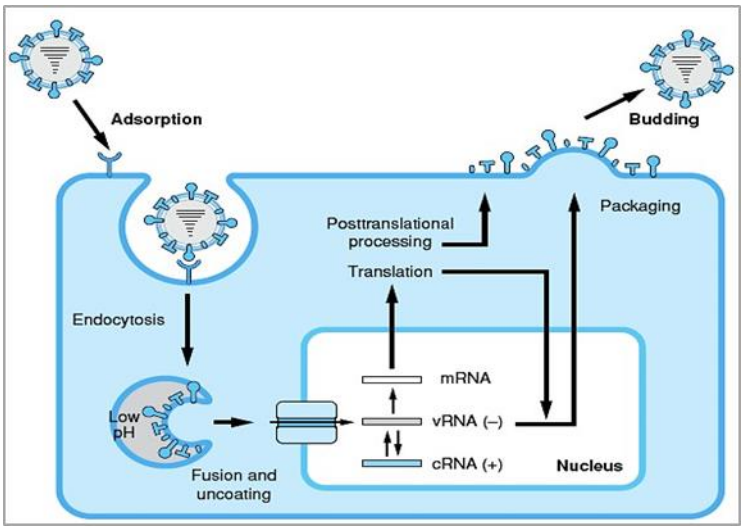

Figure 1.3. Influenza virus replication cycle. (Adapted from Fields Virology; 2007).

1.2.6 Laboratory Diagnosis of Influenza

Diagnosis of influenza is important in order to prescribe the correct antivirals, for immunoprophylaxis, and for epidemiological reasons. Successful diagnosis depends on the timing and quality of specimens collected. Clinical specimens should be taken early, preferably within 3 days of onset of symptoms since this is the time maximum virus shedding occurs. Nasopharyngeal or throat swabs combined or alone, nasopharyngeal aspirates and nasal washes are optimal specimens for virus culture and for the direct detection of viral antigens or nucleic acids. Bronchoalveolar lavage fluid and trancheal aspirate specimens may be considered if clinically warranted during serious respiratory infections.

The specimens must be placed in a suitable transport medium to stabilize the infectivity of the influenza viruses. Virus infectivity is relatively well preserved if the specimens are stored at 40C in these liquids for up to 4 days. After collection, specimens should be transported to the laboratory without delay and preferably refrigerated at -700C at all times until processing for virus isolation or nucleic acid detection (Cox 2003,). Multiple freeze-thawing of samples should be avoided since they greatly reduce the virus yield.

Although many cells have been shown to support the replication of influenza viruses, primary monkey kidney cells (PMK), and continuous cell lines such as Madin Darby Canine Kidney

(MDCK) and Rhesus monkey kidney (LLCMK2) cell lines are the most widely used. Of these, MDCK are the most commonly used (Bhatia 1999). MDCK support the growth of influenza A, B, and C viruses when 1 to 2 µg of trypsin per ml is added to the maintenance medium to sustain multiple cycles of multiplication (Meguro, Bryant et al. 1979). Appropriate treatment of patients with respiratory illness depends on accurate and timely diagnosis. The accuracy of clinical diagnosis of influenza on the basis of symptoms alone is limited because symptoms from illness caused by other pathogens can overlap considerably with influenza (Nicholson 1992, Boivin, Hardy et al. 2000, Monto, Gravenstein et al. 2000, Hardy, Eichelberger et al. 2011). Laboratory diagnosis include: virus isolation, antigen detection, nucleic acid detection and serology. Influenza virus can be readily isolated from a variety of respiratory specimens, including sputum, nose and throat swabs, and nasal aspirate or washes, early in illness.

Direct detection of influenza virus antigens in respiratory secretions is possible within hours and has been accomplished by immunofluorescence (IF), enzyme immunoassays (EIAs), radioimmunoassay and time-resolved fluoro-immunoassay. Reverse transcription-PCR (RT-PCR) is used in detecting influenza A and B virus RNAs in clinical samples.

Molecular methods are now being applied more widely in the diagnosis of influenza virus infection as well as characterization of influenza virus isolates. These methods are likely to supplant virus culture as the "gold standard" for virus detection because they are more sensitive and fast. The genetic analysis of a large number of influenza viruses isolated throughout the world each year provides information for a timely update of vaccine strains and of molecular reagents for diagnosis.

1.2.7 Epidemiology of Influenza

1.2.7.1 Global Scenario

In tropical areas, influenza occurs throughout the year. In the Northern Hemisphere, the influenza season typically starts in early fall, peaks in mid-February, and ends in the late spring of the following year. The duration and severity of influenza epidemics vary, however, depending on the virus subtype involved.

The US Centers for Disease Control and Prevention (CDC) estimates that flu-associated deaths in the US ranged from about 3000 to 49,000 annually between 1976 and 2006. The CDC notes that the often-cited figure of 36,000 annual flu-related deaths was derived from years when the predominant virus subtype was H3N2, which tends to be more lethal than H1N1.]In contrast to typical influenza seasons, the 2009-2010 influenza season was affected by the H1N1 ("swine flu") influenza epidemic, the first wave of which hit the United States in the spring of 2009, followed by a second, larger wave in the fall and winter; activity peaked in October and then quickly declined to below baseline levels by January, but small numbers of cases were reported through the spring and summer of 2010 (WHO 2010).

In addition, the effect of H1N1 influenza across the lifespan differed from that of typical influenza. Disease was more severe among people younger than 65 years than in non-pandemic influenza seasons, with significantly higher pediatric mortality and higher rates of hospitalizations in children and young adults. Of the 477 reported H1N1-associated deaths from April to August 2009, 36 were in children younger than 18 years; 67% of those children had 1 or more high-risk medical conditions (WHO 2009).

H3N2 is currently endemic in both human and pig populations. It evolved from H2N2 by antigenic shift and caused the Hong Kong Flu pandemic of 1968 and 1969 that killed up to 750,000. An early-onset, severe form of influenza A H3N2 made headlines when it claimed the lives of several children in the United States in late 2003 (Press (2005).).

The dominant strain of annual flu in January 2006 is H3N2. Measured resistance to the standard antiviral drugs amantadine and rimantadine in H3N2 has increased from 1% in 1994 to 12% in 2003 to 91% in 2005. Contemporary human H3N2 influenza viruses are now endemic in pigs in southern China and can reassort with avian H5N1 viruses in this intermediate host (WHO 2009).

As of June 2013, 630 cases of avian influenza H1N1 had been reported by the World Health Organization (WHO) worldwide, with 375 deaths. Currently, reporting from areas with poor access to health care may be limited to clinically severe cases; illness that does not fulfill WHO

diagnostic criteria is not reported. Most cases have been in eastern Asia; some cases have been reported in Eastern Europe and North Africa. Underreporting has been a concern, particularly in China, but the prevailing attitude about the need to suspect, test, and report cases of avian influenza is growing. There were cases reported in Cambodia, Vietnam, China, Egypt, and Bangladesh (WHO 2010).

Influenza B viruses cause frequent epidemics worldwide, but with no established pattern. In some year's influenza B viruses are the predominant influenza viruses isolated worldwide, and in others they are virtually absent from the human population. Both Yamagata & Victoria lineages have been observed to circulate simultaneously or individually in particular time periods and areas (McCullers, Saito et al. 2004). For example, in the United States, Vic87-lineage influenza B viruses were predominant during the 1980s, but during which time, these viruses were isolated only from sporadic outbreaks in Europe and Asia (Rota, Wallis et al. 1990). During the 1990s, Yam88-lineage influenza B viruses replaced Vic87-lineage viruses and became the predominant strains in the United States. However, both Vic87- and Yam88-lineage influenza B viruses were still detected in eastern Asia during this period. During the 2000–2001 seasons, Vic87 viruses re-emerged and spread worldwide, and both Vic87 and Yam88 viruses co-circulated in many countries (Shaw, Xu et al. 2002). Thus, the epidemics of influenza B viruses fluctuated periodically and geographically.

1.2.7.2 Bangladesh Scenario

Influenza is increasingly recognized as a cause of severe respiratory disease among healthy children in industrialized countries (Thompson, Shay et al. 2004, Azziz-Baumgartner, Smith et al. 2009). However, little information is available from developing countries like Bangladesh (Zaman, Alamgir et al. 2009). Scientists predict that the next influenza pandemic will evolve from Asia. However, it is impossible to predict how, when, and from which influenza virus the next pandemic will evolve. Bangladesh is the eighth most populous country in the world and, after only a few microstates and small island nations, has the highest population density. It also ranks second of any country for highest intrinsic risk for emergence of an avian influenza pandemic based on an index that considers risk of emergence, risk of spread, and capacity to contain an outbreak. According to health authorities, the 2009 pandemic influenza A (H1N1) virus caused

approximately 6000 deaths in Bangladesh and cost Dhaka 6.1 million United States dollars (US$) in direct medical costs to the patients (Homaira, Luby et al. 2012).

Infrequent hand washing and poor respiratory hygiene (e.g. covering mouth when coughing), limited access to care, lack of awareness of antiviral treatment and its availability, a huge shortage of influenza vaccines early in the pandemic, and a high prevalence of malnutrition may have worsened the pandemic in low-income countries such as Bangladesh. In general, the rate of hospitalization associated with seasonal and pandemic influenza in low-income countries remains largely unknown.

In a population-based study among children aged less than 5 years in urban Dhaka, the incidence of laboratory-confirmed influenza infection during 2004–2007 was estimated at 10.2 per 100 persons–years among children aged less than 5 years seeking care at ambulatory clinics. Of 3,198 nasopharyngeal wash collected from febrile or respiratory illnesses, the number of confirmed infections with influenza A and B virus were 333 and 246, respectively. The surveillances suggested that influenza season occurred during April through September (Homaira, et al. 2011). This surveillance system also identified the one human case of infection with influenza A(H5N1) in Dhaka. Surveillance date suggest the peak influenza season in human is different from the H5N1 influenza season in poultry usually in winter (Zaman, Alamgir et al. 2009).

At the beginning of April 2007, the Government of Bangladesh and icddr,b initiated national hospital-based influenza surveillance with a network of 12 surveillance hospitals throughout the country to provide nationally-representative data from all age groups. It was found Influenza prevail from 10% to 20% from July 2012 to August 2013 season with exception of October and November 2012 where influenza positive case was negative (IEDCR 2013).

While both influenza A and B while both influenza A and B virus infections were equally present among ILI cases, influenza A/H3 was predominant among SARI cases. Both strains of seasonal influenza A and influenza B were detected, while none of the cases from inpatients or outpatients were influenza A/H5 or novel strains or unsub-typeable. In Chittagong, in the south-east part of Bangladesh, influenza A was more common, whereas in Sylhet, in the northeast part,

influenza B was more common. Kriging analyses of the 2008 influenza season suggest that surveillance samples tested positive during May (month 5) in Chittagong (i.e. a large maritime port city) and Dhaka (i.e. the capital and largest city), before gradually spreading to the other parts of Bangladesh (Zaman, Alamgir et al. 2009) (Figure 1.4).

There was wide variation in the pattern of resistance to anti-viral drugs among 61 randomly selected specimens sent to CDC for antiviral resistance testing. The influenza A/(H1N1) and influenza A/ (H3N2) viruses circulated in 2007 were sensitive to the neuraminidase inhibitors oseltamivir and zanamivir, while most were resistant to the adamantane group of drugs, amantadine and rimantadine. In 2008, all the influenza A/(H1N1) viruses were resistant to oseltamivir and all the A/(H3N2) viruses were resistant to adamantanes (Zaman, Alamgir et al. 2009).

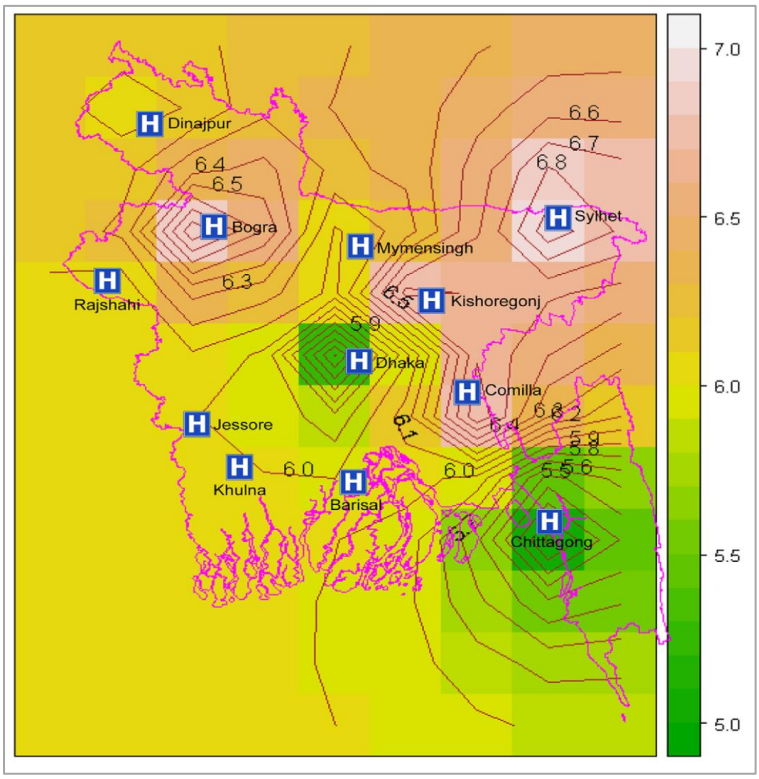

Figure 1.4. Kriging prediction of month of onset of influenza season in different parts of Bangladesh

1.2.8 Transmission and Pathogenesis

Transmission of influenza in humans probably involves both respiratory infection by aerosols and droplets together with some contact transmission from contaminated surfaces. Unfortunately, there are insufficient solid data to determine which of these is the more important and to provide guidance in protection of front-line medical staff, particularly regarding the required porosity of facemasks, during a pandemic. Nevertheless, influenza virus is relatively short-lived on most surfaces, and clear examples of aerosol transmission have been described in the literature. In addition, early experiments indicated greater infectivity by small particle aerosol than by nasal instillation of virus.

For many years, it was thought that for the efficient introduction of "novel" influenza viruses to humans, pigs were required to act as an intermediate host, or "mixing vessel". Since pigs are susceptible to both human and avian influenza viruses, the genomes of such viruses could reassort and/or adapt to this mammalian host, giving rise to new viruses with altered phenotypes that could more easily be transmitted to humans. Indeed, the pandemics of 1957 and 1968 were both caused by reassortant influenza viruses that may have been generated in pigs (Fouchier, Osterhaus et al. 2003). Moreover, several incidents of transmission of influenza viruses from pigs to humans have been described in the past decades. However, since 1996, the viruses H7N7, H5N1 and H9N2 have been transmitted directly from birds to humans but have apparently failed to spread from human to human in the human population. Such incidents are rare but demonstrate the potential of avian viruses to be transmitted directly to humans. It cannot be excluded that upon dual infection of humans with avian and human influenza A viruses, man himself may act as a "mixing vessel" for the generation of novel human-avian reassortant viruses.

After transmission to pigs, horses, humans or birds, the method of spread of influenza within the population is mainly inhalation of respiratory droplets containing the virus. Less frequently, the virus is spread by person to person contact or contact with contaminated items. Pathogenicity of influenza viruses is multifaceted and may involve viral, host and environmental factors. Inhaled virus is deposited on the mucous membranes lining the respiratory tract or directly to the alveoli, the level depending on the size of the droplets inhaled. In the former state, it is exposed to mucoproteins containing sialic acid that can bind to the virus, thus blocking its attachment to the

respiratory tract epithelial cells (Metselaar 1982). However, the action of neuraminidase allows the virus to break this bond. Specific local secretory IgA antibodies, if present from a previous infection, may neutralize the virus before attachment occurs, provided the antibody corresponds to the infecting virus type. If not prevented by one of these mechanisms, the virus attaches to the surface of respiratory epithelial cells and the intracellular replication cycle is initiated.

The major site of infection is the ciliated columnar epithelial cell. The first alteration is the disappearance of the elongated form of these cells which becomes round and swollen, the nucleus shrinks, becomes pyknotic and fragments. Vacuolization of the cytoplasm may occur. As the nucleus disintegrates, the cytoplasm shows inclusion bodies and the cilia are lost. Release of virus from the cell allows it to spread via the mucus blanket to other areas of the respiratory tract. The cell damage initiates an acute inflammatory response with oedema and the attraction of phagocytic cells. The earliest response is the synthesis and release of interferons from the infected cells; these can diffuse to and protect both adjacent and more distant cells before the virus arrives. It appears that interferons released in this way cause many of the systemic features of the "flu like" syndrome that characterize the infection.

While viral components are absorbed and trigger the immune system, the virus itself is confined to the epithelium of the respiratory tract. Specific antibody will help to limit the extra cellular spread of the virus, while T cell responses are directed against the viral glycoproteins on the surface of infected cells leading to their destruction by cytotoxic T cells and also by antibody dependent cell cytotoxicity (Greenwood 2003).

1.2.9 Antigenic Variation

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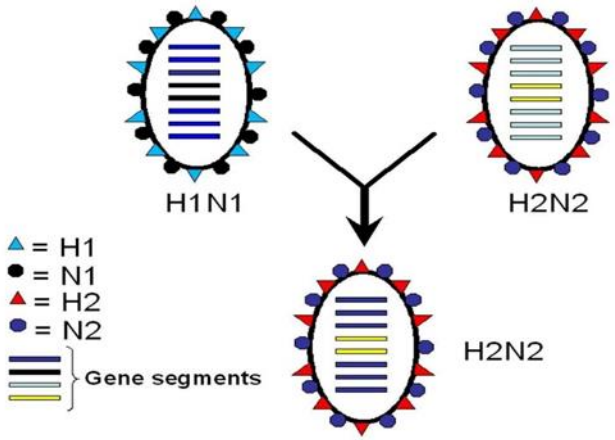


Figure 1.5. Mechanism of reassortment in influenza A viruses

1.2.10 Antivirals Used in Treatment and Control of Influenza

WHO estimates that seasonal influenza epidemics result in three to five million cases of severe illness and 250 000 to 500 000 deaths each year in the industrialized world alone (Stohr 2003). Although vaccination remains the most important measure for reducing this sizeable public health burden, antiviral drugs have been welcomed as long awaited tools for treatment and prevention. Antiviral drugs are effective in treatment as well as prophylaxis, but in prophylaxis they must be administered daily throughout the period of risk of exposure. For this reason, they may be the only intervention available during the early months of a pandemic when supplies of vaccines maybe severely limited (Monto 2003). Currently, two classes of influenza antivirals are available to manage influenza. Each class inhibits a different step in the viral replication cycle. Type A but not type B influenza viruses contain an M2 protein. This protein is responsible for uncoating the viral nucleoprotein during replication. It functions as an ion channel, preventing exposure of the viral hemagglutinin to low intracellular pH to which it is sensitive. Amantadine and rimantadine inhibit this activity and they are termed 'M2 inhibitors'. These drugs have no effect on type B influenza viruses because influenza B viruses do not possess an M2 protein (Monto 2003).

The viral neuraminidase of both type A and B viruses facilitates the release of virus from the infected cell after replication is complete and prevents the viral clumping before the next infectious cycle begins. Zanamivir (Relenza) and Oseltamivir (Tamiflu) are the “old” neuraminidase inhibitors (Nis) that have been widely used and continue to be used in influenza treatment (Barik 2012). They have been specifically designed to interrupt the replication cycle by preventing virus release and allowing virus to clump. Zanamivir is administered by inhalation, while oseltamivir is taken orally. Both classes of influenza antivirals are approximately 70-90% efficacious when used as prophylaxis (Monto 2003). Tamiflu has to be given within 48 hours of onset of clinical signs for maximum effect and is less effective if given thereafter. Recently, three new neuraminidase inhibitors have been developed and are in various stages of development. These include Laninamivir which has been approved for use in Japan, Favipiravir and Peramivir. Both Favipiravir and Peramivir are in various stages of clinical trials (Barik 2012).

1.2.10.1 Resistance to Antiviral Drugs

From the licensure of the neuraminidase inhibitors oseltamivir and zanamivir in 1999 until recently, resistance to these agents had remained at a low level, even in the countries responsible for most of their use worldwide (Monto 2006). Therapeutic use of anti-influenza drugs is frequently associated with emergence of drug resistant variants. Resistance to M2 inhibitors is generally conferred by emergence of single amino acid substitutions in the transmembrane domain of the M2 protein at positions 26, 27, 30 or 31 (Hay 1996). Common mutations associated with resistance include V27G, A/I27S/T, S31N and A30T. Resistance to neuraminidase inhibitors typically results from single amino acid changes in the NA protein active enzyme site that alters drug binding. Resistance to Oseltamivir is conferred by I117V, E119V, D198N, I222V, H274Y, R292K, N294S and I314V (N2 numbering) mutations. Double mutations with synergistic oseltamivir resistance phenotype have been noted as well. This includes the E119V+I222V double mutant, isolated from an immunocompromised child infected with H3N2 virus, and H247Y+I222V, from patients infected with influenza A(H1N1)pdm09 virus (Barik 2012). Mutations related to zanamivir resistance include, V116A, R118K, E119G/A/D, Q136K, D151E, R152K, R224K, E276D, R292K and R371K (N2 numbering).

Initial testing of the influenza A(H1N1)pdm09 virus found it susceptible to neuraminidase inhibitors (CDC 2009). However, sporadic cases of resistance to the neuraminidase inhibitors are increasingly being reported some with novel mutations besides the common H275Y mutation (Hurt, Holien et al. 2009, CDC 2009) (CDC, 2009b; Hurt et al., 2009). The pandemic virus contains the M2 inhibitors resistance-conferring change S31N in the M2 protein and is thus resistant to the M2 inhibitor drugs (Garten, Davis et al. 2009, Dawood, Iuliano et al. 2012).

1.2.11 Immunology

Influenza causes an acute infection of the host and initiates a cascade of immune reactions activating almost all parts of the immune defense system. Most of the initial innate response, including cytokine release (IFNα/β), influx of neutrophil granulocytes or natural killer cells (Mandelboim, Lieberman et al. 2001, Achdout, Arnon et al. 2003), and cell activation, is responsible for the acute onset of the clinical symptoms. Innate immunity is an essential prerequisite for the adaptive immune response, firstly, to limit the initial viral replication and

antigen load, and secondly, because the antigen-specific lymphocytes of the adaptive immune response are activated by co-stimulatory molecules that are induced on cells of the innate immune system during their interaction with viruses (Figure 1.6).

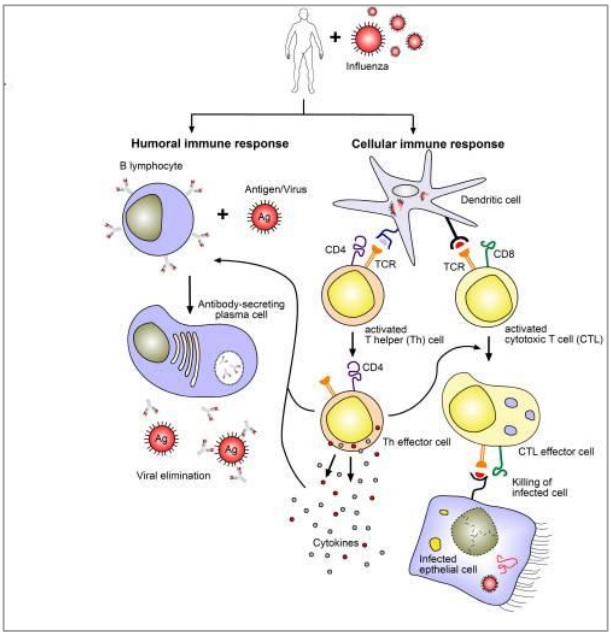


Figure 1.6. The humoral and cell-mediated immune response to influenza virus infection. (Adapted from Flint 2004).

The humoral branch of the immune system comprises B-lymphocytes which after interaction with influenza differentiate into antibody-secreting plasma cells. The cellular response starts with antigen presentation via MHC I (black) and II (blue) molecules by dendritic cells, which then leads to activation, proliferation and differentiation of antigen-specific T cells (CD4 or CD8). These cells gain effector cell function to either help directly, release cytokines, or mediate cytotoxicity following recognition of antigen.

Influenza viruses, however, encode in the non-structural protein 1 (NS1) mechanisms to evade and antagonize the IFN α/β response. NS1 is likely to sequester viral dsRNA which prevents recognition of this dangerous molecule by cellular sensors which would otherwise trigger IFN α/β release (Garcia-Sastre, Egorov et al. 1998, Garcia-Robles, Akarsu et al. 2005).

The adaptive immune response requires some days to be effective but then helps to contain the viral spread, to eradicate the virus, and finally to establish a memory response resulting in a long-lived resistance to re-infection with homologous virus. Cross-protection within a subtype of influenza has only rarely been observed and infections essentially induce no protection across subtypes or between types A and B (Treanor JJ. In: Mandell GL 2004:). Influenza infection induces both systemic and local antibody (humoral immunity), as well as cytotoxic T cell responses (cellular immunity), each of which is important in recovery from acute infection and resistance to reinfection.

1.2.11.1 Prevention and Prophylaxis of Influenza

The burden of annual influenza infection is substantial, both in terms of illness, lives lost and economic impact on society (Yewdell and Garcia-Sastre 2002). Additionally, we are eventually facing a new pandemic that could cause unprecedented levels of morbidity and mortality, both in the developed countries, but even more so in the developing countries. Particularly in a pandemic scenario, but also for the yearly influenza outbreaks, improving immunity to current influenza vaccines and developing new antiviral drugs are of vital importance. Continued and focused research efforts are needed in order to understand the immunology, epidemiology, ecology and the etiology of influenza viruses. Despite many years of studies, we still lack some basic knowledge about influenza and the infection it causes, and the subsequent immune response.

1.2.12 Current Vaccine Strategies

Although there has been reasonable success with anti-influenza treatment, vaccine development against influenza is the primary means of prophylactic therapy and preventing disease spread. To this end several different vaccine strategies have been employed, with the most commonly used strategy being injection of inactivated virus to provide immunity against potential infection. Additionally, live attenuated influenza vaccine (LAIV) has been used in various countries as well as alternative strategies such as DNA or peptide vaccinations which are currently used in experimental approaches and clinical trials.

1. 2.12.1 Inactivated Vaccines

The most commonly used vaccine strategies incorporate inactivated strains of influenza which, when exposed to the immune system, provide both antibody and cytotoxic T-cell mediated responses. Currently these types of vaccines typically combine three different strains, one from H1N1, H3N2 and B, and thus are referred to as trivalent inactivated vaccines (or TIV) (Hardy, Eichelberger et al. 2011). Generally, TIV are primarily aimed towards inducing immune protection against HA and NA, however, the antigenic nature of these proteins necessitates annual reformulation of the TIV (Poland, Rottinghaus et al. 2001). This is completed by utilizing strain surveillance programs which identify upcoming antigenic variants using the serum hemagglutinin-inhibition (HI) assay (Carrat and Flahault 2007). This strategy can predict the circulating strains up to 9-12 months in advance, thus allowing for the development of new vaccines before the predicted strains can infect a population. Inactivated vaccines are produced by first growing the selected strain in embryonated hens' eggs and then treating virus harvested in the allantoic fluid with either formalin or β propiolactone (BPL). This treatment directly inactivates the virus, but allows for the preservation of viral proteins, which when exposed to the immune system can induce a protective response to future infection (Goldstein and Tauraso 1970, Poland, Rottinghaus et al. 2001). However, many factors can affect the effectiveness of TIV, including the antigenic match between circulating and vaccine strains, the age of the recipients and their history of influenza exposure (Ambrose, Levin et al. 2011).

Recommended composition of influenza virus vaccines for use in the 2016-2017 northern hemisphere influenza season are A/California/7/2009 (H1N1) pdm09-like virus, A/Hong Kong/4801/2014 (H3N2)-like virus, B/Brisbane/60/2008-like virus.

The vaccine produced for the Southern Hemisphere 2017 season used are A/Michigan/45/2015(H1N1)pdm09-like virus, A/Hong Kong/4801/2014 (H3N2)-like, B/Brisbane/60/2008.

1.2.11.2 Live Attenuated Vaccines

As an alternative to TIV, live attenuated influenza vaccines (LAIV) have been developed by generating reassortant virus containing the HA and NA from epidemic strains, but the remainder

of the genome and proteins from attenuated cold adapted virus (Belshe, Mendelman et al. 1998). These temperature sensitive viruses cannot replicate in the upper and lower respiratory tract, but they still provide immune system exposure the targeted NA and HA proteins. LAIV are delivered by intranasal spray and are typically used for children (over the age of 24 months) and pregnant woman (Ambrose, Levin et al. 2011). LAIV can have several advantages to using a TIV. Primarily, since LAIV are delivered directly to the respiratory in addition to inducing cell-mediated responses, they can also induce mucosal immunity. LAIV has also been shown to be more protective in children when compared to the TIV and LAIV has a better efficacy rate when the predictive strains are unmatched (Ambrose, Levin et al. 2011).

1.2.13. Problems with Existing Vaccines

Vaccination has proven to be the mainstay in prevention of various deadly infectious diseases (Perrie, Kirby et al. 2007, Black, Trent et al. 2010, Bachler, Humbert et al. 2013). Historically, live-attenuated or inactivated forms of microbial pathogens (viruses, bacteria, etc.) have been used for induction of antigen-specific responses that protect the host against subsequent infections. Based on the pathogen being used, such vaccine formulations can contain anywhere between tens of to a few hundred proteins. However, protective immunity is usually dependent upon a few select proteins within such formulations, whereas the majority of proteins are unnecessary for the induction of protective immunity. Furthermore, these additional proteins may induce allergenic and/or reactogenic responses, thus emphasizing the need to eliminate them from vaccine formulations. This rationale led to an interest in subunit vaccines using single, or a select few, proteins of the microbes in vaccine formulations for induction of protective immunity (Petrovsky and Aguilar 2004, Thompson and Staats 2011). An extension of this logic would be that even single proteins contain many hundreds of antigenic epitopes, all of which are not necessary; whereas some may even be detrimental to the induction of protective immunity. This has created an interest in peptide vaccines containing only epitopes capable of inducing positive, desirable T cell and B cell mediated immune response (Sesardic 1993). Peptides‖ used in these vaccines are 20–30 amino acid sequences that are synthesized to form an immunogenic peptide molecule representing the specific epitope of an antigen. On the one hand, since epitopes are the antigenic determinants within larger proteins, these peptides are considered sufficient for activation of the appropriate cellular and humoral responses (Bijker, Melief et al. 2007, Lin, Cheng et al. 2013),

while eliminating allergenic and/or reactogenic responses. Additionally, peptide vaccines can be used for induction of broad-spectrum immunity against multiple serological variants (serovars) or strains of a given pathogen by formulating multiple non-contiguous immunodominant epitopes and/or epitopes conserved between different serovars/strains of a pathogen. On the other hand, owing to the relatively small size of peptides, they are often weakly immunogenic by themselves and therefore require carrier molecules, to add chemical stability and adjuvating, for the induction of a robust immune response (Aguilar and Rodriguez 2007, Purcell, McCluskey et al. 2007). Allergenicity and/or reactogenicity of carrier molecules themselves increase the complexity of peptide vaccine design. Manufacturing of peptide vaccines is generally considered as safe and cost effective when compared to conventional vaccines.

1.2.13.1 Limitations of Current Influenza Vaccines

Despite the annual recommendations for vaccine strain composition by the WHO, antigenic mismatches between the vaccine virus strain and the circulating strain occur that negatively influence vaccine effectiveness (Carrat and Flahault 2007). Between 1997 and 2007, there were five occurrences of mismatch, and 11 occurrences of partial mismatch across the three vaccines strains (Ansaldi, Bacilieri et al. 2008). Factors causing such mismatches include: (1) appearance of drifted viruses after the recommendation has been made; (2) inability to isolate a seed virus in egg for vaccine manufacture, or the absence of a high-growth reassortant seed; (3) co-circulation of different viruses from the same subtype. Furthermore, although influenza illness affects people of all ages, adults over 65 years of age account for approximately 90% of all influenza-related mortality (Thompson, Shay et al. 2004). However, current influenza vaccines are less effective among older adults than younger (Nichol, Nordin et al. 2003, Hannoun, Megas et al. 2004, Jefferson, Rivetti et al. 2005, Jefferson, Smith et al. 2005, Fiore AE 2008) and prevent laboratory-confirmed influenza in only 30-40% of people over 65 years of age (Hannoun, Megas et al. 2004). This is caused by changes that occur in the immune system with advancing age resulting in a reduced immune response and reduced capacity to produce antibodies (Saurwein-Teissl, Lung et al. 2002, Lazuardi, Jenewein et al. 2005).

1.2.14 Strategies to Develop an Alternate Vaccine

A variety of considerations need to be made during the design of a peptide vaccine, in context of the particular vaccine under development. First and foremost, among them is the identification of immuno-dominant domains of epitopes that are capable of inducing protective immune response in terms of humoral immunity and/or cell mediated immunity against desired antigen (Demento, Siefert et al. 2011). Immunodominant epitopes can be chosen in context of B cells, cytotoxic or helper T cells. For example, one of the most suited approaches is preparation of protective monoclonal antibody against the conserved regions, which may be helpful in designing protective as well as therapeutic vaccine against cancers (Burioni, Perotti et al. 2008). For such a vaccine, the selection of immunodominant B cell epitopes is important. On the other hand, vaccines against intracellular pathogens such as viruses or against cancers may focus on the identification of epitopes that induce cytotoxic T cell responses (Brunsvig, Aamdal et al. 2006, Testa and Philip 2012). It is to be noted that for efficient induction of either B-cell or cytotoxic T cell responses, the induction of a robust helper T cell responses is crucial (Lanier, Newman et al. 1999, Testa and Philip 2012). Thus, epitope selection will have to be directed towards not only inducing the required effector response (B cells or cytotoxic T cells), but also for induction of helper T cell responses. Among the epitopes that induce specific subsets of immune responses, the next challenge would be to identify the right epitope(s) or peptide(s) that can activate T cells to the magnitude that can confer protective immunity. Additionally, for development of a broad-spectrum vaccine against multiple serovars of a pathogen, it may be necessary to identify highly conserved immunodominant epitopes. Another issue to be considered is the processing, presentation and association of the candidate peptide vaccine by antigen presenting cells T cells in a highly MHC-heterogenous human population.

Multiple biochemical and cellular immunoassays have been designed and utilized for selection of candidate peptides for vaccines. Various strategies employing either in silico approaches or experimental approaches, or combinations of both have been followed. A variety of bioinformatics tools are used for prediction including, but not limited to, the translocation of peptides into endoplasmic reticulum (MHC-I), cleavage in lysosomal compartments (MHC-II), binding of antigen to MHC I and MHC II, HLA haplotype specificity, and recognition by T cell receptors as in figure 1.7.

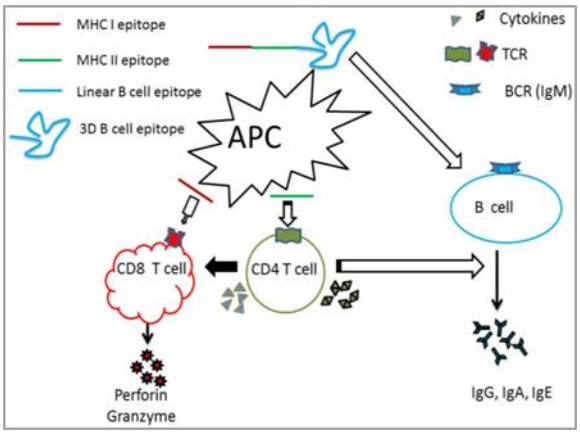


Figure 1.7. Central role of $CD4^+$ T cells in peptide vaccines.

Vaccine-induced immune response to control microbial pathogens may involve cytotoxic $CD8^+$ T cell responses, helper $CD4^+$ T cell responses, or antibody (B cell) responses. T cells recognize linear epitopes presented by antigen presenting cells, whereas B cells are capable of recognizing linear and conformational epitopes on soluble antigens. The induction of robust $CD8^+$ T cell and/or antibody responses requires cytokine help from $CD4^+$ T cells. Therefore, regardless of the nature of protective immune response required, the induction of $CD4^+$ T cell responses is critical. In case of alternate vaccine for influenza, identification of potential epitopes for IG and MHC II binding in HA and NA may be required to engineer them into peptide subunit vaccine.

1.2.15 Problem Statement

About 20% of children and 5% of adults worldwide develop symptomatic influenza A and B each year (2001) (1). The influenza pandemic has severely impacted South Asia and Bangladesh where the case fatality proportion was 0.5% during the first wave of pandemic (H1N1) 2009 (WHO 2009). Limited information is available on influenza infections from slum population in Dhaka which constitute nearly one third of the total population of the city. Bangladesh has experienced one of the highest urban population growth rates (7% per year) mainly due to migration from rural areas. Dhaka, the capital of Bangladesh, has approximately 4,500 slums of highly dense population estimated at 3.4 million with inadequate health and hygiene support systems (Democracywatch 2002, Angeles G 2006). Very high population density, poor environmental conditions and low socioeconomic status are ubiquitous characteristics promoting various types of infectious diseases

including influenza in this population. Moreover, this setting may promote potential evolution events that are likely to result in new pandemic strains (Greninger, Chen et al. 2010).

1.2.16 Significance of the Study

The SARS epidemic in 2002, influenza A H5N1 in 2003, and the current spread of pandemic H1N1 around the world are examples that highlight the need for global engagement on regional disease outbreaks (Soderstrom, Hovelius et al. 1991, Hewson-Bower and Drummond 2001, Black, Morris et al. 2003). In Bangladesh about 25% of all deaths of children aged less than 5 years and about 40% of deaths of infants are associated with pneumonia which is caused by different respiratory pathogens (Nafstad, Hagen et al. 1999, Peat, Keena et al. 2001). Much effort is placed on the diagnosis and treatment of bacterial agents, with very little effort being placed on the detection and management of viral agents. As part of the world influenza pandemic preparedness surveys and surveillance on influenza viruses will be of great public health and economical importance (8-10) (Nichol, Nordin et al. 2003, Nicholson, Wood et al. 2003). However, many questions regarding the containment of this disease remain unanswered and in these regard acquisition of knowledge on the circulatory types of influenza viruses in Bangladesh and their antiviral sensitivities, genomic analysis and antigenicity must be studied.

The finding of this study will provide invaluable information and data towards future guidelines regarding the containment of influenza viruses in Bangladesh. The genomic data will be used to design an efficient candidate peptide vaccine against locally circulating influenza viruses.

1.3 Aims and Objectives

The overall aim is to reduce the burden of influenza viruses using molecular and bioinformatics approaches.

The aim is fulfilled by achieving the following objectives:

1. To detect and characterize influenza viruses in slum areas of Dhaka city.
2. To explore genetic variations between slum strains and vaccine strains as well as globally circulating strains.
3. To assess drug susceptibility of the circulating strains.
4. To predict putative candidate peptide vaccine.

Chapter 2

Methods & Materials

2. Materials & Methods

2.1 Study Site and Design

This study is a prospective, exploratory study carried out in three densely populated slums: Rayerbazar, Hazaribagh, and Mohammadpur in Dhaka located in the southeastern periphery of the city (Figure 2.1). Samples were collected from patients with influenza-like illness (ILI) with at least one of the following symptoms: fever, cough or sore throat and only from patients who have not received antiviral drug treatment in last 7 days. All the collected specimens were tested for the presence of influenza viruses. The molecular characterization of influenza viruses was performed at the Clinical Microbiology Laboratory of Microbiology Department of Dhaka University.

Figure 2.1. Location of the slum sites from where study samples were collected.

2.2 Ethics

Before carrying out the study, a written proposal was submitted for permission to the Institutional Ethics Committee, Dhaka Medical College Ethical Committee (protocol no.# DMC-MEU/ECC/2014/17). Informed written consent was obtained from each patient or guardian (in case of children).

2.3 Sample Collection

Nasal pharyngeal swabs and or throat swabs from patients with influenza-like illness (ILI, defined as sudden onset of subjective fever, cough or sore throat). For the collection of samples, a dry cotton tipped swab was inserted into the nostril parallel to the palate and left in place for a few

seconds before being slowly withdrawn using a rotating motion. The tip of the swab was placed into a collection vial containing 2 ml viral transport medium and immediately transported to Clinical Microbiology Laboratory through maintaining the appropriate cold chain (4°C). As these samples were not processed within 48 hours, they were kept frozen at or below -80°C.

The following data were collected from the patients by a physician in a case-recorded form.
- Demographic data (age, gender)
- Clinical features and laboratory data
- Clinical diagnosis

2.4 Preparation of Specimens in the Laboratory

In the laboratory, 2ml VTM was mixed well and divided into three sterile cryogenic tubes, the first tube of 1 ml was used for RNA extraction by Qiagen viral RNA mini kit for RT-PCR, and remaining 2.5 ml tube was stored at minus -80^0 C for future use.

2.5 RT-PCR for Detection of Influenza Viruses

2.5.1 Viral Nucleic Acid Extraction

Viral nucleic acid was extracted from the collected samples using the QIAamp® Viral RNA extraction kit (Qiagen, Germany) following the manufacturer's protocol.

2.5.1.1 Reagent Preparation

- Addition of carrier RNA to Buffer AVL: 310 µl Buffer AVE was added to the tube containing 310 µg lyophilized carrier RNA to obtain a solution of 1 µg/µl. Carrier RNA–Buffer AVE than added to Buffer AVL and gently mixed by inverting the tube 10 times.
- Buffer AW1: Before using for the first time, 25 ml of ethanol (96–100%) was added as indicated on the bottle.
- Buffer AW2: Before using for the first time, 30 ml of ethanol (96–100%) was added as indicated on the bottle.

2.5.1.2 Extraction Procedure

i. 560 µl of prepared Buffer AVL containing carrier RNA was added into a 1.5 ml microcentrifuge tube.

ii. 140 µl blood sample was added to the tube and was mixed by pulse-vortexing for 15 seconds. The tube was incubated at room temperature for 10 min.

iii. Than tube was briefly centrifuged at 5000 rpm for 1 min to remove drops from the inside of the lid.

iv. 560 µl of ethanol (96–100%) was added to the sample and mixed by pulse-vortexing for 15 seconds followed by a brief centrifugation. After mixing, tube was briefly centrifuged to remove drops from inside the lid.

v. 630 µl of the solution from step 5 was transferred to the QIAamp Mini column (in a 2 ml 2-ml collection tube) without wetting the rim. Column was centrifuged at 8000 rpm for 1 min after closing the cap. Than QIAamp Mini column was placed into a clean 2 ml collection tube and the tube containing the filtrate was discarded.

vi. QIAamp Mini column was opened and remaining solution (630 µl) was added than repeated step 6.

vii. Again, QIAamp Mini column was opened and 500 µl of Buffer AW1 was added. Tube was centrifuged at 8000 rpm for 1 min after closing the cap. After centrifugation QIAamp Mini column was transferred in a clean 2 ml collection tube, and the tube containing the filtrate was discarded.

viii. QIAamp Mini column was opened and 500 µl of Buffer AW2 was added. After closing the cap tube was centrifuged at full speed 14,000 rpm for 3 min.

ix. Than QIAamp Mini column was transferred in a clean 1.5 ml microcentrifuge tube and the old collection tube containing the filtrate was discarded. 60 µl of Buffer AVE equilibrated to room temperature was added to the column and incubated at room temperature for 1 min after closing the cap. Tube was than centrifuged at 6000 x g (8000 rpm) for 1 min.

x. Extracted Viral RNA was stored at –20°C until further processing.

2.5.2 Real Time RT-PCR

2.5.2.1 Detection of Influenza Viruses

In order to identify influenza viruses, two RT-PCR protocol was performed using the AgPath-ID one-step RT-PCR kit (Applied Biosystems, Foster City, CA, USA). The first protocol was for amplification of matrix (M) gene of Influenza A and Influenza B viruses; the second protocol was for subtyping influenza A pandemic H1N1 (2009), seasonal H1N1 and H3N2. Virus positive controls were run in parallel in each batch. RNP was added in each PCR batch to test the RNA extraction procedure. Primers and probes used in rRT-PCR is shown in Table 2.1.

Table 2.1. Primer and probes used to detect Influenza viruses in this study

Primer	Sequence (5' - 3')
Flu A Forward	GACCRATCCTGTCACCTCTGAC
Flu A Reverse	GGGCATTYTGGACAAAKCGTCTACG
Flu A Probe	FAM-TGCAGTCCTCGCTCACTGGGCACG-BHQ1
Flu B Forward	TCCTCAACTCACTCTTCGAGCG
Flu B Reverse	CGGTGCTCTTGACCAAATTGG
Flu B Probe	FAM-CCAATTCGAGCAGCTGAAACTGCGGTG-BHQ1
RNP Forward	AGATTTGGACCTGCGAGCG
RNP Reverse	GAGCGGCTGTCTCCACAAGT
RNP Probe	FAM-TTCTGACCTGAAGGCTCTGCG CG-BHQ1

The components of the AgPath one step RT-PCR kit (dH_2O, 2X Buffer, detection enhancer and 25X enzyme) were mixed with the respective primers to make up a volume of 20 µl for each reaction tube. The 25 µl reaction volume for each sample contained 5 µl of extracted RNA, 12.5 µl of AgPath Kit 2X buffer, 1 µl of AgPath 25X enzyme mix, 5 pmol of Taqman probe, 10 pmol of each of the forward and reverse primers, and 6 µl of RNase-free water. PCR was achieved at 50°C for 30 min for reverse transcription, 95°C for 15 min for enzyme deactivation, 45 cycles of 94°C for 15s and 55°C for 30s. Cycle threshold ≤38 was interpreted as positive.

2.5.2.2 Subtyping of Influenza A Viruses

In order to determine the subtype, the identified Influenza viruses, subtype specific (H1N1pdm, seasonal H1N1) rRT-PCR was performed following the same protocol described in section 2.5.2.1. Primer and probes used for subtype specific RT-PCR are listed in Table 2.2.

Table 2.2. Primer and probes used to subtype Influenza viruses in this study

Primer	Sequence (5' - 3')
A/H1N1pdm 09-HA-F	AGCAAAAGCAGGGGAAAATAAAAGC
A/H1N1pdm09-HA-R	CCTACTGCTGTGAACTGTGTATTC
A/H1N1pdm09 Probe	FAM-CAGAATATACATCCRTCACAATTGGARAA-BHQ1
A/H1-HA Forward	AAGCAGGGGATAATTCTATTAACC
A/H1-HA Reverse	GTCTATCATTCCCTCCCAACCATT
A/H1-HA Probe	FAMTGAYCCAAAGCCTCTACTCAGTGCGAAAGCBHQ1
A/H3-HA Forward	AAGCATTCCYAATGCAAACC
A/H3-HA Reverse	ATTGCRCCRAATATGCCTCTAGT
A/H3-HA Probe	FAM-CAGGATCACATATGGGSCCTGTCCCAG-BHQ1

2.5.3 Characterization of Influenza Viruses in MDCK Cell Line

2.5.3.1 Isolation of Influenza Virus on Cell Culture

Selected samples that were Influenza panH1N1, Influenza seasonal H1N1, and Influenza B virus positive by RT-PCR were inoculated in Madin-Darby canine kidney (MDCK) cell line. Briefly, 200 µl of sample was added onto 70-90% confluent MDCK cells in flat sided tubes Then the cells were overlaid with 4 ml of Gibco® Dulbecco's Modified Eagles Medium (Invitrogen Corporation, NY, USA). Then inoculated cells were incubated at 33°C with 5% CO_2 and observed daily for 10 days for visual cytopathic effect (CPE) using an Olympus CKP inverted microscope (Olympus Corporation, Tokyo, Japan). When CPE was observed, the supernatant fluid was harvested in 350 µl glycerol (to a final concentration of 0.5%). Harvested cells were further used for antigenic characterization (Hemagglutination assay) and for viral RNA extraction for whole genome amplification.

2.5.3.2 Antigenic Characterization (Hemagglutination Inhibition Assay)

The hemagglutinin (HA) protein of influenza virus's agglutinates erythrocytes. Specific attachment of antibody to the antigenic sites on the HA molecule interferes with the binding between the viral HA and receptors on the erythrocytes. This effect inhibits hemagglutination and is the basis for the hemagglutination inhibition (HAI) test. In general, a standardized quantity of HA antigen is mixed with serially diluted antisera, and red blood cells are added to determine specific binding of antibody to the HA molecule.

Procedure

i. Before performing HAI, HA test was performed to ensure the HA antigen titer is high enough.
ii. 50 µl of PBS (pH 7.2) was added to wells 1B to H12 on a V-bottomed 96-well microtiter plate.
iii. 100 µl of each control antigen or field isolate was added to wells 1-10 on row A.
iv. 100 ul of the positive and negative control was added to wells A11 and A12 respectively.
v. Two-fold dilutions of each sample was performed, by transferring 50 µl from row A to B and 50 µl from row B to C and so forth. The final 50 µl was discarded.
vi. 50 µl of 0.5% RBC suspension was added to each well on the plate and mixed by using a mechanical vibrator or by manually agitating the plates.
vii. Plates were incubated at room temperature (22 to 25°C) for 30 min.
viii. Plate reading was taken on a plate reader.

2.5.3.3 Interpretation

The absence of hemagglutination will appear as a compact button or "halo". Hemagglutination is observed when the RBCs are still in suspension. The highest dilution of virus that causes complete HA is considered the HA titration end point. The HA titer is the reciprocal of the dilution of virus in the last well with complete hemagglutination. For example, if the last dilution showing complete agglutination is 1:160, then the HA titer is the reciprocal of the dilution which is 160.

2.6 Whole Genome Amplification of H1N1pdm and H3N2

2.6.1 Conventional RT-PCR

In this study, selected Influenza panH1N1 and H3N2 positive samples were subjected for whole genome amplification. Viral RNA was extracted from the harvested culture (described in section 2.5.1). All 8 gene segments were amplified and sequenced to study their molecular characteristics. rtPCR was performed following the same protocol described in section 2.5.2.1. All the gene specific respective primers to amplify the whole genome are listed in Table 2.3 and 2.4.

Table 2.3. Influenza A/panH1N1 primers used for whole genome amplification

Primer	Sequence (5' - 3')
H1pdm_PB2_F	CTCGAGCAAAAGCAGGTCAA
H1pdm_PB2_R	AGTAGAAACAAGGTCGTTTTTAAAC
H1pdm_PB1_F	AGCAAAAGCAGGCAAACCAT
H1pdm_PB1_R	AGTAGAAACAAGGCATTT
H1pdm_PA_F	AGCAAAAGCAGGTACTGAT
H1pdm_PA_R	ACCAGTAGAAACAAGGTACCTTTT
H1pdm_HA_F	ATACGACTAGCAAAAGCAGGGG
H1pdm_HA_R	ACCGTGTCAGTAGAAACAAGGGTGTTT
H1pdm_NP_F	CAGGGTAGATAATCACTCAC
H1pdm_NP_R	ACCAGTAGAAACAAGGGTATTTTTC
N1pdm_NA_F	AGCAAAAGCAGGAGT
N1pdm_NA_R	ACCCTATGACCAGTAGAAACAAGGAGTTT
H1pdm_M_F	AGCAAAAGCAGGTAG
H1pdm_M_R	ACCAGTAGMAACAAGGTAGT
H1pdm_NS_F	AGCAAAAGCAGGGTGACAAAGACA
H1pdm_NS_R	ACCAGTAGAAACAAGGGTGTTTTTTAT

Table 2.4. Influenza A/H3N2 primers used for whole genome amplification

Primer	Sequence (5' - 3')
H3_PB2_F	CTCGAGCAAAAGCAGGTCAA
H3_PB2_R	AGAAACAAGGTCGTTTTTAAA C
H3_PB1_F	AGCAAAAGCAGGCAAACCAT
H3_PB1_R	AGAAACAAGGCATTT
H3_PA_F	AGCAAAAGCAGGTACTGAT
H3_PA_R	AGAAACAAGGTACCTTTT
H3HA_F	AAAGCAGGGGATAATTCTA

H3HA_R	AGAAACAAGGGTGTTTT
H3_NP_F	CAGGGTAGATAATCACTCAC
H3_NP_R	AGAAACAAGGGTATTTTTC
H3_NA_F	AGCAAAAGCAGGAGT
H3_NA_R	AGAAACAAGGAG
H3_M_F	AGCAAAAGCAGGTAG
H3_M_R	AGMAACAAGGTAGT
H3_NS_F	AGCAAAAGCAGGGTGACAAAGACA
H3_NS_R	AGAAACAAGGGTGTTTTTTAT

2.6.2 Agarose Gel Electrophoresis and Visualization of the Amplicons

A concentration of 1.5% agarose was prepared in 1x TBE buffer. The solution was mixed by swirling gently and then heating in a microwave until all the agarose was completely dissolved. The gel was then left to cool for a few min at room temperature and ethidium bromide was added to a final concentration of 0.5µg/ml. The gel was then poured onto an electrophoretic tank containing combs and left to set for 30 min at room temperature. The combs were then carefully removed. 3µl of the PCR samples were mixed with the 2µl of the blue orange gel loading dye (Invitrogen, NY, USA) and then loaded onto the wells. A 100bp DNA ladder marker (Invitrogen, NY, USA) was loaded on the first lane of each of the wells. The tank was then connected onto a PowerPac Universal Power Supply (Bio-Rad, CA, USA) and run at 120 volts for about 30-45 min. The gel was then visualized and the gel photo photographed using the Gel Doc™ XR+ Gel Documentation System (BIO-RAD, USA).

2.7 Nucleotide Sequencing

The nucleotide sequencing was carried out in an automated ABI 3500 xL genetic analyzer (Applied Biosystems, Foster City, USA). The procedure involves three steps: PCR product purification, cycle sequencing, and BigDye XTerminator® ™ purification.

2.7.1 PCR-Product Purification

Amplified PCR products were purified using the ExoSAP-IT method prior to sequencing to remove any unconsumed dNTPs and primers.

Procedure

i. 2µl of Exo SAP-IT cleanup reagent was added directly to 5µl of PCR product to degrade primers and de-phosphorylate dNTPs that were not accumulated in the PCR reaction.

ii. Treatment was carried out for 15 minutes at 37°C and followed by a 15-minute incubation period at 80 °C. Reactions were carried out in thermo-cycler.

2.7.2 Cycle Sequencing

The cycle sequencing reaction was performed using the ABI BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA). The thermal cycling program proceeded for 25 cycles.

1. Forward and reverse sequencing reaction mixture was prepared separately in a tube on ice. For each forward or reverse reaction, the reaction mixture for cycle sequencing is as follows:

Reagents	Amount (µl)
Sequencing Buffer	1
Terminator ready reaction mixes (Big dye)	0.5
Primer (5 µl)	1
Water	5.5
Purified PCR product	2
Total volume	10

2. The reaction plate was sealed with caps which was then spin briefly.

3. The reactions were run in a thermal cycler. The thermal cycling programs used for cycle sequencing reaction is as follows:

Steps	Temperature	Time
Denaturation	96°C	10 seconds
Annealing	50°C	5 seconds
Extension	60°C	4 minutes
Hold	4°C	Hold

4. After the cycle sequencing reactions were complete, the plate was spin briefly.

2.7.3 BigDye XTerminator® ™ Purification

The BigDye® XTerminator™ Purification Kit is a fast, simple purification method for DNA sequencing reactions that improves the sequencing workflow and removes unincorporated BigDye terminators. BigDye® XTerminator™ Purification Kit removes dye blobs by capturing unincorporated dye terminators, salts and other charged molecules that may interfere with base calling and electro-kinetic sample injection. The kit contains two reagents:

- **XTerminator™ Solution**-Scavenges unincorporated dye terminators and free salts from the post-sequencing reaction.
- **SAM™ Solution**- Enhances the performance of the XTerminator™ Solution and stabilizes the post-purification reactions.

Procedure

i. After cycle sequencing, the reaction plate was centrifuged briefly, then 45 µL of the SAM™ solution was added each well of the 96-well plate.
ii. 10 µL of the XTerminator™ Solution was added into each well of the 96-well plate. XTerminator™ was agitated each time for at least 10 seconds.
iii. The plate was sealed using MicroAmp® Clear Adhesive Films, vortexed at 2000 rpm for 30 minutes and centrifuged at 200g for 2 minutes.

2.7.4 Genetic Analyzer Procedure

The sequencing plate was placed into 24-capillaries ABI 3500 XL Genetic Analyzer (Applied Biosystems). These were left to run and nucleotide sequences were obtained using the sequence analysis software (Applied Biosystems).

2.7.5 Contig Generation

From the Genetic Analyzer, chromatogram sequences for both forward and reverse sequences were obtained for each amplified segment and inspected with Chromas 2.33 (Technelysium, Helensvale, Australia). Contig was generated using the SeqMan program embedded in the DNASTAR LASERGENE Software version 10. The generated contig was then visually inspected against individual chromatograms and any ambiguities corrected.

2.7.6 Similarity Searches

In order to determine whether the obtained nucleotide sequences were similar to influenza A sequences deposited in genomic databases, similarity search against sequences in the influenza virus resource and GenBank databases was performed using the basic local alignment search tool (BLAST) with the default parameters. Any ambiguities were confirmed by comparing the original chromatogram and the similar sequences showed in NCBI GenBank Database.

2.7.7 Multiple Sequence Alignment and Phylogenetic Analysis

Multiple sequence alignment was performed using ClustalW Multiple Alignment embedded in BioEdit version 7.1.3. Briefly, a 'fasta' format file was created with study generated contigs along with the representative sequences identified by BLAST search. The gene segments of the Influenza vaccine strains were similarly downloaded and incorporated in the analysis. Then the alignment was performed using the ClustalW Multiple Alignment program. Generated multiple sequence alignment was edited to remove any gaps or discrepancies. The same procedures were followed for generating protein multiple sequence alignments. Neighbor-joining (NJ) trees were constructed by using MEGA 6 software. Reliability of the NJ trees was statistically evaluated by bootstrap analysis with 1000 replicates using Kimura 2-parameter method. Phylogenetic analysis was performed using MEGA 6 (Sinauer Associates, Inc) which is an integrated tool for conducting automatic and manual sequence alignment, inferring phylogenetic trees, mining web-based databases, estimating rates of molecular evolution, inferring ancestral sequences, and testing evolutionary hypotheses.

2.7.8 Genetic Distance Matrix Calculations

MEGA version 6 software was used to calculate the distance matrix of sample and reference sequences by p-distance model. Genetic distances were calculated between all possible pairs. By using distance matrix data, following average distances were calculated,

i. Average distances within reference sequences
ii. Average distance between reference sequences and samples sequences.

2.7.9 Reference Sequences Retrieval

The reference sequences used in the phylogenetic analysis were downloaded from the GenBank Database (http://www.ncbi.nlm.nih.gov/).

2.8 Prediction and Statistical Analysis of Potential N-glycosylation Sites

Sequon Finder was used to predict N-glycosylation sites on HA and NA and to perform statistical analysis of the glycosylation sties conservation among the viruses. Sequon Finder is a custom-made program that just simply finds all sequons (N-X-S/T, where X is not P) with in protein sequences and supposes all of sequons as potential glycosylation sites. Then it will compute the percentages of the sequon appeared at each location in all protein sequences as the conservation of the potential glycosylation site among homologous proteins. The number of glycosylation sites was obtained from a single monomer of HA and NA and the locations of the glycosylation sites on HA and NA were numbered according to the full-length HA sequence of South Carolina/1/1918 and full-length NA sequence of Brevig Mission/1/1918, respectively. Potential N-linked glycosylation sites were predicted using NetNGlyc 1.0 Server (Gupta R 2005). A threshold value of >0.5 average potential predicts glycosylation sites.

2.9 Analysis of Genetic Drift and Antiviral Drug Associated Mutations

Analysis of antigenic drift was elucidated by analyzing and comparing changes in the amino acid signature patterns of each gene segment between the study isolates and the vaccine strain. To accomplish this, the translated protein alignments of the coding region of each gene were entered in Molecular Evolutionary Genetics Analysis software (MEGA) version 6 (Tamura, Peterson et al. 2011) and analyzed.

2.10 Antiviral Assay

2.10.1 Virus Propagation and Infection in MDCK Cell Line

Influenza A virus was grown in MDCK cell to create working stocks which were used for the antiviral assay. At first two flasks (one filter flask, another normal flask) were seeded with MDCK cells (8*104 cells /ml) where one was used for control flask and another for growing virus. After 24 hours when growth was confluent, media was discarded and washed with PBS twice. Then 1.5

ml 0% DMEM media was added in both of the flask. Virus suspension (200 µl) was added in one filter flask. After that flasks were incubated for 30 minutes. 2% TPCK trypsin containing media was prepared by adding 12 µl TPCK trypsin in 4.5 ml media and added in both the flasks. After two days of incubation we got cytopathic effect and virus suspensions were harvested in 10% glycerol.

2.10.2 Virus Harvesting

At first, 10% glycerol solution was prepared by adding 1ml of glycerol in 9 ml distilled water followed by syringe filtration. Cells and suspension of the flasks were then taken in a centrifuge tube followed by centrifugation at 2000 rpm for 5 min. then the supernatant is separated in a tube and pellets were subjected to freeze-thaw and centrifugation and separated the remaining supernatant in previous tube. Then the suspension was added with 10% glycerol in a cryovial and stored at -80°C freezer.

2.10.3 Virus Titration

Virus titration was required to find out virus concentration of complete cytopathology after two days' post infection. Titration was performed by infecting 1-day MDCK culture with increasing dilutions of viral suspension on a 96-well plate. Virus suspension was diluted in serum free DMEM media. 10,20,50,100,1000-fold dilution of viral suspension was prepared concomitantly. After 2 days of incubation at 37°C, under 5% CO2, the plate was observed for complete cytopathology and expected dilution was selected for future antiviral assay.

2.10.4 Antiviral Compounds

Oseltamivir phosphate (as Tamiflu® capsules, here referred to merely as oseltamivir), the orally active prodrug form of oseltamivir carboxylate, was purchased from a local pharmacy. Ribavirin (as Cellbrain® capsules), was purchased from Incepta pharmaceuticals. Amantadine Hydrochloride (as Amantril® capsules, here referred to merely as Amantadine), was purchased from local ACI pharmaceuticals. Oseltamivir carboxylate, Amantadine Hydrochloride and Ribavirin were dissolved in cell culture medium for in vitro studies. Oseltamivir was obtained from pharmaceutical capsules that also contained other ingredients as filler material besides the drug, the contents of entire capsules minus the shell were added to sterile DMEM medium to make

up the highest µg/ml dose of drug. Lower doses of oseltamivir were made by dilution into sterile medium.

2.10.5 Preparation of Drug

The solid drug was first dissolved in serum free DMEM media containing 0.5% DMSO and 2% TPCK treated trypsin. The solution was then diluted 103,104,105and 106-fold to prepare 0.1, 1.0, 10.0, 100.0 µg/ml solution of drug. It was kept in room temperature until use.

2.10.6 Preparation of Microtitre-plate

The microtitre plate was labeled properly with the isolate ID and concentration of Amantadine drug vertically and horizontally respectively. Duplicate well was used for each well. Column 1 and 2 were used for cell control where first row of these two columns contains no drug (cell control) but other rows contain increasing dilution of drug (used for checking drugs cytotoxicity). Column 3 and 4 contains only virus (virus control). Column (5,6), column (7,8), column (9,10) and column (11,12) contains 0.1, 1.0, 10.0, 100.0 µg/ml of drug solution. Using micropipette 125 µl of cell (15*104 cells /ml) containing DMEM media (10% FBS) were seeded in each well of 96-well microplate. When growth of MDCK cells were confluent, media was removed from each well and washed with PBS twice. Then virus was diluted to appropriate concentration as selected in titration. 25 µl of virus then added in each well except those, which were selected for untreated uninfected well and drug cytotoxicity determining well. These well were added with serum free 25µl DMEM media and incubate at 37°C 5% CO2 for 1 hour. Then again, each well was washed with PBS to remove unbound virus. 0, 0.1, 1.0, 10.0, 100.0 µg/ml of drug solution were added in each infected well respectively. Untreated wells of uninfected Untreated wells of uninfected cells and untreated wells of infected cells were added with only 0% DMEM media containing 2% TPCK treated trypsin.

2.10.7 MTT assay

MTT assay was performed to determine the EC50 value of each drug. After observation of complete cytopathology in untreated wells of infected. The medium was removed from the wells and cell staining was performed using the MTT reagent. 20 µl of 2.5 mg/ml solution MTT solution (Sigma-Aldrich) were added to each well. After 2 h incubation at 37°C, 100 µl of lysis buffer: 13.5

g/100 ml SDS (POCH), 45% (v/v) DMF (Sigma-Aldrich) was added and plates were incubated 4 hours at 37°C. At test termination, absorbance was measured at $\lambda = 560$ nm using Ultramark plate reader (Bio-Rad). EC50 values were determined from the graph of absorbance vs drug concentration.

2.11 Candidate Epitope Prediction for Suitable Vaccine

2.11.1 Physicochemical Properties of HA and NA

To identify suitable vaccine candidate epitope, bioinformatics analyses were carried out based on the HA and NA proteins for both A/H1N1(pdm)09 and A/H3N2 isolates using Prot-Param (http://expasy.org/tools/protparam.html). The physicochemical properties that were investigated includes hydrophobicity, hydrophobicity, surface accessibility and electrostatic potential of the HA and NA construct protein.

2.11.2 Potential Antigenic Sites Prediction

The amino acid sequences were predicted as candidate epitope for liner B -cell epitopes using Immune Epitope Databases (IEDB) sever (http://tools.immuneepitope.org/tools/bcell/tutorial.jsp) and checked in (EMBOSS) program. The antigenic sties in the slum isolates A/H1N1(pdm)09 and A/H3N2 were determined using Kolaskar and Tonganokar antigenicity prediction method (Kolaskar and Tongaonkar 1990) based on physicochemical properties of amino acid residues (i.e. hydrophilicity, accessibility and flexibility) with about 75% accuracy. Prediction of B-cell epitopes in the protein sequences was performed based on integrating the peptide major histocompatibility (MHC) binding by using ProPred.

2.11.3 Protein Structures Prediction

Protein structure and three dimensional(3D) models or tertiary structure of the HA and NA were predicted by Phyre (http://www.sbg.bio.ic.ac.uk/phyre2/html/).

2.11.4 Homology Modeling and Visualization

The 3-D models were constructed from the sequence alignment between the constructs and the template proteins using SWISSMODEL. The 3-D model is visualized in different representation

patterns by the PyMOL viewer. Visualization and annotation of the epitopes sequences is done by using PyMOL which is open source molecular visualization system.

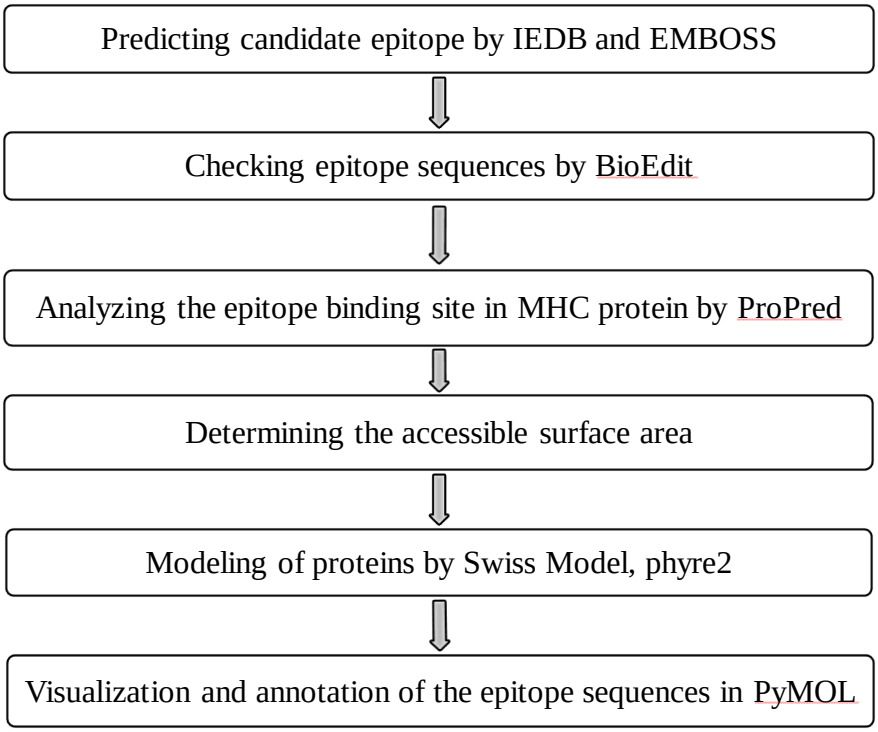


Figure 2.2. Flow chart of candidate epitope prediction

Chapter 3

Results

3. Results

In this study, we selected three densely populated slums inside Dhaka city to investigate influenza prevalence, genetic diversity, seasonal distribution among individuals who showed Influenza like illness (ILI). We performed full genomic analysis of selected Bangladeshi A/H1N1pdm/09 and A/H3N2 strains and performed bioinformatics analysis to predict putative candidate vaccine epitopes. We also investigated the susceptibility of circulating influenza strains against locally prescribed drugs.

3.1 Demographic and Clinical Manifestation of the Population

The study was conducted among 993 patients with clinically presented ILI (Influenza-like illness). The age of the enrolled patients ranged from 6 months to 69 years, mean age was 9.59 years, and the median was 4.1 years. Among ILI case patients, children under 5 years comprises 54% of total cases. Male were 453 (45.6%) and female 540 (54.4%). Demographic information of the study patients is listed in Table 3.1.

Table 3.1 Demographic information of the enrolled ILI patients

Characteristics Age (years)	Influenza-like illness patients, N=993 (%)	Patients infected with influenza viruses, N=101 (%)
0.6−<5	536 (54%)	40 (40%)
5−19	277 (28%)	35 (35%)
20−49	149 (15%)	19 (19%)
≥50	31 (3%)	7 (7%)
Mean age	9.59	1.9
Median age	4.1	2
Male	453 (45.6%)	41 (40.6%)
Female	540 (54.4%)	60 (59.4%)

3.2 Prevalence of Influenza Viruses in Three Different Slums

Real time PCR was performed to detect influenza virus RNA form nasal and throat swabs from patients with ILI. Altogether, a total of 993 ILI patients were tested for the presence of influenza viruses; Rayerbazar (n=503), Hazaribagh (n=115), and Mohammadpur (n=375). Overall Influenza

was detected in 9.2% (n=101) of the total cases. Of all positive cases, Influenza A was detected in 47% (n=47) of the patients while influenza B was detected in 53% (n=53) of the cases. In Rayerbazar, influenza B was the most dominant (91%), while in Hazaribagh and Mohammadpur, influenza A was the most predominant strain during the study period (Table 3.2 and Figure 3.1).

Table 3.2. Distribution of Influenza viruses

Slums	Total Cases	Influenza Positive, n (%)
Rayerbazar	503	54 (
Hazaribagh	115	7
Mohammadpur	375	40
Total	993	101

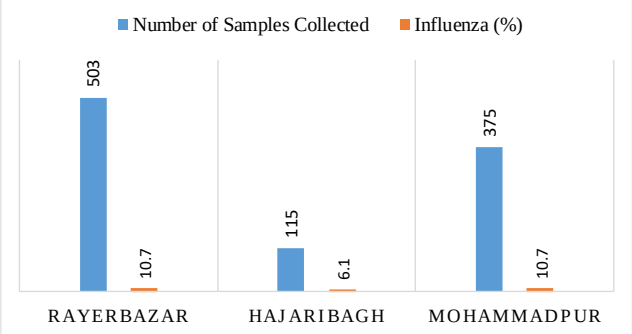


Figure 3.1. Distribution of Influenza viruses in different slums

3.3 Clinical Manifestations of Influenza Positive Patients

Among all influenza positive cases, 77% and 41% of the cases complained of runny nose and headache respectively compared to 52% and 20% cases of influenza negative ILI patients. 25% of positive patients also reported body ache compared to the 6% of the influenza negative patients. Clinical manifestations of the study patients are listed in Table 3.3.

Table 3.3 Clinical Symptoms associated with ILI patients

Symptoms	Influenza positive ILI, N = 101 (%)	Influenza negative ILI, N=892 (%)
Fever	101 (100)	892 (100)
Cough	95 (94)	820 (92)
Sore throat	26 (26)	170 (19)
Runny nose	78 (77)	460 (52)
Headache	41 (41)	180 (20)
Body ache	25 (25)	50 (6)
Malaise	5 (5)	42 (5)

3.3.1 Influenza Among Age Distribution

The proportion of influenza positive rate were higher among aged over 50 years. The influenza positive rate was about 13% in 20-49 and 5-19 years' age group. Older people (> 50 years) were found to be more susceptible to influenza (Figure 3.2).

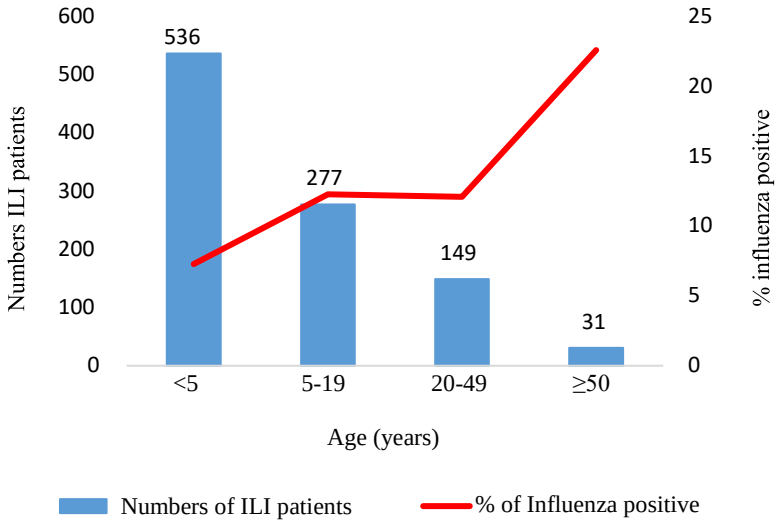


Figure 3.2 Influenza positives in different age groups

3.3.2 Seasonality of Influenza Viruses

During the study period, influenza incidence was observed throughout the year, however, the highest incidence was observed in rainy season (April to August). Incidence of influenza virus reached at peak in rainy reason but falls below 5% during winter (Figure 3.3).

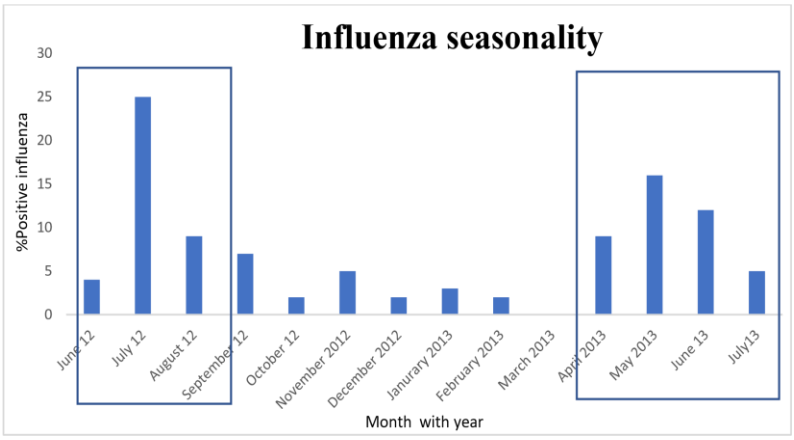


Figure 3.3. Month-wise distribution of influenza viruses

3.4 Detection of Influenza A Virus Subtypes

Positive influenza A viruses were further subtyped by using A/H1N1pdm and H3N2 specific primer and probe. Among all Influenza positives, A/H1N1pdm/09 and H3N2 accounts for 40% and 60% of the samples, respectively. No seasonal A/H1N1 strains were found during the study period. In Rayerbazar, influenza A accounted for 9% of the positive cases where A/H1N1pdm/09 and A/H3N2 represented 5% and 4% of the total influenza positive cases, respectively (Figure 3.4). In Hazaribagh slum, no influenza A/(H1N1)pdm09 strain was identified and A/H3N2 was the only circulating influenza A strain (Figure 3.4). In Mohammadpur slum, both influenza A/H1N1pdm/09, influenza A/H3N2 were prominent, only former was slight higher.

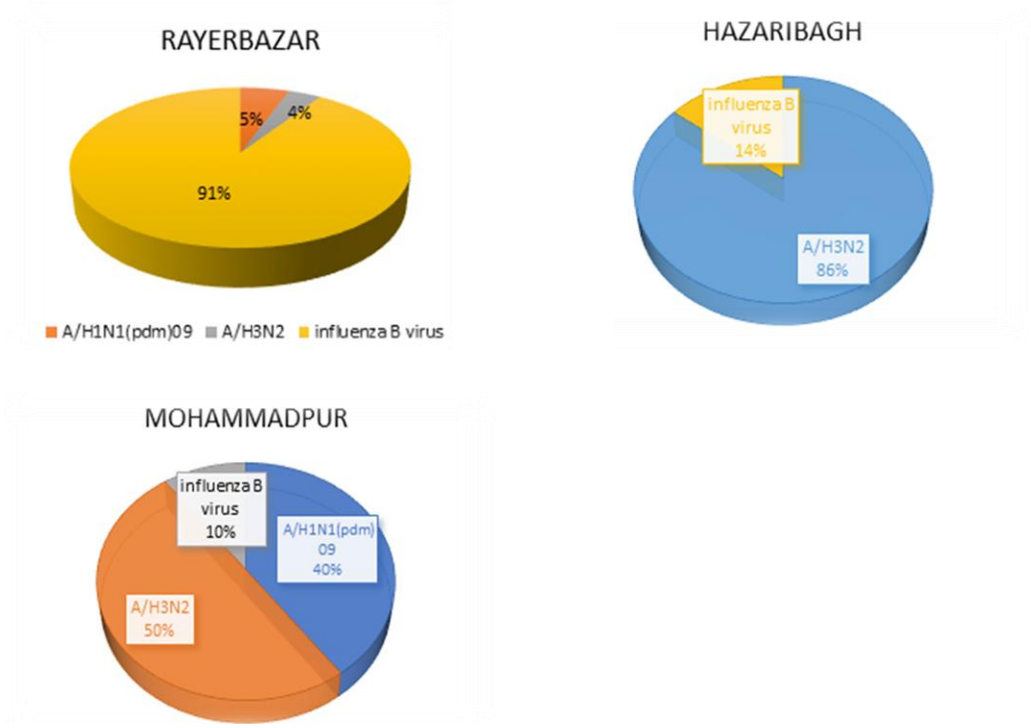


Figure 3.4. Distribution of influenza A virus in three different slums

3.5 Antigenic Characterization of Influenza Viruses

RT-PCR positive influenza A and B slum viruses were cultured in MDCK cell line. All the samples showed the desired growth in MDCK cell line. The tissue culture isolates were antigenically characterized by hemagglutination-inhibition test (HAI) using a panel of post infection with ferret antisera. 40% positive tissue culture belonged to influenza A/H1N1pdm09 and 60% belonged to A/H3N2 type. Isolate Dhaka-850/2013 showed an HAI titre of 1280 (Figure 3.5A). Isolate Dhaka-961/2013 showed HAI titre of 320 (Figure 3.5B). 74% of influenza B isolates belonged to Yamagata lineage and 26% belonged to Victoria lineage. Influenza B virus Yamagata lineage showed titre of 320 and Victoria lineage showed titre of 160 (Figure 3.5C).

3.6 Genetic Characterization of Influenza Viruses Isolated from Slums

Five sequences of the HA genes of influenza H3N2 slums isolates 542, 541, 550, 558, 472 were analyzed. Alignment of HA gene sequences of isolates 542, 541, 550, 558, 472 showed highest similarity and percent nucleotide identities (100%) (Figure 3.6). Four sequences of influenza H3N2 isolates showed similar result in similarity and percent identities in HA sequence (Figure 3.7). Due to 100% similarity in HA gene nucleotides, one representative isolate of)H1N1pdm09, H3N2 strain was selected whole genome amplification and analysis.

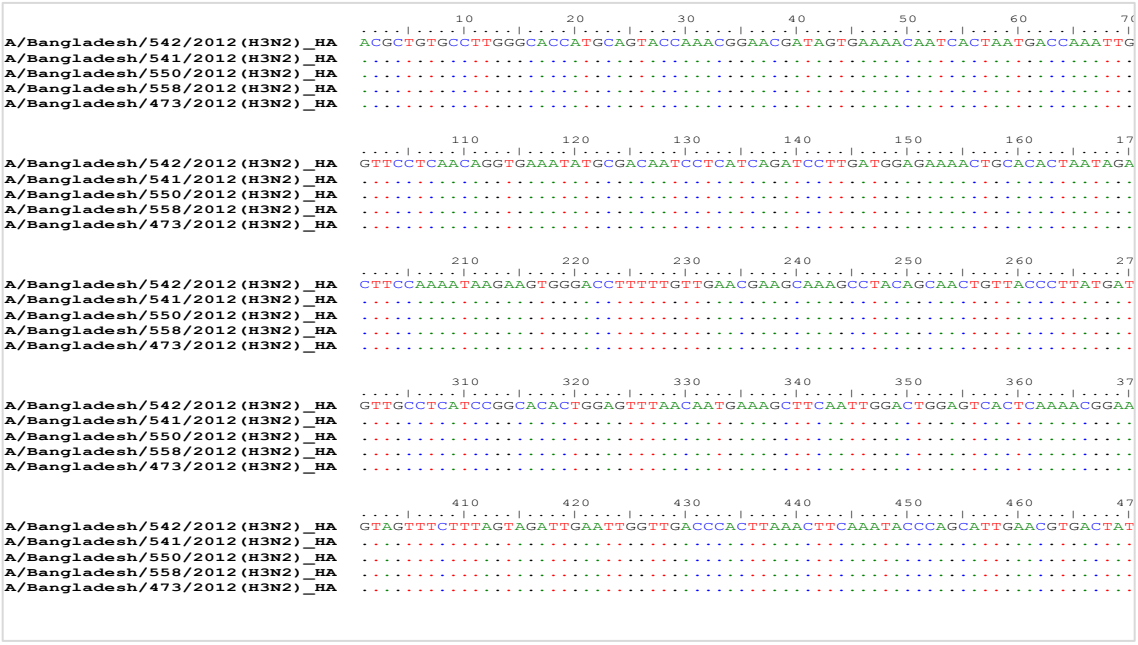
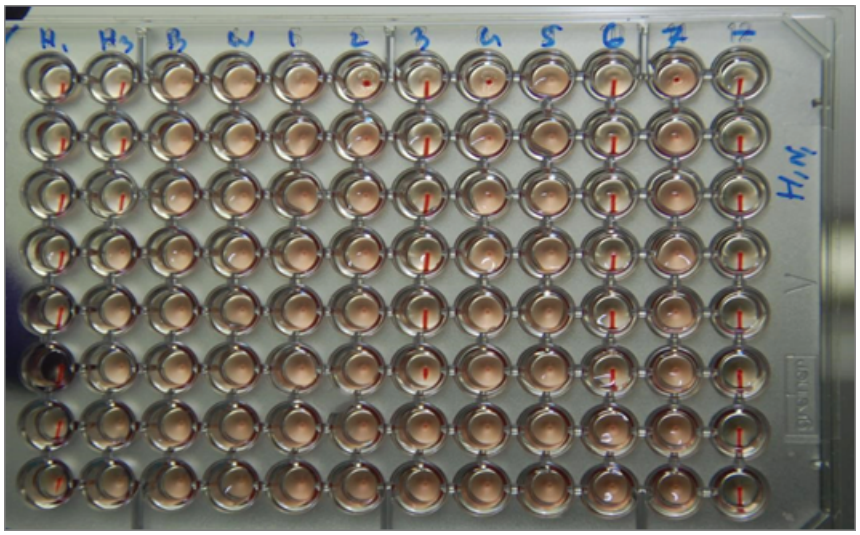
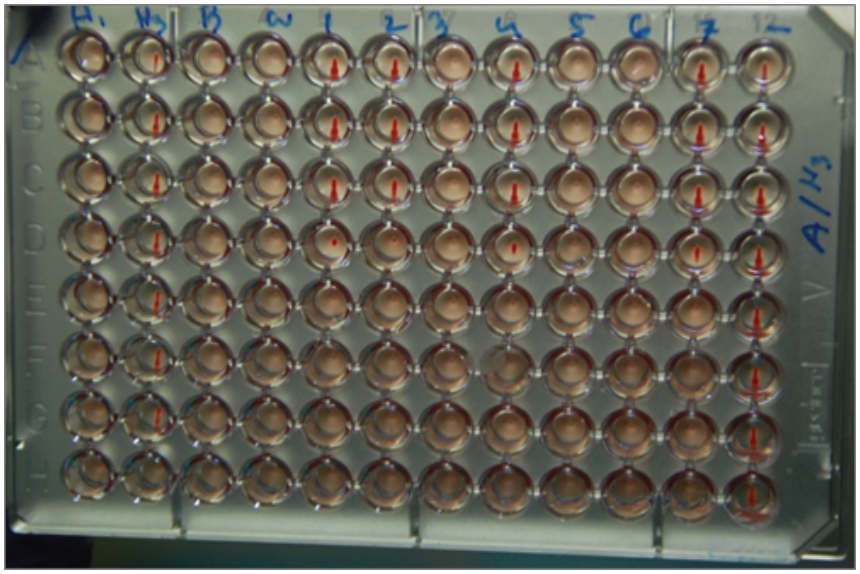


Figure 3.6. Nucleotide similarity among Influenza H3N2 virus slum isolates (HA gene)

A.



B.



C.

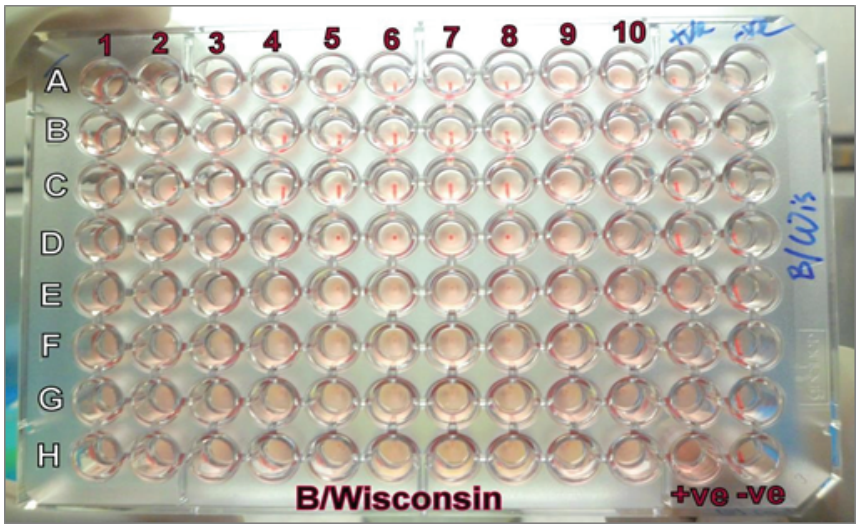


Figure 3.5. Antigenic Characterization of Influenza Viruses. A) Influenza A/H1pdm; B) Influenza A/H3; C) Influenza B

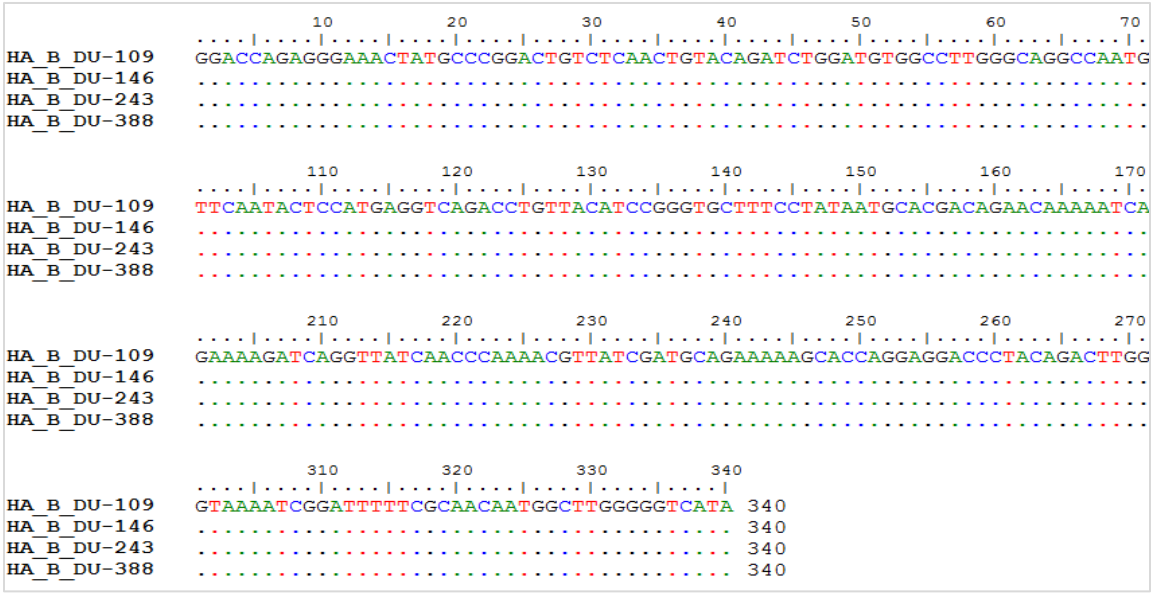


Figure 3.7. Nucleotide similarity among Influenza B virus slum isolates (HA gene)

3.7 Whole genome Amplification and Sequencing Influenza A viruses (A/H1N1pdm09 and H3N2)

One isolate of A/H1N1pdm09 (isolate A/Dhaka/850/2013) and one isolate of A/H3N2 (isolate A/Dhaka/961/2013) form cell culture was selected for the whole genome amplification and sequencing. All the 8 gene segments were amplified separately using primers specific for HA, NA, NP, PB2, PB1, PA, NS, and M.

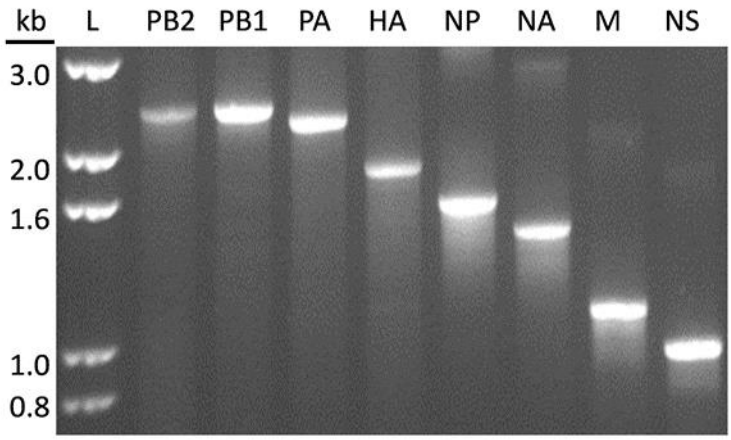


Figure 3.8. Gel photo showing the PCR amplification of the eight influenza gene segments

Lane 1, 1kb ladder DNA marker, Lane 2, PB2; Lane 3, PB1; Lane 4, PA; Lane 5, HA; Lane 6, NP; Lane 7, NA; Lane 8, M; Lane 9, NS segment.

The PCR products were sequenced in ABI prism® 3500 xL Genetic Analyzer and sequences were searched in the GenBank for sequence similarity. The eight segment namely PB2, PB1, PA, HA, NP, NA, M and NS of A/Dhaka/850/2013 showed varying degrees of similarity with multiple sequences (Figure3.8). The closest similarity in amino acid sequence in all segment was found with A/Singapore/DMS1315/2013. This isolate matches closely with vaccine strain A/California/07/2009 among the vaccine strain in GenBank. There was ≥ 97% similarity in amino acid sequence of the proteins. This influenza A isolate belonging to H3N2 lineage also shares similarity in amino acid sequence with vaccine strain A A/Victoria/361/2011 was 98.5 to 100% with exception of MP. The genome of A/Dhaka/961/2013 had 13482 nucleotides. With segment M varying significantly. H3N2 closes similarity strain was found with A/Delhi/1313/2013 strain (Table 3.4 and 3.5).

Table 3.4 H1N1pdm09 strain Identity with vaccine strain and similar strains

Segments	No of nucleotides (bp)	No of AA	% AA similarities with vaccine strain A/California/07/2009	% AA similarities with the closest strain A/Singapore/DMS1315/2013
PB2	2316	759	98.9	100
PB1	2338	755	99.2	99
PA	2199	710	99	99
HA	1751	566	97.5	99
NP	1521	497	98.8	100
NA	1410	409	98.1	99
M	992	252	98.8	100
NS	862	219	98.6	100

Table 3.5. H3N2 strain Identity with vaccine strain and similar strains

Segments	No of nucleotides (bp)	No of AA	% AA similarities with vaccine strain A/Victoria/361/2011	% AA similarities with the closest strain A/Delhi/1313/2013
PB2	2339	798	99.9	100
PB1	2341	757	99.6	99
PA	2233	716	99.6	99
HA	1736	564	98.9	99
NP	1530	498	99.8	100
NA	1411	464	98.5	97
M	1002	251	92.5	99
NS	890	230	100	100

3.8 Phylogenetic Analysis of 8 Gene Segments

Phylogenetic characterization of the nucleotide sequences of isolated local influenza viruses was carried out to compare local viruses with globally circulating viruses. The phylogenetic tree of each of the eight genes and of the concatenated genome showed similar topologies. All the trees were rooted using the nucleotide sequences of the prototype vaccine strain proposed by WHO.

3.8.1 Analysis of Individual Gene Segments

HA

In order to determine the relationship between Bangladeshi H1N1pdm09 isolate in relation to the vaccine strain, we first inferred the phylogenetic tree of the HA gene using the full coding sequence. In the phylogenetic analysis, Bangladeshi representative strain A/Dhaka/850/2013 and 40 influenza strains from different continents were included. Bangladeshi H1N1pdm09 isolate belonged to clade of 6B and showed close proximity to India, USA, South Africa and Canadian H1N1 strains (Figure 3.9 A). All the representative amino acid changes of clade 6 (D97N, S185T, S203T, E374K, S451N, K163Q, and A256T) were also observed in Bangladeshi H1N1pdm strains. On the other hand, Bangladeshi H3N2 strain clustered in clade 3C with the vaccine strain A/Victoria/361/2011 along with the concurrent circulating H3N2 strains circulating worldwide (Figure 3.9 B). Genetic analysis revealed Bangladeshi H3N2 strain shared similar amino acid changes Q33R, T128A, R142G, N145S, N278K of subclade 3C-3.

NA

In contrast to the vaccine strain A/USA/California/07/2009, Bangladeshi representative H1N1pdm09 strain (A/Dhaka/850/2013) formed a different cluster. Bangladeshi H1N1pdm09 isolate was clustered with 2013 USA strain while the vaccine strain clustered with strains circulating in 2010 (Figure 3.10 A). Representative Bangladeshi H3N2 strain (A/Dhaka/961/2013) clustered with the other concurrent strains circulating worldwide (Figure 3.10 B).

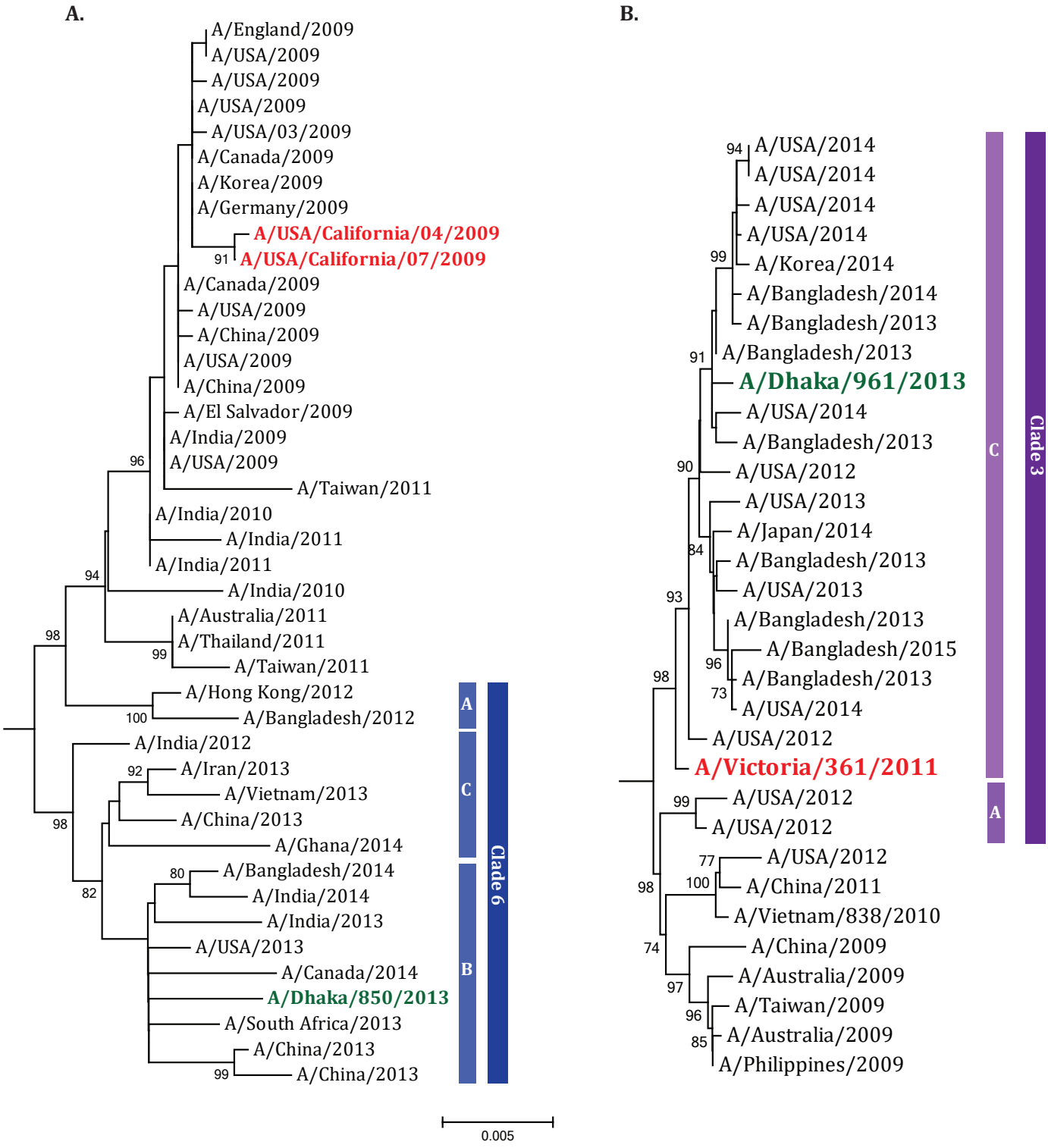


Figure 3.9. Phylogenetic tree of HA gene segment of Bangladeshi isolates.
Phylogeny was inferred with representative HA sequences of Bangladeshi A/(H1N1)pdm09 (A) and A/(H3N2) (B). Bangladeshi isolates are shown in Green and references are shown in red font. The numbers on the internal nodes indicate posterior probabilities.

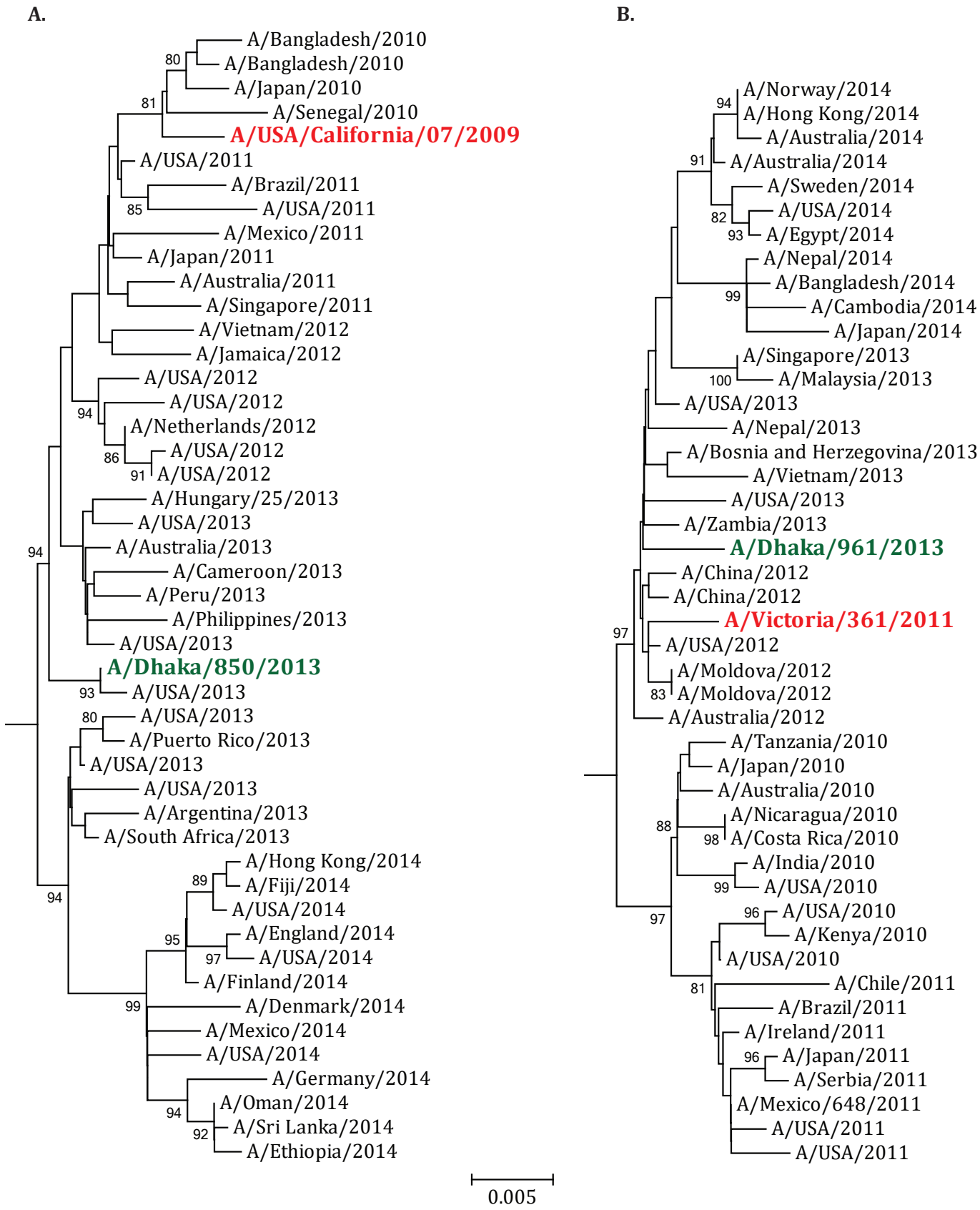


Figure 3.10. Phylogenetic tree of NA gene segment of Bangladeshi isolates.
Phylogeny was inferred with representative NA sequences of Bangladeshi A/(H1N1)pdm09 (A) and A/(H3N2) (B). Bangladeshi isolates are shown in Green and references are shown in red font. The numbers on the internal nodes indicate posterior probabilities.

Polymerase PB2, PB1 and PA genes

PB2

The phylogeny of the PB2 nucleotide sequences shows that slum H1N1pdm09 strain grouped separately in contrast to the other H1N1 circulating stains worldwide (Figure 3.11 A). Phylogenetic analysis of slum H3N2 isolate confirmed that H3N2 isolate was branched in a unique cluster to A/Victoria/361/2011. Slum isolate found that it grouped with contemporary other global strains (Figure 3.11 B).

PB1

The phylogenetic tree of the H1N1pdm09 PB1 reveals a similar topology to that of the PB2 tree (Figure 3.12 A). The PB1 sequences of early and peak phase isolates are largely distributed throughout the tree with no discernable temporal or spatial patterns while the late phase isolates group together to form a well-supported cluster. Majority of the local isolates were noted to form clusters that were interspersed with global isolates. Phylogenetic analysis of slum H3N2 isolate confirmed that H3N2 isolate was branched in a unique cluster to A/Victoria/361/2011. Slum isolate found that it grouped with contemporary strain of 2013 and 2012 strain. All clustered closely with the other global circulates of A/H3N2 (Figure 3.12 B).

PA

The phylogeny of the H1N1pdm09 PA nucleotide sequences does not show any discernable spatial and temporal patterns in the early and peak pandemic phases (Figure 3.13 A). Like the PB2 and PB1 phylogenetic trees, the PA nucleotide sequences of local isolates are interspersed with PA sequences of isolates from other countries in the world collected at the same period. Phylogenetic analysis of slum H3N2 isolate confirmed that H3N2 isolate was branched in a unique cluster to A/Victoria/361/2011. Slum isolate found that it grouped with other globally circulating strain of 2013 (Figure 3.13 B).

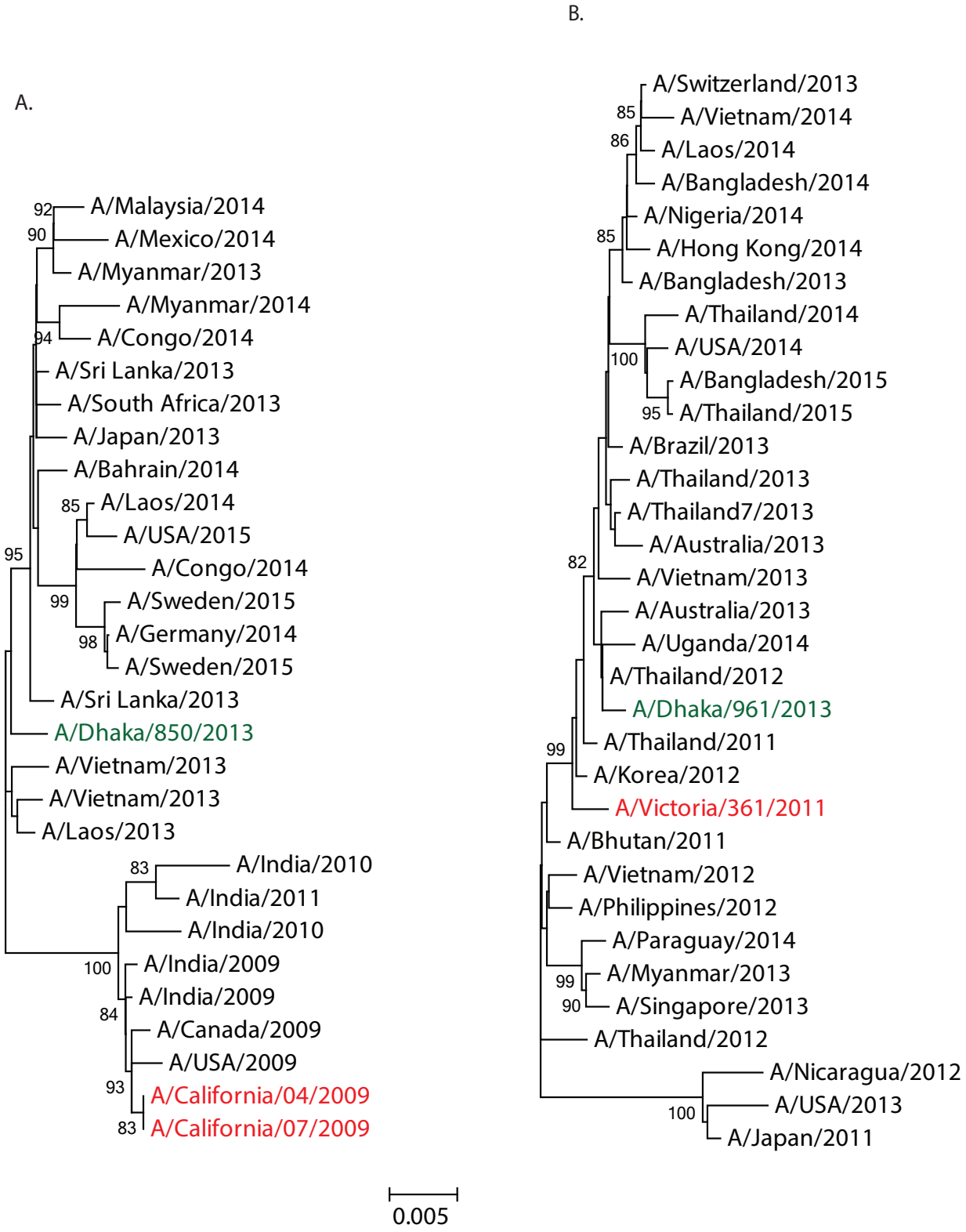


Figure 3.11. Phylogenetic tree of PB2 gene segment of Bangladeshi isolates.
Phylogeny was inferred with representative PB2 sequences of Bangladeshi A/(H1N1)pdm09 (A) and A/(H3N2) (B). Bangladeshi isolates are shown in Green and references are shown in red font. The numbers on the internal nodes indicate posterior probabilities.

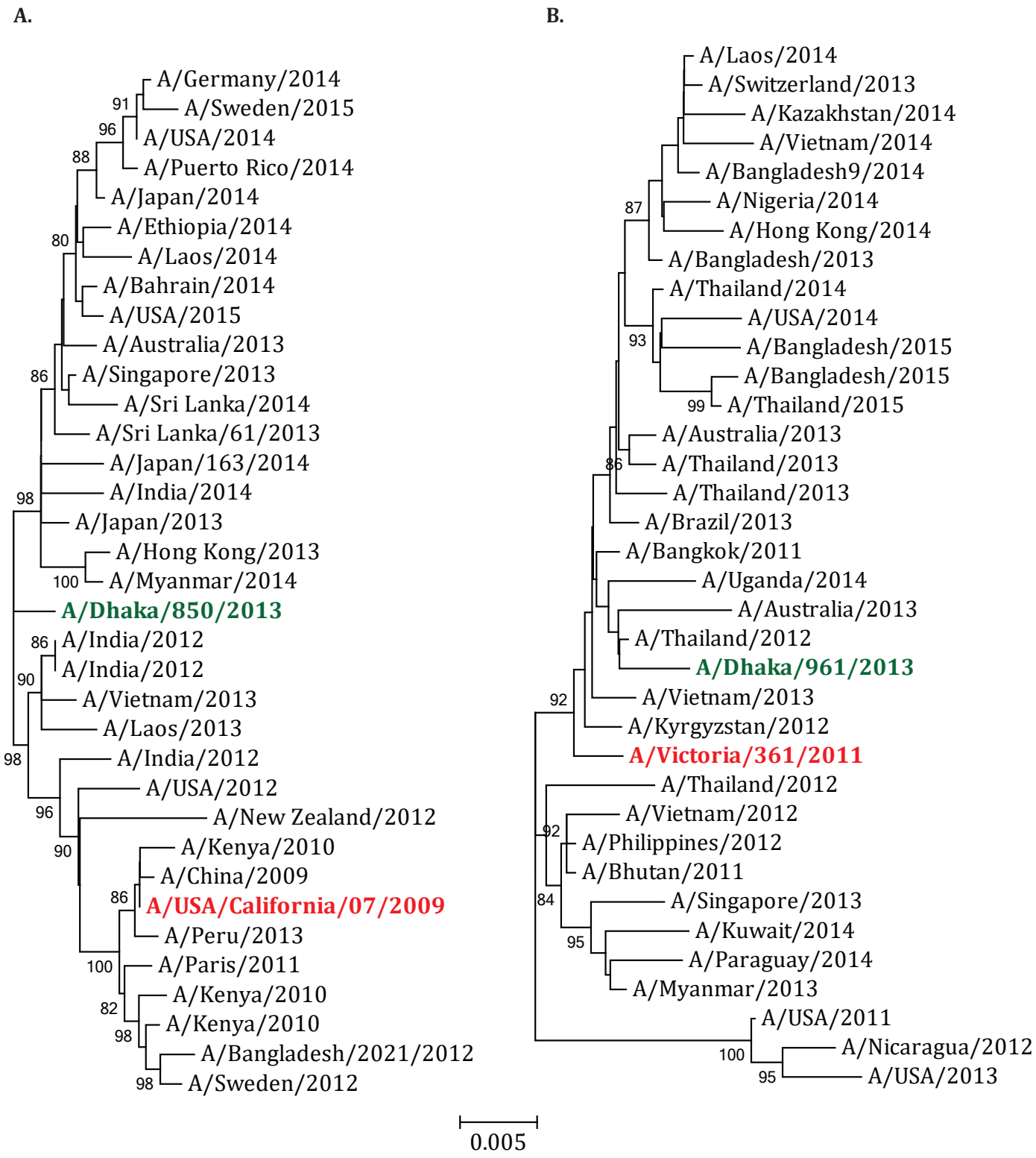


Figure 3.12A. Phylogenetic tree of PB1 gene segment of Bangladeshi isolates.
Phylogeny was inferred with representative PB1 sequences of Bangladeshi A/(H1N1)pdm09 (A) and A/(H3N2) (B). Bangladeshi isolates are shown in Green and references are shown in red font. The numbers on the internal nodes indicate posterior probabilities.

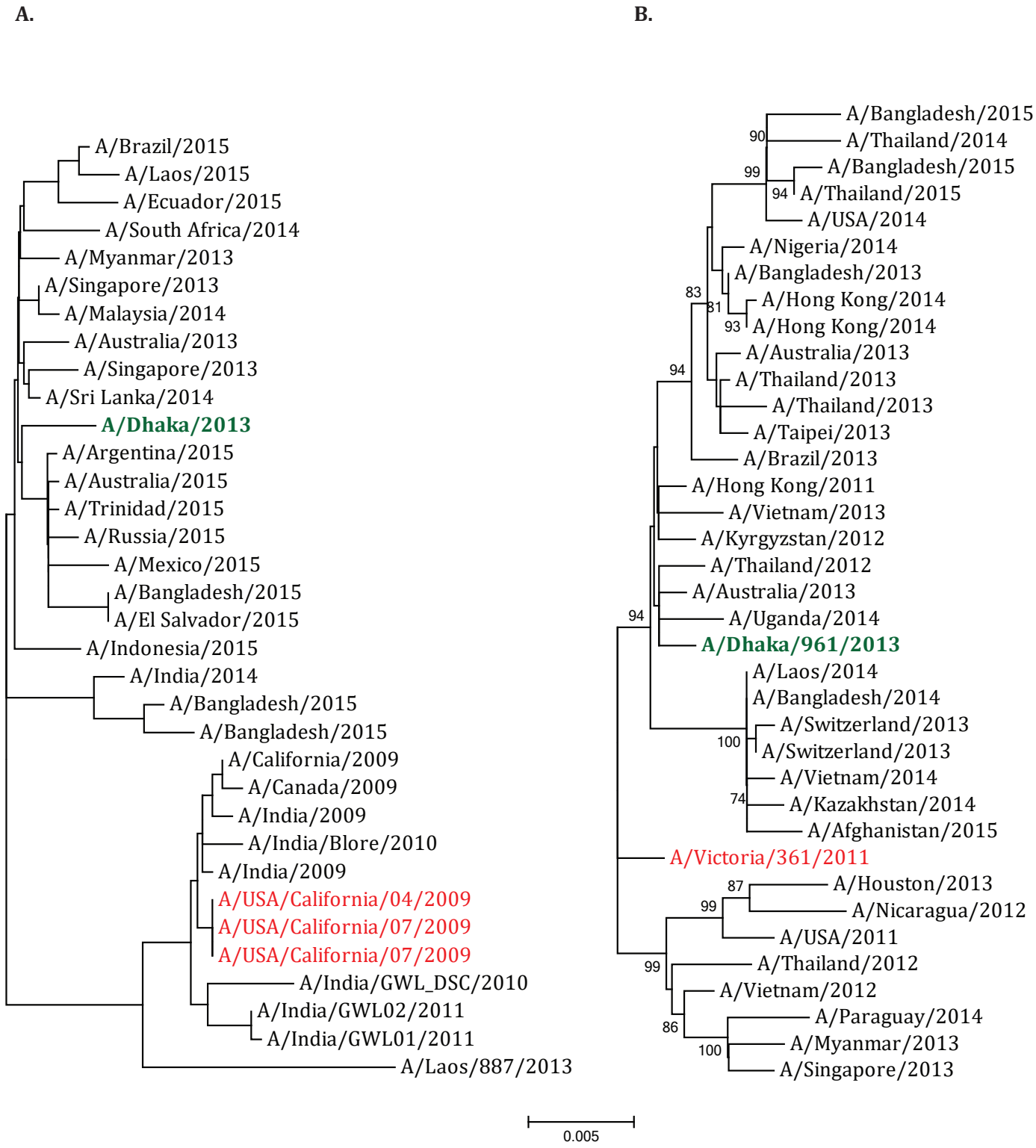


Figure 3.13. Phylogenetic tree of PA gene segment of Bangladeshi isolates.
Phylogeny was inferred with representative PA sequences of Bangladeshi A/(H1N1)pdm09 (A) and A/(H3N2) (B). Bangladeshi isolates are shown in Green and references are shown in red font. The numbers on the internal nodes indicate posterior probabilities.

NP, M and NS Genes

The NP phylogenetic tree of Bangladeshi H1N1pdm09 can also be divided into two clades, one comprising of the two earliest slum isolates A/Dhaka/850/2013 and while the second comprising all the other isolates (Figure 3.14 B). All the other local isolates did not show any spatial or temporal patterns. The NP nucleotide sequences of the local isolates were shown to be interspersed with NP sequences from other countries. Representative H3N2 strain A/Dhaka/961/2013, and other influenza virus from worldwide circulation were included. Our H3N2 isolate located in cluster of 2013 year strains and vaccine strain cluster with 2010 strains. Our strain grouped in contemporary globally circulating strains (Figure 3.14 D).

M

The phylogeny of the Bangladeshi H1N1pdm09 M nucleotide sequences of local isolates was shown to be made up of two distinct clades (Figure 3.15 A). The first comprising two of the earliest slum isolates and the second comprising of all the other isolates. The 2010 local isolates formed a well-supported cluster which also had the maximum depth of 9 nodes from the root showing greater diversification in comparison with the other isolates. The local M nucleotide sequences were also interspersed with global sequences in the phylogenetic tree though this was not as explicit as in the other nucleotide sequences. Phylogenetic analysis of slum H3N2 isolate confirmed that H3N2 isolate was branched in a unique cluster to A/Victoria/361/2011. Slum isolate found that it grouped with contemporary strain of 2013-2014. Beside our stain clustered with closely with the other global circulates of A/H3N2 (Figure 3.15 B).

NS

The phylogenetic tree of the Bangladeshi H1N1pdm09 NS nucleotide sequences of local isolates shows that they are interspersed with NS sequences from isolates from other countries (Figure 3.14 A). One cluster comprised of local isolates mostly from the peak phase, majority (67%) of the 2013 isolates cluster together to form a well-supported cluster. None of the slum isolates were found to cluster with isolates from other African countries. Phylogenetic analysis of slum H3N2 isolate confirmed that H3N2 isolate was branched in a unique cluster to A/Victoria/361/2011. Slum isolate found that it grouped with contemporary strain of 2013-2014. Beside our stain clustered with closely with the other global circulates of A/H3N2 (Figure 3.14 C).

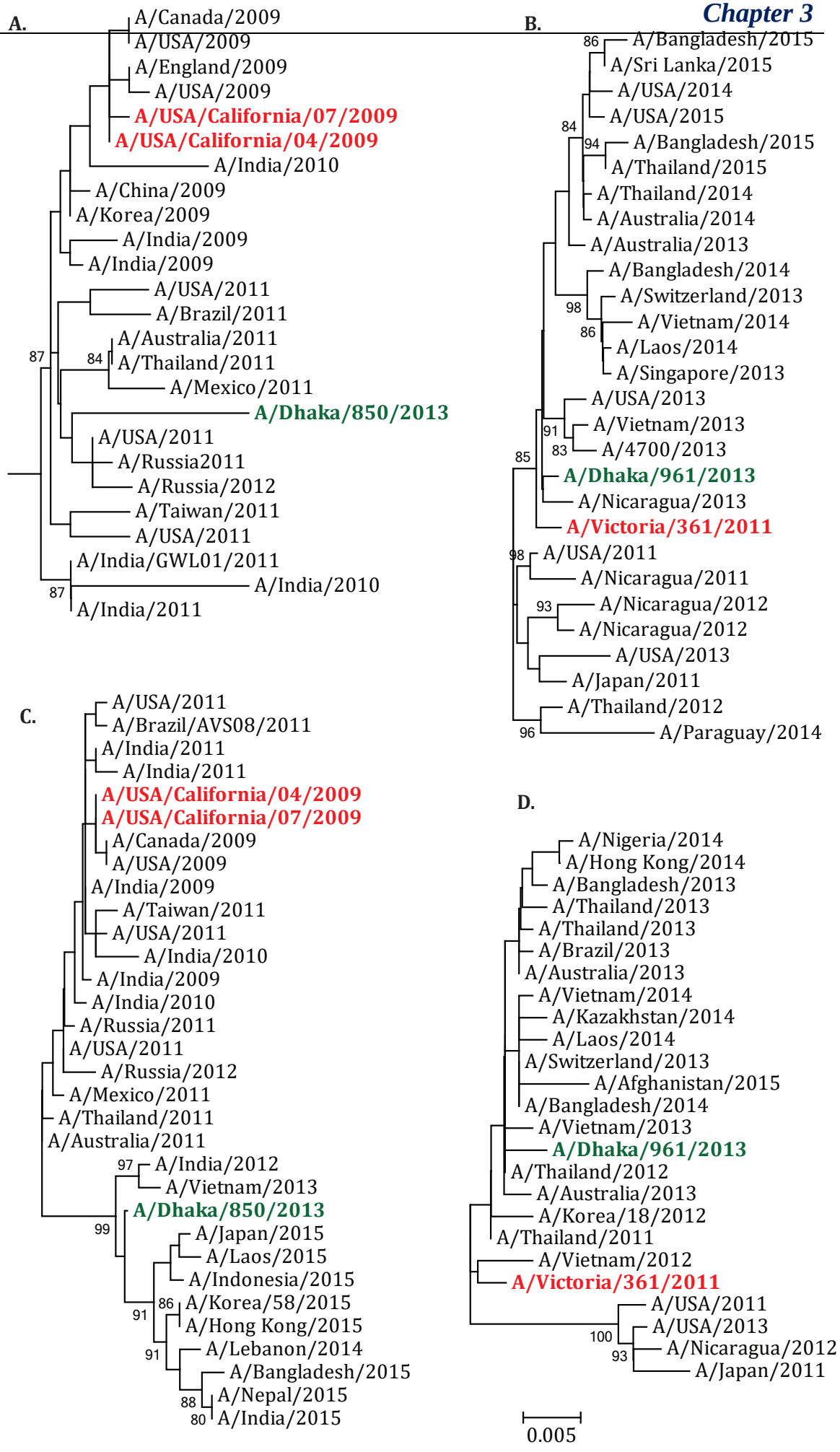


Figure 3.14. Phylogenetic tree of NS & NP gene segment of Bangladeshi isolatess.
Phylogeny was inferred with representative NS & NP sequences of Bangladeshi A/(H1N1)pdm09 (A, C) and A/(H3N2) (B, D). Bangladeshi isolates are shown in Green and references are shown in red font. The numbers on the internal nodes indicate posterior probabilities.

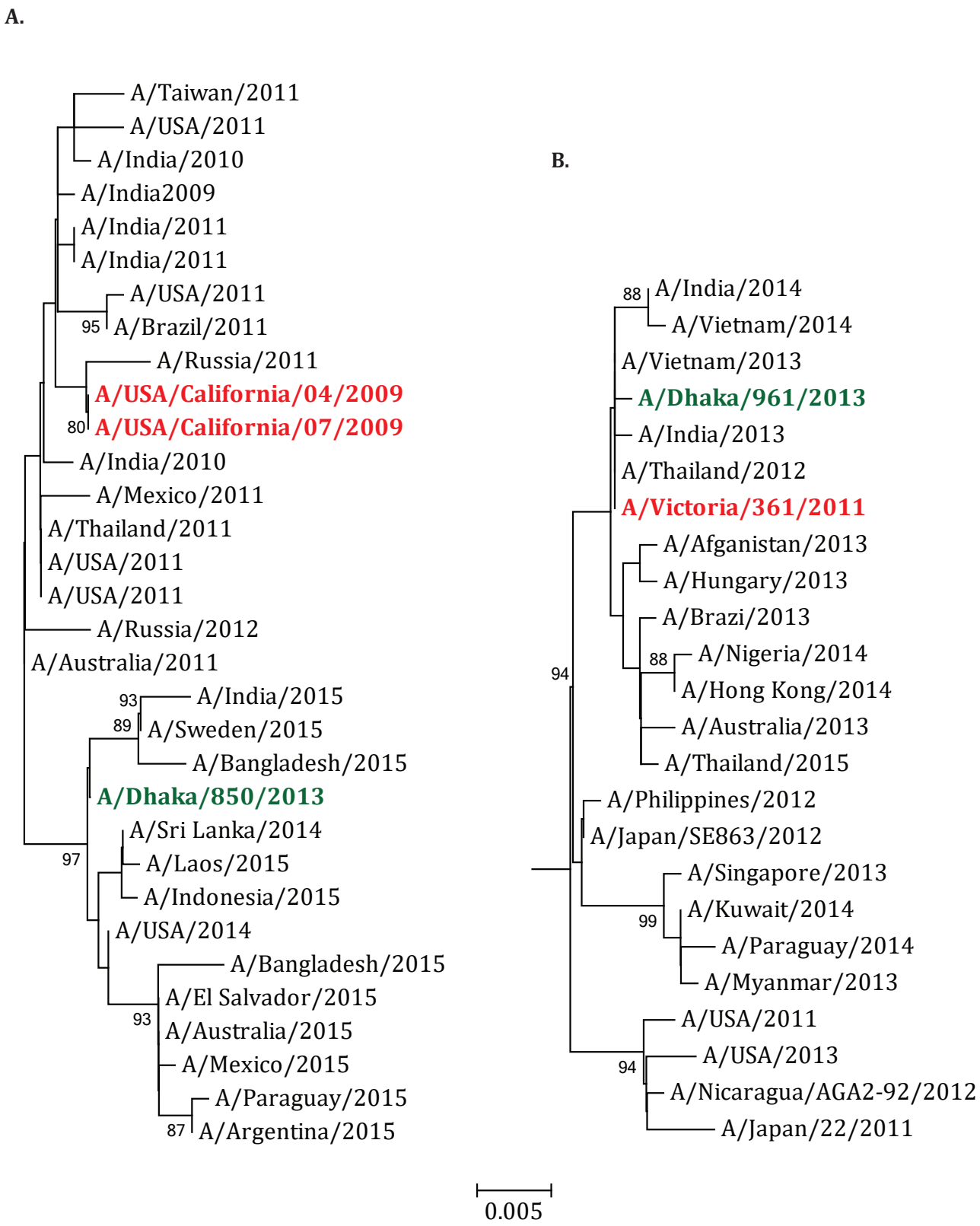


Figure 3.15. Phylogenetic tree of M gene segment of Bangladeshi isolates.
Phylogeny was inferred with representative M sequences of Bangladeshi A/(H1N1)pdm09 (A) and A/(H3N2) (B). Bangladeshi isolates are shown in Green and references are shown in red font. The numbers on the internal nodes indicate posterior probabilities.

3.9 Influenza B Virus

3.9.1 Phylogenetic Analysis of HA

Phylogenetic relationship of HA gene for two Influenza B strains, with the concurrent strains of both lineages. Our study revealed that one (Bangladesh/DUINF243/2012) of the representative strains of our study clustered with the Yamagata lineage, whereas one strain (Bangladesh/079/2012) clustered with B/Victoria/2/87 lineage strains. Co-circulation of both lineages was observed as both B/Yamagata/16/88 like (Bangladesh/079/2012) and B/Victoria/2/87 like strains (Bangladesh/079/2012) were observed. The WHO recommended vaccine strain for Northern Hemisphere of 2012-13 seasons B/Wisconsin/01/2010 of Yamagata lineage clustered closely with our study isolate (Bangladesh/DUINF243/2012). This strain also clustered with isolates from Utah and Bangladesh sampled in 2012. Strains sampled in 2007, 2008 and 2010 across the world also clustered closely with this strain. Another strain (Bangladesh/DUINF079/2012) of our study formed group with isolates from Pakistan, Huzhou, India, Utah, Wisconsin, Milano and Bangladesh circulating in 2012. It also clustered closely with the WHO recommended vaccine strains for Northern Hemisphere of 2010-11, 2011-12 seasons, Brisbane/60/2008 of Victoria lineage (Figure 3.16 a).

3.9.2 Phylogenetic Analysis of NA

Phylogenetic relationship of HA gene for two Influenza B strains, with the concurrent strains of both lineages. strains clustered with U.S.A., India and Bangladesh (Utah/02/2012, New_York/01/2012, Massachusetts/02/2008, South_Carolina/01/2008, Kol/1267/2007, Bangladesh/3333/2007).and other global contemporary stains.Phylogenetic relationship of NA gene for two Influenza B strains, with the concurrent strains of both lineages (Figure 3.16 b).

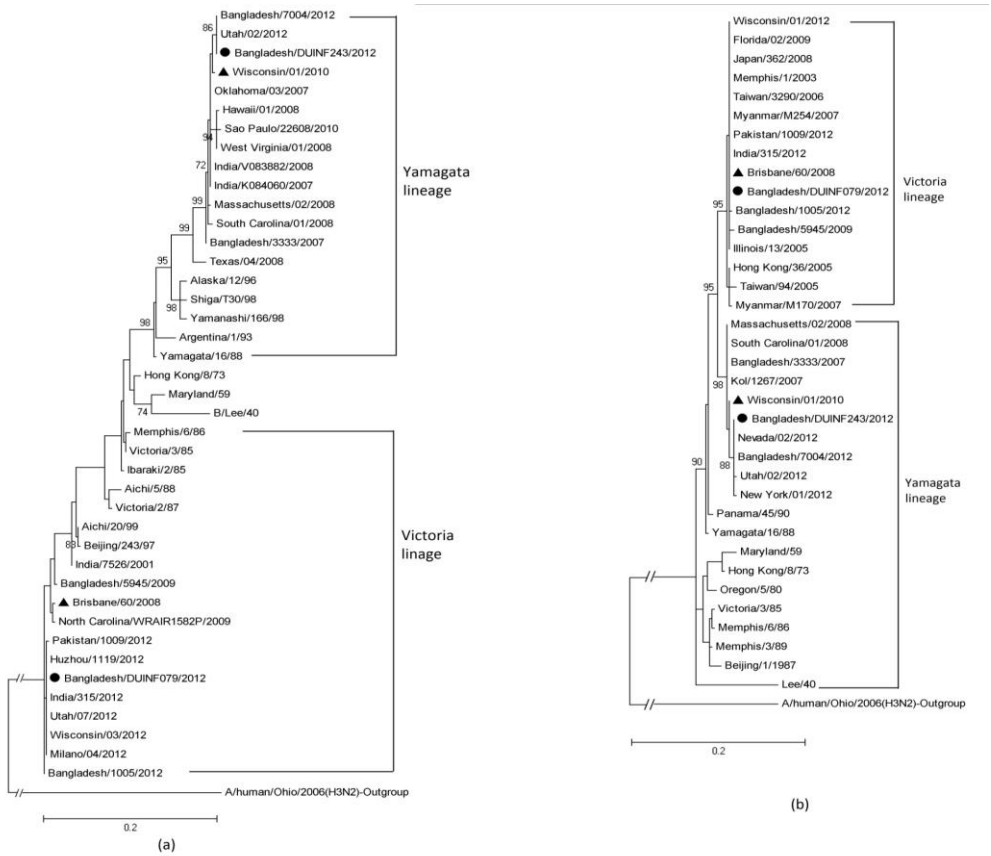


Figure 3.16. Phylogenetic tree of Influenza B based on HA and NA gene

3.10 Amino Acid Sequence Variations in Surface (HA and NA) Proteins

3.10.1 Sequence Variation in A/H1N1pdm/09

The HA gene of A/H1N1pdm isolate was compared with the contemporary vaccine strain for northern hemisphere A/California/07/2009 and found 97.5% identical at amino acid level. The three structural elements composed of 190-helix and 130-loop at the tip of the HA molecule which are considered as HA receptor binding site (RBS) were conserved in our isolate when compared with vaccine strain. However, a mutation N228D was found in 220-loop. No changes in four conserved residues, comprising Tyr98, Trp153, His183 and Tyr195 which form the base of the RBS, was not detected. Three altered amino acid were detected K163Q, S185T and S203T in the HA antigenic sites Sa, Sb and Ca respectively. Outside of these antigenic epitopes, 9 mutations- P83S, D97N, E112K, A246T, K 283E, I321V, E374K, N451D and E499K were also observed.

The Neuraminidase (NA) protein of A/H1N1pdm isolate showed 98.1 % identical at amino acid level with the vaccine strain. In NA antigenic epitopes no mutation was observed with vaccine

strain. Apart from antigenic sites, 9 mutations were detected -N44S, T48A, N200S, R220K, V241I, N248D, I321V, Y351F, N369K. However, representative the amino acid residues in the NA catalytic sites (R118, E119, D151, R152, W179, I223, R225, E277, R293, R368 and Y402) and supporting framework sites that directly interact with the substrate (R156, S180, E228, S247, E278 and N295) were highly conserved. Although most of the glycosylation sites are conserved, A/H1N1pdm slum isolates have a potential mutation in N-linked glycosylation domain NQSQ 042-044 as a result of N44S substitution.

3.10.2 Sequence Variation in A/H3N2

While comparing with vaccine strain at amino acid level A/H3N2/Victoria/361/2011, the hemagglutinin (H3N2 strain 98.9% identities with the vaccine strain. There were no changes in 190-helix, 130-loop and 200-loop in HA receptor binding site (RBS). Moreover, no changes in four conserved residues in the RBS was not detected. Two altered amino acid were detected T144A, one at antigenic A, other one R158G at antigenic B sites. 4 mutations H10Y, Q49R, N161S and N294K were observed. At, H3N2 isolates HA have a deleted N-linked glycosylation than vaccine strain. No changes in four conserved residues, comprising Tyr98, Trp153, His183 and Tyr195 which form the base of the RBS, was not detected.

In NA 98.5% similarities was found with vaccine strains at amino acid level. There were no antigenic epitopes mutation was found with vaccine strain. Apart from antigenic sites, 7 mutations Q49K, T69N, N141D, D151N, R224K, K258E, T329N were detected. However, representative the amino acid residues in the NA catalytic sites (R118, E119, D151, R152, W179, I223, R225, E277, R293, R368 and Y402) and supporting framework sites that directly interact with the substrate (R156, S180, E228, S247, E278 and N295) were highly conserved. Although most of the glycosylation sites are conserved, H3N2 isolates have a potential mutation in N-linked glycosylation domain 329NDSS as a result of T329N substitution.

Genetic analysis of influenza A/H1N1/pdm09 viruses indicated that the amino acid sequences of all genes of the Kenyan samples were largely similar (sequence similarity of 99%-100%) to that of the pandemic prototype and vaccine strain, A/California/07/2009. The largest numbers of amino acid substitutions were observed during the peak period of the pandemic in all genes except in the Matrix gene.

3.11 Amino Acid Sequence Variations in Other Internal Proteins

3.11.1 Sequence Variations in A/H1N1pdm/09

Several notable mutations were also identified in the internal genes of the H1N1pdm strain. The PB1 and PB2 genes showed 99.2-98.9% identities with the vaccine strain at amino acid level. Mutations were observed in the amino acid residues G154D, C263G, I397M, I435T, Y499S, and L509V in PB1 and R54K, M66I, D195N, R251V, R293K, V344M, I354L, and V731I in PB2 (Fig. 3, PB1 and PB2). No mutations in PB1-F2 protein related to pathogenesis such as N66S (common in HPAI H5N1 viruses) were observed. Importantly, H1N1pdm isolate sensitive to oseltamivir due to H275. For the PA gene, the mutations were G18E, V100I, P224S, N321K, I330V, K361R, R362K, A476G and P560H (Fig. 3, PA). This A/H1N1pdm09 isolate showed N31 in M2 protein, thus resistant to amantadine. PB2 gene of H1N1pdm09 slum isolate had a Glutamic acid (E) at the position of 627 (Table 3.6).

Table 3.6 Amino acid sequence variations of influenza A/Dhaka/850/2013 strain from Bangladesh compared with the vaccine strain H1N1 sequence

Gene	Vaccine Strain	AA Identity (%)	Amino Acid Substitutions
HA	A/California/07/2009	97.5	S83P, N97D, K112E, Q163K, T185S, T203S, D228D, T256A, E283K, V321I, K374E, D451N, K499E
NA	A/California/07/2009	98.1	S44N, A48T, S200N, K220R, I241V, D248N, V321I, F351Y, K369N
PB2	A/California/07/2009	98.9	R54K, M66I, D195N, R251K, R293K, V344M, I345L, V731I
PB1	A/California/07/2009	99.2	D154G, G263C, M397I, T435I, S499Y, V509L
PA	A/California/07/2009	99	E18G, I100V, S224P, K321N, V330I, R361K, K362R, G476A, H560P
NP	A/California/07/2009	98.8	I99V, E113X, Q121L, N286S, V315M, S376N, D453E
M	A/California/07/2009	98.8	I80V, K192M, R230K
NS	A/California/07/2009	98.6	I90L, V123I, S205N

3.11.2 Sequence Variations in A/H3N2

The PB1 and PB2 genes showed 99.6-99.9% identities with the vaccine strain. Mutations were observed in the amino acid residues R215K, S633G and A610T (Table 3.7) No mutations in PB1-F2 protein related to pathogenesis such as N66S (common in HPAI H5N1 viruses) were observed.

Importantly, A/H3N2 isolate sensitive to oseltamivir due to H275. For the PA gene, the mutations were observed in H7Q, D396E, and M407I position. M2 showed 92.5% similarities with vaccine strain in amino acid level. Mutations were found- C19M, N20F, D21S, S22L, D24F, H25H, and L26H. This H3N2 isolate showed resistant to amantadine due to presence of N31 in M2 protein.PB2 gene of H3N2 slum isolate was fully adapted to human as this strain had a lysine (K) at the amino acid position of 627.

Table 3.7. Amino acid sequence variations of influenza A/Dhaka/961/2013 strain from Bangladesh compared with the vaccine strain H3N2 sequence

Gene	Vaccine Strain	AA Identity (%)	Amino Acid Substitutions						
HA	A/Victoria/361/2011	98.9	Y10H	R33Q	A128T	G142R	S145N	K278N	
NA	A/Victoria/361/2011	98.5	L49Q	N69T	D141N	N151D	K224R	E258K	N329T
PB2	A/Victoria/361/2011	99.9	T601A						
PB1	A/Victoria/361/2011	99.6	K215R	G633S					
PA	A/Victoria/361/2011	99.6	Q7H	E396D	I407M				
NP	A/Victoria/361/2011	99.8	E99V	R446X					
M2	A/Victoria/361/2011	92.5	M19C	F20N	S21D	L22S	F24D	H25P	H26L
NS	A/Victoria/361/2011	98.2	K26E	D207N	N209D	E229K			
M	No change in M1	100							

3.12 Glycosylation Patterns in HA

In A/H1N1pdm09 slum strains, for HA there were eight putative N-glycosylation sites predicted at AA positions 28, 40, 104, 304, 498, 557. For NA, there were nine putative N- glycosylation sites predicted at AA positions 42, 50, 58, 63, 68, 88, 146, 235. When compared for N-glycosylation with vaccine strain A/California/07/2009 it was found number of N-glycosylation sties remain intact but for HA, slum A/H1N1pdm09 strain gained one extra sites at the AA position of 42 in NA.

In A/H3N2 slums isolates for HA there were twelve N-glycosylation sites predicted at AA position 22, 36, 52, 59, 77, 147,179, 260, 299, 497.For NA, there were eight putative N-glycosylation sites predicted at AA positions 61, 70, 86, 146, 234,367. While compared with vaccine strain A/Victoria/361/2011 for N-glycosylation sites it found that same number of N-glycosylation sites were predicted for slum H3N2 strains.

3.13 Drug Susceptibility

3.13.1 Cell Based Assay for Antimicrobial Sensitivity Testing

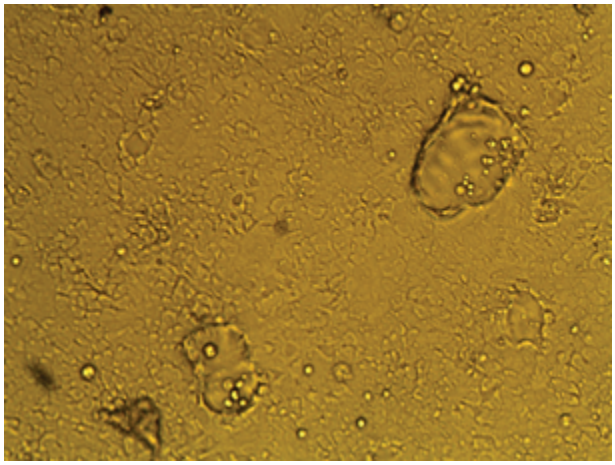
Drug susceptibility testing of influenza strains against local drugs through cell based assay was performed. The susceptibility was determined by measuring the inhibition of virus-induced cytopathic effect in MDCK cells. After two days of post infection, MTT assay was performed to determine the EC_{50} against each drug Ribavirin, Oseltamivir, and Amantadine. Results correlate with drug susceptibility obtained from signature amino acid of sequence where amino acid sequence position indicates drugs susceptibility and resistance.

3.13.2 Effectiveness of Ribavirin Drug on Influenza A Isolates

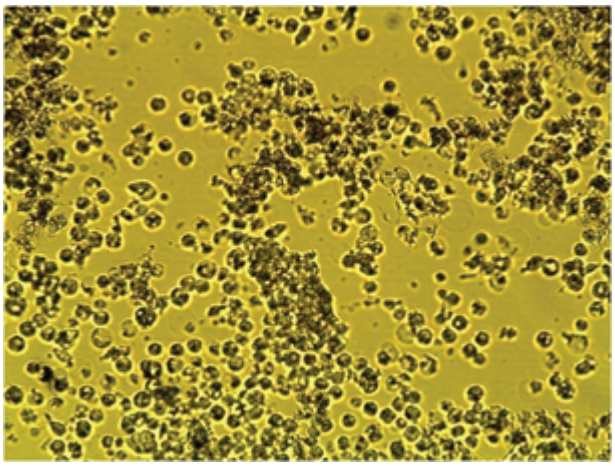
The effectiveness of ribavirin drug was examined against nine influenza A isolates (6 H1N1pdm09 and 3 H3N2) by measuring the inhibition of virus induced cytopathic effect in MDCK cells (Figure 3.17). The monolayer of MDCK cells was infected with approximately 50% cell culture infectious doses ($CCID_{50}$) of virus and infected cells were treated with ribavirin drug at varying concentrations, i.e., 0, 0.1, 1.0, 10.0 and 100 µg/ml. Three days' post infection, MTT assay was carried out to determine the 50% effective concentration (EC_{50}). All nine isolates were found susceptible to ribavirin. The survival of cells was nearly about 25%, 65% and 100% at drug concentration 0.1, 10 and 100 µg/ml, respectively (Table 3.8). The EC_{50} ribavirin for all isolates (H1N1pdm09 and H3N2) were approximately 3.0 µg/ml, which were consistent with earlier reports, suggesting that ribavirin still remains effective against influenza strains.

Table 3.8. Effectiveness of Ribavirin drug on MDCK cell line containing influenza A/H1N1& A/H3N2 virus

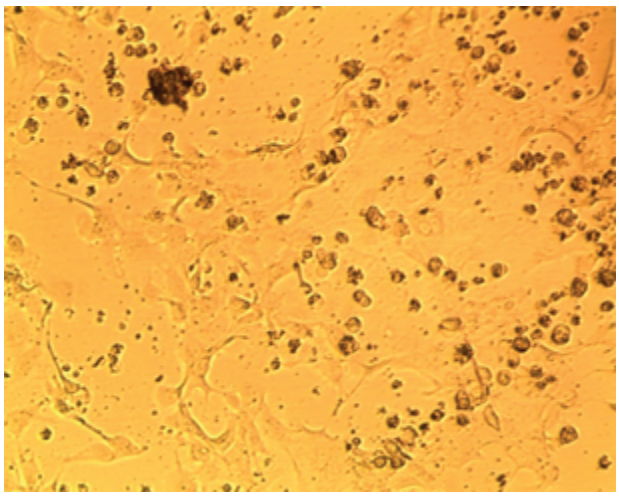
Virus	Isolates	Cell survivability (%) at drug concentration (µg/mL)				EC_{50} (µg/mL)
		0.1	1	10	100	
	Mock	100	100	100	100	
H1N1 pdm09	1	26	45	65	100	3.0
	2	25	46	66	100	2.9
	3	23	47	64	100	2.8
	4	28	44	66	100	3.0
	5	26	47	64	100	2.7
	6	25	44	42	100	3.8
H3N2	7	27	46	65	100	3.0
	8	26	44	63	100	3.6
	9	26	46	65	100	2.9



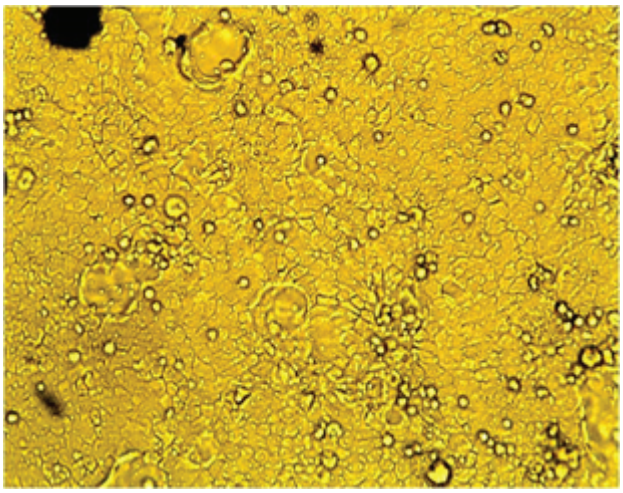
A. MDCK Cell (without drug or virus)



B. MDCK Cell line infected with virus



C. EC50 (3 days post-infection)



C. CPE at at drug concentration (3.0 µg/ml)

Figure 3.17. PDruf susceptibility assay against Ribavirin.

3.13.3 Effectiveness of Oseltamivir Drug on Influenza A Isolates

Oseltamivir drug was tested at concentrations ranging from 0.1, 1.0, 10.0 and 100 µg/ml (Table 3.9). The EC_{50} values for both A/H1N1pdm09 and H3N2 subtypes of influenza A virus was 0.35 µg/ml, which was similar to the previously reported EC_{50} value of oseltamivir drug (0.20 µg/ml).

Table 3.9. Effectiveness of Oseltamivir drug on MDCK cell line containing influenza A/H1N1 and A/H3N2 virus

Virus	Isolates	Cell survivability (%) at drug concentration (µg/mL)				EC_{50} (µg/mL)
		0.1	1	10	100	
	Mock	100	100	100	100	
	1	45	67	100	100	0.31
	2	44	63	100	100	0.39
H1N1	3	43	68	100	100	0.35
pdm09	4	42	69	100	100	0.35
	5	44	70	100	100	0.3
	6	43	69	100	100	0.35
	7	46	70	100	100	0.27
H3N2	8	45	67	100	100	0.31
	9	44	67	100	100	0.32

3.13.4 Effectiveness of Amantadine Drug on Influenza A isolates

When influenza A infection was challenged with amantadine drug, eight isolates out of nine (88%) demonstrated susceptibility to amantadine drug (EC_{50}, 0.30 µg/ml), which was consistent with prior studies. But, one A/H1N1pdm09 isolate (isolate 6) exhibited significantly higher EC50 value (21 µg/ml) than other eight isolates (Table 3.10). This reduced susceptibility clearly established the resistance of that isolate to amantadine drug. Amantadine drug itself has cytotoxic effect at 10 µg/ml.

Table 3.10 Effectiveness of amantadine drug on MDCK cell line containing influenza A/H1N1& A/H3N2 virus

Virus	Isolates	Cell survivability (%) at drug concentration (µg/mL)				EC_{50} (µg/mL)
		0.1	1	10	100	
	Mock	100	100	100	100	
	1	42	74	100	90	0.3
	2	45	82	100	86	0.2
H1N1	3	43	79	100	90	0.29
pdm09	4	42	76	100	87	0.31
	5	40	65	100	90	0.45
	6*	18	25	38	49	21
	7	40	74	100	90	0.37
H3N2	8	38	76	100	85	0.40
	9	42	75	100	90	0.30

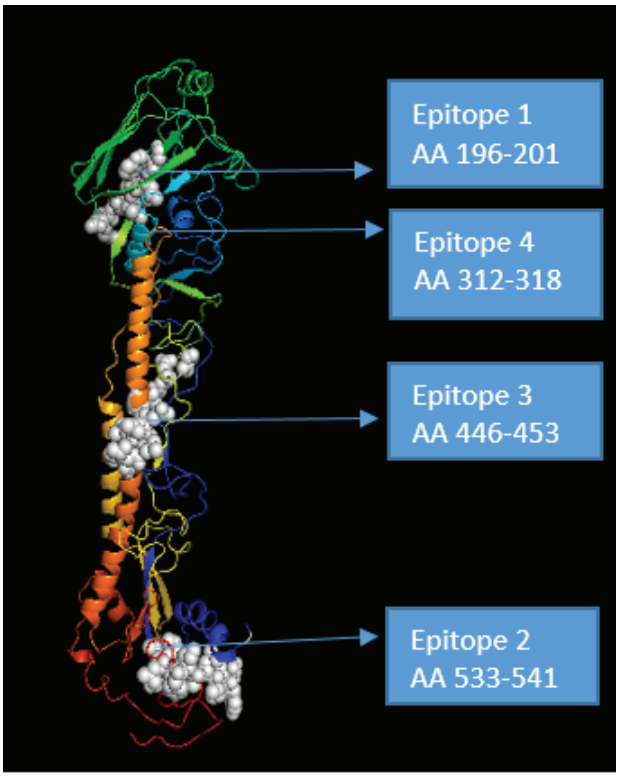
3.14 Epitope Prediction

The HA and NA sequence from both A/Dhaka/850/2013 and A/Dhaka/961/2013 were analyzed for epitope prediction with Immune Epitope Database (IEDB). The HA sequence of A/Dhaka/850/2013 showed 12 candidate epitopes through Kolaskar and Tongaonkar semi-empirical method. Four of these epitopes were likely to include antibody response because they showed MHC class II binding peptide capabilities in ProPred and EMBOSS (Figure 3.18 A). In addition, identification of multiple highly conserved region in HA indicates possibility of neutralization of this isolate with existing vaccine strain. Epitope extending between amino acid residues of 196-201, 533-541, 313-318 and 446 to 453. The NA sequence of A/Dhaka/850/2013 showed 4 highly conserved sequence epitopes with ProPred and EMBOSS. The epitopes extend between amino acid residues 92 to 100, 121 to 127, 316 to 321 and 418 to 426 (Figure 3.18 C). The HA sequence of A/Dhaka/961/2013 has 4 conserved regions between amino acid residues 38 to 49, 110 to 115, 439 to 445 and 527 to 533 as visualized in PyMoL (Figure 3.18 B). The NA of this isolates shows candidate epitope of between amino acid residues 194 to 202, 238 to 244, 418 to 432 and 436-446 (Figure 3.18 B). The identification of potential epitopes for IG and MHC II binding in HA and NA form the two isolates indicate a possibility to engineer them into peptide subunit vaccine. A successful subunit vaccine against influenza virus must overcome safety issues. In addition, in-depth experimental proof with in vitro peptides and in-vivo models in need to validate the finding of the in-silico experiment.

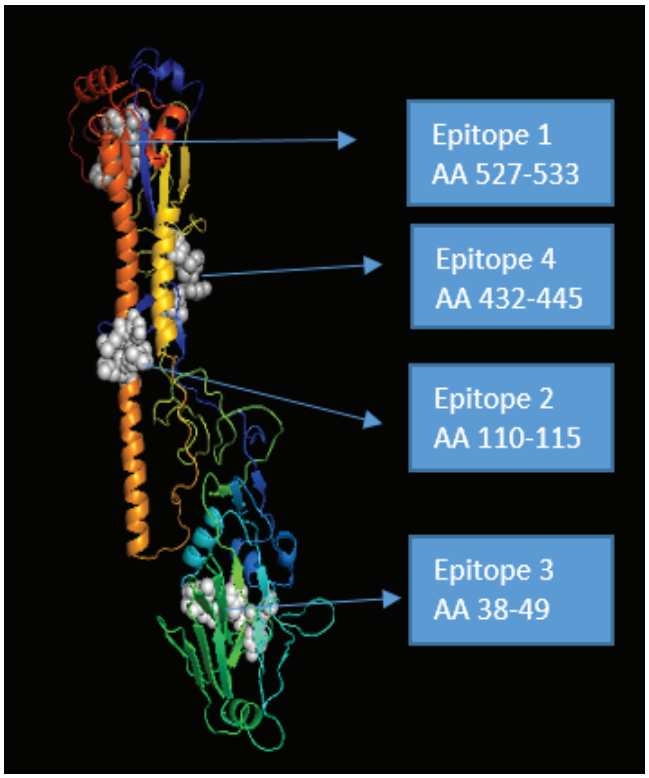
Table 3.11 Physicochemical properties, Antigenic Site Prediction of Bangladeshi Influenza Isolates

Physicochemical properties of the HA and NA

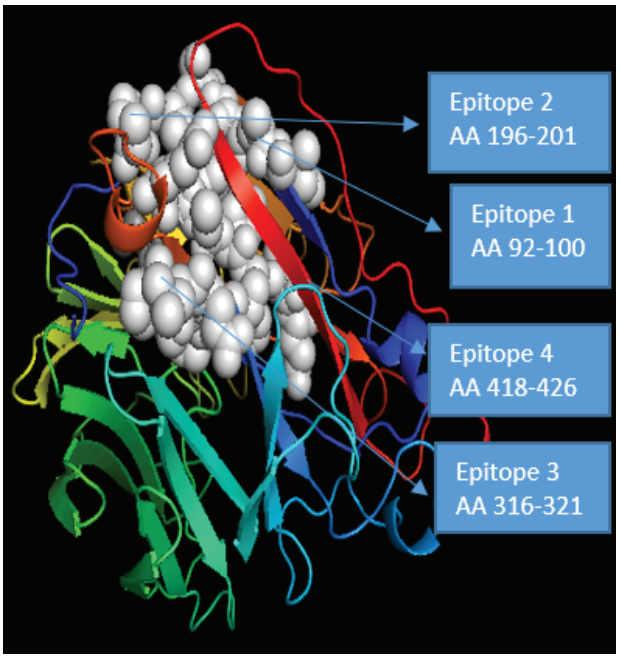
Physicochemical properties	HA of A/H1N1pdm09	NA of A/H1N1pdm09	HA of A/H3N2	NA of H3N2
Molecular Weight (kDa)	63362.75	51499.89	63552.24	53485.51
Negatively charged residue (Asp + Glu)	60	40	58	53
Positively charged residue (Arg + Lys)	61	37	63	48
Theoretical pI	7.51	6.11	8.3	6.1
Instability index	30.71	34.49	35.17	29.77
Aliphatic index	81.29	73.94	83.41	77.39
GRAVY (Grad Average of Hydropathicity)	-0.367	-0.248	-0.349	-0.281



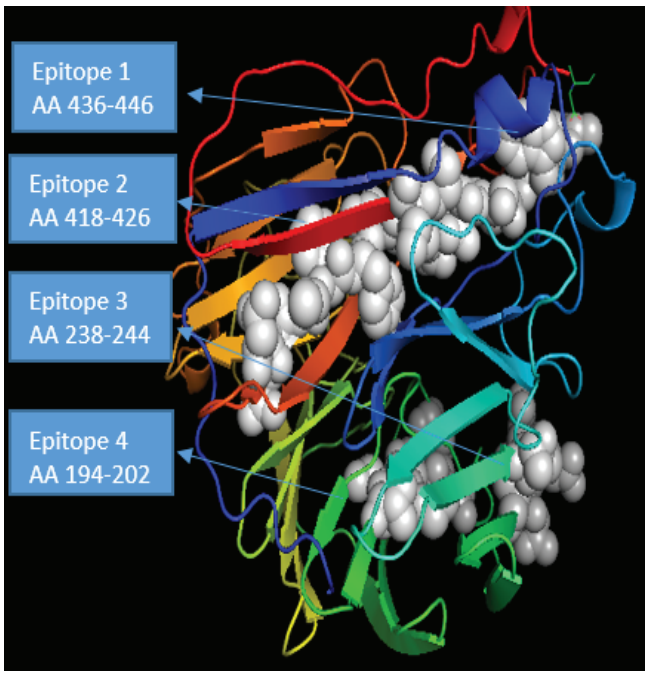
A. HA based H1N1



B. HA based H3N2



C. NA based H1N1



D. NA based H3N2

Figure 3.18. Candidate based epitope prediction, visualization, and annotation of Bangladeshi isolates.

Antigenic Site Prediction

HA

A/H1N1(pdm09)				
Amino acid sequence	Start	End	Length	Score
IYQILAIY	533	541	8	1.2
AELLVLLE	446	453	8	1.185
KEVLVLWGIHHPS	194	206	13	1.169
SLPFQNI	312	318	7	1.14

A/H3N2				
Amino acid sequence	Start	End	Length	Score
LGHHAVPNG	29	38	9	1.175
DWILWIS	527	533	7	1.2
DLWSYNAELLVALE	432	445	13	1.16
NCYPYDV	110	116	7	1.18

NA

A/H1N1(pdm09)				
Amino acid sequence	Start	End	Length	Score
NSSLCPVSGWAIY	92	100	8	1.64
FISCSPL	121	127	7	1.142
IRPCFWVEL	418	426	8	1.17
GYLCSG	316	321	6	1.164

A/H3N2				
Amino acid sequence	Start	End	Length	Score
VLWTSNSIVVF	436	446	11	1.187
INRCFYVEL	418	426	9	1.176
VVMTDGS	238	244	7	1.184
ITGDLKNAT	194	202	9	1.19

3.14.1 Result in ProPred Server of Predicted Candidate Epitopes

Another online based tool was used to predict candidate epitope for confirmation. Here we use EMBL EMBOSS to check the matching of epitopes predicted from IEDB. For analyzing candidate epitope whether it can bind with MHC to present the antigen we used online base tool ProPred (Table 3.18). All the MHC binding candidate epitope contain any of hydrophobic amino acid which include Valine (V), Leucine (L), Alanine (A) and Glutamic Acid. We checked for surface exposed amino acid also.

3.14.2 Candidate Epitope Visualization and Annotation

We predicted peptide epitope by web based tool by swiss model and confirmed it by Phyre 2. This The peptide structure was visualized and annotated by PhyMol. The HA and NA based candidate epitopes of H1N1pdm and H3N2 are shown in Figure 3.17. Physicochemical properties of HA and NA, output of antigenic site prediction, and candidate epitopes MHC binding capabilities in ProPred are shown in Table 3.12.

Table 3.12 Candidate epitopes MHC binding capabilities in ProPred

HA

A/H1N1(pdm09)		
Amino acid sequence	Start	End
IYQILAIY	533	541
AELLVLLE	446	453
KEVLVLWGIHHPS	194	206
SLPFQNI	312	318

A/H3N2		
Amino acid sequence	Start	End
LGHHAVPNG	29	38
DWILWIS	527	533
DLWSYNAELLVALE	432	445
NCYPYDV	110	116

NA

A/H1N1(pdm09)		
Amino acid sequence	Start	End
NSSLCPVSGWAIY	92	100
FISCSPL	121	127
IRPCFWVEL	418	426
GYLCSG	316	321

A/H3N2		
Amino acid sequence	Start	End
VLWTSNSIVVF	436	446
INRCFYVEL	418	426
VVMTDGS	238	244
ITGDLKNAT	194	202

Chapter 4

Discussion

4. Discussion

To control influenza infection globally, coordinated epidemiologic and virologic surveillance is a critical component of pandemic preparedness. The severity of influenza disease in Bangladesh can vary widely and is determined by the characteristics of circulating viruses, seasonality, how well the vaccine is working to protect against illness, and how many people got vaccinated. Limited information is available on influenza infections from slum population in Dhaka which constitute one third of the total population of the city. We hypothesize that the burden and genetic variation of influenza viruses in urban slum setting is very high, because of population density, unhygienic condition, rapid migrations and unawareness. Therefore, a cost effective and sustainable procedure is required to contain this disease in low income countries like Bangladesh. This study aims to investigate the burden of the disease in slum population of Dhaka city by detecting influenza viruses, to characterize circulating strains, to explore genetic variations between slum strains and vaccine strains as well as globally circulating strains, to assess drug susceptibility of the circulating strains and to predict putative candidate peptide vaccine as an alternative approach for preventing influenza viruses. We used molecular and bioinformatics approaches to achieve our objectives. Molecular phylogenies can reveal many aspects of the transmission, epidemiology, and evolution of rapidly evolving pathogens (Pybus and Rambaut 2009). Analysis of influenza virus genomes during the emergence of influenza A(H1N1)09pdm virus and A(H3N2), causing the first influenza pandemic in 40 years therefore provides a unique opportunity to track the transmission dynamics of a new influenza virus in an immunologically naïve population (Baillie, Galiano et al. 2012). The application of complete virus genome sequencing and analysis has already provided detailed insights into seasonal influenza virus infections (Ghedin, Sengamalay et al. 2005, Rambaut, Pybus et al. 2008). This analysis facilitates comparison and understanding of the evolutionary dynamics of circulating viruses and the prediction of potential evolution events that are likely to result in new strains (Greninger, Chen et al. 2010). It also allows closer examination of the importance of other genes in influenza outbreaks and vaccine selection. However, despite the availability of complete influenza genome data from several developed countries, South Asian countries including Bangladesh still provide incomplete genomes into the public domain. Most influenza sequencing in these countries has focused on the HA1 domain of the haemagglutinin gene where mutations have the greatest effect on the antigenic structure (Barr et al., 2010). In fact, by January

2013, there were only eight complete genomes of influenza A(H1N1)pdm09 virus from Africa that were publicly available in the Influenza Virus Resource database (Bao, Bolotov et al. 2008).

The current study which was conducted in three slums of Dhaka city identified influenza viruses in 10% patients with influenza-like illness. Percentage of positive influenza virus was higher in Rayerbazar and Mohammadpur (11%) than Hazaribagh slum (6%). Female (60%) were more likely to be infected by influenza viruses in this study. However, other studies did not find any significant deference with respect to influenza positivity in gender-wise distribution (Zhang, Li et al. 2009). Demographic data revealed that higher incidence rate was found in two age groups: < 5 years (40%) who are likely more venerable for viral infections and 5-19 years (35%), a majority of this group are school going children who may have exposed to viruses at higher rates than others (Greer, Tuite et al. 2010). CDC also reported that the overall attack rate was the highest among children aged 5-14 years. In 2009, A(H1N1)pdm09 strain was characterized by high morbidity in adults while mortality was higher in children and elderly ages >60 years (Greer, Tuite et al. 2010). Several studies reported that influenza A(H3N2) and influenza B infection usually affects age group >1-15 years (Greer, Tuite et al. 2010, Das, Sami et al. 2011, Broor, Krishnan et al. 2012). Others report that influenza A(H3N2) and influenza B viruses infect the people of all age group but children and elders are more prone to infection, possibly due to weaker immune system in these individuals.

Clinical manifestation due to different influenza strains may vary. Determining clinical presentation associated with different strains is useful for epidemiological comparison and help clinicians to understand effective control strategies. Some studies show that A(H1N1)pdm09 virus was pathogenic with more complicate symptoms in comparison to influenza A(H3N2) and influenza B viruses (WHO 2009. , Dawood, Iuliano et al. 2012). Since, all the samples in this study were collected from patients with influenza-like illness, no major differences in clinical symptoms could be identified. However, symptoms of runny and headache varied significantly; 77% and 41% people with influenza positive complained of runny nose and headache respectively as compared to 52% and 20% in influenza negative patients.

The study data suggest that May-September was the peak influenza season in Bangladesh. This is consistent with reported seasonality of influenza infection form the population bases surveillance in Kamalapur neighborhood of Dhaka city where the peak season was April to September during 2004-2006 (icddrb 2006). This period of the year is typically considered as rainy season in Bangladesh and it is possible that, during the monsoons, people spend more time inside homes in small poorly ventilated spaces which may increase influenza transmission [44infl]. Similar finding was observed in Pune which has a tropical climate where influenza outbreaks occurred predominately during the rainy months (Rao and Banerjee 1993, Agrawal, Sarkar et al. 2009).

One of the study goals was complete genome characterization of the circulating influenza A isolates of slums of Dhaka city, and to decipher conservative and no conservative substitutions, its comparative analysis with respect to the Bangladesh and global circulating influenza A viruses as well as the vaccine strains. Genome analysis helps understand the molecular evolution of viruses and other genetic factors unique to isolates that might help inform the formulation of vaccines for specific regions. Molecular methods used in the current study have produced an enormous amount of data on molecular characterization of specific viruses and datasets of complete genome sequences of viral isolates. Consistent with findings from previous reports, the isolates from our study showed low genetic diversity. The nucleotide sequence analysis revelated that there is no significant difference among viruses recovered from three different slums. In whole genome sequence, we successfully sequence 13,389 nt of a representative A(H1N1)pdm09 strain, A/Dhaka/850/2013 and 13482 nt of H3N2 strain, A/Dhaka 961/2013.

While the influenza A(H1N1)pdm09 strains isolated in this study had specific amino acids that defined them, they were generally similar to the reference strains, with no significant antigenic variation from the vaccine strain A/California/07/2009. Phylogenetic tree for each of the gene segments of the influenza A(H1N1)pdm09 viruses revealed seven distinct clades 1-7 (Nelson, Spiro et al. 2009). The vaccine strain A/California/07/2009 belongs to clade 1 and the Bangladeshi strain including contemporary strains belonged to clade 6 (Sharma, Joshi et al. 2013). Using global isolates whose clade identities are known, the phylogenetic analysis could show that the initial introductions of the influenza A/(H1N1)09pdm virus in the country belonged to two different global clades namely 2 and 7 (Fig3.X). Besides differences in geographical distribution, these two

clades differ genetically in the mutations they harbour relative to the prototype A/California/7/2009 strain. Clade 2 viruses have the M581L and T373I mutations in the PA and NP proteins respectively while clade 7 viruses lack these mutations but possess the mutations S220T in their HA, V100I in their NP, V106I and N248D in their NA and I123V in their NS1(Nelson, Spiro et al. 2009). In this study, clade 2 viruses were not isolated beyond the initial introduction cases.

The limited information on whole-genome sequences from H3N2 influenza virus strains from Asian countries has constrained the region's contribution toward generating the information needed to develop new vaccines, therapies, and diagnostics for influenza. There are reported mutations in the influenza H3N2 strains that can lead to increased fitness. However, on the basis of our complete genome analysis, the H3N2 isolates in our study did not carry major amino acid substitutions that are associated with efficient transmission or fitness, although biological experiments would be needed to further confirm this observation. Most of the isolates in our study shared high similarity with contemporary reference strains from other regions, which indicates that the slum viruses could be because of the introduction of new viruses into Bangladesh from the rest of the world rather than independent evolution of variants within the country. In general, we observed that in Dhaka, as in South Asia, during each influenza season, viruses cluster according to their date of collection rather than by location.

Two gene segments (HA and NA) of influenza B isolates from different slums of Dhaka city in Bangladesh were analyzed and compared with those of the WHO recommended vaccine strains B/Brisbane/60/2008 and B/Wisconsin/1/2010 for Northern Hemisphere and with other epidemiologically relevant global strains from both GenBank and GISAID databases. The similarity between Bangladeshi strains and other global strains is such that influenza B viruses are seeded from around the world rather than independent evolution within the country.

The most salient feature of influenza evolution in humans is its antigenic drift. This process is characterized by structural changes in the viruses B-cell epitopes and ultimately results in the ability of the virus to evade immune recognition and thereby re-infect previously infected hosts (Yuan and Koelle 2013). In the PB2 protein of influenza A viruses, E627K and D701N mutations are associated with increased virulence and adaptation of avian influenza viruses to the human host

(Herfst, Chutinimitkul et al. 2010). However, examination of the PB2 amino acid residues of the slum isolate A/(H1N1)09pdm influenza viruses showed that these two mutations were absent. The polymerase complex of influenza A(H1N1)09pdm virus was derived from triple-reassortant swine viruses, the PB2 gene of which was of avian origin and entered pigs around 1998 (Garten, Davis et al. 2009). The fact that this avian-origin PB2 gene did not significantly benefit from the substitutions E627K and D701N (locally and globally). But in the H3N2, slum isolates E627K was found their might avian origin PB2 gene significantly benefit from substitutions locally which allow enhanced replication. The K526R substitution in the PB2 protein was observed in the slum H3N2 isolate. The absence of K526R in H1N1pdm09 substitution beyond the early phase suggests that K526 offers transmission or replication advantage over R526 in humans.

PB1 is the core subunit for assembly of the virus RNA polymerase as the N-terminal tip of PB1 binds to the C terminus of PA while the C terminus of PB1 binds to the N terminus of PB2 (Toyoda, Adyshev et al. 1996). In the current study, no fixed amino acid substitutions were observed in the PB1 of the slum isolates. The main observation in the PB1 protein of viruses from this study was that most of the sites harboring amino acid substitutions are located towards the C-terminus of the protein which associate with the PB2 protein by binding to the N-terminal end of the PB2 protein during replication. Like the PB2, the influenza A(H1N1)09pdm virus PA is also avian-like. It contains 7 avian virus unique residues (28P, 55D, 57R, 65S, 100V, 312K, and 552T), and only 3 human virus residues (356R, 382D, and 409N). This is even though the gene was maintained in swine viruses for over a decade (Chen and Shih 2009). The study suggests that these two mutations were crucial in efficient replication of influenza A/(H1N1)09pdm slum isolate.
Mutations in HA protein have implications in receptor specificity, host range and pathogenicity. Mapping of the HA mutations observed in the slum isolates on to the known antigenic sites (Caton, Brownlee et al. 1982, Skehel and Wiley 2000) and receptor binding sites (Skehel and Wiley 2000) was carried out. Of the 13 amino acid substitutions observed in the HA protein amongst the slum A/(H1N1)pdm 09 isolate, 10 (77%) occurred in the HA1 domain while only 3 (23%) occurred in the HA2. Since HA1 is the most important in viral pathogenesis in initiating infection, the protein is a primary target for antibodies to neutralize the virus and is therefore more variable than HA2 (Skehel and Wiley 2000). Another important observation noted was that 3 of the 10 sites (i.e. at positions 163, 185 and 203) that had amino acid changes in the HA1 are located within known

antigenic sites. Of these, position 203 is located at antigenic site Ca while the other 163, 185 are located at site Sa and Sb. Outside of these antigenic epitopes, 10 mutations- P83S, D97N, E112K, N228D, A246T, K 283E, I321V, E374K, N451D and E499K were also observed. Regarding amino acid changes in the receptor binding site, only amino acid position 239 and 202 showed substitutions in two isolates. At position 239, two local isolates possessed 239E while all the remaining had 239D. At this position among influenza A(H1N1)09pdm viruses, 239D is the wild type amino acid. Studies have shown that mutations at this position alter influenza virus receptor binding specificity (Abed, Pizzorno et al. 2011). The hemagglutinin position 374 corresponds to the HA2-subunit position 47. E391K mutation has been shown to occur simultaneously with D114N (Maurer-Stroh, Lee et al. 2010). This observation was also seen in this study. This (E374K) mutation has been shown to drastically alter the oligomerization interface of HA1 and HA2 in a region that undergoes structural changes required for membrane fusion (Maurer-Stroh, Lee et al. 2010). Furthermore, this region was recently identified as a highly-conserved epitope recognized by antibodies that neutralize the closely related 1918 H1N1 virus by blocking the structural changes associated with membrane fusion (Ekiert, Bhabha et al. 2009). In H3N2 slum isolates, two altered amino acid were detected; one T144A at antigenic A, and other one R158G at antigenic B sites. In addition, 4 mutations H10Y, Q49R, N161S and N294K were observed. In both isolates, there was no receptor binding sites (RBS) remain conserved. The NP assembles with the three subunits of the polymerase into a ribonucleoprotein complex (RNP) which controls transcription and replication. In this complex, it regulates the balance between transcription and replication during the virus cycle (Portela and Digard 2002). The most dominant amino acid change in this protein of slum isolates was found to be the V100I substitution. Globally, in the pre-epidemic period only 10% of influenza A(H1N1)09pdm viruses had this valine to isoleucine change at position NP100, whereas about 57%, 80% and 93% of the virus isolates collected in the early, middle and late periods possessed this change, respectively (Pan, Cheung et al. 2010). In H3N2 slum isolate no change of amino acid was found at the position 100.

The influenza NA gene removes sialic acid from virus and cellular glycoproteins to facilitate virus progeny release and spread of infection to new cells. In NA, no antigenic catalytic mutation was observed. For both slum isolates H1N1pdm09 and H3N2, NA catalytic sites and supporting framework sites remain intact. In the slum influenza A(H1N1)09pdm virus isolate, two dominant

mutations were observed. The non-charged residue, asparagine at NA-248, mutated to a negatively charged residue, aspartic acid, which was only presented in the 1977 H1N1 pandemic viruses. Another mutation, N248D, which is a non-clade 1 defining mutation suggesting that no clade 1 viruses are circulated in the country. In addition, position 248 is in the proximity of the catalytic NA pocket (Maurer-Stroh, Ma et al. 2009). This result revealed that novel substitutions were found in slum isolated A/H1N1pdm09 strain which is in agreement with previous studies (Maurer-Stroh, Ma et al. 2009, Kao, Chan et al. 2012). Another mutation observed in the NA of local isolates is the N369K mutation, if acquired together with V241I, could potentially facilitate accommodation of the H275Y substitution without loss of fitness (Hurt, Hardie et al. 2012). This accommodation would lead to neuraminidase inhibitor resistant influenza viruses that are easily transmissible among humans. As a result, this mutation should be closely monitored in the successive seasons.

The M protein gene of influenza A viruses encodes two proteins, M1 and M2, derived by splicing of mRNA. The Ml internal protein is a major component of the virus particle with an essential role in virus assembly and budding while the M2 has a proton-selective ion channel activity and is involved in virus assembly (Garcia-Robles, Akarsu et al. 2005). It has been indicated previously that M1 is an interior virion protein (matrix) that lies just beneath the viral envelope (Garcia-Robles, Akarsu et al. 2005). On the other hand, the M2 is an integral membrane protein inserted into the viral envelope and projects from the surface of the virus as tetramers (Lamb, Zebedee et al. 1985).

An examination of the amino acid polymorphisms seen in the M1 and M2 proteins encoded by the M gene showed that there were more amino acid substitutions in the M2 (1/97) than in the M1 (3/252) protein of slum influenza A(H1N1)09pdm viruses. Thus, the M1 protein was more conserved than the M2. The host's immune response seems to have exerted stronger selective pressure on the M2 than that on the M1 protein, accounting for the increased number of amino acid substitutions in this protein in a bid to evade immune pressure (Liu and Ye 2002, Furuse, Suzuki et al. 2009). In H3N2 strain there was no change in M protein.

The NS gene segment of the influenza A virus encodes two proteins; the nonstructural protein 1 (NS1) and the nuclear export protein (NEP). NS1 is essential to inhibition of the host immune

response as it suppresses the host type 1 interferon (IFN) (Hale, Randall et al. 2008). NEP on the other hand in association with M1, mediates the nuclear export of viral ribonucleoprotein (vRNP) complexes (Neumann, Hughes et al. 2000). Deletions in the NS1 protein have been associated with increased influenza A virulence. Studies have also identified single mutations in the NS1 gene (S42P, D92E and V149A) as well as multiple mutations in the PDZ ligand domain that increase influenza pathogenicity (Li, Jiang et al. 2006, Jiao, Tian et al. 2008). In this study, the two proteins encoded by the NS gene also exhibited a different number of amino acid substitutions. Generally, the NS1 protein was more conserved than the NEP (Qu, Zhang et al. 2011).

Genome-based approaches were typically used when investigating changes in glycosylation, as well as when predicting function, due to the great simplicity of these measurements (Zhang, Gaschen et al. 2004, Vigerust, Ulett et al. 2007, Igarashi, Ito et al. 2008, Zhirnov, Vorobjeva et al. 2009). Such studies were based upon locating the sequon in the amino acid sequence predicted by the viral RNA. This method was based on assumption that all potential N-glycosylation sites were occupied. Unfortunately, the amino acid sequence is only one determinant of glycosylation because the location of glycosylation sites and the host environment also have a strong effect on glycosylation (Schulze 1997, Lin, Wang et al. 2009, Schwarzer, Rapp et al. 2009). Our results showed that two modes of protein glycosylation site alteration were involved in the evolution of human seasonal influenza viruses when compared with respective vaccine strains. The first mode was to increase the number of glycosylation sites. This mode was obvious and have been reported many times before in both human H1N1 and H3N2 viruses (Zhang, Gaschen et al. 2004, Igarashi, Ito et al. 2008, Zhirnov, Vorobjeva et al. 2009, Das, Puigbo et al. 2010). The other mode was the positional conversion of glycosylation sites (also called glycosylation site substitution), which occurs when the acquisition of a new glycosylation site in the new strain is accompanied by the loss of an existing glycosylation site. The acquisition and loss of glycosylation sites may not occur simultaneously and the acquired glycosylation sites may also not adjacent to the lost glycosylation sites in the primary structures of the proteins, as some of the exchanges occurred over an intermediate time and/or distance frame, for instance, the loss of conversion of glycosylation site 140 position on HA of H3N2 slum isolate. Our results also indicated that positional conversion of glycosylation sites might be a more effectively alteration mode. The presence of extra glycosylation of glycans on the head of 42 position of NA of H1N1pdm09, and 329 position of

NA H3N2 strains can have either positive or detrimental effects on the virus (Schulze 1997, Abe, Takashita et al. 2004). While it shields antigenic sites from immune recognition, it reduces receptor affinity of HA and enzymatic activity of NA (Schulze 1997, Abe, Takashita et al. 2004). Since only few glycans are attached at the antigenic sites and surrounding regions of HA and NA in H1N1pdm09 viruses and H3N2 strains, the acquisition of new glycosylation sites (increase in glycosylation site numbers) in these regions mainly play its positive role in escaping antibody recognition from hosts in the early evolution stages of seasonal strains, and thus it is necessary for seasonal virus to continue to prevail among humans. Therefore, alteration of glycan location (positional conversion of glycosylation sites) became a more suitable way for evolution of H1N1pdm09 viruses. By simply changing the location of the glycans, but no new glycans added, the viruses obtained the ability of escaping immune recognition from host antibodies again. So, the positional conversion of glycosylation sites may be more artful than the increase of glycosylation site numbers for the host adaption for influenza virus.

Two classes of antiviral drugs are currently in use for the treatment of influenza virus infections. These are the neuraminidase inhibitors and M2 inhibitors (Adamantanes). These drugs inhibit influenza virus replication by binding to the NA and M2 proteins respectively. While these two classes are generally effective, the emergence of drug resistant is always a constant threat. This is much so considering the error prone nature of influenza polymerases. Monitoring the susceptibility of circulating influenza viruses is therefore of utmost necessity particularly in a pandemic situation. The study therefore determined the susceptibility of the slum isolates by examining for known molecular markers of resistance in the NA and M2 proteins.

Resistance to M2 inhibitors can be achieved by a single substitution of any of the amino acid residues located at positions 26, 27, 30, 31, or 34 of the transmembrane domain of the M2 protein (Boivin, Goyette et al. 2002). In the vast majority of cases, the basis for this resistance is a single serine to asparagine amino acid replacement (S31N) in the matrix M2 protein (Wang et al., 1993). This mutation weakens (M2) transmembrane helical packing and thereby disrupts the drug binding pocket (Pielak, Schnell et al. 2009). None of slum isolates harbored mutations at positions 26, 27, 30, or 34. However, all the slum isolates had the S31N mutation and were thus resistant to M2 inhibitors. This resistance has also been found in all currently circulating influenza

A(H1N1)09pdm viruses isolated globally (Neumann, Noda et al. 2009). Resistance to neuraminidase inhibitors is conferred by any of the following mutations in the NA; I117V, E119V, D198N, I222V, H274Y, R292K, N294S, I314V, V116A, R118K, E119G/A/D, Q136K, D151E, R152K, R224K, E276D, R292K and R371K [N2 numbering] (Colman, Varghese et al. 1983). These molecular markers associated with resistance to neuraminidase inhibitors are located in the active site of the NA protein at different positions depending on the virus subtype, thus altering its sensitivity to inhibition (Hayden, Klimov et al. 2005). The study examined the slum isolates for oseltamivir and zanamivir-related mutations. No isolates did carry the resistance conferring mutations indicating their sensitivity to neuraminidase inhibitors. Thus, molecular signature result correlate with the results obtained from the drug susceptibility testing based on cell based assay.

The in-silico methods for predicting B-cell immunogenic regions focus on linear epitopes and are based on several amino acid-based propensity scales, including hydrophilicity, solvent accessibility, secondary structure, flexibility, and antigenicity (Ben-Yedidia and Arnon 2007, Wang, Wu et al. 2011, Shahsavandi, Ebrahimi et al. 2015). The total residues lying in B cell epitopes were differ from other constructs. Nearly half of amino acid residues in each predicted B cell epitopes belonging to the interaction with antibodies, the surface structures of antigenic sites that are accessible for antibodies were detected. The antigenic peptides of located in solvent accessible regions contain low complexity and high-predicted flexibility. It seems that the additional epitope can play a role in stimulating and enhancing cellular response against influenza infection but it must require the next generation of adjuvants to deliver properly. The synthesized peptides are subsequently conjugated to carrier molecules or adjuvants as required. Immunoprofiling of resultant constructs is conducted in vitro, as well as in suitable animal models for determinations of safely and efficacy, followed by progression to pre-clinical and clinical trials.

Chapter 5

Conclusion

5. Conclusion

This study demonstrates several intriguing findings towards the understanding of genetic diversity of influenza viruses in Bangladesh and prediction of alternate vaccine approaches to reduce the burden of the disease. First, at least four types of influenza viruses were circulating during the study period which justifies that a quadrivalent influenza vaccine formulation that includes influenza A, influenza B, and both lineages of influenza B viruses could be more effective to reduce influenza disease burden in the country. Second, although Bangladeshi strains and WHO recommended vaccine strains are antigenically similar, several mutations were identified. It is interesting to see whether these mutations have any impact on vaccine efficacy. Third, all slum strains were found to be sensitive to the drugs routinely used for influenza treatment. The strains were analyzed based on known molecular markers for the drug resistance which could be useful for the clinical management of the patients particularly during pandemic situation. Fourth, prediction of HA and NA based candidate epitopes on the study strains could facilitate alternate vaccine approach. Notably, in Bangladesh the influenza vaccination has not been implemented in the national vaccination schedule and the vaccine effectiveness among Bangladeshi population is remained unknown. Therefore, assessment of current vaccine effectiveness as well as efforts to assess alternate vaccine approaches are required. In summary, the findings of this study contribute to understanding the characterization of slum influenza viruses that will be useful for routine surveillance, potential drug recommendation. Moreover, candidate epitope prediction will guide for alternate and improved vaccine for the control of influenza in future.

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Appendix I

Questionnaire for Strategies in reducing the burden of influenza infections in slum, Dhaka, Bangladesh

Specimen collection Form

Identifying Information

- Serial I.D. ☐☐☐☐
- Date of Interview (dd/mm/yyyy) / / _____
- Name of Interviewee _____
- Date of Birth / /____
  - Age (years) ☐☐
  - Age (months if < 5 years) ☐☐
- Gender ☐
  Male = 1, Female = 2
- Location
  - Address _____
  - Slum _____
- **Name of Head of Household _____**
- Respondent's Name _____
- Respondent's Relation to Subject, if other than subject ☐

  Wife = 1, Husband = 2, Mother = 3, Father = 4, Daughter = 5, Son = 6, Self = 7, Other = 8 (Specify) _____

- What type of profession you are involved? ☐
  Rishwapullar = 1, Hawkar = 2, Garments worker = 3, industrial worker = 4, meson = 5,Helper = 6, Self = 7, Business = 8
  others=9 (Specify) _____
- Do you (your child) have regular income source? ☐
  1 = Yes, 2 = No, 3 = Do not know

- Which type of income source? ☐☐
  Daily=1, weekly =2 Monthly = 3

- What is your educational background?
  No Education= 1 Start schooling=2, Class five=3, Class eight=4
  S.S.C=5, H.S.C=6 ,Others=7 Speicify_____

History of Present Illness

- Chief complaint _____
  - For how many days have you (your child) had this? ☐☐
- Do you (your child) have fever? ☐
  1 = Yes, 2 = No, 3 = Do not know
  - Date of fever onset (dd/mm/yyyy) / /_____
- Do you (your child) have difficulty in breathing/Chest discomfort? ☐
  1 = Yes, 2 = No, 3 = Do not know
  - If yes, for how many days have you (your child) had this? ☐☐
- Has fever resolved? ☐
  1 = Yes, 2 = No, 3 = Do not know
  - If yes, when did it resolve? / /_____
- Duration of fever (days). ☐☐
- What was your (your child's) first symptom?
  _____
- Date of symptom onset (dd/mm/yyyy) __/__/____
- Duration of symptom (days). ☐☐
- Do you (your child) have sore throat? ☐
  1 = Yes, 2 = No, 3 = Do not know
  - If yes, for how many days have you (your child) had this? ☐☐
- Do you (your child) have running nose? ☐
  1 = Yes, 2 = No, 3 = Do not know
  - If yes, for how many days have you (your child) had this? ☐☐

- Do you (your child) have cough? 1 = Yes, 2 = No, 3 = Do not know
  - If yes, for how many days have you (your child) had this?
- How would you describe your (your child's) activity level since illness began? 1 = Normal, 2 = Less Active/Irritable, 3 = Sleepy/Difficult to get out of bed, 4 =Cannot be awoken
  - If 2 or 3, for how many days have you (your child) had this?
  - If yes, for how many days have you (your child) had this?
- Have you (your child) had immunization? 1 = Yes, 2 = No, 3 = Do not know

If yes, which type of immunization_____

Patients 15 Years and Older
Other Activities

- Do you smoke?

0 = No, 1 = Cigarettes, 2 = Biri, 3 = Hookah, 4 = Other _____

  - If yes, for how many years have you used this?
- Do you use other forms of tobacco? 0 = No, 1 = With 'pahn', 2 = 'Ghool' (powder)
  - If yes, for how many years have you used this?
- Do you (your child) have any contact with pet animal? 1 = Yes, 2 = No, 3 = Do not know
- Which type of pet animal? 1 = Chicken, 2 = Dog, 3 = Swine, 4=Duck 5=others
- Do you (your child) had contact with diseased poultry? 1 = Yes, 2 = No, 3 = Do not know
- Do you (your child) maintian hygiene after using toilet and before eating? 1 = Yes, 2 = No, 3 = Do not know
- How many people share your room that you live? One=1, Two=2, Three=3, Four=4, Five=5, Six= 6, Seven= 7, Other = 8 (Specify)_____
- Do you have any knowledge of influenza? 1 =

Appendix II

1. Virus Transport Medium

To a 500ml bottle add:

Basic Medium (100%)	482.5 mL
BSA (2.5%)	12.5 mL
Fungizone (0.8%)	4 mL

Note: Sterilize by filtration and distribute in 1.0 mL in 3.0 mL screw capped tubes or as per requirement.

2. Trypsin

Trypsin-EDTA, 0.05% 1x (Gibco, cat # 25300)

Trypsin-EDTA is aliquoted in 5 ml volumes and stored at -20C.

3. Cryoprotectant Medium

Split/Culture media+ DMSO 5% (recommended by ATCC)

E.g.: 9 ml Split media + 0.5 mL DMSO for 10 mL cryoprotectant medium

4. Fetal Bovine Serum

Fetal Bovine Serum (FBS) 90% + DMSO 10% (lab practice)

For example: 9 ml FBS + 1 mL DMSO for 10 mL freezing medium

5. Physiological Saline, 0.85% NaCl

6. Alsever's Solution

Weigh out, and dissolve in 20 ml distilled water:

Glucose	0.41 gram
Sodium Chloride	0.084 gram
Citric Acid	0.011 gram
Tri-sodium citrate	0.16 gram

Sterilize the solution by filtration through a 0.22 μm filter. Ratio of blood: Alsever's solution should be 1:1.

7. Red Blood Cells

In general Chicken RBCs are used for both HA as HAI tests. The RBCs are provided in Alsever's solution

8. Phosphate Buffer Saline

NaCl	404 gm
KH_2PO_4	4 gm
Na_2HPO_4	18.4 gm

These were dissolved in 10 liters distilled water to give a 5X concentrated solution.

11. 6X Loading Dye

Bromophenol Blue	0.25%
Sucrose (SIGMA)	30%

12. Agarose Gel

I. 1.5 g of agarose powder (GIBCO BRL, New York; USA) was weighted in a conical flask.
II. 100 ml of 1X Tris-borate EDTA (TBE) was added in measuring cylinder and poured into flask containing agarose and then melted in micro-oven for 2-3 minutes.
III. 4 µl of ethidium bromide was added (GIBCO BRL).
IV. The gel solution was poured in the gel case (in which the comb was assembled for wells) and allowed for solidification.

Appendix III

Haemagglutinin Gene (HA) for A/(H1N1)pdm09

Strain	Accession no.	Strains	Accession no.
A/Beijing/13-25/2013	KJ419937	A/Bangladesh/2021/2012	EPI41527
A/Beijing/13-5/2013	KJ419935	A/Victoria/523/2012	EPI14745
A/SAPPORO/16/2014	KG088955	A/Washington/24/2012	KC891409
A/India/P131845/2013	EPI4578542	A/Sydney/DD3-58/2011	CY092863
A/Bangladesh/3003/2013	EPI5445822	A/Thailand/CUH2911/2011	CY089467
A/Bangladesh/8027/2014	EPI4775245	A/Shanghai/3162T/2011	JN631044
A/India/6427/2014	EPI1452124	A/Georgia/07/2012	KC891299
A/Bolivia/559/2013	EPI14575212	A/New York/4735/2009	CY051670
A/South Africa/3626/2013	EPI147852	A/CanadaPQ/RV1758/2009	GQ465752
A/Stockholm/15/2013	EPI247845	A/New York/3324/2009	CY043202
A/Dhaka/850/2013	KY094978	A/Italy/127/2009	GQ392033
A/California/06/2014	KG088955	A/England/195/2009	GQ166661
A/Bangladesh/0009/2014	EPI478541	A/New York/3177/2009	CY041604
A/Quebec/12/2014	KJ396031	A/Nebraska/02/2009	GQ457496
A/Wisconsin/12/2013	KF648237	A/Oregon/13/2011	KC891354
A/Zhejiang/TZ11/2013	KC524501	A/PERTH/533/2011	KC883141
A/Ghana/DARI- 0095/2014	EPI1477744	A/HongKong/5659/2012	EPI78941
A/Tehran/35070/2013	KC842186	A/Korea/01/2009	GQ160813
A/Utah/06/2013	KF648230	A/Osaka/1/2009	GQ222046.2
A/Wisconsin/13/2013	KM408915	A/California/04/2009	FJ966085
A/India/P121716/2012	KF280683	A/California/07/2009	KU933489

Haemagglutinin Gene (HA) for A/H3N2

Strain	Accession no.	Strains	Accession no.
A/Kentucky/05/2014	KM064523	A/Bangladesh/8603/2013	EPI857412
A/Hawaii/08/2014	KM852997	A/Bangladesh/3014/2015	EPI478542
A/New York/05/2014	GQ457502	A/Bangladesh/8011/2013	EPI147852
A/Korea/3772/2014	CY187690	A/New York/07/2014	EPI147335
A/Bangladesh/10009/2014	GQ392033	A/Texas/50/2012	KC892961
A/Bangladesh/8598/2013	GQ166661	A/Victoria/361/2011	KM821347
A/Texas/06/2014	KM064457	A/Alaska/24/2012	KF790369
A/Bangladesh/12009/2013	EPI147821	A/Utah/17/2012	KC893035
A/Utah/07/2013	KM064154	A/Pennsylvania/15/2011	KC892522
A/Bulgaria/270/2013	EPI144521	A/Alabama/35/2012	KF789919
A/VICTORIA/505/2013	EPI254782	A/Guangdong/94/2011	CY099954
A/Dhaka/961/2013	Submitted	A/Laos/854/2010	KP457831
A/New York/39/2012	KF790292	A/Vietnam/838/2010	KP457372
A/Minnesota/01/2013	KF790082	A/Shanghai /190/2009	KP458242
A/Arizona/09/2012	KF790291	A/Taiwan/839/2009	KP459164
A/Alabama/07/2013	KF790150	A/Thailand/01/2009	EPI136987
A/Japan/3756/2014	CY187674	A/Perth/16/2009	GQ293082
A/Bangladesh/9269/2013	EPI126985	A/Philippines/16/2009	GQ293084
A/Oregon/01/2013	KF789977	A/Colorado/11/2014	KM064524
A/Louisiana/39/2013	KU592843		

Neuraminidase Gene for A/(H1N1)pdm09

Strains	Accession no.	Strains	Accession no.
A/Bangladesh/1685/2010	EPI874147	A/New York/02/2013	GU292353
A/Bangladesh/8003/2010	EPI417851	A/Florida/61/2013	KT274398
A/YOKOHAMA/83/2010	EPI1243256	A/PuertoRico/21/2013	GU292353
A/Senegal/024/2010	KT836708	A/Wyoming/22/2013	KT274627
A/California/07/2009	KU933489	A/Wyoming/07/2013	KM409017
A/Missouri/NHRC0001/2011	CY092424	A/Argentina/749/2013	EPI874147
A/Brazil/AVS08/2011	CY120754	A/SouthAfrica/3626/2013	EPI417851
A/New York/21/2011	KC891357	A/Hong Kong/7572/2014	EPI1243256
A/Mexico/InDRE3740/2011	CY120020	A/Fiji/19/2014	KT836708
A/Sapporo/163/2011	KY094978	A/Iowa/19/2014	KU592829
A/VICTORIA/822/2011	KG088955	A/Dhaka/850/2013	KY094979
A/SINGAPORE/62/2011	EPI478541	A/England/579/2014	EPI874147
A/Vietnam/105/2012	KJ396031	A/FLORIDA/62/2014	KU592870
A/Jamaica/764/2012	KF648237	A/Finland/473/2014	EPI1243256
A/Vermont/02/2012	KC891383	A/Denmark/50/2014	KT836708
A/Delhi/1939/2012	EPI874147	A/Mexico/2493/2014	CY088922
A/Netherlands/529/12	KG088955	A/Alaska/38/2014	KU592865
A/Texas/106/2012	KF648108	A/Berlin/81/2014	EPI874147
A/Alaska/36/2012	KF648140	A/Oman/4005/2014	EPI417851
A/Hungary/25/2013	GU292353	A/Sri Lanka/50/2014	EPI1243256
A/Delaware/09/2013	KF648123	A/Ethiopia/66/2014	KT836708

Neuraminidase Gene for H3N2

Strains	Accession no.	Strains	Accession no.
A/Norway/2178/2014	KY094978	A/Dhaka/961/2013	Submitted
A/Hong Kong/7127/2014	KG088955	A/Beijing/40430/2012	KR978493
A/Sydney/530/2014	EPI478541	A/Guangdong/947/2012	CY125692
A/Perth/1055/2014	KJ396031	A/Victoria/361/2011	KM821347
A/Stockholm/28/2014	KF648237	A/Texas/50/2012	KC892961
A/Virginia/51/2014	KY116995	A/Moldova/109/2012	EPI874147
A/Egypt/8102/2014	KC892961	A/SouthAustralia/109/2012	EPI417851
A/Nepal/1336/2014	KM821347	A/Tanzania/977/2010	EPI1243256
A/Bangladesh/10009/2014	KF790369	A/Brisbane/11/2010	KT836708
A/Cambodia/AD04410/2014	KC893035	A/Managua/2145.01/2010	CY088922
A/Chiba/61/2014	KC892522	A/Costa Rica/6696/2010	EPI874147
A/Singapore/H2013.901/2013	KF789919	A/India/3489/2010	EPI417851
A/Malaysia/22/2013	EPI874147	A/Pennsylvania/42/2010	EPI1243256
A/Nepal/1442B/2013	EPI417851	A/Alaska/02/2010	KT836708
A/Wisconsin/49/2013	EPI1243256	A/Kenya/1632/2010	GU292353
A/Nepal/1442B/2013	KT836708	A/Pennsylvania/02/2010	KC535476
A/Sarajevo/9/2013	EPI417851	A/Chile/72/2011	CY093047
A/Vietnam/13V H3-8/2013	EPI1243256	A/Brazil/1151/2011	GU292353
A/Texas/21/2013	KT836708	A/Ireland/11M27357/2011	EPI622054
A/Mexico/648/2011	GU292353	A/Osaka/10K364/2011	JN790510
A/California/03/2011	KC882485	A/Serbia/688/2011	EPI622058

PB1 gene for A/(H1N1)pdm09

Strains	Accession no.	Strains	Accession No.
A/Germany/51/2014	EPI457812	A/Dhaka/850/2013	Submitted
A/Stockholm/11/2015	EPI214578	A/India/2192/2012	EPI874147
A/Florida/62/2014	EPI654123	A/Vietnam/13V H1/2013	EPI417851
A/Puerto Rico/29/2014	EPI745212	A/Laos/828/2013	EPI1243256
A/Nagasaki/13N057/2014	EPI7453112	A/India/P121773/2012	KT836708
A/Ethiopia/1149/2014	EPI214536	A/Washington/24/2012	KC891409
A/Laos/951/2014	EPI412578	A/AUCKLAND/64/2012	GU292353
A/Bahrain/602/2014	KC891906	A/Nairobi/11/2010	GU292353
A/Hawaii/01/2015	JX444063	A/Zhejiang/89w/2009	JN207844
A/SYDNEY/82/2013	JX827392	A/California/07/2009	KU933489
A/Singapore/KK1064/201	HM628691	A/Peru/106/2013	EPI412578
A/SRI LANKA/31/2014	EU779598	A/Paris/1249/2011	KY094978
A/SRI LANKA/61/2013	EU889008	A/Kijabe/16/2010	KG088955
A/Kanagawa/163/2014	EU889008	A/Nairobi/25/2010	EPI478541
A/HIROSHIMA/19/2013	EU889016	A/Bangladesh/2021/2012	KJ396031
A/Hong Kong/5008/2013	AB027406	A/Stockholm/1/2012	KF648237

PB1 gene for H3N2

Strains	Accession no.	Strains	Accession no.
A/Laos/1115/2014	FJ966085	A/India/8448/2015	KY094980
A/Switzerland/9715293/2013	GU292346	A/Stockholm/41/2015	JZ923736
A/Kazakhstan/194/2014	JF764086	A/Dhaka/961/2013	Submitted
A/Vietnam/629/2014	GU292353	A/Vietnam/13V H3-4/2013	KY094980
A/Bangladesh/10009/2014	GQ465751	A/Sri Lanka/60/2014	JF764085
A/Nigeria/942/2014	KC781723	A/Laos/389/2015	KU933489
A/Brazil/AVS08/2011	CY120754	A/ Nihrd-Pal082/2015	FJ966085
A/Moscow/WRAIR4316N/2011	CY098056	A/Hawaii/12/2014	GU292346
A/California/04/2009	FJ966085	A/Bangladesh/1516/2015	KI965925
A/California/07/2009	KU933489	A/El Salvador/410/2015	JF765685
A/India/GWL_DSC/2010	JF764085	A/South Australia/22/2015	KC882568
A/Mexico/InDRE3740/2011	CY120020	A/Mexico/1357/2015	CY183240
A/Thailand/CU-H2911/2011	CY089467	A/Nicaragua 92/2012	CY182208
A/Missouri/NHRC0001/2011	CY092424	A/Houston/JMM_134/2013	KT889134

PB2 gene (H1N1)pdm09

Strains	Accession no.	Strains	Accession no.
A/Malaysia/11/2014	EPI457812	A/SRI LANKA/61/2013	GU292353
A/Mexico/2493/2014	EPI214578	A/Dhaka/850/2013	Submitted
A/Myanmar/13M310/2013	EPI654123	A/Vietnam/3050/2013	KY094978
A/Myanmar/14M139/2014	EPI745212	A/Vietnam/13V H5/2013	KG088955
A/Congo/2278/2014	EPI7453112	A/Laos/887/2013	EPI478541
A/SRI LANKA/30/2013	EPI214536	A/India/GWL_DSC/2010	KJ396031
A/South Africa/3626/2013	EPI412578	A/India/GWL01/2011	KF648237
A/HIROSHIMA/19/2013	EPI78421	A/India/Blore/2010	JF764086
A/Bahrain/575/2014	EPI2124562	A/Pune/NIV6447/2009	GU292353
A/Laos/952/2014	KY094978	A/CanadaRV1644/2009	GQ465751
A/Hawaii/01/2015	KG088955	A/California/06/2009	KC781723
A/Hawaii/01/2015	EPI478541	A/California/04/2009	FJ966085
A/Congo/2275/2014	KJ396031	A/California/07/2009	KU933489

PB2 gene for H3N2

Strains	Accession no.	Strains	Accession no.
A/Switzerland/9715293/2013	EPI874147	A/Mexico/InDRE3740/2011	CY120020
A/Vietnam/629/2014	EPI417851	A/Thailand/CU-H2911/2011	EPI874147
A/Laos/1115/2014	EPI1243256	A/Sydney/DD3-58/2011	EPI417851
A/Bangladesh/10009/2014	KT836708	A/India/2192/2012	EPI1243256
A/Nigeria/942/2014	GU292346	A/Thailand/VIROAF6/2012	KT836708
A/Hong Kong/4801/2014	JF764086	A/Dhaka/961/2013	Submitted
A/Bangladesh/9269/2013	GU292353	A/Bangkok/SV0471_11/2011	GU292353
A/Thailand/CU-H3680/2014	GQ465751	A/Laos/225/2015	JF764085
A/Virginia/51/2014	KC781723	A/Indonesia /2015	KU933489
A/Bangladesh/3014/2015	GU292353	A/Ulsan/58/2015	FJ966085
A/California/06/2009	KC781723	A/Singapore/TT973/2013	GU292346
A/Pune/NIV6447/2009	GU292353	A/Thailand/CU-B6309/2012	KC882568
A/Taiwan/1018/2011	JN187346	A/Nicaragua/AGA2-92/2012	CY183240
A/Boston/DOA14/2011	CY111213	A/Houston/JMM_134/2013	CY182208
A/India/Blore/2010	JF764086	A/Japan/22/2011	KT889134
A/Blore/NIV236/2009	GU292346	A/Cheboksary/IIV-92/2011	JN704795
A/India/GWL_DSC/2010	JF764085	A/Missouri/NHRC0001/2011	CY092424

Matrix Gene for A/(H1N1)pdm09

Strain	Accession no.	Strains	Accession no.
A/Taiwan/1018/2011	JN187346	A/Tomsk/IIV-19/2012	JQ768357
A/Boston/DOA14/2011	CY111213. 1	A/Sydney/DD3-58/2011	CY092863
A/India/Blore/2010	JF764086	A/India/8448/2015	JZ923736
A/Pune/NIV6447/2009	GU292353. 1	A/Stockholm/41/2015	JZ923736
A/India/GWL01/2011	JX262213	A/Bangladesh/6867/2015	EPI698532
A/India/GWL02/2011	JX262214	A/Dhaka/850/2013	KY094980
A/California/NHRC0001/2011	CY092887. 1	A/Sri Lanka/60/2014	EPI874147
A/Brazil/AVS08/2011	CY120754. 1	A/Laos/389/2015	EPI417851
A/Moscow/WRAIR4316N/2011	CY098056. 1	A/ Nihrd-Pal082/2015	EPI1243256
A/California/04/2009	FJ966085	A/Hawaii/12/2014	KT836708
A/California/07/2009	KU933489. 1	A/Bangladesh/1516/2015	KI965925
A/India/GWL_DSC/2010	JF764085	A/El Salvador/410/2015	EPI1423698
A/Mexico/InDRE3740/2011	CY120020. 1	A/SouthAustralia/22/2015	EPI26587
A/Thailand/CU-H2911/2011	CY089467. 1	A/Mexico/1357/2015	EPI78436
A/Missouri/NHRC0001/2011	CY092424. 1	A/Paraguay/1164/2015	EPI54712
A/Cheboksary/IIV-92/2011	JN704795	A/Argentina/11641/2015	EPI68423

Matrix Gene for A/H3N2

Strains	Accession no.	Strains	Accession no.
A/India/6216/2014	EPI874147	A/PERTH/258/2013	EPI874147
A/Vietnam/629/2014	EPI417851	A/Bangkok/43/2015	EPI417851
A/Vietnam/13V H3-4/2013	EPI1243256	A/Philippines/FLU /2012	EPI1243256
A/Dhaka/961/2013	Submit	A/Yokosuka/SE863/2012	KT889227
A/Delhi/1550/2013	KP412574	A/Singapore/TT973/2013	EPI874147
A/Thailand/VIROAF6/2012	KJ577193	A/Kuwait/F146/2014	EPI417851
A/Victoria/361/2011	KM821347	A/Paraguay/0097/2014	EPI1243256
A/Kabul/1514A01305430N/2013	KT889246	A/Myanmar/13M118/2013	KT836708
A/Budapest/1514A07805832T/2013	KT889259	A/Georgia/01/2011	KC882568
A/Brazil/0328/2013	JN790510	A/Houston/JMM_134/2013	CY183240
A/Nigeria/942/2014	EPI622058	A/Nicaragua/AGA2-	CY182208
A/Hong Kong/4801/2014	KY116250	A/Japan/22/2011	KT889134

Nucleoprotein Gene for A/(H1N1)pdm09

Strains	Accession no.	Strains	Accession no.
A/Canada-AB/RV1644/2009	GQ465751	A/Sydney/DD3-58/2011	CY092863
A/California/06/2009	KC781723	A/Thailand/CU-H2911/2011	CY089467
A/England/195/2009	GQ166661	A/Mexico/InDRE3740/2011	CY120020
A/South Carolina/09/2009	EPI222123	A/Dhaka/850/2013	Submitted
A/California/07/2009	KU933489	A/Missouri/NHRC0001/2011	CY092424
A/California/04/2009	FJ966085	A/Cheboksary/IIV-92/2011	JN704795
A/India/GWL_DSC/2010	JF764085	A/Tomsk/IIV-19/2012	JQ768357
A/Shanghai/143T/2009	GQ411909	A/Taiwan/1018/2011	JN187346
A/Korea/01/2009	EPI4569875	A/Boston/DOA14/2011	CY111213
A/Pune/NIV6447/2009	GU292353	A/India/GWL01/2011	JX262213
A/Blore/NIV236/2009	GU292346	A/India/Blore/2010	JF764086
A/California/NHRC0001/2011	CY092887	A/India/GWL02/2011	JX262214

Nucleoprotein Gene for A/H3N2

Strains	Accession no.	Strains	Accession no.
A/Bangladesh/1151/2015	EPI457812	A/Vietnam/13V H3-4/2013	CY089467
A/Sri Lanka/61/2015	EPI214578	A/Costa Rica/4700/2013	CY092863
A/Virginia/51/2014	EPI654123	A/Dhaka/961/2013	Submitted
A/Oklahoma/20/2015	EPI745212	A/Nicaragua/AGA2-113/2013	EPI874147
A/Bangladesh/3014/2015	EPI7453112	A/Victoria/361/2011	EPI417851
A/Bangladesh/3014/2015	EPI214536	A/Georgia/01/2011	EPI1243256
A/Thailand/CU-H3680/2014	EPI412578	A/Nicaragua/AGA2-25/2011	KT836708
A/Canberra/82/2014	EPI78421	A/Nicaragua/AGA2-110/2012	GU292353
A/BRISBANE/165/2013	EPI2124562	A/Houston/JMM_134/2013	KC882568
A/Bangladesh/10009/2014	JF764086	A/Japan/22/2011	CY183240
A/Switzerland/9715293/2013	GU292346	A/Thailand/CU-B6309/2012	CY182208
A/Vietnam/629/2014	JF764085	A/Paraguay/0097/2014	GU292353
A/Laos/1115/2014	JN704795	A/Singapore/OHC019/2013	JQ768357
A/Singapore/OHC019/2013	CY092424	A/MexicoCity/1514324N/2013	CY120020

Nonstructural Gene for A/(H1N1)pdm09

Strains	Accession no.	Strains	Accession no.
A/California/NHRC0001/2011	CY092887	A/Missouri/NHRC0001/2011	CY092424
A/Brazil/AVS08/2011	CY120754	A/Tomsk/IIV-19/2012	JQ768357
A/India/GWL01/2011	JX262213	A/Mexico/InDRE3740/2011	CY120020
A/India/GWL02/2011	JX262214	A/Thailand/CU-H2911/2011	CY089467
A/California/04/2009	FJ966085	A/Sydney/DD3-58/2011	CY092863
A/California/07/2009	KU933489	A/India/2192/2012	EPI 867542
A/Canada-AB/RV1644/2009	GQ465751	A/Vietnam/3050/2013	EPI122463
A/California/06/2009	KC781723	A/Dhaka/850/2013	Submitted
A/Pune/NIV6447/2009	GU292353	A/Niigata/14F019/2015	EPI457812
A/Taiwan/1018/2011	JN187346	A/Laos/225/2015	EPI214578
A/Boston/DOA14/2011	CY111213	A /Nihrd-Pal082/2015	EPI654123
A/India/Blore/2010	JF764086	A/Ulsan/58/2015	EPI745212
A/Blore/NIV236/2009	GU292346	A/Hong Kong/12244/2015	EPI7453112
A/India/GWL_DSC/2010	JF764085	A/Lebanon/14L66/2014	EPI214536
A/Cheboksary/IIV-92/2011	JN704795	A/Bangladesh/6867/2015	EPI412578

Nonstructural gene for H3N2

Strains	Accession no.	Strains	Accession no.
A/Nigeria/942/2014	EPI457812	A/Quebec/12/2014	KC781723
A/Hong Kong/4801/2014	EPI214578	A/Wisconsin/12/2013	GU292353
A/Bangladesh/9269/2013	EPI654123	A/Dhaka/961/2013	Submitted
A/Thailand/CUB8772/2013	EPI745212	A/Ghana/DARI-0095/2014	KC781723
A/Thailand/CUB8772/2013	EPI7453112	A/Tehran/35070/2013	GU292353
A/Brazil/0328/2013	EPI214536	A/Utah/06/2013	JN187346
A/SAPPORO/16/2014	EPI412578	A/Wisconsin/13/2013	CY111213
A/India/P131845/2013	EPI78421	A/India/P121716/2012	JF764086
A/Bangladesh/3003/2013	EPI2124562	A/Oregon/13/2011	GU292346
A/Bangladesh/8027/2014	GU292353	A/PERTH/533/2011	JF764085
A/South Africa/3626/2013	FJ966085	A/Victoria/361/2011	JN704795
A/Stockholm/15/2013	GU292346	A/Georgia/01/2011	CY092424
A/California/06/2014	GU292353	A/Houston/JMM_134/2013	JQ768357
A/Bangladesh/0009/2014	GQ465751	A/Nicaragua/AGA2- 92/2012	CY182208

Polymerase acid (PA) gene for A/(H1N1)pdm09

Strains	Accession no.	Strains	Accession no.
A/Brazil/2085/2015	JX262214	A/El Salvador/410/2015	EPI147852
A/Laos/389/2015	CY092887	A/ Nihrd-Mks101/2015	EPI247845
A/Ecuador/2375/2015	CY120754	A/India/6427/2014	KY094978
A/South Africa/3599/2014	CY098056	A/Bangladesh/861/2015	KG088955
A/Myanmar/13M310/2013	FJ966085	A/Bangladesh/6867/2015	EPI478541
A/Singapore/KK1073/2013	KU933489	A/Laos/887/2013	KJ396031
A/Malaysia/11/2014	JF764085	A/India/GWL01/2011	JX262213
A/SYDNEY/82/2013	CY120020	A/India/GWL02/2011	JX262214
A/Singapore/KK1064/2013	CY089467	A/India/GWL_DSC/2010	JF764085
A/SRI LANKA/31/2014	EPI4586552	A/California/07/2009	KU933489
A/Dhaka/850/2013	Submitted	A/California/04/2009	FJ966085
A/Argentina/22/2015	KP456680	A/Blore/NIV236/2009	GU292346
A/South Australia/22/2015	EPI417854	A/India/Blore/2010	JF764086
A/Trinidad/429/2015	EPI5445822	A/Pune/NIV6447/2009	GU292353
A/Moscow/144/2015	EPI4775245	A/ RV1644/2009	GQ465751
A/Mexico/1374/2015	EPI1452124	A/California/06/2009	KC781723

Polymerase Acid (PA) gene for A/H3N2

Strains	Accession no.	Strains	Accession no.
A/Bangladesh/1151/2015	EPI457812	A/New York/39/2012	KF790292
A/Thailand/CUH3680/2014	EPI214578	A/Minnesota/01/2013	KF790082
A/Bangkok/43/2015	EPI654123	A/Arizona/09/2012	KF790291
A/Bangladesh/3014/2015	EPI745212	A/Alabama/07/2013	KF790150
A/Virginia/51/2014	EPI7453112	A/Japan/3756/2014	CY187674
A/Nigeria/942/2014	EPI214536	A/Bangladesh/9269/2013	EPI874147
A/Bangladesh/9269/2013	EPI412578	A/Oregon/01/2013	EPI417851
A/Texas/06/2014	EPI78421	A/Louisiana/39/2013	EPI1243256
A/Bangladesh/12009/2013	EPI874147	A/Colorado/11/2014	KT836708
A/Utah/07/2013	EPI417851	A/Bangladesh/8603/2013	KC781723
A/Bulgaria/270/2013	EPI1243256	A/Bangladesh/3014/2015	GU292353
A/VICTORIA/505/2013	KT836708	A/Bangladesh/8011/2013	JN187346
A/Dhaka/961/2013	Submitted	A/New York/07/2014	CY111213
A/Utah/17/2012	KC893035	A/Texas/50/2012	KC892961
A/Myanmar/13M118/2013	KC892522	A/Victoria/361/2011	KM821347
A/Singapore/TT973/2013	KF789919	A/Alaska/24/2012	KF790369

Heamagglutinin gene for Influenza B virus

Strains	Accession no.	Strains	Accession no.
B/Wisconsin/01/2010	CY115153	B/Huzhou/1119/2012	HM628691
B/Brisbane/60/2008	CY090737	B/Sao Paulo/22608/2010	EU779598
B/India/V083882/2008	CY090687	B/Oklahoma/03/2007	EU889008
B/India/K084060/2007	EPI397484	B/Massachusetts/02/2008	EU889008
B/Bangladesh/7004/2012	CY115255	B/South Carolina/01/2008	EU889016
B/Bangladesh/3333/2007	EPI422700	B/Aichi/20/99	AB027406
B/Pakistan/1009/2012	CY115359	Carolina/WRAIR1582P/2009	K00424
B/Bangladesh/5945/2009	EPI397583	B/Maryland/59	M10298
B/Bangladesh/1005/2012	EPI395181	B/Hong Kong/8/73	DQ792897
B/India/315/2012	JX827494. 1	B/Lee/40	DQ792905
B/Utah/02/2012	KC891998	B/Yamagata/16/88	M36105
B/Utah/07/2012	KC891906	A/human/Ohio/2006	EF554795
B/Wisconsin/03/2012	JX444063		
B/Milano/04/2012	JX827392		

Neuraminidase gene for Influenza B virus

Strains	Accession no.	Strains	Accession no.
B/Brisbane/60/2008	CY115153	B/South Carolina/NHRC0001/2004	CY099452
B/Wisconsin/01/2010	CY115185	B/Kentucky/01/2012	KC891960
B/Utah/02/2012	JX27493	B/Wisconsin/01/2012	KC891938
B/Kol/1267/2007	JF965361	B/Lee/40	DQ792899
B/New York/01/2012	KC891809	B/Maryland/59	M30634
B/Nevada/02/2012	KC891956	B/Hong Kong/8/73	M30635
B/India/315/2012	EPI395180	B/Oregon/5/80	AJ249278
B/Bangladesh/1005/2012	EPI37582	B/Victoria/3/85	AF134909.2
B/Bangladesh/7004/2012	EPI397483	B/Memphis/6/86	AF129909.2
B/Pakistan/1009/2012	EPI422699	B/Memphis/3/89	AF129918.2
B/Bangladesh/5945/2009	CY115361	B/Memphis/13/03	AY582018
B/Bangladesh/3333/2007	CY115257	B/Taiwan/94/2005	EU333520
New York/09/2007	EU516010	B/Myanmar/M170/2007	FJ229870
B/Myanmar/M254/2007	FJ966242	B/Hong Kong/36/2005	EU879085
B/Taiwan/3290/2006	CY099009	A/human/Ohio/2006	EF554797