MOLECULAR EPIDEMIOLOGICAL ANALYSIS OF H5N1 HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUSES OF BANGLADESH

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ABSTRACT

Highly pathogenic avian influenza (HPAI) viruses of H5N1 subtype have been devastating poultry industry across the world since 2003. The virus also may transmit to human with high case fatality. In Bangladesh, H5N1 HPAI virus was first detected in February 2007. Since then the virus has become entrenched in poultry of Bangladesh and caused six waves of outbreaks. So far, there have been seven human cases of H5N1 HPAI infection in Bangladesh with one case fatality. The objective of the present study was to investigate the molecular epidemiology of H5N1 HPAI viruses circulating in Bangladesh since 2007. A fragment of HA gene of 21 selected isolates obtained at different time points was amplified by RT-PCR and subjected to gene sequencing and phylogenetic analysis along with reference strains of different clades and sub-clades. Full-length gene of different genome segments of selected isolates were also amplified by RT-PCR and sequenced either directly or after cloning in plasmid vectors. In addition, available gene sequences of other Bangladeshi isolates, established at international reference laboratories, were also downloaded from the GenBank. Phylogenetic tree was constructed for each genome segment. Molecular analysis was also performed on multiple alignments of deduced amino acid sequences of each protein. The results revealed that clade 2.2 virus was first introduced in Bangladesh in 2007, this was followed by the introduction of clade 2.3.2.1 and 2.3.4 viruses in 2011. Interestingly, in 2012 only clade 2.3.2.1 viruses were isolated. Phylogenetic analysis of individual full-length genome segments revealed an event of segment re-assortment. The PB1 gene of two clade 2.3.2.1 viruses has been substituted by the corresponding segment of low pathogenic H9N2 virus. Point mutations were also acquired at the potential active sites of different proteins.

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INTRODUCTION

The recent panzootic of H5N1 highly pathogenic avian influenza (HPAI) that started in the Far East and Southeast Asia towards the end of 2003 has devastated the poultry industry across the world. HPAI is now considered deeply entrenched in poultry of several countries including Bangladesh.[1] The first outbreak of H5N1 HPAI in Bangladesh was reported in 2007[2-3] and since then as many as 549 outbreaks have been reported to OIE.[4] HPAI in Bangladesh has an apparent seasonal pattern, each wave of outbreaks starting usually in the late autumn, reaching the peak in the spring and then gradually declining.[5] So far, there have been seven human cases of H5N1 avian influenza with one death in Bangladesh.[6]

Avian influenza viruses belong to Type A influenza viruses under the family *Othomyxoviridae*. Influenza virus genome consists of 8 segments of single stranded negative sense RNA. The enveloped virus particles have two surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA). Type A influenza viruses are divided into many sub-types based on the HA and NA proteins they carry on their surface. To date 17 different types of HA (H1 – H17) and 10 NA (N1 – N10) have been recognized.[7-8] The ancestor of the recent panzootic H5N1 viruses is thought to have emerged in China in 1996 and 1997.[9-11] Since then the virus has evolved into many genetic clades (Clade 0 to Clade 9), which are defined on the basis of HA gene sequences. Clade 2 viruses have further evolved into many sub-clades of second, third and fourth order.[12] Clade 2.2 H5N1 HPAI virus was introduced in Bangladesh in 2007, which was followed by the introduction of clade 2.3.2.1 and clade 2.3.4 virus in 2011.[13-15] Low pathogenic H9N2 viruses are also circulating in poultry farms of Bangladesh.[16-18]

Like other RNA viruses, avian influenza viruses are very prone to mutation and rapid evolution, leading to antigenic drift, adaptation to mammalian host, development of antiviral resistance, etc. Segmented genome of influenza viruses also favors segment re-assortment or recombination resulting from concurrent infection with more than one subtypes of the virus. Such re-assortments are feared to be the cause of possible emergence of new pandemic human influenza virus. Swine-origin 2009 pandemic H1N1 influenza virus[19] and the avian-origin 2013 H7N9 influenza virus[20] are the two recent examples of segment re-assortment events of influenza viruses causing public health concern. Routine surveillance and molecular analyses of avian influenza viruses can predict emergence of pandemic virus strain. In the present study we record some observations on molecular epidemiology of circulation H5N1 avian influenza viruses in Bangladesh during the period from 2007 to 2012, which provide evidence of apparent replacement of clade 2.2 viruses by newly introduced clade 2.3.2.1 viruses, continuing evolution of the circulating viruses, and segment re-assortment between H5N1 and H9N2 avian influenza viruses.

MATERIALS AND METHODS

VIRUS ISOLATES

A total of 21 selected H5N1 avian influenza virus isolates were subjected to molecular characterization in this study. The viruses were obtained from outbreaks in different parts of Bangladesh during the period from 2007 to 2012. They were propagated in embryonated

chicken eggs. The allantoic fluid was harvested, stored in aliquots at -70°C and used in the subsequent study.

AMPLIFICATION OF PARTIAL OR FULL-LENGTH GENOME SEGMENTS BY RT-PCR

RNA was extracted from the HPAI isolates using Qiagen RNA extraction kit. A 545 bp fragment of H5 gene and a 343 bp fragment of N1 gene were amplified with Qiagen one step RT-PCR kit using the primers H5-155F: 5'-ACA CAT GCY CAR GAC ATA CT-3' and H5-699R: 5'-CTY TGR TTY AGT GTT GAT GT-3' for H5 gene fragment[21] and N1-580-607F: 5'-TGAAGT ACA ATG GCA TAA TAA CWG ACA C-3' and N1-891-918R: 5'-CAC TGC ATA TAT ATC CTA TTT GAT ACT CC-3' for N1 gene fragment,[22] respectively. The RT-PCR products were analyzed by electrophoresis on 1.5% agerose gel stained with ethidium bromide.

For amplification of full-length genome segments from selected isolates, a two-step RT-PCR was followed. First strand cDNA was synthesized from extracted RNA using Uni-12 primer[23] and ImProm II First Strand cDNA Synthesis Kit (Promega, Madison, USA) as per manufacturer's instructions. Syntyhesized cDNA was used as the template for amplification of different full-length gene segments by PCR using gene-specific primers.[23] PCR products were analyzed by electrophoresis on 1% agerose gel stained with ethidium bromide.

CLONING AND SEQUENCING

The PCR products were purified with Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, USA) and subjected to direct sequencing with respective PCR primers. Alternatively, the cleaned PCR products were cloned by TA cloning method using pGEM®-T Easy Vector System Cloning Kit and chemically competent *E. coli* JM109 (Promega, Madison, USA). The plasmid DNA was isolated and purified with EZ Spin Column (Bio Basic Inc., New York, USA) and the cloned cDNA was sequenced with universal primers. Overlapping primers (Personal communication, C. Tosh, High Security Animal Disease Laboratory, Bhopal, India) were used to sequence longer gene segments. Sequencing was done from a commercial laboratory.

MOLECULAR ANALYSIS

The partial and full-length sequence data generated in the present study were subjected to molecular analysis. The sequence data have been submitted to the GenBank (Table 1). In addition, full-length sequence data of other Bangladeshi isolates of H5N1 avian influenza virus, which are available in the GenBank, were downloaded and used in the analysis. Multiple alignments for nucleotide and deduced amino acid sequences were performed by ClustalV method using MegAlign module of Lasergene DNAStar software and phylogenetic trees were constructed using neighbour-joining method. Any sequence appearing as distinct outgroup was subjected to blast search in GenBank database to look for its closest neighbours. Deduced amino acid alignment table for each gene segment was thoroughly examined for any substitution that might be of biological significance.

Table 1. GenBank accession number of H5 sequences of Bangladeshi HPAI virus isolates generated in the present study

Sl. No.	Name of the isolates	Host species	GenBank Accession Number
1	A/chicken/Bangladesh/BL-4/2007(H5N1)	Chicken	JN679058
2	A/chicken/Bangladesh/BL-84T/2008(H5N1)	Chicken	JN679059
3	A/chicken/Bangladesh/BL-434/2009(H5N1)	Chicken	JN679060
4	A/chicken/Bangladesh/BL-440/2009(H5N1)	Chicken	JN679061
5	A/chicken/Bangladesh/BL-469/2010(H5N1)	Chicken	JN679062
6	A/chicken/Bangladesh/BL-470/2010(H5N1)	Chicken	JQ609542
7	A/crow/Bangladesh/BL-485/2011(H5N1)	Crow	JN679063
8	A/chicken/Bangladesh/BL-499/2011(H5N1)	Chicken	JN679064
9	A/chicken/Bangladesh/BL-543/2011(H5N1)	Chicken	JN679065
10	A/chicken/Bangladesh/BL-558/2011(H5N1)	Chicken	JN679066
11	A/duck/Bangladesh/BoBL-3/2011(H5N1)	Duck	JN679067
12	A/quail/Bangladesh/Q-2/ 2011(H5N1)	Quail	JN679068
13	A/duck/Bangladesh/D-1/2011(H5N1)	Duck	JQ609543
14	A/duck/Bangladesh/D-3/2011(H5N1)	Duck	JQ609544
15	A/migratory bird/Bangladesh/P59/2011 (H5N1)	Migratory bird	KF572593
16	A/chicken/Bangladesh/ BL-660/2012 (H5N1)	Chicken	KF572587
17	A/chicken/Bangladesh/BL-670/2012(H5N1)	Chicken	KF572588
18	A/chicken/Bangladesh/BL-671/2012(H5N1)	Chicken	KF572589
19	A/chicken/Bangladesh/BL-673/2012(H5N1)	Chicken	KF572590
20	A/chicken/Bangladesh/BL-675/2012(H5N1)	Chicken	KF572591
21	A/chicken/Bangladesh/C-1/2012(H5N1)	Chicken	KF572592

RESULTS AND DISCUSSION

PHYLOGENETIC AND MOLECULAR ANALYSIS OF HA GENOME SEGMENT

Haemagglutinin gene sequences of 112 Bangladeshi isolates were analyzed, which included 88 full-length or nearly full-length HA gene sequences and 24 partial HA gene sequences. A phylogenetic tree constructed with full-length HA gene sequences of 88 Bangladeshi isolates and representative strain of each clade is presented in Figure 1. The Bangladeshi isolates were broadly divided into three clusters belonging to clade 2.2, 2.3.4 and 2.3.2.1. Year-wise distributions of different clades are shown in Table 2. The first outbreak of HPAI in poultry of Bangladesh was recorded in February 2007. Since then the HPAI has become well entrenched in Bangladesh poultry with regular outbreaks. In contrast to Southeast and Far East Asia, the virus that caused initial outbreaks in Bangladesh belonged to clade 2.2.[13-15]

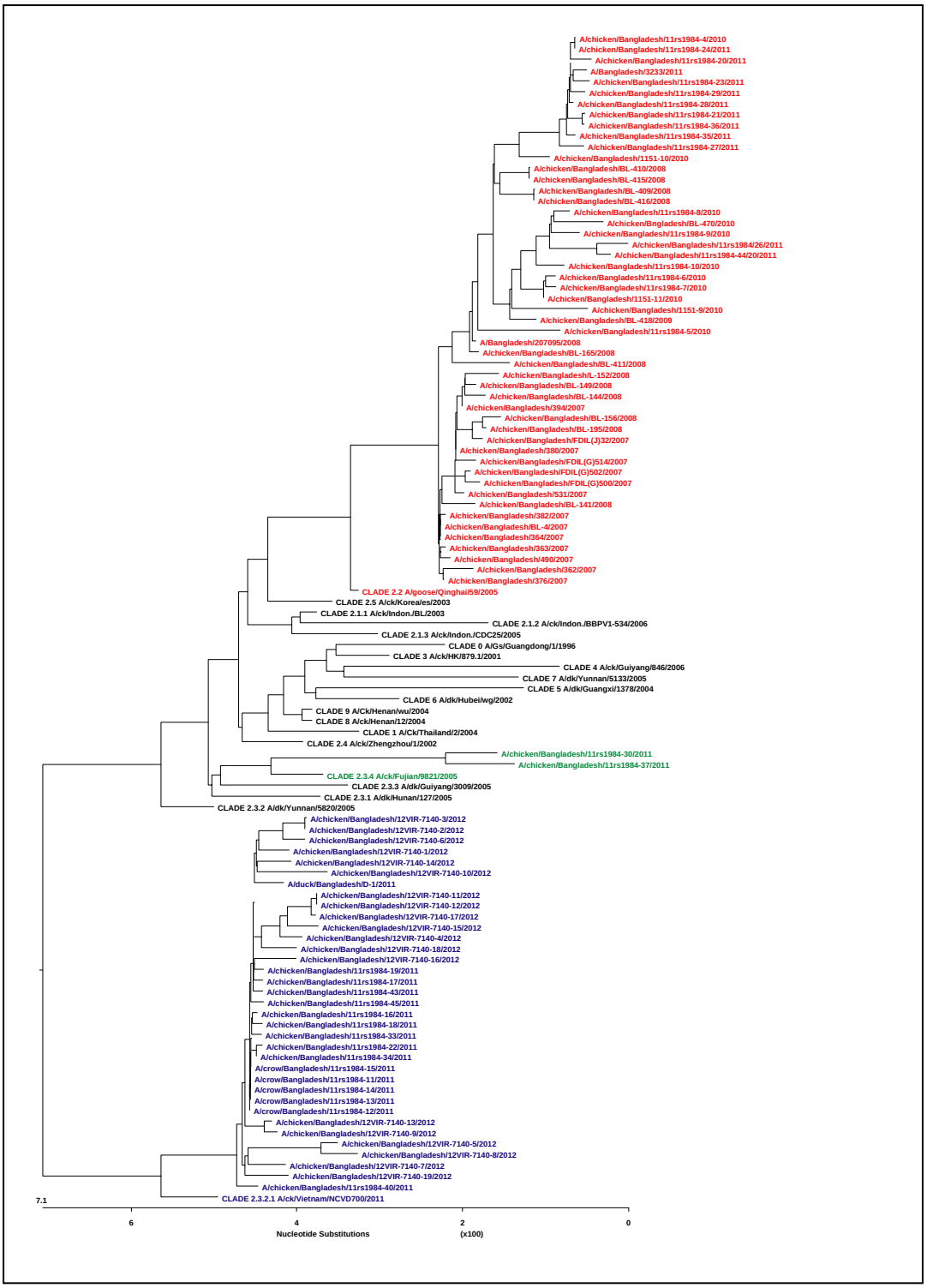

Fig. 1. HA H5 clade assignment of Bangladeshi H5N1 HPAI viruses isolated between 2007 and 2012. A total of 88 Bangladeshi isolates and representative strains of different clades were used to create the neighbour-joining tree.

Table 2. Distribution of HA clades among Bangladeshi isolates of H5N1 HPAI viruses

Year	Total Number of outbreaks*	No. of isolates sequenced§	HA clade		
			2.2	2.3.4	2.3.2.1
2007	64	20	20	-	-
2008	225	11	11	-	-
2009	35	2	2	-	-
2010	30	14	14	-	-
2011	169	43	15	3	20
2012	23	22	-	-	22
Total	546	112	62	3	42

* as reported to OIE
§ includes both partial and full-length sequences

Between 2007 and 2010, the 2.2 virus from a single introduction circulated in Bangladesh,[13] though the virus evolved into several genetic groups.[15] In 2011 viruses of clade 2.3.2.1 and clade 2.3.4 were also detected along with clade 2.2 viruses.[13,15] Interestingly, the present study revealed that all the isolates of 2012 belonged to clade 2.3.2.1 and there was no detection of any clade 2.2 or clade 2.3.4 virus in 2012 (Table 1). The reason for this apparent replacement of previously circulating clade 2.2 viruses by newly introduced clade 2.3.2.1 viruses is not clear. The clade 2.3.2.1 viruses might have some special advantages over the viruses of other clades in terms of better transmissibility and/or environmental survival fitness. Until 2011 clinical outbreaks of HPAI with clade 2.2 viruses in Bangladesh was almost restricted to chickens. On the contrary, clade 2.3.2.1 viruses were found to cause clinical disease in ducks and quails as well as cause mortality in crows[13] in addition to outbreaks in chickens. Gradual change of the dominating clades or genotypes of H5N1 in the course of time have been reported earlier in China and Vietnam.[24-25]

Deduced amino acid sequences of HA gene of 88 Bangladeshi isolates were aligned. Nucleotide sequence divergences within and between the clades are shown in Table 3. Among the three clades, Clade 2.3.2.1 appeared to be the most divergent having nucleotide divergence up to 9.2% from clade 2.3.4 and 9.6% from clade 2.2 viruses. Up to 2.4% and 2.8% nucleotide divergence were observed within clade 2.3.2.1 and clade 2.2 viruses, respectively.

Table 3. Nucleotide sequence divergence (%) between and within different clades of H5N1 HPAI viruses isolated in Bangladesh

	Clade 2.2	Clade 2.3.4	Clade 2.3.2.1
Clade 2.2	0.0 – 2.8		
Clade 2.3.4	5.8 – 8.0	1.5	
Clade 2.3.2.1	6.7 – 9.6	7.2 – 9.2	0.0 – 2.4

Two isolates of clade 2.3.4 were also 1.5% divergent from each other at nucleotide level. These data would suggest that the viruses are continuously evolving. Amino acid substitutions were observed throughout the protein sequence, which involved glycosylation sites, HA cleavage site, the receptor-binding site and antigenic sites (Table 4).

Table 4. Molecular differences in the haemagglutin of different H5N1 HPAI isolates of Bangladesh

Feature	Amino acid residues/ substitutions[a]	Presence in Bangladeshi isolates
Glycosylation site		
Conserved sites *(H5 numbering)*	^{11}NST, ^{23}NVT, ^{165}NNT, ^{286}NNS, ^{484}NGT, ^{543}NGS	All Bangladeshi isolates
Non-conserved sites *(H5 numbering)*	^{140}NSS	All (35) isolates of clade 2.3.2.1
	^{154}NNT	All (2) isolates of clade 2.3.4
	^{236}NDT	10 out of 51 isolates of clade 2.2 2 out of 35 isolates of clade 2.3.2.1
HA Cleavage site *(H5 numbering)*	^{321}PQGERRRKKR*GLF^{333}	49 out of 51 isolates of clade 2.2
	^{321}P*K*GERRRKKR*GLF^{333}	1 out of 51 isolates of clade 2.2
	^{321}PQGE*K*RRKKR*GLF^{333}	1 out of 51 isolates of clade 2.2
	^{321}ALREKRRK– – *GLF^{333}	All (2) isolates of clade 2.3.4
	^{321}PQRERRRK– – *GLF^{333}	All (35) isolates of clade 2.3.2.1
Receptor binding domain		
190 helix *(H3 numbering)*	^{190}EQTRLYQNP^{198}	All (35) isolates of clade 2.3.2.1 22 out of 51 isolates of clade 2.2
	^{190}EQT*K*LYQNP^{198}	28 out of 51 isolates of clade 2.2
	^{190}EQ*IQ*LYQNP^{198}	All (2) isolates of clade 2.3.4
130 loop *(H3 numbering)*	^{135}VSSA^{138}	All (51) isolates of clade 2.2 All (2) isolates of clade 2.3.4
	^{135}VS*A*A^{138}	All (35) isolates of clade 2.3.2.1
220 loop *(H3 numbering)*	^{221}SKVNGQSG^{228}	All (51) isolates of clade 2.2 All (2) isolates of clade 2.3.4
	^{221}SK*I*NGQSG^{228}	31 out of 35 isolates of clade 2.3.2.1
	^{221}*PKI*NGQSG^{228}	3 out of 35 isolates of clade 2.3.2.1
Base *(H3 numbering)*	^{98}Y, ^{153}W, ^{183}H, ^{195}Y	Conserved in all Bangladeshi isolates
Antigenic site		
Site 1 *(H5 numbering)*	^{136}PYQGRS^{141}	48 out of 51 isolates of clade 2.2
	^{136}PYQGR*P*^{141}	3 out of 51 isolates of clade 2.2
	^{136}PYQG*N*S^{141}	34 out of 35 isolates of clade 2.3.2.1
	^{136}*S*YQG*N*S^{141}	1 out of 35 isolates of clade 2.3.2.1
	^{136}PY*LGTP*^{141}	All (2) isolates of clade 2.3.4
Site 2 *(H5 numbering)*	^{152}KK^{153}	All (88) Bangladeshi isolates
Site 3 *(H5 numbering)*	^{125}HEASS^{129}	50 out of 51 isolates of clade 2.2
	^{125}H*K*ASS^{129}	1 out of 51 isolates of clade 2.2
	^{125}HEAS*L*^{129}	All (35) isolates of clade 2.3.2.1 1 out of 2 isolates of clade 2.3.4
	^{125}HE*TSL*^{129}	1 out of 2 isolates of clade 2.3.4

PHYLOGENETIC AND MOLECULAR ANALYSIS OF OTHER GENOME SEGMENTS

Phylogenetic trees based on PB2, PB1, PA, NP, NA, M and NS genome segments broadly followed the pattern of the tree derived with HA gene sequences (data not shown). However, a significant deviation was noticed in the phylogenetic tree based on PB1 gene sequences, where two H5N1 isolates of clade 2.3.2.1 namely A/ck/Bangladesh/12VIR-7140-16/2012(H5N1) and A/ck/Bangladesh/12VIR-7140-7/2012(H5N1), formed a separate branch (Figure 2). Blast search in the GenBank revealed that the PB1 gene of these two atypical Bangladeshi isolates were closely related to that of Bangladeshi and Indian H9N2 LPAI isolates. When two most closely related H9N2 isolates, A/ck/Bangladesh/VP01/2006(H9N2) and A/ck/Tripura (India)/105131/2008(H9N2), were included in the phylogenetic tree they clustered with the atypical Bangladeshi isolates. This clearly indicated that the PB1 gene of the atypical Bangladeshi isolates were in deed similar to that of H9N2 isolates. During the preparation of this manuscript Monne et al.[26] also reported this event of re-assortment. Further surveillance is needed to explore if these re-assortants have disappeared or established in poultry of Bangladesh.

In conclusion, the newly introduced clade 2.3.2.1 H5N1 viruses have apparently replaced previously circulating clade 2.2 viruses in Bangladesh. There has been an event of segment re-assortment between H5N1 and H9N2 viruses in Bangladesh, where H5N1 virus has acquired PB1 gene of H9N2 virus. Point mutations are accumulating in Bangladeshi isolates with potential modification of receptor binding site and antigenic sites. Extensive and continuous molecular epidemiological studies are necessary to monitor the evolution of circulating avian influenza viruses in Bangladesh.

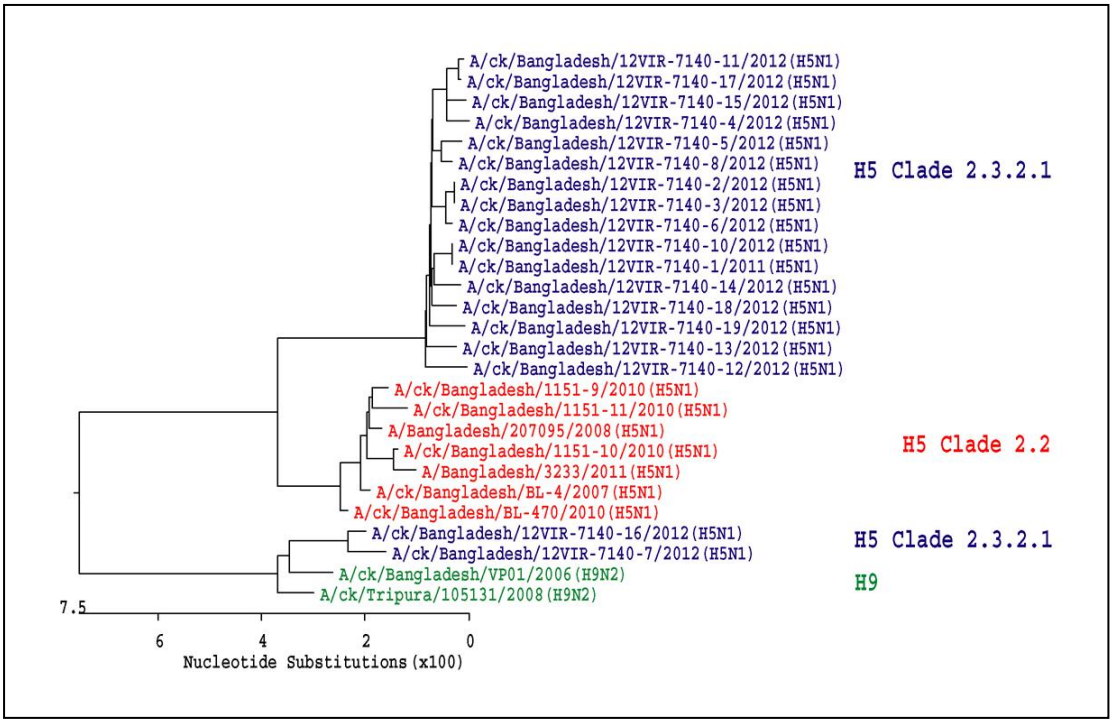

Fig. 2. Phylogenetic tree based on PB1 gene of Bangladeshi H5N1 HPAI isolates. Two H9N2 isolates are also included.

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