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Multiple reassortment events among highly pathogenic avian influenza A(H5N1) viruses detected in Bangladesh

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ABSTRACT

In Bangladesh, little is known about the genomic composition and antigenicity of highly pathogenic avian influenza A(H5N1) viruses, their geographic distribution, temporal patterns, or gene flow within the avian host population. Forty highly pathogenic avian influenza A(H5N1) viruses isolated from humans and poultry in Bangladesh between 2008 and 2012 were analyzed by full genome sequencing and antigenic characterization. The analysis included viruses collected from avian hosts and environmental sampling in live bird markets, backyard poultry flocks, outbreak investigations in wild birds or poultry and from three human cases. Phylogenetic analysis indicated that the ancestors of these viruses reassorted (1) with other gene lineages of the same clade, (2) between different clades and (3) with low pathogenicity avian influenza A virus subtypes. Bayesian estimates of the time of most recent common ancestry, combined with geographic information, provided evidence of probable routes and timelines of virus spread into and out of Bangladesh.

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Introduction

Genetic reassortment between distinct phylogenetic and antigenic lineages of highly pathogenic avian influenza (HPAI) A(H5N1) viruses and other subtypes of influenza A viruses may produce phenotypic and antigenic variants that could lead to the emergence of a pandemic virus (Garten et al., 2009; Yen and Webster, 2009). HPAI H5N1 influenza virus was detected in Southeast Asia during the 1990s and, subsequently, led to widespread poultry epizootics and morbidity and mortality in H5N1 virus infected humans (Shortridge et al., 1998). The high genetic diversity of the hemagglutinin (HA) genes of HPAI H5N1 viruses resulted in a standardized nomenclature system that unifies genetically related viruses into numbered clades (WHO, 2012). The actively circulating clades often show a geographic pattern, and enzootic circulation in specific regions has led to further classification into more discrete clades that often persist within geographically restricted areas (WHO, 2012; Younan et al., 2013). Moreover, antigenic differences between clades and subclades have been noted (Fouchier and Smith, 2010). In Bangladesh, where multiple clades of H5N1 co-circulate, the viruses have opportunities to exchange genetic material with genetically divergent H5N1 viruses and, potentially, other non-H5N1 subtypes that circulate in poultry or wild birds (WHO, 2012). Despite the first identification of H5N1 virus in Bangladesh in 2007, little is known about the genomic composition or antigenic variation of H5N1 viruses detected in this country, their geographic distribution, temporal patterns, or gene flow within the avian host population (Ahmed et al., 2012; Islam et al., 2012; OIE, 2012).

In Bangladesh, H5N1 viruses were first reported in poultry in March 2007 in northern and central regions, later spreading to other parts of the country and eventually becoming endemic (OIE,

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2012, 2013). The HA genes of the first viruses identified in the country belonged to clade 2.2 and, as of 2010, had diverged into a distinct third order clade, named 2.2.2, with restricted circulation in India and Bangladesh (Ahmed et al., 2012; WHO, 2012). The first human case of H5N1 in the country occurred in January 2008, and the isolated virus contained an HA gene with the same genetic characteristics as the circulating clade 2.2.2 H5N1 viruses detected in poultry in the country (Brooks et al., 2009). Later, two more human cases infected by viruses with the same HA were reported from Dhaka (WHO/GIP, 2013). In early 2011, after a crow outbreak in the south and central regions, H5N1 viruses from a clade previously not found in Bangladesh were detected in poultry (Islam et al., 2012; OIE, 2012, 2013). These viruses were genetically similar to clade 2.3.2.1 viruses that were circulating in Western India and Nepal since February 2011 (Nagarajan et al., 2012). Shortly after, viruses were found in poultry derived from yet another H5N1 clade, termed 2.3.4.2, that was previously identified in southeast Asian countries, such as Lao PDR, Vietnam, China, and Myanmar (Islam et al., 2012; WHO, 2013a). In 2011 and 2012, clades 2.3.2.1 and 2.2.2 viruses co-circulated in Bangladesh and spread throughout the country (OIE, 2012).

In this study, we analyzed the molecular epidemiology and full genome sequences of 40 H5N1 influenza viruses detected through virologic surveillance and outbreak investigations in Bangladesh between 2008 and 2012 to identify reassortment events that occurred within a single HA clade, as well as among different clades of H5N1 and between subtypes. To describe their micro-evolution, genotypes were named based on their HA gene clade and this convention was applied to the neuraminidase (NA) gene and the six internal genes. Bayesian estimates of the time of most recent common ancestry were combined with geographic information to find probable routes and timelines of H5N1 emergence in Bangladesh. Using ferret antisera raised against a subset of these viruses and other H5N1 HA clades, isolates collected over this four year period were characterized by hemagglutination inhibition to assess potential antigenic variability and the need to update candidate vaccine viruses for pandemic preparedness. These analyses will help public health officials better understand the potential value of continued influenza surveillance among poultry and human populations in Bangladesh.

Results

Influenza A(H5N1) virus sequencing and geographic locations

Full genome sequencing was performed for influenza A(H5N1) virus isolates including thirty-two avian isolates, five environmental isolates, and three human isolates (Table 1). The three human isolates were collected from children detected during population-based influenza surveillance in an urban community in Kamalapur, Dhaka as previously described (Brooks et al., 2010; International

Table 1
Bangladesh avian influenza A (H5N1) viruses with date of collection, clade and host information.

Virus name	Date of collection	HA clade	Host
A/Bangladesh/207095/2008	1/29/2008	2.2.2	Male, 16 months
A/chicken/Bangladesh/0912/2010	1/4/2010	2.2.2	Domestic chicken
A/chicken/Bangladesh/1012/2010	1/4/2010	2.2.2	Domestic chicken
A/chicken/Bangladesh/0411/2010	1/12/2010	2.2.2	Domestic chicken
A/poultry/Bangladesh/11255-C/2011	2/7/2011	2.2.2	Poultry
A/chicken/Bangladesh/31289-1/2011	2/20/2011	2.2.2	Domestic chicken
A/Bangladesh/5487/2011	3/7/2011	2.2.2	Male, 2 years
A/Bangladesh/3233/2011	3/9/2011	2.2.2	Female, 1 year
A/crow/Bangladesh/1008/2011	1/20/2011	2.3.2.1	Crow
A/crow/Bangladesh/1001/2011	1/20/2011	2.3.2.1	Crow
A/crow/Bangladesh/1020/2011	1/20/2011	2.3.2.1	Crow
A/crow/Bangladesh/1061/2011	1/20/2011	2.3.2.1	Crow
A/chicken/Bangladesh/11303/2011	2/4/2011	2.3.2.1	Domestic chicken
A/crow/Bangladesh/313T/2011	2/7/2011	2.3.2.1	Crow
A/crow/Bangladesh/315T/2011	2/7/2011	2.3.2.1	Crow
A/crow/Bangladesh/316T/2011	2/7/2011	2.3.2.1	Crow
A/crow/Bangladesh/1054/2011	2/13/2011	2.3.2.1	Crow
A/crow/Bangladesh/1056/2011	2/13/2011	2.3.2.1	Crow
A/crow/Bangladesh/1058/2011	2/13/2011	2.3.2.1	Crow
A/duck/Bangladesh/1849/2011	3/20/2011	2.3.2.1	Duck
A/chicken/Bangladesh/3072/2011	5/23/2011	2.3.2.1	Domestic chicken
A/chicken/Bangladesh/3075/2011	5/24/2011	2.3.2.1	Domestic chicken
A/environment/Bangladesh/1017/2011	5/29/2011	2.3.2.1	Environment
A/waterfowl/Bangladesh/33025/2011	6/29/2011	2.3.2.1	Waterfowl
A/goose/Bangladesh/4051T/2011	7/1/2011	2.3.2.1	Goose
A/chicken/Bangladesh/4058/2011	7/14/2011	2.3.2.1	Domestic chicken
A/duck/Bangladesh/4059T/2011	7/14/2011	2.3.2.1	Duck
A/chicken/Bangladesh/4070T/2011	7/15/2011	2.3.2.1	Domestic chicken
A/waterfowl/Bangladesh/31935/2011	7/17/2011	2.3.2.1	Waterfowl
A/duck/Bangladesh/4117T/2011	7/24/2011	2.3.2.1	Duck
A/duck/Bangladesh/4120T/2011	7/24/2011	2.3.2.1	Duck
A/duck/Bangladesh/4124T/2011	7/24/2011	2.3.2.1	Duck
A/environment/Bangladesh/1018/2011	9/29/2011	2.3.2.1	Environment
A/environment/Bangladesh/1011/2011	12/28/2011	2.3.2.1	Environment
A/environment/Bangladesh/1017-1/2011	12/30/2011	2.3.2.1	Environment
A/chicken/Bangladesh/42010/2012	1/8/2012	2.3.2.1	Domestic chicken
A/duck/Bangladesh/32077/2012	2/20/2012	2.3.2.1	Duck
A/environment/Bangladesh/1019-G/2012	2/28/2012	2.3.2.1	Environment
A/chicken/Bangladesh/3012/2011	2/19/2011	2.3.4.2	Domestic chicken
A/chicken/Bangladesh/11RS-1984-30/2011	6/15/2011	2.3.4.2	Domestic chicken

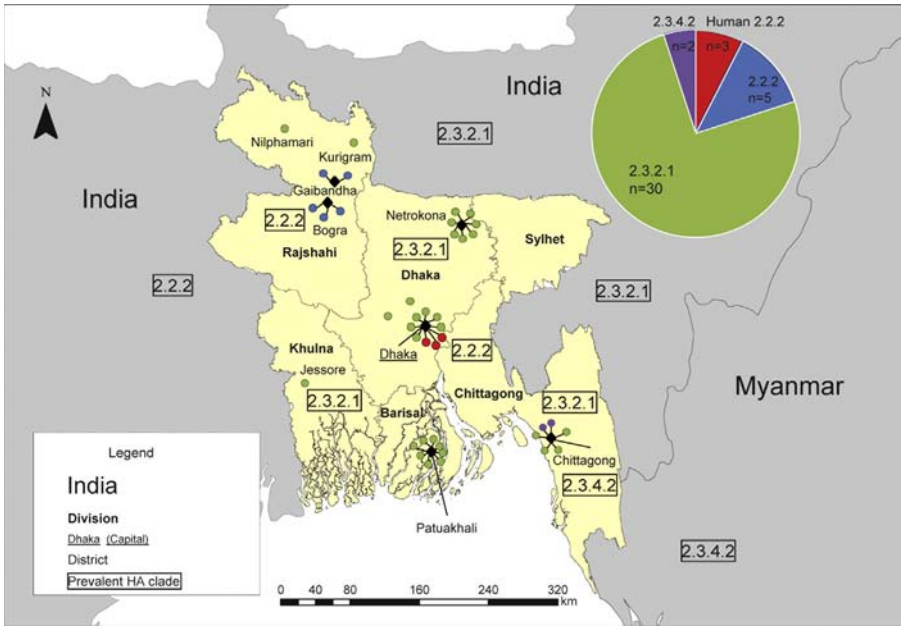

Fig. 1. Map of Bangladesh with geographic locations of each virus from this study as a colored circle (•) at or around the location and the pie chart shows the number of positive viruses per clade. The color code is for HA clade 2.2.2 (avian in blue, human in red), clade 2.3.2.1-Hubei-like (in green), and clade 2.3.4.2 (in purple). The HA gene clades of viruses found in the surrounding countries bordering with Bangladesh is shown in boxes.

Centre for Diarrhoeal Disease Research, 2011). The avian isolates included in the analysis were collected from wild birds (crows and waterfowl) and in domestic poultry (chicken, ducks) during January 2008 through February 2012, with the majority of samples collected in 2011 ($n=33$) (Table 1). Most samples were collected in central Bangladesh around the capital city of Dhaka (Division Dhaka); south of Dhaka, in the Division Barisal; in the Division Rajshahi, located in the northwest; in the southeast (Division Chittagong); and in Netrokona located in the northern part of Dhaka Division (Fig. 1).

Phylogenetic analysis, geo-temporal relationships and genotyping of viruses

Clade 2.2.2

The three viruses isolated from human cases in Dhaka and five poultry viruses (Bogra, northwest Bangladesh) that were collected between early 2010 and early 2011 clustered with previously described clade 2.2.2 viruses (Figs. 1 and 2). The sequences of A/Bangladesh/207095/2008 (H5N1) clustered outside the other Bangladeshi virus cluster for all eight gene segments (Supplementary Fig. 1A–H). Viruses that were collected in 2010 formed a cluster with Bhutan viruses from the same year in their HA gene sequences (Fig. 2). In the same cluster, the Bangladeshi viruses from 2011 shared a common node with an estimated time of most recent common ancestor (tMRCA) in mid-2011 (2011.55; highest posterior density [HPD] 95% 1.36, 1.95; Table 2). Indian HPAI H5 viruses (from public databases included in the analysis) grouped in the same larger cluster and were collected in West Bengal and Tripura districts close to the western and eastern border of Bangladesh, respectively (Figs. 1 and 2). A similar grouping was observed for the neuraminidase (NA) and the internal genes: polymerase basic protein 2 (PB2), PB1, polymerase acid protein (PA), nucleoprotein (NP), matrix (M), and nonstructural protein (NS) (Supplementary Fig. 1A–C and E–H). The tMRCAs of all 2.2.2 viruses collected in Bangladesh ranged between one to two years before their collection date (Table 2), except for the M gene, which was dated earlier (2005 to beginning of 2007). The genotype, annotated as genotype 2.2.2, was identical for all clade 2.2.2 viruses with no evidence of reassortment compared to other known 2.2.2 viruses (Fig. 3).

Clade 2.3.2.1

Thirty viruses collected during and after 2011 grouped with the clade 2.3.2.1 Hubei-like lineage with a distinct cluster sharing a common node in the phylogenetic tree (Fig. 2). For viruses within this cluster, no clear geo-temporal subgroups were defined. Low posterior probability support at the nodes within the larger cluster formed by virus isolates from Bangladesh indicated heterogeneity among these clade 2.3.2.1 viruses suggesting multiple introductions into the country or circulation of this virus for sustained periods of time (Supplementary Fig. 1A–H). In addition, HA gene sequences derived from viruses collected in different hosts (chickens, ducks, wild birds, or the environment) did not cluster into discrete groups (Fig. 2). The closest related HA sequences to the Bangladesh HA genes were from India (A/chicken/India/CA301/2011) and Nepal (A/chicken/Nepal/T1P/2012) (Fig. 2). The cluster containing these viruses was estimated to have diverged from all other 2.3.2.1 Hubei-like viruses in mid-2010 (Table 2, 2010.52; HPD 95% 1.45, 1.95; Supplementary Fig. 1D). This group shared a common node with Hubei-like viruses from Vietnam collected from 2011 to 2012 (Fig. 2), and these two groups were estimated to have diverged from each other in December 2008 (Table 2, 2008.97; HPD 95% 2.76, 3.67).

Of the thirty 2.3.2.1 Hubei-like lineage viruses, 28 grouped closely with 2.3.2.1 Hubei-like lineage viruses in their PB1, PA, and NS genes (Supplementary Fig. 1B–C and H and Fig. 3). However, their PB2 gene sequences grouped with A/Hong Kong/6841/2010-like lineage 2.3.2.1 viruses (Supplementary Fig. 1A and Fig. 3). The NA, NP and M gene sequences shared a common ancestor with 2.3.4.2 viruses and differed from all other 2.3.2.1 viruses (Supplementary Fig. 1E–G and Fig. 3). This genotype, with four genes from 2.3.2.1 Hubei-like viruses, three genes from 2.3.4.2-like viruses and one gene from 2.3.2.1 Hong Kong/6841-like viruses was referred to as Bangladesh 1 (BG1) (Fig. 3).

Among the 131 viruses in the PB1 dataset analyzed, the PB1 gene sequences of A/environment/Bangladesh/1017/2011 (H5N1) and A/

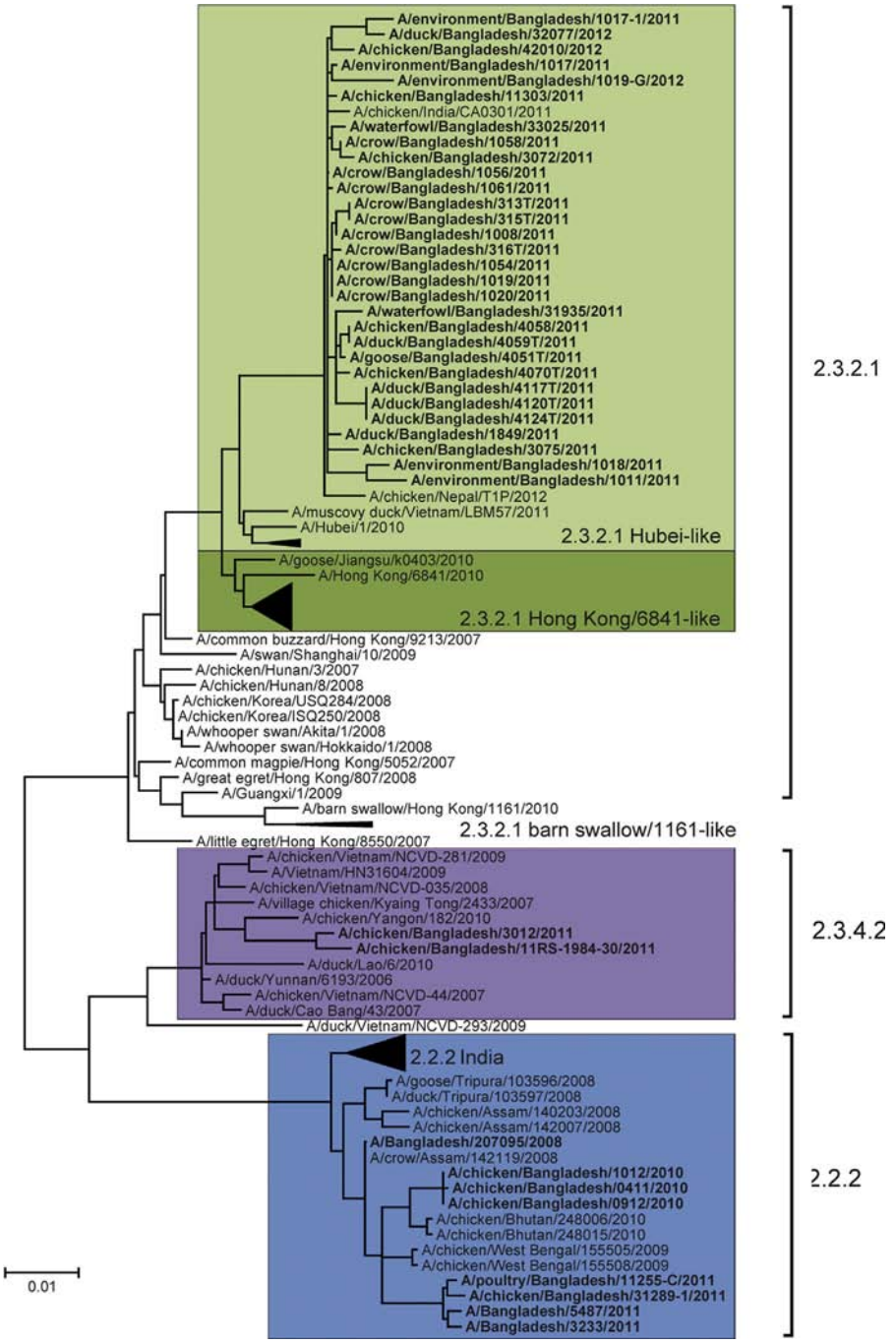

Fig. 2. Phylogenetic tree of HA gene sequences including 40 Bangladeshi viruses (in boldface) and 83 HPAI/H5N1 viruses from public databases. The clades are highlighted with color code, light green for 2.3.2.1 Hubei-like, dark green (Hong Kong/6841-like), purple for clade 2.3.4.2, and blue for clade 2.2.2. Triangles are collapsed branches that are labeled with the reference virus or lineages. This tree was inferred using the maximum likelihood method with GTR and gamma model in MEGA5 (Tamura et al., 2011).

chicken/Bangladesh/3075/2011 (H5N1) were dissimilar to all other H5N1 viruses in the dataset with 96.4% nucleotide identity to other H5N1 viruses from Bangladesh. However, they showed 99.5% nucleotide sequence identity with A/Bangladesh/0994/2011 (H9N2) and 98.5% identity with A/environment/Bangladesh/100/2010 (H9N2), both of which are low pathogenicity avian influenza (LPAI) H9N2 subtype viruses (Supplementary Fig. 1B). The two viruses clustered separately in the phylogenetic tree and differed in the tMRCA, with group 1 (A/environment/Bangladesh/1017/2011 [H5N1] and A/Bangladesh/0994/2011 [H9N2]) diverging around the end of 2010 (Table 2, 2010.75, HPD 95% 1.11, 1.93) and group 2 (A/chicken/Bangladesh/3075/2011 [H5N1] and A/environment/Bangladesh/100/2010 [H9N2]) diverging at the end of 2008 (2008.95; HPD 95% 2.41, 4.16). Thus, the genomic composition of these unique reassortants, termed Bangladesh 2 (BG2), had HA, PA and NS genes from 2.3.2.1 Hubei-like lineages (H5N1); NA, NP and M genes from clade 2.3.4.2-like lineages (H5N1); PB2 genes from 2.3.2.1 Hong Kong/6841-like lineages (H5N1); and PB1 genes from separate H9N2-like subtype viruses (Fig. 3).

Clade 2.3.4.2

The HA gene sequence of A/chicken/Bangladesh/3012/2011 (collected in Chittagong, southeast Bangladesh, Fig. 1) belonged to clade 2.3.4.2 and shared closest common ancestry with another virus recently detected from Bangladesh (A/chicken/Bangladesh/11RS-1984-30/2011) (Fig. 2). Other viruses from the same cluster

Table 2
Time of the most recent common ancestor (tMRCA) as calendar years (decimal dates) by HA clade and Bangladesh (BG) groups within a clade for each gene segment.

Gene	HA	NA	PB2	PB1	PA	NP	M	NS
Time of the most recent common ancestor – tMRCA (decimal date)								
Highest posterior density 95% (HPD 95% [upper, lower interval])								
Clade 2.2.2	2007.41	2006.67	2007.79	2006.69	2007.53	2007.21	2003.73	2007.13
	5.34, 6.32	4.9, 5.72	4.87, 5.98	5.01, 6.6	5.22, 6.4	5.17, 6.99	4.72, 6.38	4.83, 6.5
BG clade 2.2.2 group 1 (2008)	2008.94	2007.41	2008.68	2007.62	2008.42	2008.96	2004.92	2008.5
	4.1, 4.56	4.24, 4.87	4.19, 4.96	4.25, 4.96	4.39, 5.22	4.1, 4.58	4.1, 4.65	4.1, 4.43
BG clade 2.2.2 group 2 (2010)	2010.92	2009.65	2010.91	2009.16	2010.04	2010.32	2006.39	2010.28
	2.18, 2.4	2.18, 2.49	2.18, 2.43	2.63, 3.47	2.7, 3.66	2.19, 2.65	2.31, 3.4	2.19, 2.75
BG clade 2.2.2 group 3 (2011)	2011.55	2010.17	2011.56	2010.4	2011.31	2011.64	2007.75	2011.19
	1.36, 1.95	1.4, 2.21	1.36, 1.96	1.38, 2.31	1.46, 2.35	1.24, 1.94	1.15, 1.85	1.18, 2.02
Clade 2.3.2.1	2005.96	2004.96	2005.7	2005.69	2000.38	2003.72	2005.21	2006.57
	5.71, 6.75	6.57, 7.97	5.85, 7.26	5.9, 7.21	9.36, 15.03	6.27, 8.14	6.01, 8.24	5.11, 6.3
BG clade 2.3.2.1	2010.52	2010.7	2010.51	2010.52	2010.67	2009.06	2010.46	2010.57
	1.45, 1.95	1.27, 1.75	1.42, 1.98	1.45, 1.96	1.33, 1.73	1.37, 2.19	1.36, 2.17	1.32, 2
Hubei-like	2009.67	2010.15 [0.65]	2010.08	2009.72	2010.03	2008.9	2009.93	2010.26
	2.16, 2.93	1.85, 2.3	1.85, 2.49	2.04, 2.91	1.89, 2.49	1.8, 2.15	1.94, 2.69	1.8, 1.8
HK/6841-like	2009.01	2008.43	2008.52	2008.99	2008.18	2007.05	2009.7	2008.86
	2.88, 3.49	3.33, 4.2	3.24, 4.11	2.93, 3.49	3.53, 4.63	3.29, 4.46	2.07, 2.9	2.94, 3.84
BS/1161-like	2009.34	2010.19	2010.13	2009.81	2010.06	2007.89	2009.92	2010.06
	2.44, 3.31	1.94, 2.15	1.94, 2.25	2.02, 2.83	1.94, 2.49	2.39, 3.54	1.98, 2.66	1.94, 2.5
Clade 2.3.4.2	2006.16	2004.96	2005.88	2004.91	2006.02	2004.53	2005.21	2006.12
	5.78, 6.35	6.57, 7.97	5.88, 6.9	6.58, 8.08	5.81, 6.6	5.82, 6.94	6.01, 8.24	5.75, 6.57
BG clade 2.3.4.2	2010.59	2010.7	2010.49	2010.03	2010.27	2009.19	2010.64	2009.89 [0.6]
	1.28, 2	1.61, 2.8	1.34, 2.22	1.55, 2.89	1.44, 2.44	1.2, 2.29	1.06, 2.37	1.56, 3.09
BG H9N2-like group 1				2010.75				
				1.11, 1.93				
Group 2				2008.95				
				2.41, 4.16				

were collected in Southeast Asian countries, such as Myanmar, Vietnam, Lao PDR and China. Both viruses from Bangladesh were estimated to have diverged from common ancestors in mid-2010 (2010.59; HPD 95% 1.28, 2; Table 2) (Supplementary Fig. 1D). The clade 2.3.4.2 viruses grouped closely in all genes, but had long branch lengths relative to ancestors indicative of gaps in sequence data in this group. The common node ages ranged from the beginning of 2009 in their NP genes (2009.19; HPD 95% 1.2, 2.29; Table 2, Supplementary Fig. 1A–H) to late 2010 in their NA gene (2010.7; HPD 95% 1.61, 2.8; Table 2).

Antigenic characterization

H5N1 isolates were tested by hemagglutination inhibition (HAI) with ferret antiserum against representatives of the currently circulating clades of H5N1 virus and candidate vaccine viruses (Table 3; Supplementary Table 2A–D) (WHO, 2013a). Antisera generated against clades 2.2 and 2.2.2 viruses cross-reacted with Bangladesh viruses belonging to clade 2.2.2 with significantly higher HI titers than clades 2.3.2.1 and 2.3.4.2 viruses. Sera raised specifically to Bangladesh clade 2.2.2 viruses reacted with other Bangladesh viruses from the same clade within 4-fold of titers to homologous viruses indicating increased specificity of 2.2.2 viruses compared to earlier clade 2.2 reference sera (Supplementary Table 2A). The clade 2.2.2 virus isolated from a human case in 2008 was inhibited by older 2.2 antisera (i.e., A/bar headed-goose/Qinghai/1A/05) with slightly higher heterologous titers compared to 2010 and 2011 clade 2.2.2 viruses suggesting some antigenic drift among later 2.2.2 viruses. However, sera produced against the 2008 virus reacted with 2010 and 2011 viruses to heterologous titers within 4-fold or less of the homologous titer supporting the overall antigenic specificity of clade 2.2.2 viruses in Bangladesh despite several years of viral evolution (Supplementary Table 2A). Sera raised against clade 2.3.2.1 viruses reacted to higher titers with viruses from the same clade, while these sera had little to no inhibitory effects on clade 2.2.2 or 2.3.4.2 viruses (Table 3 and Supplementary Table 2A–D). Sera raised to Bangladesh viruses identified as clade 2.3.2.1, especially a recent virus from 2012, showed higher titers to related Bangladesh viruses compared to sera raised against clade 2.3.2.1 viruses from other countries (Supplementary Table 2D). Antisera generated to the A/Hubei/1/2010 candidate vaccine virus reacted with several Bangladesh clade 2.3.2.1 viruses with HI titers $\geq$8-fold different from the HI titer with homologous antigen, while antisera to the A/common magpie/Hong Kong/5052/2007 candidate vaccine reacted with these viruses within 4-fold or less compared to titers with the homologous antigen (Table 3). Expectedly, neither antiserum to these clade 2.3.2.1 vaccine candidates showed significant inhibition of clade 2.2.2 or 2.3.4.2 viruses (Table 3 and Supplementary Table 2A–D). One virus with a clade 2.3.4.2 HA gene (A/chicken/Bangladesh/3012/2011) showed little inhibition by antiserum against clade 2.2.2 or 2.3.2.1 viruses. Additionally, clade 2.3.4 sera raised to the A/Anhui/1/2005 candidate vaccine virus failed to inhibit agglutination by the 2.3.4.2 Bangladesh virus indicating substantial antigenic drift (Table 3 and Supplementary Table 2B).

Protein sequence characterization

Based on the predicted amino acid sequences, the multibasic cleavage site sequence of the clade 2.2.2 viruses was PQGERRRKKR*G (Bosch et al., 1981). All thirty 2.3.2.1 Hubei-like lineage viruses collected in Bangladesh shared an identical amino acid sequence PQRERRRKR*G at their cleavage site. The two 2.3.4.2 viruses shared identical HA cleavage site motifs (PQLRKRRKR*G) but differed from clades 2.2.2 and 2.3.2.1. One subgroup of 2.2.2 viruses (all from 2010) shared a unique glycosylation site at positions 54–56 and clade 2.3.2.1 virus A/chicken/Bangladesh/31289-1/2011 had a unique glycosylation site at positions 166–168 (mature H5 numbering). The viruses that belonged to 2.3.4.2 had a unique glycosylation site at residues 154–156 of the HA protein compared to other HA clades. The +1 alternate open reading frame in the PB1 protein (PB1-F2),

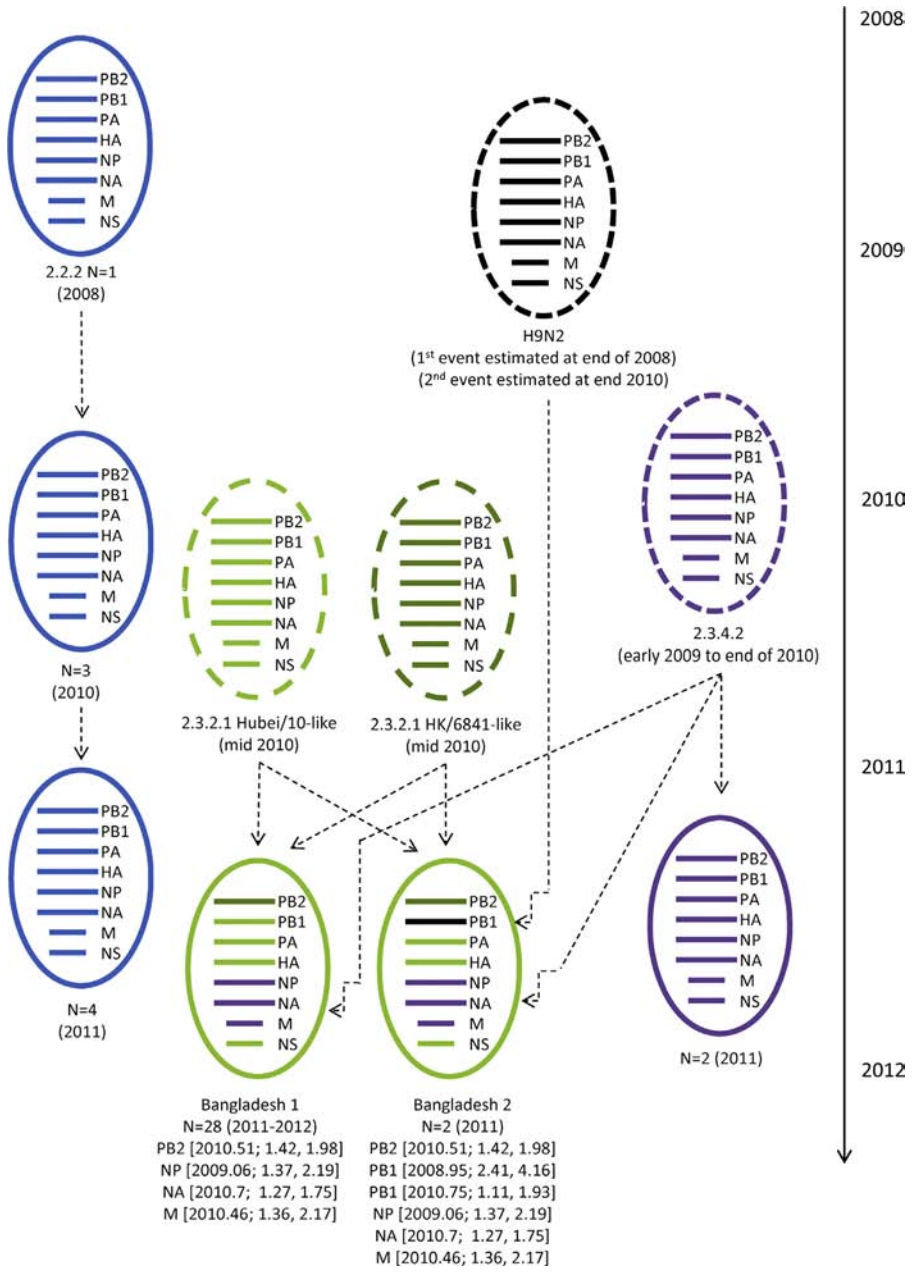

Fig. 3. Estimated timeline for the emergence of H5N1 clades and genotypes in Bangladesh. Each oval contains the eight individual gene segments as parallel lines. Viruses were color coded as follows: clade 2.2.2 (blue), 2.3.2.1 Hubei-like (light green), 2.3.2.1 Hong Kong/6841-like (dark green), 2.3.4.2 (purple) and H9N2 LPAI (black). Hypothetical ancestral viruses are shown with the same color code but with a dashed outline. Genomes reflecting virus isolates characterized for this study are shown with a solid outline. The estimated tMRCA of genes involved in reassortment are indicated below the genome by year with upper and lower HPD 95%. Abbreviations: Hubei/10-like, A/Hubei/1/10-like lineage; HK/6841-like, A/Hong Kong/6841/2010-like lineage.

associated with induction of apoptosis, encoded the 90 amino acid full length protein in all Bangladesh H5 viruses (Chen et al., 2001). All clade 2.2.2 shared the substitution glutamic acid to lysine at residue 627 (E627K) in the PB2 protein that is associated with increased pathogenicity and transmission to human hosts (Shinya et al., 2004; Subbarao et al., 1993). The M1 protein of all H5N1 viruses analyzed in this study contained 30Asp (aspartic acid) and 215Ala (alanine), which were described to increase virulence in mice (Fan et al., 2009). The M2 ion channel protein contained a double amino acid change at residues 27 (valine to alanine) and 31 (serine to asparagine) in A/environment/Bangladesh/1011/2011 and a single mutation of residue 31 (serine to asparagine) in A/environment/Bangladesh/1018/2011. These changes may reduce susceptibility to antivirals amantadine and rimantadine (Hay et al., 1986). In the NA protein no antiviral resistance markers were found (Gubareva et al., 2001).

Discussion

Upon analysis of 40 full genomes of H5N1 viruses collected in Bangladesh from 2008 through 2012, evidence of separate reassortment events was identified between two of the circulating clades of virus, 2.3.2.1 and 2.3.4.2, and between HPAI H5N1 and LPAI H9N2 viruses resulting in two different Bangladesh-specific genotypes (BG1 and BG2). Assessments of geo-temporal relationships of viruses also identified possible routes of transmission and patterns of virus spread into Bangladesh from bordering countries

Table 3
Antigenic characterization of H5N1 viruses isolated from Bangladesh representing each of the three clades detected from 2008 to 2012. Hemagglutination inhibition (HAI) titers of ferret antisera to viruses are shown and listed by HA clade. The homologous titer for each of the reference viruses/antisera is boldfaced and underlined. Italic indicate genetically related HA genes.

Clade	Reference antigens	Reference ferret antisera											
		VN/1203	IND/5	bhd/QI	tk/TK	EG/321	EG/3072	cm/HK	ws/JP	HK/6841	HUB/10 RG30	CR/BA	ANH/1
1	A/VIETNAM/1203/2004	***80***	80	< 10	80	< 10	10	20	< 10	10	80	< 10	10
2.1.3.2	A/INDONESIA/5/2005	< 10	***1280***	80	80	80	80	320	80	320	40	40	80
2.2	A/B-H GOOSE/QINGHAI/1A/05 X PR8	< 10	1280	***1280***	*1280*	*160*	*2560*	640	40	640	160	320	80
2.2.1	A/TURKEY/TURKEY/1/2005	< 10	1280	*640*	***2560***	*320*	*1280*	640	80	640	320	320	80
2.2.1	A/EGYPT/321-NAMRU3/2007	< 10	320	*320*	*320*	***320***	*640*	80	10	160	80	20	20
2.2.1	A/EGYPT/N03072/2010	< 10	320	*320*	*1280*	*160*	***2560***	160	10	640	320	40	160
2.3.2.1	A/COMMON MAGPIE/HK/5052/2007	< 10	320	< 10	40	40	80	***320***	*80*	*320*	*40*	*160*	< 10
2.3.2.1 HK/6841	A/WHOOPER SWAN/HOKKAIDO/4/2011	< 10	160	< 10	10	20	20	*320*	***640***	*640*	*80*	*160*	< 10
2.3.2.1 HK/6841	A/HONG KONG/6841/2010	< 10	80	< 10	20	10	20	*320*	*80*	***160***	*80*	*160*	< 10
2.3.2.1 Hubei	A/HUBEI/1/2010 x PR8 IDCDC-RG30	< 10	320	80	160	40	160	*320*	*160*	*320*	***320***	*160*	< 10
2.3.2.1 Hubei	A/CROW/BANGLADESH/1061/2011	< 10	80	< 10	20	10	20	*20*	*40*	*80*	*40*	***80***	< 10
2.3.4	A/ANHUI/1/2005	< 10	320	20	80	40	20	40	< 10	20	20	< 10	***320***
Clade	**Test antigens**	**Reference ferret antisera**											
		VN/1203	IND/5	bhd/QI	tk/TK	EG/321	EG/3072	cm/HK	ws/JP	HK/6841	HUB/10 RG30	CR/BA	ANH/1
2.2.2	A/CHICKEN/BANGLADESH/31289-1/2011	< 10	80	*160*	*160*	*40*	*20*	20	< 10	< 10	10	< 10	80
2.2.2	A/POULTRY/BANGLADESH/11255-C/2011	< 10	80	*320*	*320*	*40*	*20*	20	< 10	10	< 10	10	40
2.3.2.1 Hubei	A/GOOSE/BANGLADESH/4051T/2011	< 10	80	< 10	10	10	40	*160*	*80*	*160*	*40*	*80*	< 10
2.3.2.1 Hubei	A/DUCK/BANGLADESH/4059T/2011	< 10	80	< 10	< 10	10	20	*80*	*80*	*160*	*40*	*80*	< 10
2.3.2.1 Hubei	A/CHICKEN/BANGLADESH/4070T/2011	< 10	80	10	20	20	40	*160*	*160*	*320*	*80*	*160*	< 10
2.3.2.1 Hubei	A/CROW/BANGLADESH/1058/2011	< 10	160	< 10	20	20	40	*160*	*160*	*160*	*80*	*160*	< 10
2.3.2.1 Hubei	A/CROW/BANGLADESH/313T/2011	< 10	80	< 10	10	10	40	*320*	*80*	*320*	*80*	*160*	< 10
2.3.2.1 Hubei	A/CROW/BANGLADESH/315T/2011	< 10	80	< 10	10	10	40	*160*	*40*	*320*	*40*	*160*	< 10
2.3.2.1 Hubei	A/CROW/BANGLADESH/316T/2011	< 10	40	< 10	10	10	40	*320*	*80*	*320*	*80*	*160*	< 10
2.3.2.1 Hubei	A/DUCK/BANGLADESH/4120T/2011	< 10	80	< 10	10	10	20	*320*	*80*	*320*	*80*	*320*	< 10
2.3.2.1 Hubei	A/DUCK/BANGLADESH/4124T/2011	< 10	40	< 10	< 10	10	20	*160*	*40*	*320*	*40*	*80*	< 10
2.3.2.1 Hubei	A/CHICKEN/BANGLADESH/4058/2011	< 10	80	10	20	10	40	*320*	*80*	*640*	*80*	*160*	< 10
2.3.2.1 Hubei	A/CHICKEN/BANGLADESH/3072/2011	< 10	20	< 10	< 10	< 10	< 10	*80*	*40*	*160*	*20*	*40*	< 10
2.3.2.1 Hubei	A/CHICKEN/BANGLADESH/3075/2011	< 10	40	< 10	< 10	< 10	10	*160*	*40*	*160*	*40*	*80*	< 10
2.3.2.1 Hubei	A/ENVIRONMENT/BANGLADESH/1017/11	< 10	40	< 10	10	10	20	*160*	*40*	*320*	*40*	*80*	< 10
2.3.2.1 Hubei	A/CHICKEN/BANGLADESH/11303/2011	< 10	80	< 10	40	< 10	40	*320*	*20*	*320*	*80*	*160*	< 10
2.3.2.1 Hubei	A/WATERFOWL/BANGLADESH/31935/11	< 10	80	< 10	< 10	10	20	*80*	*80*	*80*	*40*	*80*	< 10
2.3.2.1 Hubei	A/WATERFOWL/BANGLADESH/33025/11	< 10	20	< 10	20	10	40	*10*	*40*	*20*	*80*	*80*	< 10
2.3.4.2	A/CHICKEN/BANGLADESH/3012/2011	< 10	160	< 10	160	40	40	80	20	10	40	< 10	*20*

and geographically close areas, as well as possible transmission from Bangladesh into northern regions. The temporal analyses indicated that the first human case detected in Bangladesh, the 2008 clade 2.2.2 virus, shared common ancestry with all other viruses from that clade. Viruses collected in both Bhutan and Bangladesh from 2010 shared joint descent and might have been introduced via trade through shared borders from India or migratory birds (Chakrabarti et al., 2009; Pawar et al., 2010). The lack of surveillance and sequence availability limited the detection of other regional sources of clade 2.2.2 viruses. The viruses clustered together for all gene segments and are described as genotype 2.2.2 (synonymous with genotype Z) (Duan et al., 2008). Thus, despite the ongoing circulation of clade 2.2.2 viruses from 2008 to 2011, this did not lead to detectable reassortment of viruses in this clade likely because viruses with different genomes were either not yet present or circulating at low levels in Bangladesh. The lack of detection of sporadic reassortants could also be attributable to fewer data available for analysis; for clade 2.2.2 viruses only 50 full genomes were available from public databases.

The majority of viruses studied here belonged to clade 2.3.2.1 (Hubei-like lineage) and were likely seeded in Bangladesh in late 2010 or early 2011 either as a single introduction or multiple introductions with related viruses. Our data suggest that this clade has since spread to different locations across the country and into different species, including chicken and crows (Islam et al., 2012). Most recent HA sequences from 2012 located at the tip of the branches in phylogenetic trees suggested that the initially introduced viruses from the beginning of 2011 continued to circulate within the country and diverged further without additional introductions. In addition, some viruses of the Bangladeshi cluster grouped closely with viruses collected in Nepal suggesting cross-border exchange either through trade or wild bird movement across northern parts of India (Nagarajan et al., 2012). A new genotype (BG1) was characterized in 28 viruses with a 4:3:1 genetic composition that has not been described previously in clade 2.3.2.1 Hubei-like lineage viruses. Full genome analysis showed that they shared the same origin in their HA, PB1, PA and NS genes (2.3.2.1 Hubei-like viruses), but grouped with HK/6841-like viruses (clade 2.3.2.1) in their PB2 genes and clade 2.3.4.2 viruses in the remaining genes (NA, NP and M).

The second genotype (BG2) identified in clade 2.3.2.1 viruses was the result of reassortment of 2.3.2.1 Hubei-like viruses with two phylogenetically distinct PB1 gene sequences derived from LPAI H9N2 subtype viruses and the remainder of the genome related to the more common BG1 reassortant (Fig. 3). The different tMRCAs of both reassortant genotypes, BG1 and BG2, suggested that these resulted from separate events. Given that both LPAI H9N2 and HPAI H5N1 viruses circulate simultaneously in Southeast Asia, including Bangladesh, the possibility of reassortment events between HPAI and LPAI viruses are more probable and likely more common than what has been described previously (Gutiérrez et al., 2009; Negovetich et al., 2011; Vijaykrishna et al., 2008; Zhang et al., 2009). It is likely that the exchange of genetic material was facilitated by the ongoing circulation of different clades of H5N1 and H9N2 viruses in Bangladesh (OIE, 2013). Recently, a LPAI A(H7N9) virus emerged in humans and birds in China in which all gene segments except for HA (H7) and NA (N9) were derived from LPAI H9N2 subtype viruses found in wild birds (Chen et al., 2013; Liu et al., 2013; Shi et al., 2013). This finding highlights that the exchange of gene segments in influenza A viruses is not restricted to a specific subtype but occurs between subtypes with different virologic features such as H5N1 and H9N2 (as in Bangladesh) or H7N9 and H9N2 viruses in China. Notably, the H7N9 viruses have caused severe and fatal disease in infected persons despite lack of symptoms in H7N9 infected birds (Chen et al., 2013; Gao et al., 2013; WHO, 2013b). Ongoing full genome monitoring for both HPAI H5N1 and LPAI viruses of diverse subtypes will be crucial to identify future reassortment in Bangladesh and the Southeast Asian region in general.

The clade 2.3.4.2 virus, A/chicken/Bangladesh/3012/2011, was collected in the Division Chittagong, which shares its southeastern border with Myanmar, and was related to other clade 2.3.4.2 viruses collected in Bangladesh and a virus detected in Myanmar. The longer branches in the phylogenetic trees combined with the estimated ancestral dates, indicated that these viruses diverged from each other at least 1–2 years before they were detected in Bangladesh and might have been introduced separately. Clade 2.3.4.2 viruses were only found in domestic chicken suggesting these viruses might have been introduced through poultry trade (Mon et al., 2012). Ancestral viruses likely emerged from bordering Myanmar or more distant locations southeast of Bangladesh like Lao PDR, China or Vietnam where this clade persisted as early as 2007 (WHO, 2012). Unlike the viruses that were derived from the other clades (2.3.2.1 and 2.2.2), 2.3.4.2 viruses did not consistently group together in their internal genes and NA genes, indicating they reassorted prior to detection in Bangladesh. The low support on some of the individual branches, however, could be attributed to the small dataset used in our analysis. Since 2011, the viruses detected in Bangladesh were derived from clade 2.3.2.1, but not from 2.3.4.2 suggesting a lack of sustained circulation of this genetic group of H5N1 viruses. Alternatively, the long branch lengths separating these viruses from other clade 2.3.4.2 viruses suggests a lack of sequence data from related viruses or surveillance gaps both in and around Bangladesh. Interestingly, clade 2.3.4.2 viruses have recently been detected in China and have caused human infection in 2013 (OIE, 2013; WHO/GIP, 2013).

H5N1 virus clades 2.3.2.1 and 2.3.4.2 have been detected in many countries, including Southeast Asian nations where both of these virus groups have circulated at high levels in recent years (Choi et al., 2013; Hu et al., 2013; Liu et al., 2010). The timeframe of estimated divergence of clade 2.3.2.1, in particular, paired with the location of the first virus detection, indicated possible transmission through bird trade, but involvement of wild birds cannot be excluded. The discovery of H5N1 outbreaks in crows in Bangladesh may also indicate a small role that peridomestic birds may play in virus dispersal (Khan et al., 2013). Viruses from Bangladesh shared common ancestors with viruses from geographically close countries such as India, Myanmar, Nepal, and Bhutan. HA gene sequences of viruses from other countries clustered closely with HA genes of viruses collected in this study confirming persistence of specific HPAI clades both inside of Bangladesh and within the region.

Results from hemagglutination inhibition testing of Bangladesh H5N1 viruses with ferret antisera raised to clades from many different countries indicated substantial antigenic drift between viruses from clades 2.2.2, 2.3.2.1 and 2.3.4.2. Furthermore, sera produced specifically against Bangladesh viruses inhibited agglutination of related Bangladesh isolates at higher reciprocal titers illustrating antigenic specificity relative to viruses collected outside the country. This data suggests that antigenic variation among H5N1 viruses exists both within Bangladesh and in neighboring regions. As such, host population immunity and existence of diverse viruses may influence poultry vaccination policy and implementation. Poultry vaccine challenge studies that assess the need for vaccine viruses to be antigenically matched should be performed. In addition, candidate vaccine viruses developed for pandemic planning purposes, such as A/Hubei/1/2010 (clade 2.3.2.1) and A/Anhui/1/2005 (clade 2.3.4), may not antigenically cover more recently circulating viruses in Bangladesh. Updates of candidate vaccine seed strains using more contemporary viruses will be important as these groups of viruses continue to diversify genetically and antigenically (WHO, 2013a). The close proximity of Bangladesh to other high-density poultry-dependent countries may also continue to increase the genetic and

antigenic diversity of viruses found in Bangladesh and require mitigation strategies at both a national and regional level (FAO, 2013a, b).

The amino acid sequence of the cleavage site in the H5 HA proteins analyzed was clade-specific, as previously observed (Zhang et al., 2012), with all viruses maintaining at least five consecutive basic amino acids. Clade 2.2.2 viruses from 2010 shared a glycosylation site at position 54 of the mature HA protein compared to other clades, and the viruses that belonged to clade 2.3.4.2 had a glycosylation site at residues 154–156. The lack of N-linked glycosylation at position 154, conserved in all but the clade 2.3.4.2 viruses, is noteworthy in that this feature, in combination with other mutations, was described for H5N1 viruses that were aerosol transmissible in a ferret model (Russell et al., 2012). As has been previously described, all clade 2.2.2 viruses detected in Bangladesh had the PB2 substitution E627K associated with increased pathogenicity and potential for mammalian adaptation (Shinya et al., 2004; Subbarao et al., 1993). The lack of this mutation in the clade 2.3.2.1 viruses detected in 2011 and 2012 and the apparent decline in the number of clade 2.2.2 viruses detected in this study may indicate a shift in the potential adaptability of these viruses for mammalian replication. It remains to be seen if the relative predominance of clade 2.3.2.1 viruses in Bangladesh will be sustained as in other countries such as Vietnam (Creanga et al., 2013). While the M1 protein of all viruses analyzed contained mutations associated with increased virulence in mice (Fan et al., 2009), no other internal gene proteins were found to possess known molecular markers of concern. In addition, no markers of reduced susceptibility to neuraminidase inhibitors were found.

This comprehensive phylogenetic study of full genome sequences from H5N1 viruses presently circulating in Bangladesh indicates that the ancestors of some H5N1 viruses reassorted with other lineages of the same clades (2.3.2.1), with other clades of H5N1 viruses (2.3.2.1 and 2.3.4.2) and even with other LPAI virus subtypes known to circulate in Bangladesh (International Centre for Diarrhoeal Disease Research, 2011). These events created a diverse genetic pool of H5N1 viruses that contribute to the dispersal of multiple variants in the country and the regions surrounding Bangladesh. Potential limitations of this study were the lack of full genome data for viruses identified in neighboring countries paired with surveillance gaps. This is particularly evident for clade 2.3.4.2 viruses that remain apparently undersampled based on available sequence data in the public domain. To achieve more accurate estimates of times of the most recent common ancestors and dispersal of viruses, exact sample collection dates in combination with locations of collection are crucial. Continuous avian influenza surveillance in poultry in Bangladesh, together with the molecular and antigenic characterization of the circulating viruses, will reveal further diversification within the virus population and help to explain virus dispersal from bordering and nearby countries in the future.

Materials and methods

Avian influenza virus surveillance and sample collection

All specimen were collected from 2008 through 2012 during multiple studies undertaken in Bangladesh by International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b), and various government partners (Institute for Epidemiology, Disease Control and Research; Department of Livestock Services; Department of Forestry) as well as international partners (Centers for Disease Control and Prevention [CDC]; EcoHealth Alliance). These projects included active live bird market surveillance, backyard poultry surveillance from nationally representative village locations and poultry and wild bird outbreak investigations. Individual oropharyngeal or tracheal swabs were collected from live birds sold at markets or backyard raised poultry or from bird carcasses collected or live birds trapped during outbreak investigations. Environmental swabs were sampled from surfaces of live bird markets, backyards where poultry flocks lived and from bird droppings around outbreak sites. Samples from H5N1-positive human cases were collected as previously described (Brooks et al., 2010; International Centre for Diarrhoeal Disease Research, 2011). Geographic distribution of samples was mapped using coordinates of the collection locations and the boundary map of Bangladesh in ArcGIS 9.3 (Environmental System Research Institute, Redlands, CA, USA).

Virus isolation, subtype detection and full genome sequencing

Original specimens were screened at icddr,b for influenza A virus using a real-time reverse transcription (RT)-PCR detection kit targeting the matrix (M) gene (CDC, 2013). Influenza A positive samples were also screened for the presence of influenza A HA subtypes H5 and H9 and tested with a H5 clade specific real-time RT-PCR (CDC, 2013; Kis et al., 2013). Influenza A positive samples were sent to CDC for further characterization. At the CDC, influenza A(H5) virus PCR-positive samples were inoculated into 10–11 day old embryonated chicken eggs (ECEs) and amniotic/allantoic fluid was harvested 24 hours post-inoculation and tested by hemagglutination assay (HA) using turkey red blood cells. All infectious materials were maintained in biosafety level 3 containment, including enhancements required by the U.S. Department of Agriculture and the Select Agents program (http://www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm). Only specimens that yielded ≥ 8 HA units were included in further analyses. Genomic RNA extracted from virus-infected amniotic/allantoic fluid using the RNeasy extraction kit (Qiagen, Valencia, CA) was used as template for generation of cDNA by random hexamer-primed reverse transcription (SuperScript®III, Life Technologies, Carlsbad, CA). The surface and internal protein genes were then amplified using influenza A virus specific primers (available upon request) as overlapping fragments with the Access Quick one-step RT-PCR kit (Promega, Madison, WI) and subsequently sequenced on an automated Applied Biosystems 3730 system using cycle sequencing dye terminator chemistry (Life Technologies, Carlsbad, CA). Contigs of full length open reading frames were generated for each gene (Sequencher 4.10.1, Gene Codes, Ann Arbor, MI). Gene sequences were submitted to GISAID (http://platform.gisaid.org) prior to publication (Accession Numbers: EPI 448024-448111, 448120-448279, 448883-448924, 353364, 353365, 353370, 353372, 353379, 353381, 314772-314779, 219467-219474, 460194-460201).

Molecular characterization

For full genome phylogenetic comparison, publicly available H5N1 sequences were included in datasets and annotated according to their HA clade designation. Sequences were aligned with the MUSCLE algorithm implemented in BioEdit (Edgar, 2004; Hall, 1999). Alignments were manually edited for frame shifts, sequence duplication and gaps. Trees to identify larger clusters were inferred using the neighbor joining (NJ) method with a Kimura 2-parameter implemented in MEGA5 (Tamura et al., 2011). Amino acid comparison was also performed in BioEdit and MEGA5. The presence of a multibasic cleavage site indicative of high pathogenicity and glycosylation sites in the HA protein were determined by comparing the coding region of each virus.

Antigenic characterization

Isolates were tested by hemagglutination inhibition assay (HAI) with ferret antisera raised against viruses representing various H5N1 clades (1, 2.1.3.2, 2.2, 2.2.1, 2.2.2, 2.3.2.1, 2.3.4) including those strains previously identified as candidate vaccine viruses (Klimov et al., 2012). Antiserum was also raised to select viruses collected during this study from different phylogenetic clades. Briefly, sera from male ferrets greater than six months of age (Triple F Farms, Sayre, PA) were tested by HAI for the presence of pre-existing antibody to seasonal influenza A viruses. To generate ferret antisera used for HAI testing, serologically naive ferrets were inoculated intranasally with 500 μl of diluted virus per nare with a range of doses depending on the virus. Ferrets were boosted with concentrated virus and adjuvant at approximately 14 days post-infection (dpi) and were exsanguinated at approximately 28 dpi. Serum was stored at −20 °C until further use. As previously described for the HAI assay, viruses were standardized to 8 HAU/50 μl and added to serially diluted, receptor destroying enzyme (RDE)-treated antisera (DENKA SEIKEN, Campbell, CA) followed by incubation at room temperature and agglutination with turkey red blood cells (Klimov et al., 2012). The HAI titers were reported as the reciprocal of the last dilution of antiserum that completely inhibited hemagglutination.

Genotyping and reassortment analysis

The dataset included full genomes of 40 H5N1 viruses collected in Bangladesh from 2008 through 2012, characterized and sequenced at CDC. For the analysis of the individual gene segments, 83 full genome sequences of genetically related viruses from public databases with at least 90% of full sequence length were included except for the PB1 gene, for which 89 virus sequences were used (Supplemental Table 1). Six additional PB1 sequences from H9N2 subtype viruses were included in the PB1 gene dataset due to the relatedness of H5N1 and H9N2 virus sequences upon basic local alignment search tool algorithm (BLAST) analysis (Altschul et al., 1990). The full genome sequences of three H5N1 viruses isolated from specimens collected from humans during community based surveillance for respiratory surveillance and pneumonia in the Dhaka (Kamalapur) were also included (Brooks et al., 2007; Brooks et al., 2010; International Centre for Diarrhoeal Disease Research, 2011). Reference virus sequences were used to represent H5N1 clades 2.2.2 and 2.3.4.2 and the three lineages of clade 2.3.2.1: Hubei-like (A/Hubei/1/2010), Hong Kong/6841-like (A/Hong Kong/6841/2010) and barn swallow/1161-like (A/barn swallow/Hong Kong/D10-1161/2010). To describe the microevolution among viruses from Bangladesh, genotypes were named based on their HA gene clade annotation and then applied to the NA and the six internal genes.

Temporal distribution using Bayesian analysis

The time of most recent common ancestor (tMRCA) was estimated using dated gene sequence alignments for each of the eight gene segments with the program package BEAST version 1.7.5 (Drummond and Rambaut, 2007). For the temporal distribution of the isolates, we used the collection date of the specimen that was available for all 40 H5N1 viruses isolated in Bangladesh. When dates were unavailable, the date was assumed to be the median of the collection year (if month was unknown) or the median of the collection month (if only day was unknown). The tMRCA is expressed as a decimal date and for each tMRCA a credible interval (Bayesian confidence interval) is given as the highest posterior density (HPD 95%) that represents an interval in the domain of a posterior probability distribution. For the Bayesian analysis, at least 50 million Monte Carlo Markov Chain (MCMC) generations were run in the SDR06 model either with HKY (Hasegawa–Kishino–Yano) or the GTR (general time reversible) model; both models were used with a gamma distribution, three partitions and a 10% burn-in removal (Shapiro et al., 2006; Yang, 1996). Effective sample size (ESS) was evaluated in Tracer for each individual run and data were only included when the ESS for molecular clock parameters were greater than 200 (Rambaut and Drummond, 2009). Tree files were generated with software included in the BEAST package and visualized in FigTree version 1.4.0 (Rambaut, 2009).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.12.023.

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