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Detection of highly pathogenic avian influenza A(H5N6) viruses in waterfowl in Bangladesh

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ABSTRACT

Bangladesh has reported repeated outbreaks of highly pathogenic avian influenza (HPAI) A(H5) viruses in poultry since 2007. Because of the large number of live poultry markets (LPM) relative to the population density of poultry throughout the country, these markets can serve as sentinel sites for HPAI A(H5) detection. Through active LPM surveillance during June 2016–June 2017, HPAI A(H5N6) viruses along with 14 other subtypes of influenza A viruses were detected. The HPAI A(H5N6) viruses belonged to clade 2.3.4.4 and were likely introduced into Bangladesh around March 2016. Human infections with influenza clade 2.3.4.4 viruses in Bangladesh have not been identified, but the viruses had several molecular markers associated with potential human infection. Vigilant surveillance at the animal-human interface is essential to identify emerging avian influenza viruses with the potential to threaten public and animal health.

1. Introduction

Highly pathogenic avian influenza (HPAI) A(H5) viruses belonging to clade 2.3.4.4 of the A/goose/Guangdong/1/1996 hemagglutinin (HA) gene lineage have spread widely in birds in Europe, Africa, Asia and the Middle East in recent years likely as a result of migratory birds and poultry trade (Lee et al., 2016; Wong et al., 2015). These viruses appeared to have first emerged in China in 2008 (Gu et al., 2011; Smith et al., 2015) and spread to Southeast Asian countries beginning in 2014 (Lee et al., 2017; Okamatsu et al., 2017; Wong et al., 2015). The expansion led to their detection in more than 23 countries with a wide geographical distribution across four continents (OIE, 2018). During this expansion, an unprecedented number of neuraminidase (NA) subtypes have replaced the N1 gene, including N2, N3, N5, N6, and N8 (Smith et al., 2015). Among the different NA subtypes of clade 2.3.4.4 viruses, A(H5N6) viruses are the only subtype that has caused human infections (Claes et al., 2016; Lei Yang, 2017; Wu et al., 2017). As of February 2019, 23 confirmed cases of A(H5N6) infection in humans have been reported; all cases were identified in China (WHO, 2018). Of these, 14 (60.9%) were fatal (Rusheng et al, 2016; WHO, 2018; Wu et al., 2017; Zhang et al., 2017).

In Bangladesh, people rely heavily on poultry for income and sustenance because of socioeconomic, cultural and religious preferences (Khan et al., 2018; Moyen et al., 2018). It is estimated that more than 300 million poultry (primarily chickens and ducks) are raised in Bangladesh and that greater than 80% of rural households raise backyard flocks (Hamid et al., 2017; Biswas et al., 2009). Although transmission of HPAI A(H5) viruses to humans appears to be rare in Bangladesh (Khan et al., 2018; Nasreen et al., 2015; Zaman et al., 2009), circulation of multiple subtypes of avian influenza viruses (AIVs) in poultry in live poultry markets (LPMs) provides opportunities for reassortment and generation of novel AIVs that could cross species barriers to infect humans (Gerloff et al., 2016; Khan et al., 2018; Md. Zakiul Hassan, 2016). Indeed, LPM workers who had close contact with poultry or their fomites had a 7.6 times higher risk for infection with AIVs compared to those who had less exposure to LPMs ($p < 0.001$) (Nasreen

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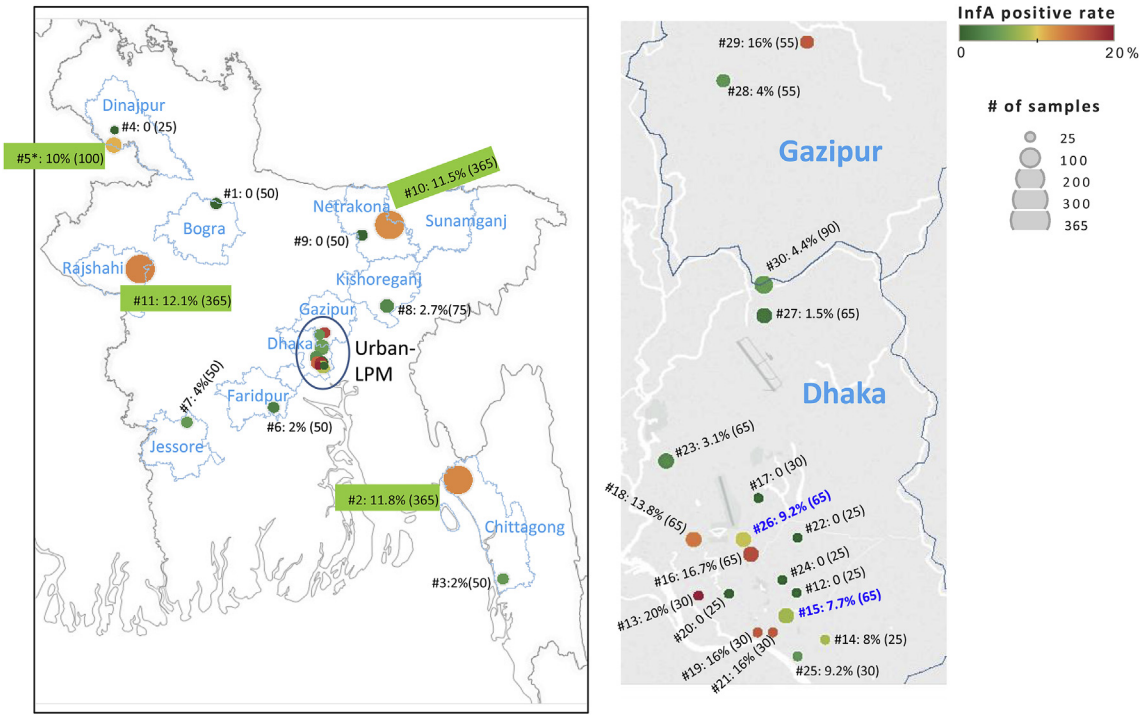

Fig. 1. Location and percent positivity for influenza A RNA in poultry among live poultry markets surveyed in Bangladesh. The market #, percentage of samples positive for influenza A RNA (excluding pooled environmental samples), and total number of samples collected from poultry in each market (in parentheses) were labeled. The mark size is proportional to the total number of samples and the mark color differentiates the positive rate of influenza A RNA ranging from 0 to 20%. **Left panel:** Location of all 30 live poultry markets, including 19 urban live poultry markets clustered in the Dhaka District (circled). The live poultry markets where waterfowl samples were collected are denoted with a green-color filled text box. * No waterfowl samples were collected from this site after Oct 2016. Also, no environmental samples were collected at this site as no poultry slaughtering was performed in this small market. **Right panel**: Location of all 19 urban live poultry markets in the Dhaka District. Two wholesale urban markets are labeled with bold blue fonts.

et al., 2015). While Dhaka and its surrounding metropolitan areas have 20–40% of the LPMs found in the country (Marinova-Petkova et al., 2016; Turner et al., 2017), a high density of LPMs are also spread throughout other regions of the country. A surveillance platform in Bangladesh LPMs was established in 2007 after detection of the HPAI A(H5) viruses in domestic poultry in order to identify emerging AIVs in poultry that pose a potential threat to human and animal health (Khan et al., 2018). This study describes the influenza A subtypes identified by this surveillance system from June 2016 to June 2017.

2. Materials and Methods

2.1. Sample collection

Avian influenza surveillance among domestic poultry in Bangladesh was conducted by the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) in collaboration with the Department of Livestock Services and U.S. Centers for Disease Control and Prevention. This surveillance platform was established in August 2007 in three LPMs. In September 2013, the surveillance was expanded to 30 LPMs (11 peri-urban LPMs and 19 urban LPMs) (Fig. 1). Due to availability of funding, the number of sites decreased after October 2016 to 10 sites (3 peri-urban and 7 urban) (S. Table 1). Specifically, the number of sites where samples were collected from domestic waterfowl decreased from four to three; five to three for backyard chickens, and twenty four to seven for commercial chickens (S. Table 1). The number of samples collected varied throughout the study period. Twenty monthly domestic waterfowl samples, ten backyard chicken samples per market were collected for the duration of the study. One environmental sample per month was collected in all but the Khanpur Bazar Market because there was no poultry slaughtering service available in the small market. For commercial chicken samples collected from June–October 2016, fifteen samples were collected per month from the Bajitpur Poura Bazar Market; ten samples were collected per month from the Gazipur Chourasta Bazar Market, the Shivebari Pouro Kancha Bazar Market, and the Tongi Bazar Market; and five samples were collected per month from the other LPMs. For commercial chicken samples collected from November 2016–June 2017, five samples were collected per month (S. Table 1). Waterfowl and backyard chicken samples were only collected in the peri-urban LPMs.

Poultry that appeared to be sick were targeted for sample collection; when no poultry were observed to be sick, samples were taken from healthy appearing poultry. When possible, samples were collected from different shops each month, with a maximum of four poultry from one farmer or vendor. Cloacal swabs were collected on cotton swabs (Thermo Fisher Scientific) and stored in individual cryovials (ThermoFisher Scientific) each containing 1 mL viral transport media (VTM). Ten environmental swabs were collected across a LPM from feces, floors, cages, waste bins, drinking water troughs, chopping boards or blood. In small LPMs where chopping boards or other relevant environment surfaces could not be identified, five environmental swabs were collected instead. All environmental swabs collected from one LPM were pooled into 50 mL falcon tubes containing 10 mL VTM. Each vial or tube was given a unique identification number, placed within an insulated cooler (4–8 °C) and transferred immediately to −80 °C for storage.

2.2. Detection and sequencing of AIVs

Total RNA was extracted from 100 µL of sample using the RNeasy kit (Qiagen) or MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche Diagnostics) following the manufacturer's instructions. The purified RNA was initially screened for the detection of the influenza A virus matrix gene using a TaqMan real-time RT-PCR assay (CDC, 2018).

Table 1
Subtypes of influenza A virus identified in live poultry markets in Bangladesh from June 2016–June 2017 (by location and sample type).

Division	District	Name of LPM	LPM#	Market location	Host	Sample type	Collection date3	Subtype4	# of samples5
Environmental samples, N = 225, Number of samples positive for influenza A = 89 (40%)									
Dhaka	Kishoreganj	Bajitpur Poura Bazzar	8	Peri-urban	ND2	Env. pool	10/4/16	H9N2	1
	Dhaka	Jatrabari Kancha Bazzar	14	Urban	ND	Env. pool	7/12/16	H9N2	1
		Kaptan Bazzar1	15	Urban	ND	Env. pool	7/12/16	**H2N3**	1
					ND	Env. pool	9/10/16	HxN1	1
					ND	Env. pool	1/25/17	H9N2	1
					ND	Env. pool	3/2/2017–4/11/17	H5N1	2
		Moulavi Bazzar	19	Urban	ND	Env. pool	7/12/16	H5N1	1
		Noya Bazzar	21	Urban	ND	Env. pool	5/9/16	HxN1	1
		Shah Ali Market Kancha Bazzar	23	Urban	ND	Env. pool	2/21/17	H9N2	1
		Tejgaon Poultry Paikari Market1	26	Urban	ND	Env. pool	7/19/16	H5N1	1
		Mohammadpur Town Hall Market	18	Urban	ND	Env. pool	1/25/17	HxN1	1
					ND	Env. pool	5/30/17	H9N2	1
	Gazipur	Shivebari Pouro Kancha Bazzar	29		ND	Env. pool	7/26/16	H9N2	1
Mymensingh	Netrokona	Mohonganj Hash Murgir Bazzar	10	Peri-urban	ND	Env. pool	9/7/16	H9Nx	1
					ND	Env. pool	6/7/17	H9N2	1
					ND	Env. pool	5/3/17	H9/H5N6	1
Rajshahi	Rajshahi	Taherpur Bazzar	11	Peri-urban	ND	Env. pool	9/5/16	H9N2	1
					ND	Env. pool	1/23/17	**H11N9**	1
					ND	Env. pool	3/20/17	**H5N3**	1
Waterfowl samples, N = 880, Number of samples positive for influenza A = 128 (15%)									
Chittagong	Chittagong	Mithachara Bazzar	2	Peri-urban	Duck	Cloacal swab	12/18/16	H3N5	1
					Duck	Cloacal swab	12/18/16	H9N2	2
					Duck	Cloacal swab	12/18/16	**H11N1**	1
					Duck	Cloacal swab	6/16/2016–12/21/2016	H5N1	6
					Duck	Cloacal swab	6/20/16–3/9/2017	HxN1	3
Mymensingh	Netrokona	Mohonganj Hash Murgir Bazzar	10	Peri-urban	Duck	Cloacal swab	12/21/16	H5N1	1
					Duck	Cloacal swab	9/7/16	**H6N2**	2
					Duck	Cloacal swab	9/7/16	**H11N2**	1
					Duck	Cloacal swab	10/5/16	H3N6	1
					Duck	Cloacal swab	10/5/16	H9N2	1
					Duck	Cloacal swab	12/21/16	HxN1	2
					Duck	Cloacal swab	1/17/17–5/3/17	H5N6	6
					Duck	Cloacal swab	8/17/2016–9/7/16	H2N3	3
					goose	Cloacal swab	12/21/16	H5Nx	2
					goose	Cloacal swab	12/21/16	H5N1	1
					goose	Cloacal swab	1/11/17	HxN6	1
					goose	Cloacal swab	1/11/17–5/3/17	**H5N6**	3
Rajshahi	Rajshahi	Taherpur Bazzar	11	Peri-urban	Duck	Cloacal swab	7/15/16	**H1N4**	1
					Duck	Cloacal swab	9/4/16	H9N2	3
					Duck	Cloacal swab	11/7/16	H4N6	4
					Duck	Cloacal swab	12/9/16	**H11N9**	1
					Duck	Cloacal swab	12/9/16	H11Nx	3
					Duck	Cloacal swab	1/23/17	**H2N3**	1
					Duck	Cloacal swab	1/23/17	H6N7	2
					Duck	Cloacal swab	5/8/17	H5N1	1
					Duck	Cloacal swab	3/20/2017–1/15/17	HxN1	3
					goose	Cloacal swab	5/8/17	HxN1	1
Rangpur	Dinajpur	Khanpur Bazzar	5	Peri-urban	Duck	Cloacal swab	6/20/16	HxN1	1
					Duck	Cloacal swab	7/22/16	H3N8	2
Commercial chicken samples, N = 1005, Number of samples positive for influenza A = 73 (7%)									
Dhaka	Dhaka	Noya Bazzar	21	Urban	Chicken	Cloacal swab	9/5/16–3/2/17	H5N1	3
		Tejgaon Poultry Paikari Market1	26	Urban	Chicken	Cloacal swab	7/19/16	H9N2	1
		Mohammadpur Town Hall Market	18	Urban	Chicken	Cloacal swab	6/14/17	H9N2	1
	Gazipur	Tongi Bazzar	30	Urban	Chicken	Cloacal swab	9/6/2016–5/3/17	H9N2	2
	Kishoreganj	Bajitpur Poura Bazzar	8	Peri-urban	Chicken	Cloacal swab	9/6/16	H9N2	1
Backyard chicken samples, N = 490, Number of samples positive for influenza A = 9 (2%)									
Rajshahi	Rajshahi	Taherpur Bazzar	11	Peri-urban	Chicken	Cloacal swab	4/24/17	H5N1	1
Dhaka	Faridpur	Bangha Bazzar	6	Peri-urban	Chicken	Cloacal swab	8/29/16	H9N2	1

[1]wholesale market.
[2]ND, not determined for pooled samples.
[3]A range in dates were given for the samples collected in consecutive days.
[4]Subtype is based on full HA and NA sequence analysis. Letter "x" denotes unknown HA or NA subtype if HA or NA sequences were not available. Subtypes in bold font represent novel subtypes that were not reported in Bangladesh previously.
[5]Twenty monthly waterfowl samples, ten backyard chicken samples, and one environmental sample per market were sampled for the duration of the study. Monthly commercial chicken samples varied over the study period (see Materials and Methods for details).

All influenza A-positive samples were subjected to full genome sequencing using Illumina MiSeq technology, as previously described (Shepard et al., 2016) and sequences were deposited in the GISAID EpiFlu database (Accession numbers EPI_ISL_332804 to EPI_ISL_333273).

2.3. Phylogenetic tree analysis

Codon-complete viral gene segments were aligned with sequences downloaded from the GISAID database following basic local alignment search tool analysis to identify closely related strains. Phylogenetic

trees of aligned gene segments were constructed using the Rainbow software package with neighbor-joining analysis and the Jukes-Cantor substitution model, as previously described (Rith et al., 2014). Each analysis was run with 1,000 bootstrap iterations for statistical support. Nucleotide sequence alignments used for trees were also translated to amino acid protein sequences to compare the HA sequence of each virus to the WHO recommended pre-pandemic candidate vaccine virus (CVV), A/Fujian-Sanyuan/21099/2017 (H5N6). Amino acid differences between the clade 2.3.4.4 viruses and this CVV were plotted on the HA phylogenetic tree at each branch node.

2.4. tMRCA analysis

Sequences for the time of most recent common ancestor (tMRCA) analysis were downloaded from GISAID using the Basic Local Alignment Search Tool (BLAST). The first 500 sequences that were closely related to the HA sequence of the A(H5N6) virus, A/duck/Bangladesh/19D857/2017 (EPI_ISL_332804), were further selected for analysis. After removing all sequences with unknown collection dates, duplicate entries, or truncation in sequences (> 60 nucleotides in the HA1 sequence or > 10% of the full length HA sequences), 363 sequences were left for the tMRCA analysis. tMRCA was estimated using the program package BEAST version 2.4.8 (Bouckaert et al., 2014). At least 130 million Monte Carlo Markov Chain generations were run with 10% burn-in removal in the general time reversible gamma nucleotide substitution model with a relaxed clock. The resulting effective sample size value for each posterior parameter was greater than 200. The maximum clade credibility trees were visualized and annotated with FigTree version 1.4.3. The tMRCA is expressed as the highest posterior density (HPD 95%) that represents an interval in the domain of a posterior probability distribution.

2.5. Antigenic characterization

Clade 2.3.4.4 H5N6-positive samples were inoculated into 10–11 day old embryonated chicken eggs and allantoic fluid was harvested 24 h post-inoculation. Isolation-positive samples (n = 6) were identified by hemagglutination assay using turkey red blood cells. Antigenic characterization of virus isolates was performed using the hemagglutination inhibition assay with polyclonal ferret immune sera produced against representative viruses and CVVs, as previously described (Amanda L. Balish, 2010).

2.6. Statistical analysis

The Fisher's exact two-sided test offered in GraphPad Prism7 (GraphPad Software Inc.) was used to compare influenza A prevalence between peri-urban LPMs and urban LPMs. The odds ratio (OR) of AIV prevalence between different hosts and different market types were calculated using Fisher's exact test.

2.7. Ethical considerations

Approval for the study was received by iccdr,b's institutional review boards: Research Review committee, Ethical Review Committee, and Animal Experimentation Ethics Committee. The surveillance components were implemented according to the protocol and all methods were performed in accordance with the relevant guidelines and regulations. During the sample collection, we described the purpose of this surveillance, expected outcome, process of sampling, and potential harm and benefits of being included in the study to the poultry owners and obtained informed consent. Animal studies performed at the Centers for Disease Control and Prevention were approved under IACUC protocol 2621DAVFERC.

3. Results

3.1. Sample collection, identification, and sequencing

In total, we collected environmental pooled samples (n = 225), domestic waterfowl cloacal swabs (n = 880), commercial chicken cloacal swabs (n = 1,005), and backyard chicken cloacal swabs (n = 490) (Table 1). Among the 2,375 poultry sampled, 2,366 (99.6%) of them were apparently healthy; nine backyard chickens appeared to be sick at the time of sampling, but no influenza A RNA was detected in these birds. Influenza A virus RNA was detected in 299 of 2,600 samples (11.5%) (S. Table 1). Influenza A virus appeared to be most prevalent among waterfowl compared to commercial chicken (odds ratio = 2.2, $p < 0.0001$) or backyard chicken (odds ratio = 9.1, $p < 0.0001$). There was no significant difference in overall prevalence of AIVs detected in commercial chickens between urban and peri-urban LPMs, or between wholesale LPMs (#15 and #26) and surrounding retail LPMs (#12–14, #17–23, #24–25) ($p > 0.2$).

All 299 influenza A positive samples were subjected to full genome sequencing. Complete or partial genome sequences were generated from 90 samples, while the others either had insufficient amplification for NGS library preparation or failed to meet internal thresholds for confident base calling. Sequence analysis identified at least 15 subtypes of AIVs (Table 1), eight of which were previously unreported in Bangladesh based on gene sequence database searches (H1N4, H2N3, H3N5, H5N3, H5N6, H6N2, H11N1, H11N9) (Table 1). Chicken samples were positive for only A(H5N1) and A(H9N2) viruses. Environmental samples and samples collected from waterfowl were positive for H1N4, H2N3, H3N5, H5N1, H5N3, H5N6, H6N2, H9N2, H11N1, and H11N9 (Table 1). A(H5N6) viral RNA was detected in ten samples; all HPAI A(H5N6) viruses were detected in waterfowl (n = 9) or in the LPM environment (n = 1) where waterfowl samples were collected (Table 1). Waterfowl samples were collected from four peri-urban LPMs (#2, #5, #10, and #11) (Fig. 1). Of note, the A(H5N6) viruses were detected in six swabs from ducks and three swabs from geese collected between Jan 17, 2017 and May 3, 2017 from the same LPM (#10) in Netrokona, Bangladesh. All A(H5N6) positive duck and geese were apparently healthy during sample collection. A(H5N6) viral RNA was also detected in an environmental sample collected on May 3, 2017 in LPM (#10) (Table 1 and Fig. 1).

3.2. Phylogenetic tree and evolution analysis

HA genes of the ten A(H5N6) viruses detected in the Netrokona LPM were related to the European/Middle Eastern/African cluster of the clade 2.3.4.4 viruses, previously described as cluster B 2.3.4.4 viruses by Lee et al.(Lee DH, 2014), with the closest nucleotide sequence similarities to A/turkey/Poland/83/2016 (H5N8) (99.46%) and A/chicken/Cameroon/17RS1661-1/2017 (H5N8)(99.18%) (Fig. 2 and S. Fig. 1). NP, M and NS gene segments were also closely related to A/chicken/Cameroon/17RS1661-1/2017 (H5N8) (nucleotide sequence identity > 99.3%) (S. Fig. 1). For the polymerase genes, the highest nucleotide identities were A/shoveler/Chany/82K/2014 (H3N8) for PB2 (97.7%), A/mallard/Chany/355/2016 (H1N1) for PB1(98.7%), and A/duck/Bangladesh/30828/2016 (H3N8) (98.7%) for PA (S. Fig. 1). The NA genes had the highest nucleotide identity with A/duck/Mongolia/127/2015 (H4N6) (98.6%), but were less than 85% similar to the closest A(H5N6) clade 2.3.4.4 CVV, A/Fujian-Sanyuan/21099/2017, suggesting that the NA genes of the Bangladesh viruses had recently reasserted with wild bird lineage viruses and were not immediately derived from A(H5N6) viruses circulating in other regions. In contrast, the Bangladesh A(H5N6) viruses shared over 97% identity in the HA, M and NS genes of A/Fujian-Sanyuan/21099/2017 (H5N6) virus. Nine of the 10 Bangladesh A(H5N6) viruses had nearly identical genomes with nucleotide identities > 99.4% for all genes except the NP (Supplemental Table 3). The NP gene of the environmental A(H5N6)

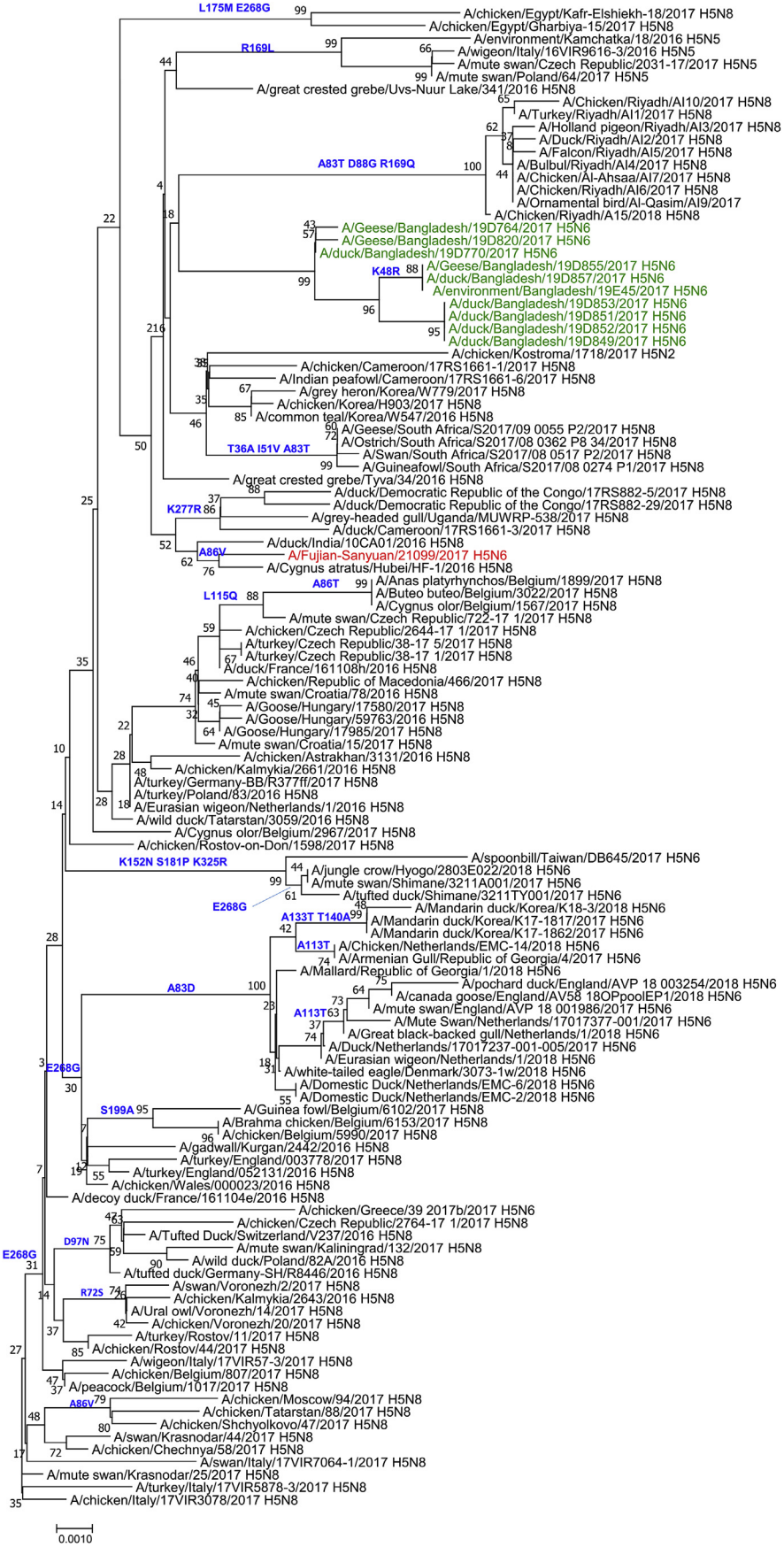

Fig. 2. Phylogenetic tree of HA gene coding sequences of the Bangladesh 2.3.4.4 viruses and closely-related viruses. A(H5N6) viruses identified in this study are in green. Clade 2.3.4.4 candidate vaccine virus, A/Fujian-Sanyuan/21099/2017, is in red. HA1 amino acid differences (numbering according to mature HA1 with signal peptide removed) compared to A/Fujian-Sanyuan/21099/2017 are shown above each node in blue. Bootstrap values were calculated from 1000 bootstrap replications. Scale bar represents number of nucleotide substitutions per site.

Table 2
Hemagglutination inhibition reactions of clade 2.3.4.4 A(H5N6) viruses detected in Bangladesh.

Reference viruses	Reference Antisera[a]					
	Subtype	ANH/1	IDCDC-RG42A	dk/ENG/36254	ck/VN/A324	ck/VN/15A59	dk/Hyogo
A/Anhui/1/2005	H5N1	**640**	< 10	20	< 10	40	80
A/Sichuan/26211/2014 x PR8 (IDCDC-RG42A)	H5N6	< 10	**160**	160	640	160	320
A/duck/England/36254/2014	H5N8	< 10	20	**80**	80	160	40
A/chicken/Vietnam/NCVD-14-A324/2014	H5N6	< 10	40	40	**320**	40	160
A/chicken/Vietnam/NCVD-15A59/2015	H5N6	< 10	20	20	320	**80**	320
A/duck/Hyogo/1/2016	H5N6	< 10	< 10	< 10	< 10	< 10	**160**
Test antigens							
A/duck/Bangladesh/19D770/2017	H5N6	< 10	20	40	40	40	40
A/goose/Bangladesh/19D820/2017	H5N6	< 10	< 10	40	40	40	40
A/duck/Bangladesh/19D849/2017	H5N6	10	10	20	20	20	10
A/duck/Bangladesh/19D851/2017	H5N6	10	10	20	20	10	10
A/goose/Bangladesh/19D855/2017	H5N6	10	10	40	40	20	40
A/duck/Bangladesh/19D857/2017	H5N6	10	10	20	20	20	20

ANH/1 = A/Anhui/1/2005; IDCDC-RG42A = A/Sichuan/26211/2014 x PR8 (IDCDC-RG42A); dk/ENG/36254 = A/duck/England/36254/2014; ck/VN/A324 = A/chicken/Vietnam/NCVD-14-A324/2014; ck/VN/15A59 = A/chicken/Vietnam/NCVD-15A59/2015; dk/Hyogo = A/duck/Hyogo/1/2016.
[a] Homologous antibody titer is marked with bold underline fonts.

virus was noticeably different from the other nine viruses with 8–10 amino acids difference relative to the other viruses (S. Table 2). Molecular clock analysis indicated the Bangladesh A(H5N6) viruses all shared a common ancestor with Asian and European H5N8 viruses, which spread to Africa, the Middle East and some Asian countries, including Bangladesh, around March 2016 (2016.278, 95% highest posterior density [HPD]: 1.948, 2.347) (S. Fig. 2). The other HPAI A(H5) viruses detected in this study were identified as clade 2.3.2.1a viruses, which have been the predominant genetic group circulating in Bangladesh since 2011 (data not shown)(Gerloff et al., 2016; Khan et al., 2018).

3.3. Protein sequence feature analysis

The HA proteins of all ten A(H5N6) 2.3.4.4 viruses had the same multibasic cleavage motif (PLREKRRKR/G) as viruses isolated from humans in China, A/Fujian-Sanyuan/21099/2017 (H5N6) and A/Sichuan/26221/2014 (H5N6) (Bi et al., 2016b). Although there have been no human infections of clade 2.3.4.4 viruses identified in Bangladesh, all HA proteins had amino acid residues HA-133A, 156A, and 235P, which were associated with increased binding of virus to the human α-2,6 linked sialic acid receptor (Wang et al., 2010; Watanabe et al., 2011; Yang ZY1, 2007). The PB2 proteins did not have the 627K residue, but did have PB2–89V, 309D, 339K, 477G, 495V, and 676T. All of these amino acids were found to be capable of compensating for PB2–627K to confer increased polymerase activity of A/wild duck/Hunan/021/2005 (H5N1) in mouse and human cells (Li et al., 2009). In addition, there were several other virulence markers in the Bangladesh A(H5N6) viral M gene (M1- 30D and 215A) and NS gene (NS1–36S, 97F, and 100M) that have been found to be important determinants for systemic infection and increased lethality of A(H5N1) virus in mice (Fan et al., 2009; Jiao et al., 2008; Kuo and Krug, 2009; Spesock et al., 2011).

3.4. Antigenic characterization

Ferret antisera raised to A/Anhui/1/2005(H5N1), A/Sichuan/26211/2014x PR8 CVV (H5N6), A/chicken/Viet Nam/NCVD-14-A324/2015 (H5N6), and A/duck/Hyogo/1/2016 (H5N6) did not inhibit the Bangladesh A(H5N6) viruses. Antisera raised to A/duck/England/36254/2014 (H5N8) and A/chicken/Viet Nam/NCVD-15A59/2015 (H5N6) inhibited the Bangladesh viruses at titers that were generally 4-fold or greater reduced compared to the homologous virus titers. Inconsistent reactivity of the Bangladesh viruses with this panel of ferret antisera indicated substantial antigenic drift of these viruses from genetically related clade 2.3.4.4 viruses and the most closely related CVVs available for testing (Table 2).

4. Discussion

In this study, we reported an average AIV prevalence of 9% among the poultry and 40% in environmental samples collected during the surveillance period. Among the poultry, AIV positive rate was higher in domestic waterfowl (15%) than in commercial (7%) and backyard chickens (2%). This study also reports the detection of clade 2.3.4.4 H5N6 viruses in Bangladesh for the first time and suggests this was a recent introduction into the country around March of 2016. Genetic analysis showed these viruses belong to the clade 2.3.4.4 viruses that were dispersed throughout Southeast Asia, European, Middle Eastern and African countries and which were also first detected in 2017.

All Bangladesh A(H5N6) viruses were identified from apparently healthy waterfowl, which is consistent with reports that waterfowl are the main reservoir of A(H5N6) virus in LPMs in other regions (Bi et al., 2016a; Li et al., 2017). The widespread hemispheric movement of this lineage of viruses suggests migratory birds might have facilitated the movement into Bangladesh (Gilbert et al., 2011; Parvin et al., 2014b). Detection in waterfowl (ducks, geese), but not chickens, also suggests the introduction might have been limited to these species and the viruses did not spread into gallinaceous birds. However, A(H5N6) virus infection may be present in gallinaceous birds in Bangladesh, but not detected in this study due to surveillance limitations. During this study, surveillance sites were decreased to 33% of the original surveillance sites and the number of samples collected from gallinaceous birds decreased during the surveillance period. In addition, HPAI A(H5N6) virus causes comparatively mild disease in ducks, but is virulent to chickens (Bi et al., 2016a; Wu et al., 2015). Therefore, chickens infected with HPAI A(H5N6) virus may have severe illness or die and may never be transported to a LPM.

The waterfowl that were positive for A(H5N6) were reared locally in the Netrokona and Sunamganj areas located in the northern part of Bangladesh along the central Asian flyway (R. El-Shesheny et al., 2017). During the winter season from October to March, thousands of wild migratory birds from different countries, such as Russia, Mongolia, China and Nepal, visit this area. Domestic ducks and geese from Netrokona and Sunamganj frequently intermingle with migratory birds during winter months (Ahmed et al., 2011; Barman et al., 2017). Therefore, it is likely that the Bangladesh A(H5N6) viruses originated from migratory birds. The timing of A(H5N6) detection also corroborates this hypothesis, as the viruses were detected during or following migratory bird arrival in Bangladesh from January to May. Consistent

surveillance in both birds and humans would allow ascertainment of whether this subtype and clade will continue to circulate in Bangladesh and whether or not this will lead to changing epidemiology of the virus in the avian host and/or the potential risk to humans.

Migratory birds, which can traverse long distances, played a major role in the geographical spread of A(H5N6) viruses (He et al., 2017; Lee et al., 2018). Viruses of clade 2.3.4.4 H5N6 appear to have first emerged in China in 2011–2012 (Hao et al., 2017; Lee et al., 2016) (Fig. 2), then spread to Lao PDR and Vietnam in 2013, and continuously to other regions most likely through migratory waterfowl (Lee et al., 2017; Okamatsu et al., 2017; Wong et al., 2015). To date, 2.3.4.4 H5N6 viruses have been detected in poultry and wild birds across Southeast Asia, the Middle East, Europe, and Africa (Beerens et al., 2018; OIE, 2018; Tun Win et al., 2017). Phylogenetic analysis of the HA and internal genes (NP, M, NS) indicated that the Bangladesh A(H5N6) viruses were related to clade 2.3.4.4 H5N8viruses detected in Africa (Fig. 2, S. Fig. 1), which may implicate movement of birds along the East Africa/West Asia flyway. NA and other internal gene (PB2, PB1, PA) analyses, however, also suggested reassortment occurred with European lineage viruses and local Bangladesh and/or Eurasian lineage viruses. Temporal analyses suggested the Bangladesh A(H5N6) viruses emerged around March 2016, likely carried by migratory birds via the East Africa/West or Central Asian flyways, given detection of clade 2.3.4.4 H5N8viruses around May 2016 in Russia and China followed by rapid expansion in Africa, the Middle East and East Asia shortly afterwards (Lee DH, 2014) (S. Fig. 2).

The wide prevalence of clade 2.3.4.4 H5 viruses in wild birds and poultry over extended periods of time (several years at least) could increase the probability of reassortment among these viruses and other AIVs (Monne et al., 2013; Parvin et al., 2014a), generating new reassortant viruses. Although A/turkey/Poland/83/2016 (H5N8) is most closely related to the HA gene of the Bangladesh A(H5N6) viruses (99.5%), its node age estimated by tMRCA analysis is younger (2016.922) than the Bangladesh viruses (2016.836) and, thus, less likely to be an ancestor of the Bangladesh viruses. A/chicken/Cameroon/17RS1661-1/2017 (H5N8) had 99.2% nucleotide identity with the Bangladesh A(H5N6) viruses, but had an older node age of 2016.526 (S. Fig. 2), indicating this or closely related viruses could be the closest ancestor to the Bangladesh viruses. In addition, other gene segments, except for the NA and polymerase genes, were also closely related to the A/chicken/Cameroon/17RS1661-1/2017 (H5N8) (> 99%), further suggesting an origin along the East African/West or Central Asian flyway where similar reassortment events have been proposed (R El-Shesheny et al., 2017). Detection of A(H5N6) viruses in LPMs where other avian influenza A subtypes were also identified (i.e., H5N1, H6N2, H11N2, H3N6, H9N2, H2N3) indicated that the LPMs in Bangladesh may facilitate onward reassortment of avian influenza viruses. It remains unclear if A(H5N6) viruses continued to circulate in Bangladesh and whether or not the endemic clade 2.3.2.1a A(H5N1) viruses will remain the predominate clade of HPAI A(H5) virus in the country. Ongoing surveillance efforts are underway to address these questions. In addition to the detection of A(H5N6) virus for the first time in Bangladesh, this surveillance also detected other subtypes (i.e., H1N4, H2N3, H3N5, H5N3, H6N2, H11N1, and H11N9) that have not been previously detected suggesting that either surveillance failed to detect these viruses in the past or that these viruses were also recently introduced.

Although no human cases with related viruses have been identified in Bangladesh (Khan et al., 2018; Zaman et al., 2009), the HA sequences of the Bangladesh A(H5N6) viruses do share high nucleotide identity (> 97%) to a virus from a human infection, A/Fujian-Sanyuan/21099/2017 (H5N6). In addition, the Bangladesh viruses had several molecular signatures of potential risk for human infection and disease. Further assessment of the genome constellation of these viruses and related clade 2.3.4.4 viruses will be important to understand the epigenetic factors that may contribute to human infection. Additional studies of the Bangladesh clade 2.3.4.4 H5N6 viruses in mammalian models would assist in determining the pathogenicity and transmissibility of these viruses and whether or not they possess phenotypic or epidemiological characteristics that differ from the currently circulating clade 2.3.2.1a viruses. In Bangladesh, poultry play a major role in AIV maintenance and spread (Khan et al., 2014). Consistent LPM surveillance for avian influenza viruses in Bangladeshis is critical to identify emerging avian influenza viruses that pose a threat to animal and public health and to determine if clade 2.3.4.4 H5N6 viruses or other HPAIs continue to circulate and evolve in the country. In addition, consistent surveillance for influenza and severe acute respiratory infection in humans in Bangladesh, especially those with contact with poultry, is important to quickly detect potential HPAI infections and implement appropriate treatment and control strategies. These findings may inform vaccine virus selection if vaccination strategies are considered in Bangladesh poultry and provide data to support the need for public health agencies to prepare for newly emerging viruses in the veterinary sector should they spillover more frequently into humans. In addition, data from this surveillance provide public health agencies with important information that can be used in selecting pre-pandemic candidate viruses for vaccine development recommendations and to prioritize pandemic planning efforts.

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Appendix A. Supplementary data

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