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The Continuing Evolution of H5N1 and H9N2 Influenza Viruses in Bangladesh

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Summary

In 2011, avian influenza surveillance at the Bangladesh live bird markets (LBMs) showed complete replacement of the highly pathogenic avian influenza (HPAI) H5N1 virus of clade 2.2.2 (Qinghai-like H5N1 lineage) by the HPAI H5N1 clade 2.3.2.1. This clade, which continues to circulate in Bangladesh and neighboring countries, is an intra- and inter-clade reassortant; its HA, PB1, PA and NS genes come from subclade 2.3.2.1a; PB2 from subclade 2.3.2.1c; and NA, NP, and M from clade 2.3.4.2. The H9N2 influenza viruses co-circulating in the Bangladesh LBMs are also reassortants, possessing five genes (NS, M, NP, PA, and PB1) from a HPAI H7N3 virus previously isolated in Pakistan. Despite frequent co-infection of chickens and ducks, reassortment between these H5N1 and H9N2 viruses has been rare. However, all such reassortants detected in 2011 through 2013 have carried 7 genes from HPAI H5N1 clade 2.3.2.1a and the PB1 gene from the Bangladeshi H9N2 clade G1 Mideast, itself derived from HPAI H7N3 virus. Although, the live birds which we sampled in Bangladesh showed no clinical signs of morbidity, the emergence of this reassortant HPAI H5N1 lineage further complicates endemic circulation of H5N1 viruses in Bangladesh, posing a threat to both poultry and humans.

Keywords

Highly pathogenic avian influenza; H5N1 clade 2.3.2.1a; H9N2 clade G1-Mideast; Bangladesh; live-bird markets; reassortants

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Introduction

Bangladesh is among the countries with the world's densest human and poultry populations (20), and its people depend heavily on poultry for food and income. In 2013 highly pathogenic avian influenza (HPAI) H5N1 viruses were still considered endemic in Bangladesh. These viruses continued to circulate, although no H5N1 outbreak had been reported to the World Health Organization for Animal Health (OIE) since March 2013 (23). Live bird markets (LBMs) are recognized sites of inter-species virus mixing (6) and probably contribute to the persistence of H5N1 viruses.

HPAI H5N1 was first introduced into Bangladesh in February 2007 (23) and was identified as Qinghai-like lineage, clade 2.2.2 (1). It circulated in the country until early 2011, causing hundreds of poultry outbreaks and 2 confirmed human cases (2). In 2011, two new H5N1 clades were detected in Bangladesh – clade 2.3.2.1a (3, 11), and clade 2.3.4.2 (11). Clade 2.3.4.2 was detected in March 2011 in Eastern Bangladesh and was successfully eradicated after the outbreak was stamped out. Clade 2.3.2.1a, first detected in January 2011, then completely replaced the previously predominant clade 2.2.2 (12). It has caused 4 human cases (21), one of which was fatal (10).

Influenza viruses of the H9N2 subtype were circulating widely in Bangladesh at the same time, having been first reported in 2006 (15). The H9N2 viruses currently circulating in Bangladesh are a novel variant that originated in China from the G1 lineage but has evolved tremendously via continuing genetic shift and by inter-subtype reassortment with HPAI H7N3 viruses (17). In-depth phylogenetic and genetic analysis showed that the H9N2 viruses isolated from the LBMs between 2009 and 2011 had acquired three internal genes (nonstructural [NS], polymerase basic 1 [PB1], and polymerase [PA]) from HPAI H7N3 viruses by reassortment (16). The hemagglutinin (HA) gene of H9N2 viruses isolated between 2011 and early 2014 in Bangladesh was derived from an Iranian H9N2 lineage (Mideast_B Iran); the neuraminidase (NA) and polymerase basic 2 (PB2) genes from Dubai H9N2 (Mideast_C Dubai); and the NS, nucleocapsid protein (NP), matrix (M), PA, and PB1 genes from HP H7N3 viruses originating in Pakistan (17). Between 2009 and 2011, these H9N2 viruses began accumulating molecular markers that favor transmission from avian to human hosts (16). In 2011, the first human H9N2 infection outside of mainland China was reported in Bangladesh (9).

The co-circulation of H5N1 and H9N2 avian influenza viruses (AIVs) in the poultry of Bangladesh raises concern about the possible emergence of novel reassortant viruses capable of human infection and transmission. For this reason, continuing influenza virus surveillance and analysis in Bangladesh is needed. Here, we report the results of avian influenza surveillance conducted monthly at LBMs and a farm in Bangladesh from January 2013 through December 2014, and the characterization of HPAI H5N1 and low-pathogenic H9N2 viruses that were circulating in poultry at this period.

Materials and Methods

Sample collection and screening

The Center of Excellence for Influenza Research and Surveillance at St. Jude Children's Research Hospital (Memphis, TN, USA) and Jahangirnagar University (Dhaka, Bangladesh) have conducted collaborative avian influenza surveillance in Bangladesh since November 2008. From January 2013 through December 2014, a monthly average of 210 virologic samples (oropharyngeal, cloacal, fecal, and water-trough samples) were collected from poultry (chickens, ducks, geese and quail) and poultry cages at 5 LBMs in Dhaka and at a LBM and poultry farm approximately 40 miles northeast of Dhaka. An additional 230 fecal samples were obtained from wild migratory waterfowl at a lake in Savar, Bangladesh in March-April 2013 and December 2014. All sampled birds appeared healthy.

Samples were collected on Dacron swabs, stored in 1 mL of glycerol transport medium (22), and transported in liquid nitrogen to St. Jude Children's Research Hospital for further testing. All samples were screened for influenza A viruses (IAVs) and then for subtype H5 by real-time reverse transcription polymerase chain reaction (rRT-PCR) with universal matrix-gene- and H5-subtype-specific primers, as previously described (14).

Virus isolation, subtyping, genome sequencing and phylogenetic analysis

Selected H5-positive and non-H5-positive influenza A samples representing each sampling period, location, and host species were propagated in 10-day-old embryonated chicken eggs for virus isolation (18). All virus isolates were definitively subtyped by endpoint RT-PCR and sequencing. Viral RNA extraction and nucleotide sequencing were performed as previously described (14, 12). Three of the HPAI H5N1 virus cultures that were co-infected with low-pathogenic H9N2 viruses, were treated with anti-H9N2 chicken antiserum to allow full genome sequencing of the H5N1 virus. The Lasergene package (DNASTar, Madison, Wisconsin, USA) was used for nucleotide sequence analysis. Full-length nucleotide sequences for all 8 gene segments were aligned and phylogenetic analyses were conducted using Maximum-likelihood method with 1000 bootstrap replicates in MEGA 5 software (19). Gene sequences were submitted to the GenBank nucleotide sequence database under the accession numbers: the numbers are pending.

Results

Influenza A and H5 screening of samples

The total of 5116 samples collected in 2013 and 2014 comprised 3717 samples from poultry (including 918 paired oropharyngeal and cloacal swabs from the same host), 1169 samples from poultry cages, and 230 fecal samples from migratory waterfowl. We found 1439 samples from 1005 birds and 395 environmental samples to contain IAV (Table 1). The highest frequency of influenza A virus was found in samples from the LBMs (39.5% of chickens and 44.4% of chicken environmental samples, 32.6% of quail and 40.4% of quail environmental samples, and 26% of ducks and 17.7% of duck environmental samples). Screening showed that IAVs had been circulating at the chicken farm at a rate just above 14%, in both the sampled birds and their environment.

Figure 1 shows the percentage of IAV-positive and H5-positive birds (Figure 1A), and environmental samples (Figure 1 B) at the LBMs in Bangladesh from January 2013 through December 2014. The birds at the LBMs showed the lowest IAV positivity (4.4%) in September 2013 and the highest (56.8%) in September 2014. Birds from which paired oropharyngeal and cloacal swabs were obtained, were considered influenza A-positive if at least one of the samples was positive. Subtype H5 was consistently detected at the LBMs between January 2013 and December 2014, with the exception of August 2013. The results obtained from the rRT-PCR screening of the environmental samples showed that in January 2013, when only 3.2% of the sampled birds (n=62) at the LBMs were H5-positive, 82.8% of the collected environmental samples (n=35) at those markets were contaminated with H5 (Figure 1 B).

Virus subtypes

We selected 702 of the 1439 influenza A-positive samples for virus isolation and recovered 307 IAV isolates. No IAV was recovered from wild-bird samples (Ct values, 28–37; cut-off Ct value= 37), and only one (an H9N2 virus) was isolated from the farm samples (Ct values, 19.8–37; cut-off Ct value= 37). We then selected 187 isolates from the LBMs to represent each sampling period, location, and host species, for further characterization. Of these, 52% (n= 97) were subtyped as H9N2 viruses; 16% (n= 30) as H5N1; 30% (n= 56) as an H9N2 and H5N1 co-infection; and 2% as non-H5, non-H9 low-pathogenic AIV isolates (1 as an H5N1, H4N6, and H9 co-infection; 1 as H6N7; and 1 as H10N1). We sequenced the complete genomes of 25 of the isolated H5N1 viruses, the hemagglutinin (HA) and neuraminidase (NA) genes of 9 H5N1 viruses isolated with co-infecting H9N2 virus, and the complete genomes of 5 H9N2 isolates (Table 2).

Molecular analysis

All 34 H5N1 isolates were found to have multiple basic amino acids at positions 321–333 (H5 numbering) of the HA cleavage site, an indicator of high pathogenicity in chickens (8), although no chickens sampled showed signs of illness. Thirty-three isolates had the amino acid motif PQRERRRK_R/GLF, and 1 (A/duck/Bangladesh/23858/2014) had R323K. All H5N1 isolates had HA 222Q and 224G, suggesting preference for α -2,3 sialic acid (avian-like) receptors (7).

Molecular analysis of the 5 sequenced H9N2 viruses isolated in the fall of 2014 showed that since 2011, they had continued to acquire molecular markers that favor mammalian transmission, especially in the PA and PB1-F2 genes (Table 3).

HPAI H5N1 phylogeny

The H5N1 HA phylogenetic tree revealed that the HA genes of all 34 contemporary Bangladeshi H5N1 viruses in this study formed a monophyletic cluster with the Bangladeshi isolates from the period 2011 to early 2013, showing that clade 2.3.2.1a (previously known as Hubei/1/10-like) is still circulating in the country and evolving locally (Figure 2). Analysis of the other 7 phylogenetic trees (not shown) revealed that overall the 2.3.2.1a lineage has been circulating relatively stably on the Indian subcontinent (Bangladesh, India, and Nepal) from 2011 through 2014. All H5N1 viruses reported in this study clustered

phylogenetically with the previously isolated 2.3.2.1a viruses from Bangladesh in all gene segments except the PB1 gene, which did not fall into the main 2.3.2.1a cluster in 3 of 25 viruses. Therefore, the current dominant 2.3.2.1a lineage circulating at the LBMs in Bangladesh is still an inter- and intra-clade reassortant with the same gene constellation as that of the viruses circulating between 2011 and early 2013 in the country. This lineage is characterized by clade 2.3.2.1a-like PB1, PA, and NS genes; a 2.3.2.1c-like PB2 gene; and 2.3.4.2-like NA, NP, and M-genes.

H9N2 phylogeny

In our phylogenetic analysis of the HA genes of 5 recently isolated H9N2 viruses, all 5 clustered as a distinct homogeneous sub-group that shared a common ancestor with A/Quail/G1/Hong Kong/G1/1997, the prototype H9N2 virus of the G1 clade (Figure 3). The HA genes of the Bangladesh H9N2 viruses were related to those of H9N2 viruses isolated in India, Pakistan, and the Middle East (Dubai/Saudi Arabia). Topologically, the Bangladesh H9N2 viruses formed a separate, well defined outgroup cluster under A/Chicken/Iran/B102/2005, the prototype H9N2 virus of the G1_Mideast clade (Figure 3), suggesting that it was the ancestor of the HA genes of our isolates. Phylogenetic analysis of the other genes (data not shown) showed that the NA and PB2 genes are related to the Mideast_C Dubai lineage and the NS, NP, MP, PA, and PB1 genes are related to the HPAI H7N3 viruses previously isolated in Pakistan. In none of our phylogenetic analyses did the Bangladesh H9N2 viruses cluster with the prototype H9N2 viruses of other lineages, suggesting that a single distinct H9N2 virus, previously isolated in Pakistan and the Middle East, is still circulating in Bangladesh.

Interactions between HPAI H5N1 and LPAI H9N2 viruses in Bangladesh

The topology of the eight H5N1 phylogenetic trees revealed that the PB1 genes of 3 of the H5N1 isolates (A/duck/Bangladesh/21269/2013 [Dk/21269], A/duck/Bangladesh/21326/2013 [Dk/21326], and A/duck/Bangladesh/21329/2013 [Dk/21329]) clustered with those of H9N2 viruses isolated in Bangladesh in 2010–2011 (Figure 4). None of these 3 isolates showed co-infection with an H9N2 influenza virus. Dk/21269 was isolated from an oropharyngeal swab collected on 24 November 2013. An NCBI BLAST alignment search revealed A/chicken/Bangladesh/18460/2012 (H9N2) and A/environment/Bangladesh/8465/2010 (H9N2) to be the genetically closest relatives of its PB1 gene, with 99%, and 98% sequence identity, respectively. The other 2 H5N1 viruses, both isolated from oropharyngeal swabs collected on 24 November 2013, had genetically identical PB1 genes that were 99% homologous to the PB1 gene of A/environment/Bangladesh/8465/2010 (H9N2). These findings suggest that the 2013 H5N1 reassortants were probably the result of a single introduction and that they transmit among chickens, although these 3 H5N1 viruses were the only reassortants identified between the Bangladeshi H5N1 and H9N2 lineages since the beginning of this surveillance project in 2008. However, 4 other H5N1 viruses, isolated in Bangladesh in 2011 and 2012, fell into the G1_Mideast cluster of our PB1 phylogenetic tree (Figure 4). The phylogenetic relationships between the PB1 genes of the 7 reported reassortant H5N1 viruses suggest that they resulted from 3 separate introductions of this genotype. Figure 5 illustrates the gene constellation of the reassortant viruses in Bangladesh and the genetic background of their H5N1 and H9N2 donors.

Discussion

Here we have reported the results of our avian influenza virus surveillance in Bangladesh in 2013 and 2014, which focused mainly on the live bird markets. In summary, our results demonstrated several main points:

First, HPAI H5N1 and low-pathogenic avian influenza H9N2 viruses were co-circulating and detected monthly at the LBMs in Bangladesh in apparently healthy birds. The chicken was the most abundant species tested in this study and apparently the most susceptible poultry species, as roughly 40% of chickens tested at the LBMs were infected with IAV. Although the last H5N1 outbreak in Bangladesh officially reported to the OIE occurred in March 2013, Bangladesh remained an H5N1-endemic country as of 1 January 2015. It was previously reported that before 2013, H5N1 viruses in Bangladesh were isolated mainly during the cold months and only sporadically between August and October (4, 12); however, we did not observe this pattern in 2013 and 2014, when H5N1 viruses were isolated during all months except August 2013. Second, the clade 2.3.2.1a HPAI H5N1 virus circulating in Bangladesh since January 2011 was the only H5N1 clade identified at the LBMs in 2013 and 2014. No new introductions were detected and all recently isolated H5N1 viruses seemed to have diverged from the local intra- and inter-clade reassortant H5N1 lineage, suggesting that poultry trade and particularly the LBMs likely contribute to the perpetuation of this genetically stable H5N1 lineage. Third, the H9N2 viruses isolated in the fall of 2014 belong to the G1_Mideast_B clade on the basis of their HA gene; their NA and PB2 genes originate from Dubai H9N2 (Mideast_C Dubai); and their remaining genes come from HP H7N3 viruses in Pakistan. These viruses continue to circulate and acquire human-like mutations in their genomes, posing potential threats to humans, especially those in contact with poultry. This indicates the need of continuous monitoring of the H9N2 viruses and their risk assessment. Finally, despite the high percentage of co-infection of single hosts with H5N1 and H9N2 viruses, reassortment between the two local lineages was very rarely detected.

The fact that in Bangladesh H5N1 virus was more often found as a co-infecting pathogen with H9N2 virus (in 30% of the analyzed IAV- positive samples) than as a single pathogen (in 16% of the analyzed IAV- positive samples), suggested that reassortment between the 2 subtypes should be anticipated. Furthermore, all sampled birds appeared healthy, regardless of the infecting pathogen(s). The absence of illness signs makes influenza outbreaks difficult to recognize and control in poultry flocks and increases the risk of infection of LBM workers in close contact with infected poultry.

Reassortants of the locally circulating H5N1 and H9N2 lineages in Bangladesh have been detected in 2011 (5), and in 2012 (13), but their total number is very low. Interestingly, all 7 detected reassortants so far (including the 3 from 2013 reported in this study) were similar 7:1 HPAI H5N1 viruses with Bangladeshi H9N2-like PB1 genes that phylogenetically grouped in the G1_Mideast cluster. The PB1 gene phylogeny showed that one of the viruses isolated in 2011, A/chicken/Bangladesh/3075/2011 is closely related to both reassortant isolates from 2012 – A/chicken/Bangladesh/12VIR-7140-7/2012 [Ck/7140-7] and A/chicken/Bangladesh/12VIR-7140-16/2012 [Ck/7140-16]. Ck/7140-7 has been isolated on a

farm, whereas Ck/7140-16 at a LBM, suggesting that this virus may possibly have been circulating at the farm and then have been introduced into the LBM by infected chickens. Apparently, in the rare event that HPAI H5N1 virus reassorts with H9N2 virus in Bangladesh, it selectively replaces the PB1 gene. This selective event may reflect some replication advantage related to the polymerase activity of the new progeny; however, in that case it is not clear why this H5N1 lineage has not become established at the LBMs in Dhaka. We hypothesize that reassortment between H5N1 and H9N2 viruses does occur in Bangladesh during mixed infection of poultry, but the only reassortant that has been detected is not maintained in poultry and has not gained predominance, suggesting that it is not the fittest H5N1 virus at the LBMs.

Monitoring of these reassortants is important, especially if they establish circulation as a dominant lineage, because of the origin of their PB1 gene and its combination with 7 genes of from HPAI H5N1 virus. The origin of the H9N2 viruses circulating in Bangladesh (the G1_Mideast lineage) shows that the PB1 gene acquired by the H5N1 reassortant viruses comes from the HPAI H7N3 viruses that have been circulating in Pakistan (Figure 5). Therefore, all 8 segments of these reassortants originated from HPAI viruses that have been reported to infect humans. The zoonotic potential of the reassortants and the effect of their H9N2-like polymerase gene on the pathogenicity of the H5N1 virus are unknown and warrant assessment.

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Abbreviations

AIV	avian influenza virus
HA	hemagglutinin
HPAI	highly pathogenic avian influenza
IAV	influenza A virus
LBM	live-bird market
M	matrix
NA	neuraminidase
NP	nucleocapsid protein
NS	nonstructural
OIE	World Organization for Animal Health

PA	polymerase
PB1	polymerase basic 1
PB2	polymerase basic 2
rRT-PCR	real-time reverse-transcription polymerase chain reaction.

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Figure 1A.

Influenza A virus positivity and H5 positivity of birds sampled at the live bird markets in Bangladesh, 2013–2014. The results are based on rRT-PCR prescreening of the original samples. Black bars indicate the percentage of influenza A- positive birds and correspond to the left y-axis of the graph. Gray bars indicate the percentage of H5-positive birds and correspond to the left y-axis of the graph. The black triangles indicate the number of sampled birds at the live bird markets and correspond to the right y-axis of the graph.

Figure 1B.

Influenza A virus positivity and H5 positivity of environmental samples collected at the live bird markets in Bangladesh, 2013–2014. The results are based on rRT-PCR prescreening of the original samples. Black bars indicate the percentage of influenza A- positive samples and correspond to the left y-axis of the graph. Gray bars indicate the percentage of H5-positive samples and correspond to the left y-axis of the graph. The black triangles indicate the number of collected environmental samples at the live bird markets and correspond to the right y-axis of the graph. ENV= environmental (refers to fecal and water-trough samples collected from poultry cages). LBMs= live bird markets.

Figure 2.

Phylogenetic relationships of the HA genes of the highly pathogenic H5N1 viruses isolated in Bangladesh in 2013 and 2014. Only bootstrap values >70 are shown. Black circles indicate viruses isolated during this study. Italicized, underlined taxa indicate prototype viruses and WHO candidate vaccine viruses.

Figure 3.

Phylogenetic relationships of the HA genes of the low-pathogenic H9N2 viruses isolated in Bangladesh in the fall of 2014. Only bootstrap values >70 are shown. Black circles indicate viruses isolated during this study. Italicized, underlined taxa indicate prototype and WHO candidate vaccine viruses.

Figure 4.

Phylogenetic relationships of the PB1 genes of the highly pathogenic H5N1 viruses isolated in Bangladesh in 2013 and 2014. Only bootstrap values >70 are shown. Black circles indicate viruses isolated during this study. Italicized taxa indicate reassortant highly pathogenic avian influenza H5N1 viruses from clade 2.3.2.1a isolated in Bangladesh, whose PB1 genes were derived from the local low-pathogenic H9N2 lineage.

Figure 5.

Genetic background of the low-pathogenic H9N2, highly pathogenic H5N1, and reassortant H5N1/H9N2 viruses circulating in Bangladesh in 2011–2014. The dashed arrow represents the hypothesis of Shanmuganatham et al, 2014 (17) that reassortment between the G1_Mideast H9N2 lineage and H7N3 viruses has occurred in Pakistan, because influenza A virus of subtype H7N3 has not been detected in Bangladesh.

Table 1
Frequency of influenza A viruses in host and environmental samples collected in Bangladesh in 2013–2014

Avian species	Total samples collected	Total birds sampled ^A	Total ENV samples	LBMs		Farm		Lake
				Birds sampled n Influenza A _B /n total (%)	ENV samples n Influenza A _B /n total (%)	Birds sampled n Influenza A _B /n total (%)	ENV samples n Influenza A _B /n total (%)	
Chicken	3127	1780	950	542/1373 (39.5%)	236/532 (44.4%)	60/407 (14.7%)	61/418 (14.6%)	–
Duck	1130	989	130	253/974 (26%)	23/130 (17.7%)	0/15 (0%)	–	–
Quail	600	460	89	150/460 (32.6%)	36/89 (40.4%)	–	–	–
Goose	29	29	–	0/29 (0%)	–	–	–	–
LWDk	230	–	230	–	–	–	–	39/230 (17%)
Total	5116	3258	1399	945/2836 (33.3%)	295/751 (39.3%)	60/422 (14.2%)	61/418 (14.6%)	39/230 (17%)

Legend: ENV=environmental; LBMs= live-bird markets; LWDk= Lesser Whistling Duck; n= number.

^ANo. of birds sampled is shown, although 2 samples (oropharyngeal and cloacal swabs) were collected from some birds.

^Bbased on rRT-PCR screening of the original samples;

Characterization of influenza A viruses isolated from poultry at the live bird markets in Bangladesh, 2013 – 2014

Table 2

	Isolate name	Sample collection date	Sample type	Virus subtype	Co-infecting subtype detected	LBMA	Gene segments sequenced	GenBank accession numbers
1	A/duck/Bangladesh/20399/2013	27-Jul-13	OP	H5N1		2	all	
2	A/duck/Bangladesh/21269/2013	24-Nov-13	OP	H5N1 ^B		2	all	
3	A/duck/Bangladesh/21326/2013	24-Nov-13	OP	H5N1 ^B		4	all	
4	A/duck/Bangladesh/21327/2013	24-Nov-13	OP	H5N1	H9	4	all of H5N1	
5	A/duck/Bangladesh/21329/2013	24-Nov-13	OP	H5N1 ^B		4	all	
6	A/duck/Bangladesh/21469/2013	27-Dec-13	OP	H5N1		3	all	
7	A/duck/Bangladesh/21814/2014	19-Jan-14	OP	H5N1		5	all	
8	A/duck/Bangladesh/21907/2014	23-Feb-14	OP	H5N1 ^C	H9N2	2	all of H5N1	
9	A/duck/Bangladesh/21908/2014	23-Feb-14	OP	H5N1	H9N2	2	HA, NA of H5N1	
10	A/duck/Bangladesh/21909/2014	23-Feb-14	OP	H5N1	H9N2	2	HA, NA of H5N1	
11	A/duck/Bangladesh/21970/2014	23-Feb-14	OP	H5N1	H9N2	4	HA, NA of H5N1	
12	A/duck/Bangladesh/21980/2014	23-Feb-14	OP	H5N1 ^C	H9N2	4	all of H5N1	
13	A/duck/Bangladesh/22115/2014	18-Mar-14	OP	H5N1	H9N2	2	HA, NA of H5N1	
14	A/quail/Bangladesh/22121/2014	18-Mar-14	OP	H5N1	H9N2	1	HA, NA of H5N1	
15	A/quail/Bangladesh/22125/2014	18-Mar-14	OP	H5N1	H9N2	1	HA, NA of H5N1	
16	A/chicken/Bangladesh/22159/2014	18-Mar-14	OP	H5N1	H9N2	4	HA, NA of H5N1	
17	A/duck/Bangladesh/22229/2014	20-Mar-14	OP	H5N1	H9N2	5	HA, NA of H5N1	
18	A/duck/Bangladesh/22399/2014	12-Apr-14	OP	H5N1		4	all	
19	A/chicken/Bangladesh/22478/2014	15-Apr-14	OP	H5N1		6	all	
20	A/chicken/Bangladesh/22482/2014	15-Apr-14	OP	H5N1		6	all	
21	A/duck/Bangladesh/22806/2014	22-Jun-14	OP	H5N1		4	all	
22	A/duck/Bangladesh/22811/2014	22-Jun-14	OP	H5N1		4	all	
23	A/duck/Bangladesh/22991/2014	17-Jul-14	OP	H5N1	NDV	4	all of H5N1	
24	A/duck/Bangladesh/22996/2014	17-Jul-14	OP	H5N1	NDV	4	all of H5N1	
25	A/duck/Bangladesh/23163/2014	17-Aug-14	OP	H5N1 ^C	H9, NDV	2	all of H5N1	

	Isolate name	Sample collection date	Sample type	Virus subtype	Co-infecting subtype detected	LBM ^A	Gene segments sequenced	GenBank accession numbers
26	A/environment/Bangladesh/23223/2014	17-Aug-14	Water (CK cage)	H5N1	NDV	4	all of H5N1	
27	A/chicken/Bangladesh/23414/2014	21-Sep-14	OP	H5N1	H9, NDV	4	HA, NA of H5N1	
28	A/duck/Bangladesh/23655/2014	18-Oct-14	OP	H5N1	H9	4	all of H5N1	
29	A/duck/Bangladesh/23656/2014	18-Oct-14	OP	H5N1		4	all	
30	A/duck/Bangladesh/23659/2014	18-Oct-14	OP	H5N1		4	all	
31	A/duck/Bangladesh/23660/2014	18-Oct-14	OP	H5N1		4	all	
32	A/duck/Bangladesh/23858/2014	16-Nov-14	OP	H5N1		4	all	
33	A/chicken/Bangladesh/23974/2014	29-Dec-14	OP	H5N1		2	all	
34	A/duck/Bangladesh/24044/2014	29-Dec-14	C	H5N1		4	all	
35	A/chicken/Bangladesh/23426/2014	21-Sep-14	OP	H9N2		4	all	
36	A/chicken/Bangladesh/23527/2014	27-Sep-14	OP	H9N2		6	all	
37	A/chicken/Bangladesh/23529/2014	27-Sep-14	OP	H9N2		6	all	
38	A/environment/Bangladesh/23571/2014	18-Oct-14	Water (CK cage)	H9N2		2	all	
39	A/chicken/Bangladesh/23618/2014	18-Oct-14	OP	H9N2		4	all	

Legend: LBM= live-bird market; NDV= Newcastle disease virus; OP= oropharyngeal; CK= chicken; HA= hemagglutinin; NA= neuraminidase.

The genomic sequences of HPAI H5N1 viruses isolated prior to 30 March 2013 have been submitted to GenBank; during the remainder of 2013 and 2014 which are not represented here, H5N1 have been detected only in co-infection with H9N2, where the last one is dominant subtype.

^AFor confidentiality, the names of the live bird markets have been replaced with numbers.

^BReassortant HPAI H5N1 virus with H9N2-like PB1 gene.

^CThis isolate was a co-infectant with H9N2 virus, which was inhibited by treatment with anti-H9N2 chicken antiserum to allow full genome sequencing of the H5N1 virus.

Table 3

Molecular genetic markers in avian influenza A(H9N2) viruses isolated from Bangladeshi poultry during 2014 that favor replication in mammals. Key amino acid substitutions implicated in mammalian transmission of Bangladesh H9N2 viruses are shown in red. New amino acid substitution not identified previously in Bangladesh H9N2 viruses are shown in red on yellow background.

Bangladesh H9N2 isolates	Hemagglutinin						M1	M2		NS		NP		PA		PB1	PB1-F2			
				HA1/HA2 cleavage site	Glycosylation site			M2 Drug Resistance	NS1	PL motif										
	191 (183)*	234 (226)*	399 (391)*		206	218	15	55	31	(227)		109	214	372	65	100	409	13	73	79
A/chicken/Bangladesh/23426/2014	H	L	K	KSKR	No	No	I	F	N	K	KPEV	V	K	D	S	V	S	P	K	Q
A/chicken/Bangladesh/23 527/2014	H	L	K	KSKR	No	No	I	F	N	K	KSEV	V	K	D	S	V	S	P	K	P
A/chicken/Bangladesh/23529/2014	H	L	K	KSKR	No	No	I	F	N	K	KSEV	V	K	D	S	A	S	P	K	P
A/environment/Bangladesh/23571/2014	H	L	K	KSKR	No	No	I	F	N	K	KPEV	V	K	D	S	A	S	P	K	L
A/chicken/Bangladesh/23618/2014	H	L	K	KSKR	No	No	I	F	N	K	KPEV	V	K	D	Y	V	S	P	R	Q

Legend:

* H3 numbering. NS= nonstructural; NP= nucleoprotein; PA and PB= polymerase genes, PL (PL motif is referred as PDZ-binding motif at the C-terminal end of the NS1 protein). Amino acids: D= aspartic acid; E= glutamic acid; I= isoleucine; K= lysine; L= leucine; R= arginine; S= serine; V= valine; H= histidine; P= proline; F= phenylalanine.