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Molecular characterization of H5N1 avian influenza virus isolated in Tripura, 2011*

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Avian influenza (AI) is a threat to poultry and public health worldwide. Currently circulating HPAI H5N1 virus, isolated from sick geese in China in 1996 (Xu *et al*. 1999), is continuing its geographical spread. Infection to poultry, other birds and sporadic zoonotic transmission to humans has raised concern about pandemic potential of the virus (Guan *et al*. 2004, Peiris *et al*. 2007). The first human infection with H5N1 HPAI virus was reported in 1997 in Hong Kong, when the H5N1 virus transmitted directly from poultry to humans infecting 18 people, of which 6 died (WHO 2012). By now the virus has spread to over 60 countries in Asia, Europe and Africa and has infected 607 people in 15 countries of which 358 have succumbed to the infection (http://www.who.int/influenza/human_animal_interface/EN_GIP_20120706CumulativeNumberH5N1cases.pdf, accessed on 7 July 2012). Economic impact of the disease is very high, as single outbreak of H5N1 virus in a remote village of Manipur, India in 2007 resulted in a loss of over 0.5% of GDP of the state (Kumar *et al*. 2008).

In India, outbreak of H5N1 HPAI in poultry was confirmed in Maharashtra in February 2006 (Pattnaik *et al*. 2006). The virus was characterized as genetic clade 2.2 of H5N1 viruses. Since then outbreaks due to clade 2.2 viruses have been reported in poultry almost every year till 2010. In 2011, a new clade 2.3.2.1 of H5N1 virus was isolated from chickens and ducks from Tripura (Nagarajan *et al*. 2012). In this study, the Tripura isolate of 2011 was characterized.

The virus, A/chicken/India/03CL488/2011, isolated from cloacal swabs of chicken and propagated in the allantoic cavities of 10-day-old specific pathogen free embryonated chicken eggs, was obtained from Avian Influenza Virus repository of High Security Animal Disease Laboratory, Indian Veterinary Research Institute, Bhopal. Viral RNA was extracted using a kit, according to the manufacturer's instructions. All the eight segments were amplified with overlapping gene-specific primers (sequences available on request) using Platinum Taq High Fidelity as described previously (Tosh *et al*. 2011). The PCR products were gel purified using gel extraction kit and sequenced using specific primers with a sequencing kit in genetic analyzer. Nucleotide sequences reported in this study were submitted to the GenBank (accession numbers JX294924- JX294931). Nucleotide sequences were aligned using the sequence alignment editor 7.0.9.0 (Hall 1999) and neighbour joining tree with 1000 bootstrap replicates were inferred using MEGA software, version 5.05 (Tamura *et al*. 2011) with Tamura Nei Model implemented in the program.

On search at National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi), A/chicken/India/03CL488/2011 showed genetic similarity (99–100% nt. homology) in all the eight genes with A/chicken/India/CA0303/2011, isolated from Tripura, suggesting that the two isolates shared a recent common ancestor. In HA, the virus possessed multiple basic amino acid motif ^{321}PQRERRRKR/G^{330} at the cleavage site, indicating highly pathogenic avian influenza virus (Chen *et al*. 2005). Amino acids G222 and Q224 (H5 numbering) in HA suggests preferential binding of the virus to α-2,3-linked sialic acid receptors, which are predominant in avian species (Li *et al*. 2004). The HA gene is highly divergent (nucleotide and amino acid p-distance of 7.4% and 6.7%, respectively) from clade 2.2.2 virus (represented by A/chicken/West Bengal/80995/2008), which is reflected by the presence of at least 32 amino acid substitutions, of which at least 10 are in the epitope/antigenic sites (Table 1). These amino acid substitutions in the HA antigenic sites could be the cause of antigenic diversity between clades 2.2.2 and 2.3.2.1 viruses reported recently (Nagarajan *et al*. 2012). Alignment of the neuraminidase (NA) sequence revealed a 20-amino acid deletion in the stalk region (position 49–68), which is an

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indicator of adaptation for efficient replication in chickens (Matrosovich *et al*. 1999). No change was also observed in the NA residues 119E, 293R, 294N and 274H and in the transmembrane domain of M2 (residues 26L, 27V, 30A, 31S and 34G) proteins that reduced susceptibility to anti-influenza drugs (Scholtissek *et al*. 1998, Suzuki *et al*. 2003). The most commonly described substitution E627K in PB2, which is involved in adaptation to mammalian host is not found in the virus (Subbarao *et al*. 1993). In NS1 gene, the virus possessed an avian-like ESEV PDZ domain-binding motif at the 3' end, which may contribute to increased virulence of the virus (Jackson *et al*. 2008).

The H5N1 viruses were classified into multiple genetic clades with average percentage pair-wise nucleotide distances between and within clades of >1.5% and <1.5%, respectively (WHOD OIE D FAO H5N1 Evolution Working Group 2011). Presently, 12 clades have been identified, and viruses from other 13 clades have not been detected since 2008 or earlier. In HA gene phylogeny, A/chicken/India/03CL488/2011 clustered with clade 2.3.2.1 viruses (Fig. 1). In the tree, viruses from Bangladesh and India (Tripura) clustered tightly (99.5–99.8% nt. homoloy) with 100% confidence, indicating an epidemiological link between these outbreaks. Bangladesh, experienced a new introduction of clade 2.3.2.1 virus in 2010, indicating spill over to poultry in India in 2011, as suggested by close similarities in the HA genes of viruses involved in both countries (FAO 2012). Further, the 2011 Bangladesh and Indian isolates shared genetic similarities (96.3–98.2% nt. homology) with the 2007–2011 H5N1 viruses isolated from chicken, bar-headed goose, ducks (tufted and mandarin), pika and swans (tundra and whooper) in China, Hong Kong SAR, Lao PDR, Mongolia, Nepal, Japan, Vietnam and Russia. The H5N1 viruses isolated during 2006–2010 in India were clustered with clade 2.2 (2008–2010 viruses belonged to new third order clade 2.2.2). It is to be seen whether the new clade 2.3.2.1 is replacing the previous one (clade 2.2.2) or co-circulating with it. From the phylogeny, it is evident that there is co-circulation of multiple clades of H5N1 viruses in the neighbouring countries. For example, A/chicken/Nepal/3–54/2010 and A/duck/Nepal/PTS60/2010 isolates belongs to clades 2.3.2.1 and 2.2.2, respectively. Similarly, viruses from three distinct clades (2.2.2, 2.3.2.1 and 2.3.4.2) were detected in Bangladesh during 2011. The H5N1 virus clade 2.3.2 was confirmed in wild waterfowls in China, Mongolia and Russia during 2009 (Li *et al*. 2011, Sharshov *et al*. 2010). Subsequently, the virus was detected in Nepal in early 2010, which was the first reported introduction of this virus into South Asia (Reid *et al*. 2011) and in Bangladesh and India in 2010 and 2011, respectively (FAO 2012, Nagarajan *et al*. 2012). In March 2010, the clade 2.3.2 virus was confirmed in European poultry and wild birds in Romania and Bulgaria, respectively (Reid *et al*. 2011). The role of wild migratory birds in the spread of H5N1 HPAI virus has been debated (Oslen *et al*. 2006, Feare 2010, Soliman *et al*. 2012). Identification of clade 2.3.2.1 virus in diverse wild birds indicates that the virus is now endemic in these birds. Further, isolation of virus from a large geographical area indicates that the virus is spreading through overlapping migratory flyways in Asia. Therefore, complete eradication of virus in near future in Asia is probably difficult as control of infection in wild birds is not a feasible option.

Table 1. Analysis of amino acids in the hemagglutinin (HA) gene of H5N1 avian influenza virus

Amino acid position[a]	Virus strains		Epitope[b]	Antigenic sites[b]
	WB/80995/08 (clade 2.2.2)	Tripura/488/11 (clade 2.3.2.1)		
	2	Q*	H	
14	E	K		
45	D	N		
53	R	K	+	
71	L	I	E	
83	I	A	E	
88	D	G		
115	Q	R	A	+
120	S	D		
133	S	A		
140	R	N	B	+
154	N	D	D	
155	D	N	D	
162	I	K		
184	A	E		
200	V	I	D	
219	V	I		
226	M	I	D	
227	E	D		
240	N	H		
252	N	Y		
269	K	R		
277	K	R		
310	R	K		
323	G	R		
446	K	R	+	
457	R	K		
473	R	K		
475	D	N		
513	T	I		
528	A	V		
533	V	A		

WB/80995/08, A/chicken/West Bengal/80995/2008. Tripura/488/11, A/chicken/India/03CL488/2011. [a]H5 numbering. [b]Designation according to Duvvuri *et al*. (2009). *Amino acids are in single letter code

SUMMARY

In this study, an avian influenza virus isolated from chicken in Tripura in 2011 was characterized by sequencing all the 8 segments of the virus. The HA cleavage site

possessed multi-basic amino acids suggesting highly pathogenic avian influenza. Based on sequence data, virus is sensitive to commonly used anti-influenza drugs. The virus belonged to clade 2.3.2.1 and is closely related to 2011 virus isolated in Bangladesh. The multiple amino acid substitutions in the HA antigenic sites could be the cause of antigenic diversity between clades 2.2.2 and 2.3.2.1 reported earlier. Co-circulation of multiple clades of H5N1 virus in neighbouring countries highlights the need for improved avian influenza surveillance in India.

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