Emergence of amantadine-resistant avian influenza H5N1 virus in India

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Abstract This study reports the genetic characterization of highly pathogenic avian influenza (HPAI) virus (subtype H5N1) isolated from poultry in West Bengal, India. We analyzed all the eight genome segments of two viruses isolated from chickens in January 2010 to understand their genetic relationship with other Indian H5N1 isolates and possible connection between different outbreaks. The hemagglutinin (HA) gene of the viruses showed multiple basic amino acids at the cleavage site, a marker for high virulence in chickens. Of greatest concern was that the viruses displayed amino acid substitution from serine-to-asparagine at position 31 of M2 ion channel protein suggesting emergence of amantadine-resistant mutants not previously reported in HPAI H5N1 outbreaks in India. Amino acid lysine at position 627 of the PB2 protein highlights the risk the viruses possess to mammals. In the phylogenetic trees, the viruses clustered within the lineage of avian isolates from India (2008–2009) and avian and human isolates from Bangladesh (2007–2009) in all the genes. Both these viruses were most closely related to the viruses from 2008 in West Bengal within the subclade 2.2.3 of H5N1 viruses.

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Introduction

Highly pathogenic avian influenza (HPAI) H5N1 virus was initially isolated from sick geese in Guangdong province, China in 1996 [1]. In the following years, due to rapid evolution of the virus, a large number of H5N1 genotypes were isolated and multiple sublineages were established in southern China [2, 3]. From late 2003, H5N1 viruses continued to expand their geographical distribution across East and South East Asian countries. In May 2005, a large-scale outbreak of H5N1 virus occurred in Qinghai Lake in China, when the virus entered from poultry into migratory birds [4]. After this outbreak, further expansion of the geographical distribution of H5N1 virus from Asia to Europe, the Middle East and Africa was observed possibly through bird migration [4–7]. The unprecedented expansion of the geographical distribution of H5N1 virus in poultry and the interspecies transmission to dogs, cats, tigers, leopards, donkeys and humans raised concern that this virus may be a potential candidate for possible next pandemic [8–13].

Currently, two classes of antiviral drugs are available to treat influenza A virus infection: the M2 inhibitors, amantadine and rimantadine, and the neuraminidase inhibitors, zanamivir and oseltamivir [14]. Monitoring the changes that affect the sensitivity of H5N1 viruses to antiviral drugs is important for the control of emerging strains. Emergence of amantadine-resistant H5N1 avian influenza viruses in several countries in South East Asia and the Middle East have been reported [15, 16], which raises concerns that a drug-resistant pandemic influenza virus may emerge in nature.

In India, H5N1 outbreak in chickens was first reported in Maharashtra State in February 2006 [17], and in the following months the virus was detected in neighbouring States of Gujarat and Madhya Pradesh. Since then, India had experienced a number of outbreaks of HPAI H5N1 in

Manipur (2007), West Bengal and Tripura (January–May 2008), and Assam, West Bengal and Sikkim (November 2008–May 2009) [18–20]. Phylogenetic analysis revealed that the isolates from these outbreaks belonged to genetic clade 2.2 [21–24]. In January 2010, H5N1 virus was reported in poultry in West Bengal for the third time since 2008 [25]. In this study, we characterized two H5N1 avian influenza virus isolates to know their relationship with other H5N1 viruses and to trace the probable source of infection. We also identified, for the first time the emergence of H5N1 strains with potential resistance to an antiviral drug (amantadine) in India.

Materials and methods

Virus isolation and identification

On 13th January 2010, three dead backyard chickens were received at HSADL from two affected villages (Nagar-2 and Hazrabati-1) of Murshidabad district in West Bengal. Initially, virus characterization from the dead birds was done by RT PCR and real time RT PCR targeting HA and M genes as described earlier [23, 26]. Virus isolation in 10-day-old specific-pathogen free embryonated chicken eggs, and confirmation of subtypes from infected allantoic fluids by hemagglutination inhibition (HI) and neuraminidase inhibition (NI) assays were done as per the protocol of OIE [27]. One virus isolate each from the above two villages were used in this study.

RNA extraction, RT-PCR and sequencing

Viral RNA was extracted from infectious allantoic fluids by the use of QIAamp Viral RNA mini kit (Qiagen). The Uni12 primer [28] was used for reverse transcription using AMV reverse transcriptase (Fermentas). PCR amplification of the eight gene segments was performed using PCR master mix (Promega) using gene-specific primers (available upon request). PCR products were purified with the QIAquick gel extraction kit (Qiagen). The purified PCR products were sequenced using BigDye terminator cycle sequencing kit, version 3.1 (Applied Biosystems) in ABI 3130 Genetic analyzer (Applied Biosystems). The sequences were assembled and edited with Lasergene program package (DNASTAR Inc, Madison, USA).

Sequence analysis

All the eight gene segments were compared with available sequences in GenBank. Sequences were aligned using BioEdit Sequence Alignment Editor 7.0.9.0 [29]. Phylogenetic trees were generated with neighbour-joining analysis using the Tamura-Nei algorithm in MEGA, version 4.0 [30]. The topology of trees was confirmed by 1000 bootstrap replicates.

Results and discussion

All the three dead chickens were tested positive for H5N1 subtype by one-step RT PCR and real time RT PCR. Viruses were isolated in chicken embryos and confirmed to be H5N1 by HI test using H5 subtype-specific serum, NI assay and subtype-specific RT PCR using H5 primers and real-time RT PCR. To study the relationship and genetic characteristics of the viruses isolated from chickens, two isolates, A/chicken/West Bengal/239020/2010 and A/chicken/West Bengal/239022/2010, were selected for the sequence analysis of the whole genome. The nucleotide sequences of the two viruses reported in this study have been deposited in the GenBank under the following accession numbers— A/chicken/West Bengal/239020/2010 (CY061291-CY06 1298) and A/chicken/West Bengal/239022/2010 (CY0612 99-CY061306).

In all eight genes, the two chicken isolates shared 99.7–100% nucleotide homology among them indicating that these viruses originated from the same progenitor viruses. The genotype of the isolates was determined by pair wise comparison of each gene segment with the corresponding sequences of reference viruses. The results indicated that the 2010 virus isolates were most closely related (>98% nucleotide homology) to other Indian H5N1 viruses that circulated in the year 2008 (data not shown). Since 2001, diverse genotypes including C, B, V, W, X, Z and Z+ of H5N1 virus have been detected in China and Southeast Asia [2]. In this study, both the virus isolates shared close genetic relationship (97–98.6% nucleotide homology) in all gene segments with genotype Z reference virus A/Bar-headed goose/Qinghai/65/2005 [31] implying that both the viruses belonged to genotype Z.

To understand the genetic origin of these viruses, phylogenetic relationships of each gene segment of both the isolates along with H5N1 sequences available in GenBank were reconstructed. To date, 10 major genetic clades of H5N1 viruses have been identified by WHO/OIE/FAO H5N1 Evolution working group, 2008 [32]. The clade 2.2 or the "Qinghai-like viruses" are further subdivided into subclades 2.2.1, 2.2.2 and 2.2.3 [16]. In the HA gene, the two viruses (A/chicken/West Bengal/239020/2010 and A/chicken/West Bengal/239022/2010) were grouped together with the other Indian isolates of the year 2008–2009 and the Bangladesh isolates of the year 2007–2009 forming a separate group (Bangladesh/India subcluster) with high (99%) bootstrap value in 2.2.3 sublineage (Fig. 1a). Interestingly, the 2007 chicken isolate from Bangladesh

Fig. 1 Phylogenetic relationships of the HA (**a**) and NA (**b**) genes of representative influenza A viruses. The sequence regions compared were nucleotide positions 29–1732 of HA and 21–1367 of NA. The trees were rooted to A/goose/Guangdong/1/1996. *Numbers* near the node indicates bootstrap values of ≥70%. *Numbers* labelled in the trees indicate WHO/FAO/OIE clade designations. *Scale bar* indicates nucleotide substitutions per site. The Bangladesh/India sub-cluster is highlighted. * Isolates sequenced in this study

(A/chicken/Bangladesh/364/2007) clustered very close to the node indicating as probable ancestor to the viruses isolated subsequently in Bangladesh and India. The above chicken isolate from Bangladesh shared close genetic relationship (99.5% nucleotide homology) with A/chicken/West Bengal/80995/2008 isolated during January 2008. It is to be noted that both the 2010 isolates were connected by a long branch in the tree. This observation could be due to longer persistence of infection indicating a greater accumulation of mutation that leads to high genetic diversity. The H5N1 viruses that circulated in early 2008 outbreaks in the State of West Bengal represented by A/chicken/India/WB-NIV2656/2008, A/chicken/India/WB-NIV28000/2008, A/chicken/West Bengal/82613/2008, A/chicken/India/WB-NIV2806/2008 and A/chicken/West Bengal/80995/2008 were closely related (an average nucleotide

sequence homology of 98.7%) to the 2010 isolates. However, the first Indian H5N1 outbreak in 2006 represented by A/chicken/Maharashtra/7979/2006 formed a distinct cluster in the tree together with 2006 swan isolates from Iran (A/Cygnus Cygnus/Iran/754/2006) and Italy (A/Cygnus olor/Italy/742/2006) (Fig. 1a). The 2007 outbreak represented by A/chicken/Manipur/NIV9743/2007 grouped with a guinea fowl isolate from China (A/guinea fowl/Shantou/1341/2006) and does not belong to EMA sublineages described here and in earlier report [22] ruling out the possible links between 2008 and 2009 H5N1 outbreaks in the country. The 2006 and 2007 Indian isolates shared overall nucleotide identity of 97.6 and 96.1%, respectively, with the 2010 isolates. The general topology of the NA tree is similar to that of the HA tree (Fig. 1b). In the NA tree, both the 2010 isolates shared close genetic similarity

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Fig. 1 continued

(b)

(average nucleotide homology of 98.1%) with chicken isolates (A/chicken/India/WB-NIV2800/2008, A/chicken/India/WB-NIV2813/2008, A/chicken/India/WB-NIV2806/2008 and A/chicken/West Bengal/82613/2008) from early outbreaks (January–May 2008) in West Bengal. Analysis of the six internal genes revealed the same broad grouping pattern as observed in the HA and NA genes (Supplementary Fig. S1a–f). Both the 2010 isolates grouped tightly to each other (with 100% bootstrap support) in all the gene segments and in turn shared close genetic relationship with 2008 outbreak strains from West Bengal. This close phylogenetic relationship indicates that the 2008 isolates could be the probable viral ancestors for 2010 isolates.

Analysis of the amino acid sequences revealed differences with A/Vietnam/1203/04 H5N1 virus, a vaccine strain in all segments except PB1 and M1. There were two amino acid alterations each in the PB2 (V220I and I292V), PB1-F2 (G22E and P33L), HA (K3T and S136N), NP (A22T and R446K) and NS1 (L214H and Y221E), and

three in NS2 (S57T, N92T and Q111L), four in PA (I13V, F105S, E327G and L336F) and five in NA (T40I, Q45H, K130N, I191V and N346K). Another amino acid substitution M495I in HA was detected in 2010 isolates was also noted in A/Indonesia/7/2005. M2 P10H and NS2 K72R mutations were shared with A/Hong Kong/212/2003 and A/migratory duck/Jiangxi/2136/2005, respectively. A recent study identified HA amino acid position 136 (120 in mature H5 numbering) as part of the newly recognized antigenic site in clade 2.2 virus (A/duck/Novosibirsk/56/2005) and is adjacent to the region corresponding to site B in H3 HA [33]. Extensive antigenic variation of the HA gene of H5N1 viruses has been described due in part to the vaccination of fowl [34]. Recently, it has been demonstrated that amino acid substitutions in natural isolates frequently occur in the HA antigenic sites of H5N1 viruses [35]. However, it is not clear what drove the amino acid mutation (S136N) of HA gene in the Indian isolates of this study. The implications of the other unique amino acid

substitutions in the isolates are not known. The HA proteins of both viruses contain multiple basic amino acids (PQGERRRKKR/G) at the HA1/HA2 junction, which is a characteristic of influenza viruses that are highly pathogenic in chickens [4]. This cleavage sequence is present in "Qinghai-like" viruses that infected the wild birds in 2005 including all the Indian isolates except the 2007 outbreak strain in Manipur (A/chicken/Manipur/NIV9743/2007), which had PQGERRR̲RKR/G. Host tropism is determined by the receptor-binding preferences. The two viruses retain amino acid residues Q222 and G224 (amino acid positions 238 and 240 at H5N1 HA gene) at the HA receptor-binding pocket that preferentially bind to avian-specific α-2,3-NeuAcGal receptors [2, 36]. A 20-amino acid deletion in the NA stalk region (position 49–68), which is similar to other genotype Z viruses [2] was observed in 2008–2010 Indian isolates. This deletion is a suggestive of adaptation of the virus from aquatic birds to terrestrial poultry, such as chicken [12]. It has been shown that amino acid mutations in the NA residues (E119V, H274Y, R292K and N294S, N2 numbering) could confer viral resistance to drug oseltamivir [37]. However, no such mutations were observed in the conserved residues in the studied H5N1 viruses, suggesting that they may be sensitive to oseltamivir. Ilyushina et al. [38] found that the IC_{50} values for amantadine-resistance in MDCK cells infected with H5N1 virus with S31N substitution in the M2 ion channel protein was ≥ 100 fold higher compared with the non mutants. Notably, the two viruses analyzed here contained amino acid N at position 31 of the M2 protein, therefore, suggesting both these viruses are resistant to amantadine. Amantadine-resistant H5N1 viruses had been reported in Saudi Arabia [16], Cambodia, China, Indonesia, Malaysia, Thailand and Vietnam [15, 39]. Use of amantadine in poultry farms in China was found to be one of the reasons for the high incidence of amantadine-resistance in H5N1 avian influenza viruses [40]. Spontaneous emergence of amantadine-resistant influenza viruses in the absence of the drug is a rare phenomenon. Previous study indicates low incidence of amantadine-resistant mutants in field isolates due to spontaneous substitutions in human influenza A viruses [41]. However, the status of the drug use in West Bengal is not known. But, the mutations observed in the M2 protein of the two isolates is important and raises concern towards the effectiveness of the antivirals in the management of avian influenza virus infection in the country.

We also analyzed the amino acids of the polymerase genes. Amino acid substitutions in the polymerase (PB2, PB1 and PA) genes have an important effect on the virulence and adaptation of H5N1 virus. For example, amino acid substitutions E627K in PB2, L13P in PB1 and N615K in PA may increase the virulence of H5N1 virus in mammalian hosts [42, 43]. Sequence analysis revealed amino acid K at 627 of PB2 in both the Indian isolates, which is associated with increased virulence of influenza H5N1 viruses in mice and highlights the risk to mammals including humans. The E627K substitution was found in several other clade 2.2 isolates including the Indian isolates except Assam, Tripua and one isolate (A/chicken/West Bengal/100879/2008) from West Bengal. One of the genetic markers in NP gene (33-Ile) of human influenza A virus [44] has also been detected in the Indian isolates that raises public health implications of the virus. D92E mutation in NS1 protein, associated with increased virulence of influenza viruses to pigs [45] was not present in any of the Indian isolates studied. The isolates possessed a ESKV amino acid sequence at the C-terminal end of the NS1 protein. This sequence was also observed in all the clade 2.2 viruses used in this study including 2008–2009 Indian isolates. Large-scale sequence analysis of avian influenza viruses indicated that the C-terminal four amino acid residues of the NS1 protein acts as a ligand that binds to PDZ domains on proteins involved in host cell signalling pathways [46]. A recent study, by using mice as a model system, showed that the PDZ-binding motif is a virulent determinant and modulates pathogenicity of influenza viruses through mechanisms not completely understood [47]. The PDZ ligand binding motifs with sequences of ESEV or EPEV in the NS1 protein were found in highly pathogenic influenza viruses of avian origin that were associated with human infections in 1997 and 2003. The low pathogenic human influenza viruses contain RSKV or RSEV in the same position. Therefore, a functional PDZ-binding domain is indicative of high virulence. The effect of the ESKV motif on influenza virus interactions is not known and needs to be determined.

In conclusion, the study provides the first molecular evidence of amantadine-resistance in Indian H5N1 isolates from chickens, which was not reported in earlier outbreaks. Phylogenetic analysis reveals that subclade 2.2.3 H5N1 virus was responsible for outbreaks in early 2010 in India. Further, the viruses from early 2008 from West Bengal were the closest genetic relative to the 2010 outbreak strains. This information, therefore, highlights the necessity of controlling the transmission of drug-resistant strains in the country.

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