Research Article

Isolation and Genetic Characterization of Influenza A (Subtype H5N1) Virus from Crows in India

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Abstract | We isolated two influenza A (subtype H5N1) viruses from crows in India, in 2011 and 2012. Nucleotide sequence of all eight genome segments of both the viruses (A/crow/India/11TI07/2011 and A/crow/India/01TR01/2012) was determined and used for analysis. The two isolates shared >99% nucleotide sequence identity in all the eight genes. Phylogenetic analysis revealed that both the viruses belonged to haemagglutinin (HA) clade 2.3.2.1 of H5N1 viruses. Within clade 2.3.2.1, the crow viruses grouped with contemporary H5N1 viruses isolated from poultry in India; poultry, crows and environmental sampleas in Bangladesh; and poultry and wild birds in Bhutan. The viruses have the characteristic feature of high pathogenicity to chickens, and possessed avian-like (α 2, 3-linked sialic acid) receptor-specificity in the HA. Analysis of amino acid sequences in the neuraminidase (NA) and M2 indicated that the crow viruses are susceptible to currently available influenza drugs.

Keywords | Highly Pathogenic Avian Influenza (HPAI), HPAI virus, subtype H5N1, Crow, Phylogeny, India

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INTRODUCTION

Highly Pathogenic Avian Influenza (HPAI) H5N1 virus, poses a serious threat to animal and public health worldwide. Since late 2003, the virus has spread to over 60 countries in Asia, Europe and Africa leading to death or culling of hundreds of millions of poultry and occasional transmission to humans revealing its pandemic potential (Li et al., 2004; Peiris et al., 2007). Till date, H5N1 virus has claimed 393 lives out of 668 laboratory confirmed human cases, as reported to WHO (2014). Presently, the H5N1 virus is endemically circulating in poultry in few countries in Asia and Africa. This ecological success could be attributed partly to the increasing host range of H5N1 viruses (Boon et al., 2007; Peiris et al., 2007; Guan and Smith 2013). The H5N1 virus, after initial outbreaks in South east Asia and China during late 2003 to early 2005, spread to Europe and Africa, and role of wild waterfowls in intercontinental migration of the virus has been extensively debated (Olsen et al., 2006; Kilpatrick et al., 2006), although trade of poultry and poultry products cannot be ruled in the virus spread. However, the land-based wild birds such as crows could play an important role in disease transmission locally due to their proximity to both human habitats and poultry chain. Therefore, understanding the molecular characteristics of H5N1 virus isolated

from crow is important for avian influenza control.

Mortality in crows *(Corvus macrorhynchos)* was observed during 2011 and 2012, in the States of Jharkhand and Odisha, respectively. Brain tissue and tracheal swab from States of Jharkhand and Odisha, respectively were received at NIHSAD, Bhopal for confirmation of avian influenza, and initial virus identification from the samples was done by real time RT-PCR and RT-PCR as described previously (Nagarajan et al., 2010; Nagarajan et al., 2012). The viruses were grown in the allanotic cavity of 9-11-days-old embryonated specific pathogen free (SPF) chicken eggs and confirmation of the HA and NA subtypes was done by hemagglutination inhibition assay (OIE, 2012) and RT-PCR (WHO, 2007), respectively. Viral RNA was extracted from infected allantoic fluid using QIAamp Viral RNA Mini Kit (Qiagen, Germany). cDNA was synthesized by AMV reverse transcriptase (Fermentas, USA) using influenza A virus universal primer Uni12 (Hoffmann et al., 2001). PCR amplification of various gene segments using segment-specific primers was carried out using Platinum *Taq* High Fidelity DNA polymerase (Invitrogen, USA) as described earlier (Tosh et al., 2011). Specific amplicons were purified with QIAquick gel extraction kit (Qiagen, Germany) and sequenced using segment-specific primers with BigDye Terminator Cycle Sequencing Kit, v3.1 (Applied Biosystems, USA) in 3130-Genetic Analyzer (Applied Biosystems, USA). Genome sequences were aligned using BioEdit Sequence Alignment Editor 7.1.3.0 (Hall, 1999). Phylogenetic tree was generated with MEGA, version 6.0, by neighbor-joining methods using Tamura-Nei algorithm (Tamura et al., 2013). Tree reliability was estimated by bootstrap tests (1000 replicates) available in the program.

The isolated viruses from the brain (A/crow/India/11TI07/2011) and tracheal swab (A/crow/India/01TR01/2012) were tested to be influenza A virus belonging to subtype H5N1. For molecular characterization, genome sequence of all eight segments of the H5N1 viruses was determined and submitted to GenBank under accession numbers KM110962-KM110969 (A/crow/India/11TI07/2011) and KM872062- KM872069 (A/crow/India/01TR01/2012). Nucleotide sequence

Table 1: Sequence homology of each gene of H5N1 virus (A/crow/India/11TI07/2011) with sequences from GenBank

Gene (nt. position of crow11TI07)	H5N1 isolates with highest homology	% nt sequence identity
PB2 (28-1548)	A/chicken/India/CL03485/2011	99.5
	A/environment/Bangladesh/12197/2011	99.6
PB1 (970-2271)	A/chicken/India/CL03485/2011	99.5
	A/environment/Bangladesh/12197/2011	99.5
PA (40-2172)	A/chicken/India/CL03485/2011	99.7
	A/environment/Bangladesh/12197/2011	99.6
HA (41-1718)	A/chicken/India/CL03485/2011	99.5
	A/chicken/Bhutan/317/2012	99.0
	A/environment/Bangladesh/12197/2011	99.6
NP (46-1527)	A/chicken/India/CL03485/2011	99.5
	A/environment/Bangladesh/12197/2011	99.7
NA (39-1310)	A/chicken/India/09CA10/2011	99.4
	A/migratory bird/Bangladesh/P18/2010	99.0
	A/chicken/Bhutan/367/2012	99.0
M (26-781)	A/chicken/India/CL03485/2011	99.7
	A/environment/Bangladesh/12197/2011	99.7
NS (27-701)	A/chicken/India/CL03485/2011	99.7
	A/environment/Bangladesh/12197/2011	99.9

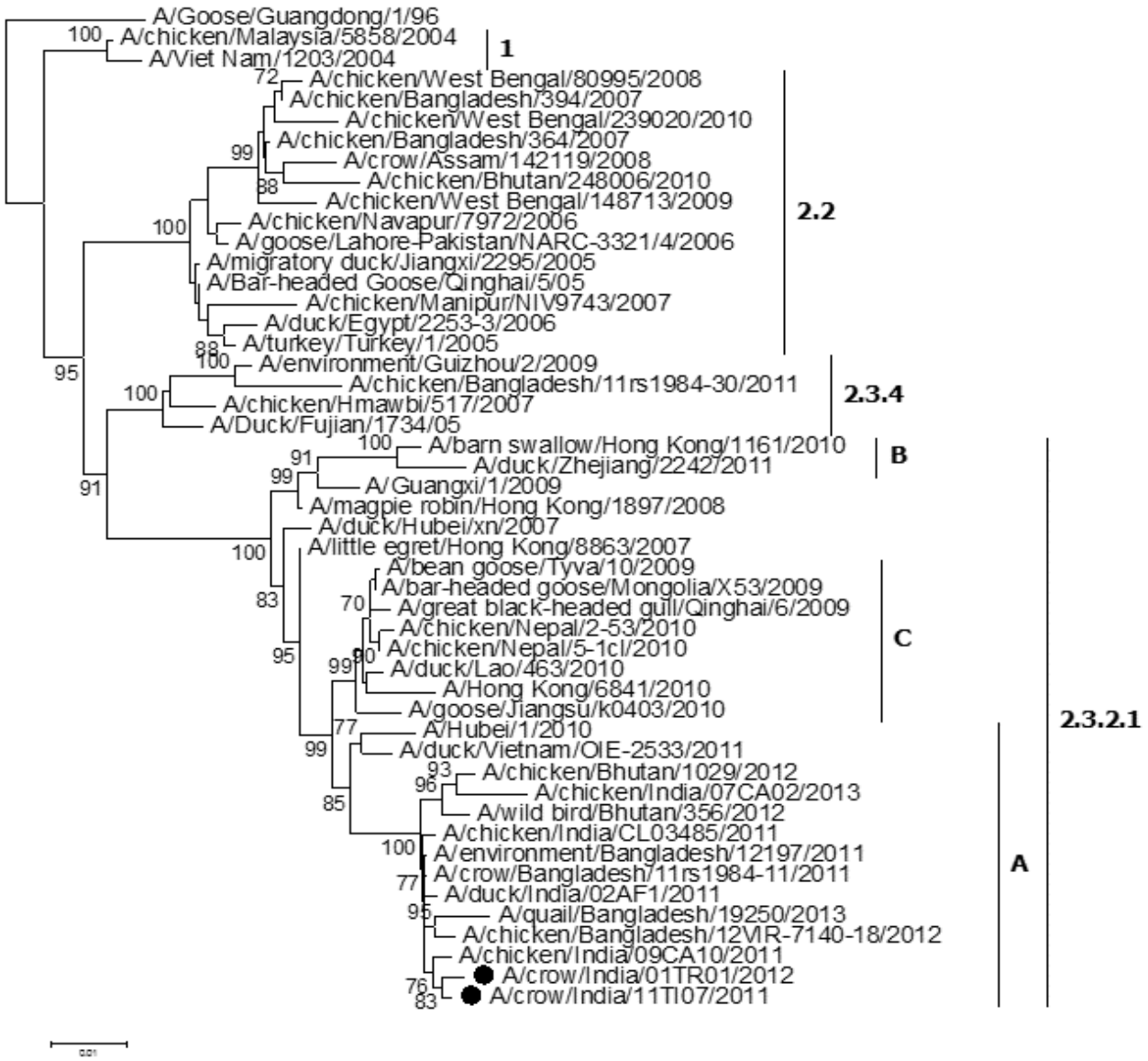

Figure 1: Phylogenetic relationship of HA sequences of H5N1 viruses isolated from crows in India. Bootstrap values (≥70%) are shown near the nodes. Major clades are shown to the right. The isolates sequenced in this study are marked with solid circle. The tree is rooted to A/Goose/Guangdong/1/96.

analysis revealed that both the isolates shared 99.3-100% sequence identity in all eight segments. Blast search (http:// blast.ncbi.nlm.nih.gov/Blast.cgi) of individual genes of crow virus (represented by A/crow/India/11TI07/2011) revealed highest homology with contemporary H5N1 viruses isolated in India, Bangladesh and Bhutan (Table 1).

Analysis of the deduced amino acid sequence identified multiple basic amino acids (^{321}PQRERRRKR/GLF^{332}) near the HA cleavage site, defining high pathogenicity to chickens. The basic amino acid motif of the crow isolates was similar to that of the H5N1 viruses isolated from domestic poultry, wild birds and environmental samples in Bangladesh, wild birds and poultry in Bhutan and poultry in India. Presence of amino acids Q222 and G224 (H5 numbering) in the receptor-binding pocket of HA1 indicated that the viruses preferentially bind to the α-2, 3-linked sialic acid receptor in avian species (Ha et al., 2001). The viruses had 20-amino acid deletion in the NA stalk region, which is characteristic of adaptation of the virus to terrestrial poultry. The crow viruses do not have amino acid substitution K627 in the PB2 protein and E92 in the NS1 protein; both mutations had been associated with increased virulence of influenza viruses (Hatta et al., 2001; Seo et al., 2002). Analysis of amino acids in the NA and M2 indicated that the crow

viruses are susceptible to currently available anti- influenza drugs adamantanes (Hay et al., 1986; Cheung et al., 2006) and neuraminidase inhibitors (Govorkova et al., 2013).

To understand origin of virus, phylogenetic relationship of the HA genes of the two crow viruses along with H5N1 HA sequences available in GenBank were reconstructed. Sequence analysis identified ten major genetic clades (clades 0-9) of H5N1 viruses, based on the viral HA gene, with subsequent detection of multiple 2nd-, 3rd- and 4th- order clades and divergent groups within it (WHO/OIE/FAO H5N1 Evolution Working Group, 2014). In the phylogenetic tree, both the crow viruses analyzed in this study were clustered with other contemporary H5N1 viruses isolated in India, Bangladesh and Bhutan within subgroup "A" of clade 2.3.2.1 or Hubei/2010-like virus group (Figure 1). Interestingly, although the crow mortalities in Jharkhand and Odisha were more than 300 km distance, both the viruses grouped closely (83% confidence) with each other indicating epidemiological link between the crow deaths. To our understanding, this is the first report of the H5N1 virus of clade 2.3.2.1 in crows in India. However, earlier we had isolated H5N1 virus belonging to clade 2.2 from dead crows near the poultry outbreak in 2008 in the State of Assam, India (Nagarajan et al., 2010). In early 2011, crow mortalities due to H5N1 virus belonging to clade 2.3.2.1 have been reported in Bangladesh (Khan et al., 2014). However, except the environmental samples, no H5N1 positivity in domesticated birds near the crow roosting site was found. In the present case, no mortality in domestic poultry was reported near both sites of crow deaths.

In conclusion, the H5N1 viruses isolated during 2011 and 2012 from crows in India are phylogenetically related to other clade 2.3.2.1 viruses isolated in India, Bangladesh and Bhutan indicating common ancestry of these viruses circulating in this region. In addition, co-circulation of a low pathogenic H9N2 avian influenza virus may allow reassortment with H5N1 virus in crows. The ubiquity and close proximity of crows to human habitats and poultry chain highlights the importance of surveillance of avian influenza in crows for emergence of any novel strain which could have a pandemic potential.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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