LETTER TO THE EDITOR

Emergence of Val27Ala mutation in M2 protein associated with amantadine resistance in highly pathogenic avian influenza H5N1 viruses in India

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The precursor of presently circulating highly pathogenic avian influenza (HPAI) H5N1 virus emerged from geese (A/Goose/Guangdong/1/96) in southern China, in 1996 (1). Since then the H5N1 virus is evolving and continuing its spread by infecting domestic poultry, other birds and sporadic zoonotic transmission to humans thereby revealing its pandemic potential (2). The H5N1 virus has spread to over 60 countries in Asia, Europe and Africa resulting in the death of millions of poultry. As on 27th July 2014, 667 laboratory-confirmed H5N1 cases of human infection have been reported to the WHO from 16 countries of which 393 had fatal outcome (3). Currently two classes of anti-viral drugs targeting two surface proteins, the neuraminidase (NA) and M2, of the virus are available for treatment and prophylaxis of influenza. Unfortunately, there is emergence of drug-resistant mutants world-wide for both the classes of drugs (4). Mutations in the drug binding sites are the most important cause of viral resistance. Resistance to the drug amantadine occurs as a result of amino acid substitutions at positions 26, 27, 30, 31, or 34 in the transmembrane domain of the M2 protein leading to reduced binding of the inhibi-

tor (4). Emergence of amantadine resistance in HPAI H5N1 viruses have been reported from mainland China, Hong Kong special administrative region, Cambodia, Vietnam, Thailand, Malaysia, Indonesia, Saudi Arabia and India with M2 Ser31Asn substitution in majority of these cases (4–8).

India is experiencing HPAI H5N1 outbreaks in poultry almost every year since its first report in 2006 (9). The H5N1 viruses isolated from different species of birds in India belonged to two phylogenetic clades: clade 2.2 isolated during 2006–2010, and the clade 2.3.2.1 isolated for the first time in 2011 and continued through the years 2012 and 2013 (10–12). Molecular sequence analysis indicated emergence of resistant strains of H5N1 viruses to anti-influenza drugs oseltamivir in 2008 (13) and amantadine in 2010 (8) in poultry outbreaks in West Bengal.

Comprehensive analysis of HPAI H5N1 viruses isolated during 200–2012 from humans and avian species revealed emergence and spread of amantadine and rimantadine resistant strains in clades 1, 1.1, 2.1.1, 2.1.2, 2.1.3, 2.2, 2.3.2 and 2.3.4 (4). As the virus is evolving and continuing its diversification, there are growing concerns that the H5N1 viruses may produce a strain having potential to cause pandemic. Therefore, monitoring of H5N1 viruses for antiviral resistance is essential to maintain therapeutic measures for control of the disease. In this study, we report the emergence

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Abbreviations: HPAI = highly pathogenic avian influenza; HA = hemagglutinin; NA = neuraminidase; M = matrix; HSADL = high security animal disease laboratory
of amantadine resistance in clade 2.3.2.1 H5N1 viruses isolated from chickens for the first time in India.

During the last week of July 2013, mortality was observed in chickens in poultry production unit, College of Veterinary Science and Animal Husbandry, Durg district, and Government poultry breeding farm, Jagdalpur (Bastar district), both are approximately 290 km apart in Chhattisgarh State, India. Post mortem findings showed tracheal congestion, epidermal haemorrhage, petechial haemorrhages on proventriculus, haemorrhagic enteritis and liver enlargement in dead birds. On 31st July 2013, 2 dead birds and pooled organs from Durg district and on next day 3 dead birds from Bastar district were received at High Security Animal Disease Laboratory (HSADL), Bhopal. Initial virus identification from the dead birds and pooled organs was done by RT-PCR and real time RT PCR targeting M and HA genes as described previously (14, 15). After isolation of virus in 9–11-day-old SPF embryonated chicken eggs, confirmation of HA and NA subtypes was carried out by hemagglutination inhibition (HI) assay (16) and RT-PCR (17), respectively. Viral RNA was extracted from infected amnio-allantoic fluids by using QIAamp viral RNA mini kit (Qiagen). The RNA was reverse transcribed into cDNA by AMV reverse transcriptase (Fermentas) using Uni12 primer (18). RT-PCR amplification of various gene segments with overlapping segment-specific primers was carried out using Platinum Taq High fidelity DNA polymerase (Invitrogen) as described previously (8). The PCR amplified products were purified using QIAquick gel extraction kit (Qiagen) and sequenced using specific primers with BigDye terminator cycle sequencing kit (Applied Biosystems) in 3130-Gene Analyzer (Applied Biosystems). Nucleotide (nt) sequences were aligned using BioEdit sequence alignment editor 7.1.3.0 (19). A maximum likelihood tree was generated using the best fit model GTR+G (gamma shape = 0.43) predicted by MEGA, version 6.0 (20).

The 2 dead birds and pooled organs of chickens from Durg district and 2 dead birds (out of three) from Bastar district were positive for H5N1 subtype by one-step RT PCR and real time RT PCR. Viruses were isolated in SPF chicken eggs and confirmed to be H5N1 by HI test using H5 subtype-specific and subtype-specific sequencing kit, v3.1 (Applied Biosystems) in 3130-Gene Analyzer (Applied Biosystems). Nucleotide (nt) sequences were aligned using BioEdit sequence alignment editor 7.1.3.0 (19). A maximum likelihood tree was generated using the best fit model GTR+G (gamma shape = 0.43) predicted by MEGA, version 6.0 (20).

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The tree is rooted to the sequences of A/Goose/Guangdong/1/96 and A/Hong Kong/156/97 viruses. Numbers near the nodes are bootstrap values (≥90%, out of 500 replications). Clade designations are shown to the right. M2-V27A denotes viruses with Val27Ala mutation. Viruses sequenced in this study are denoted with filled black circle.

Fig. 1
HA gene-based phylogenetic tree of influenza H5N1 viruses
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References: