Acta virologica **58**: 389 – 392, 2014 doi:10.4149/av_2014_03_389

LETTER TO THE EDITOR

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

C. TOSH[1], S. NAGARAJAN[1], H. V. MURUGKAR[1], M. KUMAR[1], M. PANDEY[2], D. K. JOLHE[2], S. TRIPATHI[1], S. SHUKLA[1], N. VED[1], A. MISHRA[1], R. C. GHOSH[2], D. D. KULKARNI[1]

[1]High Security Animal Disease Laboratory, Indian Veterinary Research Institute, Bhopal, India; [2]Department of Veterinary Pathology, College of Veterinary Sci. & A. H., Chhattisgarh Kamdhenu Vishwavidyalaya, Durg, India

Received March 21, 2014; accepted November 7, 2014

Keywords: highly pathogenic avian influenza; H5N1; amantadine; resistance; mutation; India

The precursor of presently circulating highly pathogenic avian influenza (HPAI) H5N1 virus emerged from geese (A/Goose/Guangdng/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 inhibitor (*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

E-mail: chakradhar.tosh@gmail.com; phone: +91-755-2750647.

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, epicardial 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, v3.1 (Applied Biosystems) in 3130-Genetic 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 serum and subtype-specific RT PCR using N1 primers. For molecular characterization, the HA, NA, and M genes of one isolate each from Durg and Bastar districts were sequenced and deposited in the GenBank under accession Nos. KJ162126-KJ162128 (A/chicken/India/07CA02/2013, Durg) and KJ162131-KJ162133 (A/chicken/India/08CA03/2013, Bastar). In the HA gene phylogeny, both isolates of the present study were grouped with clade 2.3.2.1 viruses (Fig. 1). Within clade 2.3.2.1, the two isolates along with contemporary isolates from India and Bangladesh formed a separate subgroup A with 100% confidence and is clearly distinct from the Nepal isolates of 2010 (subgroup C) indicating separate introduction of the virus into South Asia. The nt sequence analysis of the HA genes indicated that both the isolates were closely related (99.1% nt sequence identity) to each other and related by 97.3–99.0% nt sequence homology with other H5N1 viruses isolated from chickens and ducks during 2011 in India, and chickens, ducks and crows during 2011–2013 in Bangladesh. Similarly, in the NA and M genes, both isolates had >99% sequence identity with each other. Between the two isolates, 14 nt differences including 3 non-synonymous mutations leading to amino acid substitutions Asp154Gly, Ala185Gln and Pro235Glu in HA of A/chicken/India/07CA02/2013 compared to A/chicken/India/08CA03/2013 were observed. Of the three alterations, two (Asp154Gly and Ala185Gln) were predicted in the epitope D of H5N1 virus (*21*). The HA cleavage region contains multiple basic amino acid motif (Arg.Arg.Arg.Lys.Arg/Gly) indicative of HPAI (*22*). Both the isolates possessed amino acids Glu222 and Gly224 characteristic for avian receptor specificity of the virus. Amino acid alignment indicated deletion in the NA stalk region (positions 49-68), which is characteristic for adaptation of virus to terrestrial poultry (*23*). No change was observed in the NA binding sites Gln119, His275, Asp294 and Asn295 (N1 numbering) indicating sensitivity of the virus to oseltamivir. However, amino acid alteration Val27Ala was observed in M2 suggestive of reduced sensitivity of the viruses to anti-influenza drug amantadine (*4*). Emergence of amantadine resistant H5N1 viruses in avian species has been proposed to occur through drug selection pressure (*4, 5*). However, the use of amantadine in poultry farms has not been reported in India so far. The phylogenetic analysis of HA gene indicated that the 2013 Indian viruses taken in this study are closely related to A/chicken/India/09CA01/2011 (H5N1) virus isolated from chicken in West Bengal in 2011 which does not contain the Val27Ala mutation. Hence, we tried to analyse the prevalence of Val27Ala mutation among the H5N1 viruses using the sequences of M2 gene available in GenBank. Our analysis revealed presence of this mutation in only four H5N1 viruses of clade 2.3.2.1 isolated in Hong Kong in 2008, Vietnam in 2009 and Bangladesh in 2012 (Fig. 1). With only limited number of H5N1 clade 2.3.2.1 viruses reported with this mutation, it can be assumed that it could have been acquired through random mutation during replication rather than through drug selection pressure. However, non-usage of antivirals in poultry farming should not lead to an automatic assumption of reduced antiviral resistance and complacency in monitoring the emergence of H5N1 virus resistant or less susceptible to antivirals as the H5N1 subtype has the pandemic potential. Therefore, monitoring of antiviral drug susceptibility of HPAI H5N1 viruses isolated from poultry is important in making decisions for stockpiling of anti-influenza drugs for pandemic preparedness.

Acknowledgements. We thank Director, Indian Veterinary Research Institute, and Indian Council of Agricultural Research,

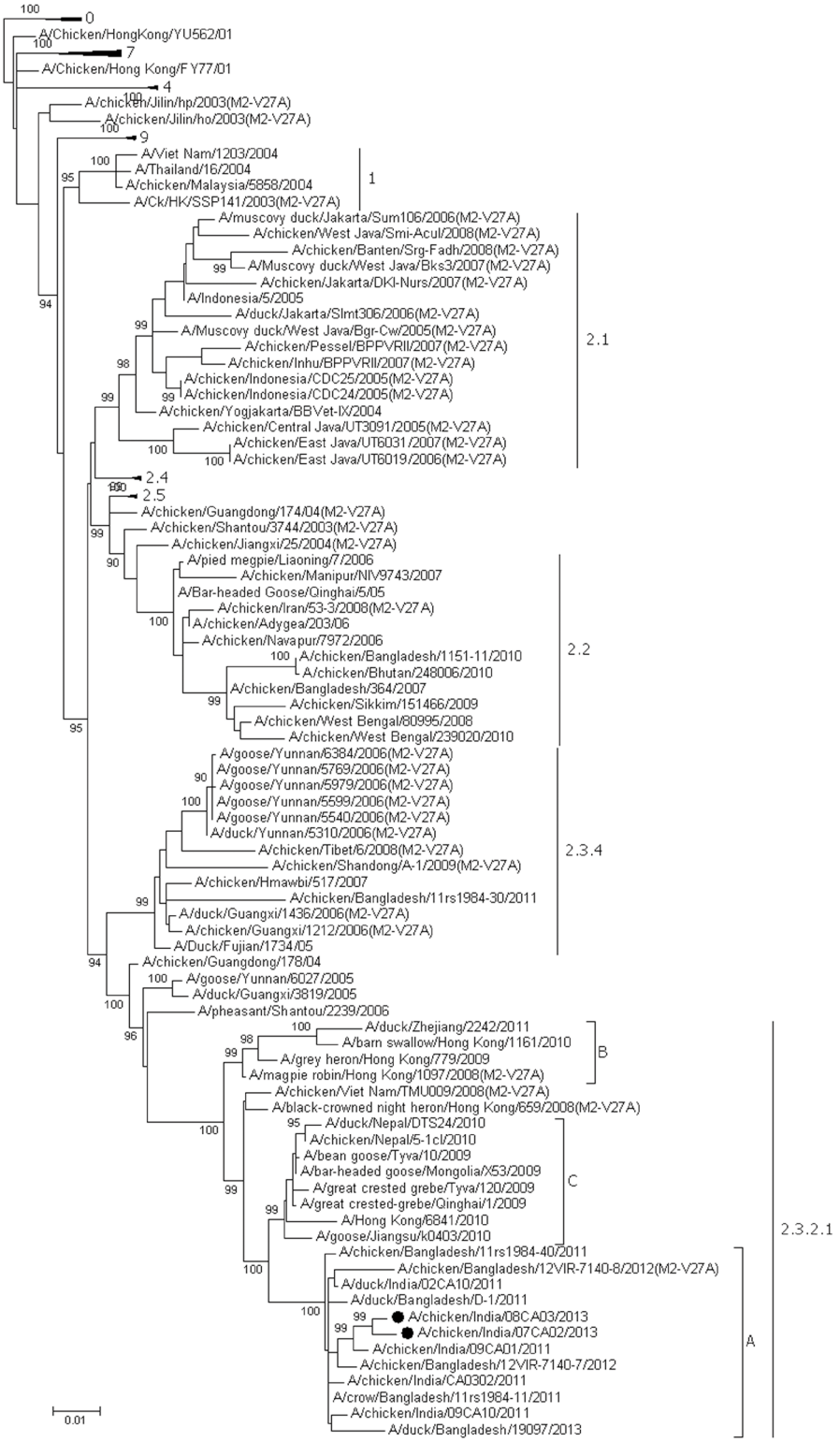

Fig. 1

HA gene-based phylogenetic tree of influenza H5N1 viruses

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.

New Delhi, for providing necessary facilities to carry out this work. We also thank the DADF, MoA, Government of India for financial support.

References:

1. Xu X, Subbarao K, Cox NJ, Guo Y, Virology 261, 15–19, 1999. http://dx.doi.org/10.1006/viro.1999.9820
2. Peiris JS, de Jong MD, Guan Y, Clin. Microbiol. Rev. 20, 243–267, 2007. http://dx.doi.org/10.1128/CMR.00037-06
3. WHO, http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/, accessed on 31st October 2014
4. Govorkova EA, Baranovich T, Seiler P, Armstrong J, Burnham A, Guan Y, Peiris M, Webby RJ, Webster RG, Antiviral Res. 98, 297–304, 2013. http://dx.doi.org/10.1016/j.antiviral.2013.02.013
5. Cheung C-L, Rayner JM, Smith GJD, Wang P, Naipospos TSP, Zhang J, Yuen K-Y, Webster RG, Peiris JSM, Guan Y, Chen H, J. Infect. Dis. 193, 1626–1629, 2006. http://dx.doi.org/10.1086/504723
6. Hurt AC, Selleck P, Komadina N, Shaw R, Brown L, Barr IG, Antiviral Res. 73, 228–231, 2007. http://dx.doi.org/10.1016/j.antiviral.2006.10.004
7. Monne I, Fusaro A, Al-Blowi MH, Ismail MM, Khan OA, Dauphin G, Tripodi A, Salviato A, Marangon S, Capua I, Cattoli G, J. Gen. Virol. 89, 2691–2697, 2008. http://dx.doi.org/10.1099/vir.0.2008/004259-0
8. Tosh C, Murugkar HV, Nagarajan S, Tripathi S, Katare M, Jain R, Khandia R, Syed Z, Behera P, Patil S, Kulkarni DD, Dubey SC, Virus Genes 42, 10–15, 2011. http://dx.doi.org/10.1007/s11262-010-0534-z
9. Tosh C, Nagarajan S, Murugkar HV, Bhatia S, Kulkarni DD, Adv. Anim. Vet. Sci. 2, 33–41, 2014. http://dx.doi.org/10.14737/journal.aavs/2014/2.4s.33.41
10. Nagarajan S, Tosh C, Smith DK, Peiris JS, Murugkar HV, Sridevi R, Kumar M, Katare M, Jain R, Syed Z, Behera P, Cheung CL, Khandia R, Tripathi S, Guan Y, Dubey SC, PLoS One 7(2), e31844, 2012. http://dx.doi.org/10.1371/journal.pone.0031844
11. WHO, Wkly. Epidemiol. Rec. 87, 401–412, 2012.
12. WHO, Wkly. Epidemiol. Rec. 88, 117–128, 2013.
13. Chakrabarti AK, Pawar SD, Cherian SS, Koratkar SS, Jadhav SM, Pal B, Raut S, Thite V, Kode SS, Keng SS, Payyapilly BJ, Mullick J, Mishra AC, PLoS One 4, e7846, 2009. http://dx.doi.org/10.1371/journal.pone.0007846
14. Nagarajan S, Rajukumar K, Tosh C, Ramaswamy V, Purohit K, Saxena G, Behera P, Pattnaik B, Pradhan HK, Dubey SC, Vet. Microbiol. 133,154–163, 2009. http://dx.doi.org/10.1016/j.vetmic.2008.06.013
15. Nagarajan S, Tosh C, Murugkar HV, Venkatesh G, Katare M, Jain R, Behera P, Khandia R, Tripathi S, Kulkarni DD, Dubey SC, Virus Genes 41, 30–36, 2010. http://dx.doi.org/10.1007/s11262-010-0477-4
16. OIE, Chapter 2.3.4, Paris, France, 436-454, 2012.
17. WHO, http://www.who.int/influenza/resources/documents/RecAIlabtestsAug07.pdf, 2007.
18. Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR, Arch. Virol. 146, 2275–2289, 2001. http://dx.doi.org/10.1007/s007050170002
19. Hall TA, Nucl. Acids Symp. Ser. 41, 95–98, 1999.
20. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S, Mol. Biol. Evol. 30, 2725–2729, 2013. http://dx.doi.org/10.1093/molbev/mst197
21. Duvvuri VR, Duvvuri B, Cuff WR, Wu GE, Wu J, Genomics Proteomics Bioinformatics 7, 47-56, 2009. http://dx.doi.org/10.1016/S1672-0229(08)60032-7
22. Chen H, Smith GJD, Zhang SY, Qin K, Wang J, Li KS, Webster RG, Peiris JSM, Guan Y, Nature 436, 191–192, 2005. http://dx.doi.org/10.1038/nature03974
23. Matrosovich M, Zhou N, Kawaoka Y, Webster RG, J. Virol. 73, 1146–1155, 1999.