Arch Virol (2007) 152: 1637–1644
DOI 10.1007/s00705-007-1002-5
Printed in The Netherlands

Archives of Virology

Analysis of the PB2 gene reveals that Indian H5N1 influenza virus belongs to a mixed-migratory bird sub-lineage possessing the amino acid lysine at position 627 of the PB2 protein

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Received 28 October 2006; Accepted 27 April 2007; Published online 11 June 2007
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Summary

Outbreaks of highly pathogenic avian influenza (HPAI) H5N1 virus were reported for the first time in India during February 2006. Herein, we have sequenced and analyzed the PB2 genes of five influenza virus isolates obtained from three affected states (Gujarat, Madhya Pradesh and Maharashtra) in India during the outbreaks. In the phylogenetic analysis, the Indian isolates were grouped in the mixed-migratory bird sub-lineage of the Eurasian lineage. From the phylogenetic tree, it is evident that viruses were probably introduced to India from China via Europe because they share a direct ancestral relationship with the Indian isolates. The virus might have spread through migratory waterfowls that survived the HPAI H5N1 infection. These viruses were able to replicate in cultured cells of avian and mammalian hosts and posses lysine at position 627 of the PB2 protein, indicating that they might be able to cross the host barrier to infect mammals.

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Introduction

Influenza viruses are members of the family *Orthomyxoviridae* and have negative-sense, single-stranded, segmented RNA in an enveloped virion [10]. The genomes encode at least 10 proteins, from the 8 RNA segments, which include two surface glycoproteins (haemagglutinin [HA] and neuramindase [NA]), nucleoprotein (NP), three polymerase proteins (PB2, PB1 and PA), two matrix (M1 and M2) proteins and two non-structural (NS1 and NS2) proteins. Influenza viruses are classified as types A, B and C based on the antigenic properties of their nucleoprotein and matrix (M1) protein. Avian influenza is caused by type A virus, which is further classified into subtypes based on the HA and NA glycoproteins. To date, globally, 16 HA (HA1-16) and 9 NA (NA1-9) subtypes have been detected in different combinations [6, 20]. In general, wild aquatic birds serve as the natural reservoir for avian influenza viruses, where the virus remains genetically stable and helps in propagating the virus to other hosts including domestic poultry and pigs. Highly pathogenic avian influenza (HPAI) virus subtype H5N1 virus was first isolated from geese in China during 1996 [21]. Subsequently,

the virus was found to cause serious losses for the poultry industry, and since 1997 it has been reported to cause human deaths [1, 14, 18].

Control of avian influenza is dependent on effective surveillance, efficient vaccination and destruction of infected populations. Development of a regional vaccine requires knowledge of the viral variant strains circulating in the target population. In India, avian influenza virus (AIV) surveillance has been continuing since 2001, and this laboratory acts as a "National Referral Facility" for avian influenza in this country. In February 2006, an avian influenza outbreak due to H5N1 infection in poultry was reported in the state of Maharashtra, western India [13]. Subsequently, it was reported in Gujarat and Madhya Pradesh, neighboring states of Maharashtra. Preliminary HA gene-based phylogenetic analysis of the isolated virus revealed genetic similarity with two swan isolates from Iran and Italy (A/Cygnus cygnus/Iran/754/06 and A/Cygnus olor/Italy/742/06) [13]. The two outbreak strains (Ck/India/7972/06 and Ck/India/8824/06) showed a nucleotide divergence as high as 3.3 and 1.2% in the HA1 and 2 regions, respectively, with the above two swan isolates. The infection was contained by culling tens of thousands of birds in the area within 10 km radius.

The viral polymerase consists of three subunits, polymerase acidic (PA), polymerase basic (PB) 1, and PB2 [5]. Previous reports have established the role of the PB2 protein in determining influenza virus host range and virulence. A single-gene reassortant virus that derived its PB2 gene from an avian influenza (A/Mallard/NY/78) virus and the remaining genes from a human influenza virus (A/Los Angeles/2/87) revealed that a substitution from glutamic acid to lysine (E → K) at position 627 of PB2 helps the virus to replicate in mammalian MDCK cells [17]. A reverse genetics study identified a substitution from glutamic acid to lysine in the same position, which increased the pathogenicity of Hong Kong 1997 avian influenza H5N1 viruses in mice [8]. Further, there is evidence that an E → K substitution at position 627 of the PB2 protein enhances replication efficiency of the virus due to increased polymerase activity [7, 15]. Thus, there is ample evidence that amino acid residue 627 of PB2 is a major determinant of replication efficiency of the virus, and the presence of lysine at this position is indicative of adaptation to mammalian hosts.

In this study, we have determined the nucleotide sequence of the coding region (approx. 2229–2256 nts) of the PB2 genes of 5 AIV H5N1 isolates recovered from outbreaks in India. The resulting sequences and other 62 sequences obtained from the international DNA databanks were compared. The reference sequences included in the analysis, obtained from the NCBI database, were selected based on BLAST search. The influenza sequence database is growing at a rapid pace, but to our knowledge, there are no sequence data available for H5N1 virus from South Asian countries including India. Hence, the present study is one step forward in this direction.

Materials and methods

Viruses and cells

The H5N1 viruses used in this study were isolated from cloacal/nasal swabs of chickens submitted for diagnosis and are available in the virus repository of this laboratory. These viruses were used in the form of chorio-allantoic fluid collected from infected embryonated chicken eggs. The viruses were subtyped using standard serological and molecular tests recommended by OIE and WHO (details not shown). Details of the five AIV H5N1 isolates used in this study are listed in Table 1. Madin-Darby canine kidney (MDCK) cells and the African green monkey epithelial cell line Vero were grown in Glasgow modified essential medium (GMEM; Sigma, USA) containing 10% fetal bovine serum (FBS; HyClone, USA). Chicken embryo fibroblast (CEF) cells were produced by digesting pieces of 11-day-old chicken

Table 1. History of the avian influenza virus isolates used in the study

Isolate no.*	Place of outbreak
A/Ck/Navapur/India/7972/06	Navapur, Nandurbar, Maharashtra
A/Ck/Surat/India/9256/06	Surat, Gujarat
A/Ck/Jalgaon/India/13840/06	Jalgaon, Maharashtra
A/Ck/Thane/India/15053/06	Thane, Maharashtra
A/Ck/Indore/India/18760/06	Indore, Madhya Pradesh

* The isolate number consists of influenza virus type/host species/place of outbreak/country of isolation/isolate number/year of isolation. *Ck* Chicken.

Table 2. Oligonucleotide primers used in the study

Primer	Sequence (5′–3′)	Source
P-PB2-F[a]	AGCGAAAGCAGGTCAATTATATTC	[11]
HSAIV_PB2_ 73F[a]	ACTCGCGAGATACTGACAAAAACC	this study
HSAIV_PB2_559R[a]	TCAATATCCTGGCTCCAACTTCAT	this study
HSAIV_PB2_445F[a]	AAAATACGCCGCAGGGTTGA	this study
HSAIV_PB2_936R[a]	TGTTGGGTTTTGTTTAAGGATGTCT	this study
HSAIV_PB2_1278R[b]	ATCACCTCGGACTGCCTTTATCAT	this study
HSAIV_PB2_1062F[a]	TACGGGCAACCTCCAAACACTGA	this study
HSAIV_PB2_1584R[a]	GCTGACCTCTTCGGGGGATAA	this study
HSAIV_PB2_1462F[a]	AGAGTTAGTAAAATGGGGGTGGATG	this study
HSAIV_PB2_1901R[a]	GGAGCTGCTGCAAATGGTAGTAGTT	this study
HSAIV_PB2_2282R[b]	TTGGTCGCTGTCTGGCTGTC	this study

[a] Primers used for PCR amplification only.
[b] Primers used for PCR amplification and sequencing.

embryos in buffered saline containing 0.25% trypsin. The cells were then cultured in GMEM with 10% FBS. All five AIV H5N1 isolates were propagated in MDCK, Vero and CEF cells and observed for cytopathic effects. Infected cells showing rounding and detachment of cells from the cultured dish, which is a characteristic of cytopathic effect, were observed from 48 h postinfection onward. All of the experiments were conducted in a biosafety level 4 facility maintained at this laboratory.

Viral RNA extraction, polymerase chain reaction and sequencing

Total viral RNA was extracted from infected allantoic fluid using a QIAamp viral RNA mini kit (Qiagen). Ten μl of RNA was reverse-transcribed in the presence of 12-mer universal primer [9] using an AMV Reverse Transcription kit (Promega) as per the instruction of the manufacturer. The polymerase basic 2 (PB2) gene (RNA segment 1) was amplified in different overlapping fragments using *Taq* DNA polymerase (Invitrogen) and the primer combinations shown in Table 2. PCR products were eluted from a 1.5% agarose gel using a QIAquick gel extraction kit (Qiagen). The purified PCR products were cloned into the pTZ57R/T vector (MBI Fermentas). Recombinant plasmids were sequenced in both strands using the fmol cycle sequencing kit (Promega) along with ^{32}P-labeled M13 forward and reverse primers; nucleotide sequences were read from autoradiograms.

Sequence analysis

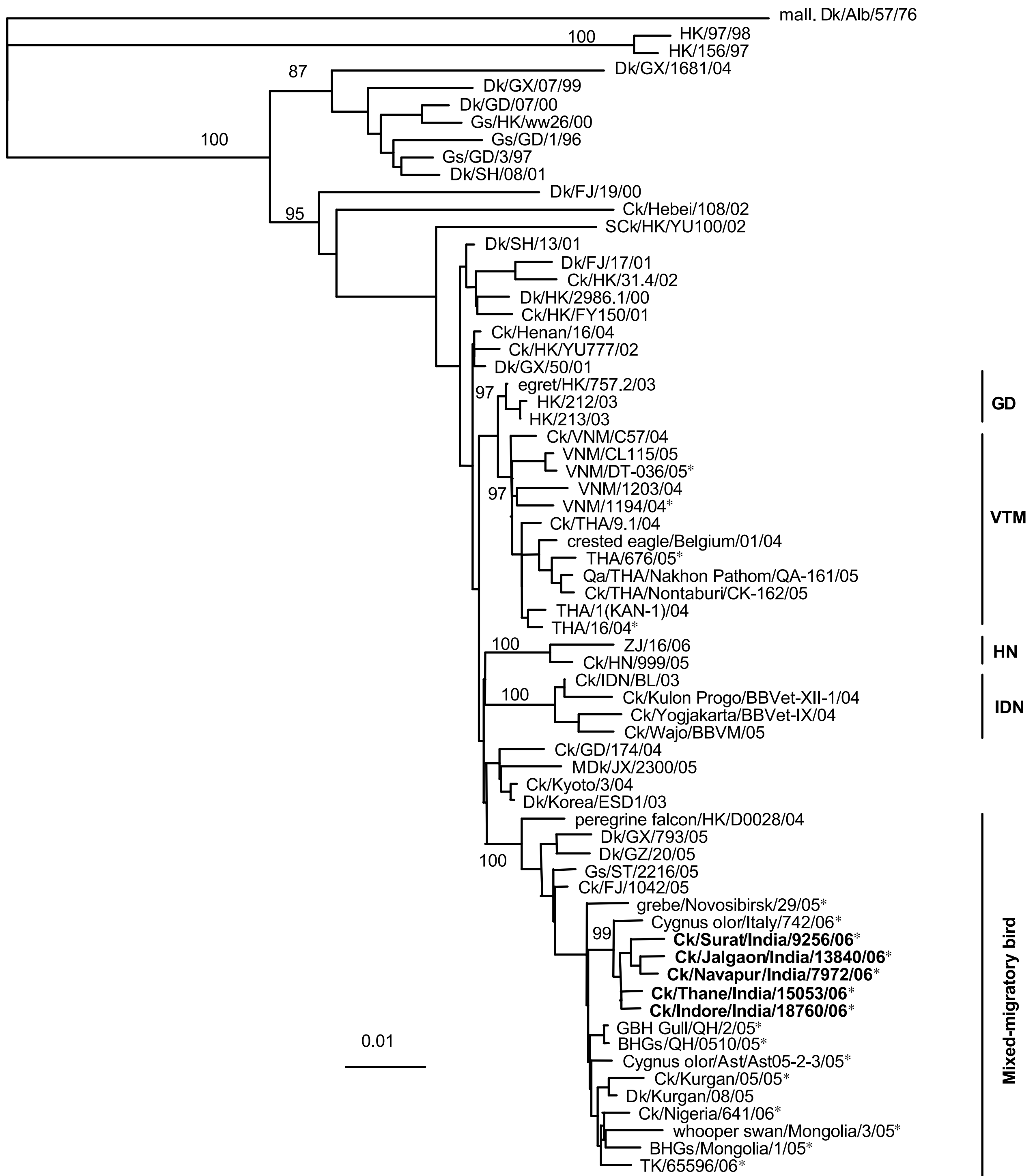
PB2 gene sequences of five H5N1 viruses determined in this study were aligned with 62 sequences from GenBank (http://www.ncbi.nlm.nih.gov) and the Influenza Sequence Database (http://flu.lanl.gov) by using the CLUSTAL X program, version 1.83 [19]. The aligned sequences were used to construct a phylogenetic tree by the neighbor-joining (Kimura 2-parameter) approach, implemented in the Phylip 3.65 package [4]. The reliability of the branching orders was estimated by bootstrapping (1000 replicates). The tree was rooted to A/Mallard Dk/Alb/57/76. The tree topology was produced in the TreeView program 1.6.6 [12]. Percentage nucleotide sequence similarity/difference was estimated using the Megalign program available in the Lasergene99 package (DNASTAR Inc., USA).

Results and discussion

Molecular epidemiology is a powerful tool in monitoring the circulation and spread of infectious agents. Worldwide, epidemiological data are available for the avian influenza H5N1 virus where the disease is prevalent, but much less is known about India. In the present study, five avian influenza H5N1 viruses isolated during February–March 2006 from 3 states (Gujarat, Madhya Pradesh and Maharashtra) of India were analyzed using the PB2 gene. Nucleotide sequences of PB2 genes of all five virus isolates were determined from 6 overlapping PCR-amplified fragments.

The five (Indian) PB2 sequences were compared with 62 PB2 sequences of avian influenza virus isolated in different countries of Asia, Europe and Africa, which were reported in the sequence database, to determine the genetic relationship among them. In the phylogenetic tree (Fig. 1), all 5 Indian isolates clustered with an avian influenza virus from Europe (A/Cygnus olor/Italy/742/06), with a nt

divergence of 0.6–1.1% among them. This Italian H5N1 isolate shared a direct ancestral relationship with the Indian isolates with a high confidence value (99%). All of the Indian isolates maintained a close genetic relationship with each other with a nt divergence of 0.5–0.9% (details not shown). In a



recent study, the H5N1 viruses could be divided genetically into distinct sub-lineages and are established in different geographical regions of South East Asia [3]. These sub-lineages [Vietnam-Thailand-Malaysia (VTM), Guangdong (GD), Hunan (HN), Indonesia (IDN) and another sub-lineage established in migratory birds including isolates from chicken, geese, ducks and humans (mixed-migratory sub-lineage)] could be well differentiated in the phylogenetic tree derived from PB2 gene sequences, with high node values of 97–100% (Fig. 1). The Indian isolates grouped within the mixed-migratory bird sub-lineage. Except for one 2004 isolate (A/peregrine falcon/HK/D0028/04) and two duck isolates (Dk/Guangzhou/20/05 and Dk/Guangxi/793/05) from 2005 from China, all other isolates from the mixed-migratory bird sub-lineage, including the 5 Indian isolates, showed a maximum nt divergence of 2.0% among themselves, indicating a close epidemiological link among them. These observations show that the H5N1 virus had likely been introduced to India by migratory birds from China via Europe as (1) the viruses from China and Europe shared an ancestral relationship with the Indian isolates, and (2) the Indian isolates were clustered in the mixed-migratory bird sub-lineage in the phylogenetic tree (Fig. 1). Although the means of transfer of virus to India could not be directly ascertained, transfer by migratory waterfowl that survived the HPAI H5N1 infection cannot be ruled out. Dissemination of HPAI H5N1 virus in China over long distances has been linked to migratory birds [2]. Further, it has been shown that H5N1 influenza virus has continued to spread from its established source in southern China, the hypothetical influenza epicenter [16], to other regions through transport of poultry and bird migration. This study supports our previous observation on HA-gene-based analysis [13] in which two Indian H5N1 viruses isolated from the Nandurbar (Ck/India/7972/06) and Jalgaon (Ck/India/8824/06) districts of Maharashtra were phylogenetically grouped with an Italian isolate included in the present study (A/Cygnus olor/Italy/742/06) and another from Iran (A/Cygnus cygnus/Iran/754/06), both isolated from swan during 2006. It was proposed in the same report, based on the sharing of a phylogenetic relationship of the Indian isolates with H5N1 viruses from Qinghai (central China) and Jingxi (southern China), that the H5N1 virus from either or both of these two places in China might have spread, possibly through migratory birds, to India and other parts of Asia, Europe and Africa during 2005–06.

A maximum nt sequence divergence of 16.9% among the Indian and the remaining in group sequences in the tree was observed between Ck/India/15053/06 and a human isolate HK/97/98; similarly, minimum nt sequence divergence of 0.6% was observed between Ck/India/15053/06 and Cygnus olor/Italy/742/06.

Alignment of the nucleotide and deduced amino acid sequences of the PB2 gene revealed common substitutions that are unique to the Indian cluster in the tree. One synonymous substitution at nt position 780 C $\rightarrow$ A was found in the Indian isolates compared to rest of the in-group sequences in the tree. Similarly, there are nt signature residues shared by the Indian cluster, including the Italian H5N1 sequence (A/Cygnus olor/742/06), making them distinct from rest of the isolates in the tree. For example, there were two silent nt substitutions at

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Fig. 1. Phylogenetic tree showing genetic relationships among the avian influenza H5N1 viruses. The tree was drawn from the alignment of the complete coding region of the PB2 gene. The tree was rooted to A/mall. Dk/Alb./57/76. The scale bar indicates genetic distances (nucleotide substitution/site). Numbers near the major nodes indicate bootstrap probabilities (percentage of 1000 replicates). Not all supports are shown due to space constraints. The isolate number consists of host species/country of isolation/isolate number/year of isolation. Major sub-lineages are shown to the right; Vietnam-Thailand-Malaysia (*VTM*), Guangdong (*GD*), Hunan (*HN*), Indonesia (*IDN*). Sequence data generated in the present study are shown in bold, and sequences marked with *have lysine at 627 of PB2. *Ck* Chicken; *Dk* duck; *MDk* migratory duck; *Gs* goose; *SCk* silky chicken; *Qa* quail; *mall Dk* mallard duck; *BHGs* bar-headed goose; *GBH gull* great black-headed gull; *Alb* Alberta; *HK* Hong Kong; *GD* Guangdong; *SH* Shanghai; *VNM* Vietnam; *THA* Thailand; *IDN* Indonesia; *JX* Jiangxi; *HN* Hunan; *QH* Qinghai; *Ast* Astrakhan; *Tk* Turkey; *ZJ* Zhejiang; *FJ* Fujian; *ST* Shantou; *GX* Guangxi; *GZ* Guangzhou

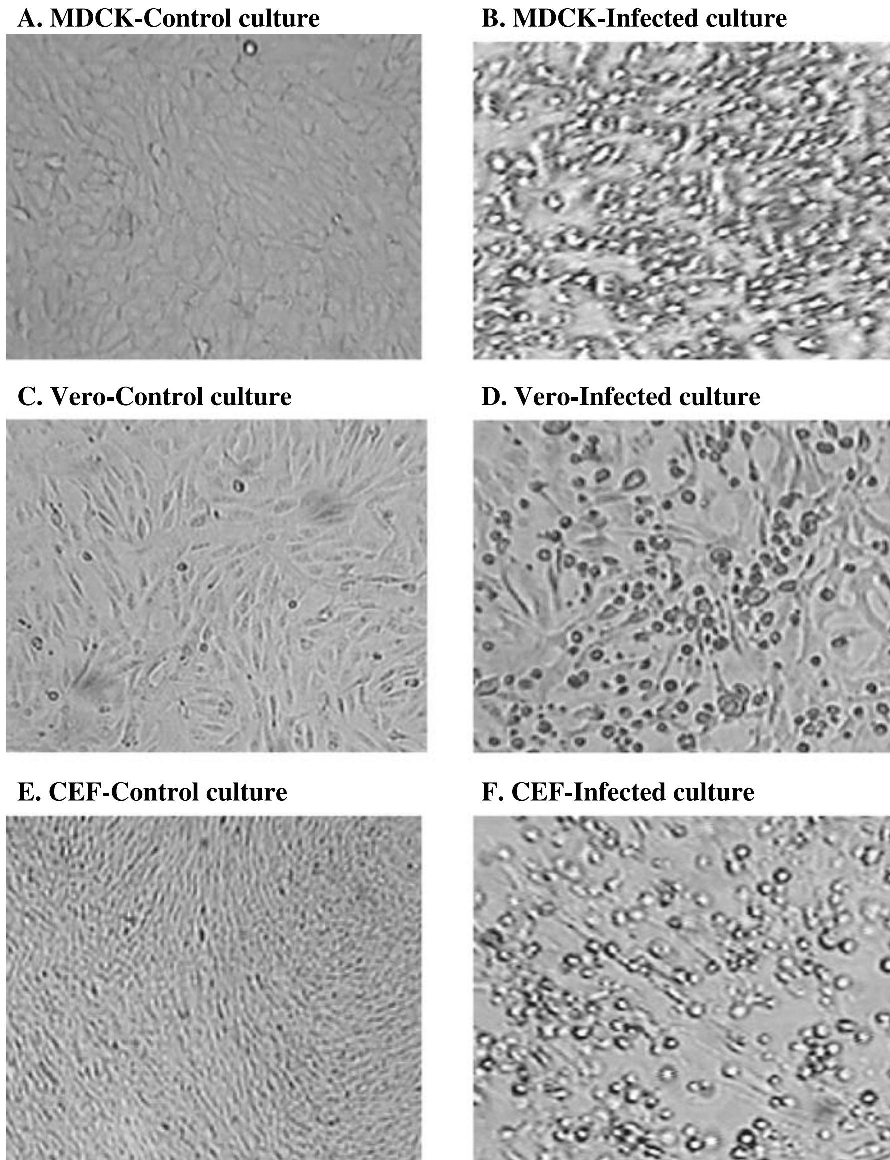

Fig. 2. Monolayer culture of MDCK, Vero and CEF cells showing cytopathic effects at 72 h of infection with H5N1 virus (A/Ck/Navapur/India/7972/06). Magnification ×100

positions 1461A → G and 1698A → G, and one non-synonymous substitution at position 377A → G (resulting in amino acid substitution K → R at position 126; data not shown) in all the Indian isolates and the isolate A/Cygnus olor/Italy/742/06 that were absent in all of the remaining isolates included in the study. This observation further supports the direct epidemiological link between the Indian and European viruses.

We studied the growth of all five AIV H5N1 isolates in avian and mammalian cells of monolayer cultures (Fig. 2). The virus showed characteristic cytopathic effect including rounding and detachment of the cells from the culture dish, which indicates pathogenic potential of H5N1 viruses to the above hosts. Another observation was that all five Indian H5N1 isolates posses a lysine residue at 627 position of the PB2 protein, which mimics human-influenza-virus-like character. In the mixed-migratory bird sub-lineage including the 5 viruses from India (Fig. 1), of the 21 isolates 15 were from China, Russia, Nigeria, Mongolia, and Turkey and had lysine at this position (627). This human-influenza-virus-like amino acid (K627) was also found

in the chicken H5N1 viruses of 1997 isolated from Hong Kong [8] and migratory bird H5N1 viruses of 2005 from Qinghai Lake (central China) [2, 22]. Previously, it has been shown that the amino acid residues at position 627 of the PB2 protein are a determinant of host specificity [17], and adaptation of influenza virus to a mammalian host arises from resultant increase in the activity of the polymerase complex [7]. Increased virulence of H5N1 viruses in mice has been attributed to substitution from glutamic acid to lysine at position 627 [8]. Influenza H5N1 viruses with lysine at position 627 of the PB2 protein (HK483RG and HK6PB2-627K) showed widespread distribution of the virus and neurovirulence in mice, while viruses with glutamic acid at this position (HK3PB2-627E and HK486RG) were localized to respiratory organs and are nonlethal [15]. Of the three H5N1 influenza viruses from human fatal cases studied, two possess lysine and one glutamic acid at PB2 position 627 [14]. In the present investigation, of the 13 human isolates included in the analysis, 5 had lysine, and the remaining one had a glutamic acid residue at 627 of the PB2 protein. This observation indicates that the Indian H5N1 viruses, which also have K at this position of the PB2 protein, might cross the species barrier and infect mammals, which is of immense public health concern.

Conclusion

In summary, the HPAI H5N1 virus isolated from poultry in the recent outbreaks in India belongs to the mixed-migratory bird sub-lineage within the Eurasian lineage of avian influenza viruses. From the phylogenetic tree it is evident that virus had likely been introduced to India from China via Europe as the Italian isolate shares a direct ancestral relationship with the Indian isolates. The virus might have spread through migratory waterfowl that survived the HPAI H5N1 infection. All five Indian isolates studied were able to replicate in cell monolayers of mammalian and avian hosts and possess the amino acid lysine at position 627 of the PB2 protein, indicating that that these viruses might be able to cross the host barrier and infect mammals, indicating a possible threat to human health.

Acknowledgements

We thank Indian Council of Agricultural research, New Delhi and Department of Animal Husbandry, Dairying and Fisheries (Ministry of Agriculture), Government of India for providing necessary facilities to undertake the work. RPK thanks the Indian Veterinary Research Institute (Bareilly, Uttar Pradesh) for his M. V. Sc fellowship.

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