Phylogenetic analysis revealed genetic similarity of the H5N1 avian influenza viruses isolated from HPAI outbreaks in chickens in Maharashtra, India with those isolated from swan in Italy and Iran in 2006

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India was free from highly pathogenic avian influenza (HPAI) virus H5N1 till January 2006. In February 2006, our laboratory diagnosed two H5N1 outbreaks in chickens in the neighbouring districts of Nandurbar (first outbreak) and Jalgaon (second outbreak) of Maharashtra, India. Both the outbreaks occurred in a span of 12 days with heavy mortality in affected chicken populations. From the first outbreak, AIV H5N1 was isolated from cloacal swab of chickens and in case of second outbreak the virus was isolated from dead chickens in embryonated chicken eggs. Both the H5N1 viruses met all the criteria of highly pathogenic avian influenza virus. In the present investigation an attempt has been made to trace the origin of both the Indian H5N1 viruses by comparing their partial nucleotide sequences in the HA1 and HA2 regions of the HA gene with 30 other H5N1 viruses that were isolated in different countries of Asia, Europe and Africa during the years 1997 (02), 2003 (02) and 2004–2006 (26). From the study it is clear that the H5N1 virus that caused outbreak in chickens in Jalgaon did not originate from the neighbouring district of Nandurbar where the first outbreak occurred; instead both the HPAI outbreaks in Maharashtra were due to two different populations of the virus introduced at two different times. Genetic heterogeneity was observed between the H5N1 viruses isolated in 2006 in different countries; the two viruses of Malaysia and Laos formed a sublineage different from the one consisting of the viruses of India, Iran, Iraq, Italy, Nigeria and Egypt.

Keywords: Avian influenza virus, H5N1, highly pathogenic avian influenza virus, phylogeny.

INFLUENZA viruses are enveloped, pleomorphic and negative sense segmented RNA viruses belonging to the family Orthomyxoviridae. These viruses have been isolated from a wide range of hosts including humans, pigs, birds, horses and sea mammals, and are classified as types A, B and C based on the antigenic differences in two of their gene products, nucleoprotein (NP) and matrix (M1). Avian influenza is caused by type A influenza virus, which is further classified into subtypes on the basis of two surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA). Accordingly, 16 HA (HA1–HA16) and nine NA (NA1–NA9) subtypes have been identified[1]. All the 16 HA subtypes of AIV have been isolated from wild, aquatic birds, which are a primordial reservoir of all influenza A viruses[1,2]. However, these viruses (Avian Influenza Virus; AIV) normally do not make wild birds sick, but once transmitted to poultry (mostly chicken, turkey and domestic ducks) can cause serious economic losses arising from high mortality and trade embargo[3].

HA gene product is predominantly responsible for pathogenicity of AIV. This gene is about 1778 bp long with an open reading frame of 1704 bases (568 amino acid residues). Based on the pathogenicity of AIVs in domestic poultry, these are classified into two pathotypes, viz. highly pathogenic avian influenza (HPAI) and non-highly pathogenic avian influenza (nHAPI) including mild pathogenic, low pathogenic and non-pathogenic AIV[4]. Cleavage of HA molecule (HA0), by host cell proteases, into HA1 and HA2 subunits is essential for infectivity, and AIVs with high and low levels of pathogenicity differ in their cleavage sequence. Highly pathogenic AIVs possess polybasic amino acid residues (at least six) in their (HA) cleavage sequence making it readily recognizable by a wide range of cellular proteases[5–7]. Therefore, AIVs containing multiple basic amino acid residues in the HA cleavage region have multiple sites of virus replication and produce more severe infection in birds and human beings[7,8]. Highly pathogenic AIVs are restricted to H5 (H5N1 strain) and H7 (H7N7 and H7N3 strains) subtypes, and these are capable of causing severe respiratory disease and high mortality in infected chickens and can be transmitted directly to humans[6]. Of these, HPAI virus H5N1 has been responsible for the death of millions of domesticated poultry (and also human deaths arising from direct or close contact with infected poultry or contaminated surfaces) in East Asian countries, since the first H5N1 outbreak in 1997 in poultry in Hong Kong. Since December 2003 till date, H5N1 infections in poultry or wild birds have been reported in six African countries, 17 Asian countries including East Asia, South-East Asia and near East (China, Cambodia, Hong Kong, Indonesia, Japan, Korea, Laos, Malaysia, Mongolia, Myanmar, Thailand, Vietnam, India, Iraq, Iran, Israel and Jordan) and 25 European (including Eurasia) countries. New H5N1 outbreaks in poultry, beginning in June 2004, were reported in several countries of Asia including Cambodia, China, Indonesia, Malaysia, Thailand and Vietnam. Since May 2005, H5N1 outbreaks in poultry were reported in China, Kazakhstan, Romania,

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Table 1. Per cent nucleotide divergence of the two Indian H5N1 viruses of 2006 with other H5N1 viruses isolated elsewhere in the world

ISDN*/NCBI accession number of the H5N1 viruses	Description of the virus	% nucleotide divergence with Indian H5N1 viruses in HA1 and HA2 regions				Remarks
		A/Ck/Nandurbar/MH/India/7972/2006		A/Ck/Jalgaon/MH/India/8824/2006		
		HA1 region	HA2 region	HA1 region	HA2 region	
AF082035	Ck/Hong Kong/1997	9.7	4.2	8.3	4.0	
AF098543	Dk/Hong Kong/1997	9.9	4.0	8.5	3.8	
AY639405	Gs/China/2004	9.9	4.6	8.5	4.4	
DQ095628	Gs/China/2005	8.7	3.2	7.4	2.9	
AY651353	Ck/Hong Kong/2003	7.5	3.0	6.2	2.8	
DQ334776	Ck/Thailand/2005	8.5	3.6	7.2	3.4	H5N1 isolates clustered in Group A
ISDN40925	Ck/Laos/2004	7.9	3.2	6.6	3.0	
AY651343	Ck/Vietnam/2004	8.1	3.4	6.8	3.2	
ISDN122143	Ck/Cambodia/2005	8.9	3.1	7.6	2.9	
ISDN122147	Gs/Cambodia/2005	9.3	3.3	7.9	3.1	
ISDN124032	Dk/Vietnam/2005	8.5	3.8	7.2	3.6	
ISDN124036	Ck/Vietnam/2005	8.5	3.6	7.2	3.4	
AY609312	Ck/GD/China/2004	6.4	1.7	5.1	1.3	H5N1 isolates clustered in subgroup B1
ISDN49016	Ck/YG/Japan/2004	6.6	1.6	5.3	1.4	
ISDN40921	Ck/Korea/2003	6.4	1.6	5.1	1.4	
DQ32098	Ck/Indonesia/2005	6.8	3.8	5.5	3.6	H5N1 isolates clustered in subgroup B2
AY651324	Ck/Indonesia/2004	6.8	3.0	5.5	2.8	
DQ095625	Ck/YN/China/2005	7.9	2.8	6.6	2.5	H5N1 isolates clustered in subgroup B3
ISDN138756	Ck/Malaysia/2006	7.7	2.6	6.0	2.3	
ISDN138780	Dk/Laos/2006	7.9	2.6	6.2	2.3	
DQ320920	MDk/JX/China/2005	3.7	1.2	2.6	1.0	H5N1 isolates clustered in subgroup B4
DQ440535	Swan/Iran/2006	3.3	1.2	2.2	1.0	
DQ412997	Swan/Italy/2006	3.3	1.0	2.2	0.8	
India 7972/2006	Ck/Nandurbar/7972/2006	–	–	3.5	0.6	
India 8824/2006	Ck/Jalgaon/8824/2006	3.5	0.6	–	–	
DQ095612	BH goose/QH/China/2005	3.5	0.8	2.4	0.6	
DQ406728	Ck/Nigeria/2006	4.0	0.8	3.0	0.6	
DQ323672	Ck/Kurgan/Russia/2005	4.0	1.0	2.9	0.8	
DQ435202	Human/Iraq/2006	4.0	1.0	3.1	0.8	
AB233320	Swan/Mongolia/2005	4.2	1.0	3.1	0.6	
DQ407519	Turkey/Turkey/2005	4.7	1.0	3.7	0.8	
DQ447199	Ck/Egypt/2006	4.7	1.4	3.7	1.2	

*Influenza Sequence Database Number.
Ck, Chicken; Dk, Duck; GD, Guangdong; Gs, Goose; JX, Jiangxi; MDk; Migratory duck; QH, Qinghai; YN, Yunnan; YG, Yamaguchi.

Russia and Ukraine. Death of wild birds was also reported during this period in China, Croatia, Mongolia and Romania. Since February 2006 alone, the first cases of H5N1 infections in poultry were detected in 34 countries, including India, spread over Asia, Europe and Africa. India reported the first case of H5N1 infection in chickens in Nandurbar district (taluk Navapur) of Maharashtra on 18 February 2006. Subsequently, the infection was detected in backyard poultry in Jalgaon district (taluk Yaval, village Marul) of Maharashtra. Diagnosis of these H5N1 infections/viruses using standard serological, virological and molecular techniques, as described in the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (*Mammals, Birds and Bees*), 5th edition (2004), Office International des Epizooties (OIE; World Organization of Animal Health), was done at the High Security Animal Disease Laboratory (HSADL), Bhopal (details not shown). This laboratory acts as the national centre for diagnosis of avian influenza in India.

The present investigation was carried out to trace the origin (by targeting HA gene sequence) of the first two Indian H5N1 viruses isolated from chicken in two neighbouring districts of Maharashtra (one from district Nandurbar (A/Ck/Navapur/Nandurbar/Maharashtra/India/7972/2006) and the other from district Jalgaon (A/Ck/Marul/Yaval/Jalgaon/Maharashtra/India/8824/2006) by assessing their phylogenetic relationship with other H5N1 viruses (Table 1) which circulated in several East and south-east Asian countries, Iraq, Iran, Russia, Italy, Turkey, Egypt and Nigeria during 2004–06. Two Hong Kong H5N1 viruses of 1997, and one each of Korea and Hong Kong isolated in 2003 were also included in the study. Phylogenetic analysis

using HA gene sequences has revealed distinct regional sublineages of H5N1 virus in Asia[9].

The H5N1 virus 7972/2006 was isolated from cloacal swab of chickens (received at this laboratory on 11 February 2006) and the other virus 8824/2006 was isolated from a dead chicken (received at this laboratory on 23 February 2006) in embryonated chicken eggs following standard procedure. Both these H5N1 viruses belong to HPAI virus group as revealed by (i) high mortality in affected chicken populations, (ii) presence of polybasic amino acid residues (^{341}RRRKKR^{346}) at the HA cleavage site, (iii) cytopathogenicity in the absence of trypsin in MDCK cells, and (iv) intra-venous pathogenicity (IVP) test in chickens (IVP index 2.90) (details not shown).

Partial nucleotide (nt) sequence of HA1 (nt 276–866) and HA2 (nt 1184–1666) gene of the two Indian H5N1 viruses 7972/2006 and 8824/2006, isolated during Februray 2006, was determined by cycle sequencing using the Sanger's di-deoxy chain termination technique and ^{32}P labelled M13 forward and reverse primers (*fmol* DNA Cycle Sequencing System, Promega). These sequences were derived from 1.43 Kb HA–H5 fragment of both the isolates generated by one-step RT–PCR (Access RT–PCR System, Promega) using RNA template extracted from chorio-allantoic fluid of infected embryonated chicken egg and gene-specific primers [(+) 5′ ^{266}TGCCGGA ATGGTC TTACATAGTG^{288} 3′ and [(–) 5′ ^{1695}TCTG CATTGTAACGACCCATTG^{1674} 3′], and cloned in pGEM-T Easy vector (Promega). Nucleotide sequence in the HA1 and HA2 regions (nucleotide residues 276–866 and 1184–1666, respectively) of both the Indian H5N1 viruses is shown in Figures 1 and 2.

Partial nucleotide sequence in the HA1 and HA2 regions of both the Indian H5N1 viruses was compared with that of 30 selected H5N1 viruses reported from different countries of Asia including East and South-East Asia (26), Europe and Eurasia (02), and Africa (02). Of these H5N1 viruses, seven are of 2006 [one each from Malaysia (Chicken; Ck), Laos (Ck), Nigeria (Ck), Iraq (human), Iran (swan; *Cygnus cygnus*), and Italy (swan; *Cygnus olor*)], 13 are of 2005 [four from China isolated from chicken/ goose/migratory duck, one each from Mongolia (swan), Russia (Ck), Indonesia (Ck), Thailand (Ck) and Turkey (turkey), two each from Cambodia (goose and duck) and Vietnam (Ck and duck)], six are of 2004 [two from China (Ck and goose), and one each from Indonesia (Ck), Vietnam (Ck), Laos (Ck) and Japan (Ck)], two are of 2003 [one each from Hong Kong (Ck) and Korea (Ck)], and the rest two (one each from chicken and duck) are from Hong Kong isolated during 1997. The sequences in the HA1 and HA2 regions were separately aligned using 'CLUSTAL W' and analysed by 'dnadist' and 'Neighbour-joining (NJ)' programs available in PHYLIP 3.65 package ((http://evolution.genetics.washington.edu/phylip.html). Phylogenies were estimated by performing 1000 NJ 'bootstrap' replicates using 'seqboot' program available in the PHYLIP 3.65 package and consensus phylogenetic trees (one for HA1 and the other for HA2 regions) showing node values were obtained. Phylogenetic trees were plotted using 'TreeView 1.6.6' program (http://taxonomy.zoology.gla.ac.uk/rod/ treeview.html).

Neighbour-joining trees based on HA1 and HA2 gene sequences are shown in Figures 3 and 4 respectively. Both the phylogenetic trees reveal clustering of the viruses in two major groups, named A and B. Seven viruses isolated in East Asian countries of Vietnam, Laos, Thailand and Cambodia during the years 2004 and 2005 (AY651343, DQ334776, ISDN122143, ISDN122147, ISDN124032, ISDN124036 and ISDN40925) are clustered into one group (group A). Out of these, six viruses were isolated from chicken, and the remaining one (ISDN122147) from goose. The two H5N1 isolates of 2006 from Malaysia (ISDN138756) and Laos (ISDN138780) did not cluster in this group (group A), with the viruses of Vietnam, Thailand, Laos and Cambodia isolated during 2004 and 2005, though in a recent study[9] H5N1 viruses of Vietnam, Thailand and Malaysia (VTM) isolated during the period 2003–05 were found grouped together in a sublineage (VTM sublineage). This could be due to continuous mutation process occurring in HA gene of H5N1 viruses during circulation in birds resulting in evolution of new sublineages in 2006. Multiple sublineages of H5N1 virus have been identified in Asia[9].

^{276}TGCCGGAATGGTCTTACATAGTGGAGAAGATCAATCCAGCCAATGACCTCTGTTACCCAGG
GAATTTCAACGACTATGAAGAACTGAAACACCTATTGAGCAGAATAAACCATTTTGAGAAAA
TTCAGATCATCCCCAAAAGTTCTTGGTCAGATCATGAAGCCTCATCAGGGGTGAGCTCAGCAT
GTCCATACCAGGGAAGGTCCTCCTTTTTTAGAAATGTGGTATGGCTTATCAAAAAGAACGATG
CATACCCAACAATAAAGAGAATTTACAATAATACGGGGGGGAGAGACCCCCCGGTACTGTGG
GGGATTCACCATCCAAATGATGCGGCAGAGCAGACAAGGCTCTATCAAAACCCAACCACCTA
TATTTCCGTTGGGACATCAACACTAAACCAGAGATTGGGACCAAAAATAGCTACTAGATCCAA
GGTAAACGGGCAAAGTGGAAGGATGGAGTTCTTTTGGACAATTTTAAAACCGAATGATGCAA
TAAACTTTGAGAGTAATGGAAATTTCATTGCTCCAGAAAATGCATACCAAATTGTCCAGAAAG
GGGACTCAACAATCATGAAAAGTGAATTGG^{866}-----**HA1**-------//-------**HA2**------^{1184}TGGAGTCACC
AATAAGGTCAACTCGATCATTGACAAAATGAACACTCAATTTGAGGCCGTTGGAAGGGAATTT
AATAACTTAGAAAGGAGAATAGAAAATTTAAACAAGAAGATGGAAGACGGATTCCTAGATGT
CTGGACTTATAATGCTGAACTTCTGGTTCTCATGGAAAATGAGAGAACTCTAGACTTTCATGA
CTCAAATGTCAAGAACCTTTACGACAAGGTCCGACTACAGCTTAGGGATAATGCAAAGGAGC
TTGGTGACGGTTGTTTCGAGTTCTATCACAGATGTGATAATGAATGTATGGAAAGTGTAAGAA
ACGGAACGTATGACTACCCGCAGTATTCAGAAGAAGCAAGATTAAAAAGAGAGGAAATAAGT
GGAGTAAAATTGGAATCAATAGGAACTTACCAAATACCGTCAATTTATTCAACAGTGGCGAG
CTCCCTAGCACTGGCAATCATGGTGGCTGGTCTATCTTTATGGATGTGCTCCAATGGGTCGTTA
CAATGCAGAA^{1666}

Figure 1. Partial nucleotide sequence in HA1 and HA2 regions (reverse complementary strand) of H5N1 virus 7972/2006.

^{276}TGCCGGAATGGTCTTACATAGTGGAGAAGATCAATCCCGCCAATGACCTCTGTTACCCAGG
GAATTTCAACGACTATGAAGAACTGAAACACCTATTGAGCAGAATAAACCATTTTGAGAAAA
TTCAGATCATCCCCAAAAGTTCTTGGTCAGATCATGAAGCCTCATCAGGGGTGAGTTCAGCAT
GTCCATACCAGGGAAGGTCCTCCCTTTTTAGAAATGTGGTATGGCTTATCAAAAAGAACGATG
CATACCCAACAATAAAGAGAAGTTACAATAATACCAACGGGGAAGATCCTCTGGTACTGTGG
GGGATTCACCATCCAAATGATGCGGCAGAGCAGACAAGGCTCTATCAAAACCCAGCCACCTA
TATTTCCGTTGGGACATCAACACTAAACCAGAGATTGGTACCAAAAATAGCTACTAGATCCAA
GGTAAACGGGCAAAGTGGAAGGATGGAATTCTTTTGGACAATTTTAAAACCGAATGATGCAA
TTAACTTTGAGAGTAATGGAAATTTCATTGCTCCAGAAAATGCATACAAAATTGTCAAGAAAG
GGGACTCAACAATCATGAAAAGTGAAATGG^{866}-----**HA1**-------//-------**HA2**------^{1184}TGGAGTCACC
AATAAGGTCAACTCGATCATTGACAAAATGAACACTCAATTTGAGGCCGTTGGAAGGGAATTT
AATAACTTAGAAAGGAGAATAGAAAATTTAAACAAGAAGATGGAAGACGGATTCCTAGATGT
CTGGACTTATAATGCTGAACTTCTGGTTCTCATGGAAAATGAGAGAACTCTAGACTTTCATGA
CTCAAATGTCAAGAACCTTTACGACAAGGTCCGACTACAGCTTAGGGATAATGCAAAGGAGC
TTGGTAACGGTTGTTTCGAGTTCTATCACAGATGTGATAATGAATGTATGGAAAGTGTAAGAA
ACGGAACGTATGACTACCCGCAGTATTCAGAAGAAGCAAGATTAAAAAGAGAGGAAATAAGT
GGAGTAAAATTGGAATCAATAGGAACTTACCAAATACTGTCAATTTATTCAACAGTGGCGAGC
TCCCTAGCACTGGCAATCATGGTGGCTGGTCTATCTTTATGGATGTCCTCCAATGGGTCGTTAC
AATGCAGAA^{1666}

Figure 2. Partial nucleotide sequence in HA1 and HA2 regions of H5N1 virus 8824/2006.

Twenty H5N1 viruses, including the two Indian viruses, are clustered into another group (group B). This group is highly heterogeneous with viruses isolated from 15 different countries of Asia (including India), Europe and Africa during the years 2003–06. The Asian isolates in this group are from East Asian countries of China, Japan, Laos, Korea and Mongolia, South Asian country of India, South-East Asian countries of Malaysia and Indonesia, and near East countries of Iran and Iraq. The European isolates in the group are from Italy, Turkey and Russia (Eurasia). The African isolates are from Nigeria and Egypt. This cluster (group B) could be subdivided into four subgroups, named B1–B4. The subgroup B1 comprised of three H5N1 viruses of East Asia [one chicken isolate each from Korea (ISDN40921; 2003), China (AY609312; 2004) and Japan (ISDN49016; 2004)] isolated during the years 2003 and 2004. The subgroup B2 comprised of two H5N1 isolates of chicken (of 2004 and 2005) from Indonesia (South-East Asia) (AY651324; 2004 and DQ320928; 2005). The subgroup B3 comprised of two east Asian isolates, one chicken isolate of 2005 (China, DQ095625) and another duck isolate of 2006 (Laos, ISDN138780), and one South-East Asian (chicken) isolate of 2006 (Malaysia, ISDN138756).

The subgroup B4 was most heterogeneous in group B, and comprised of 12 H5N1 viruses isolated from chicken/turkey/migratory duck/swan/goose/human during 2005 and 2006 in countries of Asia (China, Mongolia, India, Iraq and Iran), Europe (Italy and Turkey), Eurasia (Russia) and Africa (Nigeria and Egypt), including one H5N1 virus (DQ095612) isolated from a (bar-headed) goose of Qinghai (QH) Lake in central China where an outbreak of H5N1 occurred[9] in migratory waterfowl in May, 2005. In addition, all the 12 H5N1 viruses in subgroup B4 appear to have originated from the H5N1 virus that was isolated from chicken in Guangdong (GD), China during 2004 (AY609312) (Figures 3 and 4). The HPAI H5N1 virus was initially isolated from geese in the Guangdong province of (southern) China in 1996, and HA of all H5N1 viruses analysed was found to have originated from this virus[9]. The two Indian viruses (isolated from chicken in 2006) in this subgroup (B4) are genetically closer (2.2–3.3% divergence in HA1 region and 0.8–1.2% divergence in HA2 region) to the two H5N1 viruses isolated from swan in Italy and Iran during 2006 (DQ412997 and DQ440535), so also to the Qinghai (central China) and Jiangxi (JX) (southern China) isolates of 2005 [DQ095612 (from bar-headed goose) and DQ320920 (from migratory duck), respectively] with a nucleotide divergence of 2.4–3.7% and 0.6–1.2% in HA1 and HA2 regions, respectively (Figures 2 and 3 and Table 1). The rest of the eight viruses in this subgroup (B4) are also related to the above two H5N1 isolates of 2005 of China. Therefore, it is evident that the H5N1 from either

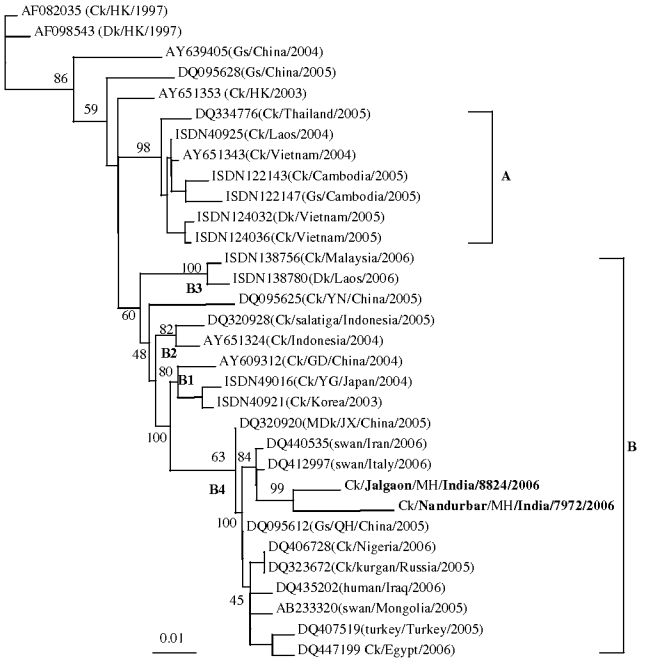

Figure 3. Neighbour-joining tree based on HA1 gene (nt 276–866). Figures (in percentage) near the nodes indicate bootstrap values. Tree was rooted to AF082035 (Ck/HK/1997). Scale bar indicates nucleotide substitution/site. Two Indian isolates are shown in bold. Major lineages (A and B) are shown to the right of the tree. NCBI accession numbers/Influenza sequence database number (ISDN) of the viral sequences are shown for reference. MH, Maharashtra; HK, Hong Kong.

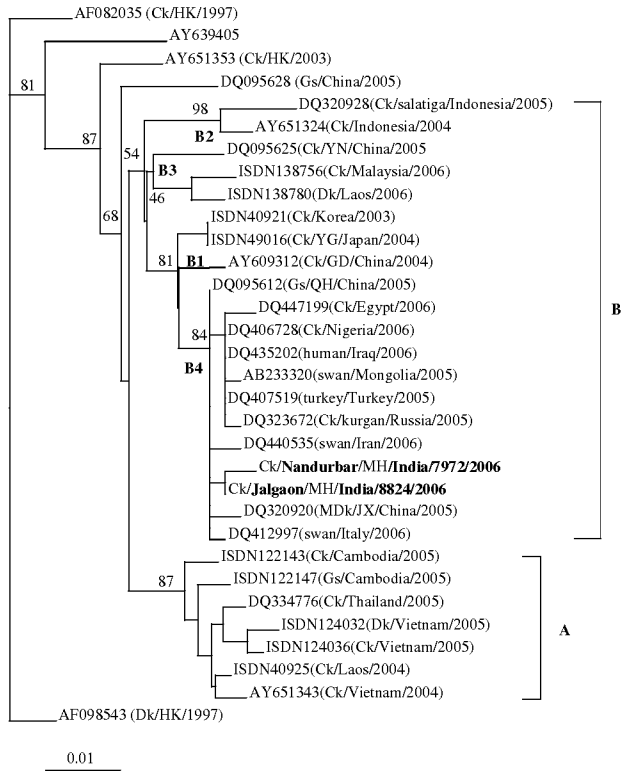

Figure 4. Neighbour-joining tree based on HA2 gene (nt 1184–1666). Figures (in percentage) near the nodes indicate bootstrap values. Tree was rooted to AF082035 (Ck/HK/1997). Scale bar indicates nucleotide substitution/site. Two Indian isolates are shown in bold. Major lineages (A and B) are shown to the right of the tree. NCBI accession numbers/Influenza sequence database number (ISDN) of the viral sequences are shown for reference. MH, Maharashtra; HK, Hong Kong.

or both of these two places (Jiangxi and Qinghai) in China might have spread, possibly through migratory birds, to different parts of Asia (including India), Europe and Africa during 2005–06. Li *et al.*[10] observed that domestic ducks in southern China are responsible for the generation and maintenance of HPAI H5N1 virus, and that wild birds might have contributed to its spreading in Asia. Recently, Chen *et al.*[9] have isolated H5N1 virus from apparently healthy migratory birds in southern China. They have further observed that H5N1 virus has continued to spread out from its established source in southern China through transport of poultry and bird migration. Li *et al.*[10] demonstrated that HPAI virus H5N1 that caused disease outbreaks in poultry in China and seven other East Asian countries between 2003 and 2004 as well as human deaths in Thailand and Vietnam originated from the 1997 H5N1 virus of Hong Kong. This new virus was classified as genotype 'Z' that was responsible for new outbreaks in East Asia[10] during 2003–04.

Per cent nucleotide divergence, in HA1 and HA2 regions, between the two Indian viruses and the other 30 viruses (sequences) used in the study was calculated using 'Megalign' program available in the 'Lasergene99' (DNAstar Inc.) software package (Table 1). Sequence in the HA1 region was less conserved than that in the HA2 region. Haemagglutinin (HA) was synthesized as a polyprotein precursor (HA0) that was post-translationally cleaved into two subunits, HA1 and HA2. It may be seen that the two Indian H5N1 viruses, isolated from affected chickens from two different HPAI outbreaks, occurring in a span of 12 days in February 2006, in two neighbouring districts of Maharashtra (first in Nandurbar and then in Jalgaon) were not identical; they were 3.5% divergent in the HA1 region and 0.6% divergent in the HA2 region from each other (Table 1). This nucleotide divergence, between the two Indian viruses, in the HA gene might have occurred during transmission/circulation of the virus in chickens. But, the level of nucleotide sequence divergence between the two Indian viruses and 30 other H5N1 viruses, isolated elsewhere in the world (Table 1), shows that the virus 8824/2006 isolated from Jalgaon (second outbreak) must have evolved earlier than the virus 7972/2006 isolated from Nandurbar (first outbreak). In comparison to the virus of the first outbreak (7972/2006), the virus 8824/2006 (of second outbreak) is less divergent from all the 30 H5N1 viruses compared. Therefore, it is quite possible that the H5N1 virus 8824/2006 that caused the outbreak in chickens in Jalgaon did not originate from the neighbouring district of Nandurbar where the first outbreak occurred; instead both the HPAI outbreaks in Maharashtra were (possibly) due to two independent populations of the virus introduced at two different times. It may be mentioned here that the suspected clinical materials (cloacal swab/dead chicken) from Nandurbar was received at this laboratory on 11 February 2006 and those from Jalgaon on 23 February 2006.

Nucleotide sequence comparison with the seven H5N1 viruses isolated during 2006 in different countries revealed that (in the HA1 region) both the Indian H5N1 viruses are genetically closer (2.2–3.3% divergence) to the two H5N1 viruses isolated from swan in Italy and Iran which is suggestive of spread of the virus to distant places through wild, aquatic bird migration[9,10], compared to 3–4.7% divergence from the three viruses of Iraq (human), Egypt (Ck) and Nigeria (Ck), and 6–7.9% divergence from the remaining two viruses of Laos (Dk) and Malaysia (Ck). This observation shows genetic heterogeneity among the H5N1 viruses isolated in different countries, and existence of different sublineages during the year 2006. The two viruses of Malaysia and Laos formed one sublineage, whereas the viruses of India, Iran, Iraq, Italy, Nigeria and Egypt formed the second sublineage (Table 1, Figures 3 and 4). In a recent study[9], existence of multiple sublineages has been reported among the H5N1 viruses isolated in Asia during 2003–05.

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