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A novel reassortant avian influenza H4N6 virus isolated from an environmental sample during a surveillance in Maharashtra, India

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***Background & objectives*: Low pathogenic avian influenza (LPAI) viruses cause mild clinical illness in domestic birds. Migratory birds are a known reservoir for all subtypes of avian influenza (AI) viruses. The objective of the study was to characterize AI H4N6 virus isolated from an environmental sample during surveillance in Maharashtra, India.**

***Methods*: AI surveillance in wild migratory birds was conducted during the winter migratory bird season (2016-2017) in Pune, India. AI H4N6 virus was isolated from the faecal droppings of a wild migratory waterbird. Virological and molecular characterization of the isolated virus was carried out. Virus titration, haemagglutination inhibition assay, receptor specificity assay, intravenous pathogenicity index and neuraminidase inhibition assays were performed. Full genome sequencing, molecular and phylogenetic analyses were also conducted.**

***Results*: The virus was found to be of low pathogenicity, with avian type receptor specificity, and was susceptible to neuraminidase inhibitors. Phylogenetic and molecular analysis revealed that the present virus is a result of extensive reassortment with AI H8N4, H6N2, H4N3 and H3N6, predominantly as donor viruses among others.**

***Interpretation & conclusions*: This is the first report of the isolation and characterization of an LPAI H4N6 virus from an environmental sample from India. The present study showed that the H4N6 virus is a novel reassortant and divergent as compared with the reported H4N6 viruses from poultry in India, indicating independent introduction. This highlights the role of wild and migratory birds in the transmission of AI viruses and necessity of such studies at the human-animal interface.**

Key words Avian influenza - birds - environment - H4N6 - phylogeny - reassortment

Avian influenza (AI) viruses are a highly heterogeneous group with varying pathogenicity in different species[1]. AI viruses are classified into two forms based on the severity of the illness caused in poultry. Low pathogenic AI (LPAI) viruses cause mild clinical symptoms whereas highly pathogenic AI (HPAI) viruses cause severe respiratory illness and death among infected chickens[1]. The global circulation of AI viruses is a major concern due to their ability to acquire high pathogenicity from low-pathogenic strains and/or reassortment with each other to form novel AI viruses with varying pathogenicity[2]. AI viruses can

also emerge as HPAI viruses from an LPAI virus even in the absence of reassortment[3].

Besides their natural host reservoirs such as wild birds, waterfowl, gulls and shorebirds, AI viruses also circulate in poultry[4]. In addition to posing a threat to human health, LPAI outbreaks lead to severe economic losses to the poultry industry. There have been several reports of the LPAI H4N6 from poultry and wild ducks across the world[5-7]. Genetically, divergent reassortant H4N6 viruses have been reported as a result of intermixing among different AI subtypes[8]. Isolations of AI H4N6 and other mammalian adapted H4 viruses of the avian lineage from pigs have been reported from the USA, China and Canada[9]. These viruses possessed substitutions in the HA protein which enabled adaptation to mammalian type sialic acid receptors (alpha-2,6-linked sialic acid). There have been speculations about the potential for cross-species transmission of H4 viruses on the basis of two amino acid substitutions each in HA and PB2 proteins[10]. There have also been reports of the seroprevalence of antibodies against AI H4 subtypes in poultry workers[11]. Liang *et al*[12] reported that in addition to possessing dual receptor binding specificity, AI H4 viruses also possessed limited airborne transmissibility, suggesting the potential threat posed to public health.

In India, apart from outbreaks of HPAI, LPAI viruses such as H4N6, H9N2 and H11N1 in poultry and wild migratory birds have also been reported[13-15]. Wild migratory birds congregate at different water bodies, and several sites have been recognized for their arrival. The winter migratory bird season in India is from October to March. The first report of LPAI H4N6 in India was in the year 2010 from domestic chickens and ducks[15], however, not from wild birds. So far, there are no reports of the isolation of an H4N6 virus from environmental samples from India. The present study reports the isolation, virology and molecular characterization of a reassortant AI H4N6 virus from India.

Material & Methods

Collection of specimens and virus isolation: AI surveillance was undertaken during the winter migratory bird season spanning from December 2016 to March 2017 in Pune district, Maharashtra, India. Water bodies such as backwater of dams, lake and reservoirs which are known to host wild and migratory birds. The surveillance sites having water bodies included Bhigwan, Kumbhargaon, Dhumalwadi, Pimpri and Lonand (18.1758° N 74.4516° E, 18.2677° N 74.7864° E, 17.8756° N 74.4767° E, 18.6298° N 73.7997° E and 18.0417° N 74.1862° E, respectively). A total of 222 environmental samples (213 droppings + nine water samples) were collected in Viral Transport Medium (VTM; 2 mL) containing antibiotics (penicillin - 2000 U/mL, streptomycin - 0.2 mg/mL, gentamycin - 0.25 mg/mL). *p*H of the droppings was recorded using *p*H indicator strips. The samples were transported to the laboratory in cold-chain. They were processed further in the laboratory and all the samples were inoculated in ten-day-old embryonated white-leghorn clean chicken eggs (Venkateshwara Hatcheries Ltd., Pune) as per the protocol mentioned previously[16]. Embryonated chicken eggs are preferred for virus isolation as most of the AI viruses grow readily in these[17]. Briefly, the inoculated eggs were incubated at 37°C for 72 h with daily observation. The allantoic fluids from all the inoculated eggs were harvested irrespective of the mortality, and haemagglutination assay performed using 0.5 per cent Turkey red blood cells as per the WHO protocol[17]. The allantoic fluids showing HA titre <2 were considered negative.

Haemagglutination and neuraminidase subtyping: For identification of the HA subtype, haemagglutination inhibition assay was performed[17] (WHO, 2002). Viral RNA was extracted using QIAamp viral RNA mini kit (Qiagen, Germany) according to manufacturer's instructions, and one-step reverse transcription-polymerase chain reaction (RT-PCR) was performed using the NA diagnostic primers to identify the NA subtype[18].

Virus titration: The 50 per cent egg infectious dose (EID_{50}) titration was performed as per the method mentioned by Klimov *et al*[19]. Briefly, ten-fold serial dilutions of the virus isolate were performed in PBS and inoculated in four eggs per dilution. The eggs were incubated for 72 h, allantoic fluids were harvested and haemagglutination assay titres were determined. The EID_{50} titres were then calculated using the Reed and Muench 50 per cent endpoint determination method.

Sialidase assay: The sialidase assay was performed to determine the receptor-binding specificity of the viruses as described previously[20]. Briefly, 50 µl of one per cent goose red blood cell suspension prepared in PBS was treated with 1.25 units of alpha 2,3-sialidase enzyme (Takara Bio Inc., Japan) for 1 h at 37°C. These

sialidase enzyme-treated RBCs were then used in HA assay using standard protocol[17].

Intravenous pathogenicity index: The Intravenous Pathogenicity Index (IVPI) was determined. Briefly, 5-6-wk-old white-leg-horn chickens (n=10) were infected by intravenous route with 0.2 ml of 1:10 diluted virus allantoic fluid containing $10^{3.33}$ EID_{50} virus. The chickens were housed in an animal isolator (Montair Process Technology, The Netherlands). The control chickens (n=2) received 0.2 ml PBS and were housed separately. Chickens were observed daily for clinical signs and symptoms for 10 days. Faecal droppings were collected and were processed for virus isolation in 10-day-old embryonated chicken eggs. Intravenous pathogenicity index was determined as described in the WHO Manual on Animal Influenza Diagnosis and Surveillance[17]. All experiments were approved by the Institutional Animal Ethics Committee (IAEC).

Fluorescence-based neuraminidase inhibitor assay: The susceptibility to NA inhibitors oseltamivir and zanamivir was determined using the fluorescence-based neuraminidase inhibition (NAI) assay[21]. The NAIs of oseltamivir carboxylate and zanamivir were used in the assay. The viruses, namely A/Fukui/20/2004 wild type H3N2 and A/Fukui/45/2004 (H3N2) 119V mutant (kindly provided by the WHO Collaborating Center for Reference and Research on Influenza, Melbourne, Australia) were used as reference standards for susceptibility and resistance, respectively, since no other reference viruses were available for the NA subtype N6. The plots and the IC_{50} values were calculated for the sample and standards by using the curve fitting software JASPER (CDC, USA).

One-step reverse transcription-polymerase chain reaction (RT-PCR) and full genome sequencing: All the eight gene segments were amplified by one-step RT-PCR using SuperScript III Platinum one-step RT-PCR system (Invitrogen, USA) reagents and specific primers. Full genome sequencing was carried out using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The sequences were determined using an automated 3130 XL Genetic Analyzer (Applied Biosystems, USA). Nucleotide sequences were assembled and aligned using 'SeqScape' software v 2.5.0 (Applied Biosystems, USA) and edited using 'BioEdit' (v 7.0.9.1, CDC, Atlanta, USA). The sequences of all the eight gene segments have been submitted to GenBank (accession numbers 'MK453333 to MK453340').

Phylogenetic and molecular analysis: Since the isolate belonged to the subtype H4N6, the dataset for gene-wise phylogenetic analysis was assembled by retrieving the available sequences of H4N6 viruses from the Global Initiative on Sharing AI Data (GISAID) EpiFlu database[22]. Furthermore, to determine the strains which had closest identity with the present isolate BLAST was performed, and sequences with higher percentage nucleotide identities were included in the study (Table I). Multiple sequence alignment and construction of phylogenetic trees were performed using MEGA v5.05. Molecular analysis was performed using 'BioEdit' (v 7.0.9.1, CDC, Atlanta, USA).

Results

Specimen collection, virus isolation and haemagglutination assay: Among a total of 24 wild bird species which were observed, nine were migratory, ten resident and five resident-migratory bird species (Table II). The *p*H of the droppings ranged between 6.5 to 8.0. No mortality was observed in any of the inoculated eggs. Out of 222 harvested allantoic fluids tested in HA, virus was isolated from one faecal dropping of a water bird, which showed an HA titre of 32 HAU. All the other allantoic fluids were negative in HA assay.

Haemagglutinin and neuraminidase subtyping: The virus subtype was identified as H4N6 based on haemagglutination inhibition assay and NA diagnostic RT-PCR. The isolated H4N6 virus was assigned the nomenclature: 'A/migratory bird/India/1722760/2017 (H4N6)'; hereafter referred to as 'H4N6-1722760'.

Virus titration, sialidase assay, intravenous pathogenicity index and neuraminidase inhibition assay: EID_{50} titre of H4N6-1722760 was $10^{4.33}$/0.2 ml. Sialidase assay revealed avian type receptor specificity. The IVPI score of the infected chickens was 0.00 indicating low-pathogenicity. Faecal droppings of infected chickens were tested at the end of the experiment on day 10 and were positive for virus isolation. H4N6-1722760 was susceptible to both oseltamivir carboxylate and zanamivir with IC_{50} values 0.71 and 0.03 nM, respectively.

Table I. List of birds observed during surveillance

Common name	Family	Scientific name	Status (migratory/resident/RM)
Bar-headed goose	*Anatidae*	*Anser indicus*	Migratory
Ruddy shelduck	*Anatidae*	*Tadorna ferruginea*	Migratory
Northern shoveller	*Anatidae*	*Anas clypeata*	Migratory
Indian spot-billed duck	*Anatidae*	*Anas poecilorhyncha*	Resident
Eurasian wigeon	*Anatidae*	*Anas penelope*	Migratory
Little egret	*Ardeidae*	*Egretta garzetta*	Resident
Grey heron	*Ardeidae*	*Ardea cinerea*	Resident
Cattle egret	*Ardeidae*	*Bubulcus ibis*	Resident
Intermediate egret	*Ardeidae*	*Egretta intemedia*	Resident
Red-wattled lapwing	*Charadriidae*	*Vanellus indicus*	Resident
Painted stork	*Ciconiidae*	*Mycteria leucocephalus*	RM
Asian openbill	*Ciconiidae*	*Anastomus oscitans*	Resident
Brown-headed gull	*Laridae*	*Larus brunnicephalus*	Migratory
Pallas's gull	*Laridae*	*Larus ichthyaetus*	Migratory
Western yellow wagtail	*Motacilidae*	*Motacilla flava*	Migratory
Little cormorant	*Phalacrocoracidae*	*Phalacrocorax niger*	Resident
Greater flamingo	*Phoenicopteridae*	*Phoenicopterus ruber*	RM
Black-winged stilt	*Recurvirostridae*	*Himantopus himantopus*	RM
Little stint	*Scolopacidae*	*Calidris minuta*	Migratory
Western black-tailed godwit	*Scolopacidae*	*Limosa limosa*	Migratory
Whiskered tern	*Sternidae*	*Chlidonias hybrida*	RM
Indian river tern	*Sternidae*	*Sterna aurantia*	Resident
Glossy ibis	*Threskiornithidae*	*Plegadis falcinellus*	Resident
Eurasian spoonbill	*Threskiornithidae*	*Platalea leucorodia*	RM

RM, resident migratory

Molecular analysis: The sequences of H4N6-1722760 were analysed for their genetic characteristics. All the gene segments were screened for known molecular markers. There was an absence of multibasic aminoacids at the HA cleavage site, indicating that it was an LPAI virus. The HA sequence had 226Q and 228G (H3 numbering) at the receptor-binding region, implying avian virus-like receptor specificity (sialic acid -2,3-NeuAcGal)[23]. No amino acid deletion in the NA stalk region was observed. These results corroborated findings of the sialidase assay and intravenous pathogenicity index (IVPI). The avian-associated residues 158E, 271T, 590G, 591Q, 627E and 701D were observed in PB2. The substitutions in the M2 protein which confer amantadine resistance were absent. H4N6-1722760 showed that a full-length PB1-F2 protein of 90 amino acids, wherein *N66S* mutation, known to increase the virulence of influenza A virus by inhibiting the early interferon response, was present[24]. The NS1 protein had an alanine (A) residue at position 149, which is known to enable antagonization of interferon induction in chick embryo fibroblast cells[25]. At the C-terminal of the NS1 protein, the ESEV motif was observed. The presence of this motif is known to cause increased virulence and pathogenicity in experimentally infected mice, similar to that reported in HPAI H5N1 viruses[26]. However, pathogenicity in the mammalian model was not tested.

The per cent nucleotide identity (PNI) of the isolated strain H4N6-1722760 with representative H4N6 strains isolated from poultry in India in the year 2010 was compared. The *PB2, PB1, PA, NP, NA* and *M* genes ranged between 92 and 97 per cent. The PNIs of the *HA* genes of the three previous H4N6 isolates with H4N6-1722760 were 84 per cent. The PNIs of the *NS* genes of previous H4N6 isolates with H4N6-1722760

Table II. BLAST (Basic Local Alignment Search Tool) results for H4N6-1722760

Gene	Virus	Accession number	Subtype	PNI
PB2	A/mallard/Chany/126K-2/2014	EPI1388622	H8N4	98
	A/mallard/Chany/126K/2014	EPI884262	H5N3	98
	A/mallard duck/Netherlands/56/2015	EPI1306909	H3N2	98
PB1	A/duck/Hubei/ZYSYG3/2015	EPI942215	H6N2	98
	A/northern shoveler/Egypt/MB-D-695C/2016	EPI1581322	H7N3	98
	A/mute swan/Croatia/102/2016	EPI873617	H5N5	98
PA	A/duck/Bangladesh/31227/2016	EPI1099011	H6N2	98
	A/black-necked crane/Zhaotong/ZT-12/2013	EPI1133530	H1N2	98
	A/duck/Hokkaido/X9/2016	EPI1510517	H8N4	98
HA	A/duck/Mongolia/17/2011	EPI1153577	H4N3	96
	A/duck/Mongolia/118/2015	EPI704283	H4N6	95
	A/duck/Bangladesh/25891/2015	EPI965331	H4N6	93
NP	A/teal/Egypt/MB-D-487OP/2016	EPI1581280	H7N3	99
	A/teal/Egypt/MB-D-621C/2016	EPI1581314	H7N9	99
	A/pintail/Egypt/MB-D-384C/2015	EPI1581278	H3N6	99
NA	A/mallard/Toguchin/19/2017	EPI1328510	H4N6	98
	A/duck/Sichuan/04.08 CDLQ033-O/2015	EPI659686	H4N6	97
	A/duck/Mongolia/146/2010	EPI1153565	H3N6	97
M	A/gadwall/Chany/315/2016	EPI925991	H1N1	98
	A/mallard/Chany/126K-2/2014	EPI1388625	H8N4	98
	A/northern shoveler/Egypt/MB-D-695C/2016	EPI1581326	H7N3	98
NS	A/duck/Hubei/ZYSYG8/2015	EPI942277	H6N2	99
	A/duck/Mongolia/499/2015	EPI704567	H10N7	99
	A/duck/Mongolia/278/2011	EPI1134008	H3N8	99

PNI scores obtained from GISAID BLAST. Representative sequences of H4N6 viruses, as well as those of other subtypes sharing maximum identity with H4N6-1722760. Multiple nucleotides and amino acid sequence alignments for all the eight gene segments were performed using MEGA (v5.05). Top three representative hits showing maximum identity have been included. PNI, per cent nucleotide identity, GISAID, Global Initiative on Sharing Avian Influenza Data

were 74 per cent for chicken isolates and 96 per cent for the duck isolate (Table III).

Phylogenetic analysis: Phylogenetic trees for all the eight gene segments revealed that H4N6-1722760 belonged to the Eurasian lineage (Fig. 1). The HA of the present virus showed divergence from the HA genes with the maximum per cent nucleotide identity being 96 per cent for A/duck/Mongolia/17/2011(H4N3); and 95 and 93 per cent for H4N6 viruses A/duck/Mongolia/118/2015 and A/duck/Bangladesh/25891/2015, respectively (Fig. 1A). For the NA gene, H4N6-1722760 bore closest resemblance to N6 viruses from Mongolia, Sichuan province in China and from Toguchin, Russia (Fig. 1B). In the phylogenetic tree of PB2 gene, H4N6-1722760 closely grouped with the PB2 of H8N4 and H5N3 viruses from Chany, Russia and an H3N2 virus from the Netherlands (Fig. 1C), whereas for PB1, the closest isolates were those from Hubei (H6N2), Egypt (H7N3) and an HPAI H5N5 virus from Croatia (H5N5) (Fig. 1D). In the phylogenetic tree of the *PA* gene, H4N6-1722760 was clustered with the viruses from Bangladesh (H6N2), China (H1N2) and Japan (H8N4) (Fig. 1E). For *NP*, H4N6-1722760 closely grouped with H7N3 and H7N9 viruses from Egypt and an HPAI H5N8 virus from Aghakhan, Iran (Fig. 1F). For the *M* gene, H4N6-1722760 was closely related to H8N4 and H1N1 viruses from Chany, Russia and an H7N3 virus from Egypt (Fig. 1G). For the *NS* gene, H4N6-1722760 grouped in the allele B (Fig. 1H), usually encountered in avian viruses, with resemblance to an H6N2 virus from Hubei, China, and

Table III. Per cent nucleotide identities of H4N6-1722760 with AI H4N6 viruses previously isolated from India

Gene	Virus	Accession number	PNI
PB2	A/Duck/India/10736/2009	MK453341.1	97
	A/chicken/India/101006/2009	MK453347.1	93
	A/chicken/India/101018/2009	MK453354.1	93
PB1	A/Duck/India/10736/2009	MK453342.1	94
	A/chicken/India/101006/2009	MK453348.1	95
	A/chicken/India/101018/2009	MK453355.1	95
PA	A/Duck/India/10736/2009	MK453343.1	94
	A/chicken/India/101006/2009	MK453349.1	96
	A/chicken/India/101018/2009	MK453356.1	96
HA	A/Duck/India/10736/2009	JX310059.1	84
	A/chicken/India/101006/2009	JX310061.1	84
	A/chicken/India/101018/2009	JX310062.1	84
NP	A/Duck/India/10736/2009	MK453344.1	94
	A/chicken/India/101006/2009	MK453350.1	92
	A/chicken/India/101018/2009	MK453357.1	92
NA	A/Duck/India/10736/2009	JX310060.1	96
	A/chicken/India/101006/2009	JX310063.1	96
	A/chicken/India/101018/2009	JX310064.1	95
M	A/Duck/India/10736/2009	MK453345.1	97
	A/chicken/India/101006/2009	MK453351.1	97
	A/chicken/India/101018/2009	MK453358.1	97
NS	A/Duck/India/10736/2009	MK453346.1	96
	A/chicken/India/101006/2009	MK453352.1	74
	A/chicken/India/101018/2009	MK453359.1	74

PNI scores obtained using NCBI BLAST. PNI, per cent nucleotide identity; NCBI, National Center for Biotechnology Information

H10N7 and H3N8 viruses from Mongolia. It has been shown experimentally that although its introduction in mammalian hosts is rare, allele B does not attenuate viral replication[27]. Hence, the possibility of infection in mammals cannot be ruled out.

Phylogenetic analysis and high per cent nucleotide identities with viruses of varied subtypes indicated that the present virus is a result of complex and extensive reassortment events, as illustrated in Fig. 2. The *PB2* and *M* genes of H4N6-1722760 were contributed by A/mallard/Chany/126K-2/2014(H8N4)-like viruses; *PB1, PA* and *NS* genes by A/duck/Hubei/ZYSYG3/2015(H6N2)-like; *HA* gene by A/duck/Mongolia/17/2011(H4N3)-like; *NP* gene by A/pintail/Egypt/MB-D-384C/2015(H3N6)-like and *NA* gene by A/duck/Sichuan/04.08 CDLQ033-O/2015(H4N6)-like viruses.

Discussion

So far, there are no reports of isolation of H4N6 virus from the environmental samples from wild migratory birds in India, although LPAI H4N6 has been isolated from domestic poultry in the year 2010[15]. The environmental sample from which the present virus was isolated was from the vicinity of a water body where mixed flocks of migratory water birds were present. It has been reported that the rates of isolation of AI viruses from environmental samples are low[12,13,24,25]. The isolated virus was a novel reassortant and phylogenetically divergent than the earlier H4N6 viruses from India. AI H4N6 virus isolated from India in the year 2010 belongs to the Eurasian lineage[13]. Their *PB2, PB1, PA, NP, NA* and *M* genes showed high PNIs with H4N6-1722769, indicating similarity and/or common ancestry. However, the PNIs of *HA* for the previous isolates were low, and they formed a separate group as compared to

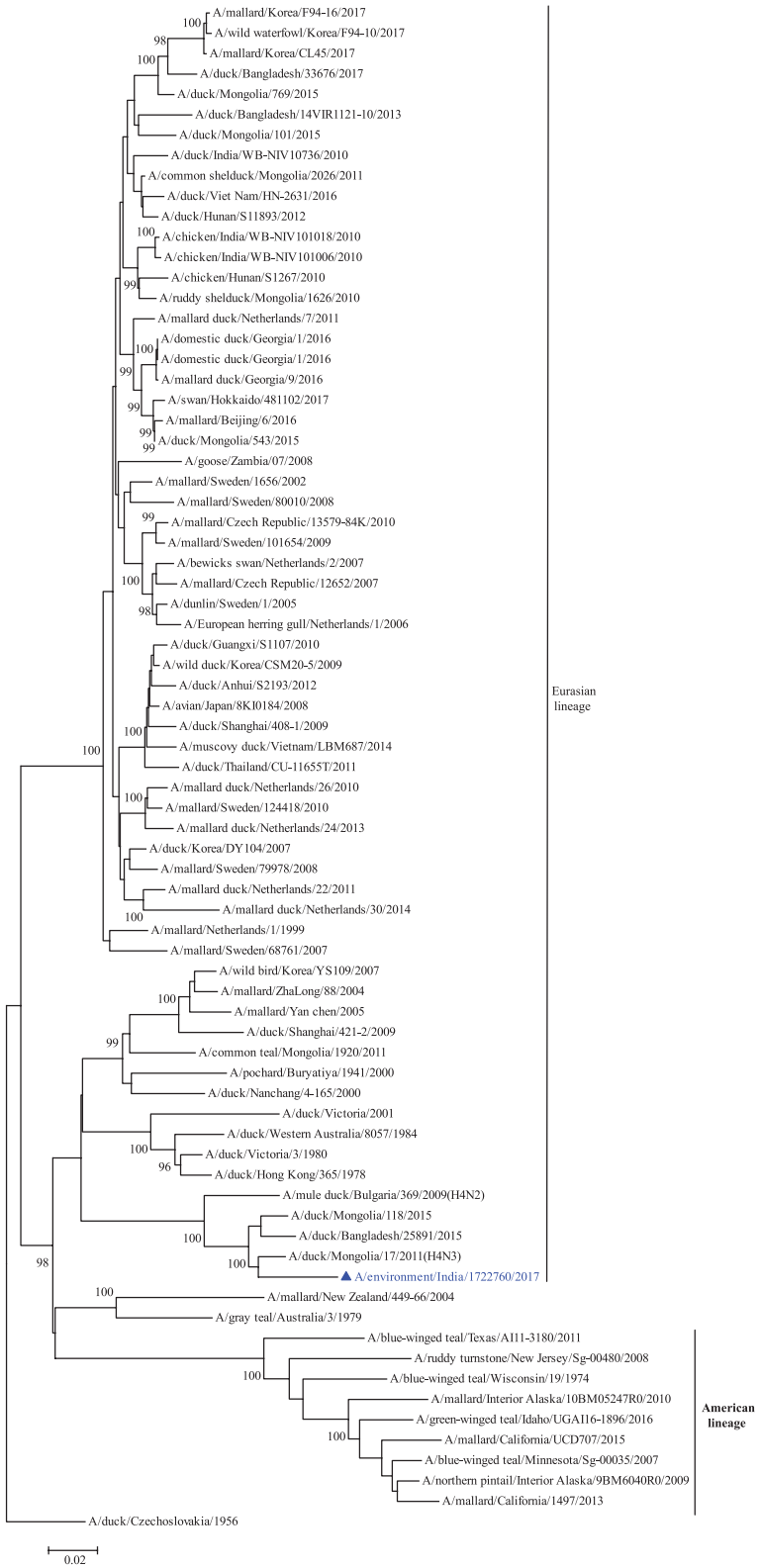

Fig. 1. Phylogenetic trees for all the eight gene segments of H4N6-1722760 constructed using the neighbour-joining method with the Kimura 2-parameter distance model and 1000 bootstrap replicates in MEGA v5.05. A/migratory bird/India/1722760/2017 (H4N6) is shown in blue with a triangle. (**A**) *HA* gene phylogenetic tree rooted to the virus A/duck/Czechoslovakia/1956, the Eurasian and American lineages have been labelled.

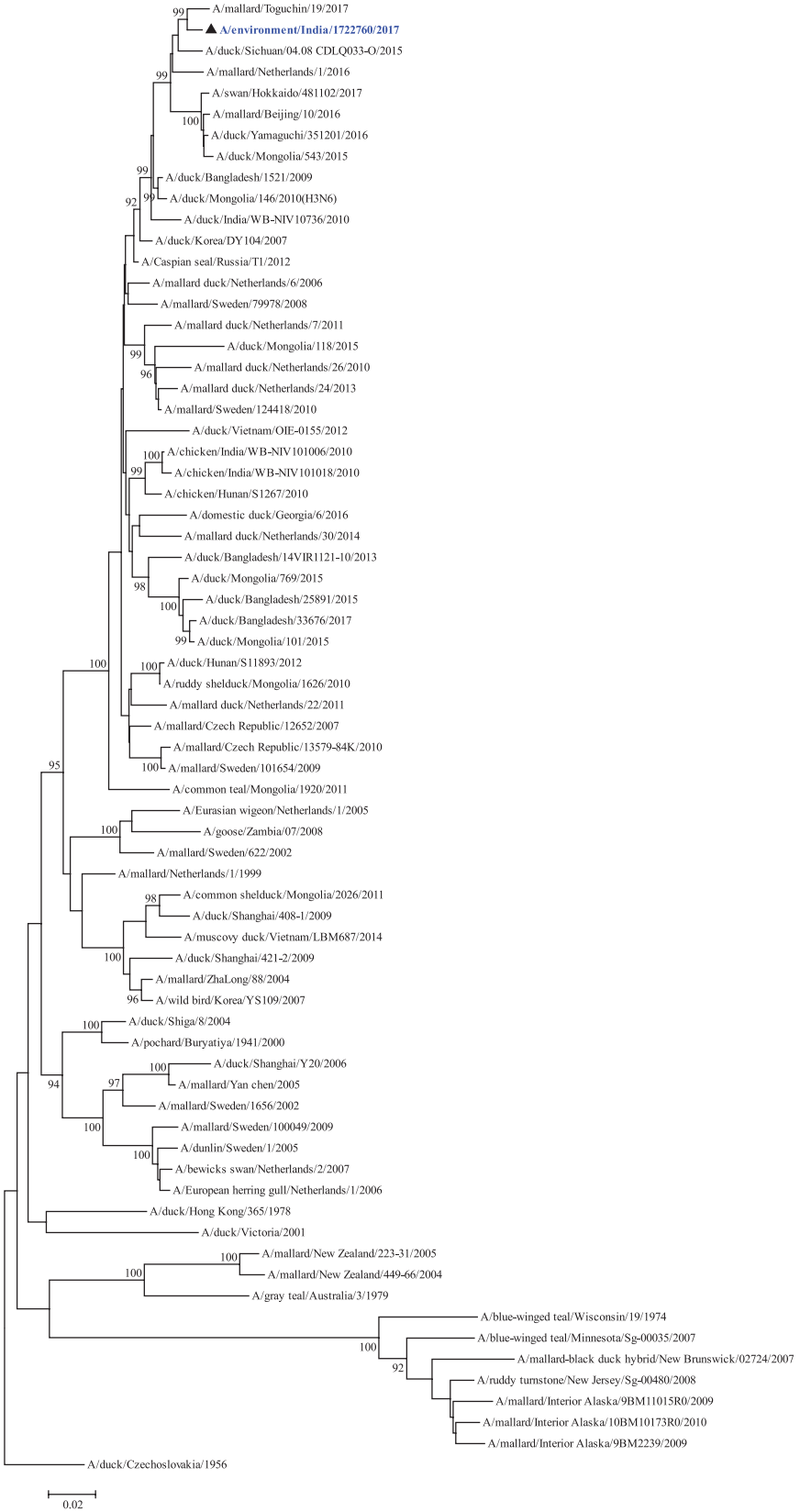

Fig. 1. (**B**) *NA* gene phylogenetic tree rooted to A/duck/Czechoslovakia/1956.

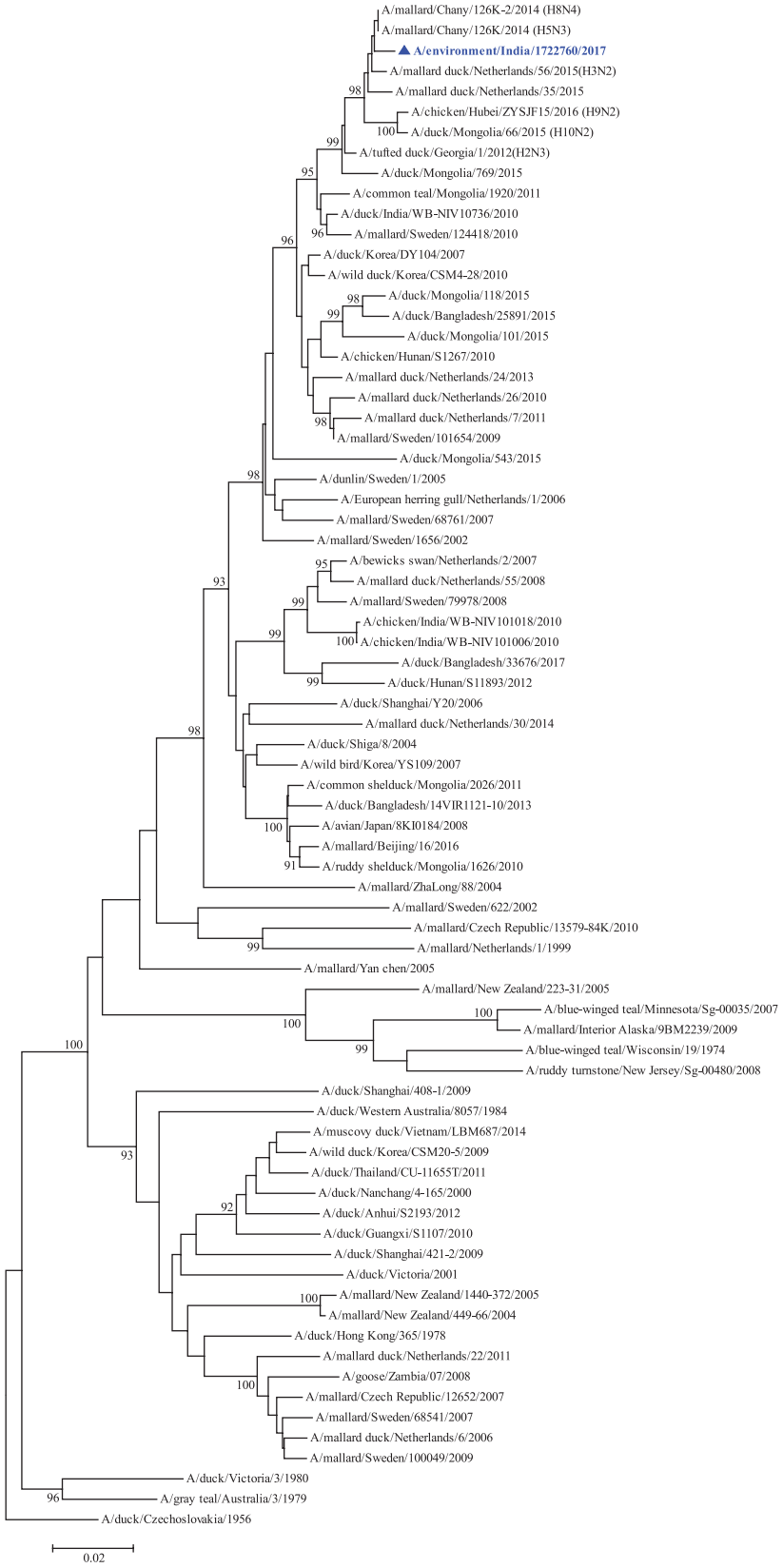

Fig. 1. (**C**) *PB2* gene phylogenetic tree rooted to A/duck/Czechoslovakia/1956.

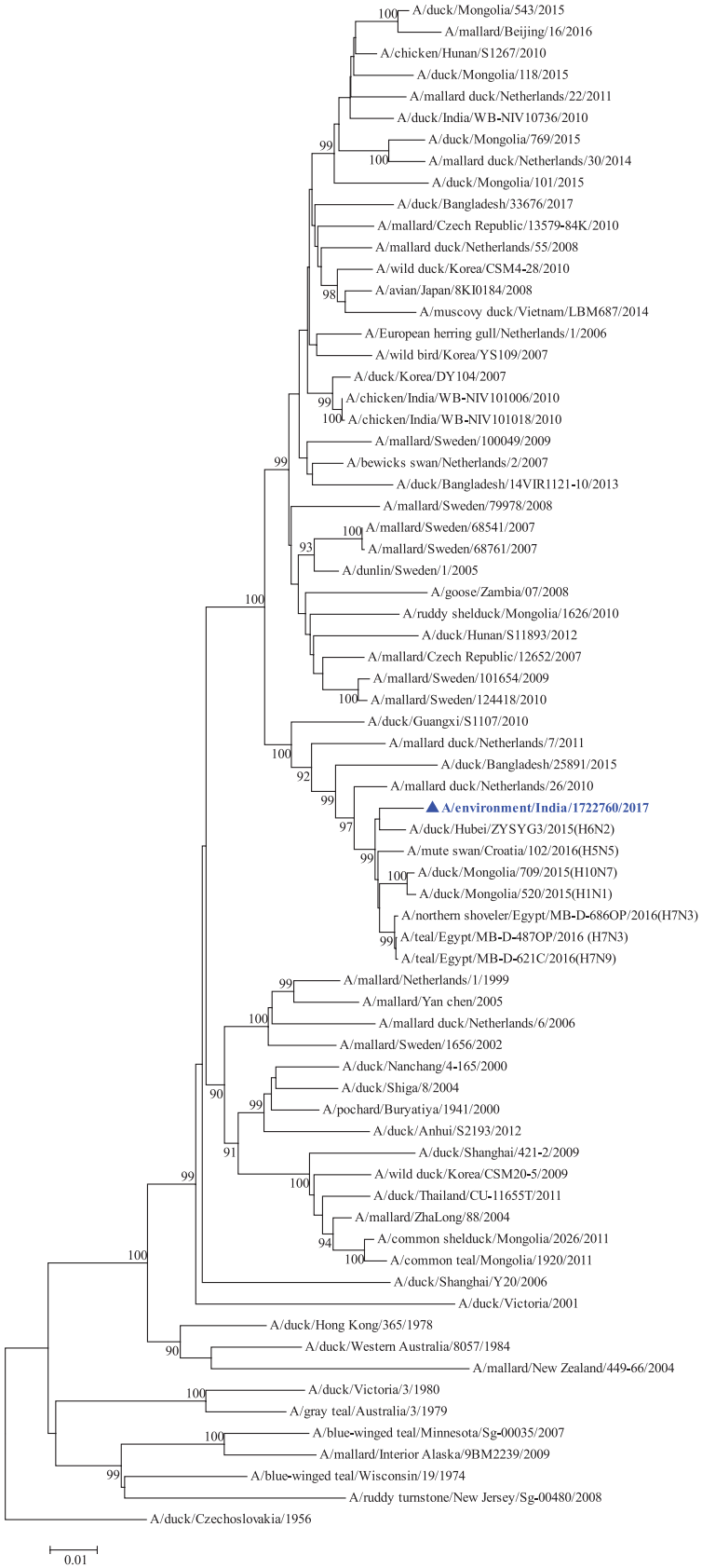

Fig. 1. (**D**) PB1 gene phylogenetic tree rooted to A/duck/Czechoslovakia/1956.

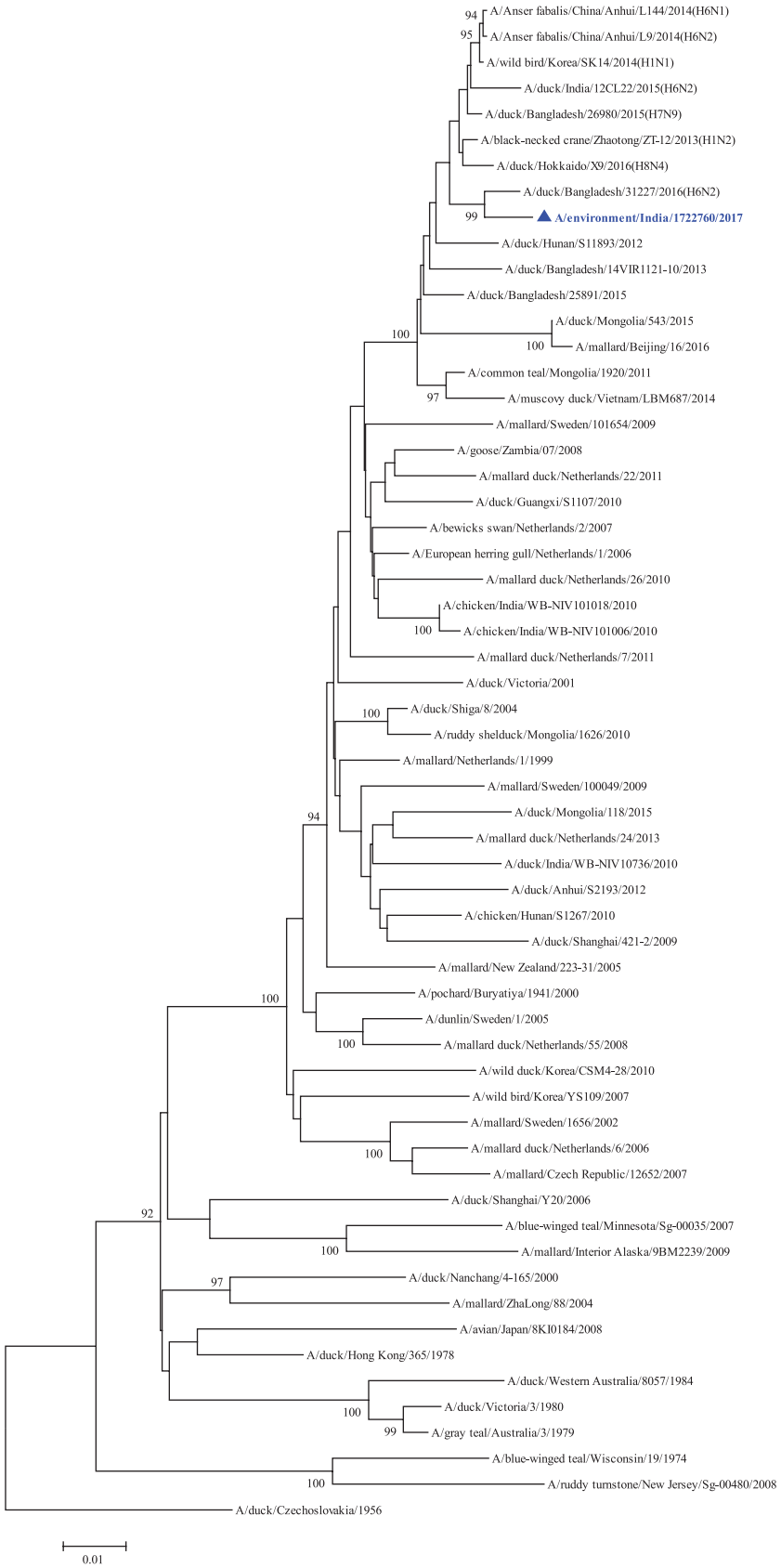

Fig. 1. (**E**) *PA* gene phylogenetic tree rooted to A/duck/Czechoslovakia/1956.

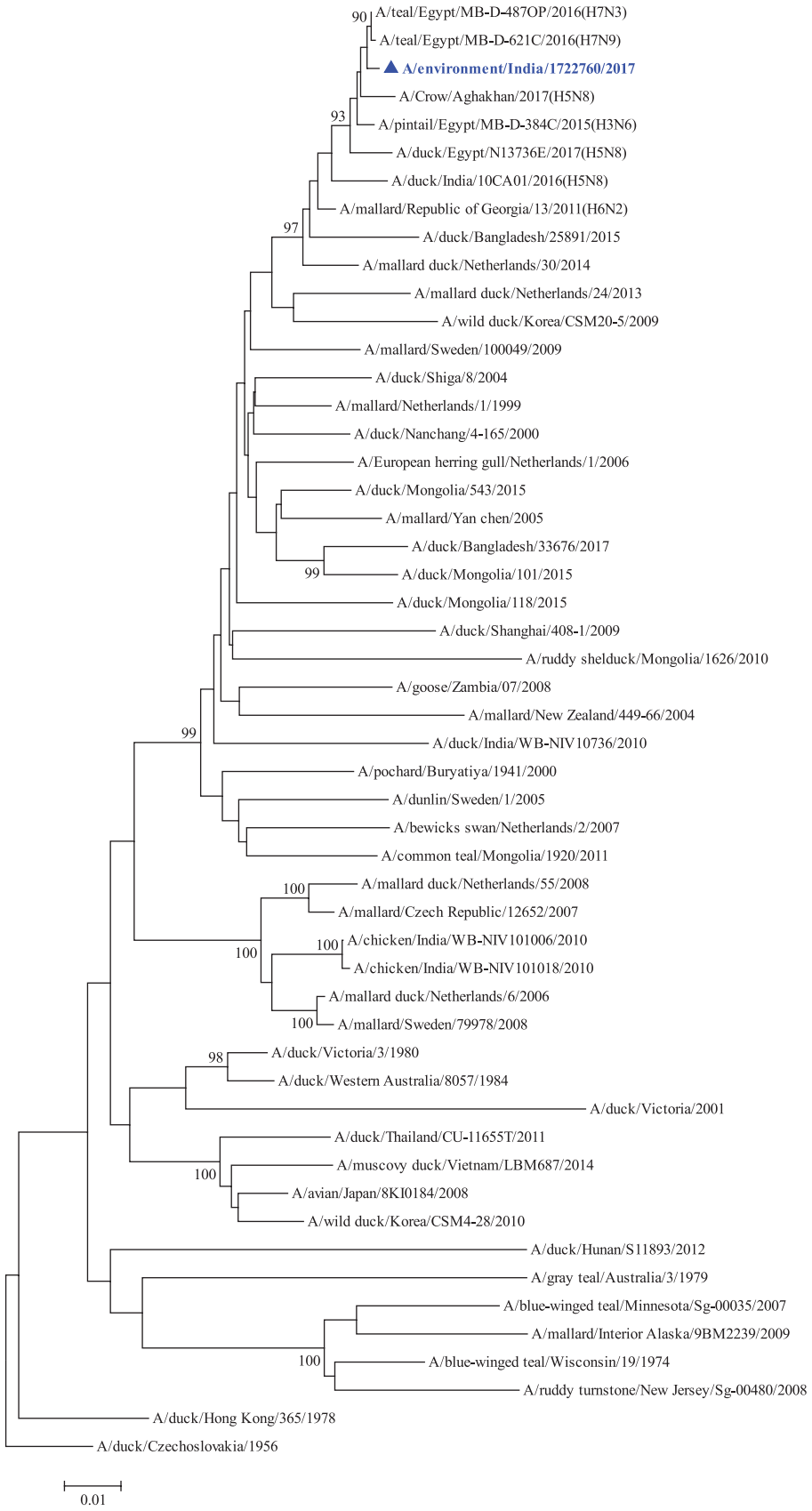

Fig. 1. (**F**) *NP* gene phylogenetic tree rooted to A/duck/Czechoslovakia/1956.

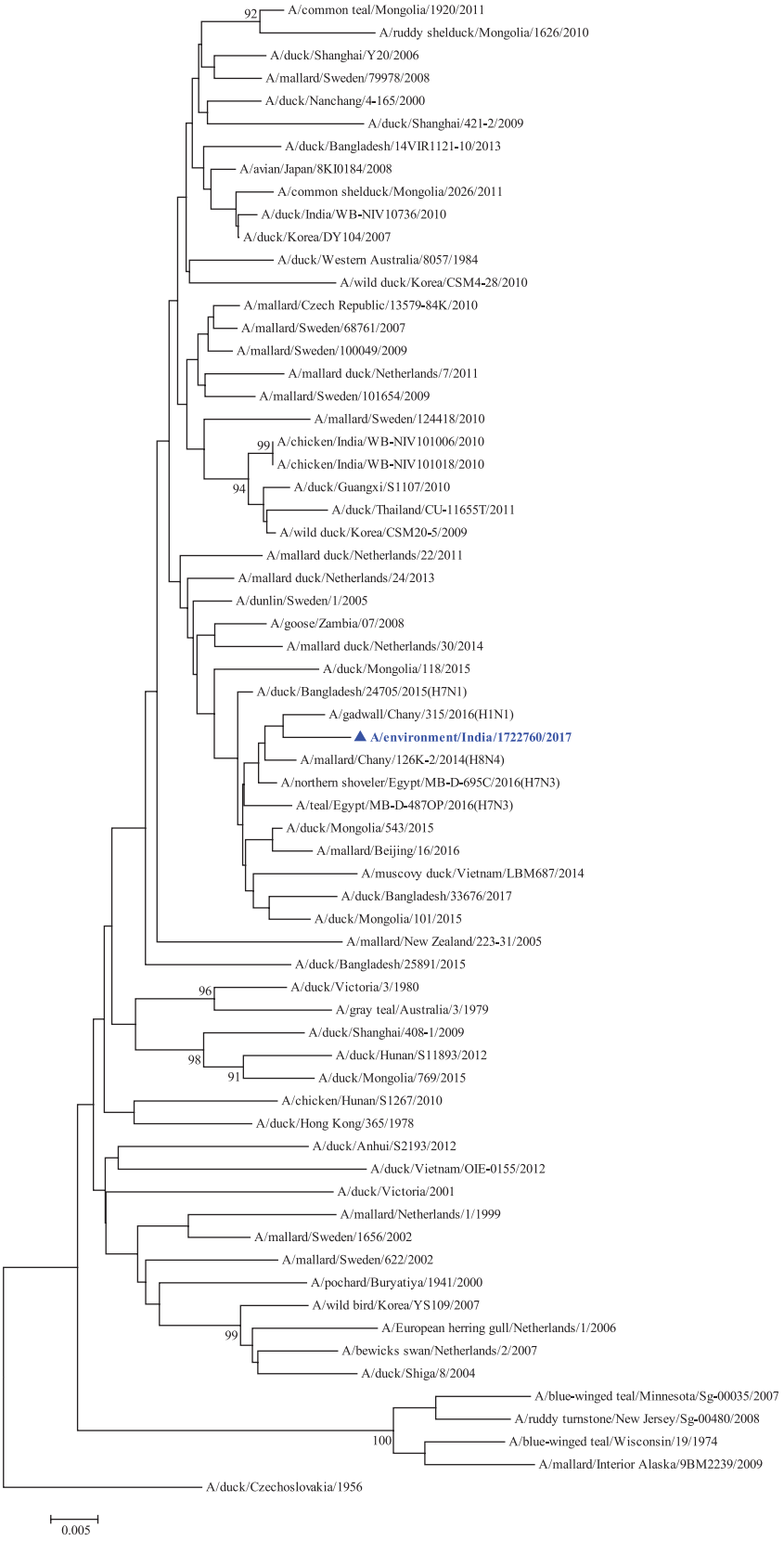

Fig. 1. (**G**) *M* gene phylogenetic tree rooted to A/duck/Czechoslovakia/1956.

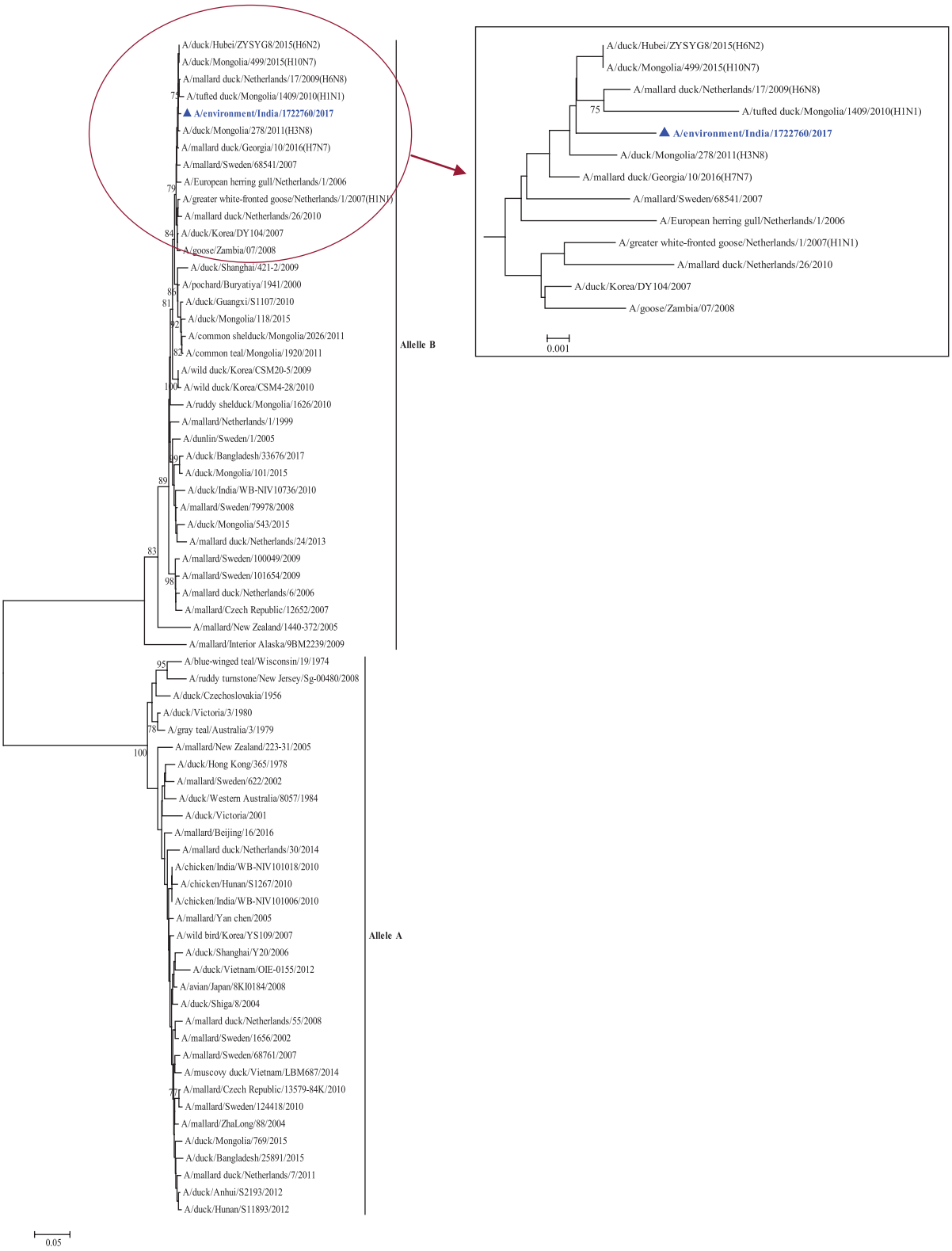

Fig. 1. (**H**) *NS* gene phylogenetic tree, the two alleles *A* and *B* have been labelled, the cluster containing A/migratory bird/India/1722760/2017 (H4N6) has been enlarged in the inset.

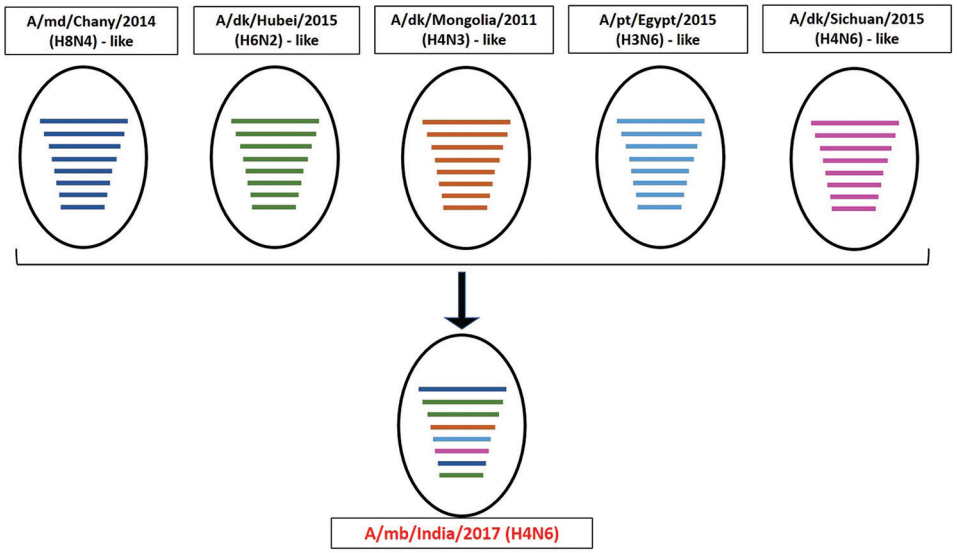

Fig. 2. Schematic diagram for proposed origin of the reassortant H4N6-1722760. The proposed genetic constellation of H4N6-1722760 has been represented schematically, depicting the genesis of the reassortant. The abbreviations md, dk and pt stand for mallard, duck and pintail, respectively. A/mb/India/2017 (A/migratory bird/India/1722760/2017) represents the reassortant virus in the present study. The circles represent virus strains, whereas coloured horizontal lines represent the individual gene segments of the respective viruses (*PB2, PB1, PA, HA, NP, NA, MP, NS* genes). The virus A/mallard/Chany/126K-2/2014 (H8N4) contributed *PB2* and *M* genes (represented in blue), A/duck/Hubei/ZYSYG3/2015 (H6N2) contributed *PB1, PA* and *NS* genes (represented in green), A/duck/Mongolia/17/2011 (H4N3) contributed the *HA* gene (represented in light brown), A/pintail/Egypt/MB-D-384C/2015 (H3N6), *NP* gene (represented in sky blue) and A/duck/Sichuan/04.08 CDLQ033-O/2015 (H4N6) contributed the *NA* gene (represented in pink).

H4N6-1722760 in the phylogenetic trees indicating divergence (Table III and Fig. 1A). For the *NS* genes, the PNIs of the two chicken isolates were low and they belonged to the allele A, also indicating divergence. Interestingly, the PNI of the *NS* gene from the duck isolate, A/Duck/India/10736/2009, showed high similarity with H4N6-1722760 and grouped within the same allele (allele *B*), this indicated common ancestory for the *NS* gene (Table III and Fig. 1A). Previous reports of H4N6 virus from across the globe indicate isolation from ducks such as Teals, Mallard and Muscovy, and even from pigs[9]. It has been shown that the *p*H of the faecal droppings may affect virus isolation and that acidic *p*H has detrimental impact on AI virus isolation[16]. The *p*H of droppings shows variation among different bird species based on diet. It has been experimentally shown that acidic *p*H hampers virus isolation. The *p*H of the dropping from which the virus was isolated was basic (*p*H 8.0) favouring survival of the virus in faecal dropping enabling virus isolation.

The close relatedness of H4N6-1722760 with viruses from Asia and Europe indicated the probable role of migratory birds in its transmission since India lies in the central Asian flyway, which facilitates intermixing of different migratory bird species. Notably, ringing and tracking studies conducted on Brown-headed gulls have shown that the birds migrate from their breeding grounds in China to overwinter in Thailand and Cambodia; with Bangladesh, India, Myanmar and Vietnam as stopover sites[28]. In addition, ring recovery studies report a direct migratory connection between India and Russia and between India and Europe[29]. During the present surveillance activity, migratory, resident, and also resident migratory bird species were observed at the study site. This assertively indicates intermingling of different types of bird species which might lead to the possible transmission of the virus, since it is believed that interactions between migratory birds and resident domestic poultry in close proximity could be a factor in the spread of AI viruses[30].

It has been demonstrated experimentally that avian H4 virus can adapt to mammals by point mutations in PB2 or HA[10]. Furthermore, the seroprevalence of antibodies against AI H4 viruses in poultry workers highlights a potential threat to public health[11]. In this regard, there is a dearth of extensive surveillance studies on migratory birds and serosurveillance studies among at-risk populations from India. The limitation of the present study was that the sample size was small.

In conclusion, the LPAI H4N6 virus isolated from Maharashtra, India, was found to be a

reassortant and of low pathogenicity. Similarity of the virus with other Eurasian viruses signifies the possible role of wild and migratory birds in the transmission and spread of LPAI viruses in India. Further, molecular deviation of the current H4N6 virus from the previously isolated viruses from poultry indicates the possibility of independent introduction. Since the possibility of human infections with H4 viruses cannot be ruled out, there is a need for continuous AI surveillance in wild and migratory birds along with poultry, in India to study and monitor continuing virus evolution at the animal-human interface.

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References

1. Alexander DJ. A review of avian influenza in different bird species. *Vet Microbiol* 2000; *74* : 3-13.
2. Abdelwhab el-SM, Veits J, Breithaupt A, Gohrbandt S, Ziller M, Teifke JP, *et al.* Prevalence of the C-terminal truncations of NS1 in avian influenza A viruses and effect on virulence and replication of a highly pathogenic H7N1 virus in chickens. *Virulenc*e 2016; *7* : 546-57.
3. Monne I, Fusaro A, Nelson MI, Bonfanti L, Mulatti P, Hughes J, *et al.* Emergence of a highly pathogenic avian influenza virus from a low-pathogenic progenitor. *J Virol* 2014; *88* : 4375-88.
4. Kawaoka Y, Chambers TM, Sladen WL, Webster RG. Is the gene pool of influenza viruses in shorebirds and gulls different from that in wild ducks? *Virology* 1988; *163* : 247-50.
5. Hu J, Xu X, Wang C, Bing G, Sun H, Pu J, *et al.* Isolation and characterization of H4N6 avian influenza viruses from mallard ducks in Beijing, China. *PLoS One* 2017; *12* : e0184437.
6. Kalonda A, Saasa N, Nkhoma P, Kajihara M, Sawa H, Takada A, *et al.* Avian influenza viruses detected in birds in Sub-Saharan Africa: A systematic review. *Viruses* 2020; *12* : 993.
7. Parvin R, Kabiraj CK, Mumu TT, Chowdhury EH, Islam MR, Beer M, *et al.* Active virological surveillance in backyard ducks in Bangladesh: Detection of avian influenza and gammacoronaviruses. *Avian Pathol* 2020; *49* : 361-8.
8. Khalil AM, Nishi N, Kojima I, Fukunaga W, Kuwahara M, Masatani T, *et al.* Transition in genetic constellations of H3N8 and H4N6 low-pathogenic avian influenza viruses isolated from an overwintering site in Japan throughout different winter seasons. *Arch Virol* 2020; *165* : 643-59.
9. Abente EJ, Gauger PC, Walia RR, Rajao DS, Zhang J, Harmon KM, *et al.* Detection and characterization of an H4N6 avian-lineage influenza A virus in pigs in the Midwestern United States. *Virology* 2017; *511* : 56-65.
10. Xu G, Wang F, Li Q, Bing G, Xie S, Sun S, *et al.* Mutations in PB2 and HA enhanced pathogenicity of H4N6 avian influenza virus in mice. *J Gen Virol* 2020; *101* : 910-20.
11. Kayali G, Barbour E, Dbaibo G, Tabet C, Saade M, Shaib HA, *et al.* Evidence of infection with H4 and H11 avian influenza viruses among Lebanese chicken growers. *PLoS One* 2011; *6* : e26818.
12. Liang L, Deng G, Shi J, Wang S, Zhang Q, Kong H, *et al.* Genetics, receptor binding, replication, and mammalian transmission of H4 avian influenza viruses isolated from live poultry markets in China. *J Virol* 2016; *90 :* 1455-69.
13. Chakrabarti AK, Pawar SD, Cherian SS, Koratkar SS, Jadhav SM, Pal B, *et al.* Characterization of the influenza A H5N1 viruses of the 2008-09 outbreaks in India reveals a third introduction and possible endemicity. *PLoS One* 2009; *4* : e7846.
14. Pawar S, Chakrabarti A, Cherian S, Pande S, Nanaware M, Raut S, *et al.* An avian influenza A(H11N1) virus from a wild aquatic bird revealing a unique Eurasian-American genetic reassortment. *Virus Genes* 2010; *41* : 14-22.
15. Pawar SD, Kale SD, Rawankar AS, Koratkar SS, Raut CG, Pande SA, *et al.* Avian influenza surveillance reveals presence of low pathogenic avian influenza viruses in poultry during 2009-2011 in the West Bengal State, India. *Virol J* 2012; *9* : 151.
16. Pawar SD, Pande SA, Tare DS, Keng SS, Kode SS, Singh DK, *et al.* Morphological and biochemical characteristics of avian faecal droppings and their impact on survival of avian influenza virus. *Food Environ Virol* 2018; *10* : 99-106.
17. World Health Organization. *WHO manual on animal influenza diagnosis and surveillance*. Geneva: WHO; 2002.
18. Tsukamoto K, Ashizawa T, Nakanishi K, Kaji N, Suzuki K, Shishido M, *et al.* Use of reverse transcriptase PCR to subtype N1 to N9 neuraminidase genes of avian influenza viruses. *J Clin Microbiol* 2009; *47 :* 2301-3.
19. Klimov A, Balish A, Veguilla V, Sun H, Schiffer J, Lu X, *et al.* Influenza virus titration, antigenic characterization, and serological methods for antibody detection. In: Kawaoka Y, Neumann G, editors. *Influenza virus methods in molecular biology: Methods and protocols*. New York, USA: Humana Press; 2012. p. 25-51.
20. Pawar SD, Parkhi SS, Koratkar SS, Mishra AC. Receptor specificity and erythrocyte binding preferences of avian influenza viruses isolated from India. *Virol J* 2012; *9* : 251.
21. Hurt AC, Barr IG, Hartel G, Hampson AW. Susceptibility of human influenza viruses from Australasia and South East Asia to the neuraminidase inhibitors zanamivir and oseltamivir. *Antiviral Res* 2004; *62* : 37-45.

22. Shu Y, McCauley J. GISAID: Global initiative on sharing all influenza data – From vision to reality. *Euro Surveill* 2017; *22* : 30494.

23. Shi Y, Cui H, Wang J, Chi Q, Li X, Teng Q, *et al.* Characterizations of H4 avian influenza viruses isolated from ducks in live poultry markets and farm in Shanghai. *Sci Rep* 2016; *6* : 37843.

24. Conenello GM, Tisoncik JR, Rosenzweig E, Varga ZT, Palese P, Katze MG. A single N66S mutation in the PB1-F2 protein of influenza A virus increases virulence by inhibiting the early interferon response *in vivo*. *J Virol* 2011; *85* : 652-62.

25. Li Z, Jiang Y, Jiao P, Wang A, Zhao F, Tian G, *et al.* The NS1 gene contributes to the virulence of H5N1 avian influenza viruses. *J Virol* 2006; *80 :* 11115-23.

26. Jackson D, Hossain MJ, Hickman D, Perez DR, Lamb RA. A new influenza virus virulence determinant: The NS1 protein four C-terminal residues modulate pathogenicity. *Proc Natl Acad Sci U S A* 2008; *105* : 4381-6.

27. Turnbull ML, Wise HM, Nicol MQ, Smith N, Dunfee RL, Beard PM, *et al.* Role of the B allele of influenza A virus segment 8 in setting mammalian host range and pathogenicity. *J Virol* 2016; *90* : 9263-84.

28. Ratanakorn P, Wiratsudakul A, Wiriyarat W, Eiamampai K, Farmer AH, Webster RG, *et al*. Satellite tracking on the flyways of brown-headed gulls and their potential role in the spread of highly pathogenic avian influenza H5N1 virus. *PLoS One* 2012; *7* : e49939.

29. Verhagen JH, van der Jeugd HP, Nolet BA, Slaterus R, Kharitonov SP, de Vries PP, *et al.* Wild bird surveillance around outbreaks of highly pathogenic avian influenza A(H5N8) virus in the Netherlands, 2014, within the context of global flyways. *Euro Surveill* 2015; *20* : 21069.

30. Pandit PS, Bunn DA, Pande SA, Aly SS. Test-modeling highly pathogenic avian influenza transmission in wild birds and poultry in West Bengal, India. *Sci Rep* 2013; *3* : 1-8.

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