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Molecular detection and characterization of Influenza 'C' viruses from western India

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ABSTRACT

Since 2003, India has had a well-established influenza surveillance network, though Influenza C virus was not the focus of study. We therefore retrospectively analyzed clinical samples from Pune, western India collected during January 2009 to August 2015, by real-time RT-PCR. Three of 2530 samples of patients with influenza-like illness (ILI) or severe acute respiratory illness (SARI) showed positivity for Influenza C virus infection, while 105 and 31 samples were positive for Influenza A and B viruses respectively. Influenza C viruses were successfully isolated using the embryonated egg system and whole genomes were sequenced and analyzed phylogenetically. HE gene-based phylogeny showed that two viruses C/India/P119564/2011 and C/India P121719/2012 clustered with the C/Sao Paulo/378/82 (SP82) lineage, whereas C/India/P135047/2013 clustered with the C/Kanagawa/1/76 (KA76) lineage. The internal gene of these viruses grouped in two lineages. The PB1, PB2, M and NS genes of the study viruses grouped with C/Yamagata/26/81 (YA81), while the P3 (PA) and NP genes grouped with C/Mississippi/80 (MS80). Bayesian clock studies conclude that the Indian strains may have emerged through multiple reassortment events.

1. Introduction

Influenza C virus infections normally result in mild upper respiratory disease in young children, but, it can also cause lower respiratory illness in children < 2 years leading to hospitalizations (Matsuzaki et al., 2006). Recurrent infections with this virus frequently occur in children as well as in adults and seroepidemiological studies have documented wide distribution of the virus. (Salez et al., 2014; Kaji et al., 1983; Manuguerra et al., 1994). Non-primate mammals like dogs (Manuguerra et al., 1993) and pigs (Guo et al., 1983; Kimura et al., 1997) are also shown to be infected by Influenza C viruses, and chances of human infection in spite of the cross species barrier are undeniable. Lack of a suitable cell line attributes to low isolation frequency of the virus ranging from 0.43% to 1.73% (Matsuzaki et al., 2014). The detection of Influenza C viruses using molecular methods has been reported from many countries including Finland, France, Cuba, Spain, Japan, Italy, and India (Kauppila et al., 2014; Gouarin et al., 2008; Ramos et al., 2008; Antón et al., 2011; Matsuzaki et al., 2012; Principi et al., 2013; Roy Mukherjee et al., 2013). Long term monitoring of Influenza C viruses in children in Japan revealed that winter or early summer outbreaks occur at 1 or 2-year intervals (Matsuzaki et al., 2002; Matsuzaki et al., 2003; Matsuzaki et al., 2014). On the basis of antigenic and hemagglutinin-esterase (HE) gene sequence analysis, Influenza C virus is represented by six antigenic lineages namely C/Taylor/1233/47 (Taylor47), C/Kanagawa/1/76 (KA76), C/Mississippi/80 (MS80), C/Aichi/1/81 (AI81), C/Yamagata/26/81 (YA81), and C/Sao Paulo/378/82 (SP82) (Matsuzaki et al., 2016). Except for reassortment and accumulation of point mutations as the probable means of evolution, otherwise Influenza C viruses are antigenically stable (Matsuzaki et al., 1994, 2003). Co-circulation or mixed infection of two different lineages of influenza C virus in a single host can lead to emergence of a reassortant virus with exchange of genomic segments. An early study from Japan suggests that reassortment between two different Influenza C virus strains occurs frequently in nature and the genome composition of Influenza C viruses may influence their ability to spread in humans (Peng et al., 1994).

At present > 300 complete Influenza C virus genomes are available at NCBI influenza resource (https://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi) as well as in the EpiFlu Database of the Global Initiative on Sharing All Influenza Data (GISAID) (www. gisaid.org). Recent studies based on 106 complete genomes between 1947 and 2014, determined the genetic lineages and reassortment events in Influenza C viruses and revealed that the internal genes are divided into two major lineages: C/Mississippi/80 and C/Yamagata/26/81. Multiple reassortment events occurred between 1992 and 1996 among the viruses of Mississippi and Yamagata lineages (Matsuzaki et al., 2016).

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Currently, patients diagnosed with Influenza A or B virus infections are treated with a neuraminidase inhibitor; however, this drug is ineffective against Influenza C virus infection. (Matsuzaki et al., 2006). The patient management of Influenza C virus infections necessitate accurate diagnosis based on reliable virological tests. Conventionally cell culture techniques helped in detecting certain outbreaks successfully (Matsuzaki et al., 1994, 2000, 2002). MDCK and HMV-II cells have been used for the isolation of Influenza C virus, though the rate of recovery is very low. Amniotic inoculation of embryonated hen eggs remains the most sensitive technique to isolate this virus. Recently, real-time reverse-transcriptase PCR (rRT-PCR) has proved to be a more rapid and sensitive method for laboratory-based diagnosis with the nucleoprotein (NP) or matrix (M) gene as a choice for the target (Matsuzaki et al., 2012; Pabbaraju et al., 2013).

The only study from India during 2011–12 showed a 0.18% prevalence rate for Influenza C virus (Roy Mukherjee et al., 2013). Whole

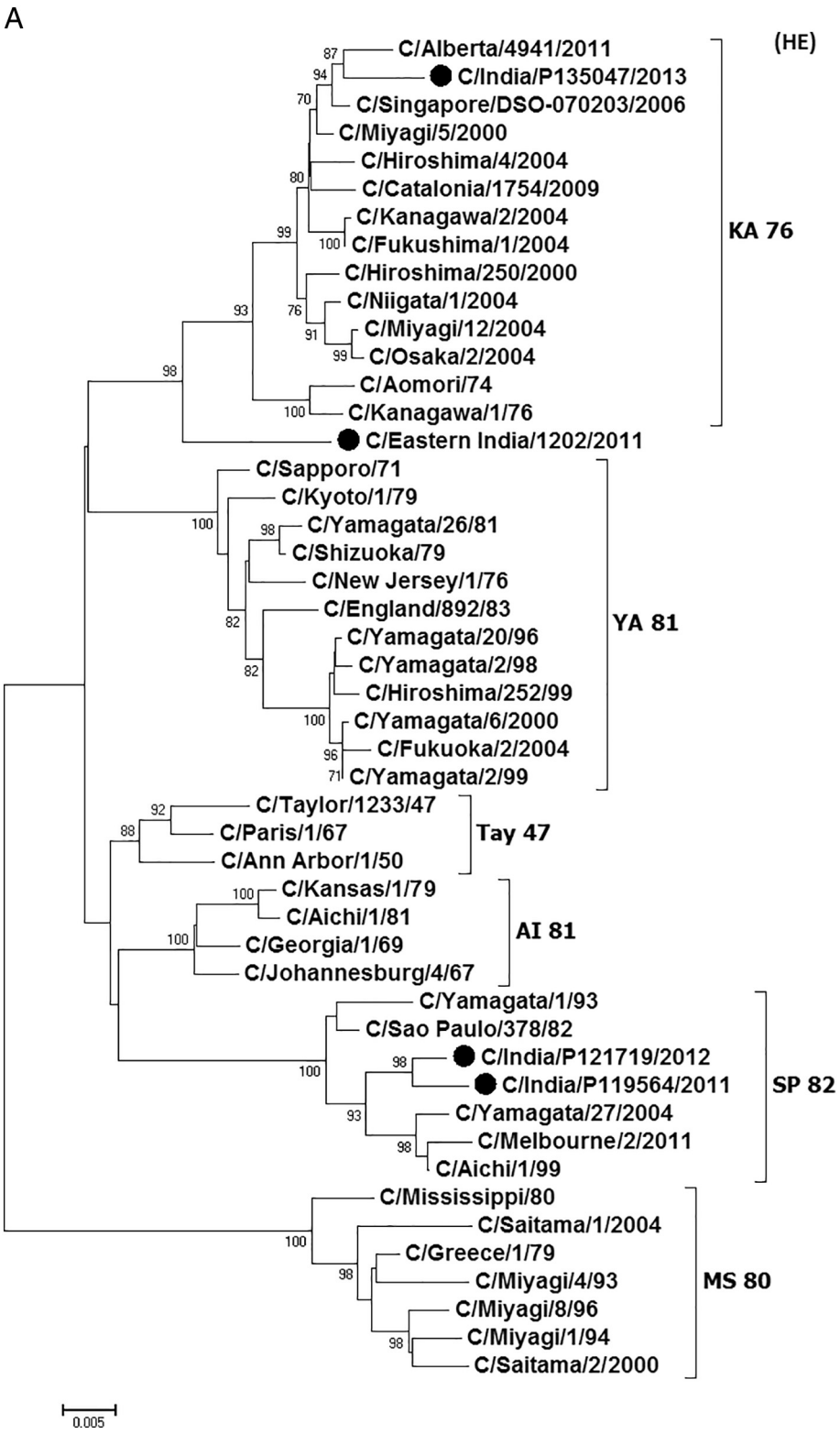
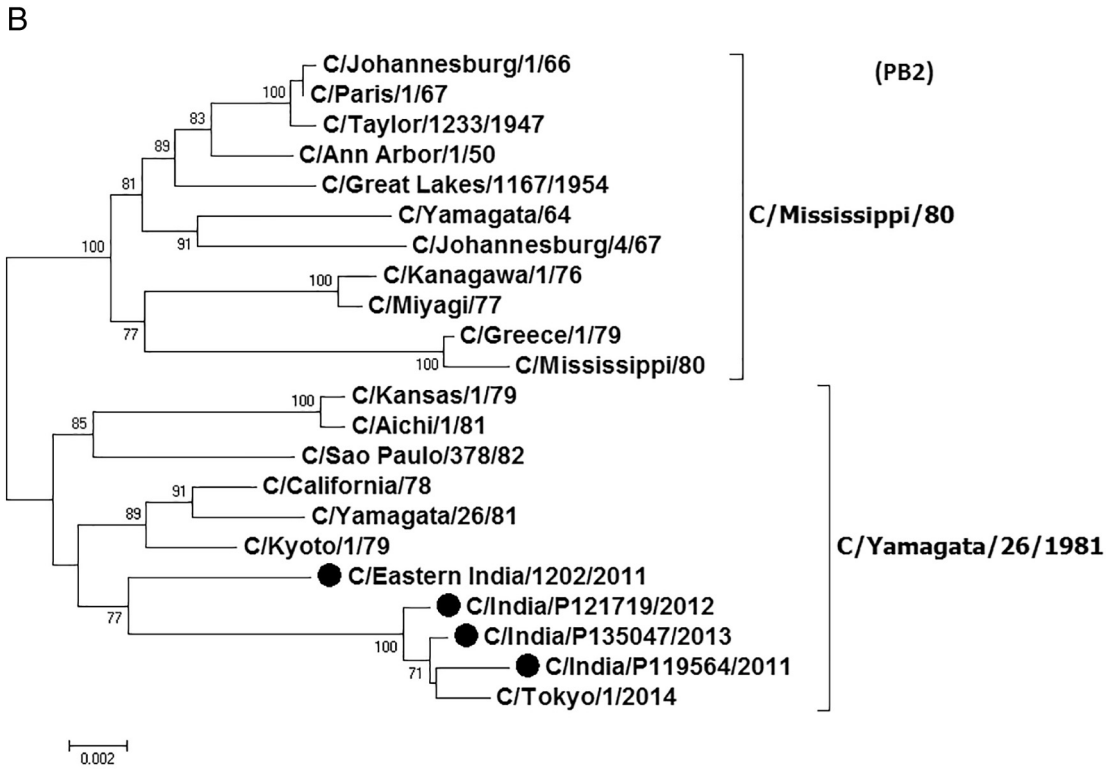
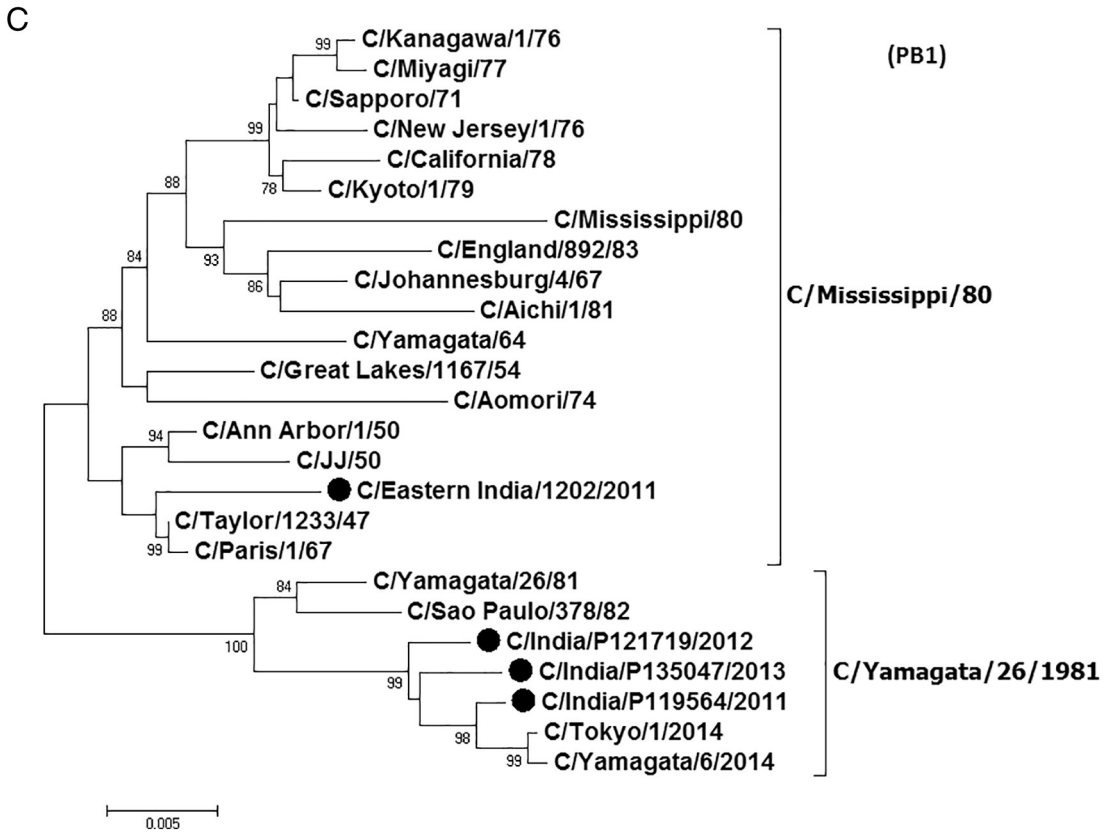


Fig. 1. A: Phylogenetic tree of Influenza C virus hemagglutinin-esterase (HE) genes; the numbers above the branches are the bootstrap probabilities (> 70%) for the each branch determined using the MEGA software (version 6). The study strains are marked in solid circle including Eastern India strain. B-G: Phylogenetic tree of Influenza C virus PB2(1B), PB1(1C), P3(1D), NP(1E), M(1G) NS(1F), genes; the numbers above the branches are the bootstrap probabilities (> 70%) for the each branch determined using the MEGA software (version 6).





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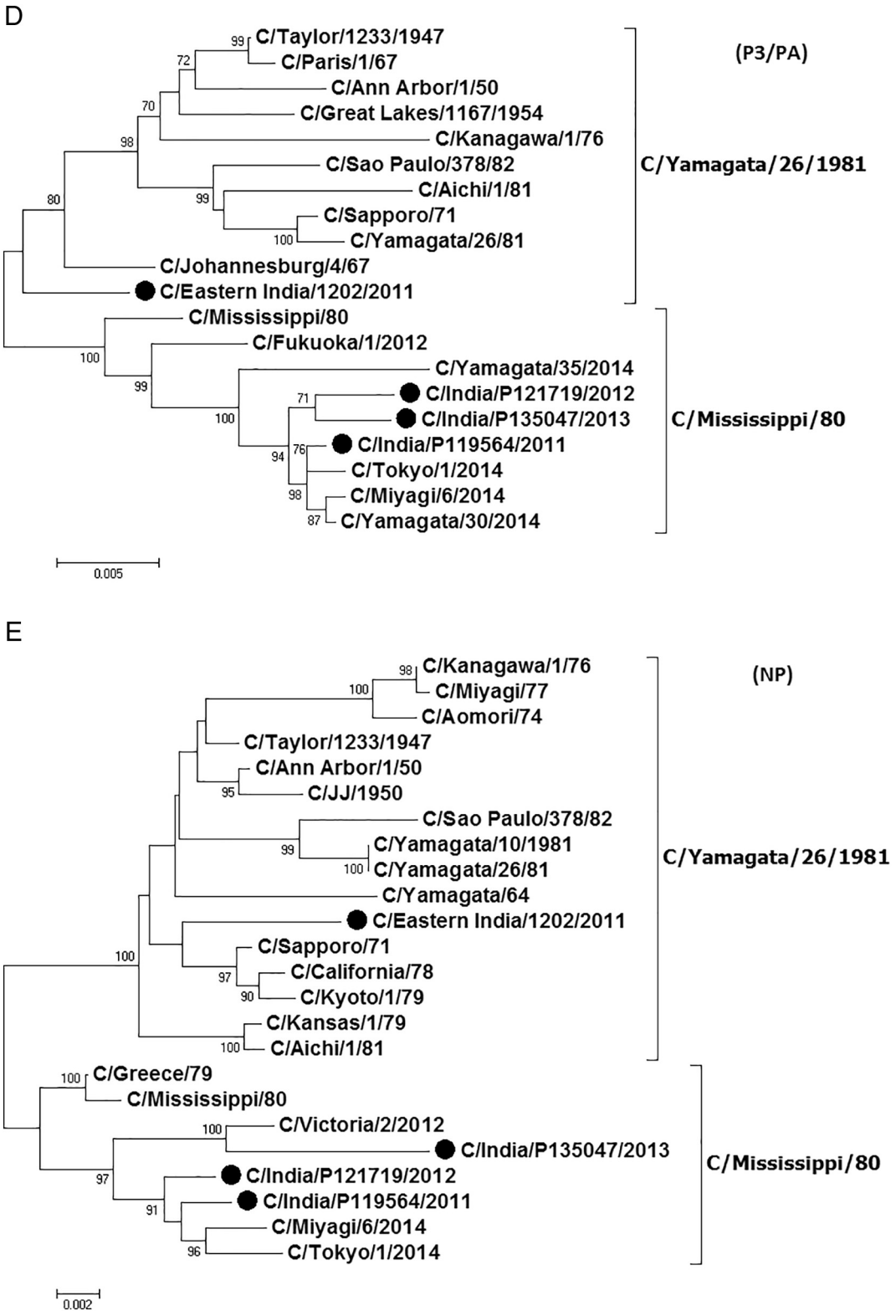

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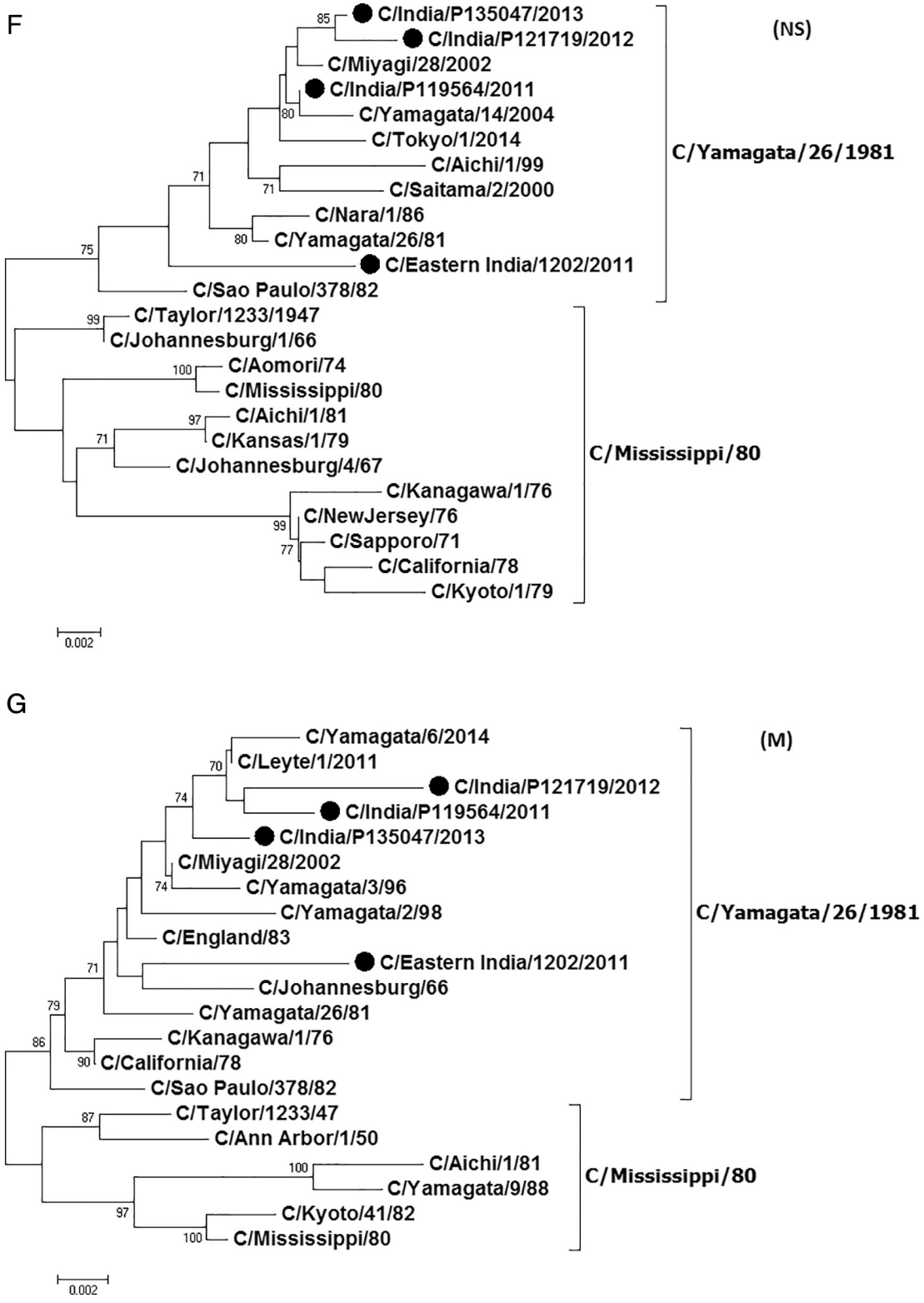

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genome sequencing of a single Influenza C virus isolate showed the occurrence of multiple reassortment events. To enhance the understanding of the evolution of Influenza C viruses, we carried out molecular detection, isolation and whole genome sequence analysis of Influenza C viruses retrospectively for clinical samples obtained during the years 2009–2015.

2. Materials and methods

An existing outpatient based sentinel system that has been established by the National Institute of Virology (NIV) was used for the study, and both severe as well as mild cases of ILI were included. During January 2009 to August 2015, 1025 nasal and throat swabs (NS and TS) were obtained from children having ILI, manifested with sudden onset

Table 1
Genomic composition of Study viruses (Bold and underlined) & Eastern India strain along with HE gene lineage viruses Kanagawa and Sao Paulo.

Lineage	Virus strain isolated in the study	RNA segment						
		PB2	PB1	P3	HE	NP	M	NS
KA176	**C/Kanagawa/1/76**	MS80	MS80	YA81	**KA76**	YA81	YA81	MS80
	C/India/135047/2013	YA81	YA81	MS80	**KA76**	MS80	YA81	YA81
	C/Eastern India/1202/2011	YA81	MS80	YA81	**KA76**	YA81	YA81	YA81
SP82	**C/Sao Paulo/378/82**	YA81	YA81	YA81	**SP82**	YA81	YA81	YA81
	C/India/119,564/2011	YA81	YA81	MS80	**SP82**	MS80	YA81	YA81
	C/India/121719/2012	YA81	YA81	MS80	**SP82**	MS80	YA81	YA81

The abbreviations used are as follows: MS80: Mississippi Lineage C/Mississippi/80; YA2681: Yamagata Lineage C/Yamagata/26/81; KA176: Kanagawa Lineage C/Kanagawa/1/76; SP82: Sao Paulo Lineage C/Sao Paulo/378/82.

of fever (> 38 °C), running nose, cough, breathlessness and/or fatigue attending outpatient dispensaries (OPD) and 1505 hospitalized children presenting with severe pneumonia, bronchitis or having severe acute respiratory illness (SARI), mainly characterized by history of fever or measured fever ≥ 38 °C and cough with onset within the last 7 days and requiring overnight hospitalization (IPD) referred to NIVPune for pandemic H1N1 testing from five hospitals from Pune were included in the study. The reported age range, for both SARI and ILI was 0.0 to 14.9 years; mean age was 1.2 years. The study was approved by the National Institute of Virology Institutional Human ethics committee. Written informed consent was obtained from all participants or next of kin, and caretakers or guardians on behalf of minors/children enrolled in the study. Prior approvals for the use of clinical material were obtained from the Institutional Review Board (IRB). The samples were well stored at − 80 °C and not freeze-thawed for more than two times.

2.1. Laboratory diagnosis

All the NS and/or TS samples from OPD and IPD were retrospectively analyzed for influenza viruses by real time PCR. Detection of Influenza C virus was carried out using published protocols based on the NP and M gene (Matsuzaki et al., 2012; Pabbaraju et al., 2013). In addition, the clinical samples were tested by rRT-PCR for Influenza A viruses including pandemic H1N1 viruses using WHO protocol (CDC, 2009). Detection of Type B and seasonal H3N2 was done using WHO protocol for molecular diagnosis of influenza virus in humans – update November 2012 (http://www.who.int/influenza/gisrs_laboratory/molecular_diagnosis_influenza_virus_humans_update_201211.pdf) (Accessed on August 2014). A confirmed influenza case was defined as a patient meeting the ILI/SARI case definition and positive for Influenza by rRT-PCR.

2.2. Virus isolation

Influenza C virus rRT-PCR positive samples were inoculated into Madin-Darby canine kidney (MDCK) cells. Attempts were made to isolate the virus using embryonated chicken eggs by amniotic and allantoic routes as per the 2011 WHO Manual for the laboratory diagnosis and virological surveillance of influenza (http://apps.who.int/iris/bitstream/10665/44518/1/9789241548090_eng.pdf) (Accessed on July 2012) (World Health Organization, 2011).

The culture supernatant and amniotic fluid were tested by a hemagglutination (HA) test using chicken or guinea pig erythrocytes. Influenza C viruses cause agglutination with chicken RBCs but does not cause agglutination of guinea pig cells (Chakraverty, 1978). Isolation was suspected if the HA test was positive with chicken but negative with guinea pig erythrocytes. Influenza C virus isolates were thereafter confirmed by rRT–PCR using Influenza C virus NP and M gene specific primers (Matsuzaki et al., 2012; Pabbaraju et al., 2013).

2.3. Nucleotide sequencing and phylogenetic analysis of Influenza C viruses

Whole genome amplification and sequencing was done as per previously published protocols (Roy Mukherjee et al., 2013; Matsuzaki et al., 2007 & 2016). Briefly, nucleotide sequencing and analyses were carried out as follows: viral RNA was extracted from 100 µl of original clinical material using the MagMAX™ Viral RNA isolation kit (Ambion® Life Technologies - Thermo Fisher Scientific). Individual segments of Influenza C virus were amplified by RT-PCR using TaKaRa® PrimeScript One Step RT-PCR Kit using gene-specific primers for HE (1926 bp), M (1124 bp), NP (1698 bp) and NS (923 bp) as described by Roy Mukherjee et al., 2013. Full-length polymerase genes PB1 (2264 bp), PB2 (2320 bp) and P3 (2129 bp) were amplified using published primers (Matsuzaki et al., 2016).

Amplicons were purified using PCR purification kits (Qiagen) and sequenced using Big Dye Terminator version 3.1 kit (Applied Biosystems, Foster City, CA). The sequencing was done on ABI 3730 DNA analyzer (Applied Biosystems). The best fit Tamura-Nei nucleotide substitution model was selected to generate a Neighbor-joining tree, based on the sequences of this study together with previously reported cognate sequences of corresponding genes from global database, including those from India (Roy Mukherjee et al., 2013; Matsuzaki et al., 2000, 2002, 2003; Odagiri et al., 2015; Muraki et al., 1996; Peng et al., 1994; Tada et al., 1997), using MEGA version 6 (Tamura et al., 2013). Six classic reference strains: Taylor47 KA76, MS80, AI81, YA81, and SP82 were also included. Gaps and missing data were eliminated by using pair-wise deletion option. Statistical support for branching was tested by bootstrapping with 1000 replicates.

2.4. Divergence times for the Influenza C viruses

Divergence times for Indian influenza C viruses along with 80 other sequences were estimated using the Bayesian Markov chain Monte Carlo (MCMC) approach implemented in the Beast package version 1.8.1 (Drummond et al., 2012). The relaxed uncorrelated lognormal clock was employed with the Bayesian skyline tree prior, and the general-time-reversal model with gamma-distributed rates of variation among sites and a proportion of invariant sites (GTR + G + I) as the best-fit nucleotide substitution model. The MCMC chains were run for two billion iterations for the HE gene and three hundred million iterations for the internal genes and samples were obtained every steps. Three independent runs of the chain were combined and the convergence was assessed using TRACER version 1.5. The maximum clade credibility (MCC) tree was visualized using FigTree version 1.3.1.

2.5. Nucleotide sequence accession numbers

The nucleotide sequences were submitted to the GenBank database and the assigned accession numbers are: KT232082 to KT232088, KM507552 to KM507558 and KM507545 to KM507551.

3. Results

3.1. Detection by rRT-PCR

Respiratory specimens screened by Taqman real -time RT-PCR for the presence of Influenza A, B and C viruses, showed 3 (0.12%) out of 2530 samples to be positive for Influenza C virus; 105 (4.1%) were positive for Influenza A, and 31(1.23%) for Influenza B. Influenza C virus was not detected in any of the IPD cases. The three samples that

tested positive were from a 3-year-old male, 4-year-old female, and a 2.5-year-old male child who had visited the OPD during the months of June 2011, February 2012 and April 2013 respectively. The Ct values for the samples were 32, 30 and 36 respectively (Supplementary Fig. 1A, B, C).

All the positive samples were processed for isolation using MDCK cell line and embryonated chicken eggs. Two virus strains C/India/P119564/2011and C/India/P121719/2012 were successfully isolated from amniotic fluid and confirmed by the HA test using chicken RBCs (Supplementary Fig. 2A–B) as well as by real-time RT-PCR. The observed Ct values for the isolate were 30 and 29 in passage 2. The sample C/India/P135047/2013 having a late Ct value of 36 was followed up to passage level 3, but could not be isolated.

3.2. Nucleotide sequencing and phylogenetic analysis of influenza C virus

Phylogenetic analysis of the HE gene (Fig. 1a) showed six distinct lineages represented by Taylor47, KA76, YA81, AI81, SP82, and MS80. The C/India/P135047/2013 strain grouped with the KA76 lineage which also includes the earlier reported C/Eastern-India/1202/2011

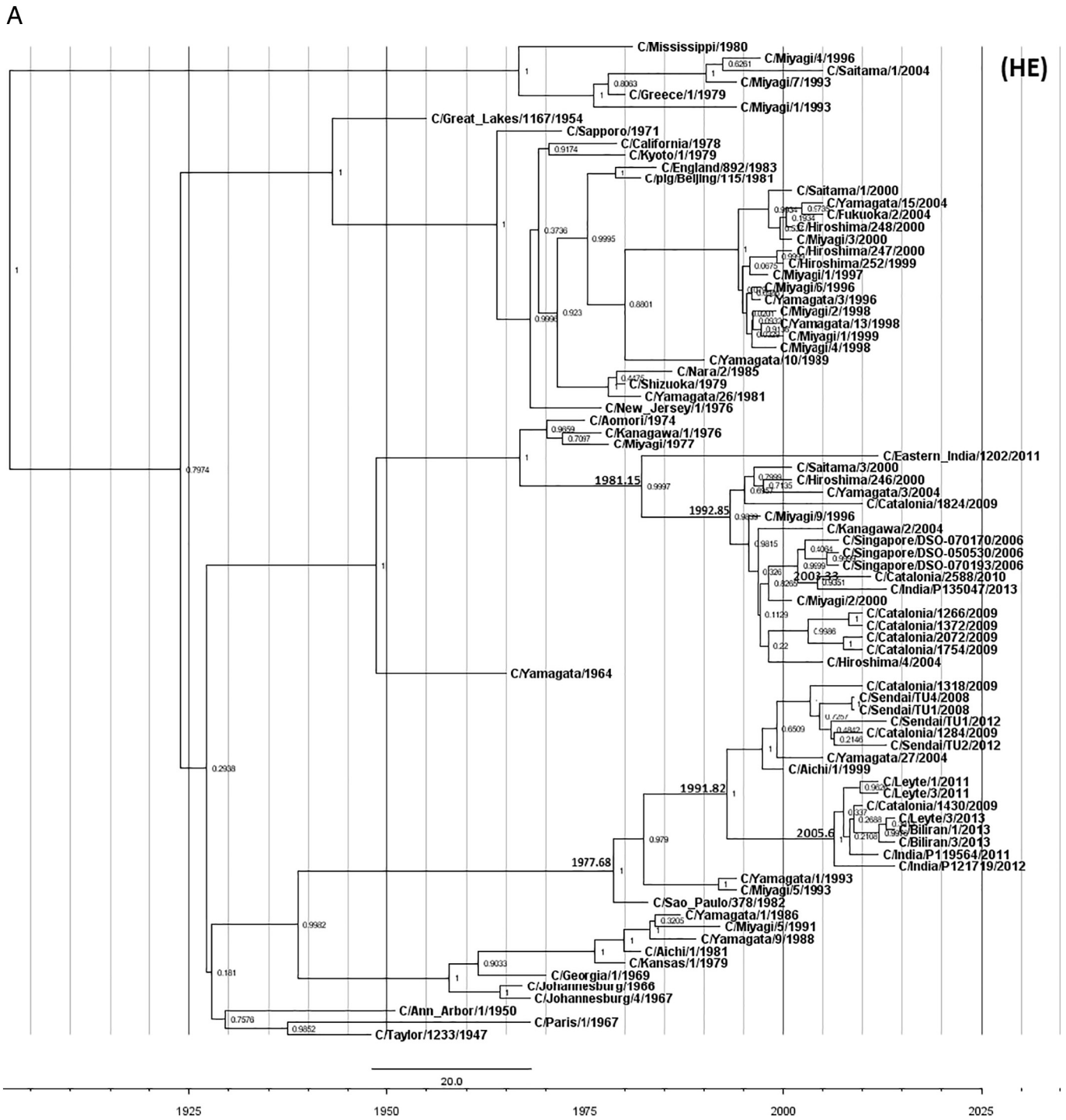
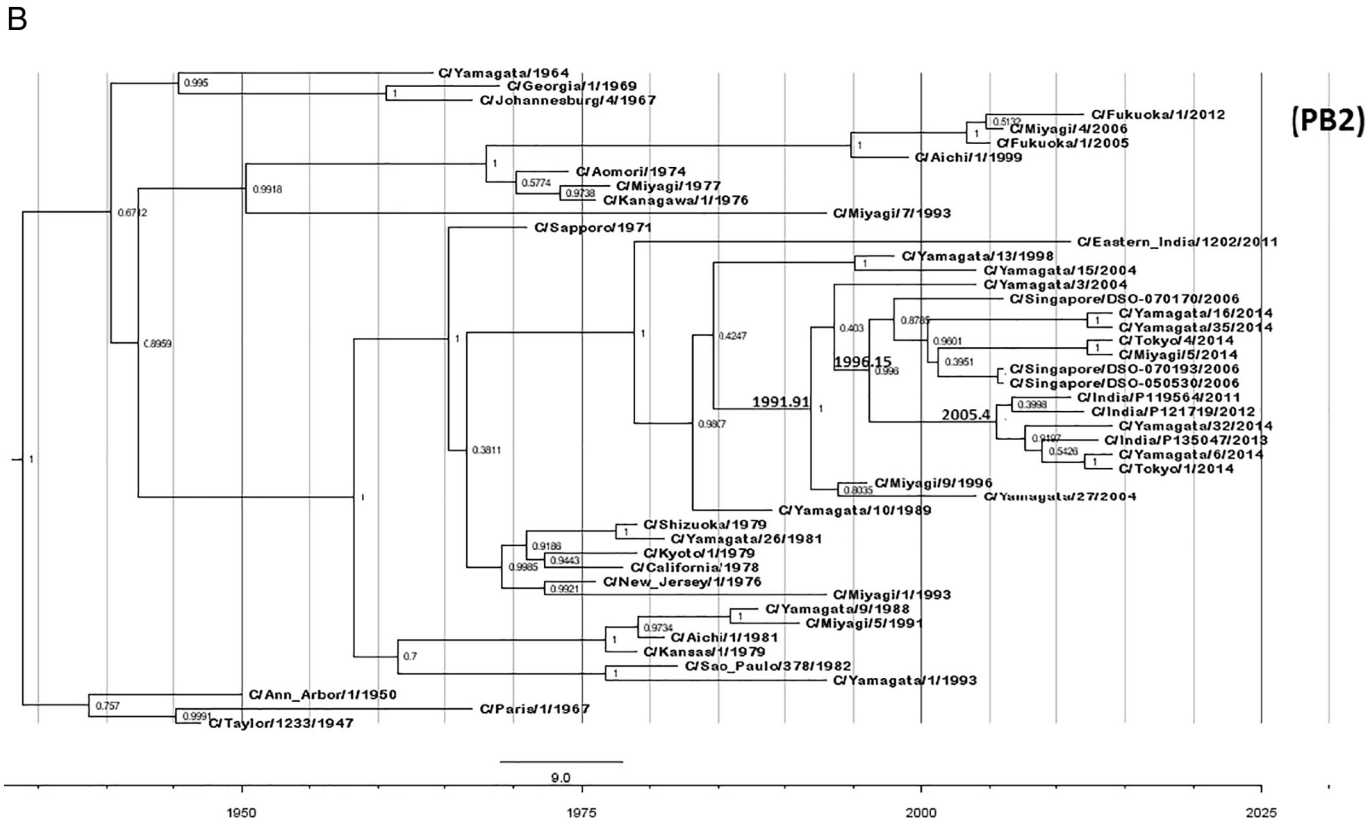
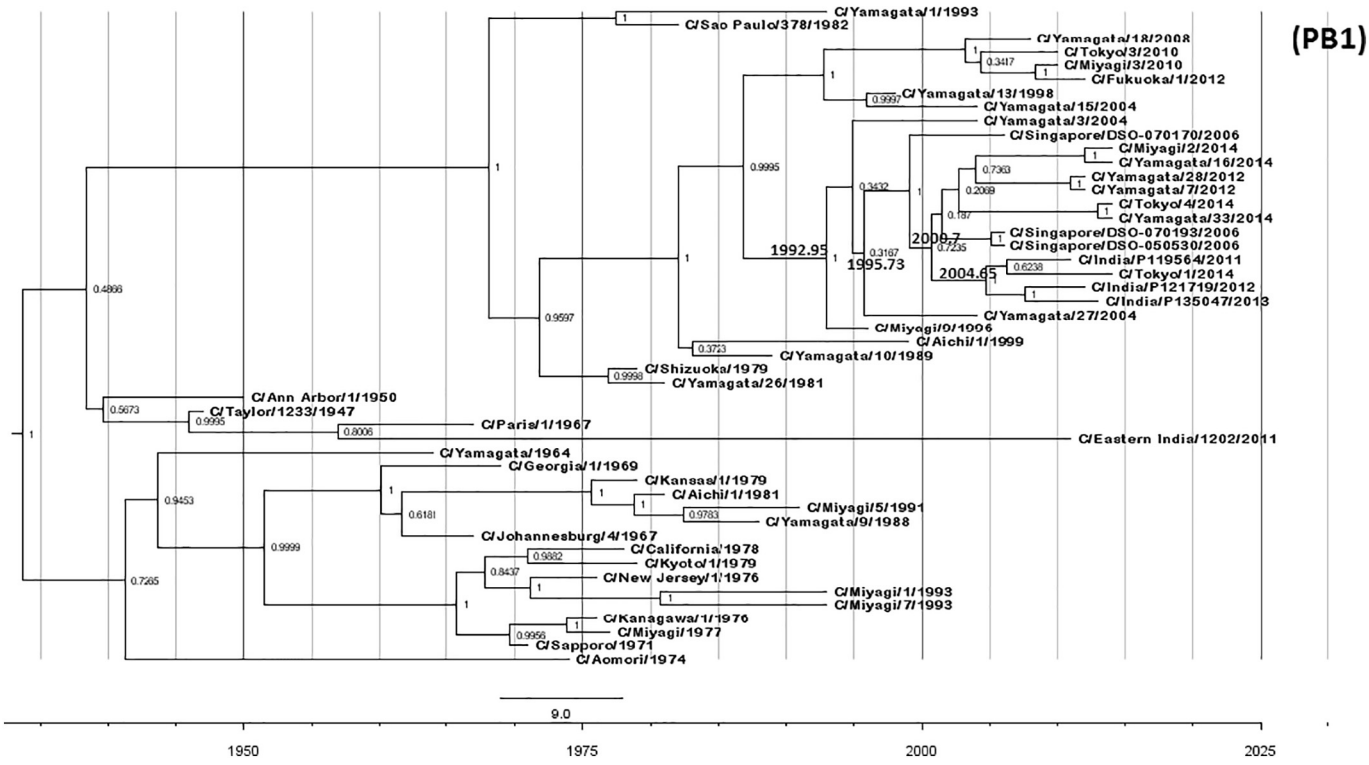


Fig. 2. A: Bayesian evolutionary tree of Influenza C virus based on the nucleotide sequence of the HE gene. The evolutionary years for Indian strains are reported on respective node. This Bayesian tree was generated using the BEAST software. B-G: Bayesian evolutionary tree of Influenza C virus based on the nucleotide sequence of the PB2(2B), PB1(2C), P3(2D), NP(2E), M (2G) NS(2F), gene.





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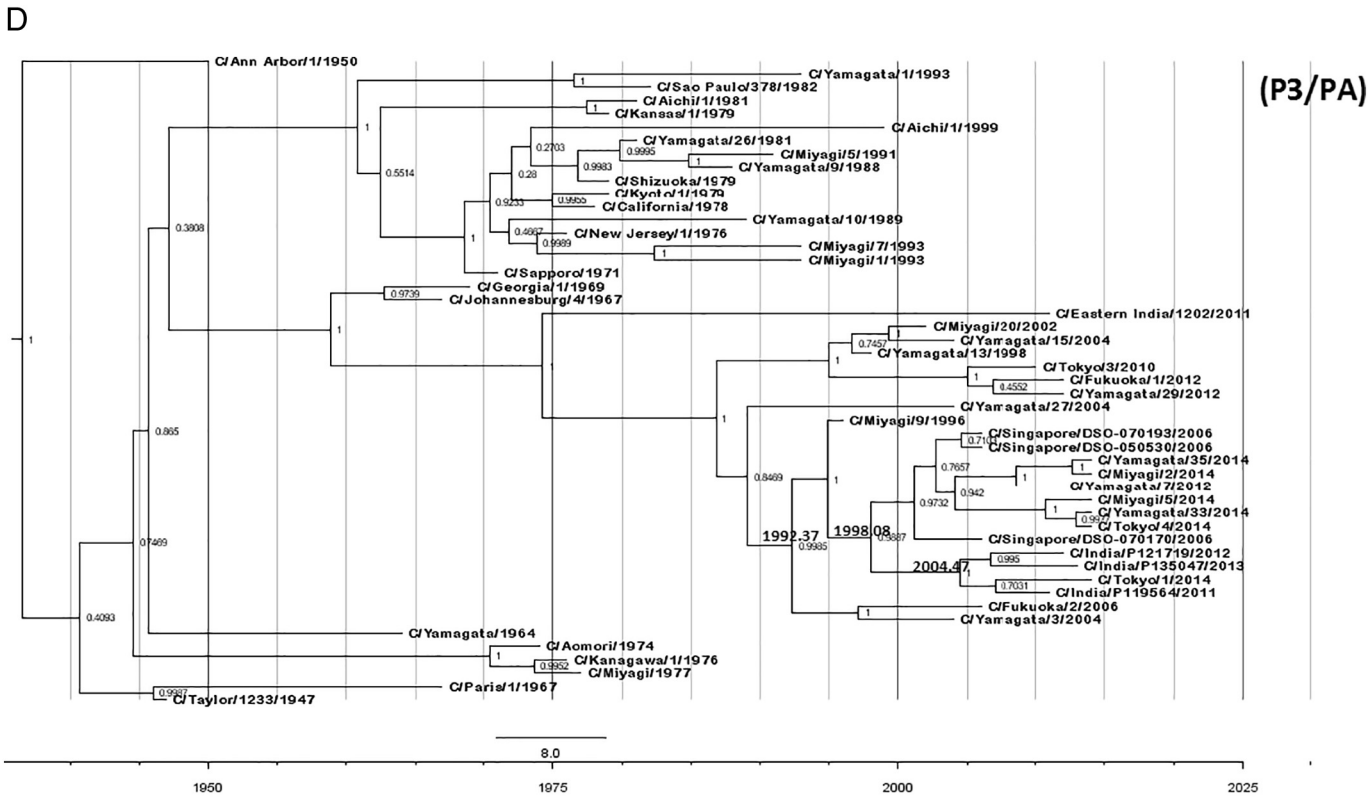

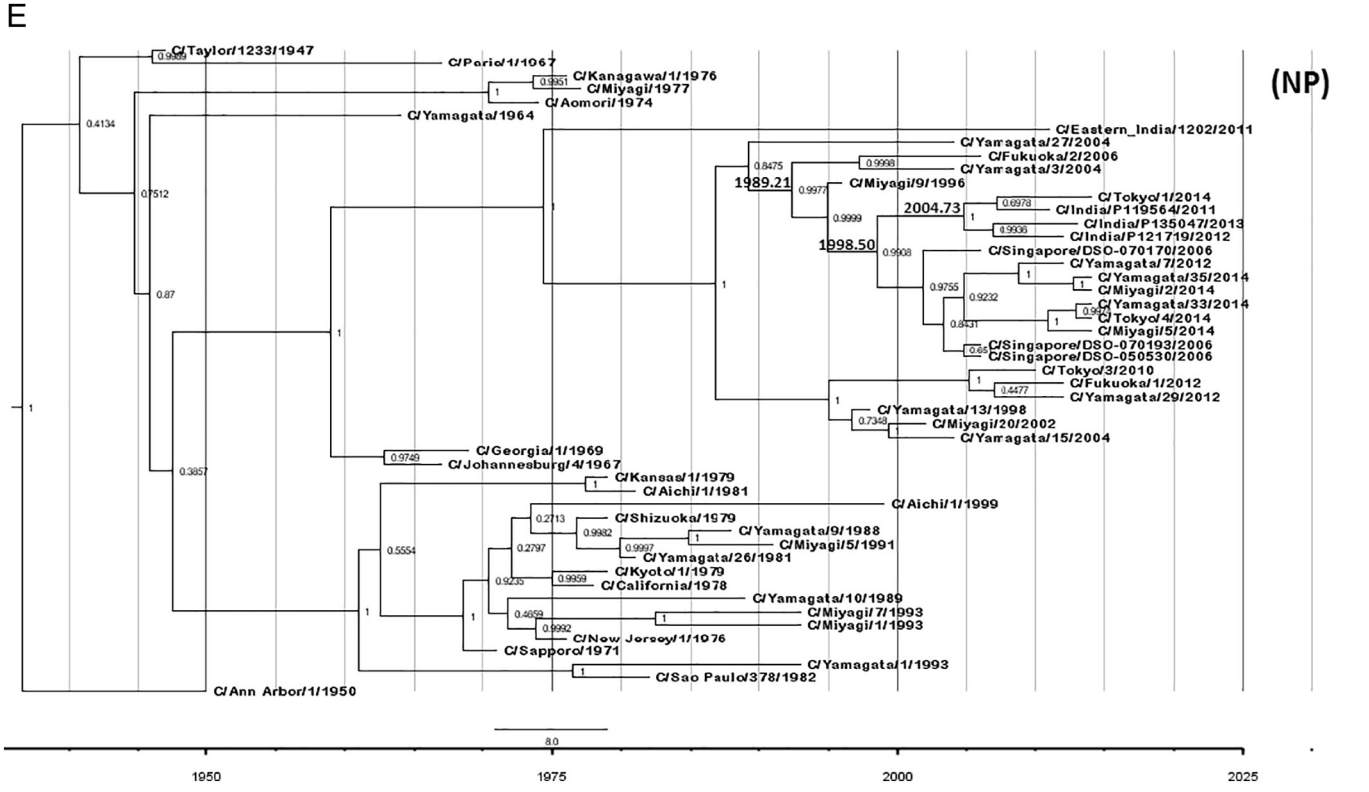

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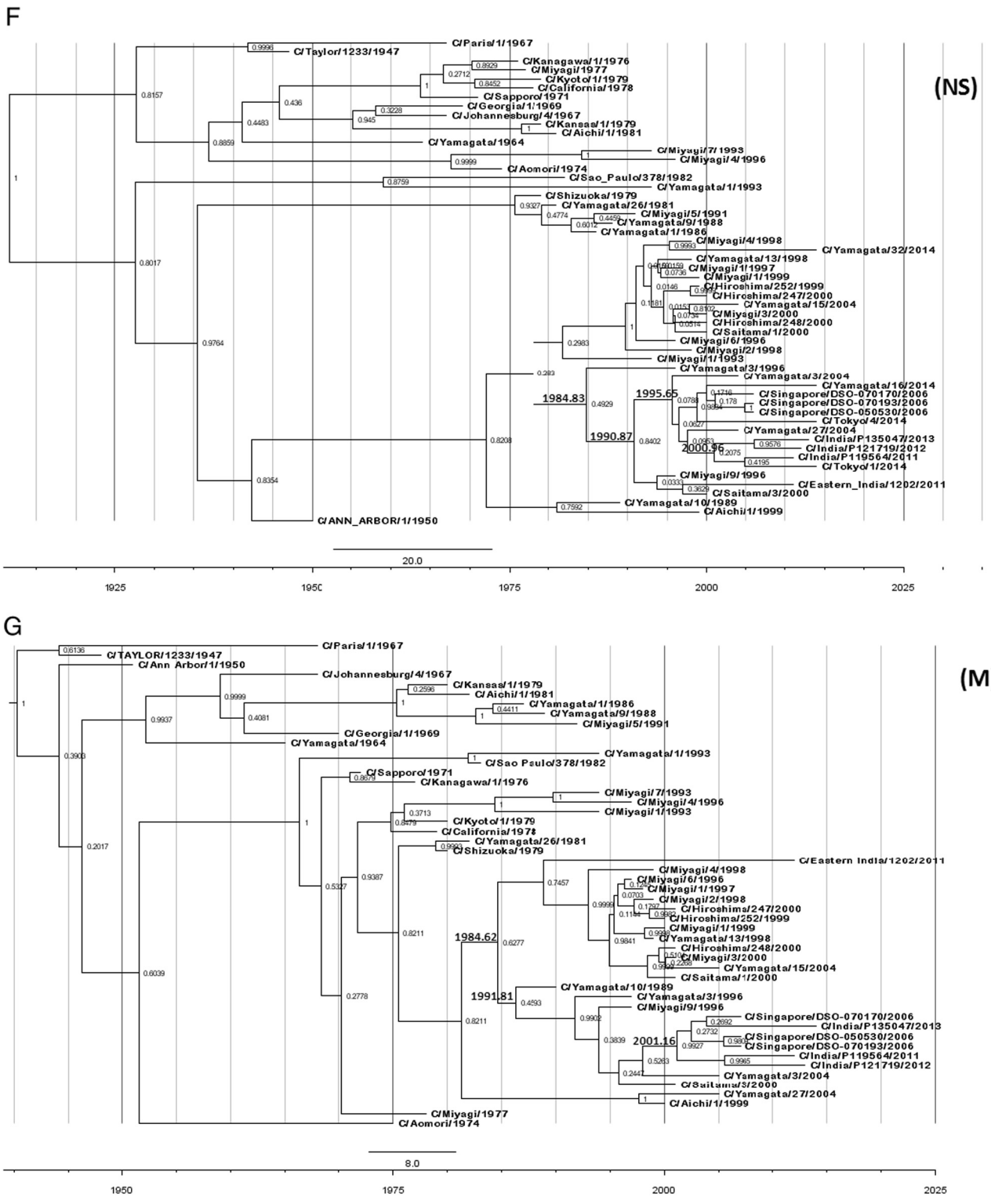

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strain. Further, the two strains C/India/P119564/2011, C/India/P121719/2012 grouped with the SP82 lineage. The nucleotide identity of the HE genes of 2011, 2012 and 2013 strains showed 93.47 to 99.20% among themselves (Supplementary Table 1). To further determine the genomic composition of the Indian strains and occurrence of reassortment event(s), phylogenetic analysis of the internal genes was carried out (Fig. 1b to 1g).

Phylogenetic analysis based on the PB2, PB1, M, and NS genes, showed that the viruses belong to the YA81-related lineage, while the phylogeny based on the P3 and NP genes showed that they belonged to the MS80 (Fig. 1B–G) lineage. The genome compositions of the study strains determined by the phylogenetic analyses are summarized in Table 1. The results showed that all the study strains were reassortants having multiple lineage genes. Thus during the study period Influenza C viruses belonging to the KA76 and SP82 lineages were found to be co-circulating with similar internal genome composition.

Bayesian evolutionary tree for all the genes were generated to determine when the study strains diverged from their last common

Table 2
Evolutionary Rates of All genes with 95% HPD and emerging year of Indian strains from common ancestor.

Genes	Evolution rate	95%HPD	Common ancestor emerging year
HE	$5.0004 * 10^{-4}$	$[4.13 * 10^{-4}, 5.90 * 10^{-4}]$	2005.6 (SP82 lineage), 2003.3 (KA76 lineage)
M	$3.86 * 10^{-4}$	$[2.9906 * 10^{-4}, 4.7943 * 10^{-4}]$	2001.16
NP	$4.24 * 10^{-4}$	$[3.4364 * 10^{-4}, 5.0724 * 10^{-4}]$	2004.7
NS	$2.64 * 10^{-4}$	$[1.753 * 10^{-4}, 3.5559 * 10^{-4}]$	2000.9
P3	$4.21 * 10^{-4}$	$[3.4408 * 10^{-4}, 5.0622 * 10^{-4}]$	2004.4
PB1	$3.86 * 10^{-4}$	$[3.1063 * 10^{-4}, 4.6261 * 10^{-4}]$	2004.6
PB2	$3.69 * 10^{-4}$	$[2.9412 * 10^{-4}, 4.5439 * 10^{-4}]$	2005.4

ancestor. As shown in Fig. 2a, the HE gene, of the Indian strains diverged from their last common ancestor at the estimated node age of ~2005 for the SP82 lineage strains and 2003 for the KA76 lineage virus. The estimated divergence time periods for the HE and internal genes (Fig. 2B to 2G) are summarized in Table 2.

4. Discussion

In this study we isolated two Influenza C viruses out of three positive clinical samples from amniotic fluid of embryonated egg. Overall the Influenza C virus detection rate was very low and positive patients had mild illness (ILI). None of the IPD samples from the SARI cases were positive for Influenza C virus. A previous report from Japan revealed that the seasonality of Influenza C virus is different than Influenza A, and epidemics of Influenza C usually occur after peaks of Influenza A (Matsuzaki et al., 2014). In India distinct seasonality of Influenza A and B viruses is observed (Chadha et al., 2015) and the western part of the country has peak activity during the monsoon months, June to September. The Influenza C virus detection in this report was from the month of February, April and early June, implying that the Influenza C virus activity is observed during the inter-seasonal period. However to determine the seasonality of Influenza C viruses, long-term studies need to be conducted. In India, outbreaks or epidemics due to influenza C virus have never been reported.

Genetic characterization of the HE gene of the study viruses from western India suggests that the 2011 and 2012 viruses belong to the SP82 lineage and the single virus of 2013 belongs to the KA76 lineage as the eastern India 2011 strain (Roy Mukherjee et al., 2013). Earlier studies suggested that the KA76 antigenic group and the SP82 antigenic group were the dominant strains in Japan, between 2002 and 2014 (Matsuzaki et al., 2016). Co-circulation of the KA76 and SP82 antigenic lineages were reported from Catalonia, Spain (2009–2010 season) (Antón et al., 2011), Milan, Italy (2008–2009 and 2009–2010 seasons) (Principi et al., 2013), Alberta, Canada (2010–2011 season) (Pabbaraju et al., 2013), and Yamagata, Japan (2011–2012 season) (Odagiri et al., 2015; Matsuzaki et al., 2016) with SP82 as the dominant lineage. Our study demonstrates co-circulation of both these lineages in 2011 as reported in Canada and Japan. However, we could not confirm the reported isolates at the antigenic level, due to non-availability of group/lineage-specific antiserum

Matsuzaki et al. (2016) reported that worldwide, before 1990 the Aichi, Mississippi, Kanagawa, Sao Paulo and Yamagata lineage viruses were in circulation; however the Aichi lineage viruses departed in 1992 and Kanagawa along with Sao Paulo lineages became dominant from 2002 to 2014. During 2000–2014 the viruses of Kanagawa, Sao Paulo and Yamagata lineage were highly homologues at the internal genome composition having Yamagata lineage-like composition. Multiple reassortment events of the Mississippi and Yamagata lineage viruses have also hypothesized to have occurred between 1992 and 1996 (Matsuzaki et al., 2016). Our study similarly showed that the Indian strains had internal genes belonging to multiple lineages which may have resulted in P3 and NP genes from the Mississippi-like virus with the other internal genes of the Yamagata-like virus.

The Bayesian evolutionary analyses based on the HE gene, showed that the strains of KA76 lineage diverged from a common ancestor in 1965 and further evolved into two groups, the eastern Indian strain of 2011 in 1981 and Miyagi group ~1992. The C/India/P135047/2013 virus which belongs to the Miyagi group further diverged in 2003. The SA82 lineage viruses diverged from a common ancestor ~1977. The Indian viruses of 2011 and 2012 along with the cluster of Philippine and Catalonia viruses diverged ~2005. The long branches of deep divergence represent the time periods with lack of virus samples. Bayesian evolutionary analysis of the internal genes showed that the time to the most recent common ancestor of the Indian strains was ~2001 based on the M and NS genes while ~2005 based on the NP, P3, PB1 and PB2 genes. Thus multiple evolutionary time frames were observed for the internal and surface genes. Divergence times of the Indian viruses when compared with those from other countries may reflect a long-term persistence of influenza C virus inside India, though the possibility of their recent introductions cannot be ruled out. The estimated evolutionary rate for the HE gene of the Influenza C virus was found to be 5.0004×10^{-4} substitutions/site/year which is agreeable with the earlier report (Matsuzaki et al., 2016).

The study showed a very low detection frequency of Influenza C virus in the Indian population. The internal gene composition of the two lineages in circulation were identical though differing from the Eastern Indian strain and suggests that chances of reassortment in the community is very low and the virus may have been seeded from outside India. The Bayesian evolutionary analyses further confirmed that the Indian strains may have evolved through past reassortment events and may have been seeded in the country from external sources.

In conclusion, we report detection of Influenza C viruses from mild ILI cases from western India where we had limited epidemiological information for Influenza C. We also estimated the divergence times from common ancestors. This study along with the previous report from Eastern India still do not represent the various geographically distinct areas in a vast country like India and hence multisite long-term monitoring of Influenza C viruses is necessary to define the epidemiology and seasonality. Likewise it is necessary to monitor the evolutionary patterns of Influenza C virus in India.

5. Addendum

V A Potdar and MS Chadha: Study design and execution of the study at the National Institute of Virology, Pune, preparation of the manuscript.

M Dakhave, A Manchanda, D Hinge, N Jadhav: Carried out the study including identification of patients, virus detection, characterization and phylogenetic analysis,

P Kulkarni: carried out virus isolation

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Conflict of interest

None to declare.

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