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BACTERIAL, FUNGAL AND VIRUS MOLECULAR BIOLOGY - SHORT COMMUNICATION

Molecular investigation of human metapneumovirus in children with acute respiratory infections in Chennai, South India, from 2016–2018

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Abstract

Human metapneumovirus (hMPV) has emerged as a frequent cause of acute respiratory infections (ARI) among young children. The prevalence and genetic diversity of hMPV circulating in Chennai, Southern India, has not been studied yet. Hence, this study was aimed to investigate the prevalence, co-infection with other respiratory viruses like HRSV A and B, influenza A and B, hRV and HPIV 1–4 viruses, socio-demographic associations, and genotypes of hMPV among children in Chennai. A total of 350 nasal swab specimens were collected from children with ARI during April 2016 to August 2018 and tested for hMPV by real time PCR method. In this study, hMPV was detected in 4% (14/350) of the samples. One hMPV positive sample was found to be co-infected with influenza B virus. The mean and median ages of the children with hMPV infection were 61.5 months (5.1 years) and 83 months (6.9 years), respectively. Phylogenetic analysis of the partial F gene revealed the presence of A2c subcluster among the study strains as well as with B1 and B2 lineages. The prevalence data obtained in this study is important in evaluating the role of hMPV in childhood ARI and emphasizes the importance of routine viral diagnosis in hospitals. To the best of our knowledge, this is the first study to report the prevalence, seasonality, and genetic diversity of hMPV in Chennai as well as the first study to report A2c subcluster of hMPV among children in India.

Keywords hMPV · ARI · Real time PCR · Fusion gene · Phylogeny · A2c subcluster

Introduction

Human metapneumovirus (hMPV) is an enveloped, single-stranded RNA virus belonging to the family Pneumoviridae and *Metapneumovirus* genus [1]. It was first isolated in 2001 in Netherlands [2]. Since then, it has been documented as a frequent cause of both upper respiratory tract infection and lower respiratory tract infection among people of all age groups, predominantly affecting the children, elderly, and immunocompromised individuals [3–5]. The epidemiological scenario of hMPV is understudied in India. The first case of hMPV in India was reported in 2004 from Pune [6]. Other studies have been conducted in India at Lucknow, New Delhi, and Vellore, where children were the study population [7–9]. Premature birth, pre-existing nosocomial infection, young age, and underlying chronic heart, pulmonary, or neural disorders are some of the risk factors associated with severe hMPV infection [10]. hMPV circulates as two distinct genotypes — hMPV A and B [3, 11]. Based on the phylogenetic analysis of the nucleocapsid (N) and F gene sequences, Genotype A is further classified into A1 and A2 and Genotype B is divided into B1 and B2 lineages [12]. Lineage A2 is divided into A2a and A2b [12, 13], A2b1 and A2b2 subclusters [14], while lineage B2 consists of B2a and B2b [15]. Recently, an additional A2c subcluster was revealed in Japan, Malaysia, and Croatia [16–18]. It is important to determine the lineages of circulating hMPV strains which could aid in hMPV preventive measures. However, there is limited data on the prevalence and genetic diversity of hMPV in Chennai, South India. This study describes the prevalence of hMPV and its association with demographic factors like age, gender, and seasonality

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during April 2016–August 2018 in Chennai, South India. The circulating lineages of hMPV strains were determined, and mutational analysis was performed.

Materials and methods

Study population and sample collection

Children below 18 years of age and visiting the pediatric outpatient department of three tertiary care hospitals (ESI hospital, K.K. Nagar, Chennai, Institute of Child Health, Egmore, Chennai and Rabindran's Health Care Centre, Ambattur, Chennai) and one pediatric clinic (Triplicane, Chennai) with symptoms of acute respiratory infection such as fever, cough, nasal congestion, rhinorrhea, headache, sore throat, myalgia, and dyspnea were enrolled in the study during April 2016–August 2018. Children with co-morbidities including cystic fibrosis, asthma, bronchiectasis, pulmonary hypertension, congenital heart disease, congestive heart failure, neutropenia, and babies born prematurely were excluded from the study. The samples were collected from children experiencing symptoms for ≤ 21 days.

The children were grouped [19] as follows: infants (1 month–2 years), 121/350, 34.6%; young children (2–6 years), 148/350, 42.3%; older children (6–12 years), 68/350, 19.4%; and adolescents (12–18 years) 13/350, 3.7%.

Seven children had repeated infection and hence, three hundred and fifty nasal swabs were collected from three hundred and forty-three children. The nasal swabs were immediately transported to laboratory in HiViral Transport medium (HiMedia, Mumbai). Informed consent was obtained from their parents/guardian.

Detection of human metapneumovirus

RNA was extracted from 150 µl of the sample using NucleoSpin RNA virus kit (Macherey Nagel, Germany) according to the manufacturer's instructions. The extracted RNA was quantified using NanoDrop Spectrophotometer ND-1000 (Thermo Fisher Scientific, USA) and immediately subjected to reverse transcription. Reverse transcription was carried out using RevertAid first strand cDNA synthesis kit (Thermo Fisher Scientific, USA) according to manufacturer's protocol and stored at − 20 °C until use.

Real time PCR was performed using previously published primers targeting the highly conserved nucleoprotein (N) gene and capable of identifying all the four lineages [20]. The 10-µL real time PCR master mix consisted of 5 µL of 5 × FastStart Universal Probe Master (ROX) (Roche Diagnostic, Germany), 0.2 µL each of forward and reverse primers (10 µM), 0.2 µL of probe (25 µM), 2 µL of cDNA, and 2.4 µL of nuclease free water. The cycling conditions were standardized at 95 °C for 10 min followed by 45 cycles of at 95 °C for 15 s and 60 °C for 1 min. Extension was carried out for 10 s at 72 °C.

Real time PCR was carried out in StepOnePlus Real Time instrument (Thermo Fisher Scientific, USA).

Analysis of co-infection and seasonality

The samples were further screened for the presence of other respiratory viruses namely, influenza viruses A and B [21], human respiratory syncytial virus [22], human parainfluenza viruses types 1 to 4, and human rhinovirus. The details of primers, PCR reaction mixture, and cycling conditions for hPIV and hRV are given in Supplement Table 1. Temporal distribution of hMPV was determined by plotting number of hMPV detected against total number of samples collected.

Sequencing and phylogeny

For phylogenetic analysis, the partial F gene of hMPV strains was amplified by heminested RT-PCR using primers previously described [23]. For the first round PCR, 2 µL of cDNA was added to 18 µL master mix containing 2 µL of 10 × PCR buffer, 0.5 µL each of forward and reverse primer (HRSV common primers) (10 µM), 0.3 µL of 10 mM dNTPs, and 0.025 U of Taq polymerase (5U/µL). Thermal cycling conditions for the first round were as follows: initial denaturation of 94 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 10 min.

The second round was performed with 1 µL of the first-round product as template with same reaction mixture and thermal cycling conditions except that the annealing temperature was set at 58 °C, and the PCR was carried out for 20 cycles.

PCR products were purified using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) and sequenced using ABI 3731 × L DNA sequencer (Applied Biosystems, USA) at AgriGenome Labs Pvt Ltd, Kerala, India. Nucleotide sequences of partial F gene of hMPV strains representative of A1 (isolate 00–1) [2], A2a (CAN97-83) [13], A2b (JPS03-240) [24], A2c (hMPV/OkinawaJPN/137/10) [16], B1 (NL/1/99 and CAN97-82) [25, 26], and B2 (NL/94/01) [27] lineages were retrieved from the GenBank for reference. All the sequences were contig aligned using ClustalW and phylogenetic analysis was done using the MEGA 6 software (Pennsylvania, USA) [28]. Approximately 291 bp of the partial F gene was used in the phylogenetic analysis. The best model for constructing phylogenetic tree was estimated using the Find Best DNA/Protein Models (ML). Using this method, it was found that the K2 + I (Kimura-2-parameter + evolutionarily invariable sites) contained the lowest

BIC (Bayesian Information Criterion) score and hence, was the appropriate model (Supplement Fig. 1). The statistical robustness and reliability of the tree topology were analyzed using 1000 bootstrapping replicates. The evolutionary distances were computed using the p-distance method, which was given in the units of the number of base differences per site.

Mutational analysis of the hMPV strains

RNA viruses can accrue genetic differences when outbreaks occur, which leads to modulations in virulence and evolvability [29]. Hence, the deduced amino acid sequences from partial F gene of hMPV strains were compared with reference strains CLZ0741 for A2c lineage, NL/94/01 for B2 lineage and NL/1/99 and CAN97-82 for B1 lineage for mutational analysis. Synonymous and nonsynonymous mutations were analyzed by the method of Nei and Gojobori using SNAP (Synonymous/Nonsynonymous Analysis Program) provided by the HIV database website. The O-glycosylated residues and potential N-glycosylation sites (Asn–X–Ser/Thr, where X is any amino acid except Proline) were predicted using the NetOGlyc version 4.0 server (http://www.cbs.dtu.dk/services/NetOGlyc/) and NetNGlyc version 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/) software, respectively.

Sequence data were registered under accession numbers MK080266, MK080267, MK080268, MK080269, MK080270, MK080271, MK080272, MK080273, MK080274, MK080275, MK080276, MK080277, and MK080278 at the NCBI GenBank.

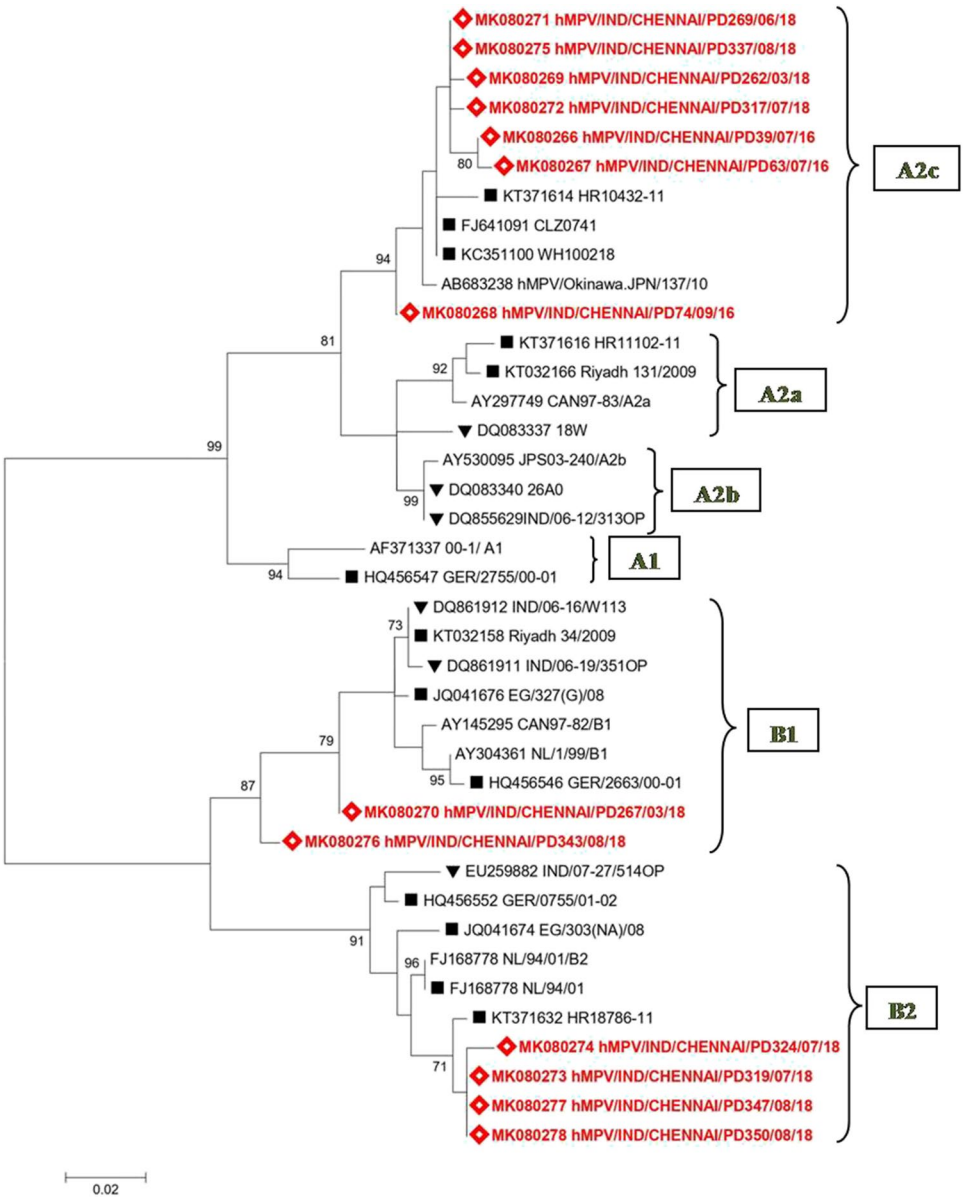

Fig. 1 Phylogenetic analysis of the human metapneumovirus strains obtained in this study. Phylogenetic tree of hMPV based on the partial F gene was constructed using maximum likelihood method using 1000 bootstrapping replicates. Values greater than 70% are shown. Sequences of reference strains and strains from other countries were obtained from NCBI GenBank. Sequences obtained in this study are indicated with a lozenge symbol. Other Indian strains are indicated with a black down-pointing triangle symbol. Strains from other countries are represented with a black square symbol

Results and discussion

Among three hundred and fifty samples collected, fourteen samples (14/350, 4%) were positive for hMPV by real time PCR (Table 1). The 4% positivity rate was comparable with other studies from India; 5% in Pondicherry; 3% in Kolkata; and 3.6% in Lucknow [30–32]. A high prevalence rate of 12.7% was observed in a study from Vellore, South India [9]. Prevalence rates of as low as 1.7% and as high as 27% have been reported globally [33, 34]. This variation in the prevalence rates could be attributed to the different study population, ununified sampling methods, different techniques employed for viral detection, and the study period. hMPV is reported to be responsible for 4–16% of ARI-hospitalizations in children [35]; hence, the prevalence data obtained in this study is valuable in evaluating the role of hMPV in childhood ARI and emphasizes the importance of routine viral diagnosis in hospitals.

Among the fourteen samples positive for hMPV, only one sample (1/14, 7.1%) was found to be co-infected with influenza B virus. In a study from India, hRSV and human rhino virus were frequently detected with hMPV [36]. The association between such dual infections and disease severity is an ongoing debate. Nandhini et al. found that two hMPV patients co-infected with HRSV presented with severe infection requiring hospitalization [30]. While some studies have documented increased risk of severe infection in cases of co-infection [37, 38], other studies have shown no impact on severity of infection [39, 40].

A male:female ratio of 3:4 was observed among the patients with hMPV infection. The mean and median ages were 61.5 months (5.1 years) and 83 months (6.9 years), respectively. Similar studies from other countries have reported the median of children with hMPV infection as 17.2 months [41] and 24 months [42]. In these studies, a male preponderance was observed which was in contrast to the results of present study. Studies have documented higher prevalence of hMPV in children less than 5 years of age [31, 41]. In the current study, hMPV was more prevalent in young children (57.1%, 8/14) which could be attributed to a more immature immune system when compared to older children and exposure to infections in the school environment.

With respect to the annual distribution of hMPV in our study, three cases were detected in 2016 (21.4%) and eleven cases in 2018 (78.6%). No hMPV was detected among samples collected in 2017. This data is in accordance with earlier observations documenting high and low incidence of hMPV in alternating years [30, 43]. This biennial pattern has also been reported globally [18, 41, 44, 45].

Most of the hMPV strains were detected during monsoon season (July–August) in this study. In contrast to these results, the peak incidence of hMPV occurred in the month of February in North-Western India [46]. Narayanan et al. found that the hMPV-positive ARIs were more common during the cooler and wetter months of July to January [9]. Similarly, Agrawal et al. reported that majority of the hMPV positive samples were detected during July–November, although low frequency of hMPV was observed throughout the year [47].

The partial F gene sequencing (219–342 aa) was carried out for 13/14 (92.9%) hMPV positive strains. Phylogenetic analysis of the thirteen strains revealed that three different lineages of hMPV circulated in our region during the study period (Fig. 1); B1 (2/13, 15.4%), B2 (4/13, 30.8%), and A2c (7/13, 53.8%). Three hMPV samples detected in 2016 were found to belong to the A2c subcluster. In 2018, hMPV

Table 1 Socio-demographic and molecular data of hMPV positive patients

S. no	Age/sex	Location	Year of detection	Lineage	Accession number
1	3.5Y/F	Ammayathoppu	July 2016	A2c	MK080266
2	3Y 8 m/F	Manali Mathur	July 2016	A2c	MK080267
3	2Y 1 m/M	Anna Nagar	September 2016	A2c	MK080268
4	9Y/F	Triplicane	March 2018	A2c	MK080269
5	6Y/M	Triplicane	March 2018	B1	MK080270
6	2Y/M	Egmore	June 2018	A2c	MK080271
7	7 m/F	Thirumullaivoyal	July 2018	Not Applicable*	-
8	13Y/F	Ambattur	July 2018	A2c	MK080272
9	3Y/F	Annanur	July 2018	B2	MK080273
10	10Y/M	Menambedu	July 2018	B2	MK080274
11	11 m/F	Thirumullaivoyal	August 2018	A2c	MK080275
12	6Y/M	Senthil Nagar	August 2018	B1	MK080276
13	6Y/M	Annanur	August 2018	B2	MK080277
14	6Y/F	Ambattur	August 2018	B2	MK080278

*Sequencing could not be performed

strains belonging to all the three lineages (B1, B2, and A2c subcluster) were co-circulating. Two out of the ten samples (20%) belonged to the B1 lineage; four (40%) samples each were of B2 lineage and A2c subcluster. Co-circulation of hMPV lineages is a familiar trend which has been documented from countries all over the world [18]. None of our hMPV strains belonged to the A1 lineage, A2a, and A2b subclusters, though they have been previously reported from India [43, 48, 49]. However, this is the first report of the A2c subcluster in India. This subcluster was earlier reported from epidemiologic studies in Japan, Malaysia, Croatia, and recently in Bangladesh [16–18, 50] and our A2c strains closely matched with strains from these countries (Fig. 1).

The strains belonging to the A2c subcluster in our study clustered with a significant bootstrap value of 94%. The intragenotypic *p* distance was found to be less than 0.07 between all the members of the same cluster (Supplement Table 2) suggesting very little divergence among the strains. This finding is similar to previous studies which suggest minimal progressive drift and considerable stability of genetic lineages over a period of time [51].

On comparing the A2c, B1, and B2 strains obtained from our study with their respective reference strains, it was found that the A2c strains in the present study had 97.1–97.9% and 100% identity at nucleotide and amino acid level, respectively. The two B1 strains in this study had 95.8% and 97.6% identity at nucleotide level and 100% identity at amino acid level. The identity of the B2 strains in this study ranged from 97.4 to 98.2% at nucleotide level and 99.2% at amino acid level due to the D280N mutation observed among all our B2 strains.

The F protein of all the analyzed strains had a length of 127 amino acids. The deduced amino acid sequence alignment of the groups A and B hMPV strains revealed that they were highly conserved with very few lineage specific mutations (Supplement Figs. 2, 3, and 4). Two mutations were observed among all the strains when compared with the hMPV reference isolate 00–1; F258I and G294E. The N233Y, V286I, and Q312K mutations were observed among all the B1 (Supplement Fig. 3) and B2 lineage strains (Supplement Fig. 4); they were however absent among the A2c strains (Supplement Fig. 2). The K296N and K296D point mutations were present only in B1 and B2 lineage strains, respectively. We also observed the D280N mutation in our B2 strains which was previously reported from other countries. Selection pressure analysis revealed that this region of F gene was under high negative selection with a dS/dN ratio of 49.13.

In agreement with previous studies, the partial F gene sequences from our study were highly conserved, especially at the amino acid level [18, 51]. This could be due to the involvement of this gene in host immunity and viral functions. These sequences also lacked potential O-linked and N-linked glycosylation sites, which have been hypothesized to be involved in escaping the host immune response in other viruses [52].

In India, in spite of the fact that a number of children die due to ARI every year, community-based studies documenting the contribution of circulating respiratory viruses are very sparse. Our study is the first epidemiologic study on hMPV from Chennai in South India. This study underlines the importance of hMPV as an etiology of ARI in children and emphasizes the need for routine laboratory diagnosis of this virus. This study also provides significant understanding of the circulating hMPV strains in Chennai. This is also the first report on the presence of the subcluster A2c in India.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s42770-022-00689-2.

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Declarations

Ethics approval The study was approved by the Institutional Human Ethics Committee of Dr. ALM Post Graduate Institute of Basic Medical Sciences, University of Madras (Approval No: UM/IHEC/12–2014-II and UM/IHEC/07–2017-I).

Conflict of interest The authors declare no competing interests.

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