



Whole genome sequencing and evolution analyses of *Human metapneumovirus*

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Abstract

Human metapneumovirus (HMPV) is a major pathogen of acute respiratory tract infections (ARTIs) in children. Whole genome sequence analyses could help understand the evolution and transmission events of this virus. In this study, we sequenced HMPV whole genomes to improve the identification of molecular epidemiology in Beijing, China. Nasopharyngeal aspirates of hospitalized children aged < 14 years old with ARTIs were screened for HMPV infection using qPCR. Fourteen pairs of overlapping primers were used to amplify whole genome sequences of HMPV from positive samples with high viral loads. The epidemiology of HMPV was analysed and 27 HMPV whole genome sequences were obtained. Sequence identity and the positional entropy analyses showed that most regions of HMPV genome are conserved, whereas the G gene contained many variations. Phylogenetic analysis identified 25 HMPV sequences that belonged to a newly defined subtype A2b1; G gene sequences from 24 of these contained a 111-nucleotide duplication. HMPV is an important respiratory pathogen in paediatric patients. The new subtype A2b1 with a 111-nucleotide duplication has become predominate in Beijing, China.

Keywords *Human metapneumovirus* · Whole genome sequence · Genetic diversity

Introduction

Human metapneumovirus (HMPV) is a member of the *Pneumoviridae* family and was first discovered in 2001 from a patient with a respiratory infection [1]. HMPV mainly causes upper and lower respiratory tract infections in children with mild symptoms. However, HMPV can also occur in concentrated outbreaks among susceptible people, and even cause death in critically ill patients [2]. Notably, in an

Iranian case report, three children co-infected with HMPV and severe acute respiratory coronavirus 2 (SARS-COV-2) were found to have died, suggesting that HMPV may directly or indirectly influence susceptibility to and pathogenicity of SARS-CoV-2 [3].

HMPV is a single-stranded RNA virus with a genome of approximately 13 Kb, containing eight genes encoding nine proteins. Based on genomic characteristics, HMPV can be divided into four subtypes, A1, A2, B1, and B2; subtype A2 can be further classified into two additional subtypes A2a and A2b [4]. Although several HMPV subtypes can be prevalent at the same time every year, there is a dominant subtype. Recently, a new genotype of A2 was defined in several studies [5–7], and a novel mutant that contained a 180-nucleotide (nt) or 111-nt duplication (dup) in the G gene has been detected since 2015 [8–10]. The clinical role and genotype of this new mutant is still unclear, and whole genome sequence monitoring is necessary.

In this study, positive HMPV respiratory infection samples collected from children in Beijing between 2017 and 2019 were Sanger sequenced. A total of 27 HMPV whole genome sequences were obtained and analysed to study the sequence characteristics of HMPV whole genomes in Beijing, China.

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Materials and methods

Study population and specimen collection

Nasopharyngeal aspirates (NPAs) were collected from children (aged < 14 years old) with acute respiratory tract infections (ARTIs) at the Beijing Friendship Hospital (China) between April 2017 to March 2019. Clinical characteristics were recorded. All samples were collected in tubes containing viral transport medium and kept at -80°C until use.

Detection of HMPV

Total viral nucleic acid was extracted from 200 μL of each clinical specimen using a QIAamp MinElute Kit (Qiagen, Germany). HMPV detection was performed by quantitative real-time polymerase chain reaction (qPCR) assay using a One-step RT-PCR Kit (Ambion, USA). The specific primers (Forward primer: 5'-CATATAAGCATGCTATATTAAGAGTCTC-3'; Reverse primer: 5'-CCTATTTCTGCAGCATATTTGTAATCAG-3') and probes (5'-FAM-TGYAATGATGAGGGTGTCACTGCGGTTG-3'-TAMRA) for the HMPV N gene were used as previously described [11]. Samples with a cycle threshold (Ct) < 37 were regarded as positive.

Sequencing

HMPV-positive samples with high copy numbers (Ct < 30) were further used for whole genome sequencing. Viral nucleic acid was reverse transcribed using SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific, USA) to synthesize cDNA. Whole genomic sequences were amplified by fourteen pairs of overlapping primers that were used as previously described [12]. PCR was performed with Ex-Taq (TaKaRa, China), and amplification products were sequenced by Beijing Tsingke Biological Technology. For fragments that were difficult to amplify, new nested primers were designed for amplification. Obtained sequences were assembled using Sequencher 5.0 software.

Phylogenetic and evolutionary dynamics analysis

Sequence comparisons were made using published GenBank HMPV sequences, and 30 HMPV whole genome sequences and 52 HMPV gene F sequences were chosen for phylogenetic analysis. Neighbor-joining (NJ) trees were constructed using MEGA 7.0 with 1000 bootstrap replicates. The sequence identity and the entropy values of amino acid residues encoded in the whole genome sequences were calculated using BioEdit. Recombination events were detected by Simplot. In addition, 554 G gene sequences of HMPV

genotype-A from 1982 to 2019 were retrieved from GenBank database (Supplementary Table 1). Phylogenetic and evolutionary dynamics analyses on HMPV genotype A were performed using the Bayesian Markov chain Monte Carlo (MCMC) methods as implemented in BEAST v1.10.4. Two independent runs were performed for 100 million generations, sampling every 10,000 MCMC step, under a Tamura-Nei (TN93) + Int substitution model and the log-normal relaxed molecular clock. The population dynamics of HMPV genotype-A sequences were estimated by means of Bayesian skyline reconstruction.

Statistical analysis

Data analysis was performed using SPSS 13.0, and the significance of the difference in rates among categorical data was tested by chi-squared and Fisher's exact tests. Independent-samples *t*-test was used to analyze continuous variables. Two-tailed $P < 0.05$ was considered statistical significance.

Results

Epidemiology of HMPV

From April 2017 to March 2019, we collected a total of 2,848 NPAs in the Beijing Friendship hospital (China), and 176 samples (6.18%, 176/2828) were shown to be HMPV positive via qPCR. The male/female ratio of HMPV infection was 1.55 (107:69), which has no significant difference ($p = 0.128$). The median age of HMPV-infected children was 3 years (IQR: 0–14 years), and the detection rates have no significantly different among different age groups

Table 1 Population demographic of HMPV-positive specimens from April 2017 and March 2019

Variable	Number of patient	Number of HMPV positive children	Prevalence of HMPV (%)	<i>p</i> value
Gender				0.128 ^a
Male	1574	107	5.42	
Female	1274	69	6.80	
Age(years)				0.093 ^a
≤ 1	760	42	5.53	
> 1 to ≤ 2	341	27	7.92	
> 2 to ≤ 3	609	46	7.55	
> 3 to ≤ 4	316	24	7.59	
> 4 to ≤ 5	177	5	2.82	
> 5 to ≤ 7	266	15	5.64	
> 7 to ≤ 14	379	17	4.49	
Total	2848	176		

^a χ^2 -test

($p=0.093$) (Table 1). However, the HMPV prevalence of patients aged ≤ 4 years (139/2026, 6.86%) was significantly higher than that of patients aged 4–14 years (37/822, 4.50%) ($p=0.018$). HMPV infection from April 2017 to March 2019 has significant seasonal differences, mainly occurred in spring (42.05%, 74/176) and winter (37.50%, 66/176) ($p<0.001$). In contrast, the HMPV detection rate in summer and autumn is 9.66% (17/176) and 10.80% (19/176), respectively.

Clinical characteristics of HMPV infections

All 176 HMPV-infected patients were diagnosed with a lower respiratory tract infection (LRTI). The average length of hospital stay was 6.47 ± 2.45 days, and 61.93% HMPV-infected children were discharged within 7 days. The main clinical symptoms included cough (96.02%, 169/176), fever (≥ 38 °C; 91.48%, 161/176), rhinorrhoea (51.14, 90/176), and other common flu-like symptoms and gastrointestinal symptoms. None of the patients developed severe respiratory problems, such as dyspnea. The mean viral load of HMPV-positive samples was 6.7×10^4 copies/ μ L, and there was no significant difference in viral load between patients aged 0–4 years and patients aged 5–14 years, and HMPV infected patients under 4 years of age were more likely to have respiratory symptoms than patients aged 5–14 years, such as rhinorrhoea (58.99% vs 21.62%, $p<0.001$) and nasal obstruction (29.5% vs 16.22%) (Table 2).

Whole genome analysis of HMPV

General PCR was performed on HMPV positive samples with a Ct value < 30 , and the products were Sanger sequenced. The fragments were assembled to produce whole genome sequences, and 27 HMPV genomes were obtained. The sequence lengths were between 13,281 and 13,448 nt in length, and the sequences have been submitted to GenBank,

the GenBank accession numbers are provided in Supplementary Table 2. The sequence identity among all the analysed sequences was 0.799–0.999 and was 0.801–0.999 among the sequences identified in this study (Table 3). The sequence positional entropy (SPE) was calculated for each residue position of the aligned sequences. Most protein coding regions exhibited low SPE values, whereas the amino acid residues encoded by the G gene had high SPE values (Fig. 1). The Simplot analysis showed no evidence of recombination for any of the sequences in this study. The HMPV F protein is an important viral protein that mediates the membrane fusion reaction, which mainly relies on two functional structural sites (cleavage site: RQSR and integrin $\alpha\beta 1$ binding site: RGD) [13, 14], the SNP analysis of these two functional structure sites showed no mutation among the 27 HMPV F genes.

Phylogenetic analysis of HMPV

A phylogenetic tree of 27 HMPV whole genome sequences was constructed using NJ with the Tamura-Nei model. Based on this, HMPV can be divided into six sub-lineages (A1, A2a, A2b1, A2b2, B1, and B2). Among the 27 sequences, 25 (92.59%) were grouped into the A2b lineage, and the other two sequences were grouped into the B1 lineage (Fig. 2). Phylogenetic analyses of HMPV F gene sequences had the same results as the whole genome phylogenetic analyses. There are three sub-lineages in A2 (A2a, A2b1, and A2b2), and all 25 detected A2 sequences were classed as the A2b1 subtype. The alignment results showed that 96% of obtained A2b1 sequences (24/25) contained a 111-nt-dup in the G gene.

To understand the demographic history of HMPV A genotype, 554 global HMPV G gene sequences were collected from GenBank, and the Bayesian Maximum Clade Credibility (MCC) trees were defined Fig. 3). The Bayesian skyline population estimate indicated that the population dynamic

Table 2 Clinical characteristics of 176 HMPV-infected patients

Clinical characteristics	Age (years)		<i>p</i> value	Total (n=176) %
	0 to 4 (n=139) %	5 to 14 (n=37) %		
Cough	133, 95.68	36, 97.3	1 ^b	169, 96.02%
Fever	126, 90.65	35, 94.59	0.665 ^b	161, 91.48%
Rhinorrhoea	82, 58.99	8, 21.62	$<0.001^a$	90, 51.14%
Nasal obstruction	41, 29.5	6, 16.22	0.157 ^a	47, 26.7%
Shiver	17, 12.23	4, 10.81	0.373 ^b	21, 11.93%
Vomit	14, 10.07	2, 5.41	0.578 ^b	16, 9.09%
Sneeze	30, 21.58	3, 8.11	0.062 ^a	33, 18.75%
Diarrhea	6, 4.32	0, 0	0.438 ^b	6, 3.41%
Viral load (copies/ μ L)	7.25×10^4	4.37×10^4	0.678 ^c	–

^aPearson Chi-Square; ^b Continuity correction; ^c t test

Table 3 Identity of 27 HMPV whole genome sequences

Gene	Nucleotide sequence identity		Amino acid sequence identity	
	All the sequences	Our sequences	All the sequences	Our sequences
N	0.852–1	0.865–1	0.946–1	0.949–1
P	0.792–1	0.795–1	0.829–1	0.836–1
M	0.837–1	0.852–1	0.952–1	0.968–1
F	0.826–1	0.834–1	0.936–1	0.936–1
M2-1	0.845–1	0.856–1	0.935–1	0.946–1
M2-2	0.842–1	0.847–1	0.873–1	0.887–1
SH	0.676–1	0.692–1	0.553–1	0.564–1
G	0.475–1	0.48–1	0.225–1	0.242–1
L	0.837–1	0.839–1	0.935–1	0.937–1
Whole genome sequence	0.799–0.999	0.801–0.999	–	–

of the HMPV A genotype peaked between 2000 and 2003, and then plateaued from 2003 to 2009; after 2009, there was a further decline in population size. All G-180-nt-dup and G-111-nt-dup sequences were grouped into the A2b1 subtype. The A2b1 lineage most likely originated around 1992, whereas the A2b2 lineage probably originated from 1995. The time to the most recent common ancestor (tMRCA) of the HMPV genotype A2b1-180-nt-dup sequences and A2b1-111-nt-dup sequences was estimated at approximately 7.283 and 4.768 years, respectively. The A2b1-111-nt-dup sequences were derived from A2b1-180-nt-dup sequences. The evolutionary rate of HMPV genotype-A G gene was approximately 3.654×10^{-3} substitution/site/year (95% highest probability density of 3.1303–4.0357). The sequence identity between A2b1 and A2b2 was 0.615–0.968, and the

sequence identity between A2b1-180-nt-dup and A2b1-111-nt-dup was 0.847–0.89.

Discussion

The serological study showed that HMPV has been globally circulating since 1958 and was identified late in 2001 because of the difficulty encountered in isolating and culturing this virus in vitro [1]. Recently, the novel variants with G-111-nt and G-180-nt dup variants have been detected in countries around the world, including Japan, Spain, Croatia, and China [8–10, 15–17], highlighting the risks of HMPV epidemics. In this study, we obtained 27 HMPV genome

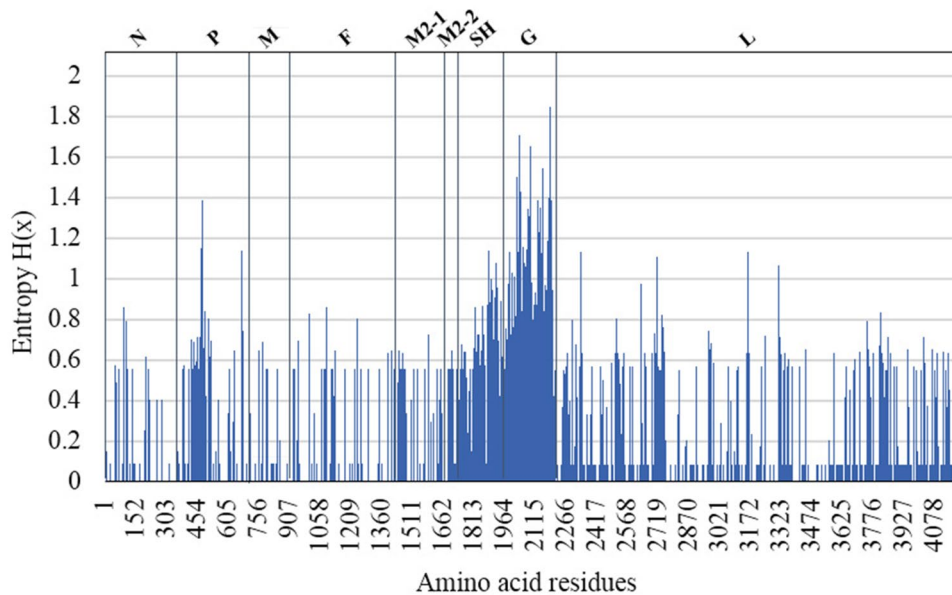


Fig. 1 Sequence positional entropy (SPE) analyses of amino acid residues of the HMPV protein coding region

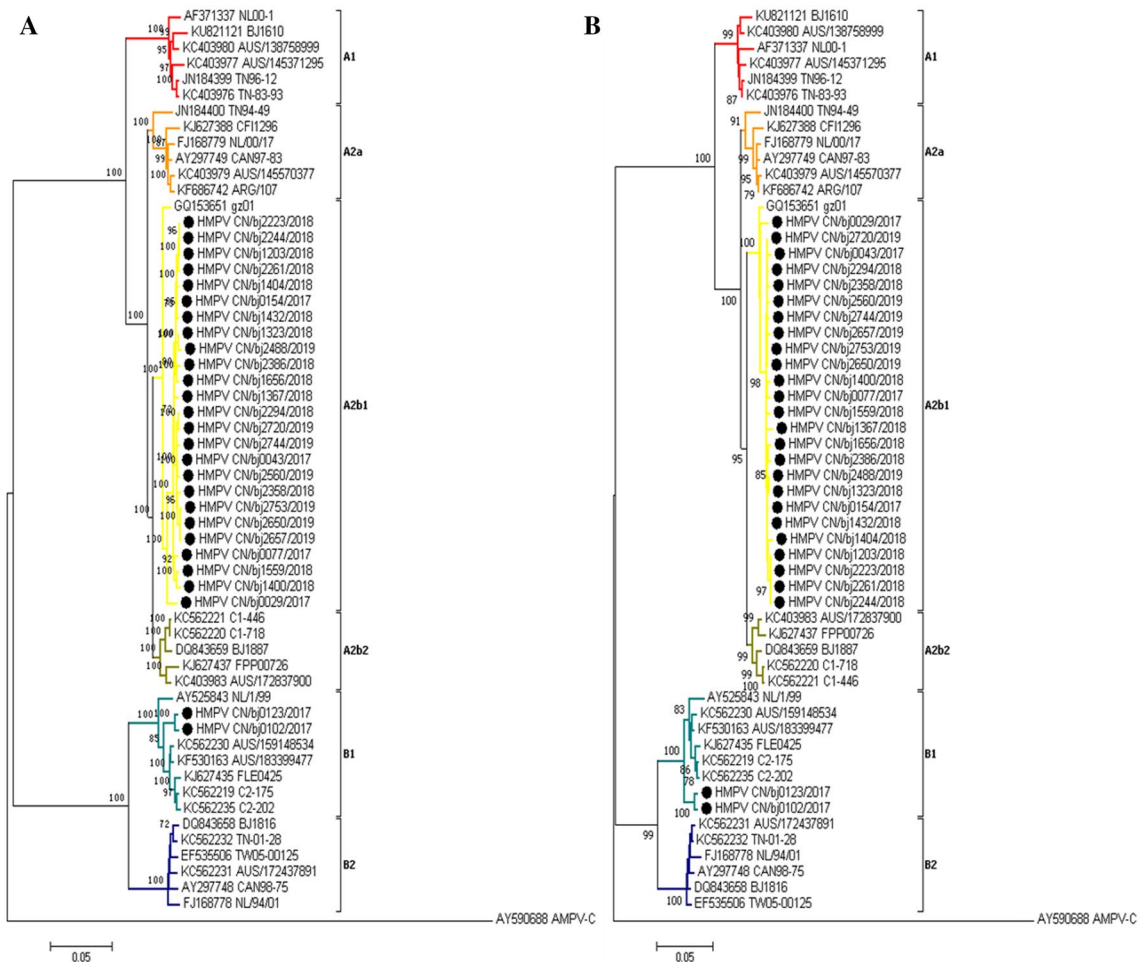


Fig. 2 Phylogenetic relationships of 27 HMPV strains. **A** Neighbor-joining (NJ)-tree based on the whole genome sequences. **B** NJ-tree based on the F gene sequences. The strains obtained in this study are marked with ●

sequences from NPAs of hospitalized children with ARTIs and, using PCR and Sanger sequencing, analyzed the phylogenetics of the HMPV sequences, especially the HMPV A genotype.

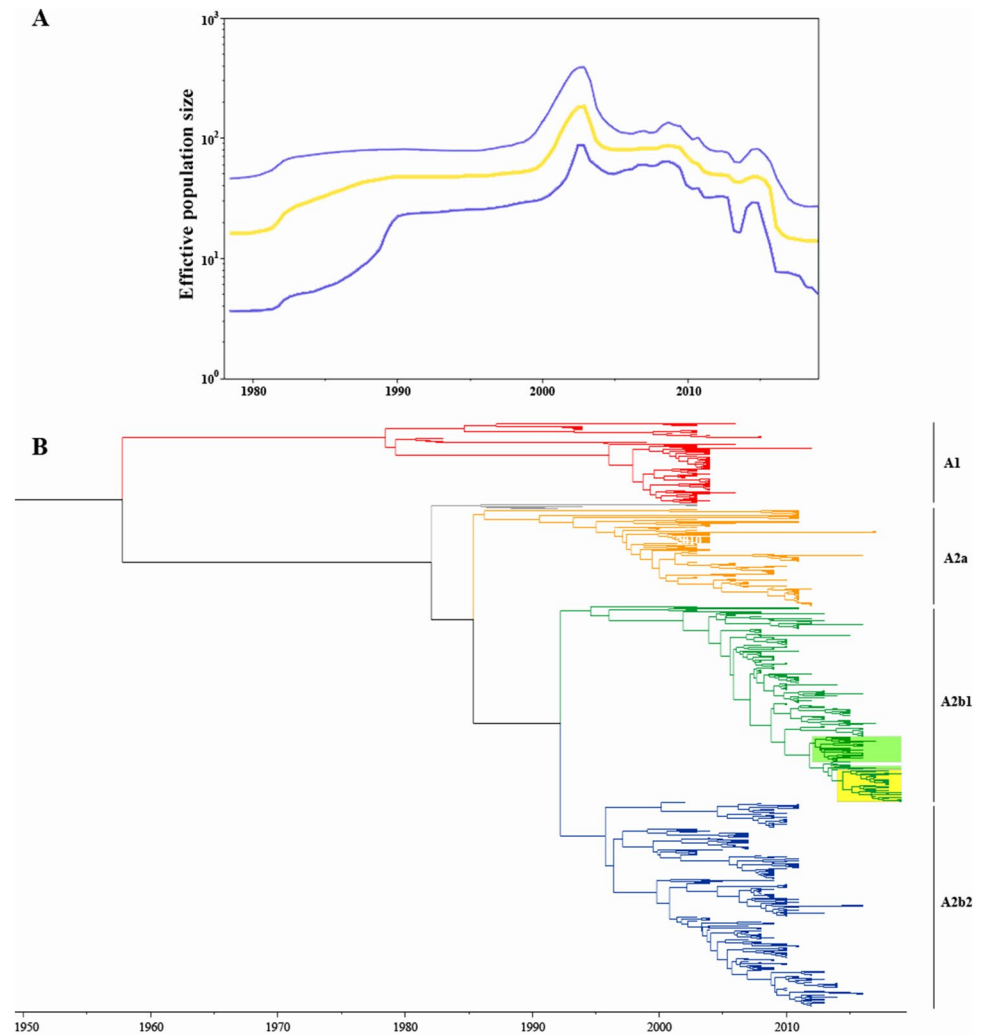
HMPV mainly causes respiratory infection in children between the ages of 2 and 5, although reinfection can occur throughout life [18]. HMPV mainly causes upper and/or lower respiratory tract infections while the clinical characteristics are difficult to distinguish from those of influenza-like symptoms. Lower respiratory tract infections due to HMPV can lead to pneumonia, bronchiolitis, and acute asthma exacerbations [19]. In this study, we screened samples from hospitalized children with respiratory infections over two years, and the HMPV infection rate was 6.18% (176/2828). HMPV infection has no gender significant difference. The median age of HMPV-infected children was 36 months, and the HMPV prevalence of patients aged ≤ 4 years was significantly higher than that of patients aged 4–14 years (6.86% vs 4.50%, $p = 0.018$). The season distribution result showed

that HMPV infection mainly occurred in spring (42.05%) and winter (37.50%), which was consistent with prior studies [20, 21] ALL HMPV positive patients were diagnosed with LRTI, main clinical symptoms included cough, fever and rhinorrhoea. HMPV infected patients under 4 years of age were more likely to have respiratory symptoms than patients aged 5–14 years, such as rhinorrhoea (58.99% vs 21.62%, $p < 0.001$) and nasal obstruction (29.5% vs 16.22%).

The sequence identity and the sequence positional entropy analyses showed that HMPV genome sequences have low genetic diversity, and no recombination event was detected in 27 obtained sequences. The two protein coding regions most susceptible to mutation are the G and SH genes, which is consistent with a previous report [22, 23]. The HMPV F gene has two important functional structural sites (cleavage site: RQSR and integrin $\alpha\beta 1$ binding site: RGD), and no mutation was detected in this study.

THMPV has five sub-genotypes (A1, A2a and A2b, B1, and B2). In recent years, the novel subtypes A2c, A2b1, and

Fig. 3 Demographic history of HMPV G gene. **A** Population growth inferred via a Bayesian Skyline coalescent tree. The blue lines represent the 95% highest probability density of the effective population size while the yellow line indicates the median of the size and genetic diversity of the population. **B** Maximum clade credibility tree of the HMPV G gene constructed using BEAST (version 1.10.5) under the skygrid nonparametric coalescent model. The green box indicates A2b1 180-nt-dup strains, and the yellow box indicates A2b1 111-nt-dup strains



A2b2 were proposed based on a short portion of the F gene and limited numbers of HMPV strains [6, 24], and these subtypes have subsequently been identified by a variety of methods. However, Nao, et al. [25] have demonstrated that subtypes A2b2 and A2c are separate descriptions indicating the same subtype, and the detailed evolutionary relationships between these subtypes are still unclear.

In this study, a NJ-Tree was constructed using HMPV whole genome sequences, the result showed that A2b can be divided into 2 clusters, A2b1 and A2b2. In the 27 obtained sequences, 25 (92.59%) grouped into the A2b1 lineage, and the other two sequences grouped into the B1 lineage. Phylogenetic analyses of HMPV F gene sequences showed the same results, and 96% of these sequences contained an 111-nt dup in the G gene, which is consistent with results from Japan and Luohe, China [17, 26]. Although the G protein is unnecessary for HMPV infection [27], it may improve the transmission of the HMPV subgroup A2b strain [26]. The same nucleotide duplication events have also been seen in the G gene of the human respiratory syncytial virus, another

member of the *Pneumoviridae* family, which has become the dominant strain globally [28–30]. The MCC tree results showed that the A2b genotype formed two branches as early as 1992, and the A2b1-180-nt dup lineage most likely originated in 2011, which is consistent with a previous study [8]. A2b1-111-nt-dup sequences were derived from A2b1-180-nt dup sequences around 2014. The evolutionary rate of the HMPV A genotype G gene was approximately 3.654×10^{-3} substitution/site/year as shown previously [8]. The demographic population history of HMPV genotype A implied outbreaks in effective population size since 2001; this may have been caused by the discovery of HMPV in 2001, and many studies have focused on the evolution of HMPV since then. However, after 2009, there was a decline in HMPV genotype A population size, this may be due to the improve of medical and health conditions in many places.,.

In conclusion, HMPV is an important virus in paediatric patients, especially in children between 2 and 5 years old. In this study, 27 HMPV whole genome sequences were obtained, and the character of their whole genome sequences

were analysed. Most regions of the HMPV genome were conserved, although those of the G gene was the most variable. Phylogenetic analysis showed that 25 obtained HMPV sequences belong to a newly defined subtype A2b1 and that the G gene of 24 sequences contained a 111-nt duplication. The role of the 180-nt-dup and 111-nt-dup remains unclear, which is a reminder for us to continuously monitor this new subtype and investigate the pathogenicity of the new subtype.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11262-023-02001-2>.

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Data Availability Availability of data and materials condensed anonymized data are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors declare no competing interests.

Ethical approval The project was approved by the Ethical Committee of National Institute for Viral Disease Control and Prevention, China CDC, and the committee's reference number is IVDC2017-021.

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