Genetic Analysis of Human Parainfluenza Virus Type 4-Associated with Severe Acute Respiratory Infection Among Children in Luohe City, Henan Province, China, During 2017-2018

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Abstract

During 2017–2018, Nasopharyngeal aspirates (NPAs) from 627 hospitalized patients with SARI at Luohe Center Hospital were tested by RT-PCR for human parainfluenza virus 4(HPIV-4). 14 (2.2%) of 627 samples were positive for HPIV-4. The complete nucleotide sequence of the HN gene from 9 positive samples was amplified and sequenced successfully. Genetic analysis showed that the HPIV-4 strains circulating in Luohe city are more closely related to HPIV-4A. Our study indicated that there were multiple lineages of HPIV-4 circulating in Henan Province in China during the study period, which will improve our understanding of the epidemiological and clinical characteristics of HPIV-4.

Main Text

Human parainfluenza viruses (HPIVs), belonging to the family *Paramyxoviridae*, are enveloped, negative, single-stranded RNA viruses [1, 2]. Based on genetic and antigenic variation, HPIVs have been divided into four serotypes into 2 different genera: *Respirovirus* (HPIV-1 and HPIV-3) and *Rubulavirus* (HPIV-2 and HPIV-4) [3, 4]. Globally, HPIVs account for a significant proportion of acute respiratory infections (ARIs) among children under the age of 5 years [5, 6]. HPIV-4 was first identified in 1959 by Johnson et al. [7] and was formerly associated with mild respiratory illness in young people. However, recent studies indicate that it can cause more severe infections, such as pneumonia and bronchiolitis, in children and elderly individuals [8-12] and even in immunocompetent individuals and critically ill patients [13-15].

HPIV-4 is subdivided into two subtypes, HPIV-4A and HPIV-4B, based on hemagglutination inhibition and neutralization tests [16]. However, although studies associated with HPIV-4 infection have increased globally, possibly due to improved and increased diagnostic testing, the molecular characteristics of regional and global circulating HPIV-4 strains have not been fully elucidated.

In this study, 627 nasopharyngeal aspirates (NPAs) were collected and screened for HPIV-4 infection from hospitalized patients with severe acute respiratory infection (SARI) in Luohe city, Henan Province, China, during 2017-2018. Informed consent was obtained from patients or their guardians for the donation of their samples involved in this study. This study was approved by the second session of the Ethics Review Committee of the National Institute for Viral Disease Control and Prevention (IVDC) of the Center for Disease Control and Prevention (CDC) in China, and the methods were conducted according to the guidelines. SARI cases were identified according to the sentinel surveillance program for hospitalized SARI cases in China (http://www.gov.cn/zwgk/2011-02/11/content_1801649.htm).

The samples were transported to the IVDC of the China CDC under cold chain for further identification. Specimens were stored in sterile minimal medium at -20 or -80°C pending molecular analysis. All methods were performed in accordance with the relevant guidelines and regulations.

Viral RNA was extracted from NPAs samples using a QIAgen viral RNA mini kit(QIAgen, Valencia, CA, USA), and HPIV-4 positive samples were screened by one-step real-time RT-PCR using a PrimeScript^{TM} RT-PCR Kit (Takara Biotechnology Dalian, China, cat: DRR064A) as described previously [17]. The complete

hemagglutinin-neuraminidase (HN) gene (1740 nt) of HPIV-4 was amplified by nested RT-PCR for genotyping HPIV-4. The first round of RT-PCR was performed using the following in-house designed primer pair: forward primer, 5'- ATAGGGGGGAACRCACTTCTCAGC-3'; reverse primer, 5'-GGCRGRTTGTTTRTYGAGGACC-3'. The nested PCR was carried out with the inner primers forward primer, 5'-AACAATCCAGARRGACRTCACATCAA-3' and reverse primer, 5'- TCTTTCAGTGGATGGTTGAGGA-3'. The RT-PCR products were purified for sequencing using a QIAgen quick gel extraction kit (QIAgen, Hilden, Germany), and the amplicons were sequenced on an ABI PRISM 3100 DNA Sequencer (PerkinElmer, Beijing, China). Nucleotide sequences were assembled and edited using Sequencher (Gene Codes Corporation, Ann Arbor, MI, USA). The sequences of HPIV-4 in our study have been submitted to GenBank (the accession numbers are MT681670-MT681678).

Pairwise distances of nucleotide and deduced amino acid sequences were aligned with 43 HPIV-4 strains from GenBank using the ClustalW algorithm implemented in MEGA software version 7.0. Phylogenetic analyses based on complete HN gene nucleotide sequences were performed (MEGA version 7.0) by the maximum likelihood method using Kimura 2-parameters as the substitution model, with statistical significance of phylogenies estimated by bootstrap analysis with 1,000 replicates.

From October 2017 to December 2018, a total of 627 NPAs from inpatients (391 males and 236 females) who met the SARI case definition were collected from Luohe Central Hospital in Henan Province. The hospitalized patients with SARI were aged from 0 to 91 years, among which children under 6 years old accounted for 78.0% (489/627). The median age was 7.9 years.

Of the 627 patients, 14 (2.2%, 95% CI 1.1-3.4) patients were positive for HPIV-4 by real-time RT-PCR. The cycle threshold (CT) values for the HPIV-4-positive samples ranged from 25 to 35. Most of the CT values of the positive samples ranged from 29 to 33. The ratio of males to females was 1.7:1. Among the 14 patients (median 2.5 years) with determined HPIV-4, all of them were younger than 7 years old, and 50% ranged from 0 to 3 years of age (Table 1). Among the pediatric patients, 10 (71.4%) were diagnosed with bronchopneumonia, 3 (21.4%) with bronchiolitis, and 1 (7.1%) with mucocutaneous lymph node syndrome (MCLS).

The seasonality of HPIV-4 was observed throughout the study period. The positive rates of HPIV-4 in spring, summer, autumn, and winter were 1.2% (1/85), 17.5% (10/57), 0.5% (1/199), and 0.7% (2/286), respectively (Figure 1). The highest detection rates of HPIV-4 were found in summer, and the detection rate of HPIV-4 varied significantly between seasons ($\chi^2 = 67.456$, $P = 0.000$).

The complete HN gene sequences of 9 HPIV-4 (CT values between 25 and 33) isolates amplified from 14 HPIV-4-positive specimens were analyzed. The HN gene failed to amplify from the remaining 5 HPIV-4-positive specimens, which had CT values ranging from 27 to 35 in the real-time RT-PCR. This could be due to mismatches in the primer-binding sites or low viral load.

All HPIV-4 HN gene sequences available in GenBank (43 sequences) were downloaded and aligned with the 9 sequences obtained in this study. Among the 9 HPIV-4 HN gene sequences, 7 sequences clustered

with the HPIV-4A reference strains, and the remaining 2 clustered with the HPIV-4B reference strains (Figure 2). By convention, the prototype of HPIV-4, which was isolated in Japan in 1959, formed a single lineage designated cluster Ⅰ. The strains from Denmark in 2013, Japan in 2010, and Australia in 2008 consisted of cluster Ⅱ, with a mean nucleotide divergence of 1.7%. The most viruses from Asia, with a mean nucleotide divergence of 1.7%, were clustered together and designated cluster Ⅲ. The mean distance between the three clusters is 5.4% and is larger than the mean distance within the three clusters (1.7%), in reference to a report that described phylogenetic analysis of HPIV-3 [18]. Cluster III strains were further grouped into four lineages (lineages 1, 2, 3 and 4) with 1.8-2.7% nucleotide divergence. In our study, 6 HPIV-4A strains belonged to the lineage 4 group together with the previously reported 4 strains from Japan. The remaining HPIV-4A strain was placed into lineage 3 with 7 strains from Japan. Additionally, 2 strains found in this study were identified as HPIV-4B, which has been rarely reported in recent decades.

The divergence between the 7 HPIV-4A strains in our study and the prototype strain M-25 was approximately 6.6%-6.9% and 10.1%-11.0% in the nucleotide and amino acid sequences, respectively. The two HPIV-4B isolates revealed 4.9%-5.0% nucleotide divergence and 8.0%-8.2% amino acid divergence compared with the prototype strain. Notably, the length of the HN gene sequence (1740 nt) in our 9 strains is longer than that of the prototype strain (1725 nt) by 15 nucleotides (5 amino acids) at the carboxy-terminus. Compared with other strains, the divergence of nucleotides and amino acids among HPIV-4A was 1.0%-3.7% and 0.3-3.4%, respectively. Among HPIV-4B, it was 0.5-4.9% and 0.5-8.4%, respectively. Briefly, there are two common subtypes of HPIV-4 in China, HPIV-4A and HPIV-4B, of which HPIV-4A includes two lineages of cluster Ⅲ.

In the present study, 14 samples positive for HPIV-4 from 627 (2.2%) SARI patients collected between October 2017 and December 2018 were analyzed to determine the epidemiology of HPIV-4 in Luohe city, Henan Province, China, by using q-RT-PCR. All patients with HPIV-4 infection were less than 7 years old, 57% of who were younger than 3 years, indicating that children of this age group are the main HPIV-4-susceptible population. This result was similar to the results of previous reports. The positive rate of HPIV-4 was 1.2% among 0.5-month-old to 16-year-old patients, especially 3-5-year-old children in Beijing, China, and the median patient age was 4.1 years old in Colorado between 2009 and 2012 [19, 20]. Therefore, HPIV-4 may be gradually becoming an important pathogen that causes SARI in children, which is more severe than previously thought.

In our study, HPIV-4 infections occurred during summer and autumn, especially in summer, which is inconsistent with published studies from other countries. In Japan, Abiko et al. [8] described an outbreak of HPIV-4 infections during the 2011-2012 winter season. The same result was reported in Canada during 2004-2005 [21]. This finding may be attributed to different geographical regions and research times. However, as the number of positive samples is limited in this study, a large-scale investigation of the HPIV-4-positive rate is needed to understand the seasonal patterns of HPIV-4. Additionally, considering that only strains from hospitalized patients were analyzed in this study, the possibility that other underestimated strains could be circulating in the general population cannot be ruled out.

Previous studies reported that HPIV-4 strains were detected at different times in different regions [21-23]. In our study, we characterized the HN gene from HPIV-4-positive samples from patients with SARI and further divided all of the HPIV-4A strains into three clusters. This study is the first report that has described the phylogeny of HPIV-4 based on the complete HN sequence. Compared with the HPIV-4 prototype strain M-25, 9 strains in our study, including other strains from GenBank, demonstrated significantly different characteristics. A five amino acid insert in the HN protein of Luohe strains may result in changes in the antigen-binding site and have a subsequently severe influence on viral replication and transmission. This finding also indicates that the viruses are probably evolving.

Phylogenetic analysis revealed 5 (Henan-SA20180251/247/303/268/292) of 7 HPIV-4A strains that might share a chain of transmission. The remaining two HPIV-4A strains were similar to the Japanese strains between 2010 and 2011. As the phylogenetic tree shows, it is suggested that the domestic HPIV-4A strains belonged to lineage 4 of cluster □, which seems to be prevalent. Moreover, two HPIV-4B strains were associated with three USA HPIV-4B strains isolated during 2015-2016. This result reveals that the HPIV-4 strains were undergoing global circulation and more closely related to HPIV-4A than HPIV-4B, based on the HN gene sequences in our study. However, additional sequences are needed to expand the dataset, and additional studies are required to confirm the relative importance of HPIV-4A and HPIV-4B.

In conclusion, we reported the HN gene sequences of 9 HPIV-4 strains isolated from SARI patients. The divergence among HPIV-4 strains indicated that these viruses have circulated in the environment for many years and have undergone evolution. To better recognize its clinical importance and seasonal patterns, HPIV-4 should be included in the routine panels of respiratory virus detection, although most clinical laboratories do not screen for HPIV-4 [23, 24]. This report provides valuable information about HPIV-4 isolates to help prevent HPIV-related respiratory diseases.

Declarations

Acknowledgments

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Authors' contributions

SSZ, NYM and WBX prepared the manuscript. RPH, JX and WBX designed and coordinated the study. YZ, ALC and ZZ collected the specimens and performed the experiments. SSZ, NYM, YZ, ALC, ZZ and WBX performed the data analysis. All authors read and approved the final manuscript.

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Data availability

All data included in this study are available upon request from the corresponding author.

Conflict of interest

The authors declare that they have no competing interests.

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Table

Table 1. Sex and age distribution of HPIV-4-positive patients

	Number of total specimens	Number positive for HPIV-4 (%)
Sex		
Males	391	8 (2.0%)
Females	236	6 (2.5%)
Age group (years)		
<1	77	1 (1.3%)
≥1 to <3	238	7 (2.9%)
≥3 to <6	174	5 (2.9%)
≥6 to <14	67	1 (1.5%)
≥14	71	0
Total	627	14 (2.2%)

Figures

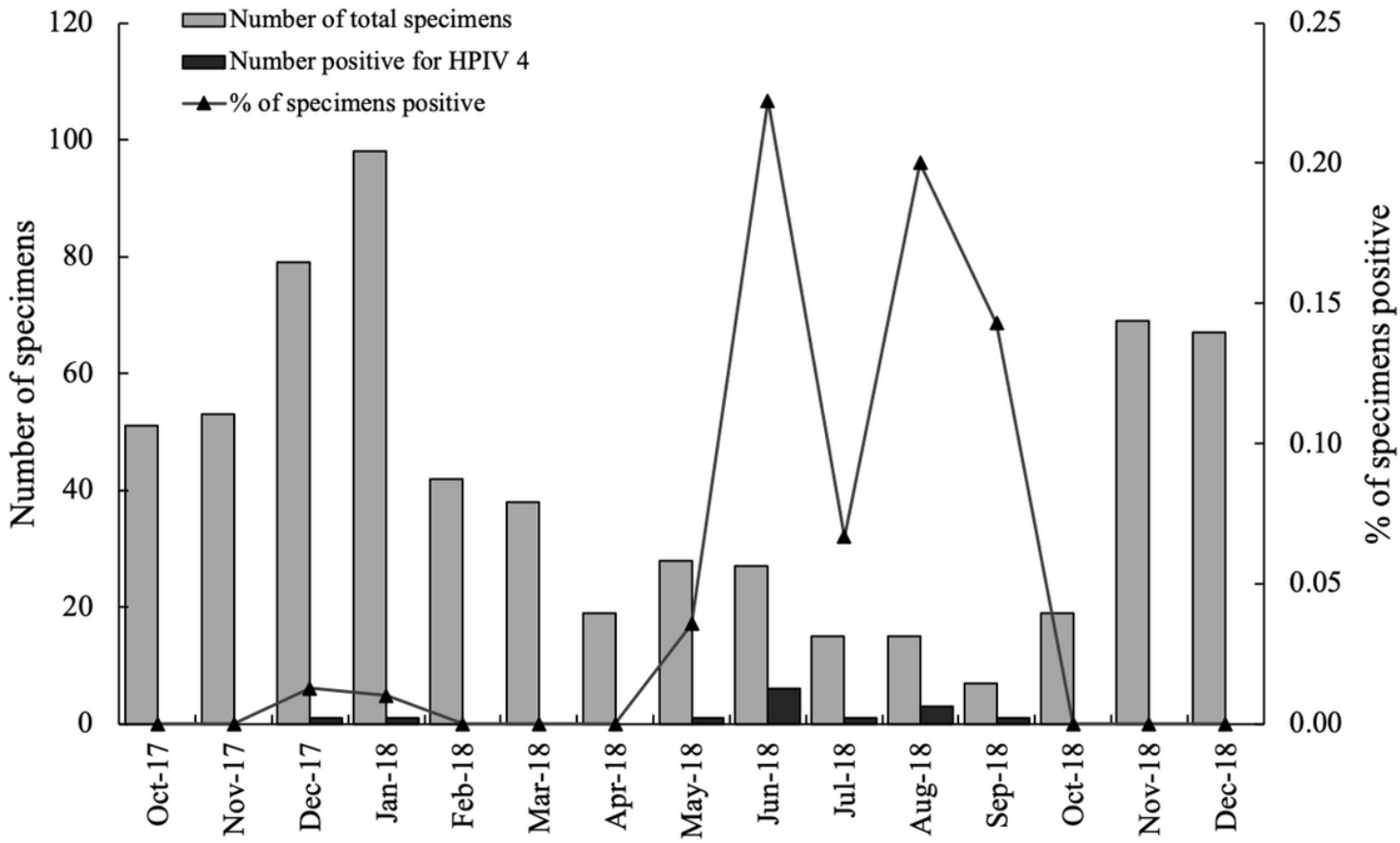

Figure 1

Monthly distribution of human parainfluenza virus 4 (HPIV-4) infections

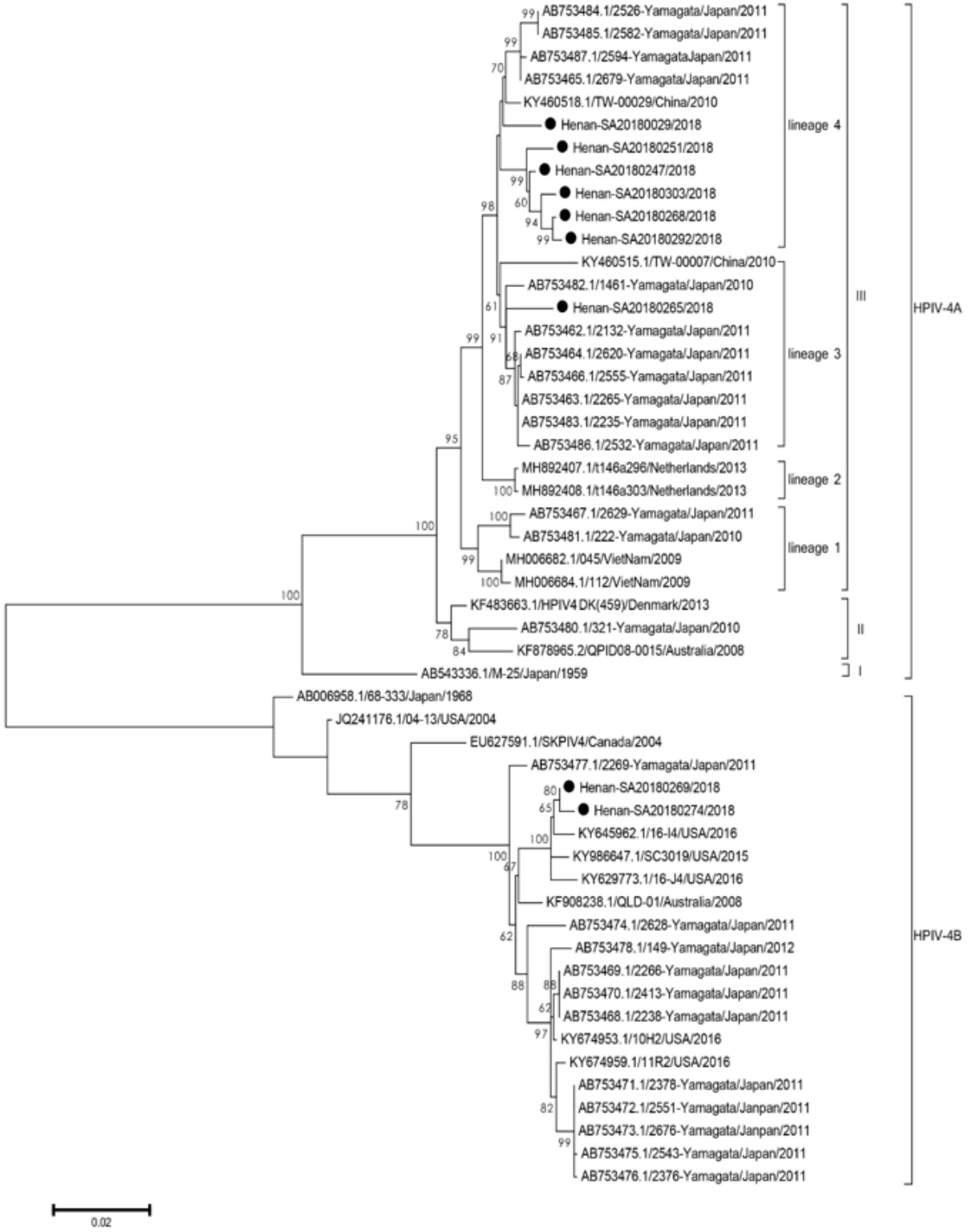

Figure 2

Phylogenetic tree based on complete HN gene nucleotide sequences of HPIV-4 strains

Supplementary Files

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- Submission.txt