Detection of enterovirus 68 as one of the commonest types of enterovirus found in patients with acute respiratory tract infection in China

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INTRODUCTION

Human enterovirus 68 (HEV-68) is an enterovirus associated with respiratory illness. In China, no information about HEV-68 is available for children yet. This study aimed to investigate the presence of HEV-68 in mainland China between 2009 and 2012 and to explore the migration events of HEV-68 across the world. Among 1565 samples tested from children, 41 (2.6 %) were positive for HEV and 223 (14.3 %) for human rhinovirus (HRV). Seven (17.1 %) of 41 HEVs were HEV-68. Two HEV-68- and five HRV-positive samples were detected in 585 adult samples. HEV-68 is the predominant type of enterovirus in children with acute respiratory tract infection (ARTI), followed by HEV-71 and coxsackievirus A6. Three HEV-68-infected children presented with severe pneumonia and one presented with a severe asthma attack. The viruses were attributed to two novel distinct sublineages of HEV-68 based on phylogenetic analysis of partial VP1 gene sequences. Migration events analysis showed that the USA and the Netherlands were possible geographical sources of HEV-68, from where three strains migrated to China. In conclusion, HEV-68 may play a predominant role among the enteroviruses associated with ARTI in children. Additional surveillance is needed to clarify the reason why HEV-68 causes such a wide spectrum of disease, from asymptomatic to severe respiratory disease and even death.

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Abbreviations: ARTI, acute respiratory tract infection; CV, coxsackievirus; EBV, Epstein–Barr virus; HEV, human enterovirus; HRV, human rhinovirus.

The GenBank/EMBL/DDBJ accession numbers for the HEV-68 sequences determined in this study are JQ924862–JQ924867 and JX898784–JX898786.

One supplementary table and four supplementary figures are available with the online version of this paper.
HEV-68 is an enterovirus associated with respiratory illness that shares epidemiological and biological features with human rhinoviruses (HRVs) (Oberste et al., 2004). Although isolated cases of HEV-68 have been reported since the virus was first described in 1962, clusters of cases have been recognized only recently. During 2008–2010, case clusters of HEV-68 infection were reported in Asia, Europe and the USA associated with respiratory illness ranging from relatively mild illness to severe illness requiring intensive care and mechanical ventilation, or even to death. Currently, HEV-68 has been highlighted as an increasingly recognized cause of respiratory illness (CDC, 2011; Hasegawa et al., 2011; Imamura et al., 2011; Rahamat-Langendoen et al., 2011; Jacobson et al., 2012; Meijer et al., 2012). Although several phylogenies in the article have labelled HEV-68 clades by geographical region, none has analytically inferred the history of the virus's migration.

During 2009–2012, we performed a retrospective surveillance study on hospitalized children and outpatient adults with ARTI to analyse the epidemiological and clinical features of HEV-68-related respiratory tract diseases in China. Moreover, a phylogeographical analysis was carried out to infer migration patterns of HEV-68.

**METHODS**

This retrospective study was conducted at three hospitals as part of a viral surveillance of ARTI cases between June 2009 and June 2012. ARTI was determined based on acute onset and syndromes of cough, rhinorrhoea and/or dyspnoea and/or fever of more than 37.5 °C. Nasopharyngeal aspirates were collected from children who were hospitalized for ARTI at respiratory clinics at the Children’s Hospital, Chongqing Medical University, Chongqing, China. Nasopharyngeal swabs were collected from adult outpatients with ARTI in two hospitals located in Beijing and Tianjin.

A standardized questionnaire was designed to obtain information on the hospitalized children from medical records, including demographic characteristics, underlying medical conditions, symptoms and signs, laboratory tests, radiographic findings, sputum culture results and disease outcome. The study protocol was reviewed and approved by the Ethics Review committee of the three hospitals in which the study was performed. Written informed consent was acquired from parents or guardians of all participants. Information on cytomegalovirus, Epstein–Barr virus (EBV), Chlamydia pneumoniae and Mycoplasma pneumoniae infection was obtained by testing IgM and/or IgG antibodies using ELISA or passive particle agglutination [Cytomegalovirus IgG antibody diagnostic kit and Cytomegalovirus IgM antibody diagnostic kit Zhuhai S.E.Z. Haitai Biological Pharmaceuticals Co.; Anti-EBV-CA-ELISA (IgM) and anti-EBV-CA-ELISA (IgG), EUROIMMUN Medizinische Labordiagnostika AG; Chlamydia pneumoniae IgM antibody diagnostic kit, DIA.PRO Diagnosis Bioprobes Srl; Diagnostic kit for Measurement of Antibodies to Mycoplasma pneumoniae, Fujiirebio].

RNA was extracted from each specimen using a QIAamp MinElute Virus Spin kit (Qiagen) and the cDNA sample was synthesized by using a SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). HRV and HEV were detected by PCR, and amplicons were further sequenced using the primers of 5’ non-translated sequences from a previous study (Imamura et al., 2011). The samples were also screened for influenza virus (A, B and C), metapneumovirus, respiratory syncytial virus (RSV), parainfluenza virus (types 1–4) and coronaviruses (229E and OC43) using molecular methods described previously (Tiveljung-Lindell et al., 2009).

The VP1 gene sequence was amplified and sequenced as described previously (Rahamat-Langendoen et al., 2011). The genomic sequences were assembled using Lasergene’s DNA SeqMan software version 7.1.0 (DNA Star). All comparison alignments were performed and a phylogenetic tree was reconstructed by the neighbour-joining method with 1000 bootstrap pseudoreplicates using CLC Genomic Workbench 5. Similarities between strains were calculated using BioEdit version 7.1.3 (http://www.mbio.ncsu.edu/bioedit/bioedit.html). All statistical analyses were conducted using SAS version 9.13 (SAS Institute).

From GenBank, we obtained all available partial HEV-68 VP1 gene sequences with a minimum length of 369 nt isolated between 1962 and 2012 across 12 geographical locations, comprising Japan (Osaka and Yamagata), the USA, the Netherlands, South Africa, New Zealand, Gambia, Senegal, Italy, the UK, France and China (detailed information is available in Table S1 available in the online Supplementary Material). Character state changes were ordered and phylogenetic trees reconstructed with maximum-parsimony using PAUP version 4.0b10. The statistical phylogeography was performed using Statistical Analysis of Migration Events through a Phylogeny (MigraPhyla), as reported previously (Wallace et al., 2007). A Monte Carlo test of 10 000 trials was conducted to determine the probability that the frequencies of migration events between each pair of localities in the original migration tree were more than expected when the localities were randomly distributed across the tree’s tips. Delayed transformation (DELTTRAN) was used to infer the migration events among localities through the gene tree. The nominal type I error rate was corrected for multiple tests (family) across locality pairs to test the significance of the $P$ values. For greater power generated by reducing the number of comparisons, the stratified false discovery rate (sFDR) was performed to minimize the fraction of wrong rejections among rejected hypotheses (Buonaguro et al., 1986).

**RESULTS**

A total of 1565 nasopharyngeal aspirates (145 from 2009, 446 from 2010, 698 from 2011 and 276 from 2012) were collected from children aged 1 month to 14 years (median: 9 months). Altogether 585 nasopharyngeal swabs (203 from 2009, 290 from 2010 and 92 from 2011) were obtained from adult outpatients aged from 17 to 96 years (median: 28 years). The proportion of males was 66.1% (1034) in children and 58.6% (343) in adults.

Among the paediatric patients, 41 (2.6%) were detected as positive for HEV and 223 (14.3%) for HRV. The proportion distribution of serotypes is shown for HEV in Fig. 1. HEV-68 was detected in seven (17.1%) of the 41 paediatric patients that were HEV positive and thus was the predominant type of enterovirus in children with ARTI, followed by HEV-71 and CV-A6. In adult patients, two HEV-68- and five HRV-positive samples were detected.

The clinical and epidemiological characteristics of patients with HEV-68-positive detection are shown in Table 1. All paediatric patients had an admission diagnosis of pneumonia, accompanied by asthma exacerbation in three patients. Three children had respiratory failure. Five
HEV-68 infections were detected concurrently with other viruses, mainly with RSV. Three and five HEV-68-positive cases were found between August/October and December in 2010 and 2011, respectively. One case was found in March 2012.

For HEV-68-positive patients, the most common clinical signs and symptoms were fever, cough, moist rales, expectoration and diarrhoea, which were indistinctive from other HEV infection symptoms (Table 2). The hospital duration ranged between 3 and 9 days. All seven paediatric patients presented abnormal chest radiographic findings, including increased lung markings and/or interstitial change and/or overinflation. In contrast to the children, both adults had sore throat, headache and fatigue as well as myalgia. All nine patients recovered well, with no deaths reported. For HRV infections, cough, expectoration and moist rales were the main clinical manifestations, accompanied by a high white blood cell count and a high rate of co-infection with other pathogens.

Sequence analysis of VP1 revealed that the HEV-68 isolates detected in the study had nucleotide sequence similarities of 69.5–99.8% with each other, and similarities of 70.8–92.4% with NCY403/JPOC10-378 strains (GenBank accession numbers JX101846/AB601883). On the phylogenetic tree based on VP1 gene nucleotide sequences of 756 bp, all modern HEV-68 strains grouped into two separate lineages (lineages 1 and 2, Fig. 2) and the two lineages formed five and four sublineages, respectively (Fig. 2, detailed expansion in Fig. S1). The CQ5585, CQ5584, CQ5735 and CQ5759 strains formed a group and then clustered with KC763162 (Italy 2012), which fitted into sublineage 1.3. The three strains BJ5234, TJ3395 and CQ5914, together with the CQ4278 and AB667899 (Yamagata 2008) strains were clustered in sublineage 2.2. The CQ5378 strain was located in sublineage 2.3 branch. On adding the strains from the UK, the strains from 2009 and 2010 were grouped into sublineages 1.2 and 2.3, respectively (Fig. S2), and no obvious changes were found on the new phylogenetic tree constructed with 601 bp of VP1 gene nucleotide sequences compared with the former. When adding other sequences of the strains from China in 2006, these grouped into sublineage 2.2 observed from the 369 bp phylogenetic tree

Table 1. Characteristics of patients with positive HEV-68 detection between 2010 and 2012, China

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age/sex</th>
<th>Disease onset date</th>
<th>Signs/symptoms</th>
<th>Diagnosis</th>
<th>Co-detected pathogens</th>
</tr>
</thead>
<tbody>
<tr>
<td>CQ-3578</td>
<td>2 years/M</td>
<td>Sep 2010</td>
<td>Fever, cough, nasal discharge and sputum production</td>
<td>Pneumonia</td>
<td>None</td>
</tr>
<tr>
<td>CQ-4278</td>
<td>1 years/M</td>
<td>Dec 2010</td>
<td>Fever, cough, sputum production and diarrhoea</td>
<td>Pneumonia, asthma</td>
<td>HBoV, RSV and Streptococcus pneumoniae</td>
</tr>
<tr>
<td>CQ-5584</td>
<td>10 years/M</td>
<td>Oct 2011</td>
<td>Fever, cough, wheezing, sputum production and respiratory failure</td>
<td>Severe pneumonia, severe asthma</td>
<td>None</td>
</tr>
<tr>
<td>CQ-5585</td>
<td>3 years/M</td>
<td>Oct 2011</td>
<td>Fever, cough and sputum production</td>
<td>Pneumonia</td>
<td>EBV, CP and MP</td>
</tr>
<tr>
<td>CQ-5735</td>
<td>10 years/M</td>
<td>Dec 2011</td>
<td>Fever, cough, wheezing, chest pain and respiratory failure</td>
<td>Severe pneumonia, moderate asthma</td>
<td>RSV, CMV and CP</td>
</tr>
<tr>
<td>CQ-5759</td>
<td>4 months/F</td>
<td>Dec 2011</td>
<td>Fever, cough, wheezing, sputum production, diarrhoea and respiratory failure</td>
<td>Severe pneumonia</td>
<td>RSV</td>
</tr>
<tr>
<td>CQ-5914</td>
<td>2 months/M</td>
<td>Mar 2012</td>
<td>Fever, cough and diarrhoea</td>
<td>Pneumonia</td>
<td>Flu-A, RSV and CMV</td>
</tr>
<tr>
<td>TJ-3395</td>
<td>20 years/M</td>
<td>Aug 2010</td>
<td>Fever, cough, nasal discharge, sore throat, sputum production, chest pain, headache, fatigue and myalgia</td>
<td>Upper respiratory tract infection</td>
<td>None</td>
</tr>
<tr>
<td>BJ-5234</td>
<td>51 years/F</td>
<td>Aug 2011</td>
<td>Fever, sore throat, sputum production, headache, fatigue and myalgia</td>
<td>Upper respiratory tract infection</td>
<td>None</td>
</tr>
</tbody>
</table>

M, male; F, female; HBoV, human bocavirus; RSV, respiratory syncytial virus; EBV, Epstein–Barr virus; CMV, cytomegalovirus; CP, Chlamydia pneumoniae; MP, Mycoplasma pneumoniae; Flu-A, influenza A virus.
of VP1. Sublineages 1.3 and 2.2 were the main epidemic genotypes in China, different from other counties (Fig. S3; detailed expansion in Fig. S4).

The migration events inferred across the parsimony genetic phylogenies for the 187 VP1 sequences (601 bp) of HEV-68 from 13 locations among five continents, and their concatenation showed that the USA and the Netherlands acted as the geographical source (Table 3). The Monte Carlo analysis generated frequency distributions for each entry in the resulting migration matrix. sFDR correction applied to multiple comparisons tests identified one pathway with migration significantly greater than expected under the Monte Carlo distributions: from the Netherlands to the UK. Migration events also appeared in Japan, between Osaka and Yamagata. In China, three strains migrated from the USA, whilst one strain from Yamagata (AB667899) probably came from China.

**DISCUSSION**

In this study, we found that HEV-68 was the most prevalent enterovirus type in children in our samples from China, and was also detected in adults. However, the proportion distribution of the main enterovirus types in the children from our study differed from that in adults reported by Xiang et al. (2012) in China. Most prevalence studies on HEV-68 so far have been carried out on children with acute respiratory tract disease, and a prevalence ranging between 0.32 and 12.37% has been observed (CDC, 2011), with the exception of a higher rate (74.28%) in children with asthma exacerbation (Hasegawa et al., 2011). The prevalence of 0.3% in the current study is lower than the rates reported previously for children (2.57–3.64%) (CDC, 2011), although comparable with those reported by studies with a large sample size (Meijer et al., 2012). The discrepancies could be accounted for by differences in the characteristics of the study populations, as well as the seasonality of different HEV types. HEV-71 and CV-A6 were present at a higher proportion, although not statistically higher than the proportion of HEV-68. These may be also important pathogens causing ARTI in children.

HEV-68 is considered to occur disproportionately among children, with an especially high prevalence among children less than 5 years of age according to a previous study (CDC, 2011); however, a broad-range screen of HEV-68 in the Netherlands disclosed the highest prevalence of HEV-68-positive patients among persons aged 50–59 years (Meijer et al., 2012). Serocellular epidemiological studies in Finland showed that most adults may have been infected previously with HEV-68 (Smura et al., 2010). Our study also found HEV-68 infection in adults, providing further evidence that people of all ages are likely to be infected with HEV-68. Future serological studies are needed to determine the infection rates from both symptomatic and subclinical infections.

Information on the clinical characteristics of HEV-68 infections is limited, but recent studies show that HEV-68 infection presents from non-symptomatic, mild upper respiratory symptoms to severe respiratory disease requiring hospitalization, disease of the central nervous system and even death (Kreuter et al., 2011; Rahamat-Langendoen et al., 2011; Jacobson et al., 2012). In our study, three children with positive HEV-68 detection presented with severe pneumonia, in which two had both RSV and HEV-68 detected and the other had a single infection with...
HEV-68, although combined with a severe asthma attack. Whether there is a synergistic or combined effect of RSV and HEV-68 co-infection needs further investigation. HEV-68 infection may induce severe asthma attacks, especially in atopic children without a history of asthma or treatment (Hasegawa et al., 2011), which might act as a plausible explanation for the severe disease manifestation in this patient. Despite the positive detection, the causal inference between HEV-68 and the clinical manifestations warrants further investigation based on the serological study of patients with single virus infection. In contrast, body pain was the main symptom in adults, and this difference should be considered in the clinical diagnosis of HEV-68.

According to previous studies, the seasonality of HEV-68 infection falls between August and November, within or after the typical enterovirus season (CDC, 2011). The HEV-68 infections in China were also observed with a similar seasonality, which coincided with the temporal distribution of other respiratory viruses in the same area.

Previous studies by Meijer et al. (2012) and Tokarz et al. (2012) illustrated three clades or lineages of HEV-68 based on the VP1 nucleotide sequences of HEV-68. However, when more sequences collected from GenBank and the strains from China were also included, two lineages of the phylogenetic tree were formed, as found by Lauinger et al. (2012). In every lineage, several sublineages appeared, and the strains from China were located in three sublineages, also including other Chinese strains detected by Xiang et al. (2012). Sublineages 1.3 and 2.2 were expanded by the Chinese strains, or, rather, the strains from China formed the two sublineages. Other strains in the two sublineages may have migrated from China.

The migration events of HEV-68 across the world were confirmed by the spatial dynamics analysis in our study.

Fig. 2. Phylogenetic analysis of HEV-68 based on VP1 constructed using a 756 nt sequence corresponding to nt 2389–3144 in the Fermon strain, from the USA (GenBank accession no. AY426531). A tree with 1000 bootstrap replicates was reconstructed using the neighbour-joining method with the rgw CLC Genomic Workbench 5 software package based on nucleotide sequences. The location and year of collection are shown in parentheses. The strains detected in this study are in red. Bar, nucleotide substitutions per site.
The USA and the Netherlands were considered as the sources of migration events of HEV-68 and one pathway passed the sFDR cut-off point. Many of the other pathways accumulated considerable support although their \( P \) values did not reach the sFDR cut-off point. However, HEV-68 strains from additional countries, especially countries in Europe and South America, are needed to further analyse the migration events.

One limitation of the study should be addressed. A different target population could cause discrepancies in prevalence and the clinical spectrum of HEV-68, as hospitalized patients are more prone to present with more severe disease. Therefore, different target populations, especially patients in the community, should be screened in the future to provide a more comprehensive understanding of HEV-68 infection in China.

In conclusion, HEV-68 may play a predominant role in the aetiology of ARTI caused by enteroviruses in China. These findings add to the developing evidence that HEV-68 infections have become widespread throughout the world and need to be considered in patients with worsening respiratory tract infection. The detected HEV-68 in China formed some new sublineages. Their gene diversity was based mainly on the geographical subdivision, as well as migration events from the USA and the Netherlands. Additional surveillance is needed to clarify the reason why HEV-68 causes such a wide spectrum of disease from asymptomatic to severe respiratory disease and even death.

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