Arch Virol (2014) 159:971–977
DOI 10.1007/s00705-013-1915-0

ORIGINAL ARTICLE

Molecular detection of human parechovirus in children with acute gastroenteritis in Guangzhou, China

Huifang Chen · Yuexian Yao · Xie Liu · Ni Xiao · Yuan Xiao · Yanhui Huang · Qing Chen · Shouyi Yu

Received: 9 August 2013 / Accepted: 29 October 2013 / Published online: 13 November 2013

Abstract Human parechoviruses (HPeVs) are widespread pathogens causing a wide spectrum of diseases. The prevalence and genetic diversity of HPeV in children with acute diarrhea in China is not well known. The purpose of this study was to investigate the epidemiological characteristics of HPeV in Guangzhou, China. A total of 328 stool specimens collected from children under the age of 5 years with acute diarrhea were tested for the presence of HPeV. Of these, 44 (13.4 %, 44/328) were HPeV positive, with the majority of the infected children (97.7 %, 43/44) being younger than two years of age. HPeV was more frequently detected during July and August. The epidemiological profile of co-infections was similar to that observed in a previous study. Six different HPeV genotypes, including HPeV1, -3, -4, -5, -6, and -14, were identified, and of these, HPeV14, a rarely reported genotype, was reported for the first time in children with acute gastroenteritis in China. In summary, this study clearly demonstrated that HPeV circulating in Guangzhou, China, is genetically diverse, including six genotypes, and it provides useful epidemiological data on the features of HPeV infection in this area.

Introduction

Human parechoviruses (HPeVs) are members of the large and growing family *Picornaviridae* [1, 2]. Sixteen HPeV genotypes have been described worldwide [3–10]. Infections with HPeVs are widespread, but different HPeV genotypes have different epidemiological and clinical characteristics.

HPeV1 is believed to be a common pathogen that mainly affects young children with mild gastrointestinal or respiratory symptoms, whereas HPeV2 infections rarely occur and are mostly associated with gastrointestinal symptoms [6, 7, 11, 12]. HPeV3 infection causes more-severe diseases, such as sepsis or sepsis-like illness and meningitis or encephalitis [13–16]. Although HPeV genotypes 4–16 have been identified in a number of different countries [17–23], their clinical role remains to be clarified.

In China, epidemiological surveillance of HPeV infection in children with diarrhea has been reported since 2009 [24]. Little is known about this viral agent causing acute gastroenteritis in China, except for two studies of HPeV detection in acute gastroenteritis in infants and children less than 5 years old [25, 26]. Thus, the role and effect of HPeV infection in acute gastroenteritis is unclear.

The aims of this study were to investigate the prevalence and distribution of HPeV in Guangzhou, China, by direct screening and typing of stool samples from children with acute diarrhea under the age of 5 years during the 2012 to 2013 season, and to characterize the circulating strains by sequence and phylogenetic analysis.

Materials and methods

Sample collection

Three hundred twenty-eight stool specimens were collected from children under 5 years of age with diarrhea who were being treated as outpatients. Stool samples analyzed in this study were collected from June 2012 through May 2013 and

H. Chen · Y. Yao · X. Liu · N. Xiao · Y. Xiao · Y. Huang · Q. Chen · S. Yu (✉)
Department of Epidemiology, School of Public Health and Tropical Medicine, Southern Medical University, Guangzhou, China
e-mail: epidemiologymmu@126.com

stored at −20 °C until further analysis. All collected specimens were also screened for other diarrheal viruses including human group A rotavirus, norovirus (NoV), astrovirus, sapovirus (SaV) and adenovirus [27–30]. Informed consent was obtained from the parents of all children who provided specimens. The study protocol was approved by the ethics committees of the School of Public Health and Tropical Medicine of Southern Medical University.

RNA extraction and parechovirus testing

Total RNA was extracted from supernatants of 10 % (w/v) of stool specimens in PBS using TRIzol (Invitrogen, Carlsbad, CA) as described in the manufacturer's instructions. Extracted RNA was stored at −80 °C and reverse transcribed into cDNA using a reverse transcription system (Promega, Madison, WI). cDNA was either used directly for PCR or stored at −20 °C.

Testing for HPeV was carried out using highly conserved 5' untranslated region (5'-UTR) primers [31] under the following reaction conditions: 94 °C for 1 min, followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final incubation at 72 °C for 7 min. Positive samples producing a amplicon with a predicted size of 243 bp were identified by visualization under UV light after 2 % agarose gel electrophoresis and ethidium bromide staining.

Parechovirus genotyping and phylogenetic analysis

For parechovirus genotyping, samples testing positive by PCR of the 5'-UTR were amplified by nested PCR using primers from the VP3/VP1 junction region as described elsewhere [31]. The two rounds of amplification were performed under the following cycling conditions: 40 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s, and a final incubation at 72 °C for 7 min. The final products of 304 bp were identified by agarose gel electrophoresis as described above and were purified by using a QIAquick kit (QIAGEN).

Sequencing was carried out using a Big-Dye Terminator Cycle Sequencing Kit and an ABI 3730 XL DNA Analyzer (Applied Biosystems). The sequences were compared with those of reference strains available in the NCBI GenBank database using the BLAST server (http://www.ncbi.nlm.nih.gov/blast). Sequences were assembled with Sequencher software (version 4.6, Gene Codes Corp., Ann Arbor, MI), and genetic identity was determined by comparing the sequence with standard strains in GenBank. Multiple sequence alignment was conducted using ClustalW [32], and a phylogenetic tree was constructed from partial nucleotide and deduced amino acid sequences of the VP3/VP1 region using MEGA version 5.0 (http://www.megasoftware.net/) [33].

Nucleotide sequence accession numbers

Partial nucleotide sequences of HPeV VP3/VP1 junction region identified in the present study were deposited in the GenBank database. The accession numbers are KF489908 - KF489951.

Results

Study group

The female-to-male gender ratio was 0.84 (150/178). The age of children with acute gastroenteritis ranged from 10 days to 58 months, with a mean age of 11 months, and the difference in the age distributions of the boys and girls was not statistically significant ($P = 0.887$, $\alpha = 0.05$).

Epidemiology of HPeV infections

Of the 328 specimens, 44 (13.4 %, 44/328) were HPeV positive. The details of these 44 samples are shown in Table 1. Twenty-four (54.5 %, 24/44) were from boys, and 20 (45.5 %, 20/44) were from girls. Taking into account the gender ratio, the difference in the prevalence of HPeV was not statistically significant ($P = 0.968$, $\alpha = 0.05$). The age range of HPeV-positive patients was from 1 month to 48 months (median, 7 months; mean age, 8.97 months); 43.2 % (19/44) were younger than 6 months, 47.7 % (21/44) were 7 to 12 months old, 6.8 % (3/44) were 13 to 24 months old, and 2.3 % (1/44) were 48 months old. HPeV was detected throughout the entire year except February and December, with the peak incidence in July (30.8, 12/39) and August (32.1 %, 9/28) (Fig. 1).

Among of 44 positive samples, co-infection with other enteric viruses was found in 23 (52.3 %) pediatric patients, while monoinfection by HPeV alone was detected in 21 (47.7 %) cases. Coinfection with rotavirus was found most frequently (12 samples, 27.3 %), while another specimens revealed coinfection with norovirus (5 samples, 11.4 %), adenovirus (4 samples, 9.1 %), astrovirus (4 samples, 9.1 %) or sapovirus (1 sample, 2.3 %). HPeV showed an 11.8 % (21/178) positive rate in stool samples that were negative for all of the other five enteric viruses.

Phylogenetic analysis

For genotyping using nucleotide sequences, all HPeV-positive samples were successfully amplified and sequenced. Phylogenetic analysis of the VP1/VP3 region of reference HPeV strains and the isolates studied here showed that the strains could be identified as HPeV1 (32 strains), HPeV3 (4 strains), HPeV4 (2 strains), HPeV6 (4

Table 1 Summary of positive samples

Sample code	Age	Gender	Collection date	Other virus	HPeV type
GZ-595	9 Mo	M	06-Jun-2012	Adenovirus	3
GZ-601	6 Mo	F	12-Jun-2012	Negative	3
GZ-637	8 Mo	F	27-Jun-2012	Astrovirus	1B
GZ-640	10 Mo	F	04-Jul-2012	Negative	1B
GZ-641	5 Mo	M	04-Jul-2012	Negative	1B
GZ-647	2 Yr	F	05-Jul-2012	Astrovirus	1A
GZ-648	2 Yr	F	06-Jul-2012	Negative	1B
GZ-651	6 Mo	M	09-Jul-2012	Negative	1B
GZ-653	10 Mo	M	09-Jul-2012	Norovirus	1B
GZ-656	5 Mo	M	10-Jul-2012	Norovirus	1B
GZ-658	2 Yr	F	11-Jul-2012	Negative	1A
GZ-665	7 Mo	M	19-Jul-2012	Norovirus	6
GZ-675	7 Mo	M	26-Jul-2012	Rotavirus	1A
GZ-678	6 Mo	M	30-Jul-2012	Norovirus	1A
GZ-681	4 Yr	F	30-Jul-2012	Rotavirus	6
GZ-684	4 Mo	M	06-Aug-2012	Negative	1A
GZ-688	1 Yr	M	07-Aug-2012	Negative	1A
GZ-692	3 Mo	M	08-Aug-2012	Rotavirus	3
GZ-704	8 Mo	M	18-Aug-2012	Rotavirus	1B
GZ-705	4 Mo	M	18-Aug-2012	Rotavirus	1B
GZ-707	7 Mo	M	20-Aug-2012	Adenovirus	1A
GZ-708	6 Mo	F	21-Aug-2012	Astrovirus, Adenovirus	1A
GZ-710	4 Mo	M	22-Aug-2012	Rotavirus	1B
GZ-718	7 Mo	M	30-Aug-2012	Norovirus	1B
GZ-722	5 Mo	M	04-Sep-2012	Rotavirus	1A
GZ-726	8 Mo	F	06-Sep-2012	Negative	1A
GZ-731	5 Mo	F	12-Sep-2012	Negative	1A
GZ-751	1 Yr	F	09-Oct-2012	Negative	6
GZ-764	8 Mo	F	22-Oct-2012	Negative	1A
GZ-771	8 Mo	M	15-Oct-2012	Negative	1B
GZ-789	6 Mo	F	30-Oct-2012	Negative	1A
GZ-795	2 Mo	F	31-Oct-2012	Rotavirus	1B
GZ-802	10 Mo	F	02-Nov-2012	Rotavirus	3
GZ-810	5 Mo	M	07-Nov-2012	Negative	1A
GZ-825	1 Mo	F	20-Nov-2012	Negative	6
GZ-901	7 Mo	M	28-Jan-2013	Rotavirus	1B
GZ-919	6 Mo	M	19-Jan-2013	Rotavirus, Astrovirus	14
GZ-974	10 Mo	F	29-Mar-2013	Adenovirus, Sapovirus	1A
GZ-990	1 Yr	F	24-Apr-2013	Rotavirus	5
GZ-997	5 Mo	M	06-May-2013	Negative	1A
GZ-1005	8 Mo	M	20-May-2013	Negative	4
GZ-1006	1 Yr	F	21-May-2013	Negative	1A
GZ-1024	4 Mo	M	28-May-2013	Negative	1A
GZ-1030	7 Mo	F	31-May-2013	Negative	4

Abbreviations: Mo, month; Yr, year; M, male; F, female

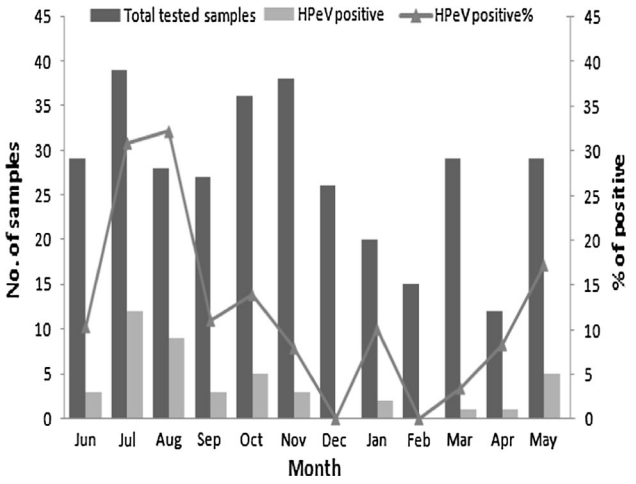

Fig. 1 Seasonal distribution of HPeV infections in children in Guangzhou, China, 2012–2013. Bars represent the number of acute diarrhoea cases and HPeV-positive cases. The line indicates the percentage of HPeV-positive samples in each month

strains), and HPeV14 (1 strain) (Fig. 2). HPeV1 was the predominant genotype, and more than half of the viruses identified in this study clustered with the prototype Harris strain. Of the 32 HPeV1 isolates, one (GZ-684) was divergent from the rest of the group. The divergence was 10.4 % and 23.4 %, respectively, when compared with the VP3/VP1 junction region of HPeV1A (SH 401, JX441355) and the remaining HPeV1B clade. Since the divergence for a new genotype assignment has to exceed 25 %, the GZ-684 strain was clustered with HPeV1. HPeV1 isolates could be grouped into two clusters of Harris-like clade 1A and clade 1B [34]. Therefore, the isolate GZ-684 was assigned to genotype 1A.

Notably, isolate GZ-990 was segregated to a single branch that could not be assigned to a specific HPeV cluster (Fig. 2). GZ-990 had highest nucleotide sequence identity, 77.5 %, to the prototype strain HPeV5 CT86-6760 (AF055846). The second-best match was 73.9 % identity to HPeV4 K251176-02(DQ315670). Importantly, the pairwise distances (0.192–0.298) between isolate GZ-990 and other reference strains was over the threshold of 18 % divergence for nucleotides for the division of intra- and intertype categories [31]. When amino acid sequences were analyzed, isolate GZ-990 was characterized as HPeV type 5 (the pairwise distance between this isolate and HPeV5 CT86-6760 was 0.051, lower than the 8 % divergence threshold) (Fig. 3).

Discussion

Human parechoviruses (HPeVs) have been classified into sixteen serotypes based on neutralization test or molecular identification. Clinical manifestations that have been

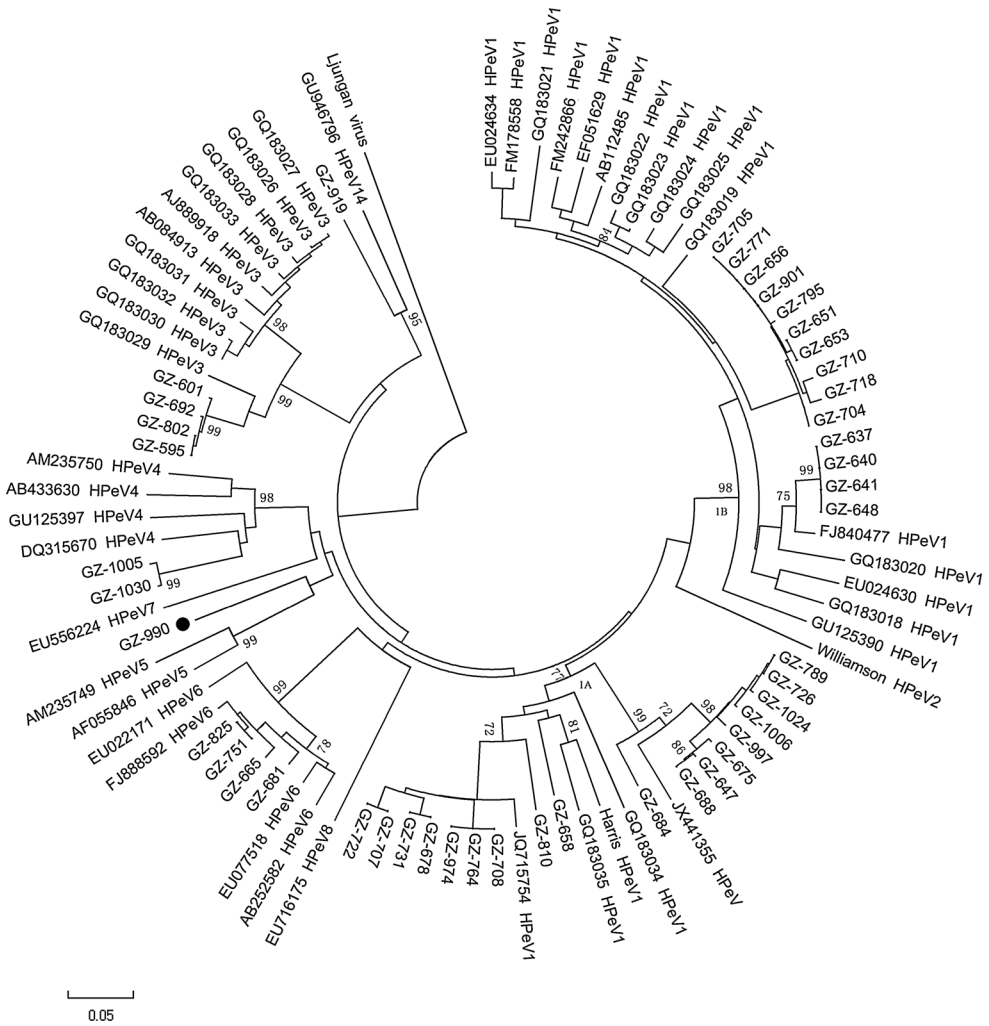

Fig. 2 Phylogenetic analysis based on the nucleotide sequence of the VP3/VP1 junction region. The tree was constructed using the neighbor-joining method with the p-distance model. A bootstrap test was replicated 1000 times, and only bootstrap values over 70 are shown. A sample (GZ-990) that could not be assigned to a specific HPeV cluster is indicated by a black circle

associated with HPeV infections include gastroenteritis, respiratory diseases, aseptic meningitis, lymphadenopathy, myocarditis, sepsis-like syndromes with necrotizing enterocolitis, and sudden infant death syndrome (SIDS), notably in young children. [15, 17, 19, 35–43]. Epidemiological studies of HPeV are required to further our understanding of disease causation and geographic distribution.

We tested stool specimens collected from children less than 5 years old for HPeV between 2012 and 2013 in Guangzhou, China. The percentage of HPeV-positive specimens (13.4 %) was lower than that found in other studies in China (55 % in Shanghai [25] and 25.3 % in Lanzhou [26]). The discrepancy might be due to whether the samples were negative for other gastroenteritis-associated viruses, the geographical location, the specific year of sample collection, or the use of different PCR methods.

Nearly all of the infections (97.7 %) occurred in the first 2 years of life. This age distribution is consistent with previous studies [7, 8, 44, 45]. The gender-neutral distribution of HPeV infection is also in agreement with studies from Thailand [32], Lanzhou [26] and Shanghai, China [25]. In Guangzhou, HPeV infection had a distinct seasonal distribution that peaked during July and August. Similar seasonal distributions were also found in Shanghai, China [25].

Coinfection with other enteric viruses was found in 23 (52.3%) pediatric patients. A 52.3 % coinfection rate, together with the previously reported 64.4 % from Lanzhou [26], suggests that coinfection is common for HPeV. Rotavirus was the most common partner. By screening fecal samples known to be negative for common enteric viruses, this study provides one more piece of evidence that HPeV infection is associated with acute gastroenteritis. With a detection rate of 11.8 % (21/178) among the samples tested that were negative for common enteric viruses, this study demonstrates that HPeV-related diarrhea among children with acute gastroenteritis is not rare in

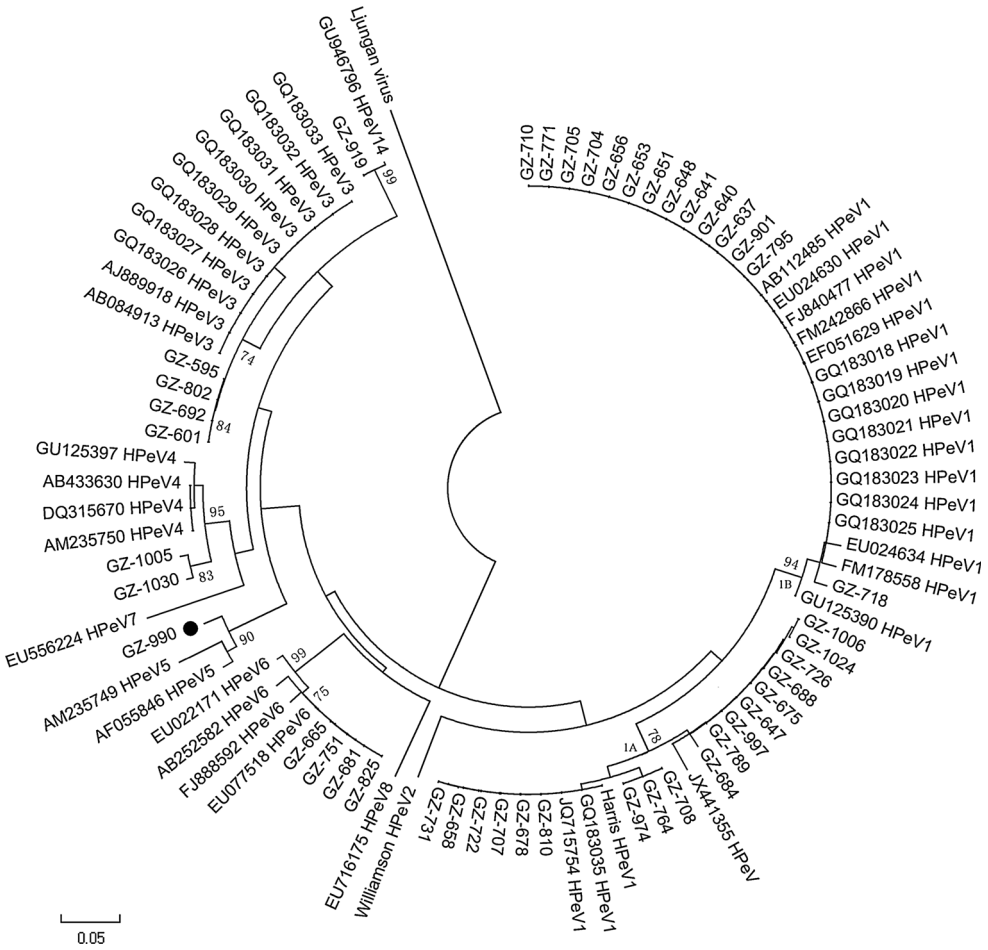

Fig. 3 Phylogenetic analysis based on the deduced amino acid sequence of the VP3/VP1 junction region The tree was constructed using the neighbor-joining method with the p-distance model. A bootstrap test was replicated 1000 times, and only bootstrap values over 70 are shown. A sample (GZ-990) that could not be assigned to a specific HPeV cluster is indicated by a black circle

Guangzhou, China. Comparing our results to those from two other studies, which have shown detection rates of 2.0 [12] and 8.1 % [8], respectively, the difference in detection rate might well be due to study populations and geographical locations.

In this study, six different HPeV genotypes, HPeV1,-3, -4, -5,-6, and -14, were present in infants and children with acute gastroenteritis in the Guangzhou region. The HPeV1 genotype was predominant over other genotypes, which is consistent with the fact that HPeV1 is the major genotype worldwide [14, 46–52]. In our study, most of the HPeV1 isolates (56.25 %, 18/32) clustered with HPeV1A, which has seldom been found in recent years [7, 45, 53]. Changes in the rate of HPeV1A infection in Guangzhou could not be investigated due to the lack of data from previous years; however, this study could confirm that genotype HPeV1A was circulating in Guangzhou, China.

The inconsistent assignment of isolate GZ-990 in phylogenetic trees based on nucleotide and amino acid sequences was due to the fact that most of the differences in the nucleotide sequences between GZ-990 and HPeV5 reference sequence occurred at synonymous sites. In this research, infection with HPeV8, which was detected in other areas of China, was not detected, indicating that HPeV8 is most likely absent in Guangzhou. More studies are needed to confirm the occurrence for HPeV8 in China. It is noteworthy that HPeV14, a rarely reported genotype, was detected unexpectedly in this study. As far as we know, this is the first report of the presence of HPeV14 in China to date.

In conclusion, with the identification of HPeV in fecal specimens, six different genotypes were found. In addition, this is the first report of HPeV14 infection in patients with acute gastroenteritis in China. This study provides useful epidemiological data for future disease control and prevention by documenting the distribution of genotypes, as well as age and seasonal patterns in Guangzhou, China.

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