

SCIENTIFIC REPORTS



OPEN

Large outbreak of herpangina in children caused by enterovirus in summer of 2015 in Hangzhou, China

Received: 08 January 2016
Accepted: 28 September 2016
Published: 18 October 2016

Wei Li*, Hui-hui Gao*, Qiong Zhang, Yu-jie Liu, Ran Tao, Yu-ping Cheng, Qiang Shu & Shi-qiang Shang

Herpangina, usually caused by coxsackie virus A, is prevalent in children spreading through the fecal-oral transmission and the respiratory droplets dissemination. Also, it is mostly asymptomatic and self-limiting. In our study, we found that large outbreak of herpangina in children occurred in the summer of 2015 in Hangzhou, China. From May 1th to August 31th, a total of 10 210 children were diagnosed with herpangina in Children's Hospital of Zhejiang University School of Medicine. 2 310 throat swabs were collected and tested for enterovirus detection by real-time RT-PCR, while 1 651 cases were positive with the rate of 71.5%. Based on VP1 gene or 5'UTR region sequences, Coxsackievirus A2, A4, A6, A10, B2, B4 and echovirus 30 were detected in these cases. More importantly, Coxsackievirus A2 may be the major subtype of enterovirus resulting in children with herpangina in Hangzhou, China.

Enteroviruses (EVs), belonging to the picornaviridae family, include coxsackievirus A (CVA) and coxsackievirus B (CVB), echoviruses, polioviruses and the numbered enteroviruses^{1,2}. EVs is one of the most common viruses that causes diseases in human³. Particularly, it is the major pathogen that causes herpangina, hand-foot-mouth disease (HFMD) and neurological diseases in children throughout the world⁴⁻⁶. A large outbreak of HFMD occurred in 2008 in China, with EV71, CA16, and CVA6 identified as the major pathogens causing HFMD⁷⁻⁹. Interestingly, in our previous study, we found EV71 and CA16 were decreasing after 2010, while other types of EVs caused herpangina were increasing in Hangzhou⁵. Herpangina, generally considered as one of the asymptomatic and self-limiting infectious diseases in patients with mild clinical complications, can be cured completely within 5-7 days after infection¹⁰. Outbreaks of herpangina were reported in many cities, what's more, many subtypes of EVs were identified as the pathogens causing herpangina, such as CVA, CVB, and echovirus¹⁰⁻¹². In this article, we displayed a large outbreak of herpangina in children in the summer of 2015 in Hangzhou, China.

Results

Patients' characteristics. From May 1, 2015 to August 31, 2015, 10 210 patients were diagnosed with herpangina at outpatient department in our hospital. In temporal distribution, the herpangina patients comprised 1 433 children (14.0%) in May, 5 257 children (51.5%) in June, 2 871 children (28.1%) in July, and 649 children (6.4%) in August.

Children enrolled in this study were with a median age of 1.75 years old (ranging from 0.03 to 13.5 years old) at the onset of herpangina and a male to female ratio of 1.26. 9 352 out of 10 210 (91.6%) children were less than 5 years, with constitution ratios of 23.2%, 46.6%, 21.8%, 6.7% and 1.7% in children aged 0-1 year, 1-3 years, 3-5 years, 5-9 years and >9 years, respectively.

All children with herpangina were divided into three groups by age and performed with routine blood test and C-reactive protein (CRP) assay. Our results showed that the median number of white blood cells were $7.4 \times 10^9/L$, $8.9 \times 10^9/L$, $8.2 \times 10^9/L$, the median percentage of lymphocyte were 46.4%, 38.2%, 30.3%, the median percentage of neutrophil were 58.5%, 54.3%, 63.2%, and the median level of CRP were 25.1 mg/ml, 15.3 mg/ml, 14.8 mg/ml in each age group (Table.1).

Department of Clinical Laboratory, Children's Hospital of Zhejiang University School of Medicine, Hangzhou 310013, PR China. *These authors contributed equally to this work. Correspondence and requests for materials should be addressed to S.-Q.S. (email: shangshiqiang33@163.com)

Age	white blood cells	Lymphocyte(%)	Neutrophil(%)	CRP(mg/L)
<3 years	$7.4 \times 10^9/L$	46.4%	58.5%	25.1
3–5 years	$8.9 \times 10^9/L$	38.2%	54.3%	15.3
>5 years	$8.2 \times 10^9/L$	30.3%	63.2%	14.8

Table 1. Routine tests of children with herpangina.

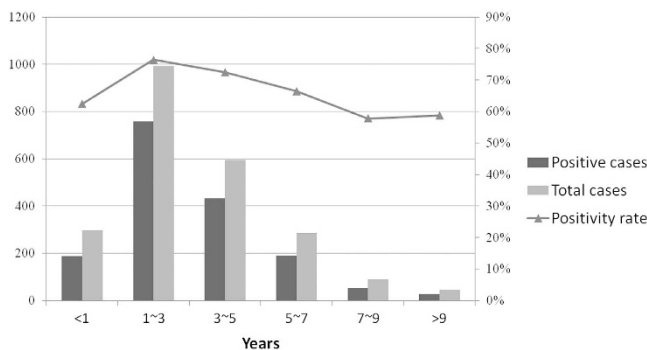


Figure 1. Age distribution of enterovirus infections among children with herpangina.

Results of enterovirus assay. To confirm the major pathogen causing herpangina, 2 310 throat swabs were randomly collected from patients and tested by enterovirus one-step real-time RT-PCR. As a result, 1 651 samples were tested positive for enterovirus with the rate of 71.5%. Positive rate for enterovirus was the highest among children aged 1–3 years (76.4%), and steadily declined with age increase or decrease (Fig. 1). However, children in other age groups, the detection rates of enterovirus infection were still as high as 62.4%, 72.5%, 66.4%, 57.8% and 58.7% in <1 year, 3–5 years, 5–7 years, 7–9 years, and >9 years, respectively.

VP1 gene based phylogenetic analysis. To confirm the subtypes of enterovirus, 35 clinical strains were randomly collected and the VP1 and 5'UTR gene of the EV were amplified by the conventional RT-PCR (Figure S1). The amplification products were purified, sequenced and then used for phylogenetic analysis. After comparing the VP1 and 5'UTR genes with different referenced EV strains, the sequence homology was displayed as 90.9–99.5% (nucleotide), which met the serotype identification criteria for homologous serotypes. The molecular typing results showed that the major EV isolates belonged to the human EVA and EVB species, including 22 samples with Coxsackievirus A2, 3 samples with Coxsackievirus A10, 1 sample with Coxsackievirus A6, 2 samples with Coxsackievirus B2, 1 sample with Coxsackievirus B4 and 3 samples with Echovirus 30. The last 3 samples were negative for VP1 PCR and were identified as Coxsackievirus A4 by 5'UTR PCR and sequencing. Based on the partial VP1 gene and 5'UTR region, the homologous comparison results for the isolates from the same serotypes were 92.5–99.4% (nucleotide) (Table S1).

Based on the VP1 gene and 5'UTR region sequences, phylogenetic analysis for the EVs of this study was done by comparison with all available VP1 gene sequences from the Genbank. From the constructed phylogenetic tree (Fig. 2), we can find all clinical isolates from Zhejiang belonged to the human EVA and EVB species. The CVA2, CVA10 and CVB4 isolates were most closely related to the stains from Shenzhen (Southern China). CVB2 isolate was most closely related to the stains from Shanghai (Southern China) and E30 isolates were most closely related to the stains from Shandong (Northern China).

Discussion

Herpangina, associated with various enterovirus serotypes, is a commonly prevalent illness in young children^{13,14}. In the summer of 2015, large outbreak of herpangina happened in Hangzhou, China. From May to August, 10 210 children were diagnosed with herpangina with the highest prevalence rate in June. Among the infected children, 91.6% were less than 5 years with high rate in 1–3 years children. We also found that the level of CRP slightly increased in children with herpangina during the study period.

Enteroviruses infection occurred in summer throughout the world¹⁵. To confirm whether enteroviruses are the major pathogens causing herpangina in Hangzhou, China, real-time RT-PCR targeting to highly conserved region in the human enteroviruses (HEV) genome was performed to detect the enterovirus. Among 2 310 throat swabs, 1 651 (71.5%) were tested positive for enterovirus. The children aged 1 to 5 years old were the most susceptible population with a peak incidence in 1–3 years, which was similar to all herpangina aging incidence. Most importantly, children older than 3 years also exhibited high infection rate in Hangzhou. Among patients belonged to kindergarten and primary school age, cross infections occurred. To control infection, a short-term suspension was conducted in some schools.

As reported in a previous study, herpangina was associated with different strains of enteroviruses, such as CVA2 in Taipei in 2008¹⁶, CVA5 in Korea in 2009¹⁰, CVA6 and CVA10 in France in 2010¹⁷, Coxsackievirus A8 in Thailand in 2012¹¹, Coxsackievirus A9 in Brazilian Amazon in 2014¹² and in Japan during 2000–2005. There

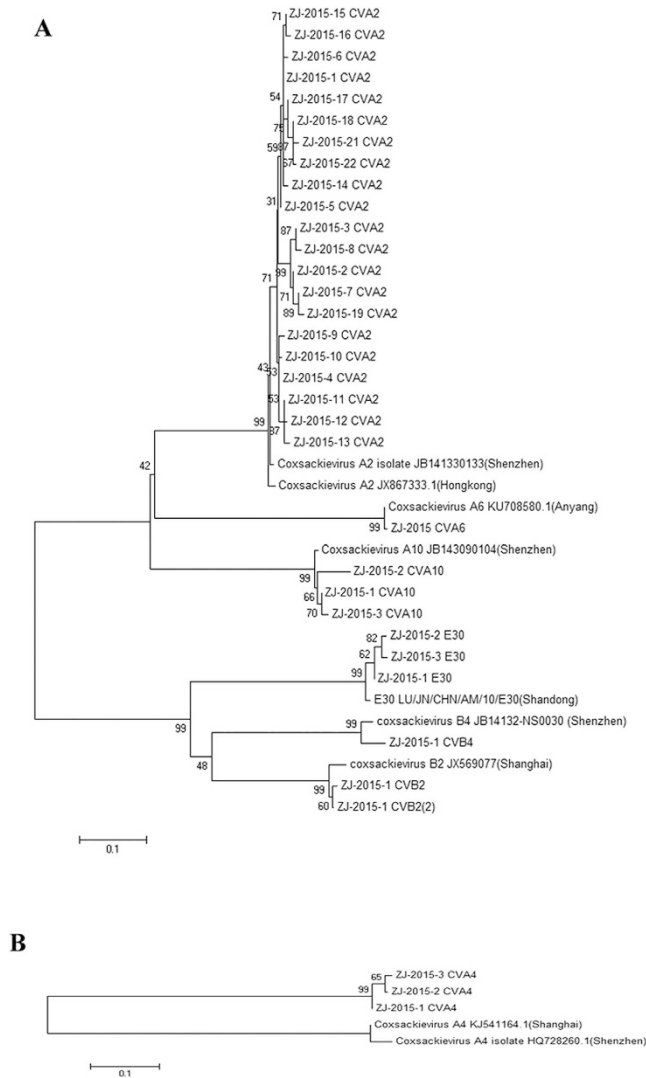


Figure 2. Phylogenetic analysis of the enterovirus isolates based on the partial VP1 gene or 5'UTR sequences. (A) Phylogenetic analysis of the enterovirus isolates based on the partial VP1 gene sequences; **(B)** Phylogenetic analysis of the enterovirus isolates based on the partial 5'UTR sequences.

are also some reports about enterovirus infections caused by CVA5, CVA6 and CVA10¹⁸. Our study has established CVA2 as the most possible prevalent cause of herpangina in Hangzhou in 2015. Our study investigated distinct clusters of CVA2, CVA4, CVA10 and E30 strains in relation to their geographic origins. Phylogenetic analysis showed that the Thai CVA2 strains were closely related to strains isolated from Shenzhen and Hongkong, China, in 2012. Most importantly, CVA2 was reported caused 2 children death with respiratory symptoms in Hongkong¹⁹. In our surveillance, we didn't find death cases among children for diagnosis of herpangina. Besides surveillance of herpangina, our hospital has also conducted the surveillance of enterovirus-associated encephalitis. Different from herpangina, we found E30 was the major pathogen causing enterovirus-associated encephalitis and none of CVA2 samples were found (data not shown). These results indicated that E30 may more easily invade to brain system of children.

This is the first report about the large outbreak of enterovirus herpangina among children in hangzhou, China. And the associated major subtype of enterovirus may be HEV A with high incidence of CVA2. In further studies, we will sequence more clinical enterovirus samples from EV-positive children with herpangina which would be helpful to further surveillance of enterovirus.

Materials and Methods

Patients and definitions. The Children's Hospital of Zhejiang University School of Medicine is the largest comprehensive center for pediatric health care in Zhejiang province. This was a retrospective study conducted from May 2015 to August 2015 in the hospital. Patients who met the following criteria were enrolled: (1) age less than 14 years old, (2) all children who visit our hospital in the study period, (3) all children who were diagnosed

with herpangina (well-characterized multiple vesicular exanthema and ulcers of the soft palate with presentation of fever, sore throat and anorexia) in our hospital in the study period.

Ethical approval and informed consent. This retrospective study and method were approved by the medical ethics committee of the Children's Hospital of Zhejiang University School of Medicine (NO. 2015-PRIB008) and all experiments were performed in accordance with relevant guidelines and regulations. Informed consent was obtained from all subjects.

Routine blood test and C-reactive protein. White blood cell (WBC) counting, the proportion of lymphocyte and neutrophils were measured by Sysmex blood cell instrument (Sysmex 800i, Japan). Concentrations of C-reactive protein (CRP) were measured by the QuikRead go instrument with QuikRead go CRP kits (Orion Diagnostica, Finland).

Detection of enterovirus. Throat swabs were collected from children with the symptoms of herpangina. RNA was extracted by Nucleic acid automatic extraction instrument (Zhi-jiang company, Shanghai, China). The detection of EV was performed in ABI Stepone plus system by commercial one-step real-time RT-PCR assay kit (Zhi-jiang company, Shanghai, China). The real time RT-PCR was conducted under these conditions: 15 min at 50 °C, 5 min at 95 °C, and then followed by 40 cycles of 15 sec at 94 °C and 45 sec at 55 °C. Samples with CT value less than 35.0 were identified positive.

VP1 and 5'UTR gene sequencing and phylogenetic analysis. All primers used in VP1 gene amplification were based on previous study²⁰. Reverse transcription PCR (RT-PCR) kit (Invitrogen, Shanghai, China) was used to perform enterovirus cDNA synthesis. The total volume per RT-PCR was 10 µL which included 2 µL buffer (5×), 0.4 µL dNTP (10 mM), 1 µL DTT, 0.2 µL primer mix (Zhi-jiang company, Shanghai, China), 0.5 µL Superscript III, 5 µL Enterovirus RNA, 0.4 µL RNasin. The reaction was under these conditions: 22 °C, 10 min; 45 °C, 45 min; 95 °C, 5 min. The first round PCR of the VP1 gene was carried out in a mixture with a total volume of 50 µL that included 10 µL of the RT-PCR product, 0.5 µL DSC Taq (Enzymatics), 2.5 µL outer primers (Zhi-jiang company, Shanghai, China). The amplification was under the following conditions: 95 °C, 5 min; 40 cycles × (95 °C, 30 s; 42 °C, 30 s; 60 °C, 45 s); 72 °C, 10 min. The second round PCR was carried out in a mixture with a total volume of 50 µL that included 1 µL of the first round PCR product, 0.5 µL DSC Taq (Enzymatics), 2.5 µL outer primers (Zhi-jiang company, Shanghai, China). The amplification was under following conditions: 95 °C, 6 min; 40 cycles × (95 °C, 30 s; 60 °C, 20 s; 72 °C, 25 s); 72 °C, 10 min. The PCR product was sequencing in Majorbio (Shanghai, China). For VP1 PCR negative samples, we used 5'UTR primers (Zhi-jiang company, Shanghai, China) to amplify and sequence 5'UTR region. The protocol is the same to VP1 PCR. VP1 gene and 5'UTR DNA sequences of the EV isolated were contrasted with the National Center for Biotechnology Information (NCBI) database through BLAST. Based on the sequences of the VP1 gene, phylogenetic analysis was done by using the Mega 5.1 software. The tree was constructed by using the neighbor-joining method. Significance of phylogenies was investigated by bootstrap analysis with 1,000 pseudoreplicate data sets. Bootstrap values of are indicated on the tree.

References

- Puenpa, J. *et al.* Prevalence and Characterization of Enterovirus Infections among Pediatric Patients with Hand Foot Mouth Disease, Herpangina and Influenza Like Illness in Thailand. *PLoS One* **9**, e98888 (2014).
- Lee, C. J. *et al.* Clinical features of coxsackievirus A4, B3 and B4 infections in children. *PLoS One* **9**, e87391 (2014).
- Abzug, M. J. The enteroviruses: problems in need of treatments. *J. Infect.* **68**, S108–S114 (2014).
- Liu, C. C., Chow, Y. H., Chong, P. & Klein, M. Prospect and challenges for the development of multivalent vaccines against hand, foot and mouth diseases. *Vaccine* **32**, 6177–6182 (2014).
- Li, W. *et al.* Epidemiology of childhood enterovirus infections in Hangzhou, China. *Viol. J.* **12**, 58 (2015).
- Xiao, H. *et al.* Molecular characterization of echovirus 30-associated outbreak of aseptic meningitis in Guangdong in 2012. *Viol. J.* **10**, 263 (2013).
- Xu, M. *et al.* Genotypes of the Enterovirus Causing Hand Foot and Mouth Disease in Shanghai, China, 2012–2013. *PLoS One* **1**, e0138514 (2015).
- Guo, W. P. *et al.* Fourteen types of co-circulating recombinant enterovirus were associated with hand, foot, and mouth disease in children from Wenzhou, China. *J. Clin. Virol.* **70**, 29–38 (2015).
- Lu, J. *et al.* Hand, foot and mouth disease in Guangdong, China, in 2013: new trends in the continuing epidemic. *Clin. Microbiol. Infect.* **20**, O442–O445 (2014).
- Park, K. *et al.* Enteroviruses isolated from herpangina and hand-foot-and-mouth disease in Korean children. *Viol. J.* **9**, 205 (2012).
- Puenpa, J. *et al.* Prevalence and characterization of enterovirus infections among pediatric patients with hand foot mouth disease, herpangina and influenza like illness in Thailand, 2012. *PLoS One* **9**, e98888 (2014).
- Oliveira, D. B. *et al.* Outbreak of herpangina in the Brazilian Amazon in 2009 caused by Enterovirus B. *Arch. Virol.* **159**, 1155–1157 (2014).
- Tebruegge, M. & Curtis, N. Enterovirus infections in neonates. *Semin. Fetal Neonatal. Med.* **14**, 222–227 (2009).
- Jubelt, B. & Lipton, H. L. Enterovirus/picornavirus infections. *Handb. Clin. Neurol.* **123**, 379–416 (2014).
- Stalkup, J. R. & Chilukuri, S. Enterovirus infections: a review of clinical presentation, diagnosis, and treatment. *Dermatol. Clin.* **20**, 217–223 (2002).
- Lee, M. H. *et al.* Molecular diagnosis and clinical presentations of enteroviral infections in Taipei during the 2008 epidemic. *J. Microbiol. Immunol. Infect.* **44**, 178–183 (2011).
- Mirand, A. *et al.* Outbreak of hand, foot and mouth disease/herpangina associated with coxsackievirus A6 and A10 infections in 2010, France: a large citywide, prospective observational study. *Clin. Microbiol. Infect.* **18**, E110–E118 (2012).
- Yamashita, T., Ito, M., Taniguchi, A. & Sakae, K. Prevalence of coxsackievirus A5, A6, and A10 in patients with herpangina in Aichi Prefecture, 2005. *Jpn. J. Infect. Dis.* **58**, 390–391 (2005).
- Yip, C. C. *et al.* Recombinant coxsackievirus A2 and deaths of children, Hong Kong, 2012. *Emerg. Infect. Dis.* **19**, 1285–1288 (2013).
- Nix, W. A., Oberste, M. S. & Pallansch, M. A. Sensitive, seminested PCR amplification of VP1 sequences for direct identification of all enterovirus serotypes from original clinical specimens. *J. Clin. Microbiol.* **44**, 2698–2704 (2006).

Acknowledgements

This study was supported by Key Projects of the National Science & Technology Pillar Program (2012BAI04B05) and Medical Scientific Projects from Health department of Zhejiang Province (2015KYA119).

Author Contributions

Conceived and designed the experiments: W.L. Performed the experiments: W.L., Y.-J.L. and Q.Z. Analyzed the data: Y.-J.L., Y.-P.C. and R.T. Contributed reagents/materials/analysis tools: W.L., Y.-P.C., S.-Q.S. and Q.S. Wrote the paper: W.L. and H.-H.G.

Additional Information

Supplementary information accompanies this paper at <http://www.nature.com/srep>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Li, W. *et al.* Large outbreak of herpangina in children caused by enterovirus in summer of 2015 in Hangzhou, China. *Sci. Rep.* **6**, 35388; doi: 10.1038/srep35388 (2016).



This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>

© The Author(s) 2016