Prevalence of Nonpolio Enteroviruses in the Sewage of Guangzhou City, from 2009 to 2012

Huanying Zheng,a Jing Lu,a,Yong Zhang,b Hiromu Yoshida,c Xue Guo,a Leng Liu,a Hui Li,a Hanri Zeng,a Ling Fang,a Yanling Mo,a Lina Yi,a Toru Chosa,d Wenbo Xu,b Changwen Kea
Guangdong Provincial Center for Disease Control and Prevention, Panyu District, Guangzhou, China; WHO WPRO Regional Polio Reference Laboratory and Ministry of Health Key Laboratory for Medical Virology, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Changping District, Beijing, China; Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan; Bureau of International Cooperation, International Medical Center of Japan, Tokyo, Japan; Guangdong Provincial Institution of Public Health, Guangdong Provincial Center for Disease Control and Prevention, Panyu District, Guangzhou, China

The human-pathogenic viruses in urban sewage have been extensively monitored to obtain information on circulating viruses in human communities. Enteroviruses (EVs) excreted by patients who present with diverse clinical syndromes can remain infectious in the environment for several weeks, and limited data on circulating environmental EVs are available. A 4-year (2009 to 2012) surveillance study was conducted to detect nonpolio enteroviruses (NPEVs) in the urban sewage of Guangzhou city, China. After the viruses in the sewage samples were concentrated and isolated, molecular identification was used to detect and type the NPEVs. During the 4-year study, 17 different NPEV serotypes were identified in the sewage of Guangzhou city. The most common serotypes were echovirus 11 (ECHO11), ECHO6, ECHO7, and ECHO12 and coxsackie group B viruses 5 (CVB5) and CVB3. The predominant serotypes were influenced by spatial and temporal factors and differed each year. CVB5 was commonly detected in 2009 and 2010 but was rarely isolated in 2011 and 2012. In contrast, CVB3 was not observed in 2009 and 2010 but was increasingly detected in 2011 and 2012. This study provides an overview of the serotype distribution and circulation patterns of NPEVs in the sewage of Guangzhou, China. In the absence of a systematic EV disease surveillance system, the detection and characterization of sewage-borne NPEVs will help us better understand the changes in EV disease trends and the epidemic background of circulating EVs, which could help interpret the EV trends and warn of future outbreaks in this area.

Human enteroviruses (EVs) are members of the genus *Enterovirus* within the order *Picornavirales*, family *Picornaviridae*, and consist of 4 species: *EV-A*, *EV-B*, *EV-C*, and *EV-D* (1). Based on their pathogenicity in humans, EVs were initially classified into 4 subgroups: polioviruses, type 1 (PV1) to PV3; coxsackie group A viruses, type 1 (CVA1) to CVA22 and CVA24; coxsackie group B viruses, type 1 (CVB1) to CVB6; and echoviruses, type 1 (ECHO1) to ECHO7, ECHO9, ECHO11 to ECHO27, and ECHO29 to ECHO34 (2). EVs are associated with diverse clinical syndromes, ranging from mild fever; headache; herpangina; and hand, foot, and mouth disease (HFMD) to severe and potentially fatal illnesses, such as aseptic meningitis, encephalitis, myocarditis, and acute flaccid paralysis (3–6). In recent years, outbreaks of different EV infections and related diseases have been frequently reported in China (7–10); however, only limited data are available on the circulation patterns of EVs in the environment (11, 12).

Although different serotypes of EVs can cocirculate, the predominant serotype is determined by spatial and temporal factors. For instance, in Beijing, China, CVA21 and EV-D68 were the predominant serotypes in patients with acute EV respiratory infections from 2006 to 2010 (13). However, in France and Spain, ECHO11 and ECHO6 are the most frequently detected agents in human-EV-positive adults with acute respiratory tract infections (14, 15). The high incidence of aseptic meningitis that occurred in Alberta, Canada, in 2010 was caused by CV9A, and an aseptic meningitis outbreak in Korea in 2008 was mainly caused by ECHO6 and ECHO30 infections (16, 17). For this reason, determining the temporal and geographic patterns of EV circulation, especially the dynamics of EV serotype shifts, is critical.

The presence of human-pathogenic viruses in urban sewage has served as an indicator of their existence in a given population (18–21). In urban populations with no or questionable surveillance, monitoring the viruses in circulating sewage can provide valuable supplementary information, especially when persistent virus circulation or frequent reintroduction is suspected. In this study, we provide an overview of the nonpolio enteroviruses (NPEVs) circulating in urban sewage of Guangzhou, China, from 2009 to 2012. During this 4-year period, 17 cocirculating NPEVs were isolated from sewage samples, and the circulation patterns of the predominant NPEVs (ECHO6, ECHO7, ECHO11, ECHO12, and CVB5) were described. To our knowledge, this is the longest, most systematic study of EV prevalence in sewage in China, and these results will help us to better understand the changes in enteric disease trends and the potential risk of an enteric disease epidemic.

MATERIALS AND METHODS

**Sewage sample collection.** Raw sewage samples were collected monthly from January 2009 to December 2012 from the primary sedimentation...
Mix Kit (Qiagen) with 0.5 M (24). In general, a pending EV was classified as the same serotype as the Technology Information (NCBI), and the serotype of each isolate was aligned using the Search Tool (BLAST) server at the National Center for Biological Information. Positive products (245 to 400 nucleotides [nt]) were purified using a Qiagen QIAquick PCR purification kit (Qiagen) and sent for sequencing using a positive products. After 40 cycles of amplification (95°C for 30 s, 60°C for 30 s, and 72°C for 45 s), the PCR products were analyzed on 1.2% agarose gels, and the highly conserved motif in the VP3 and VP1 regions, respectively. After 40 cycles of amplification (95°C for 30 s, 42°C for 30 s, and 72°C for 45 s), 2.5 μl PCR1 products were used as a template in the second-round PCR with 0.5 μl (each) primers AN88 and AN89, targeting a partial VP1 region. After 40 cycles of amplification (95°C for 30 s, 60°C for 30 s, and 72°C for 45 s), the PCR products were analyzed on 1.2% agarose gels, and the positive products (~350 to 400 nucleotides [nt]) were purified using a Qiagen Quick PCR purification kit (Qiagen) and sent for sequencing using primers AN88 or AN89. The sequences were analyzed with the Basic Local Alignment Search Tool (BLAST) server at the National Center for Biotechnology Information (NCBI), and the serotype of each isolate was determined according to a previously described molecular typing method (24). In general, a pending EV was classified as the same serotype as the prototype strain if it had >75% nucleotide identity and >85% amino acid sequence identity in the VP1 coding region; the pending EVs were classified into different serotypes if they had <70% nucleotide identity and <85% amino acid sequence identity.

**RESULTS**

In total, 947 positive isolates were collected, comprising 916 NPEV and 31 nontypeable viruses. Seventeen NPEV serotypes were identified based on the molecular typing of a 340-bp fragment sequence in the VP1 region, and the number of EV serotypes ranged from 10 to 16 during the period from 2009 to 2012. The distribution of serotypes identified in EVs is presented in Table 1. Overall, the 6 most commonly identified EVs were ECHO11 (26.4% of all isolates), ECHO6 (24.1%), ECHO7 (13.4%), ECHO12 (13.3%), CAYB5 (9.5%), and CAYB3 (3.1%).

To investigate the circulating NPEVs according to season, the number of NPEV isolates detected each month was determined. As shown in Fig. 1, from 2009 to 2012, the number of NPEVs typically increased from February on and generally peaked in July, except in 2010, when a large number (35 isolates) of NPEVs were isolated in June and only a few (9 isolates) were detected in July. However, some variations in the seasonal prevalence of NPEVs were observed. In 2009, there was another peak in April, and 36 (16.7%) NPEVs were isolated. In 2010 and 2011, numerous NPEVs were isolated in October (32 isolates) and November (26 isolates), but a similar pattern was not observed. Interestingly, our results differ from those of other studies (25, 26), in which the number of NPEVs in Guangzhou sewage samples did not remain high during the summer and fall and a sharp decrease in the number of NPEVs was observed in June, July, and August of 2009, 2010, and 2011, respectively. The circulation pattern of each NPEV serotype also differed. As shown in Fig. 2, a large number of ECHO6 and ECHO12 viruses were detected in June, whereas most ECHO7 and ECHO11 viruses were isolated around October.

The distribution patterns of NPEV serotypes also varied over the years, even though the total numbers of NPEVs isolated from sewage each year were similar. Among these NPEV serotypes, 10 were detected every year, and another 2 serotypes (ECHO1 and CAYB4) were detected in only a single year. The predominant serotypes also differed each year. As shown in Table 1 and Fig. 2, ECHO6 and ECHO11 were the most prevalent serotypes during

---

**Table 1** NPEV serotypes detected in the sewage of Guangzhou City, China, each year from 2009 to 2012

<table>
<thead>
<tr>
<th>Serotype</th>
<th>2009</th>
<th>2010</th>
<th>2011</th>
<th>2012</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECHO1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1 (0.1)</td>
</tr>
<tr>
<td>ECHO3</td>
<td>7</td>
<td>5</td>
<td>6</td>
<td>6</td>
<td>23 (2.5)</td>
</tr>
<tr>
<td>ECHO6</td>
<td>38</td>
<td>101</td>
<td>42</td>
<td>40</td>
<td>221 (24.1)</td>
</tr>
<tr>
<td>ECHO7</td>
<td>40</td>
<td>28</td>
<td>22</td>
<td>33</td>
<td>123 (13.4)</td>
</tr>
<tr>
<td>ECHO11</td>
<td>59</td>
<td>71</td>
<td>59</td>
<td>53</td>
<td>242 (26.4)</td>
</tr>
<tr>
<td>ECHO12</td>
<td>20</td>
<td>21</td>
<td>46</td>
<td>35</td>
<td>122 (13.3)</td>
</tr>
<tr>
<td>ECHO13</td>
<td>4</td>
<td>5</td>
<td>2</td>
<td>5</td>
<td>16 (1.7)</td>
</tr>
<tr>
<td>ECHO19</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>12 (1.3)</td>
</tr>
<tr>
<td>ECHO20</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>4 (0.4)</td>
</tr>
<tr>
<td>ECHO24</td>
<td>5</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>14 (1.5)</td>
</tr>
<tr>
<td>ECHO29</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2 (0.2)</td>
</tr>
<tr>
<td>ECHO30</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>4 (0.4)</td>
</tr>
<tr>
<td>CVB1</td>
<td>0</td>
<td>1</td>
<td>9</td>
<td>0</td>
<td>10 (1.1)</td>
</tr>
<tr>
<td>CVB2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>4 (0.4)</td>
</tr>
<tr>
<td>CVB3</td>
<td>0</td>
<td>1</td>
<td>6</td>
<td>21</td>
<td>28 (3.1)</td>
</tr>
<tr>
<td>CVB4</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>3 (0.3)</td>
</tr>
<tr>
<td>CVB5</td>
<td>34</td>
<td>43</td>
<td>2</td>
<td>8</td>
<td>87 (9.5)</td>
</tr>
</tbody>
</table>

*Most common serotypes: 2009, ECHO11 (27.6%), ECHO7 (18.4%), and ECHO6 (17.5%); 2010, ECHO6 (34.8%), ECHO11 (24.5%), and CVB5 (14.8%); 2011, ECHO11 (29.4%), ECHO12 (22.9%), and ECHO6 (20.9%); 2012, ECHO11 (22.7%), ECHO6 (17.1%), and ECHO12 (15.0%).
were isolated from RD cells. 59.8% of CVBs were isolated from HEp-2 cells, whereas 40.2% were isolated from RD cells while only 16.9% were isolated from HEp-2 cells. Conversely, HEp-2 cells seemed to be better for CVB detection, since RD cells were more sensitive to echoviruses, with 83.1% of echoviruses isolated from RD cells. Our results indicated that RD cells were more sensitive to NPEVs. Our results demonstrated that the newly emerged ECHO30 was the most common serotype in sewage samples from 2009 to 2012. In this study, different cell lines also exhibited different sensitivities to NPEVs. Our results indicated that RD cells were more sensitive to echoviruses, with 83.1% of echoviruses isolated from RD cells while only 16.9% were isolated from HEp-2 cells. Conversely, HEp-2 cells seemed to be better for CVB detection, since 59.8% of CVBs were isolated from HEp-2 cells, whereas 40.2% were isolated from RD cells.

**DISCUSSION**

Circulation of EVs in sewage is a proven indicator of their presence in a given community. Therefore, sewage surveillance is regarded as a complementary approach to determine the prevalence and duration of epidemic EVs in a human population (11, 22, 26, 27). For instance, in a serotype-based surveillance study performed by Sedmak et al., clinical isolates were compared with sewage isolates in Wisconsin from August 1994 to December 2002 (26). The study showed that the most commonly detected EV serotypes in sewage were similar to the most commonly detected EV serotypes in clinical samples. Also, the annual peaks in sewage EV titers were accompanied by peaks in clinical cases, which occurred in late summer or early fall. Moreover, the high sequence similarity between EVs from sewage and clinical samples provides substantial evidence at the molecular level. A study conducted by Iwai et al. from 2002 to 2003 demonstrated that the nucleotide sequences of ECHO13 isolated from sewage water were closely related to those isolated from patients with aseptic meningitis in Toyama Prefecture, Japan (22).

In this study, we reported an overview of NPEV prevalence in the sewage of Guangzhou city, the capital city of Guangdong Province, China. During the 4-year surveillance study, 10 common circulating NPEV serotypes were identified in Guangzhou, which were (in descending order) ECHO11, ECHO6, ECHO7, ECHO12, CVB5, CVB3, ECHO3, ECHO13, ECHO24, and ECHO19. In contrast, ECHO1, ECHO20, ECHO29, ECHO30, CVB1, CVB2, and CVB4 were only occasionally detected in sewage samples.

Currently, no surveillance system is set up to monitor NPEV clinical infection in China. Because most people infected with enterovirus do not show clinical symptoms, or show only mild symptoms, clinical data on NPEV infection are very limited and mainly from testing of patients with meningitis or meningoencephalitis and HFMD. ECHO6, a predominant NPEV in the sewage of Guangzhou, was also isolated in other regions of China, including Yunnan, Shandong, and Henan Provinces (11, 12, 28, 29). Correspondingly, outbreaks of ECHO6-associated aseptic meningitis and HFMD were reported in Anhui and Shandong Provinces in 2005 and 2011 (7, 30). CVB5, which was commonly detected in 2009 and 2010 in the sewage of Guangzhou, was reported to be the etiologic agent for an aseptic meningitis outbreak in Shandong Province, China, in 2009 (31). More direct evidence was provided by ECHO30. In this surveillance, ECHO30 was not detected until 2010. Although the number of positive isolates was low, the continuous identification of ECHO30 from 2010 to 2012 suggested its circulation in the environment in Guangzhou. Consistently, an outbreak of aseptic meningitis occurred in Luoding city, which adjoins Guangzhou city, in 2012. Our recent study revealed that the newly emerged ECHO30 was the most commonly isolated EV serotype in cerebrospinal fluid samples from the patients (32, 33). These cooccurrences of NEPV in sewage and clinical samples prove the value of environmental surveillance for enteroviruses.

The results of this study also suggest that the circulation patterns of individual EVs change, along with temporal and spatial factors. Due to the different features of each EV, the seasonal pattern of the EVs differs over time, and the circulation patterns for the different serotypes might vary. In Guangzhou city, ECHO11 and ECHO6 were the most common serotypes in sewage during our 4-year surveillance study. Meanwhile, the months during which the largest numbers of ECHO6 and ECHO11 viruses were detected differed. Comparing our surveillance data with those from similar reports, we found that the NPEVs detected in this study were also identified in sewage surveillance studies from other areas, such as Shandong Province, China; Iran; France; and the United States, whereas the predominant NPEV serotypes in these different geographical regions varied (11, 25, 26, 34). These findings demonstrate the value of environmental surveillance of EVs in this area.

In the present study, the detection results using 2 cell lines with different sensitivities to the different EV serotypes are more convincing (26). Therefore, it is reasonable to assume that our sewage
testing is a reflection of local EV activity. However, it should also be noted that some EVs, especially EV-As, such as EV-A71, might have been missed in our study, because their growth rates in cells are lower than those of EV-Bs. Molecular typing of EVs is better for identifying strains that might have been classified as “untypeable” by the conventional neutralization method. Moreover, the sequences obtained allowed us to further analyze the evolution of the circulating NPEVs through phylogenetic assays.

In the absence of a systematic EV disease surveillance system in China, our study on the prevalence of sewage-borne NPEVs has at least two advantages. First, our study provides relevant area-specific epidemiological data on potential waterborne pathogenic viruses that will help public health practitioners determine the long-term circulation patterns of individual EVs and health-based targets (i.e., water or food quality targets for pathogens). Second, since NPEVs are occasionally related to serious diseases, such as myocarditis and aseptic meningitis, our description of the predominant NPEVs in sewage provides an epidemic background of the circulating EVs that can be used to interpret the trends in EV prevalence and to provide warning of possible enteroviral disease outbreaks.

ACKNOWLEDGMENTS

This project was funded by Sasagawa Medical Awards in Aid for the Japan-China Cooperation Project; a grant for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labor and Welfare of Japan; and the Bill and Melinda Gates Foundation (project no. OPP1039272).

We declare that we have no competing interests.

REFERENCES


