Occurrence of Hand-Foot-and-Mouth Disease Pathogens in Domestic Sewage and Secondary Effluent in Xi’an, China

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Hand, foot and mouth disease (HFMD), caused by a group of enteric viruses such as Enterovirus 71 (EV71), Coxsackievirus A16 (CVA16) and Coxsackievirus A10 (CVA10), is heavily epidemic in East Asia. This research focused on investigating the occurrence of HFMD pathogens in domestic sewage and secondary effluent before disinfection in a wastewater treatment plant (WWTP) in Xi’an, the largest megacity in northwest China. In order to simultaneously detect all three HFMD pathogens, a semi-nested RT-PCR assay was constructed with a newly designed primer set targeting conservative gene regions from the 5’ untranslated region (UTR) to VP2. As a result, 86% of raw sewage samples and 29% of the secondary effluent samples were positive for the HFMD viral gene, indicating that HFMD pathogens were highly prevalent in domestic wastewater and that they could also persist, even with lower probability, in the secondary effluent before disinfection. Of the three HFMD pathogens, CVA10 was positive in 48% of the total samples, while the occurrences of CVA16 and EV71 were 12% and 2%, respectively. It could thus be stated that CVA10 is the main HFMD pathogen prevailing in the study area, at least during the investigation period. High genetic diversity in the conservative gene region among the same serotype of the HFMD pathogen was identified by phylogenetic analysis, implying that this HFMD pathogen replicates frequently among the population excreting the domestic sewage.

Key words: Enterovirus, hand-foot-and-mouth disease, phylogenetic analysis, semi-nested RT-PCR, wastewater

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Human enterovirus (HEV), a genus of positive-sense single-stranded RNA virus, is associated with a number of clinical infections (14). Most cases of enterovirus infection are asymptomatic or mild, and can usually recover without any special medication (24). Hand, foot and mouth disease (HFMD) is an acute enterovirus infection, characterized by a brief febrile illness and vesicular lesions on the hands, feet, mouths and buttocks of infected people (22). In recent years, numerous large outbreaks of HFMD have occurred in the Asia-Pacific region, including China (29, 30), Singapore (1, 21, 25), South Korea (10), Malaysia (3), Vietnam (22), and Japan (6, 8).

Enterovirus 71 (EV71), Coxsackievirus A16 (CVA16) and Coxsackievirus A10 (CVA10), members of Human enterovirus A (HEV-A) species, are major etiologic agents of HFMD (15), and have caused significantly high morbidity and mortality in China (27, 28). Enteroviruses are excreted from infected individuals with feaces for up to 11 weeks (4), thereby possibly existing in domestic wastewater at high titers. Since enteric viruses are not removed efficiently by conventional wastewater treatment processes, and viral contamination of water environment may occur due to the discharge of effluent that contains human pathogenic viruses if proper disinfection is not performed (19, 23). Although the contamination level of environmental waters with various pathogenic viruses including EV71 has been revealed (2, 7, 9, 12, 20), few studies have been conducted by far for a comparison of the occurrences of different HFMD viral pathogens in the water environment.

This study thus aims to establish a general molecular biological method with a newly designed primer set for detecting and comparing the occurrence of EV71, CAV10, and CAV16, the three main HFMD viral pathogens. The method was then applied to monitor the occurrence of these HFMD viral pathogens in a domestic wastewater treatment plant (WWTP) in Xi’an, the largest megacity in northwest China, in a seven-month period. The extent of HFMD pathogen occurrence was evaluated by the frequency of positive samples with HFMD genes from domestic sewage and secondary effluent before disinfection. The main HFMD pathogens were identified by gene sequence determination, and phylogenetic analysis of the acquired gene was performed to elucidate the genetic diversity of HFMD pathogens in the investigation area.

Materials and Methods

Design of specific primers

A primer set was designed based on the conservative gene regions of whole genome sequences of CAV10, CAV16 and EV71 to detect these HFMD viral pathogens simultaneously. The complete nucleotide sequences of five EV71 strains, five CVA16 strains and one CVA10 strain were retrieved from the National Center for Biotechnology Information (NCBI) database, GenBank, and multiple alignment was created with Clustal X 1.83 to design a primer set with its feasibility checked using Primer3 (v. 0.4.0) (http://frodo.wi.mit.edu/primer3/).
cDNA of target viruses were applied to semi-nested RT-PCR in the specificity test. Ten-fold serial dilutions of the viral sensitivity test. All PCR products were visualized as described above.

Polyethylene glycol (PEG) precipitation was employed to recover viruses from wastewater samples according to Lewis and Metcalf (13) with some modifications. PEG6000 and sodium chloride were added to yield the final concentrations of 8% (w/v) and 2.3% (w/v), respectively. The mixture was stirred gently at about 80 rpm, incubated at 4°C overnight and then centrifuged at 9,000×g for 30 min at 4°C. The supernatant was discarded and the pellet was suspended in 1 mL deionized distilled water (DDW) with a vortex mixer.

Water sample processing

The constructed method was applied to investigate the occurrence of HFMD pathogens in a WWTP in Xi’an, China where a conventional activated sludge process is applied for treating domestic wastewater with a capacity about 150,000 m³/d. Water sampling was conducted for about 7 months from November 2010 to May 2011 at 10-day intervals so that 21 batches were collected, each including the raw sewage and secondary effluent before disinfection by chlorine. The sampling period almost covered the spring season (from March to May) when HFMD was prevailing in general (28).

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Results and Discussion

Primer design

A primer set for semi-nested RT-PCR was designed in relatively highly conserved regions among the three HFMD viruses. The appropriate sites for simultaneous amplification of the three HFMD viral pathogens were observed both in their 5' untranslated region and capsid protein-coding regions (from VP4 to VP2) (Fig. 1). The primer sequences are: 546F (1st round forward primer), 5'-CGGAACCGACTACTTTGG-3'; 1090R (reverse primer), 5'-GCARTASKMRG-3'; 592F (2nd round forward primer), 5'-TGGCTGCTTATG-3'.

The raw sequence data were analyzed with Chromas software (version 2.31) to obtain the final sequence to compare with those published in the NCBI database using the Basic Local Alignment Search Tool (BLAST) program. The phylogenetic tree of the acquired viral genes was constructed using Clustal X 1.83, and depicted with Njplot (http://pbil.univ-lyon1.fr/software/njplot.html).

Nucleotide sequence accession numbers

The nucleotide sequences of the semi-nested PCR products acquired in this study have been deposited in the Genbank database under accession numbers JN086565 to JN086604.

Detection of HFMD viral pathogens in wastewater samples from a WWTP

As a result of semi-nested RT-PCR analysis, 86% (18...
of 21) of raw sewage samples and 29% (6 out of 21) of secondary effluent samples were found to be positive for the HFMD viral gene, indicating that HFMD pathogens were prevalent in domestic wastewater and could also persist, even with lower probability, in the secondary effluent. Figure 3 shows the monthly variation of their occurrence in raw sewage and secondary effluent during the sampling period. For raw sewage, the positive ratio was 3/3 in December 2010 and March, April, and May 2011, while 2/3 in the other months. For the secondary effluent, the positive ratio was 2/3 in November 2010 and May 2011, followed by 1/3 in December 2010 and March 2011. The frequent occurrence of HFMD viral pathogens in the secondary effluent (before disinfection) implies the high stability of HFMD pathogens during biological treatment in the WWTP. If the effluent is not effectively disinfected before being discharged into receiving water (as sometimes happens in China), the infection risk cannot be ignored.

**Phylogenetic analysis of HFMD pathogens**

Figure 4 shows the occurrence of HFMD viruses in the collected water samples. For raw sewage, CVA10 was detected from 71% (15 of 21) of the samples, while CVA16 and EV71 were detected from 19% (4 of 21) and 5% (1 of 21) of the collected samples, respectively. One sewage sample (collected on May 5th) showed the coexistence of CVA10 and CVA16, and another sample (collected on November 23rd) showed the coexistence of CAV10 and EV71. For the secondary effluent, CVA10 was detected from 24% (5 of 21) of the samples, and CVA16 was detected from 5% (1 of 21) of the samples, while EV71 was not detected. CVA10 could thus be suspected as the main HFMD virus occurring in domestic wastewater in the study area, at least during the investigation period. The nucleotide identity between each HFMD virus isolated in this study and HFMD strains previously isolated in China was found to be about 97%, 95%, and 90% for CVA10, CVA16 and EV71, respectively.

At the amino acid level, the identity ranged from 92% to 99%. These comparisons suggest that HFMD viral strains isolated in this study were genetically close to those previously reported in China.

Overall, 40 sequences were obtained from wastewater samples so a phylogenetic tree, as shown in Fig. 5, could be constructed using the neighbor-joining method. Bootstrap analysis was performed by resampling the data sets 1,000 times. A single sequence was obtained from four sewage samples (collected on November 14th, April 1st, and May 15th and 25th, respectively), and multiple sequences were obtained from a single sample in more than half of all sewage
samples (14 of 21). The sequences originating from CVA10 were highly diverse with shared nucleotide identity from 94% to 99%. The high mutation rate in the viral RNA genome is mainly owing to the lack of a proofreading function of RNA-dependent RNA polymerase during replication in human cells (17), implying that this serotype replicates frequently among the populations who have excreted sewage into the WWTP.

The frequent detection of diverse CVA10 is not expected because CVA10 has been a relatively minor pathogen for HFMD infections largely occur in the investigation period, which is observed even in the non-epidemic season (January and February in general). It is postulated that asymptomatic patients as reservoirs and sources of enteric viruses could excrete a larger amount of replicated virions than symptomatic patients, although norovirus belongs to a totally different viral family from enteroviruses; however, asymptomatic patients as reservoirs and sources of enteric viruses in populations (5) should be highlighted to understand the environmental epidemiology of HFMD.

A more accurate description of different genotypes should be determined from the VP1 gene (18) in order to understand the characteristics of the epidemiological mechanism and genetic evolution of CVA10. Nevertheless, the primer set established in this study (targeting VP4 to VP2 region) could be useful for screening all three HFMD pathogens (EV71, CVA10 and CVA16) from wastewater samples. Although the existence of HFMD viruses in domestic wastewater may indicate the possibility of disease transmission by water media because the treated effluent from the WWTP, if not sufficiently disinfected, may finally enter a water body, the direct infectivity of HFMD pathogens in sewage and treated effluent is still unknown. Such a topic may need further investigation.

**Conclusion**

The semi-nested RT-PCR developed in this study can provide a tool for rapid and sensitive detection of HFMD viral pathogens from wastewater samples. The simultaneous detection of three HFMD pathogens, EV71, CVA16 and CVA10, was achieved with the specific primer set targeting the conservative gene regions from 5'UTR to VP2. The high positive rate in raw sewage (86%) and treated wastewater (29%) indicated that HFMD viral pathogens were highly prevalent in the investigation area. CVA10 might be the main HFMD pathogen in the investigation area, as was revealed by sequence determination and phylogenetic analysis of the acquired viral gene. Further studies are needed on the infectivity of HFMD viral pathogens and the health risks posed by these pathogens in water.

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