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Original Article

Molecular Detection of Human Enteroviruses in Wastewater Treatment Plant

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Abstract

Background and Aims: Enteroviruses are spread worldwide that cause many diseases, including gastrointestinal infections. These viruses are considered as an important pathogen in wastewater and therefore their presence in treated wastewater can cause disease transmission. We aimed to investigate the molecular prevalence and characterization of human enteroviruses in wastewater samples of a wastewater treatment in Tehran, Iran.

Materials and Methods: From November 2017 to April 2018, a total of 15 samples were collected by Grab sampling method from three chambers including the chamber adjacent to the aeration tank and the pre-chlorination effluent and the post-chlorination effluent. The samples were analysed for the presence of human enteroviruses by an integrated cell culture/polymerase chain reaction (ICC/PCR) technique. Then, the isolated enteroviruses were evaluated using sequencing analysis.

Results: The results showed that the rate of infection with enteroviruses using culture method and RT-PCR technique in wastewater samples was 80% and 40%, respectively. Also, human enteroviruses in the samples were Coxsackievirus B5 (50%), poliovirus type 1 vaccine (33%) and Echovirus 11 (16%).

Conclusion: Human enteroviruses were detected in wastewater samples and the highest rate with coxsackievirus B5 (50%) and then with poliovirus type 1 vaccine (33%) and Echovirus 11 (16%).

Keywords: Wastewater, ICC-PCR, Human Enteroviruses

Introduction

The reuse of treated wastewater is one of the most important issues in water resources management (1,2). Irrigation of plants and agriculture with treated wastewater is very useful due to the presence of nutrients in the effluent, but there are concerns about the presence of toxic agents and possible pathogens in the wastewater (3). Enteric viruses such as adenoviruses, noroviruses, rotaviruses, and enteroviruses are the most important viral pathogens in wastewater (4,5). The viruses do not completely disappear in wastewater treatment and can remain active in a humid environment for a long time (3).

Human enteroviruses include polioviruses, coxsackieviruses A and B, ecoviruses and newer enteroviruses which can cause a variety of diseases, including respiratory infections, meningitis, acute hemorrhagic conjunctivitis, and gastrointestinal diseases (6-9).

Enteroviruses are found in sewage, seas and rivers, as well as in swimming pools (10-12). These viruses are resistant to a wide range of pH and temperature and increase under conditions such as health poverty, population density, and inadequate sewage disposal systems (13, 14). On the other hand, isolation of polio virus by environmental monitoring is very important because it can provide useful information about the rotation of intestinal viruses, determine the spread and duration of virus circulation in the population, evaluate the effectiveness of vaccination, and demonstrate the circulation of wild-type and vaccine-

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derived polio virus (15,16).The aim of this study was to isolate and determine the types of enteroviruses in the wastewater treatment plant located in Tehran, Iran.

Methods

Sample collection and concentration: From November 2017 to April 2018, a total of 15 samples were collected by Grab sampling method from three chambers including the chamber adjacent to the aeration tank and the pre-chlorination effluent and the post-chlorination effluent. All samples were collected in sterilized tubes and transported under refrigeration to the laboratory.

The concentration of samples was performed based on the method described by Minor et al (17). Briefly, samples were centrifuged at 4000 rpm for 25 minutes at 4°C, and the resulting supernatant was then transferred to a new tube. Thereafter, 3.9 ml Dextran, 3.5 ml NaCl, and 2.84 ml polyethylene glycol 6000 were added to each 500 mL of the supernatant. The final mixture was centrifuged at 4000 rpm for 2 hours at 4°C, the supernatant was discarded, and the pellet was suspended in phosphate-buffered saline (PBS), and stored at −80°C until use (17, 18). The samples were then subjected to the detection of human enteroviruses by the integrated cell culture-polymerase chain reaction (ICC-PCR).

Cell culture: In this study, the Vero cell line was used for ICC-PCR. The cells were cultured in DMEM (Dulbecco's Modified Eagle Medium), supplemented with 8-10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C and 5% CO2 atmosphere. Before inoculation into the Vero cell line, the samples were filtered using 22 µm filters. The onset of cytopathic effects in enteroviruses has been reported to be 24-48 hours. In this study, infected cells were examined for one week The cultures were then subjected to freeze-thaw cycles to release viral particles from the cells. The lysate was then centrifuged, and the supernatant was used for viral RNA extraction.

RNA extraction: RNA extraction was performed by Favorprep viral nucleic acid extraction kit (Favorgen, Taiwan), according to the manufacture's protocol. The concentration of the extracted RNA was determined using NanoDrop(ND-1000, Thermo Scientific, USA).

Virus detection by PCR: At first, the complementary DNA (cDNA) obtained from RNA strands using a cDNA synthesis kit (Yekta Tajhiz Azma, Iran) according to the manufacturer's instructions. Then PCR was used to amplify a 434 bp amplicon of 5' NTR region. The forward and reverse primers were 5'-CAAGCACTTCTGTTTCCCC-3' and 5'-ATTGTCACCATAAGCAGCCA-3',
respectively. PCR was performed in a thermal cycler machine model 9700A (Applied Biosystems, USA).

The total volume of the reaction mix was 20 µL, and it contained the following components: 8µL of master mix , 0.5 µL of each primer, 5µL of cDNA template, and 6 µL of double-distilled water (ddH2O). Thermal PCR cycles were as follows: The initial denaturation step was carried out at 95°C for 5 minutes, followed by 40 cycles of 95°C (30 seconds), 58°C (30 seconds), and 72°C (30 seconds), and an extension of 72°C for 5 minutes. PCR products were separated on a 1.5% agarose gel stained with safe stain and visualized under UV light.

Sequencing and Phylogenetic Tree: The PCR products were sequenced by Sanger sequencing. The sequences were aligned with the human enteroviruses reference sequence by the CLC Main Workbench 5.5 software(CLC bio, Boston, MA, USA). The phylogenetic was carried out using MEGA version 10.

Results

A total of 15 samples were collected by Grab sampling method from three chambers including the chamber adjacent to the aeration tank and the pre-chlorination effluent and the post-chlorination effluent. The results of cell culture and PCR at different sampling dates are shown in Table 1. 80% of the samples showed cytopathic changes in cell culture. All samples were then tested by PCR, which was positive in only 6 cases (40%).

Table 1. ICC-PCR results of the wastewater

Samples	Dec 2017		Jan 2018		March 2018		Apr 2018		May 2018	
	Cell culture	PCR	Cell culture	PCR	Cell culture	PCR	Cell culture	PCR	Cell culture	PCR
Aeration tank	+	_	+	+	+	_	+	_	+	_
Pre-chlorination	+	_	+	+	+	+	+	+	+	_
Post-chlorination	+	+	+	+	_	_	_	_	_	-

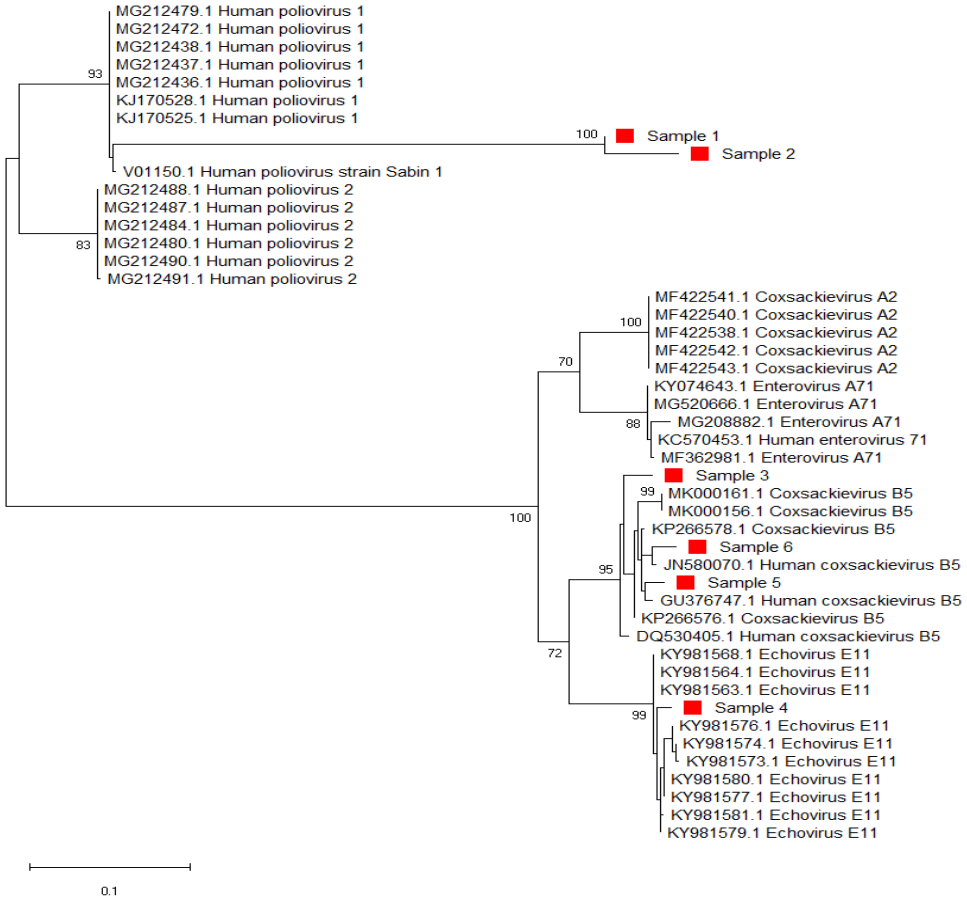


Fig. 1. The phylogenetic tree obtained by Nighberhood-joining method with Bootstrap equivalent to 1000. The wastewater samples is shown with the red squares.

The analysis of the sequence alignment showed that the detected human enteroviruses in the samples were Coxsackievirus B5 (50%), poliovirus type 1 vaccine (33%) and Echovirus 11 (16%), and the results of which can be seen in figure 1.

Discussion

One of the wastewater treatment aims is to reduce the circulating pathogens. Entroviruses are stable in the humid environments and can be transmitted through oral-fecal route. The current study investigated the enteroviruses

distribution and molecular characterization in a wastewater from Tehran. Common methods for detecting active viruses in effluent samples are based on cell culture (19). In this study, ICC-PCR method was used to detect viruses in the samples due to the advantages of this method over PCR or cell culture alone (18). In addition to increasing the speed of diagnosis, this method reduces the limitations of each of the above methods. For example, one of the limitations of PCR in environmental samples is lack of differentiation between infectious and non-infectious viruses.

Gantzeret et al. showed that the presence of the enterovirus genome in untreated wastewater samples is not a reason for the presence of the virus capable of replication in these samples (20).

In addition, the use of direct PCR in such samples has many false negative results due to the presence of enzyme inhibitors (21).

In the present study, PCR test was positive in 6.6% (2 out of 30 samples) of samples that did not have CPE for culture. The results of the present study showed that the rate of enterovirus infection using ICC-PCR was 40% in the effluent samples. In some samples, cell culture is positive and PCR is negative. It appears that the observed CPE may be related to another virus. To prove this, the nested PCR assay can be used to identify false positives. Also, the highest rate was with Coxsackie B5 (50%), followed by poliovirus type 1 vaccine (33%) and echovirus 11 (16%).

In a study by Katayama et al. (22), the rate of enterovirus contamination in samples from treated wastewater was 65%. Also in the study of Pusch et al. (23) this rate is 75%. In the Pusch study conducted in 2005 in Germany, quantitative PCR methods were used, and in the Katayama study in Japan in 2008, PCR and RT-PCR were used to detect viruses (22, 23). In a study conducted by Amdiouni et al. In 2012 in Morocco using ICC-PCR method and culture on two cell lines of rhabdomyosarcoma tumor tissue (RD) and Hep2, the rate of enterovirus infection was determined in 33% of the treated wastewater samples (18).

Nikaeen et al. (24) showed that enteroviruses were found in 40% of the 30 samples. In another study, Moazeni et al. (25) investigated the presence of enteroviruses in effluent samples obtained from the Isfahan treatment plant..

In our study, the highest pollution was related to April. The differences seen in the results of the above studies can be justified according to the sample size and different conditions of the sample. The majority of studies have shown a similar level of contamination, which is especially evident in relation to studies conducted in Iran.

The limitation of the present study is the lack of quantitative examination of this viruses in wastewater samples.

Conclusion

In conclusion, the results showed that the rate of infection with enteroviruses using culture method and RT-PCR technique in wastewater samples was 80% and 40%, respectively.

Acknowledgment

None.

Conflict of interest

No conflict of interest is declared.

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