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ORIGINAL ARTICLE

Detection of echovirus 7 in effluent sewage treatment plant Ahvaz city, Iran

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ABSTRACT

Background and Objectives: The enteroviruses may lead to conditions such as aseptic meningitis, encephalitis, acute flaccid myelitis, epidemic pleurodynia (Bornholm disease), hemorrhagic conjunctivitis, and myopericarditis among pediatric populations. The present study was undertaken to identify the presence of enteroviruses within sewage treatment systems.
Materials and Methods: 24 composite effluent sewage samples (500 ml each) were collected, centrifuged (1000 rpm for 30 minutes), and the first supernatant was saved. The precipitate was resuspended in 10 ml of supernatant, treated with 10% chloroform, and centrifuged again (1000 rpm for 5 minutes) to collect a second supernatant. The first and second supernatants were combined, treated with 2.2% sodium chloride and 7% polyethylene glycol 6000, and the mixture was agitated at 4°C overnight before being centrifuged for two hours at 2000 g. After discarding the supernatant, the pellet was resuspended at a 1:100 dilution. Each sample was then inoculated into RD and HeLa cells for virus isolation, followed by detection via RT-PCR. A phylogenetic tree was constructed to determine the genotypes of the isolated enteroviruses.
Results: Enterovirus was detected in 10 of 24 (41.7%) sewage effluent samples. Phylogenetic analysis of five randomly chosen positive samples identified echovirus 7.
Conclusion: The removal of enteroviruses during the sewage treatment process is of paramount importance, necessitating heightened attention to this critical phase of wastewater management.

Keywords: Wastewater; Environment; Enterovirus; Polymerase chain reaction

INTRODUCTION

Enteroviruses (EVs) represent a group of highly transmissible pathogens that are responsible for a diverse spectrum of diseases, encompassing hand, foot, and mouth disease (HFMD), encephalitis, aseptic meningitis, myocarditis, conjunctivitis, as well as afflictions of the respiratory and gastrointestinal

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systems (1). Furthermore, enterovirus 71 has been identified as a causative agent of HFMD, whereas EV-D68 is implicated in respiratory conditions and acute flaccid myelitis (AFM) (2, 3). EVs are non-enveloped, positive-sense, single-stranded RNA viruses belonging to the Picornaviridae family. Their virions exhibit icosahedral symmetry and are approximately 22-30 nm in diameter (4). The classification of EVs includes polioviruses (PV, comprising three distinct serotypes), Coxsackie A viruses (CAV, consisting of twenty-three serotypes), Coxsackie B viruses (CBV, with six serotypes), echoviruses (E, totaling twenty-eight serotypes), as well as enteroviruses 68-71 (5). Human enteroviruses are categorized into four species (EV-A to EV-D). Serotypes known to induce sepsis-like illnesses in children include six from species A (CAV2, CAV4, CAV6, CAV10, CAV16, and EV71) and four from species B (E6, E9, E30, and CAV9) (6, 7). Enterovirus serotyping is based on sequencing and phylogenetic analysis of the VP1 hypervariable region (8). The viruses are environmentally stable, including resistance to acidic conditions (pH 3.0) (9).

Enteroviruses, which are prevalent viruses excreted in feces, are introduced into the sewage, leading to the release of virulent enteroviruses into the ecological system. The processes of activated sludge treatment and chlorination exhibit varying degrees of efficacy in the removal or inactivation of enterovirus genotypes (10). Activated sludge treatment and effluent chlorination are standard unit processes employed in wastewater management that facilitate the decrease of infectious virus titers (10-12). The environmental spread of enteroviruses occurs through routes such as groundwater, sewage effluent, and contaminated drinking water. Transmission follows the fecal-oral route, with primary replication occurring in the host's gastrointestinal system (13). Furthermore, this pathogen is implicated in the manifestation of acute disease states (14).

Various enteric viruses, including multiple enterovirus serotypes (e.g., coxsackievirus B, echovirus 30), poliovirus, and reovirus type 1, have been detected in the effluent of activated sludge wastewater treatment plants (WWTPs) (15-19). Accordingly, the present study was undertaken to detect and characterize enterovirus serotypes in the effluent of a sewage treatment plant in the city of Ahvaz, Iran.

Ethic status. This research project received ethical approval (number IR AJUMS.MEDICINE.REC.1400.077) from the ethics committee of Ahvaz Jundishapur University of Medical Sciences, located in Ahvaz, Iran.

MATERIALS AND METHODS

Monthly, two separate 500 ml wastewater samples were collected from the Ahvaz wastewater treatment plant. The samples were transported on ice to the virology laboratory and stored at 4°C. This sampling was conducted from October 2022 to October 2023 in Ahvaz, Iran. Over a one-year period, 24 sewage samples were collected and concentrated according to an established protocol (20). Sample processing was performed as follows: Twenty-four composite sewage effluent samples (500 ml each) were centrifuged at 1000 rpm for 30 minutes. The primary supernatant was retained. The resulting pellet was resuspended in 10 ml of this supernatant, treated with 10% chloroform, and centrifuged again at 1000 rpm for 5 minutes to yield a secondary supernatant. The first and second supernatants were combined and treated with 2.2% (w/w) sodium chloride (Sigma) and 7% (w/w) polyethylene glycol 6000 (Merck, Germany). The mixture was then agitated at 4°C overnight and centrifuged for 2 hours at 2000 g. After discarding the supernatant, the pellet was resuspended in phosphate buffer at a 1:100 dilution (of the initial volume) and passed through a 0.22 µm filter. Each sample was inoculated onto RD and HeLa cell monolayers for virus isolation. Viral detection was performed by RT-PCR, and a phylogenetic tree was constructed for enterovirus genotyping.

Inoculation of the concentration suspension in cells cultures. RD cells (human rhabdomyosarcoma) were cultured at 37°C with 5% CO2 in high-glucose Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) supplemented with 10% fetal bovine serum (FBS, Life Technologies), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B. Both RD (human rhabdomyosarcoma) and HeLa (Henrietta Lacks) cells were cultured under identical conditions. HeLa cells were maintained at 37°C with 5% CO_2 in high-glucose Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) supplemented with 10% fetal bovine serum (FBS, Life Technologies), 100 U/ml

penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B. For the assay, cells were seeded in 24-well plates and grown to confluence prior to inoculation.

RNA extraction and RT-PCR for enterovirus 5UTR detection. After a cytopathic effect (CPE) was observed in either RD or HeLa cells, the supernatant was collected. Viral RNA was extracted from the supernatant using an RNA extraction kit (Sinaclone, Iran) according to the manufacturer's instructions. Complementary DNA (cDNA) was then synthesized using a commercial kit (Takara, Japan). The detection of the enterovirus genome was accomplished through a semi-nested polymerase chain reaction (PCR). The formulation of the PCR reaction mixture included 2.5 µl of PCR buffer, 0.5 µl of dNTP, 20 pmol of each primer, EV-1 (F1): 5´-CAAGCACTTCTGTTTCCCCGG-3´ and EV-R: 5´- ATTGTCACCATAAGCAGCCA-3´, 0.75 µl of MgCl2, 0.12 µl of Taq polymerase, 400 ng of template, and deionized water (D/W) to a final volume of 25 µl. Furthermore, both positive and negative controls were subjected to amplification in a thermocycler (TC-512, Techne, UK) across 35 cycles (with the following conditions: 94°C for 45 seconds, 54°C for 45 seconds, 72°C for 45 seconds, and a final elongation at 72°C for 10 minutes). Subsequently, 1 µl of the resultant product served as the template for the second amplification cycle, which comprised 2.5 µl of PCR buffer, 0.5 µl of dNTP, 20 pmol of each EV-F2 (5´- TCCTCCGGCCCCTGAATGCG-3´) and EV-R primers (5´- ATTGTCACCATAAGCAGCCA-3´), 0.75 µl of MgCl2, 1 µl of the template, 0.12 µl of Taq polymerase, and 18.62 µl of distilled water, subjected to 35 cycles of amplification (94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds, followed by a final extension at 72°C for 5 minutes). The final 155 bp product was electrophoresed on a 2% agarose gel, and its presence confirmed a positive result (21).

Detection of VP1 region of enterovirus. The VP1 region of enterovirus was amplified from samples that were positive for the 5' untranslated region (5'UTR). The 25 µl PCR reaction mixture contained 2.5 µl of 10X PCR buffer, 0.5 µl of dNTPs, 20 pmol each of primers VP1-F (GCRTGCAATGAYTTCTCWGT) and VP1-R (GCICCIGAYTGITGICCRAA), 0.12 µl of Taq polymerase, 350 ng of template DNA, and nuclease-free water to a final volume of 25 µl. Amplification was carried out for 35 cycles under the following conditions: 94°C for 45 s, 54°C for 45 s, 72°C for 45 s, with a final extension at 72°C for 10 min. PCR products were analyzed on a 2% agarose gel. The presence of a 1000 bp band indicated a positive sample (22).

The analysis of 5UTR and VP1 sequencing and phylogenetic tree. For sequencing, five positive PCR products from each target region (5'UTR and VP1) were randomly chosen. These amplicons were sent to Bioneer Company (South Korea) for bidirectional Sanger sequencing.

The analysis of isolates through partial 5UTR and VP1 sequencing was performed by contrasting them against 5UTR consensus nucleotide sequences derived from the reference enterovirus serotypes, utilizing SnapGene software (version 3.2.1) alongside the NCBI enterovirus database (https://blast.ncbi.nlm.nih.gov). The sequences of five positive 5'UTR and five positive VP1 enterovirus amplicons were subsequently submitted to GenBank to obtain accession numbers.

A phylogenetic tree was generated for the partial VP1 region to ascertain the serotypes of the enterovirus, employing the Maximum Likelihood method under the Kimura 2-parameter distance model with 1000 bootstrap replicates (23). The implementation of these methodologies was facilitated by MEGA software version 6.

RESULTS

Out of 24 effluent samples, 10 (41.66%) exhibited positive results for both the 5'UTR and VP1 regions of the human enterovirus. The five sequences corresponding to the partial 5'UTR have been documented in GenBank with the following accession numbers: OR587957, OR587958, OR587959, OR587960, and OR587961. Similarly, the five sequences from the partial VP1 region have been deposited in GenBank under accession numbers OR594642–OR594646. Analysis of the VP1 region—supported by consensus reference comparison and phylogenetic reconstruction—identified all five isolates as echovirus 7 (Fig. 1).

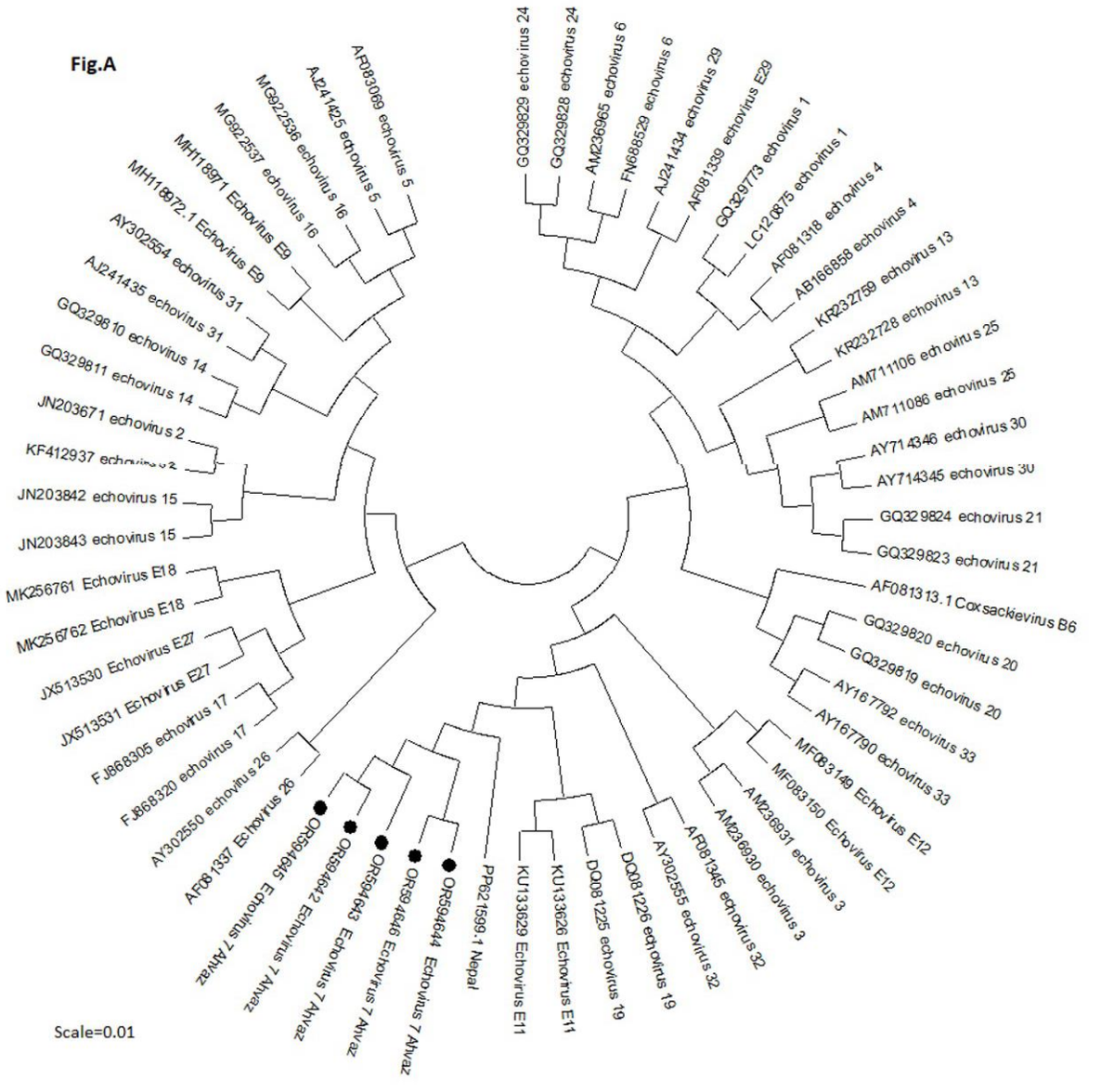

Fig. 1. presents a phylogenetic tree of the VP1 region from enteroviruses detected in sewage effluent. The tree was constructed using the Maximum Likelihood method. The VP1 isolates (OR594642–OR594646) were compared with reference echovirus serotypes from GenBank. Analysis of the tree showed that the isolates, marked by black circles, cluster with the echovirus 7 reference sequence PP621599 (Nepal). Coxsackievirus B6 was used as the outgroup.

DISCUSSION

Wastewater possesses the potential to harbor a myriad of chemicals, microbiological pathogens, and other pollutants that pose significant risks to both human health and aquatic ecosystems (24, 25). Among these contaminants, enteroviruses (EVs) represent the most ubiquitous viral pathogens affecting the human population. These viruses can elicit a spectrum of acute manifestations, ranging from relatively benign respiratory infections akin to the common cold to severe systemic afflictions such as meningitis, myocarditis, and flaccid paralysis (26).

The present investigation elucidated that among 24 sewage effluent samples, 10 (41.66%) were identified as positive for enterovirus. The analyses of nucleotide and amino acid sequences, in conjunction with the evaluation of the phylogenetic tree for five ran-

domly selected samples, revealed that all five identified samples corresponded to echovirus 7. Phylogenetic analysis showed that the VP1 sequences of the isolates (OR594642–OR594646), marked by black circles, clustered with echovirus serotype 7 (accession PP621599, Nepal).

Few studies have reported the isolation of echovirus 7 from sewage. It has been detected in sewage samples from Shandong Province, China (Tao et al., 2016) (27), and Argentina (Farías et al.) (28) and Senegal (Ndiaye et al.) (29). To the best of my knowledge, this is the first report of echovirus 7 detection in a sewage treatment plant in Ahvaz, Iran.

Although rare, severe manifestations of echovirus 7 infection have been documented in neonates and children (30-32). Systemic echovirus 7 infection can involve multiple organ systems, leading to conditions such as meningoencephalitis, encephalomyelitis, sepsis, pneumonia, acute gastrointestinal injury, and coagulopathy (33, 34).

Enterovirus virions exhibit substantial stability, with the majority of enteroviruses demonstrating considerable resistance to acidic conditions, enduring pH levels as low as 3.0 (9, 26).

Kyriakopoulou et al. (35) detected echovirus 7 in river water and from a sewage treatment plant in Larissa, Greece. Rhodes et al. (36) detected echovirus 7 from different water matrices using hollow-fiber ultrafiltration and celite, Cincinnati city, Ohio, USA. Karim et al. (37) have detected echovirus 7 from large volumes of water using inexpensive electropositive cartridge filter, the NanoCeram filter, Cincinnati city, Ohio, USA. Amdiouni et al. detected echovirus 7 in Casablanca, Morocco (38). The polyethylene glycol 6000 (PEG 6000) method has been used for the recovery and concentration of viruses, including echovirus 7 (EV7), from water and wastewater (39, 40).

The existing literature on the detection of human enteroviruses in Iran is exceedingly sparse. Moazeni et al. identified a concerning level of contamination risk associated with enteroviruses in the context of reclaimed wastewater utilized for agricultural activities, asserting that the presence of these viruses could constitute a significant public health concern in Isfahan, Iran, as of 2017 (15).

A limitation of this study is that it did not screen for other viral pathogens, such as human adenovirus, hepatitis A and E viruses, astrovirus, norovirus, human rotavirus, and human papillomavirus. The presence of these viruses in the same samples warrants further investigation (41-45).

Despite the existence of over 280 serotypes of human EVs, only four have been associated with licensed vaccines, specifically those targeting the three serotypes of poliovirus and EV-A71 (9, 46). Currently, there are no antiviral therapies available to address EV infections, and efforts to implement comprehensive global surveillance for these viruses have lacked effective coordination (9).

The resilience of enteroviruses (EVs) makes their eradication a significant challenge. Their ability to resist inactivation and persist in water and sewage systems facilitates their spread and complicates public health efforts (24). Consequently, the removal of enteroviruses during the sewage treatment process is of paramount importance, necessitating heightened attention to this critical phase of wastewater management.

CONCLUSION

This research elucidates a notable prevalence rate of 41.66% for enterovirus identified within samples obtained from treated sewage facilities. Notably, echovirus 7 emerged as the predominant enterovirus present within these treated sewage specimens. The eradication of enteroviruses poses significant challenges due to their inherent resilience to inactivation and their ability to persist in aquatic environments and sewage systems for extended durations. Consequently, it is imperative to prioritize the formulation of vaccines and antiviral agents to counteract the repercussions of EV outbreaks. Therefore, ensuring effective enterovirus removal during sewage treatment is critical and requires greater focus and optimization.

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