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ScienceDirect

Journal of the Chinese Medical Association xx (2018) 1−8

www.jcma-online.com

Original Article

Complete genome sequence of a multi-recombinant echovirus 6 strain isolated from CSF in Ahvaz, Southwestern Iran

Samaneh Abbasi [a,b], Manoochehr Makvandi [a,b,*], Ali Teimoori [a,b], Alireza Samarbaf-Zadeh [a,b]

[a] *Health Research Institute, Infectious and Tropical Disease Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran*
[b] *Department of Virology, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran*

Received February 13, 2017; accepted June 13, 2017

Abstract

Background: Echovirus 6 (E6), is one of the main enteroviral serotypes, was initially isolated from patients with aseptic meningitis (AM) and is a major cause of hospitalization among children and adults worldwide.
Methods: A cerebrospinal fluid (CSF) sample was collected from patient with clinically suspected aseptic meningitis (AM) in August 2011. Following detection of a virus and subsequent virus serotyping, the whole genome sequence was determined. The sequence of the VP1 region of the isolated strain E6 RA/E6/Ahvaz/Iran/2011 showed 79% (>75%) nucleotide and 94% (>85%) amino acid homology with prototype strain D'Amori. The isolated strain was identified as an E6 serotype. A specimen was cultured in a human rhabdomyosarcoma (RD) cell line. Following propagation, the virus was further analyzed using the plaque assay technique, reverse transcription PCR (RT-PCR), rapid amplification of CDNA ends (RACE), TA cloning, sequencing, phylogenetic analysis, Simplot and boot scanning analyses (ver. 3.5) were applied to find evidence of recombination in the isolated strain.
Results: The isolated Echo6 strain RA/E6/Ahvaz/Iran/2011 has been recorded in GenBank with a partial and complete genome accession numbers (KX619440) (KX198605), respectively. The complete genomic sequence was 7435 nt, with a 742 bp 5′ UTR, 117 bp 3′ UTR, and an open reading frame (ORF) encoding a polypeptide of 2191 amino acids. The nucleotide analysis of the VP1 and structural genomic regions of the isolated strain showed high similarity with strain E6-10887-99 isolated from patient with facial nerve paresis in Russia in 1999. The recombinations evidence were observed in the isolated strain E6 RA/E6/Ahvaz/Iran/2011 and found to have a high levels of inter-serotypic exchanges in 2C and 3A-3C genomic regions with Echovirus13 and Echovirus14, respectively.
Conclusion: Full genome sequence analysis of enteroviral is required to understand the epidemiological pattern and to evaluate the new enterovirus circulating in community.

Keywords: Enterovirus B; Iran; Molecular sequence data; RD cells; Recombination

1. Introduction

The new order *Picornavirales* includes the *Picornaviridae* family, and the *Enterovirus* (EV) genus is one of the best characterized genera in this family.[1,2] Human enteroviruses (HEVs) are divided into four species, HEVs A to D, and consist of more than 110 serotypes.[1,3] They are non-enveloped, small icosahedral and single-stranded (+) sense RNA viruses with a genome about 7.5 kilobases (kb) in length, containing a single open reading frame (ORF) encoding a polyprotein. The coding region is divided into three sub-regions: P1 encodes the structural proteins (VP4, VP2, VP3, and VP1) and the P2 and P3 regions encode non-structural proteins. In the P2 region, 2A encodes a protease and

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

* Corresponding author. Dr. Manoochehr Makvandi, Department of virology, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.
E-mail address: manoochehrmakvandi29@yahoo.com (M. Makvandi).

https://doi.org/10.1016/j.jcma.2017.06.026

2B–2C encodes two proteins involved in replication of RNA and inhibition of host cell gene expression. Region P3 contains 3A (encodes membrane-anchored factors and an RNA-binding protein), 3B (encodes a genome-linked protein called VPg), 3C (encodes a viral protease), and 3D (encodes an RNA-dependent RNA polymerase).[3,4]

Human enteroviruses are very contagious; they are typically spread by the fecal-oral route and can also be spread by respiratory routes and from mother to infant in the peripartum period.[5] Human enteroviruses infect an estimated billion or more persons each year worldwide.[3,6] More than ninety percent of enterovirus infections are benign, self-limiting, and asymptomatic; but a fraction of enteroviruses cause severe and life-threatening illnesses, such as aseptic encephalitis, aseptic meningitis (AM), summer colds, epidemic myalgia, acute myocarditis, acute flaccid paralysis (AFP), acute hemorrhagic conjunctivitis (AHC), acute childhood viral exanthema, and hand, foot, and mouth disease (HFMD), which are public health problems worldwide.[5,7,8]

Enteroviruses were traditionally typed by neutralization reaction tests, but the results were often ambiguous. Today, typing is based on the sequence of the VP1 capsid protein and the typing protocol has been replaced by molecular methods that provide a comprehensive genetic characterization of each enterovirus serotype.[8,9] The definitions of enterovirus serotypes are based on nucleotide and amino acid similarities; thus, the same serotype is defined as having >75% nucleotide similarity or >85% amino acid sequence similarity in the VP1 coding sequence.[9] High rates of mutations and recombinations have been reported among the different enteroviruses serotypes, providing mechanisms for frequent enterovirus evolution.[8] The rate of genetic recombination is correlated with the endemicity of virus populations and the repeated or time-correlated cycles of emergence of HEVs.[7,10]

Currently, no effective medicines, antiviral treatments, or vaccines are available for many of the enteroviral infections in the *Picornaviridae* family.[11] In the present study, a cerebrospinal fluid (CSF) sample was collected from a patient with aseptic meningitis. The aim was to detect the virus, to perform serotyping, and to determine the whole genome sequence. Full genome analysis is necessary for a more comprehensive understanding of epidemiological events involving enteroviruses serotypes.[7] This is the first description of the complete genome sequence of the E6 strain causing AM in Iran.

2. Methods

2.1. Patient and clinical sample

A CSF sample was obtained from a female infant with clinical signs and symptom of AM who was hospitalized at the Razi University Hospital (Ahvaz, Iran) in August 2011. The sample was delivered to the virology laboratory and stored at −70 °C. First diagnosis showed positive for enterovirus by amplification of the 5′ untranslated region (UTR) by Reverse transcription polymerase chain reaction (RT-PCR).[12] Subsequently, the VP1 RT- PCR amplification assay was used to identify molecular typing of the isolated strain.[9]

2.2. Cell culture and plaque assay

2.2.1. Preparation of virus stocks

The CSF sample was inoculated and grown on a human rhabdomyosarcoma (RD) cell line at 37 °C in a 5% CO_2 atmosphere for 5–6 days. The cells were observed for signs of cytopathic effects (CPEs) for 24 h. The medium in each well was then decanted, the wells were washed twice with phosphate-buffered saline (PBS, Bio-Idea, Iran), and then the medium was replaced with fresh 1x Dulbecco's Modified Eagle Medium (DMEM; Bio-Idea, Iran) supplemented with 10% heat inactivated fetal bovine serum (FBS; Bio-Idea, Iran) and 1% penicillin (100 U/mL)/streptomycin (100 mg/ml) (Pen/strep, Bio-Idea, Iran). Subsequently, when the CPEs were observed, suspensions of pure-culture virus were prepared by multiple freezing (−20 °C) and thawing (37 °C) cycles and a final vortexing.

2.2.2. Double layer agar plaque assay

Pure virus was isolated using a plaque assay on double layer agar (DLA). A 150 µl volume of tenfold serially diluted virus particles (10^{-4} to 10^{-8} dilutions) was seeded, along with a negative control, into 6-well plates (Sigma–Aldrich) containing confluent monolayers of RD cells (1×10^5 cells/cm^2). After absorption of the viruses at 37 °C for 1 h, 3 ml of 2x DMEM (Bio-Idea, Iran) was mixed with an equal volume of 1.5% purified cell culture grade agar (Sigma–Aldrich, cat NO 1296) supplemented with 1% heat inactivated fetal bovine serum (FBS, Bio-Idea, Iran), overlaid as a monolayer in the 6-well plates (Sigma–Aldrich), and incubated overnight at 37 °C in 5% CO_2. Another 3 ml of 2x DMEM containing 1.5% agar was overlaid onto the 6-well plate and stained with 0.01% neutral red (Sigma–Aldrich, cat. NO. 4638). The plate was sealed with aluminum foil to protect it from light and incubated overnight at 37 °C in 5% CO_2. Three plaques were picked up and passaged three times in a 25 cm^2 flask containing confluent RD cells. Incubation was continued until CPEs were observed, and the culture was then stored at −70 °C for further study.[13]

2.3. RNA extraction, RT-PCR, and rapid amplification of cDNA ends (RACE)

Viral RNA was extracted from cell culture supernatant using a QIAamp viral RNA minikit (Qiagen, Westburg, The Netherlands) according to the manufacturer's instructions. In this study, whole genome sequences were obtained by designing some primers according to 16 conserved regions of several complete genome strains of E6 retrieved from GenBank (Table 1). All primers were synthesized by Metabion (Martinsried, Germany). First, the cDNA was prepared using a RevertAid First Strand cDNA Synthesis Thermo Kit (cat. NO. K1622) according to the manufacturer's instructions. The RT-PCR was then carried out for conserved

Please cite this article in press as: Abbasi S, et al., Complete genome sequence of a multi-recombinant echovirus 6 strain isolated from CSF in Ahvaz, Southwestern Iran, Journal of the Chinese Medical Association (2018), https://doi.org/10.1016/j.jcma.2017.06.026