Virus Research 183 (2014) 75–80

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Molecular typing and characterization of a new serotype of human enterovirus (EV-B111) identified in China

Yong Zhang[a,1], Mei Hong[b,1], Qiang Sun[a,1], Shuangli Zhu[a], Tsewang[b], Xiaolei Li[a], Dongmei Yan[a], Dongyan Wang[a], Wenbo Xu[a,*]

[a] 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, Beijing, People's Republic of China
[b] Tibet Center for Disease Control and Prevention, Lhasa City, Tibet Autonomous Region, People's Republic of China

ARTICLE INFO

Article history:
Received 14 October 2013
Received in revised form 7 January 2014
Accepted 8 January 2014
Available online 3 February 2014

Keywords:
New serotype enterovirus
EV-B111
Characterization
Seroepidemiology

ABSTRACT

Molecular methods, based on sequencing the region encoding the complete VP1 or P1 protein, have enabled the rapid identification of new enterovirus serotypes. In the present study, the complete genome of a newly discovered enterovirus serotype, strain Q0011/XZ/CHN/2000 (hereafter referred to as Q0011), was sequenced and analyzed. The virus, isolated from a stool sample from a patient with acute flaccid paralysis in the Tibet region of China in 2000, was characterized by amplicon sequencing and comparison to a GenBank database of enterovirus nucleotide sequences. The nucleotide sequence encoding the complete VP1 capsid protein is most closely related to the sequences of viruses within the species enterovirus B (*EV-B*), but is less than 72.1% identical to the homologous sequences of the recognized human enterovirus serotypes, with the greatest homology to EV-B101 and echovirus 32. Moreover, the deduced amino acid sequence of the complete *VP1* region is less than 84.7% identical to those of the recognized serotypes, suggesting that the strain is a new serotype of enterovirus within *EV-B*. The virus was characterized as a new enterovirus type, named EV-B111, by the Picornaviridae Study Group of the International Committee on Taxonomy of Viruses. Low positive rate and titer of neutralizing antibody against EV-B111 were found in the Tibet region of China. Nearly 50% of children ≤5 years had no neutralizing antibody against EV-B111. So the extent of transmission and the exposure of the population to this new EV are very limited. This is the first identification of a new serotype of human enterovirus in China, and strain Q0011 was designated the prototype strain of EV-B111.

1. Introduction

Currently, human enteroviruses (EVs) are classified into 4 species: *EV-A*, *EV-B*, *EV-C*, and *EV-D* (Knowles et al., 2011). The species *EV-B* comprises 60 serotypes: coxsackievirus group B (CVB: serotypes 1–6), coxsackievirus group A (CVA: serotype 9), echovirus (serotypes 1–7, 9, 11–21, 24–27, 29–33), EV-B69, and recently identified novel EV serotypes to be designated EV-B73–B75 (Norder et al., 2002; Oberste et al., 2001, 2004b), EV-B77–B88 (Norder et al., 2003; Oberste et al., 2007; Sun et al., 2013; Tao et al., 2013), EV-B93 (Junttila et al., 2007), EV-B97–B98 (Oberste et al., 2007; Smura et al., 2007; Yamashita et al., 2010), EV-B100–B101 (Oberste et al., 2007), EV-B106–B107 (Yamashita et al., 2010), EV-B110 (Harvala et al., 2011), and simian enterovirus SA5.

The viruses in species *EV-B* belong to the genus *Enterovirus* in the family *Picornaviridae* and order *Picornavirales*. Picornaviruses are small, non-enveloped human EVs comprising 60 copies each of the capsid proteins VP4, VP2, VP3, and VP1, which enclose a positive-sense, single-stranded RNA genome. The viral RNA contains a long open reading frame flanked by a 5′-untranslated region (UTR) and a 3′-UTR.

In this study, we describe a newly discovered EV serotype within species *EV-B*, which was named EV-B111 by the Picornaviridae Study Group of the International Committee on Taxonomy of Viruses (ICTV) (www.picornastudygroup.com). The virus was isolated from a patient with acute flaccid paralysis (AFP) during virological surveillance supporting global polio eradication in China in 2000. To the best of our knowledge, EV-B111 has not been reported elsewhere, and its pathogenic role, disease association, and global occurrence are unknown. The aim of this study was to characterize the newly discovered serotype EV-B111.

* Corresponding author at: 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, No. 155, Changbai Road, Changping District, Beijing 102206, People's Republic of China. Tel.: +86 10 58900187; fax: +86 10 58900187.
E-mail address: wenbo_xu1@aliyun.com (W. Xu).
[1] These authors contributed equally to this work.

0168-1702/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.virusres.2014.01.002

2. Materials and methods

2.1. Clinical specimens

The newly discovered EV serotype (EV-B111, strain Q0011/XZ/CHN/2000, hereafter referred to as Q0011) was isolated from a stool sample collected from a 2-year-old girl who presented with AFP on September 2000 in Lhasa City (population: ~474,500) of the Tibet Autonomous Region, China, during the course of poliovirus surveillance activities in support of the global polio eradication initiative. Epidemiological data was collected prospectively by the attending physician using an anonymous standard questionnaire after the oral informed consents were obtained.

For an antibody seroprevalence study to EV-B111, 50 health children ≤5 years of age were surveyed. Fifty serum samples were collected randomly, with informed parental consent, in 2010 by the Tibet Center for Disease Control and Prevention: 25 collected in Lhasa City and 25 collected in Xigaze Prefecture. All children had no sign of disease at the time of sample collection.

2.2. Viral isolation and primary identification

A stool sample from the AFP patient was collected and processed according to the standard procedures recommended by the World Health Organization (WHO, 2004). The samples were then inoculated into 2 cell lines, human rhabdomyosarcoma (RD) and a mouse cell line carrying the human poliovirus receptor (L20B), used to observe the development of EV-like cytopathic effects, and the virus grew only in the RD cell line. Isolates were initially characterized by a micro-neutralization assay using poliovirus type-specific rabbit polyclonal antisera and pooled horse antisera against the most frequently isolated echoviruses and coxsackieviruses (National Institute for Public Health and the Environment [RIVM], Bilthoven, The Netherlands) (WHO, 2004).

2.3. Neutralizing antibody detection

Neutralizing antibodies against EV-B111 were detected with a neutralization test by microtechnique on human RD cell line, as previously described with some modifications (Zhu et al., 2010). Serum samples were inactivated at 56 °C for 30 min before use, and sample dilutions of 1:4–1:512 were assayed. Twenty-five microliters of virus, with a tissue culture infective dose ($TCID_{50}$) of 100, was mixed with 25 μl of the appropriate serum dilution and incubated. After incubation for 7 days, the highest dilution of serum that protected 50% of the cultures was recorded. A serum sample was considered positive if the neutralization antibody level was presented at a dilution of 1:8.

2.4. Viral RNA extraction and reverse transcription

Viral RNA was extracted from the viral isolate using a QIAamp Viral RNA Mini Kit (Qiagen) and stored at −80 °C for further use. SuperScript II RNase H-reverse transcriptase (1 μl, 200 U; Invitrogen) was used to produce single-stranded cDNA from 5 μl of purified viral RNA. The cDNA syntheses were primed by primers 7500A and E492 (Table 1), respectively, and performed at 42 °C for 2 h, followed by incubation at 60 °C for 15 min to inactivate the enzyme. Finally, RNA in an RNA:DNA hybrid was specifically degraded with 1 μl of RNase H (Promega) at 37 °C for 30 min.

2.5. Full-length genome amplification

Two long-distance PCR amplifications were performed using the TaqPlus Precision PCR system (Stratagene). Reactions contained 5 μl of cDNA (see above), 0.1 mM of each dNTP, 10 μl of TaqPlus buffer, 1.0 ng μl^{-1} of a forward (0001S48 or E490) and reverse (E492 or 7500A) primer (Table 1), and 5 U of TaqPlus enzyme in a 100-μl reaction. The amplification was carried out with 32 cycles of 94 °C (40 s), 60 °C (40 s), and 72 °C (5 min), followed by final incubations at 94 °C (1 min) and 72 °C (15 min).

2.6. Nucleotide sequencing

Two long-distance PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen). Cycle sequencing reactions were carried out using BigDye terminator chemistry (ver. 3.1; Applied Biosystems), and the amplicons were sequenced by a 'primer-walking' strategy. The primers used in PCR and the sequencing reaction are listed in Table 1. Sequencing was performed in both directions using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems), and every nucleotide position was sequenced at least once from each strand. The 5′ rapid amplification of cDNA ends (RACE) core set (Takara Biomedicals) was used to determine the

Table 1
PCR and sequencing primers.

Primer	Nucleotide position (nt)	Primer sequence (5′–3′)	Orientation	Reference
0001S48[a]		GGGGACAAGTTTGTACAAAAAAGCAGGCTTTAAAACAGCTCTGGGGTT	Forward	Yang et al. (2003)
EVB111-858S	858–877	ACTCAGCAAACAGGCAGGAT	Forward	This study
EVB111-1077A	1058–1077	TTGAGATATTCGGGCCACTC	Reverse	This study
EVB111-1740S	1740–1759	CAGTAATGGCCACTCCAGGT	Forward	This study
EVB111-1939A	1920–1939	CCGGTACGCATCCATACTTT	Reverse	This study
E490[a]	2226–2248	TGIGTIYTITGYRTICCITGGAT	Forward	Oberste et al. (2006)
EVB111-2619S	2619–2638	CTCGGTCGGAATCGAGTATT	Forward	This study
EVB111-2835A	2816–2835	CCTTGGCCCTGATATGATGT	Reverse	This study
E492[a]	2953–2934	GGRTTIGTIGWYTGCCA	Reverse	Oberste et al. (2006)
EVB111-3544S	3544–3563	TGTCTTTGAGGGTCCAGGTC	Forward	This study
EVB111-3686A	3667–3686	CAACAACTCCGTGCTCACAC	Reverse	This study
EVB111-4471S	4471–4490	AAAATCTGTGGCGACCAATC	Forward	This study
EVB111-4634A	4615–4634	GTGACACGTCCTTCCCATCT	Reverse	This study
EVB111-5381S	5381–5400	AAGGTTCAGGGACCAGCTTT	Forward	This study
EVB111-5598A	5579–5598	AGATTGGTGCCATCCTTGTC	Reverse	This study
EVB111-6294S	6294–6313	CTTATGTTGCCCTTGGCATT	Forward	This study
EVB111-6481A	6462–6481	CAGACTCGATGCCTCAATCA	Reverse	This study
EVB111-7038S	7038–7057	TGACATGGACCAACGTCACT	Forward	This study
EVB111-7193A	7174–7193	ATAAGGAGCGCACATGATCC	Reverse	This study
7500A[a]		GGGGACCACTTTGTACAAGAAAGCTGGG$(T)_{24}$	Reverse	Yang et al. (2003)

[a] The primer pairs 0010S48/E492 and E490/7500A were used for long-distance PCR, with expected amplicons of 3.00 kb and 5.20 kb, respectively.