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LETTER

Molecular typing of non-polio enteroviruses isolated from acute flaccid paralysis cases in Iran from 2010 to 2015

Dear Editor,

Acute flaccid paralysis (AFP) is a complex syndrome often caused by polioviruses. While most countries have eradicated wild polioviruses by vaccination, AFP still remains a health problem in these countries. Most studies have highlighted non-polio enteroviruses (NPEVs) as main isolates from patients with AFP. Type identification of NPEVs isolated from AFP cases in wild poliovirus-free countries, such as Iran, is crucial. To achieve this, virus isolation in cell culture and typing of the nontypeable isolates are standard methods for identification of NPEV serotypes. Since reference antisera are not available for all NPEV types, some enteroviruses are frequently found to be untypeable. Molecular typing of NPEVs has been very useful for identification of many NPEV isolates. In this study, we aimed to achieve molecular typing of untyped NPEVs from patients with AFP.

In this study, the NPEVs isolated from 29 AFP cases that failed in neutralization test (NT) were identified by nested polymerase chain reaction (PCR), partial VP1 sequencing, and phylogenetic analysis. The results showed that all NPEVs belonged to human enterovirus B (HEV-B). Echovirus 3, echovirus 33, and echovirus 21 were the predominant genotypes. Enterovirus 75 (EV-B75) was detected in two cases of AFP, representing the first separation in Iran. Phylogenetic analysis was performed for geographical clustering of echovirus 3 and enterovirus 75, showing remarkable clustering with previous isolates in India and suggesting that a specific isolate circulated in these regions. Thus, this study highlighted the different HEV-B types in AFP cases and revealed that surveillance of NPEVs was essential in polio-free countries.

Enteroviruses (belonging to the genus *Enterovirus* of the family *Picornaviridae*) have a positive-sense RNA genome enclosed in a naked, icosahedral capsid. These viruses infect individuals through fecal-oral transmission. Over 250 enterovirus serotypes capable of infecting humans have been identified (Bodian, 1955; Stanway, 2013). Determining the serotype of HEV is important for epidemiology, prevention, and control. Despite elimination of polio in Iran, there are still numerous cases of AFP observed annually that are not associated with poliovirus (Kapusinszky et al., 2010; Saeed et al., 2007; Trallero et al., 2010).

Enterovirus detection and serotyping can be achieved by conventional methods of RD cell culture and microneutralization tests (Oberste et al., 2000); however, these methods are limited by a lack of reference antisera for all enterovirus serotypes and the inability to detect new isolates. For direct identification of all enterovirus serotypes, reverse transcription PCR (RT-PCR) of the VP1 region and sequencing are often applied. Thus, we assessed HEV strains circulating in Iranian children with AFP by molecular techniques.

Twenty-nine samples that displayed cytopathic effects in RD cells but were negative in microneutralization tests were used as templates. RNA was extracted from the RD cell lysate. The RNA was eluted in 50 μL DEPS water and stored at –70 °C until use. cDNA synthesis was then carried out in with avian myeloblastosis virus reverse transcriptase at 42 °C for 45 min. Then, VP1 nested RT-PCR was performed using 224 and 222 primers for the first round synthesis and AN88 and AN89 primers for the second round, as previously described (Rahimi et al., 2009; Oberste et al., 2006). PCR products were directly sequenced and analyzed using the GenBank database; the highest identity score was detected as the serotype of the strain (Table 1).

The BLAST results showed that all NPEVs belonged to HEV-B. The majority of the 29 sequenced isolates were echoviruses, and the type of coxsackievirus was not determined. All 29 isolates from NPEVs included E-3 (34%), E-24 (13%), E-33 (10%), E-21 (10%), E-11 (6%), EV-B75 (6%), E-6 (3%), E-19 (3%), E-14 (3%), E-30 (3%), and E-13 (3%). The frequency of echovirus 3 among all PCR-positive NPEVs was highest. Notably, using VP1 sequence analysis, HV-B75 was identified for the first time in Iran.

To investigate the genetic relatedness of echovirus 3 and EV-B75 isolates in this study with globally circulating types, phylogenetic analyses of partial VP1 sequences with prototypes representing different types available in GenBank were performed. The results showed that the strains in this study had monophyletic clusters and that

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Table 1. Enterovirus types detected by partial VP1 sequencing

	Sample name	Accession No.	Serotype	Identity(%)
1	318-2010-IRI	MF002015	E-6	90
2	425-2010-IRI	MF002033	E-3	91
3	254-2011-IRI	MF002016	E-21	95
4	261-2011-IRI	MF002019	E-24	92
5	261-2011-8-IRI	Similar 261-2011	E-24	92
6	261-2011-9-IRI	Similar 261-2011	E-24	92
7	261-2011-11-IRI	Similar 261-2011	E-24	92
8	459-2011-IRI	MF002017	E-33	96
9	512-2011-IRI	MF002018	E-19	95
10	549-2011-IRI	MF002031	E-3	92
11	225-2011-20-IRI	MF002032	E-3	91
12	225-2011-19-IRI	Similar 225-2011	E-3	91
13	95-2012-IRI	MF002020	E-21	94
14	137-2012-IRI	MF002034	E-3	92
15	185-2012-IRI	MF002021	E-14-IRAN	90
16	310-2012-IRI	KT245156	EV-B75	91
17	379-2012-IRI	MF002035	E-3	92
18	411-2012-IRI	KT245155	EV-B75	91
19	558-2012-IRI	MF002022	E-30	95
20	71-2013-IRI	MF002030	E-3	92
21	215-2013-IRI	MF002029	E-3	90
22	405-2013-IRI	MF002023	E-11	96
23	691-2013-IRI	MF002024	E-11	95
24	90i-2014-IRI	MF002025	E-21	94
25	28-2015-IRI	MF002026	E-33	94
26	30-2015-IRI	MF002028	E-3	91
27	189-2015-IRI	MF002036	E-3	90
28	339-2015-IRI	Not determined	E-13	91
29	723-2015-IRI	MF002027	E-33	94

both echovirus 3 and EV-B75 isolates (accession nos.: KT245155.1 and KT245156.1) clustered with close geographical regions (India isolates; Figures 1A, 1B).

The gold standard for the detection and typing of EV infections is virus isolation from cell culture, followed by analysis with neutralization tests based on the Lim Benyesh-Melnick (LBM) pool. However, pools of antisera for neutralization tests are limited to detection of all enterovirus serotypes. In this study, samples that failed in serological typing (untypeable enteroviruses) were analyzed by molecular tests and sequence analysis.

Previous studies have shown that most NPEVs isolated from AFP cases were related to coxsackievirus B; however, only echovirus strains were isolated in our study (Battistone et al., 2014; Oyero et al., 2014). This could be because HEp-2 cells were widely replaced with RD cells in 2003 by national polio laboratories, and RD cells have a low sensitivity for isolation of coxsackieviruses (World Health Organization (WHO), 2004; Prim et al., 2013). Our molecular results also showed that RD cells were not appropriate for separation of coxsackieviruses and NPEVs.

In this study, nine different isolates of echoviruses were detected, with echovirus 3 being the most commonly

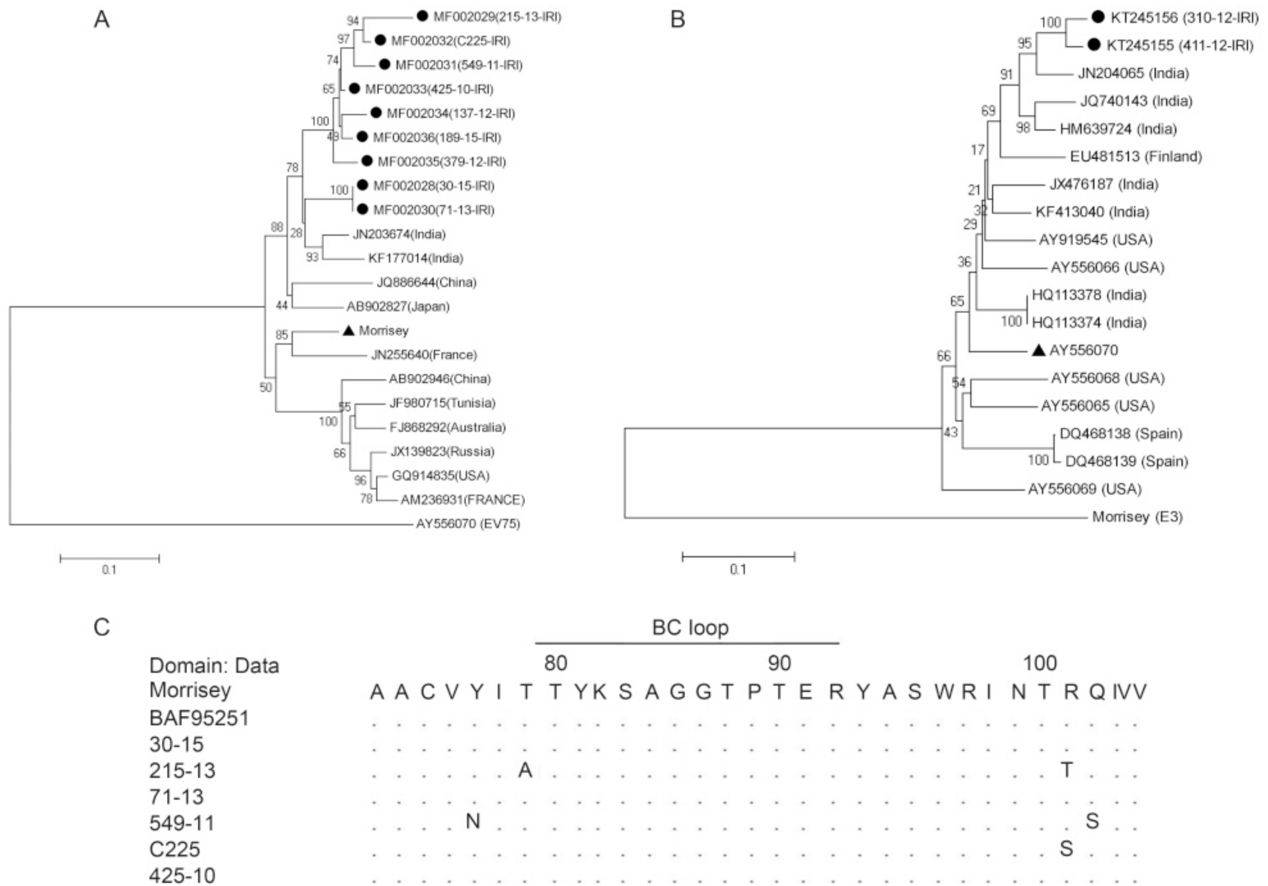

Figure 1. (A) Phylogenetic tree of echovirus 3 partial VP1 sequences from Iran and reference sequences from GenBank. The tree was constructed by the neighbor-joining algorithm implemented in the MEGA-5 program using p-Distance. Sequences from Iran and the prototype are indicated with black circles and black triangles, respectively, and reference sequences are indicated by accession number (country). (B) Neighbor-joining phylogenetic analysis of VP1 enterovirus 75. Closed circles indicate EV-B75 isolates identified in this study (accession nos.: KT245155.1 and KT245156.1) and other previously reported EV-B75 sequences (from 1789 to 2119). GenBank accession numbers and origin of country are reported. The scale bar indicates the number of nucleotide substitutions per site. The significance of phylogenies was investigated by bootstrap values, with 500 pseudoreplicates; datasets of greater than 75 are indicated at the branch nodes. The prototype is indicated with a black triangle. (C) Echovirus 3 multiple amino acid alignment of VP1 partial of sequences of the Morrisey reference sequence (from 2538 to 2855) and six untypeable echovirus 3 strains. Each dot indicates a position where there was no difference in the corresponding amino acid residue from the reference strain.

isolated serotype, consistent with a previous study (Rahimi et al., 2009). In contrast, previous studies have shown that E-6, E-11, and E-13 have been recorded more frequently than other echovirus strains (Battistone et al., 2014; Oyero et al., 2014). Thus, it is likely that this type is the most common environmental circulating echovirus strains in recent years. Further studies involving monitoring of wastewater and sewage surveillance are needed.

Sequencing showed that the most prevalent echovirus was echovirus 3. We assumed that mutations occurred in the VP1 antibody binding site (BC loop) and affected strain identification by neutralization assays. However, bioinformatics analysis was performed to investigate mutations in the BC loop, and no changes were detected in this region (Figure 1C).

Notably, we detected EV-B75 in two patients with AFP. Although EV-B75 was isolated from patients with AFP in a previous study (Lewthwaite et al., 2010), this was the first report of EV-B75 isolation from patients with AFP in Iran (accession nos.: KT245155.1 and KT245156.1). Additionally, phylogenetic analysis showed that the two strains were similar; however, compared with the different strains from GenBank, separate clusters were formed, and the strains were closely related to the JN204065

strain isolated from India (Figure 1C).

In summary, our findings showed that for correct identification of NPEVs, cell lines other than RD cells must be used. In addition, neutralization tests did not show high sensitivity for identification of all NPEVs. Finally, establishment of direct molecular tests with high sensitivity and specificity is needed to identify NPEV from patient and environmental samples.

FOOTNOTES

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