

Original Article

Molecular assay and genotyping of childhood enterovirus infection in central nervous system

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Received January 11, 2016; Accepted May 20, 2016; Epub January 1, 2017; Published January 15, 2017

Abstract: Enterovirus (EV) belongs to the picornavirus family and is common pathogen in clinics. Early study regarding EV infection mainly utilized pathogen culture, but retarding diagnosis and treatment. Recent progress of molecular biology has made progress for pathogen diagnosis. This study thus investigated the expression of EV in cerebrospinal fluid (CSF) of children with central nervous infection, in an attempt to discuss the clinical significance of EV genotyping. 94 children with aseptic encephalitis (or meningitis) were enrolled in parallel with 37 controls. Fluorescent RT-PCR was used to screen EV from CSF, with positive results obtained from those samples with CT values less than 30. After primary screening of EV, amplification of VP1 and VP4 segment was then performed, followed by sequencing of positive fragments and genotyping. No EV RNA was detected in 37 control patients, while 69 out of 94 aseptic encephalitis patients had EV RNA expression. Among those 53 samples were positive for VP1 segment while 47 samples were VP4-positive. Sequencing of those segments showed more than 96% homology with Gen Bank EV standard viral strain. Fluorescent RT-PCR can detect EV RNA from CSF of central nervous infection children with efficiency and accuracy. The combined genotyping of VP4 sequence can further improve sensitivity of EV typing.

Keywords: Enterovirus, VP1, VP4, RT-PCR

Introduction

Enterovirus (EV) belongs to the picornavirus family and is common pathogen in clinics [1]. Currently discovered EVs consists of 71 serotypes including poliovirus (PV), Coxsackievirus (CV), entero cytopathic human orphan virus (ECHO) and new type enterovirus [2]. EV has small viral particle of icosahedron shape, in which single positive strand RNA resides [3]. EV RNA encodes 4 structural proteins, namely VP1 to VP4 [4], which form the capsid surrounding viral RNA. VP1 to VP3 locate outside the capsid while VP4 resides inside [5]. Study has found that outside EV viral capsid there were multiple antigen determinants, which are related with specific immune response [6]. The polymorphism of genes encoding outer surface of capsid determines specificity of antigen determinants and genetic polymorphism of all serotypes of virus [7]. VP4 protein, which locates inside the capsid, ensures the stability of virus

[8]. Epidemiology study showed worldwide distribution of EV infection, especially in those regions with warm, humid environment and crowded population. China is one high-incidence area of EV. With popular application of vaccine, PV infection is quite rare in recent years, whilst CV and ECHO are still common pathogens for both local and systemic inflammation [9]. EV infection can occur in all age groups, among which adult patients frequently present as minor or asymptomatic features due to complete immune functions, while children infection may develop into systemic symptoms even affecting major organs such as brain, heart and liver, causing unfavorable prognosis or even death [10]. An incomplete survey showed that EV infection accounts for 80% of all aseptic encephalitis (or meningitis) [11]. Due to its requirement for host cells, isolation and culture of viral pathogens is relatively difficult, causing time lag of pathogen diagnosis, thus retarding clinical application. Recent progress

Genotyping of enterovirus infection

Table 1. Primer sequence

Primer	Sequence
Universal	Forward TCCGGCCCCTGAATGCGGCTAAT
	Reverse CACCGGATGGCCAATCCA
	Probe GAAACACGGACACCCAAAGTA
VP1	Forward MIGCIGYIGARACNG
	Reverse CICCIGGIGGIAYRWACA
	Probe CCGAGGATGCAAGGCTTGTTCAGA
VP4	Forward CARGTYTCHGTNCCRTTYATGTCACC
	Reverse GCTGTYTTCGMYTTRAYCCADGC
	Probe CGAAGGATGCCAGAAGGTACCCGT

Note: IUB standard codes were used for mixed base pairs in primer sequence. I, hypoxanthine; D=G, A or T; R=A or G; Y=T or C; N=A, T, C or G; M=A or C.

in molecular biology has made advancement in pathogen diagnosis of EV. Fluorescent RT-PCR revolutionized classical pathogen diagnosis approach. Due to its safety, rapidness, high replicability, sensitivity and specificity, fluorescent RT-PCR has become an important tool in molecular biology [12]. This study thus employed fluorescent RT-PCR to describe the expression of EV in cerebrospinal fluid (CSF) of EV infected children, in an attempt to elucidate the clinical significance of EV genotyping in central nervous system (CNS) infected children.

Materials and methods

Research objects

A total of 94 aseptic encephalitis (meningitis) children from January 2011 to December 2014 were enrolled in Dongying Municipal People's Hospital. 1 ml CSF samples were collected at the day of admitting. There were 74 patients at acute phase and 20 children at recovery phase. There were 61 males and 33 females, with aging between 0.5 and 10 years (average age = 5.82 ± 3.81 years). Another cohort of 37 children (24 males and 13 females, average = 6.73 ± 3.25 years) who received brain surgery at the same time period were enrolled as the negative control. They had no clinical symptom of infection, with normal blood and biochemical indexes. Positive control was employed using standard viral strain of COXB3, ECHO30 and EV71 provided by Kunming Institute, Chinese Academy of Sciences. CSF samples were stored at -80°C . This study has been approved by ethical committee of Dongying Municipal People's Hospital and has obtained written consents from all research subjects.

Viral RNA extraction

QiampMinelute Virus Spin Kit (QIAGEN, Germany) was used to extract viral RNA. 0.5 ml CSF was mixed with 50 μl proteinase K and 200 μl AL buffer, and was vortexed for 30 s. The mixture was then incubated at 60°C for 15 min. 0.5 ml absolute ethanol (Baotaike, China) was added for 30 s vortex, followed by 5 min room temperature incubation. The mixture was transferred to the column and was centrifuged at 12000 g for 30 s at 4°C . The filtrate was discarded, followed by 0.5 ml buffer I and 0.7 ml buffer II (each followed by 30s centrifugation). 0.1 ml RNase-free water was then added into the column for 1 min centrifugation to obtain RNA solution, which was kept at -80°C for further use.

Fluorescent RT-PCR

One step RT-PCR kit reverse transcription kit (ABI, USA) was used for RNA synthesis. 500 ng RNA was incubated at 65°C for 5 min, followed by ice cool down for 2 min. PCR primer and Taqman Universal Master Mix (ABI, USA) including reverse transcription buffer, polymerase and water were prepared in a 20 μl system. PCR amplification was performed on VIIA 7 real-time fluorescent quantitative PCR cycler (ABI, USA) under the following conditions: 95°C for 5 min, followed by 40 cycles each containing 95°C 15 s, and 60°C 60 s. Universal primers and typing primers were shown in **Table 1**. Universal primer was designed based on 5'-non coding region of EV genome, while typing primers were located at VP1 and VP4 regions.

Target gene fragment purification

Quick Gel Extraction Kit (QIAGEN, Germany) was used to purify target gene fragments. Agarose gel bands containing target gene were weighted and mixed with QG solutions (100 mg gel with 300 μl buffer). After heating at 50°C plate, with inversion every 3 min until complete resolving of agarose gel, the mixture was added with equal volume of isopropanol (Baotaike, China). After vortex, the mixture was transferred to the column for 12000 g centrifugation at 4°C for 1 min. The infiltrate was discarded and 0.5 ml QG buffer was loaded onto the column, which was re-centrifuged for 12000 g at 4°C for 1 min. 0.75 ml PE buffer was again added and was centrifuged for 12000 g at 4°C for 1 min. The column was transferred and

Genotyping of enterovirus infection

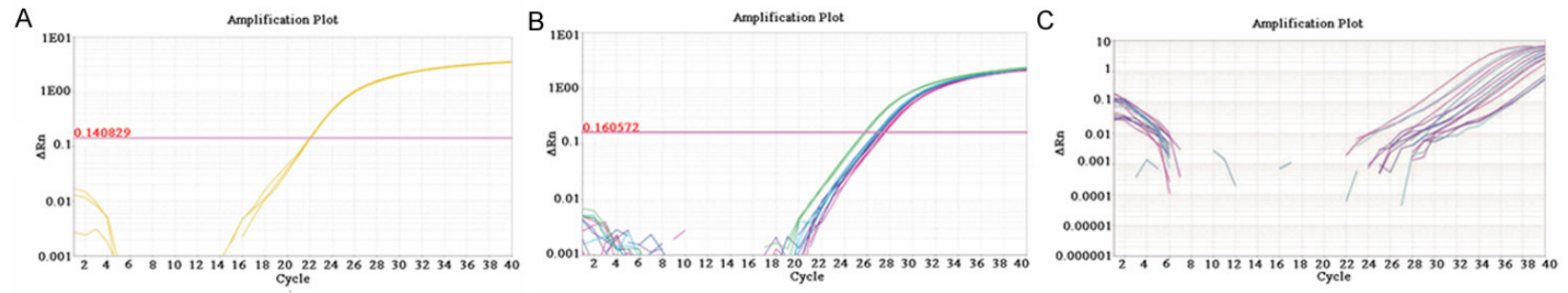


Figure 1. RT-PCR amplification curve using universal primers. A. Standard viral strains; B. Positive CSF samples; C. Negative CSF samples.

Genotyping of enterovirus infection

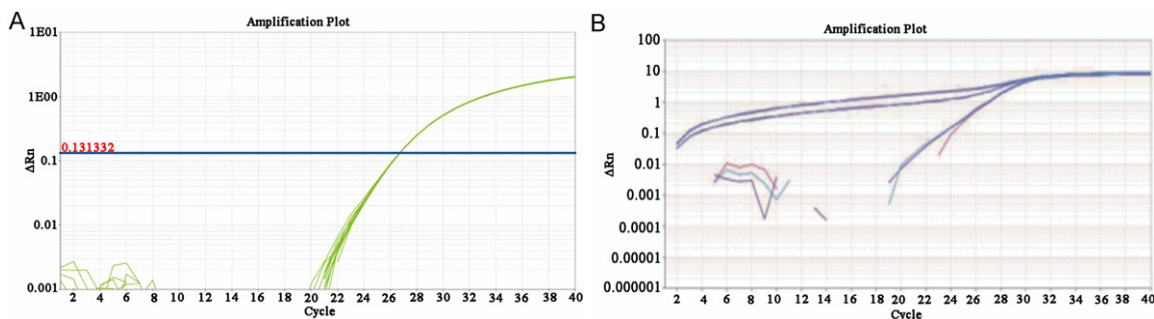


Figure 2. Fluorescent RT-PCR amplification curves by VP1 primer. A. Positive CSF samples; B. Negative CSF samples.

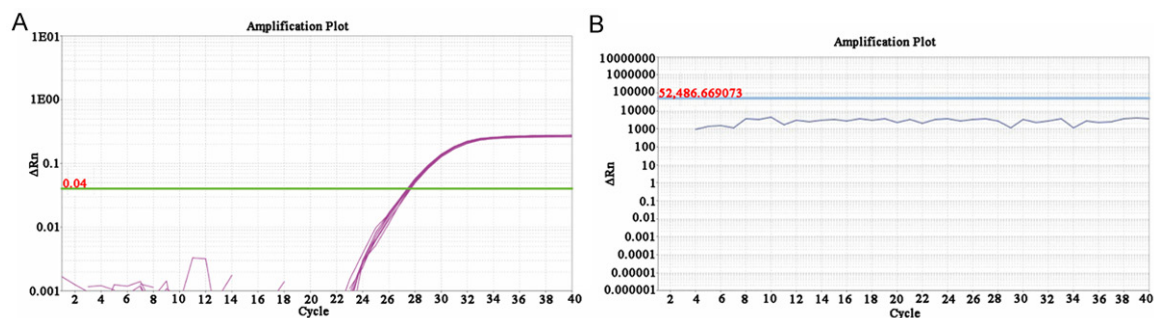


Figure 3. Fluorescent RT-PCR amplification curve by VP4 primers. A. Positive CSF samples; B. Negative CSF samples.

loaded with 100 μ l elution buffer for extracting RNA solution by 1 min centrifugation. RNA solution was stored at -20°C for further use.

Transformation and sequencing of recombinant plasmid

10 μ l solution containing target gene fragment was incubated on ice, and was mixed with 2 μ l pBR322 vector, 1 μ l T4 DNA ligase and 6 μ l ligation buffer for 4°C overnight incubation. On the next day, competent cells stored at -80°C were thawed on ice, and were mixed with 10 μ l ligation products for 30 min iced incubation. Cells were transferred to 42°C water-bath (Changyuan, China) for 90 s, followed by ice incubation for 2 min. 1 ml LB (without ampicillin) medium was added for 37°C incubation for 60 min. 0.4 ml mixture was inoculated onto culture dish containing LB (with ampicillin) medium. After 37°C incubation for 12 h, bacterial colony was inoculated into 5 ml tube containing 4 ml LB medium with ampicillin. The tube was incubated at 37°C for 12 h. 1 ml bacterial solution was then extracted for plasmid DNA and sequenced by BGI (China). DNA star sequence analysis software was used to align sequences.

Results

Fluorescent RT-PCR using universal primers

We used fluorescent RT-PCR to test standard viral strains of EV (including COXB3, ECHO30 and EV71) using universal primers for amplification. CT value of the amplification curve was about 22, showing single curve (**Figure 1A**). We further tested CSF samples from 94 aseptic encephalitis (meningitis) and 37 control children using universal primers. Amplification curves of positive samples were shown in **Figure 1B**. 69 out of 94 patients were positive for EV RNA by fluorescent RT-PCR, making positive rate at 73.4%. In all 37 control samples, fluorescent RT-PCR was negative for EV RNA (**Figure 1C**).

Fluorescent RT-PCR using VP1 primers

Universal primer showed 69 out of 94 CSF samples from aseptic encephalitis (meningitis) children were positive for EV RNA. We then genotyped those 69 samples regarding VP1 segment. Fluorescent RT-PCR amplification curve was shown in **Figure 2**. Results showed a total

Genotyping of enterovirus infection

Table 2. VP1 and VP4 typing results

Group	Positive	Negative	Statistics
VP1	58	11	$\chi^2=1.151, P=0.283$
VP4	53	16	
VP1+VP4	65	4	$\chi^2=8.271, P=0.016$

Table 3. Homology between clinical and standard strains of EV

Sample size	EV standard strain	Sequence homology
6	COX A7	97%
12	COX A9	97%
7	COX A11	99%
9	COX B3	98%
6	COX B5	96%
5	ECHO 12	98%
2	ECHO 30	96%

of 58 positive (CT value less than 30) samples, making the positive rate at 84.1%.

Fluorescent RT-PCR results by VP4 primers

VP4 segment genotyping was performed in 69 positive samples with EV RNA. Fluorescent RT-PCR amplification curve of target genes were shown in **Figure 3**. There were 53 cases with RT-PCR positive (Ct values less than 30), making the positive rate at 76.8%.

Comparison between VP1 and VP4 typing

In all 69 cases with positive EV RNA expression in CSF, there were 58 VP1 positive samples and 53 VP4 positive samples. No significant difference existed between two typing methods ($\chi^2=1.151, P=0.283$, **Table 2**). We then combined VP1 and VP4 typing, and found 63 positive cases (with either of VP1 or VP4 positive). Comparing to single typing of VP1 and VP4, there was significant difference of positive rate ($\chi^2=8.27, P=0.016$).

Sequence results of genotypes

We sequenced 47 samples with dual positive in VP1 and VP4 typing. Results were aligned in parallel with sequence generated by DNA star sequence analyzing software. We found over 96% homology of sequence in 47 positive samples against EV standard strains (**Table 3**).

Discussion

EV is one common pathogen in humans and can lead to various diseases [13]. Epidemiology survey showed worldwide distribution of EV infection, with high incidence, morbidity and mortality, thus severely affecting the public health [14, 15]. EV infection can affect multiple tissues/organs, especially in central nervous system such as encephalitis or meningitis [16, 17]. Obserste et al generated genotyping approach of EV based on VP1 gene sequence by VP1 genotyping [18]. VP4 protein resides inside the capsid, and has 207nt length, which ensures the stability of EV gene sequence [19]. Some studies found different viral gene structures and pathogenic ability across different EV serotypes, plus variations of clinical symptoms in patients, suggesting the implication of EV typing in both diagnosis and treatment of EV infection [20]. However, classical method had difficulty regarding pathogen diagnosis of virus, plus the inability to determine certain viral strains, thus severely impeding diagnosis and treatment of EV infection. Recently, with the wide application of fluorescent RT-PCR, the long-time consuming of pathogen diagnosis has been resolved. Based on this, we proposed to investigate the application of fluorescent RT-PCR in genotyping EV, and its clinical implication in typing of EV gene from CSF in children with CNS infection.

Experimental results showed that fluorescent RT-PCR approach can rapidly identify EV. Using universal primers in standard viral strains, 94 cases of aseptic encephalitis (meningitis) CSF samples and 37 negative control samples, it was found that single amplification curve occurred in positive controlled standard viral strains without specific amplification, no amplification curve in all 37 negative control samples, and amplification curves in 69 out of 94 CSF samples with aseptic encephalitis (meningitis), making the positive rate of EV RNA at 73.4% as consistent with previous study [21]. Study showed that universal primers had better specificity in fluorescent RT-PCR amplification, further suggesting that EV is the major pathogen for aseptic encephalitis or meningitis in children. We further amplify 69 CSF samples that were positive for universal primers using sub-typing primers. Results showed that 58 (or 53) out of 69 samples were positive for VP1 (or

VP4) segment gene. There was no significant difference between two types of genotyping approaches ($\chi^2=1.151$, $P=0.283$). Combined assay of VP1 and VP4 found 65 positive cases, with significant difference compared to other two typing methods ($\chi^2=8.27$, $P=0.016$). These results suggested that both VP1 and VP4 could genotype EV with satisfactory efficiency, with improved efficiency by combined assay. By sequencing of VP1 and VP4 positive products, and sequence alignment by DNA star software, we found positive sequence in all 47 CSF samples, which had more than 96% homology against EV standard viral strains. These results indicated that the genotyping of VP1 and VP4 in conjunction with sequence alignment with EV standard strain, may benefit typing of EV.

In summary, fluorescent RT-PCR can rapidly identify EV in CSF samples of aseptic encephalitis (or meningitis) children. Sequencing of VP1 or VP4 can achieve genotyping of EV pathogen, while combined assay of VP1 and VP4 can expand positive amplification rate. These genotyping data suggested that, in the sub-typing of EV pathogen, one can perform VP1 genotyping first, followed by VP4 genotyping in negative samples, in order to improve the positive rate of EV pathogen.

Disclosure of conflict of interest

None.

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Genotyping of enterovirus infection

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