

Original Article

Analysis on the sequence of the whole genome of an isolated enterovirus 71 strain

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Abstract: An enterovirus 71 (EV71) strain Query was isolated from a patient specimen in 2015. In order to know about its genetic evolution, this study amplified gene fragment of the isolated strain by RT-PCR and carried out sequencing of the total genome. The homology and genetic evolution of the gene sequence of the virus strain in the study were analyzed. The results showed that the isolated EV71 strain in this study had higher homology of nucleotide sequence and amino acid sequence with other virus strains, which was 80%-97% and 88% to 92%, respectively, but it had lower homology with Cox.A16 (homology of nucleotide sequence and amino acid sequence of Cox.A16 was 81% and 79%, respectively). Compare of homologous sequence at the encoding region VP1 demonstrated that the experimental isolated strain EV71 had higher homology of amino acid sequence at VP1 region with other virus strains. Genetic evolution of nucleotide sequence at VP1 region of the identified strain and other EV71 strains was analyzed, and the results demonstrated gene sequence at VP1 region and 5'UTR region of the isolated strain and SDLY017 strain was at the same branch, both of which belonged to C4a, a subtype of type C4.

Keywords: EV71, genome, homology, genetic evolution analysis

Introduction

Enterovirus 71 (EV71) is a member of Picornaviridae Enterovirus, and its infection mainly causes hand-foot-and-mouth disease (HFMD) [1-3]. Under general conditions, it is difficult to differentiate the clinical symptoms of HFMD caused by EV71 infection and those caused by Coxsackie virus A16 (CA16) [4]. However, not only HFMD but also aseptic meningitis, brain-stem encephalitis and poliomyelitis-like paralysis and other diseases related to central nervous system can be caused by EV71 infection, bringing patients greater damage compared to CA16 [5-7]. According to the difference of nucleotide sequence at VP1 region, EV71 are divided into three gene types, A, B and C, and type B and type C are further divided into B1, B2, B3 and B4, C1, C2, C3 and C4 [8].

In 1974, Schmidt et al. firstly reported EV71 was isolated from a California patient with a disease of nervous system symptoms [9]. Subsequently, EV71 was reported to be epidemic by many countries and regions. Currently,

EV71 has caused disease outbreaks and prevalence for many times throughout the world, and the prevalence at the Asian-Pacific region exhibits rising tendency [10-14]. HFMD prevalence was firstly reported in Shanghai in 1981, which was then reported in Beijing, Hebei, Fujian, Jilin, Shandong, Hubei, Xining, Guangdong, etc. [15]. Therefore, conducting related study about EV71 was significant for prevention and control of the virus outbreaks and prevalence. Genetic evolution of the whole gene sequence of EV71 (also named Query) isolated from one patient specimen was analyzed in the paper.

Materials and methods

Virus isolation

Virus was isolated from the patient specimen. Inoculate the specimen on healthy rhabdomyoma (RD) cells with good morphology and a single layer after pretreatment, and set up the cell control. All specimens were passaged blindly for two generations, and it was judged to be negative if cytopathic (CPE) did not occur after

Sequence of enterovirus 71 strain

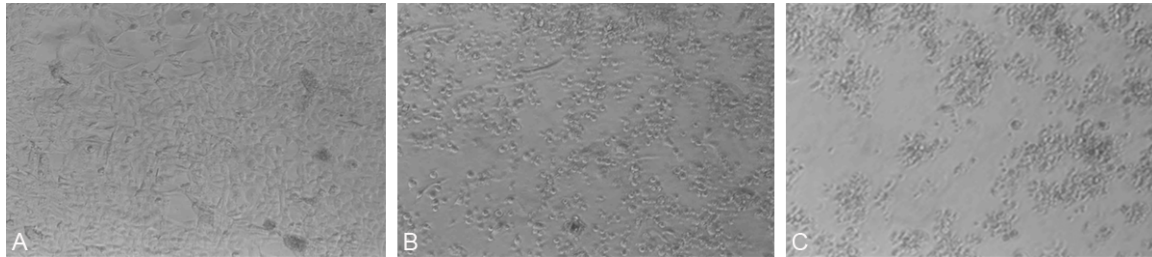


Figure 1. Virus isolation on RD cells. A: 0 day after virus infection. B: 7 days after virus infection; C: 9 days after virus infection.

Table 1. Comparison of homology of nucleotide and amino acid sequence between the identified strain and other EV71 strains

| | Genome | Ployprotein |
|----------------|--------|-------------|
| U22521 (A) | 80 | 88 |
| ETU22522 (B) | 81 | 90 |
| DQ341361 (C1) | 82 | 90 |
| AF119795 (C2) | 81 | 89 |
| DQ341356 (C3) | 83 | 90 |
| EU703813 (C4a) | 97 | 92 |
| AF302996 (C4b) | 90 | 88 |
| EU527983 (C5) | 82 | 90 |
| U05876 (CA16) | 81 | 79 |

the second generation. The procedures of cell culture and virus isolation followed the standard procedures in the further edition of Laboratory Operating Procedures of Poliomyelitis issued by WHO.

Extraction of virus ribonucleic acid (RNA) and reverse transcription polymerase chain reaction (T-PCR)

Nucleic acid of the virus was extracted by QIA amp Viral RNA Mini Kit (TAKARA). Take 200 µl of the virus isolation suspension, and operate according to the kit instructions. RT-PCR of the extracted RNA was carried out immediately, with the method referring to DNA extraction kit instructions of TAKARA. PCR products were analyzed by 0.8% agarose gel electrophoresis.

Sequencing of nucleic acid

The recycled products were sent to a sequencing company for sequencing. The employed instrument was ABI PRISM 3730, the sequencing reagent was Big Dye terminator v3.1, and Sanger sequencing method was used.

Sequence analysis

The sequencing results were edited by DNA-MAN8.0 software. Online assemble software CAP3 was used to montage the virus strain sequence, and the sequence after montage was compared with Blastn in NCBI website to determine whether it was the sequence of EV71. Blastn software was also used to match homology of the virus nucleotide and amino acid. Neighbor-Joining method in MEGA 5.2 software was employed to construct the evolution tree.

Results

EV71 Query strain isolated

The specimen was continuously passaged on RD cells for two generations, and they were harvested when the cells shrunk and dropped obviously (**Figure 1**). EV71 was primarily identified by RT-PCR, which was named Query strain.

Sequencing of the whole virus genome and homology compare

It was found by nucleotide sequencing that length of the whole genome of the identified Query strain in the study (excluding poly-A tail) was 7425 bp, among which A, C, G, and T as well as GC accounted for 27.1%, 24.4%, 23.7% and 24.7%, 48.1% respectively. A total of 2197 amino acids were encoded. Compare of homology of nucleotide and amino acid sequence in the virus genome are listed in **Table 1**. The results showed that the isolated strain had higher homology of nucleotide and amino acid sequence with other EV71 strains, which was 80%-97% and 88% to 92%, respectively, but it had lower homology with Cox.A16 (homology of nucleotide sequence and amino acid sequence

Sequence of enterovirus 71 strain

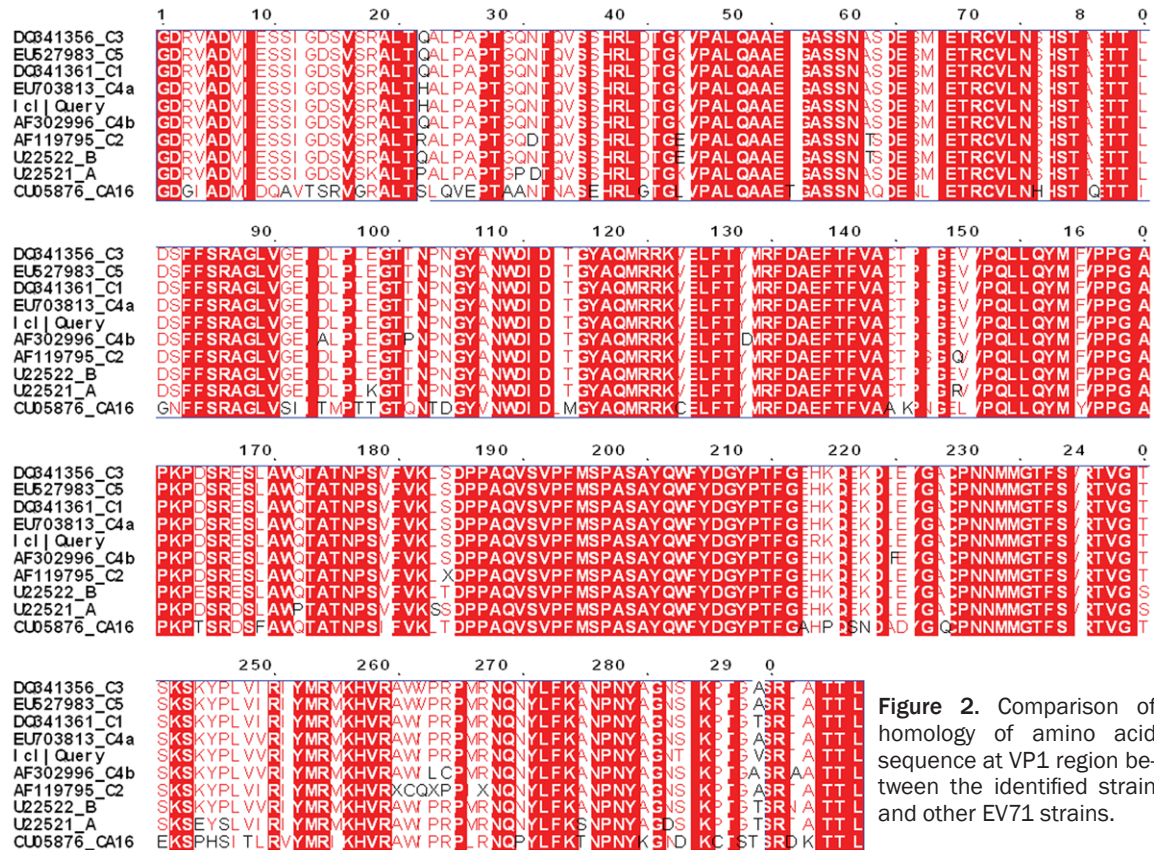


Figure 2. Comparison of homology of amino acid sequence at VP1 region between the identified strain and other EV71 strains.

of Cox.A16 was 81% and 79%, respectively). Compare of homologous sequence at the encoding region VP1 demonstrated that the experimental isolated strain EV71 had higher homology of amino acid sequence at VP1 region with other virus strains (**Figure 2**).

Genetic evolution analysis of the virus gene

Analysis on genetic evolution of the nucleotide sequence at VP1 region in the identified strain and other EV71 strains demonstrated that sequence at VP1 region of the identified strain gathered at C4a, a subtype of type C4, and it was located at the same branch with SDLY107 virus strain, indicating they had close relationship (**Figure 3**). Nucleotide sequence at VP1 region of other EV71 strains gathered at individual classes.

Analysis on genetic evolution of nucleotide sequence at 5'UTR region of the identified strain and other EV71 strains demonstrated that the identified strain and SDLY107 virus strain were on the same branch, and farther from other types of representative strains.

Discussion

Enterovirus 71 (EV71) is a member of Picornaviridae Enterovirus, which is a single stranded positive RNA virus with a genome length of about 7400 bp [8]. In the recent years, EV71 showed a tendency of outbreaks in China and dead cases of EV71 were reported in several provinces. Molecular genetics study of poliomyelitis virus demonstrated that change of a single nucleotide or amino acid at a specific region of the genome, such as 5'UTR and VP1 region, was critical for neurotoxicity of the virus [17]. Therein VP1 gene is most valuable in study, which determines antigenicity of the virus. It is not only the classification basis of various serum types of enterovirus, but also the classification reference of various members of Picornaviridae [18]. During the recent years, recombination and mutation of virus genes frequently occurred, leading to outbreaks of EV71 diseases with various symptoms in various regions. Therefore, it is significant to investigate and analyze the whole genome sequence of EV71. In the study, the whole genome of one

Sequence of enterovirus 71 strain

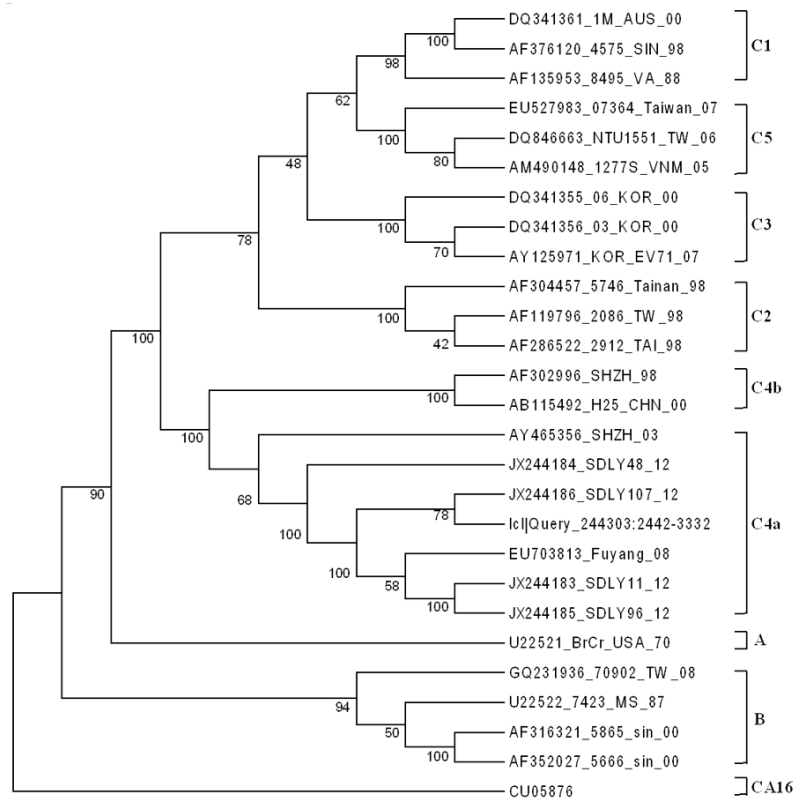


Figure 3. Analysis on genetic evolution of the nucleotide sequence at VP1 region in the identified strain and other EV71 strains.

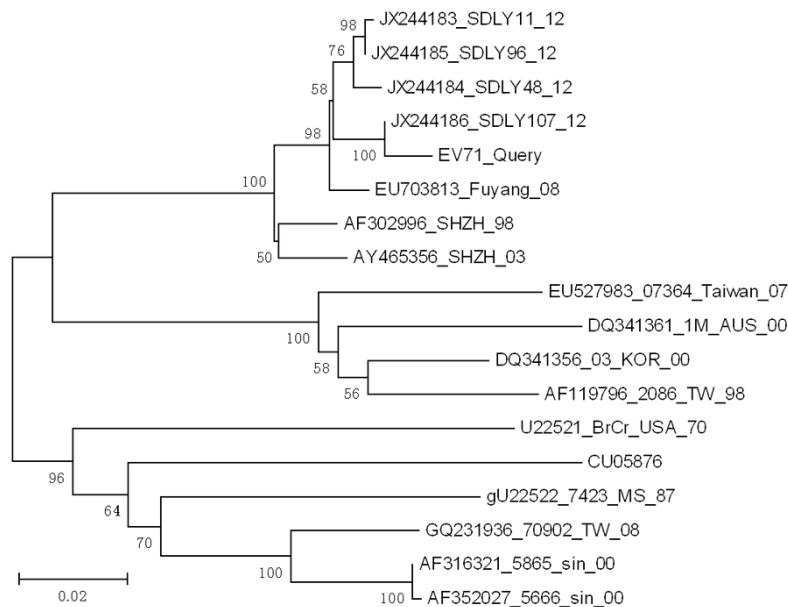


Figure 4. Analysis on genetic evolution of nucleotide sequence at 5'UTR region of the identified strain and other EV71 strains.

EV71 strain (Query strain) was sequenced and the homology was analyzed. Moreover, the tree of genetic evolution was plotted, and the rela-

(representative strain of type B) and BrCr/70 (representative strain of type C) (**Figures 3 and 4**), suggesting they had farther relationship.

tionship of the tested sequence was analyzed.

The whole genome of the identified strain Query in the study had a length of 7425 bp, encoding 2197 amino acids in total. Sequences of the representative strains of type A, B and C of EV71 were downloaded from Gen Bank, and homology was analyzed, with the tree of relationship system constructed by Neighbor-Joining method. The results of homology analysis demonstrated that the isolated strain in the study had higher homology with the various representative strains of EV71, but it had lower homology with Cox.A16 (**Table 1**). Compare of the homology of amino acid at VP1 region between Query and other EV71 strains showed they had higher homology (**Figure 2**). Thus the results above confirmed that the isolated Query in the study was EV71 strain.

The relationship tree of gene sequences in the isolated Query strain in the study and representative A, B and C strains of EV71 was constructed based on the encoding region of VP1 and 5'UTR region. The results demonstrated that nucleotide sequence at VP1 region and 5'UTR region of the isolated Query strain in the study was closest to SDLY107 strains genetically, indicating they had the closest genetic evolution relationship. The identified strain in the study had a farther distance with MS/87

The previous study demonstrated that sDLY107 strains had closer relationship with Beijing strains, Henan strains, Guangxi strains, Shenzhen strains, Lanzhou strains, Fuyang strains, Chongqing strains, and Zhejiang strains in China mainland, which were classified into C4 subtype according to the traditional VP1 gene typing method. Query and C4 strains, subtype of EV71, were in a cluster, suggesting their closer relationship. Therefore, the isolated Query strain in the study belonged to C4 strains, subtype of EV71. C4 subtype is the main gene subtype prevalent in China mainland [18]. sDLY107 strain was the EV71 which was isolated by Wen Hongling et al. from RD cells in a dead child patient at Linyi People's Hospital in Shandong in 2011 [18], which may be related to Query. Frequent individual flow among regions was beneficial for the transmission of EV71 between two regions. The results of the study also demonstrated that there may be no obvious antigenic drift and variation of the prevalent C4 strains at present in China, which were relatively stable. Moreover, C4 subtype virus of EV71 may have been widely prevalent and transmitted in China mainland. The previous study indicated that amino acid mutation of E947D and K1873R of sDLY107 occurred, possibly related to neurotoxicity of EV71 [18]. Whether there is amino acid loci mutation and whether there is neurotoxicity still require further study.

Conclusion

Currently, the mechanism of neurotoxicity of EV71 is not clear. In the study, the whole genome of the isolated strain Query was sequenced and the genetic evolution was analyzed. The results demonstrated that the virus strain belonged to C4a genotype, enriching gene bank of national EV71 and providing significant reference basis for the establishment of EV71 prevention and control strategies. At present, C4 subtype may still be the predominant genotype of the prevalent EV71 strain in China mainland. Thus it is necessary to focus eyes on its genetic evolution and monitor continuously.

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Disclosure of conflict of interest

None.

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