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Molecular Epidemiological Analysis of Echovirus 19 Isolated From an Outbreak Associated With Hand, Foot, and Mouth Disease (HFMD) in Shandong Province of China[1]

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Objective To elucidate the genetic characterization and molecular epidemiological features of Echovirus 19 (E19) isolates collected from an outbreak associated with hand, foot and mouth disease (HFMD) in Tai'an city of Shandong Province of China from July to September, 2003. **Methods** Thirty seven Echovirus 19 isolates were isolated from stool specimens and throat swabs collected during the outbreak, then major capsid (VP1) genomic sequence was determined, and phylogenetic tree was done based on the VP1 sequences among these 37 and other E19 viruses deposited in the Genbank. Also a representative strain named CHN-SD03-TN12 was selected for sequencing of 5'-untranslated regions (5'-UTR). **Results** The identity rate was about 98.9%-100% among all these 37 E19 viruses. The genetic relationships between these 37 E19 isolates and other strains reported were also depicted. The identity rate was about 78.4%-78.9% compared with E19 reference strain Burke. The substitutions in the sequence of 5'-UTR resulted in changes in the conjectural properties of 5'-UTR of E19 viruses. **Conclusion** The genetic features of E19 viruses isolated during the outbreak in Shandong Province in 2003 may be associated with a genetic and antigenic drift that changes the virulence of the Shandong isolates, but the molecular changes in Shandong E19 viruses contributing to their phenotype remain to be further illuminated. However, the sequences described in this paper substantiate the changes taken place in capsid VP1 and 5'UTR regions. These substitutions may contribute to their tropism and virulence, and play a significant role in pathogenesis and clinical manifestations of the disease.

Key words: Enterovirus 19(E19); Hand, foot, and mouth disease (HFMD); Molecular epidemiology; 5' Untranslated regions (5'-UTR); B-C loop

INTRODUCTION

Human enteroviruses (HEVs) are common pathogens in human beings over the world and human beings are the only known reservoir of HEVs. HEV infections are associated with a broad spectrum of clinical features, including paralysis, myocarditis, aseptic meningitis, encephalitis, conjunctivitis, or systemic infections, especially in newborn babies[1]. All human enteroviruses (HEVs) are grouped into four clusters (HEV-A, HEV-B, HEV-C, and HEV-D) according to the nucleotide sequence analysis of the major capsid protein (VP1) gene[2-3]. The HEV-B cluster contains all echoviruses, all 6 Coxsackie B viruses, Coxsackie A9 virus, enterovirus type 69, and some new types of enterovirus, such as type 73[4], 74, 75, 77, and 78[5].

The genomic RNA of enteroviruses is of positive polarity and about 7500 nucleotides in length, which contains a single open reading frame (ORF) encoding structural and nonstructural proteins, structural protein encode 4 capsid proteins, VP4, VP2, VP3, and VP1. VP1 is the most important protein among these 4 proteins containing main epitopes that are exposed on the surface of the virion, and the BC loop in VP1 region is of prime importance for the reactivity with type-specific antibodies, although little is known about the exact antibody-binding sites for E19 viruses at present[6]. 5'-untranslated region (5'-UTR) of E19 genome is highly structured, in general containing seven predicted secondary structures, and some of which act as internal

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ribosome entry site (IRES)[7-8], which has important functions to viral life cycle. Also, 5'-UTR has been shown to influence viral replication and the virulence of several enteroviruses, such as poliovirus, Coxsackie B virus, and ECHO virus[9-11].

The enteroviruses are frequently associated with human infections, although most of them are asymptomatic or recessive infection. Some enteroviruses can cause distinct clinical symptoms. It is well known that an outbreak of hand, foot, and mouth disease (HFMD) occurred in Taiwan from 1998 to 2000[12]. HFMD is mostly related to infections with Coxsackie A16 (CA16) and some other enteroviruses, such as CA4, VA5, CA9, CA10 and EV_{711} infections have been reported[13], whereas E19 virus infection was seldom reported due to associated with HFMD but sometimes with uveitis[14], epidemic neuromyasthenia[15], gastroenteritis, myalgia, and acute flaccid paralysis syndrome[16], especially systemic infection in neonates[17]. Furthermore, few E19 infections have been reported in USA in recent years[18-19].

In this paper, we report a prevalence of HFMD in Shandong Province in 2003. This large HFMD outbreak affected more than 150 children with obvious symptoms, most of them had eruptions on mouths, feet, and hands. Nearly half of the patients had subsequent syndromes such as myocarditis, aseptic meningitis, encephalitis, and herpangina. In addition to EV71 and ECHO30 (E30) viruses, E19 virus was identified from the cases of HFMD, representing the most frequent isolates in the outbreak. E19 has been previously considered as a weak pathogen causing only mild symptoms. However, too many E19 viruses were isolated (accounting for about 72.1% of the patients with HFMD complicated by myocarditis and aseptic meningitis). To our knowledge, E19 infection has not been reported to have relationship with HFMD. In this study, a detailed analysis of the genetic features of Shandong E19 virus and other E19 viruses was performed, including the comparison of its nucleotide sequence and deductive amino acid sequence of VP1 region.

MATERIALS AND METHODS

Clinical Specimens and Viral Isolation

All 37 samples were collected from children under 6 years of age. All the children had fever and eruptions in their mouths, hands, feet, and hips, but mouths and hands were the most affected locations, and nearly one third of the children were complicated by certain syndromes. Eleven children (28.6%) had myocarditis, 1 child (2.6%) had aseptic meningitis, and 1 child (2.6%) had herpangina. Viruses were isolated in Center for Disease Control and Prevention of Shandong Province using three different cell lines (RD, Vero, and HEp-2 cells). The cytopathic effect (CPE) only appeared in RD cells (a human rhabdomyosarcoma cell line, ATCC CCL136). Thirty-five E19 viruses were isolated from fecal specimens and another 2 E19 viruses were isolated from throat swab specimens. Cell culture flasks (25 cm^2) containing confluent monolayer of RD cells grown in Eagle's minimum essential medium (MEM) supplied with 10% fetal bovine serum were infected with these E19 viruses. When CPE appeared, the infected cells were harvested by frozen and than three times, and stored at -20℃.

Neutralization Test

Horse antiserum pools against the most frequently non-polio enteroviruses were prepared by the Institute of Medical Biology, Chinese Academy of Medical Sciences in Kunming (KMB). Anti-enterovirus pools A-H were diluted according to the manufacturer's instructions.

Microplate neutralization test was done using 96-well plates, with cell control and virus control in each plate, and the back titration was done to confirm the number of viruses used in the test that was within the range of 32 to 320 $CCID_{50}$.

Then the viruses were grown in monolayer of RD cells, and another neutralization test was done using rabbit polyclonal antiserum to prototype strain Burke (also obtained from KMB).

Viral RNA Extraction and RT-PCR

Nucleic acid was extracted from the viruses using Trizol extraction method (Gibco BRL, NY, USA). By taking 250 μL viral suspension, 3 volumes of Trizol extraction solution and 100 μL chloroform were added. After incubated for 10 min at room temperature, they were centrifuged at 13 000 RPM for 10 min and 500 μL isopropanol was added into the supernatant. After incubated for 10 min at room temperature, they were centrifuged at 10 000 RPM for 10 min at 4℃. After the supernatant was discarded, additional 950 μL of 70% ethanol was added after mixing well, then centrifuged at 10 000 RPM for 20 min at 4℃, and the pellet was dried at room temperature and resuspended in 20 μL RNase-free water.

In order to amplify the amplicons for sequencing, RT-PCR was done after RNA extraction. The primer pairs used[20] are listed as follows:

008: 5'- GCRTGCAATGAYTTCTCWGT-3'
013: 5'-GGIGCRTTICCYTCIGTCCA-3'
012: 5'- ATGTAYGTICCICCIGGIGG-3'
011: 5'- GCICCIGAYTGITGICCRAA-3'

First, reverse transcript was done using downstream primer at 37℃ for 1 h. After reverse transcriptase was inactivated, PCR was performed at 94℃ for 3 min, at 94℃ for 30 s, at 42℃ for 45 s, at 72℃ for 60 s, for 35 cycles, and a final extension at 72℃ for 10 min. The products were identified by gel electrophoresis on 1.7% agarose gels.

The length of the first amplicon between primers 008 and 013 was about 640 bp, at the 5' terminal of the VP1 gene, including BC loop sequence. The length of the second amplicon between primers 012 and 011 was about 460 bp at the 3' terminal of the VP1 gene, and these 2 amplicons were used for sequencing.

Nucleotide Sequencing

The products were purified using the QIAquick gel purification kit (Qiagen, Tokyo, Japan), and about 40 ng of the purified products was used for sequencing reaction. Cycle sequencing reactions were performed using BigDyeTM terminator V3.0 cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA). In order to ensure the reliable results, both sense and antisense strands were sequenced and then the products were purified using Centri-Sep spin column protocol (Princeton Separations Adelphia, NJ, USA). Sequences were determined using the dideoxy chain termination method by 3100 genetic analyzer (ABI, Tokyo, Japan) following the manufacturer's instructions.

Get the 5'-UTR Sequence of E19 Strain

The 5'-UTR sequence of the representative strain CHN-SD03-TN12 was obtained by two methods synchronously. One was acquired by RT-PCR with viral specific primers followed by sequencing. The sequences of the primers are as follows:
0001S: 5'-TTAAAACAGCTCTGGGGTT-3';
PE2: 5'-TCCGGCCCCTGAATGCGGCTAATCC-3';
PE1: 5'-ACACGGACACCCAAAGTAGTCGGTCC-3';
1018Z: 5'-TTGAATTGCCCAGAGTTAGCTGCA-3'.

The primers PE1 and PE2 used here were reported before[21] and the other 2 were designed according to the sequence of E19 reference strain Burke.

The other method was acquired by exact sequencing 5' terminus of the genome using a rapid amplification of cDNA end (5'-RACE) method (TaKaRa Biotechnology, Dalian, China).

The first strand cDNA was synthesized by reverse transcription reaction using the primers decorated with phosphate acid at the 5' terminus, and then the RNA hybridized with cDNA was hydrolyzed in the presence of RNase H (Promega, USA), followed by precipitation and purification with ethanol. The pellet was re-suspended using ligation buffer, and add 40% PEG#6000, 1 μL T4 RNA ligase. Ligation of each single strand cDNA to a circinal one was carried out overnight at 16℃. Using the circinal cDNA as template, the first amplification of the ligated 5' terminus of the viral genome was performed at a 50 μL reaction consisting of template, 10×PCR buffer, dNTPs, 1st PCR primers, Taq polymerase, dH2O, and then nested PCR, in which the template was ten-time diluted of the 1st PCR products in the same condition, but the 2nd primers were used instead. Finally, sequencing was done to get the sequence of 5' terminus of the viral genome.

Sequence Cleaning and Other Relevant Analysis Software

Sequence data were saved as standard chromatogram format (.ab1) files and analyzed using Sequencher software version 4.0.5 (Gene Codes, Ann Arbor, Michigan, USA). An identity search was conducted using the BLAST service at the National Center for Biotechnology Information (National Library of Medicine, USA). Multiple sequence alignments and neighbour-joining phylogenetic tree were made using BioEdit Sequence Alignment Editor Program version 5.0.9 (Tom Hall, North Carolina State University, Carolina, USA), and MEGA software version 2.0 (Sudhir Kumar, Arizona State University, Arizona, USA). Phylogenetic tree was conducted by Tree View software, version 1.6.0.

Nucleotide Sequence Accession Number

Thirty-seven E19 strains reported in this paper are available in the Genbank. The accession number is from DQ081190 to DQ081226.

RESULTS

Neutralization Test

All the 37 viral isolates were identified as E19 by microplate neutralization test using horse antiserum pool, and the viral attack titers ranged from 32 to 320 $TCID_{50}$/0.025 mL. Eight viral isolates were selected randomly and neutralized at 100 $TCID_{50}$/0.025 mL by standard antiserum to E19 virus, but the neutralization titers of 8 viral isolates were lower than those of prototype strain Burke except for SD03-TN45 and SD03-TN48 (Table 1).

Molecular Typing of Shandong Isolates

The VP1 genomic sequences of all 37 viral isolates were determined, which were very similar, and the identity rate was about 98.9%-100%. When a identity search was conducted using the BLAST service

TABLE 1

Identified Serotype and Neutralization Titers of Selected 8 Viruses

ID	Identified Serotype	Neutralization Titer
E19 Strain Burke	/	1∶5120
SD03-TN-12	E19	1∶2560
SD03-TN-37	E19	1∶2560
SD03-TN-38	E19	1∶2560
SD03-TN-45	E19	1∶5120
SD03-TN-48	E19	1∶5120
SD03-TN-50	E19	1∶2560
SD03-TN-54	E19	1∶1280
SD03-TN-56	E19	1∶2560

at the National Center for Biotechnology Information, the maximal similarity reached about 87% compared with E19 strain 87SD140 (Genbank number AJ241438) and 78.9% compared with Burke strain. The second-maximal similarity was about 72%, compared with E11 strain IRQ/2000-10001 (Genebank number, AY121423)[22]. All the 37 isolates were classified as E19, which was in agreement with the results of the neutralization test.

Comparison With Other Available E19 Isolates

There are only 3 E19 VP1 sequences that have been reported and can be downloaded from the Genbank. Within all of the sequences we studied, a phylogenetic tree (Fig.1) was constructed by maximum-likelihood method.

FIG. 1. Phylogenetic tree of the VP1 region of E19 viruses, showing the relationship between the Shandong isolates and other ones. The tree was constructed with the maximum-likelihood method, and E11 strain Gregory was selected as the root.

From the phylogenetic tree, we can see that the VP1 sequences of all these 37 isolates were quite close and clustered into one evolutionary lineage. Compared with the sequence reported, the most similar one to 37 Shandong isolates was isolated in France in 1999 (87SD140, Genbank number AJ241438). The second strain was isolated in Finland in 2003 (K/542/81, Genbank number AY167107). The E19 prototype strain, strain Burke, have the lowest similarity.

A representative Shandong strain CHN-SD03-TN12 was selected for further analysis because all these 37 Shandong sequences were clustered into one evolutionary lineage.

Amino Acid Sequence Comparison Between Strain CHN-SD03-TN12 and Other Available E19 Strains

The deduced amino acid sequences of VP1 of strain CHN-SD03-TN12 compared with other available E19 strains were aligned using BioEdit software (Fig. 2). The amino acid sequence of strain CHN-SD03-TN12 showed the highest homology to strains 87SD140 and K542/81, with the similarity rate of 98.6% and 96.9%, respectively. The similarity rate was about 92.8% compared with E19 prototype strain, strain Burke. B-C loop located in VP1 region formed a part of the epitope on the surface of the virion, which is important for the reactivity with neutralizing antibodies[23]. B-C loop was located from amino acid 82 to 92 in the study (Fig. 2), and there were 2 substitutions between strain CHN-SD03-TN12 and E19 prototype strain, one was glutamine (Q) to lysine (K) in amino acid 82, and the other was aspartic acid (D) to glutamic acid (E) in amino acid 85. Even with these 2 substitutions, it seemed that the property of epitope site B-C loop reactivating with neutralizing antibodies remained unchanged by and large.

5'-UTR Sequence Comparison Between Strain CHN-SD03-TN12 and E19 Prototype Strain Burke

The sequences of 5'UTRs of strain CHN-SD03-TN12 and E19 prototype strain Burke were aligned using BioEdit software. It was noted that the two regions had a higher sequence variation. Within the first high variation region, strain CHN-SD03-TN12 had 2 base deletions at positions 88 and 103, but had a base insertion at position 125, compared with the 5'UTR sequence of E19 prototype strain Burke (Fig. 3).

In 5'-UTR, an oligopyrimidine tract (OPT) is a crucial element for efficient cap-independent initiation of translation[24]. The sequence of OPT element of E19 was located from nt564 to nt583, and both strain CHN-SD03-TN12 and E19 prototype strain Burke had the same sequence of 5' terminus of OPT 5'TTTCCTTTT3', but the sequence of 3' terminus of OPT was highly diverse.

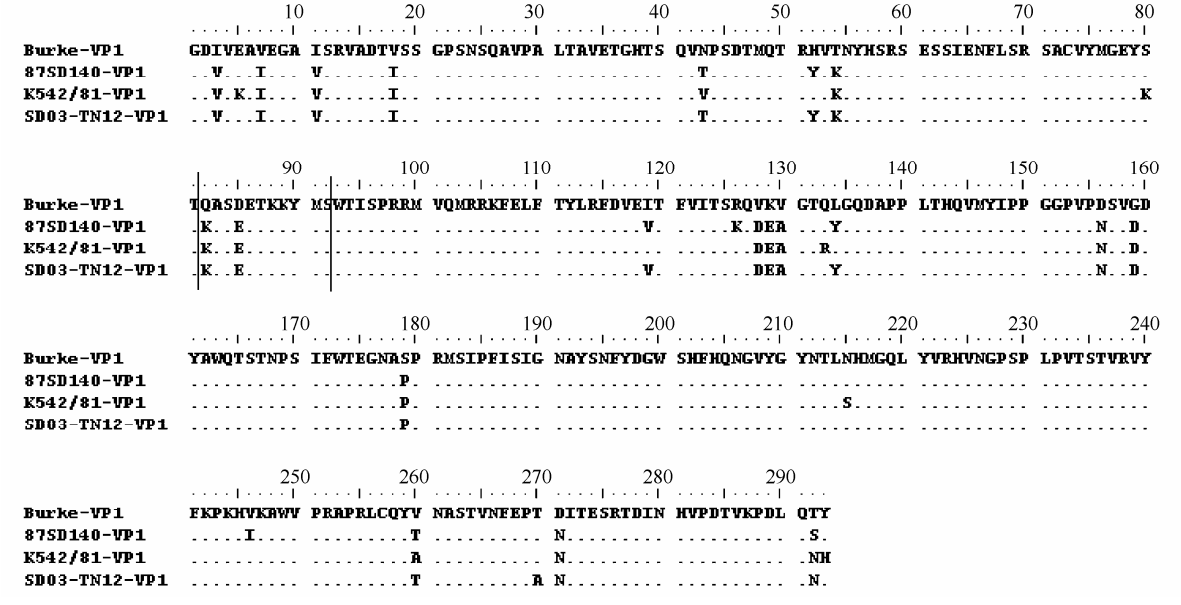

FIG. 2. Alignment of the deduced amino acid sequences of the VP1 polyprotein of strainCHN-SD03-TN12 and other E19 strains, with the sequences between two vertical lines showing B-C loop region.

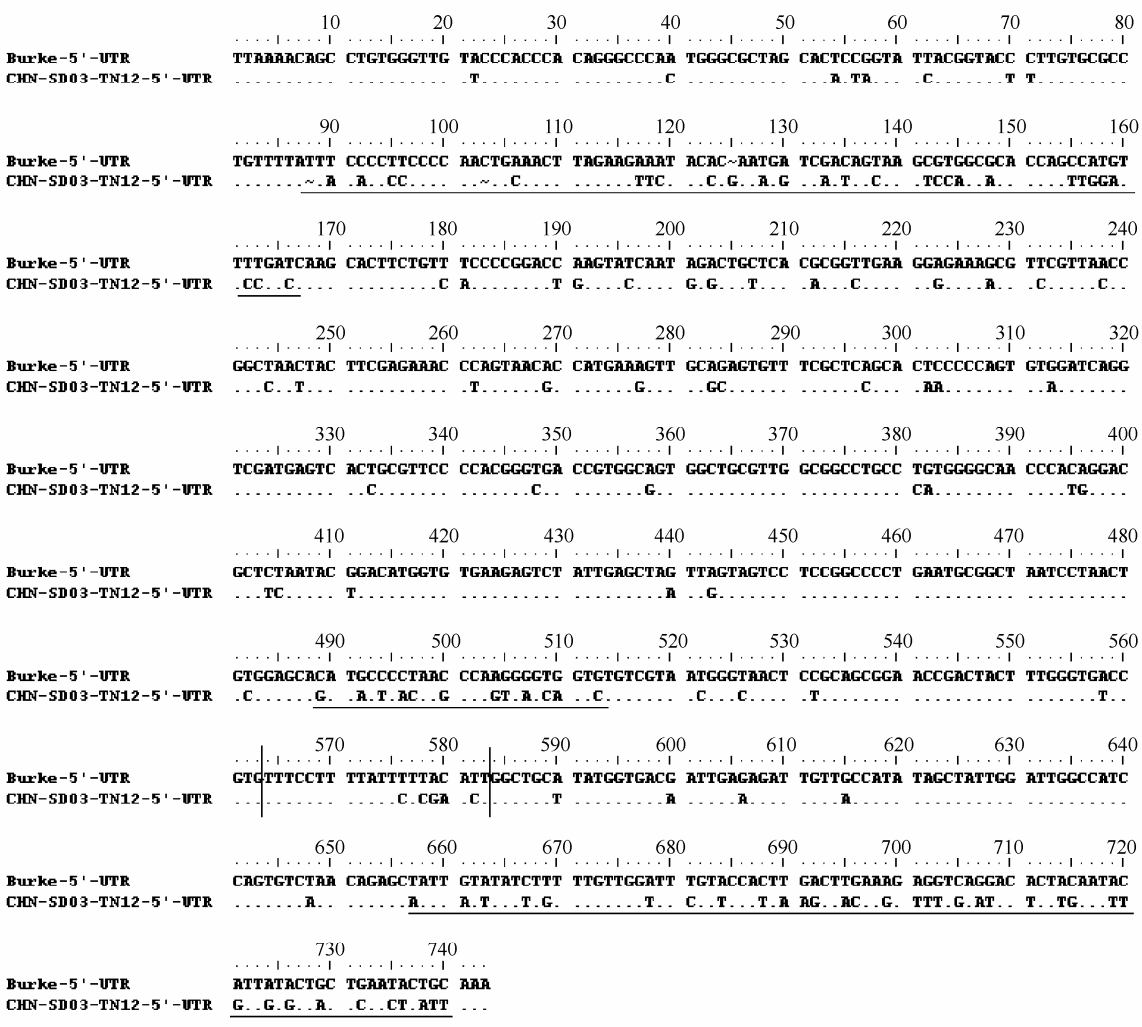

FIG. 3. Alignment of 5'UTR regions of strain CHN-SD03-TN12 and E19 prototype strain Burke. Regions with a higher nucleotide variability are underlined; with the sequences between two vertical lines showing OPT element.

DISCUSSION

A large HFMD outbreak occurred in Shandong Province of China in 2003, which affected more than 150 children with obvious symptoms, and most of them had eruptions on mouths, feet, and hands, nearly half of them developed subsequent syndromes, such as myocarditis, aseptic meningitis, encephalitis, and herpangina. E19 viruses were the most frequently isolated from cases of HFMD, accounting for about 72.1% of all the viruses isolated, including familiar EV71 and E30 virus. E19 has been considered as a weak pathogen causing mild symptoms, but during that outbreak, a large number of E19 viruses were isolated from the patients with HFMD complicated by myocarditis and aseptic meningitis. To our knowledge, E19 infection is reported for the first time to be related with HFMD. The only reports about E19 in the past was involved with uveitis, epidemic neuromyasthenia, gastroenteritis, and myalgia, especially systemic infection in neonates, *etc*.

All the 37 viral isolates were confirmed as E19 by neutralization tests and molecular sequencing typing. Eight randomly selected viruses could be neutralized by standard antiserum to E19 virus, but the neutralization titers of 8 viral isolates were lower than those of prototype strain Burke except for SD03-TN45 and SD03-TN48, showing that the epitopes of these 8 viruses are similar to E19 reference strain Burke, with certain variations.

Compared with E19 strain 87SD140 and E19 reference strain Burke, the maximal similarity is about 87% and 78.9% respectively, suggesting that there is no molecular epidemiological link within

these strains. Molecular typing is a useful and reliable tool, giving a good correlation with the traditional method.

Although all the 37 Shandong strains belong to E19 virus in cluster B enterovirus genus, the Shandong isolates have drifted significantly from those of the prototype strain Burke. Comparison of the sequences of Shandong strains with other E19 sequences available in Genbank could show about a 87% nucleotide similarity and a 98.6% amino acid similarity to the recently reported strain 87SD140.

B-C loop in VP1 region has been shown to be important for the reactivity of type-specific antibodies, substitutions of residues 84 and 85 in the B-C loop can abolish neutralizing reactivity to the virus[6]. In the Shandong strains, B-C loop is located in VP1 region from amino acids 82 to 92, in which two amino aid substitutions occur compared with E19 prototype strain Burke. It seemed that these varieties could not significantly affect the antigenic properties.

An oligopyrimidine tract (OPT) lies in 5'-UTR and the sequence of OPT element of E19 is located from nt564 to nt583. The sequence of 5' terminus of OPT, 5'-TTTCCTTTT-3', is crucial for efficient initiation of translation, and 5' terminus of OPT contains complementary sequences within the host 18S rRNA[8]. It was reported that echovirus serotypes which frequently cause infections of the central nervous system have the oligopyrimidine tract sequence 5'-TTTCCTTTT-3', and less neurovirulence serotypes exhibit variations in this sequence[11]. Moreover, in highly neurovirulence poliovirus, such an identical motif has been observed (data not shown). Since strain CHN-SD03-TN12 also has such an identical motif, it can be speculated that it is also a neurovirulent strain.

To our knowledge, HFMD is rarely associated with E19 infections. This paper provides the first description of the genetic features of E19 from China, and the outbreak in Shandong Province may due to a genetic drift that changed the virulence of the Shandong isolates, but the molecular changes in Shandong E19 viruses contributing to their phenotype remain to be further illuminated. However, the sequences described in this paper substantiate the changes occurred in capsid VP1 and 5'UTR regions, and these substitutions may play a significant role in pathogenesis and clinical manifestations of the disease.

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