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A Comparison of the Biological Characteristics of EV71 C4 Subtypes from Different Epidemic Strains*

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Abstract: The comparative analysis of the biological characterization and the genetic background study of EV71 circulating strains is commonly recognized as basic work necessary for development of an effective EV71 vaccine. In this study, we sequenced five EV71 circulating strains, isolated from Fuyang, Hefei, Kunming and Shenzhen city of China and named them FY-23, FY-22, H44, K9 and S1 respectively. The sequence alignment demonstrated their genotypes be C4. The genetic distance of the VP1 gene from these isolates suggested that they were highly co-related with genetic identity similar to other previously reported EV71 strains in China. Additionally, these strains were identified to display some obvious proliferation dynamics and plaque morphology when propagated in Vero cells. However, a distinctive difference in pathogenic ability in neonatal mice was found. Some differences in cross neutralization test & immunogenic analysis were also found. All these results are related to the biological characterization of circulating EV71 strains in China and aid in the development of an EV71 vaccine in the future.

Key words: Enterovirus 71 (EV71); Subtype C4; Epidemic strain; Hand-foot and mouth disease (HFMD)

The major infectious pathogen of Hand-foot and mouth disease (HFMD) [2], enterovirus 71 (EV71) is a member of the genus *Enterovirus* in the family *Picornavirida*[1]. Since it was first reported in 1969[14], large outbreaks of HFMD have been reported worldwide[7]. Lately, increasing attention has been drawn to HFMD outbreaks as the number of infected people and the severity of cases has increased [6, 11]. Due to etiological analyses and the identification of three EV71 genotypes and more than 10 sub-genotypes[20], the study of the epidemiological characteristics of these strains on pathogenesis is of great importance. Indeed, a large outbreak of HFMD in the Anhui Province of China (Fuyang County) in March 2008 was associated with a rush for etiological studies and EV71 vaccine development[18]. However,

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epidemiologically, EV71 infection has been reported in Chinese population for many years [21], although different descriptions of the EV71 infectious strains in different regions have been reported in previous papers[5,12]. Thus, there is a need to conduct a comparative biological analysis of the epidemic strains in different regions to facilitate the development of an effective EV71 vaccine. This will in turn aid investigation of the pathogenic characteristics of EV71 and their epidemiological significance, as well as provide basic data for related vaccine research. This study is based on the preliminary analysis of EV71 strains collected in different regions of China since 2006. In this research, a comparative analysis of the biological characteristics of five C4 subtypes of endemic EV71 strains from different regions of China was carried out, and the relevant data for understanding the features of EV71 endemic strains are presented. This work will provide a direct base for the ongoing research and development of an EV71 vaccine in China.

MATERIALS AND METHODS

Cells and viruses

Cell substrates included Vero cells (passage 43-146) and KMB-17 cells (passage 28-32, preserved by the Institute of Medical Biology, CAMS). Cell monolayer was cultured statically in MEM supplemented with 8% bovine serum. The EV71 type A standard strain BrCr was provided by the World Health Organization (WHO).

Virus isolation and adaptive passage of cells

Clinical samples were isolated from the throat swabs and blood secretions of the respiratory tracts of HFMD patients from Fuyang and Hefei in Anhui Province, Kunming in Yunnan Province, and Shenzhen in Guangdong Province in May 2008. Virus samples were sterilized, diluted with phosphate buffered saline (PBS), filtered through a 0.2-μm membrane, and stored at −20 ℃. Samples were then inoculated into Vero cells, the pathogenic effect (CPE) was observed, and isolated viruses were harvested after second generation blind passages. After KMB-17 cells (passage 28) grew into a monolayer in small square bottles, the original media was discarded, 1 mL of EV71 cultural medium harvested from the fifth passage in Vero cells was added, cells were incubated at 37 ℃ for 30 min, cell maintenance media (MEM containing 1% bovine serum) was added, and the cells were cultured at 37 ℃ until 80% of them displayed CPE. The harvested viruses were stored at −60 ℃ for future use or were passaged by the same methods.

Virus infectiousness assay

EV71 harvests were serially diluted 10-fold into 96-well culture plates (100 μL/well). Each diluent was inoculated into 8 wells and Vero or KMB-17 cells were added to 1×10^4 cells/100 μL/well. CPE was observed and recorded on the third and seventh days. “CPE positive” was defined as more than 50% of wells being cytopathic. Finally, the infectious titer was calculated by Karber’s method.

Analysis of virus infection and proliferation dynamics

The viruses, in 0.05-0.1 MOI ratios, were inoculated into 85% confluent Vero cells and adsorbed for 30 min at room-temperature. DMEM without serum was added, and cells were cultured at 37 ℃. Samples were collected every four hours for 72 h. All collected samples were frozen and thawed 3 times, and the virus titration assay was then performed to detect the proliferation dynamics curve of the virus in cells. The growing dynamics curve of different

generations of virus in cells was detected using the same method.

Virus neutralization test

Anti-EV71 specific anti-serum prepared in a rabbit that was immunized with the EV71 standard strain (genotype A) was diluted 1:8. EV71 harvests cultured in Vero cells were collected as well to be diluted into 1000-2000 $CCID_{50}$/50 μL, mixed with 50 μL of diluted serum, and cultured in 96-well plates at 37 ℃ and 5% CO_2 for 1 h. Next, 1×10^4 Vero cells/100 μL medium was added and incubated at 37 ℃ and 5% CO_2 for three to five days. The CPE was observed. The control groups of serum, virus, cell and EV71 (genotype A) were set. At the same time, mice were immunized in the abdominal cavity with the virus prepared in Vero cells, with a dose of 10^6 $CCID_{50}$/100 μL on the zero, second and fourth weeks, separately. Blood samples were collected from the eye after reimmunization one week later. A neutralization test was conducted for the above virus with supernatants by the same method.

Virus plaque morphology analysis

The harvests from the second passage in Vero cells were diluted 10-fold to 10^{-5}, virus dilutions of 10^{-4} and 10^{-5} were inoculated into a 6-well plate, adsorbed at room temperature for 30 min, and DMEM culture medium with 0.9% agar (5% bovine serum, 42 ℃) was added. The plates were kept at room temperature until solidification, and then incubated with turning at 37 ℃ and 5% CO_2 for five to seven days. The agar was removed and the cells were stained with crystal violet to observe plaque morphology.

Extraction, reverse transcription and cDNA sequence determination of viral genome nucleic acids

The viral genomic RNA of the related generation was extracted using the Qiagen standard kit according to the manufacturer's protocol. Reverse transcription of the genomic RNA was conducted with an RT-PCR kit, and specific primers were designed according to the annotated EV71 genome sequence. A total of 10 pairs of primers were used (details omitted). A forward primer (5'-CCGGAATTCAAGGATGCTAGT GATATCCT-3') and a reverse primer (5'-CGCGGAT CCCATTGTGAGTGGCAAGGT-3') were used to amplify the VP1 gene of EV71 five isolated strains. The PCR temperature profile included denaturation at 95 ℃ for 5 min, followed by 30 cycles of denaturation at 95 ℃ for 30 sec, annealing at 52 ℃ for 30 sec and extension at 72 ℃ for 1 min, followed by 10 min of heating at 72 ℃. The *Eco*R I site and *Bam*H I site (underlined) were included in the forward and reverse primers, for the purpose of cloning. Acquired cDNA gene fragments were cloned into the pMD18-T vector and individually sequenced.

A comparative analysis of virus gene sequences

The complete cloned gene sequence of the EV71 strains was analyzed using MeGa3.1 software. A comparison with other sequences in GenBank was performed, and the pedigree charts were mapped.

Analysis of virus immunogenicity

The viral harvests of the 15th passage, which were already adapted to KMB-17 cell culture, were subcutaneously inoculated into ICR mice, at a dose of 10, 20 or 30 μg of protein per mouse. There were six mice in each group. Serum was isolated from collected venous blood to detect its neutralizing antibody titer four weeks later.

The pathological effect in neonatal mouse brain by different strains

Two to three day-old neonatal mice were inoculated in the brain with a dose of 10^5 $CCID_{50}$/20 μL/mouse, and with a dose of 20μL PBS/mouse in the control

group, a separate strain was used to inoculate each group of mice. The number of neonatal mice in each group of FY23, FY22, K9, S1, H44 and BrRr strains was 10, 14, 12, 11, 18 and 11 respectively, and it was 13 in the control group. The mouse survival situation was observed and recorded each day after injection.

Statistical analysis

All data were analyzed using Sigma Software.

RESULTS

Isolation and detection of EV71 strains from different regions

A routine viral isolation procedure was conducted on 44 viral samples collected by throat swab and respiratory tract blood secretions from patients from Hefei, Fuyang, Shenzhen, and Kunming. Twenty samples inoculated into Vero cells tested CPE positive (see Materials and Methods). After the neutralization test with anti-EV71 serum (anti-BrCr strain), 16 samples were positively identified as EV71. Five EV71 strains from different regions were then selected for further study (Table 1).

Analysis of the growing proliferation dynamics tendency of five different isolated strains

A preliminary study on the proliferation of the five isolated strains in Vero cells suggested that each could grow well in this milieu. In the process of cell passage for 5 generations, an increasing tendency in replicating efficiency was observed (Fig. 1A). However, there was no significant difference for the BrCr strain (which was adapted to Vero cells) while passaging over five generations (Fig. 1A). For the 5 test strains, the infectious titers of the fifth generation proliferated in Vero cells were all higher than 10^6 $CCID_{50}$/mL. The proliferation dynamic curve of Vero cell passage on the fifth generation further indicated that when the

Table 1. Basic information of five EV71 strains

Strain ID	Isoloation			Patients			Adaptability to Vero-cell	Identified by anti-EV71serum
	date	place	sample	years	gender	Clinical symptoms		
FY-23	05/11/2008	Fuyang	throat swab	1.7	male	severe	+	+
FY-22	05/11/2008	Fuyang	throat swab	0.6	male	fever	+	+
H44	05/01/2008	Hefei	throat swab	1.6	male	fever	+	+
K9	06/13/2008	Kunming	throat swab	3.8	male	heat rash	+	+
S1	05/27/2008	Shenzhen	throat swab	1.9	male	heat rash	+	+

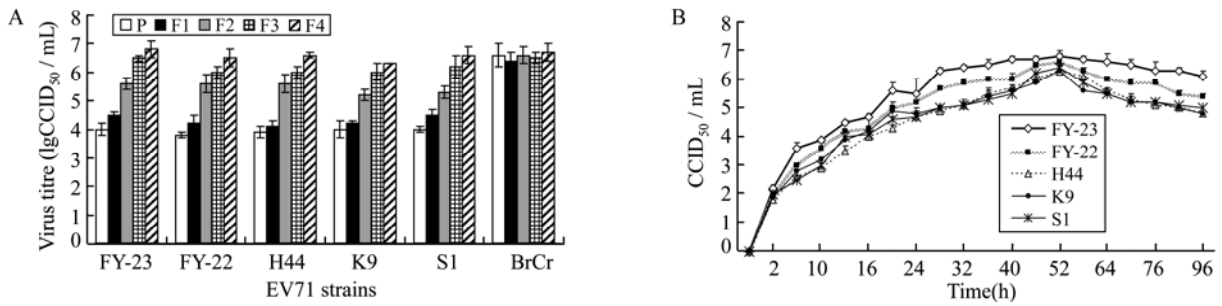

Fig. 1. The proliferation dynamic tendency of the isolated EV71 strains in Vero cells. A: Increasing tendency of replicating efficiency in the process of cell passage for 5 generations. B: The proliferation dynamic curve of cell passage on the fifth generation during the first 96 h.

five strains were inoculated into cells with a concentration of 0.05-0.1 MOI, their proliferation values peaked during the first 54-60 hours. The highest proliferation value (up to $10^{6.8}$ $CCID_{50}$/mL) was found with the FY23 strain from Fuyang; the lowest (up to $10^{6.25}$ $CCID_{50}$/mL) was the H44 strain from Hefei. Subsequently, titers of all strains showed a slight decrease. However, the positive control genotype A BrCr strain showed a higher proliferation titer (Fig. 1B).

A plaque morphology analysis of the five isolated strains

To further understand the biological characteristics of EV71 strains from different regions, we conducted a plaque morphology analysis of all viral harvests proliferated on the fifth generation of Vero cells. The results suggested that the basic plaque formation of these isolates was similar to that of the genotype A BrCr strain. All were 2-4 mm in diameter with irregular shapes (Fig. 2).

Analysis of genetic characteristics of the five isolated strains

Based on the basic biological characteristics of the different isolates, a gene sequence analysis of viral structural protein VP1 was done for each of the strains. VP1 cDNA was amplified by RT-PCR, and the results indicated that certain differences in the VP1 coding region exist among these five strains (Fig. 3A). However, these polymorphisms do not cause amino acids changes. The VP1 sequences of FY23 and FY22 strains were submitted to the GenBank database and were given accession No.EU812515 and No.EU913466 respectively, and the VP1 sequences of K9, S1 and H44 strains have not been submitted. A subsequent comparative analysis of the VP1 gene among the five strains and other annotated strains suggested that our isolates belong to subtype C4, a cluster with small genetic distance (Fig. 3B).

An analysis of pathogenicity of neonatal mice inoculated with the isolated strains

An effective animal model of EV71 infectious pathology was not provided in previous studies[3], but the biological characteristics of EV71 suggested that neonatal mice may be suited for such a purpose[19]. Therefore, a pathological test was performed by injecting the isolated viruses into the brains of neonatal mice. The FY-23, FY-22, and S1 strains caused hind limb paralysis and eventual death in two-to three-day-old neonatal mice. However, no similar phenomenon happened with the H44 and K9

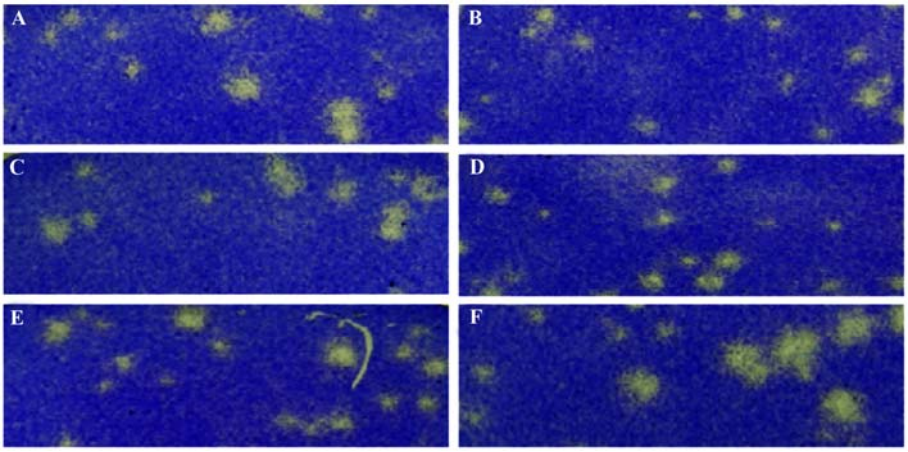

Fig. 2. Plaque formation of EV71 isolates in Vero cells. A, FY23; B, FY22; C, H44; D, K9: E, S1; F, BrCr.

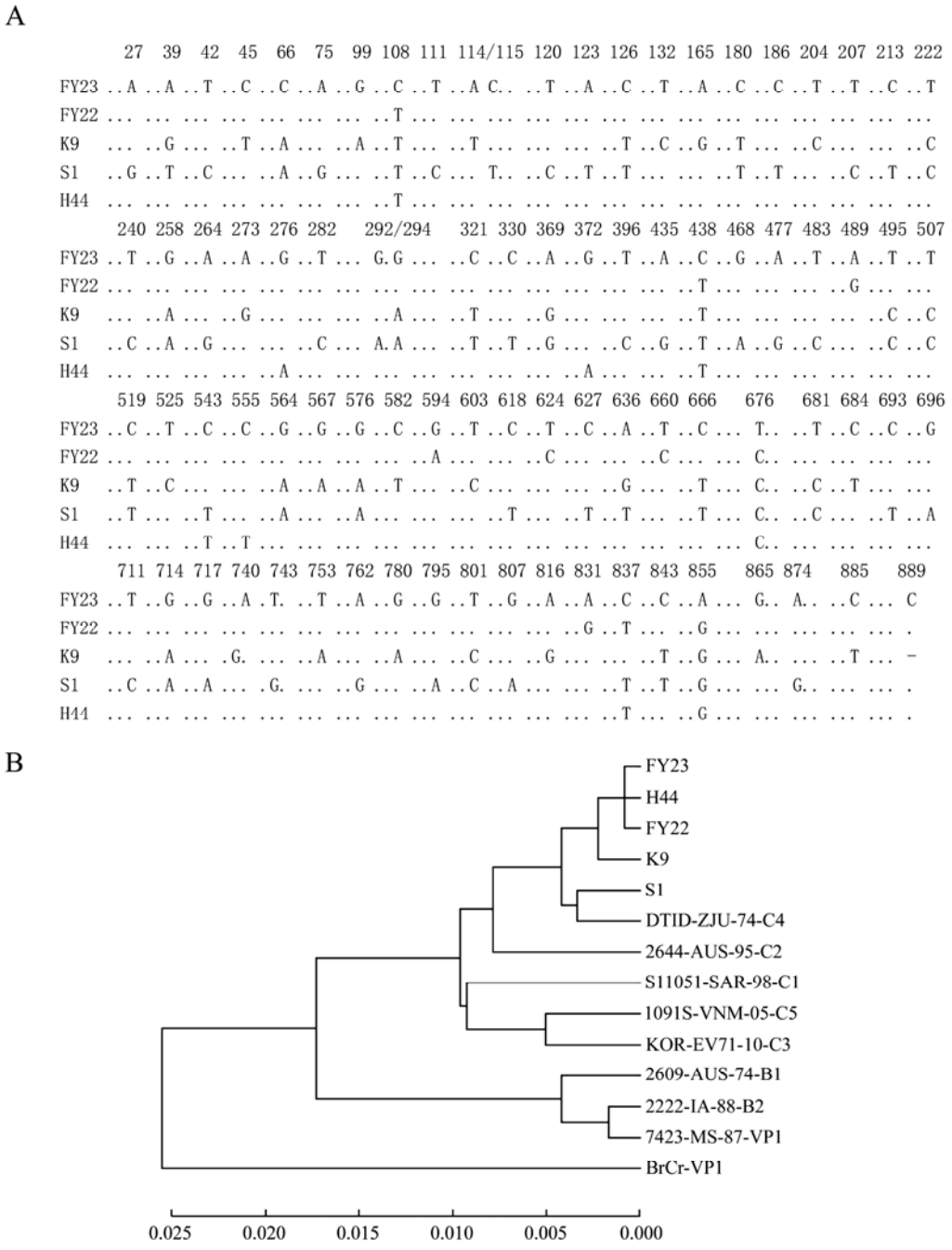

Fig. 3. Analysis of genetic features of the five isolated strains. A: Comparative analysis of the sequences of the VP1 gene among the five strains, only show the different bases based on the sequence of FY23. B: The genetic distance of the VP1 gene among the five strains and other annotated strains.

strains (Fig. 4), and there were no deaths in the control group (data was not shown). These results suggested that the five isolated EV71 strains have no significant differences in their biological characteristics, but they are genetically different and thus lead to variable nervous system lesions in neonatal mice. The pathogenic ability of FY-22, FY-23, and S1 strains would decline by serial passage on KMB-17 cells, but not on Vero cells.

Cross neutralization test of different strains

The various strains of genotype C isolated from different regions (as mentioned above) displayed

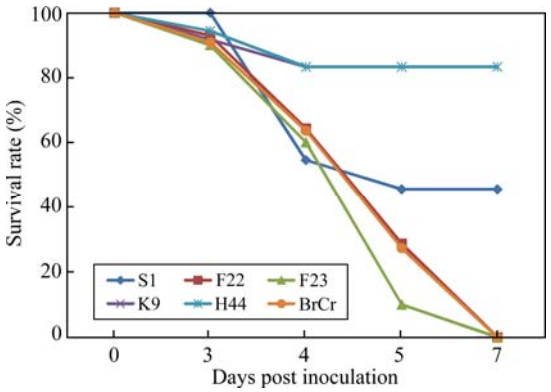

Fig. 4. Comparison of the pathogenicity of the isolates in a neonatal mouse nervous system model.

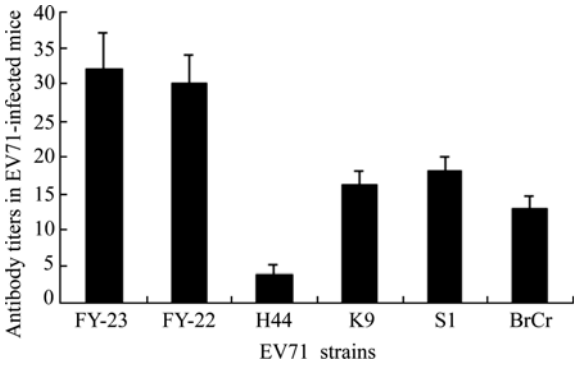

Fig. 5. Immunogenicity analysis of EV71 isolates in mice.

similarities and differences in their biological and genetic characteristics. To determine their antigenicity, a test was designed using anti-A genotype BrCr and anti-YF23 sera to attempt to neutralize each of the five isolated strains and the BrCr strain. The results showed that either anti-A genotype serum or anti-C genotype serum had the ability to neutralize A or C genotype viruses, but their neutralizing titers were different (Table 2).

Immunogenicity analysis of different isolated strains

Immunology analyses of the EV71 isolates were performed. Mice were subcutaneously immunized twice, and each strain showed a remarkable neutralizing antibody response on the 14th day after immunization (Fig.5). Among them, the neutralizing antibody titers induced by the FY-22 and YF-23 strains were the highest, up to 1:2500; the titers induced by the S1 strain were lower but still over 1:1 000. However, the H44, K9, and BrCr strains were similar, only inducing titers of 1:128 to 1:256. This indicated that the immunogenicity and ability to induce neutralizing antibodies differs among the isolates.

Table 2. Cross-neutralization test of genotypes A or C anti-serum against different strains

Antibody \ Virus	FY-23	K-9	K-12	H-44	S-1	BrCr
Anti-A	1:64	1:32	1:32	1:16	1:128	1:128
Anti-C	1:64	1:8	1:16	1:8	1:64	1:16

DISCUSSION

Since the 1990s, large-scale epidemics of HFMD caused by EV71 have frequently occurred in Asia [4, 16]. The recent outbreaks in China have generated renewed interest in EV71 etiology and development of a vaccine[15]. From the first report of the EV71 genotype A strain in 1969 [14], to the genotype B strain epidemic in Asia, to the appearance of endemic strains of the C4 subtype in China in 2008 [9], severe HFMD cases caused by EV71 infection have been increasing. Urgent attention is needed to further understand the difference between EV71 genotypes and the biological consequences of such differences among isolates of the same genotype. As such, this work focused on the analysis of the biological characteristics of EV71 strains isolated from different cities in China to probe the relationship between genetic backgrounds and immunological features. It is our hope that the results presented here will further our understanding of the etiology of the pathogenic characteristics of endemic EV71 strains in China and provide a direct basis for the research and development of a vaccine.

Recent molecular epidemic analyses have suggested that there is no obvious biological characteristics in

the geographical distribution for the HFMD epidemic caused by EV71 infection in China [10]. The prevalent EV71 strain in most regions of the country is subtype C4 [8]. In this study, 20 viral samples were successfully isolated in Vero cells from dozens of HFMD patients in different cities. Among them, 16 were identified as EV71 virus, and five were identified as subtype C4 (Fig. 3). Most of other identified viruses were subtype A [13]. An analysis of the genetic distance of the VP1 gene from the five C4 isolates suggested that the strains from different regions are highly related (Fig. 3B) and genetically similar to other EV71 strains reported in China thus far [9]. This seems to indicate that the known domestic EV71 isolates share a similar genetic origin. On this basis, a further biological analysis supported this inference to a certain extent; the five test strains could adapt and grow in Vero cells with similar proliferation dynamics. The time to reach peak replication and to fulfill the proliferation cycle was roughly the same among the isolates (Fig. 1), and plaque formation/morphology was also similar (Fig. 2). However, it should be noted that the plaque formation of the positive control BrCr genotype A strain in Vero cells was also similar. This phenomenon seems to suggest that plaque morphology may not be a significant indication of EV71 biological characteristics. Meanwhile, no other description of significant differences among EV71 strains has been reported so far [17]. Interestingly, we did discover an obvious difference in terms of the generation of lesions in the nervous system of a neonatal mice model among the control and five test isolates. The FY-22, FY-23, and S1 strains could cause paralysis and death in neonatal mice, but the K9, and H44 strains only exhibited this ability weakly (Fig. 4). This suggests that strains of the same genetic subtype can still display varying pathogenic effects, especially, FY22 and H44 have a great difference in this aspect of pathogenic ability while their main sequences of VP1 have a great similarity. However, the genetic basis for this difference remains to be elucidated. Similarly, the strains also displayed different immunological characteristics. Despite cross-neutralization tests indicated that anti-genotype A and anti-subtype C4 sera could neutralize both the A and C4 strains, their neutralization titers in serum were different (Fig. 1). The EV71 strains also had different abilities to induce neutralizing antibody production in mice (Fig.5). Although this difference may be partially due to the fact that different strains were used in the antibody detection process (the FY-23 strain was used for detection), this result still indicates an immunogenic difference among these strains. The data in this paper provide a stepping stone for further research on the development of an EV71 vaccine. Of course, the results would possess the basis of specific genetics change. Thus, further investigation is required. In any event, the results reported here will contribute to the study on EV71 etiology and research.

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