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Correspondence
Minetaro Arita
minetaro@nih.go.jp

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Temperature-sensitive mutants of enterovirus 71 show attenuation in cynomolgus monkeys

Minetaro Arita,[1] Hiroyuki Shimizu,[1] Noriyo Nagata,[2] Yasushi Ami,[3] Yuriko Suzaki,[3] Tetsutaro Sata,[2] Takuya Iwasaki[4] and Tatsuo Miyamura[1]

[1,2,3]Department of Virology II[1], Department of Pathology[2] and Division of Experimental Animals Research[3], National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo 208-0011, Japan

[4]Division of Clinical Investigation, Institute of Tropical Medicine, Nagasaki University, Nagasaki 852-8523, Japan

Enterovirus 71 (EV71) is one of the major causative agents of hand, foot and mouth disease and is sometimes associated with serious neurological disorders. In this study, an attempt was made to identify molecular determinants of EV71 attenuation of neurovirulence in a monkey infection model. An infectious cDNA clone of the virulent strain of EV71 prototype BrCr was constructed; temperature-sensitive (*ts*) mutations of an attenuated strain of EV71 or of poliovirus (PV) Sabin vaccine strains were then introduced into the infectious clone. *In vitro* and *in vivo* phenotypes of the parental and mutant viruses were analysed in cultured cells and in cynomolgus monkeys, respectively. Mutations in 3D polymerase ($3D^{pol}$) and in the 3′ non-translated region (NTR), corresponding to *ts* determinants of Sabin 1, conferred distinct temperature sensitivity to EV71. An EV71 mutant [EV71(S1-3′)] carrying mutations in the 5′ NTR, $3D^{pol}$ and in the 3′ NTR showed attenuated neurovirulence, resulting in limited spread of virus in the central nervous system of monkeys. These results indicate that EV71 and PV1 share common genetic determinants of neurovirulence in monkeys, despite the distinct properties in their original pathogenesis.

INTRODUCTION

Enterovirus 71 (EV71) belongs to the genus *Enterovirus* of the family *Picornaviridae* and possesses a single-stranded, positive-sense RNA genome of approximately 7500 nt in length (Brown & Pallansch, 1995; Schmidt *et al.*, 1974). Genetically, EV71 is classified as a species A human enterovirus along with some coxsackie A (CA) viruses, such as CA10 and CA16 (Brown & Pallansch, 1995; Pulli *et al.*, 1995). As well as CA10 and CA16, EV71 causes hand, foot and mouth disease (HFMD) and herpangina, which are common and self-limiting diseases that typically occur in children. However, EV71 infection sometimes causes severe neurological diseases, such as brainstem encephalitis and polio-like paralysis (Chumakov *et al.*, 1979; Wang *et al.*, 2003), mainly in infants and young children (McMinn, 2002). A number of fatal encephalitis cases were reported in large-scale HFMD outbreaks in Malaysia in 1997 (Abubakar *et al.*, 1999; Shimizu *et al.*, 1999) and in Taiwan in 1998 and 2000 (Ho *et al.*, 1999; Lin *et al.*, 2003; Lu *et al.*, 2002; Wang *et al.*, 2002). Furthermore, sporadic HFMD cases with severe neurological manifestations have been reported in the Western Pacific region, e.g. in Australia, Singapore, Hong Kong and Japan (Ahmad, 2000; Chan *et al.*, 2000; Fujimoto *et al.*, 2002; Herrero *et al.*, 2003; Komatsu *et al.*, 1999; Lum *et al.*, 1998; McMinn *et al.*, 1999, 2001b). Numerous factors (e.g. virus genotypes or specific mutations, herd protective immunity, individual immunity or association with other infectious agents) could lead HFMD to become a more serious disease. From molecular epidemiological studies of EV71, McMinn *et al.* (2001a) suggested that an amino acid change at position 170 of VP1 (from Ala to Val) is involved in the virulence of EV71. Non-structural proteins of EV71 (2A and 3C proteinases) were responsible for the induction of apoptosis in infected cells *in vitro* (Kuo *et al.*, 2002; Li *et al.*, 2002). However, crucial epidemiological or experimental evidence to identify critical factors of EV71 pathogenesis has yet to be provided (Shimizu *et al.*, 1999).

The occasional association of EV71 infection with serious neurological manifestations suggests that EV71 is highly neurotropic, like poliovirus (PV), which is the causative agent of poliomyelitis. The molecular determinants of the neurovirulence of PV have been studied extensively on the vaccine strains (Sabin 1, 2 and 3) (reviewed by Minor, 1992) in monkeys, as well as in transgenic mice carrying the

The GenBank/EMBL/DDBJ accession numbers for the complete genomic sequences of EV71(BrCr-TR) and EV71(BrCr-ts) are AB204852 and AB204853, respectively.

human PV receptor gene (Horie *et al.*, 1994; Koike *et al.*, 1993; Ren *et al.*, 1990). In contrast, the molecular basis of EV71 neuropathogenicity remains poorly understood, partly due to the lack of appropriate infection models.

Recently, we established an experimental EV71 infection of cynomolgus monkeys by using intravenous inoculation (Nagata *et al.*, 2004). This new experimental system of EV71 consistently induced typical neurological manifestations similar to those observed in human cases, including tremor, ataxia and polio-like paralysis (Nagata *et al.*, 2004). These disorders were caused by encephalomyelitis, involving both the pyramidal and extrapyramidal systems, in monkeys. These neurological manifestations were difficult to assess in current mouse models, where some clinical symptoms, including rash and hind-limb paralysis, were observed and adaptive mutations of EV71 played a critical role in the virulence (Chen *et al.*, 2004; Wang *et al.*, 2004). Not all of the EV71 isolates, irrespective of their clinical backgrounds, could achieve infection in mice (N. Nagata, H. Shimizu & T. Iwasaki, unpublished data). Therefore, we applied a monkey infection model for the evaluation of genetic determinants of EV71 neurovirulence.

In this study, we established an infectious cDNA clone derived from the prototype BrCr strain of EV71 and examined the effect of temperature-sensitive (*ts*) mutations on neurovirulence in a monkey infection model. We analysed a *ts* variant of the BrCr strain [EV71(BrCr-ts)] with an attenuated phenotype (Hagiwara *et al.*, 1983; Hashimoto & Hagiwara, 1983) to identify the critical mutations for its *ts* phenotype. We examined the effect of *ts* determinants of Sabin strains in the context of the EV71(BrCr) genome on the attenuation.

METHODS

Cells and viruses. Vero cells (derived from African green monkey kidney cells) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5 % fetal calf serum (FCS) and were used for virus preparation, titration and measurement of growth kinetics and temperature sensitivity. *ts* and temperature-resistant (*tr*) variants of the prototype BrCr strain (Schmidt *et al.*, 1974), EV71(BrCr-ts) and EV71(BrCr-TR), were isolated previously in cynomolgus monkey kidney (CMK) cells (Hagiwara *et al.*, 1983; Hashimoto & Hagiwara, 1983). EV71(BrCr-ts) showed an attenuated phenotype and EV71(BrCr-TR) retained the neurovirulent phenotype of the BrCr strain in cynomolgus monkeys (Hashimoto & Hagiwara, 1983; Nagata *et al.*, 2002, 2004). The variants used in this study were obtained after further plaque purification in Vero cells from the original virus stock. The viral genomes of plaque-purified variants had mutations compared with the parental BrCr strain. The virus stocks were prepared in Vero cells by RNA transfection of the transcripts derived from corresponding infectious clones.

RNA extraction, RT-PCR and sequencing. Viral genomic RNA was extracted from the culture fluid of infected cells by using a High Pure viral RNA purification kit (Roche). RT-PCR was performed by using RevatraAce reverse transcriptase (Toyobo) for reverse transcription and either Advantage 2 polymerase (Clontech) or *Tbr* EXT DNA polymerase (Finnzymes) for PCR. PCR products were purified by using a PCR purification kit (Qiagen). Direct sequence analysis was carried out on the full-length genomic sequences of EV71(BrCr-TR) or EV71(BrCr-ts), using DNA fragments amplified by RT-PCR as the templates of the sequence reaction. The sequence of the 5′ end of the viral genome was determined by using a 5′ RACE (rapid amplification of cDNA ends) system, ver. 2.0 (Invitrogen), according to the manufacturer's instructions. The sequence of the 3′ end of the viral genomes was determined from an RT-PCR product obtained with primers 7200F+ and *Eco*RI-3END− (Table 1). DNA sequencing was performed by using a BigDye Terminator v3.0 cycle sequencing ready reaction kit (Applied Biosystems) and then analysed by an ABI PRISM 310 genetic analyser (Applied Biosystems).

General methods of molecular cloning. Two *Escherichia coli* strains were used for the preparation of plasmids. The TOP10 strain (Invitrogen) was used for direct cloning of PCR products, using a TOPO XL PCR cloning kit (Invitrogen). The XL10gold strain (Stratagene) was used for the preparation of other plasmids. Ligation of DNA fragments was performed by using a Quick Ligation kit (New England Biolabs). Site-directed mutagenesis was performed by using KOD plus DNA polymerase (Toyobo) (Sambrook & Russell, 2001).

Construction of the infectious cDNA clone of EV71(BrCr-TR). A DNA fragment containing 6 kb of the 3′ region of the viral genome was amplified by RT-PCR using Advantage 2 polymerase (Clontech) from the viral genome of EV71(BrCr-TR) with primers EV71-1500F+ and EV71-A2− (Table 1). The resultant cDNA fragment was cloned into plasmid pCR-XL-TOPO by using a TOPO XL PCR cloning kit (Invitrogen). Next, the 5′ end sequence of EV71(BrCr-TR) was amplified by RT-PCR with primers *Pvu*T7-45+ and 1595R− and then cloned into the above construct following digestion by *Pvu*I and *Mun*I. However, the RNA transcript derived from the resultant full-length cDNA of EV71(BrCr-TR) did not produce any viable viruses after RNA transfection into Vero cells (data not shown). Therefore, to remove possible lethal mutation(s) in the construct, the 3′ part of the cDNA fragment of EV71(BrCr-TR) was obtained by RT-PCR using *Tbr* EXT DNA polymerase (Finnzymes) with primers A2*Bam*HI− and EV71-1500F+ (Table 1) and then cloned into the *Bam*HI site of the above construct. Transfection of the RNA transcript derived from this cDNA clone produced viable viruses in Vero cells. This infectious clone of EV71(BrCr-TR) was digested with *Sna*BI and *Mlu*I, and then cloned into plasmid P/H d40 (a generous gift from Dr E. Wimmer) (Zhao *et al.*, 2000). In this construct, to introduce *Mlu*I and *Sna*BI sites, part of the plasmid vector was obtained by PCR using *Tbr* EXT DNA polymerase (Finnzymes) with primers *Mlu*I-vec+ and *Sna*BI-vec−, using plasmid P/H d40 as the template DNA. There were five nucleotide differences between the sequence of the EV71(BrCr-TR) genome and that of the resultant infectious clone. To restore the sequence of the clone to the consensus sequence of the EV71(BrCr-TR) genome, the 5′ fragment was amplified again by RT-PCR with primers *Sna*BI-T7-EV71+ and 1595R−, using the viral genome of EV71(BrCr-TR) as the template. The obtained fragment was digested with *Mun*I and *Sna*BI and then ligated into the infectious clone. To restore other mutated sites, DNA fragments obtained with primers EV71-1500F+ and 71/3393−, with primers EV71-2800F+ and tr-6300R− or with primers E2 CF2+ and tr-6300R− were digested with *Mun*I and *Xma*I, with *Xma*I and *Sal*I or with *Sal*I and *Spe*I, respectively, and then ligated sequentially into the infectious clone. Finally, the resultant infectious clone was sequenced and confirmed to have the consensus sequence of the EV71(BrCr-TR) genome. This infectious clone of EV71(BrCr-TR) was designated pEV71(BrCr-TR).

Construction of *ts* mutants. We constructed *ts* mutants of EV71 by introducing the mutations of a *ts* variant of the BrCr strain [EV71(BrCr-ts)] (Hagiwara *et al.*, 1983; Hashimoto & Hagiwara, 1983) (Fig. 1). For the construction of a cDNA clone of EV71(ts-TR), a cDNA sequence was amplified from the EV71(BrCr-ts) genome by

Table 1. Primers used for the construction of the infectious clone of EV71(BrCr-TR)

Primer name	Sequence (5′–3′)
1595R−	TCCAGCGGGCTGATAGGCACCACC
2784+	CATAACCGGTCATGCGCAGATG
2784−	CATCTGCGCATGACCGGTTATG
3564−	CTCACTAGCTTCTACAAACACCAAACTAGG
6153C+	GAACCTGATGAGCACGTGACACAGGC
6153C−	GCCTGTGTCACGTGCTCATCAGGTTC
7023AT+	GATAAATCACCTATTTTCAATGAGGTTAC
7023AT−	GTAACCTCATTGAAAATAGGTGATTTATC
7200F+	AACACTCAAGATCACGTGCGCTCCC
71/3393−	GGCGGTTRACCACYCTDAAGTTGCCCAC
7409G−	AAAAACGCGTTTTTTTTTTTTTTTTTTTTTTTTTTCGCTATTCTGGTTATAAC
A2*Bam*HI−	AAAAGGATCCTTTTTTTTTTTTTTTTTTTTTTTTTTGCTATTCTGG
E2 CF2+	GAGCAAACACCGTATTGAACCTGTATG
*Eco*RI-3END−	ACTGGAATTCTTTTTTTTTTTTTTTTTTTTTTTTTV
EV71-1500F+	GGATTAATCTNCGNACCAACAA
EV71-2800F+	TTCACNTACATGCGCTTTGANGC
EV71-A2−	CCATCGATGGTTTTTTTTTTTTTTTTTTTTTTTTTTGCTATTCTGG
*Mlu*I-vec+	TCAAACGCGTTTGAAGACGAAAGGGCCTCG
*Pvu*T7-45+	AAATTCGATCGAAATTAATACGACTCACTATAGGTTAAAACAGCCTGTGGGTTGCA-CCCACTCACAGGGCCCACTGGGC
S472+	GAATGCGGTTAATTCTAACTGCGGAGCAC
S472−	GTGCTCCGCAGTTAGAATTAACCGCATTC
S480+	CCTAACTGCGGGGCACATACCCT
S480−	AGGGTATGTGCCCCGCAGTTAGG
S481+	CCTAACTGCGGAACACATACCCTTAATC
S481−	GATTAAGGGTATGTGTTCCGCAGTTAGG
*Sna*BI-vec−	AAATTACGTAATTTCGATAAGCCAGTCGAG
*Sna*BI-T7-EV71+	TTAATACGTATTAATACGACTCACTATAGGTTAAAACAGCCTGTGGGTTGCACC
TR2784+	GACATAACCGGTTATGCGCAGATGCGC
TR2784−	GCGCATCTGCGCATAACCGGTTATGTC
TR5000F+	ATAGCGTCGACACCGTGGTATCGG
tr-6300R−	AGGATGTGTCTTTTCTTGATGCCC

RT-PCR with primers *Sna*BI-T7-EV71+ and 1595R− and then cloned into pEV71(BrCr-TR) after partial digestion with *Sna*BI and *Bam*HI. This cDNA clone was designated pts-TR. For the construction of a cDNA clone of EV71(ts-ts-TR), a DNA fragment was obtained from the EV71(BrCr-ts) viral genome by RT-PCR with primers EV71-1500F+ and 3564− and then cloned into pts-TR after partial digestion with *Xma*I and *Bam*HI. This cDNA clone of mutant ts-ts-TR was designated pts-ts-TR. For the construction of a cDNA clone of EV71(BrCr-ts), a DNA fragment was obtained from the EV71(BrCr-ts) viral genome by RT-PCR with primers EV71-1500F+ and A2*Bam*HI− and then cloned into pts-ts-TR after digestion with *Xma*I and *Spe*I. This cDNA clone of mutant EV71(BrCr-ts) was designated pEV71(BrCr-ts). cDNA clones of EV71(ts2784) and EV71(TR2784) were prepared by site-directed mutagenesis by PCR with primers 2784+ and 2784− or with primers TR2784+ and TR2784−, using pEV71(BrCr-TR) or pEV71(BrCr-ts) as the template.

Next, we constructed cDNA clones of other *ts* mutants by introducing the corresponding mutations of the attenuation and *ts* determinants of PV Sabin strains (Fig. 3). For the construction of a cDNA clone of EV71(3′), site-directed mutagenesis was performed by PCR with primers 6153C+ and 6153C−, using pEV71(BrCr-TR) as the template. The clone obtained was subjected to site-directed mutagenesis with primers 7023AT+ and 7023AT−. Next, a DNA fragment was obtained by PCR with primers TR5000F+ and 7409G−, using the obtained clone as the template, and then cloned into pEV71(BrCr-TR) following digestion with *Spe*I and *Mlu*I. This cDNA clone of EV71(3′) was designated pEV71(3′). A cDNA clone of EV71(S1-3′) was obtained by site-directed mutagenesis by PCR with primers S480+ and S480−, using pEV71(3′) as the template. cDNA clones of EV71(S1), EV71(S2) and EV71(S3) were obtained by site-directed mutagenesis with primer set S480+ and S480−, with S481+ and S481− or with S472+ and S472−, respectively, using pEV71(BrCr-TR) as the template.

RNA transfection. RNA transcripts were obtained by using a RiboMAX large-scale RNA production system–T7 kit (Promega) with *Mlu*I-linearized DNA as the template. The *in vitro*-synthesized RNA transcripts were transfected onto the monolayer of Vero cells in six-well plates (Falcon) by the DEAE–dextran method and incubated at 35 °C in 2 ml 5 % FCS/DMEM per well (Lu *et al.*, 1995). Cytopathic effects (CPE) on Vero cells were observed at 24 h post-transfection (p.t.). The cells were harvested when all of the cells exhibited CPE (4–7 days p.t.) and stored at −70 °C. The titres of recovered EV71 mutants were 10^5–10^6 50 % cell culture infectious dose ($CCID_{50}$) ml^{-1}.

Virus titration. Virus titre was determined by measuring $CCID_{50}$ by the microtitration assay in Vero cells, as described previously

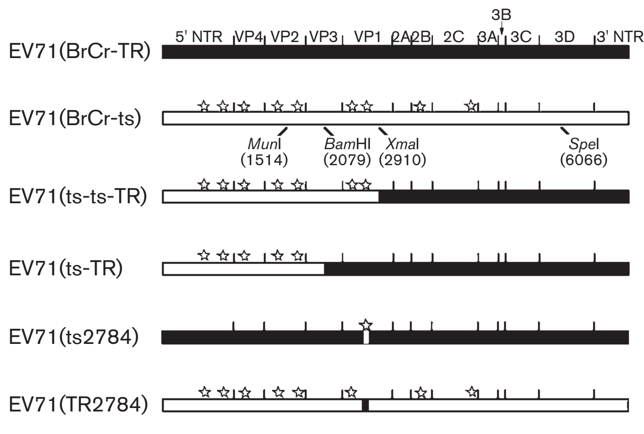

Fig. 1. Schematic diagram of the genomes of EV71 mutants. The sequences derived from the parental EV71(BrCr-TR) genome are represented as closed boxes and the sequences derived from the EV71(BrCr-ts) genome are represented as open boxes. The mutations observed in the EV71(BrCr-ts) genome are shown as open stars on each mutant genome. Restriction-enzyme sites are shown on the genome of EV71(BrCr-ts).

(Nagata *et al.*, 2002). Briefly, inoculated Vero cells were cultured at 36 °C for 10 days and observed for CPE. The value of $CCID_{50}$ was calculated according to the Behrens–Kärber method (Kärber, 1931).

Growth kinetics of EV71 mutants. Vero-cell monolayers were cultured in 96-well plates (Stripwell Plate; Corning) containing $2{\cdot}4\times10^4$ cells per well. EV71 mutants were inoculated into the cells at an m.o.i. of 1·0 ($2{\cdot}4\times10^4$ $CCID_{50}$ virus per well) and then incubated for 1 h at 36 °C. Inoculated cells were washed three times with 5 % FCS/DMEM and then 100 µl 5 % FCS/DMEM was added per well. The cells were incubated at 36 °C and harvested at the times indicated, from 2 to 12 h post-infection. The titre of virus was determined by $CCID_{50}$ measurement.

Plaque assay. The plaque assay was performed in 12-well plates (Falcon) containing a Vero-cell monolayer. Tenfold dilutions of virus solution were inoculated at 100 µl per well and incubated for 30 min at 36 °C. Then, 1 ml 2 % FCS/modified Eagle's medium (MEM) containing 0·5 % agarose ME (Iwai Kagaku) was added per well and incubated at 36 °C. After incubation for 4 days, 0·5 % agarose ME in 2 % FCS/MEM was overlaid on the first layer of agarose gel and further incubated for 3 days at 36 °C. The cells were fixed in formaldehyde and then stained with 0·5 % crystal violet.

Temperature sensitivity. The temperature sensitivity of viruses was evaluated by determining the virus titre in Vero cells at 36 °C, which we used for the isolation of EV71 from clinical samples, and at a supraoptimal temperature, 39 °C. Temperature sensitivity was expressed as logarithmic difference of the $CCID_{50}$ values at 36 and 39 °C ($\Delta CCID_{50}$). We defined temperature sensitivity from 2·0 to 2·75 logarithmic difference as a slight *ts* phenotype, and those with more than 2·75 logarithmic difference as a strong *ts* phenotype.

Monkey neurovirulence test. Eight 17–21-year-old female cynomolgus monkeys were used for the determination of neurovirulence of EV71 mutants. All animal procedures were approved by the Committee for Biosafety and Animal Handling and the Committee for Ethical Regulation of the National Institute of Infectious Diseases, Japan. Animal care, breeding, virus inoculation and observation were performed in accordance with the guidelines of the committees.

Under light anaesthesia with ketaral and xylazine, 1 ml of each virus solution (containing 10^7 $CCID_{50}$ virus) was inoculated intravenously into the right tibial vein. Neurological manifestations of monkeys were checked daily for 10 days and autopsy was performed on day 10 post-inoculation (p.i.) after anaesthesia. Moribund monkeys before 10 days p.i. were sacrificed under deep anaesthesia. At autopsy, various parts of the central nervous system (CNS) were sampled for histopathological and virological analyses. The method of scoring the histological changes of the CNS (lesion score) was described previously (Nagata *et al.*, 2002). For virus isolation, a portion of excised tissues was stored at −80 °C. After freezing and thawing, 10 % (w/v) tissue homogenates in MEM containing 2 % FBS were centrifuged at 10 000 ***g*** for 10 min to remove cell debris. Supernatants were subjected to virus isolation in Vero cells. The cells were checked for CPE for 1 week and then blind passage was conducted for CPE-negative samples after freezing and thawing of the first-round passage. If CPE was not observed in the first- or second-round cultures, the result of virus isolation was recorded as negative.

RESULTS

Identification of the *ts* determinant of EV71(BrCr-ts)

To map the critical *ts* mutation of a *ts* variant of EV71 [EV71(BrCr-ts)], the entire genome sequence was determined and compared with that of a *tr* variant [EV71(BrCr-TR)]. The EV71(BrCr-ts) genome had nine nucleotide changes compared with that of EV71(BrCr-TR) and three of them were non-synonymous (Table 2). The three amino acid changes were located in capsid proteins VP2 and VP1 and in non-structural protein 2C (Table 2). We introduced these mutations into the infectious clone of EV71(BrCr-TR) to generate EV71 mutants, as described in Methods (Fig. 1). Viable viruses were recovered from all six clones, although their virus titres were different (Table 3).

To identify the *ts* determinant of EV71(BrCr-ts), temperature sensitivity was analysed for the parental and mutant

Table 2. Mutations of the EV71(BrCr-ts) genome

Mutations are from the BrCr-TR to the BrCr-ts genome.

Nucleotide position	Site of mutation	Nucleotide change	Amino acid change
491	5′ NTR	U to C	–
681	5′ NTR	U to C	–
848	VP4	C to U	–
1154	VP2	U to C	–
1707	VP2	G to A	Ala253 to Thr
2693	VP1	U to C	–
2784	VP1	U to C	Tyr116 to His
4034	2B	A to G	–
4990	2C	C to U	Thr305 to Ile

Table 3. Temperature sensitivity of EV71 mutants

EV71 mutant	Titre* at:		Δ36/39 °C
	36 °C	39 °C	
BrCr-TR	4·25	3·0	1·25
ts-TR	3·75	1·75	2·0
ts-ts-TR	2·5	ND (<0·5)	>2·0
BrCr-ts	3·5	ND (<0·5)	>3·0
ts2784	3·25	ND (<0·5)	>2·75
TR2784	5·0	3·0	2·0
S1	4·25	2·25	2·0
S2	4·0	1·75	2·25
S3	3·5	1·25	2·25
3′	4·0	ND (<0·5)	>3·5
S1-3′	4·0	ND (<0·5)	>3·5
3′ (4511, spinal cord)	4·0	ND (<0·5)	>3·5
3′ (4511, brainstem)	4·13	ND (<0·5)	>3·6
3′ (4512, brainstem)	4·0	ND (<0·5)	>3·5
S1-3′ (4514, spinal cord)	4·13	ND (<0·5)	>3·6

*Virus titre represents $\log_{10}(CCID_{50})$ in 10 μl virus sample.
ND, Not detected.

viruses by measuring the virus titre at 36 and 39 °C. Although the virus titres of EV71(ts2784) and EV71(ts-ts-TR) were much lower than that of the cDNA-derived EV71(BrCr-TR), even at 36 °C, the two mutants did not grow at 39 °C as well as EV71(BrCr-ts) (Table 3).

Introduction of a single nucleotide substitution at nt 2784 (U to C) into the EV71(BrCr-TR) genome resulted in impaired virus growth at 39 °C (Δ36/39 °C, >2·75 log) (Table 3). In contrast, a reciprocal substitution (C to U) at the same position of the EV71(BrCr-ts) genome resulted in only a slight *ts* phenotype [mutant EV71(TR2784); Δ36/39 °C, 2·0 log]. The plaque sizes of EV71(BrCr-TR) (cDNA-derived), EV71(ts-TR) and EV71(TR2784) were similar to that of the parental strain (Fig. 2a). On the other hand, EV71(ts-ts-TR) and EV71(ts2784) viruses showed smaller plaques, as well as EV71(BrCr-ts). These results indicate that a single nucleotide substitution at nt 2784 (VP1-Tyr116) is mainly responsible for both the *ts* and small-plaque phenotypes of EV71(BrCr-ts).

Construction of EV71 mutants carrying the *ts* determinants of PV Sabin strains

To generate *ts* mutants that could show growth comparable with that of the parental strain *in vitro*, we examined the effect of the *ts* determinants of PV Sabin strains. As illustrated in Fig. 3, we constructed a series of EV71(BrCr) mutants carrying corresponding nucleotide substitutions.

Firstly, we focused on the mutations in the 5′ non-translated region (NTR) within domain V of the genomes of Sabin strains (at nt 480, 481 and 472 for Sabin 1, 2 and 3, respectively; Fig. 3c), which act as a *ts* determinant and also as the major determinant of attenuation. The 5′ NTR of the EV71(BrCr) genome had a type I internal ribosome entry

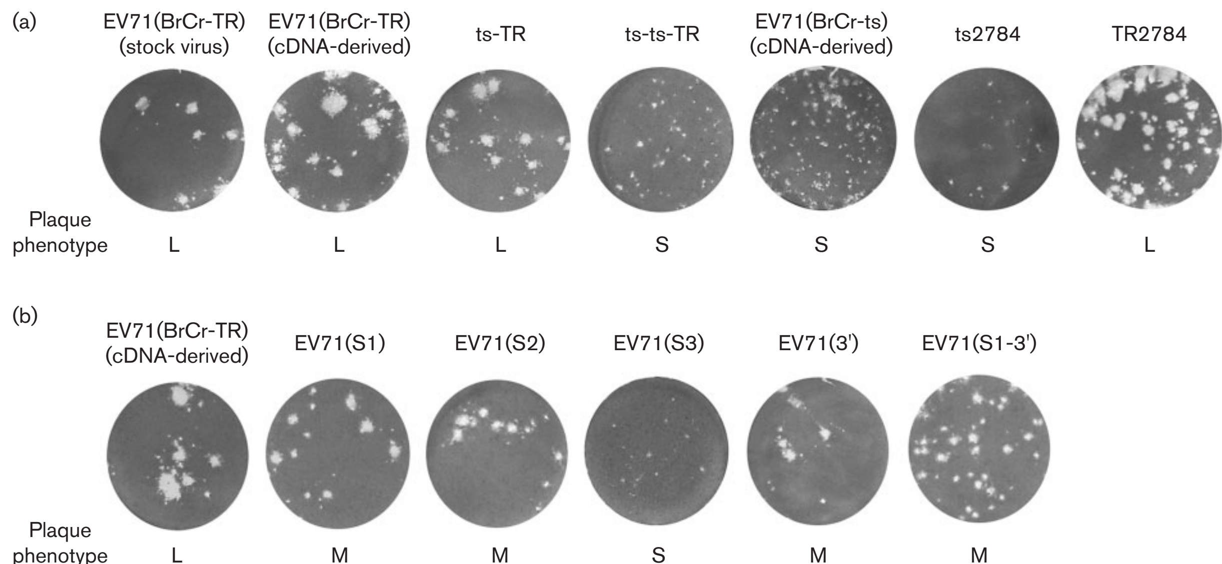

Fig. 2. Plaque phenotype of EV71 mutants. (a) Plaque phenotype of EV71 mutants derived from EV71(BrCr-ts). (b) Plaque phenotype of EV71 mutants carrying mutations of Sabin strains. Mutants EV71(S1), EV71(S2) and EV71(S3) have a mutation of the 5′ NTR of Sabin 1, 2 or 3, respectively. The EV71(3′) mutant has mutations in the $3D^{pol}$-coding region and 3′ NTR. The EV71(S1-3′) mutant contains all the mutations of the EV71(S1) and of EV71(3′) mutants. The assay was performed on Vero-cell monolayers incubated at 36 °C. L, Large-plaque phenotype; M, medium-plaque phenotype; S, small-plaque phenotype.

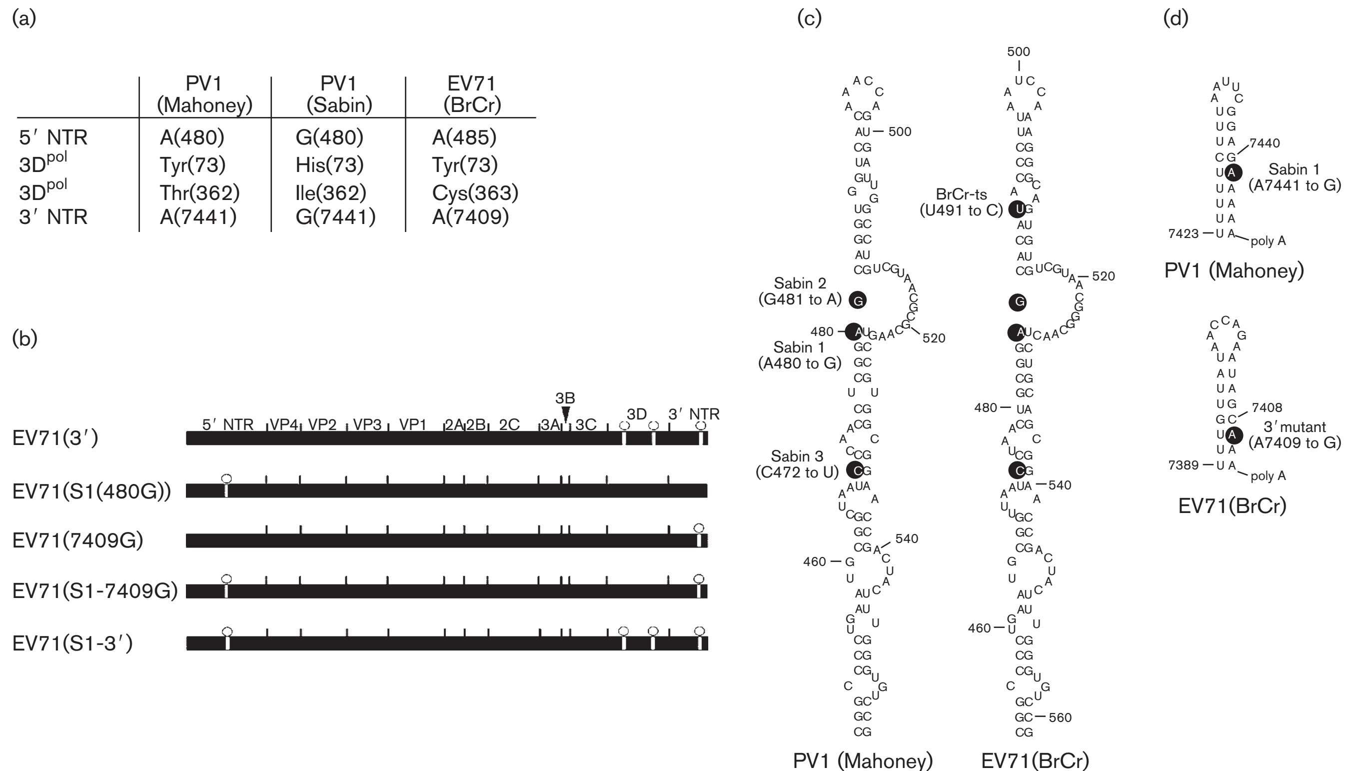

Fig. 3. EV71 mutants containing the corresponding mutations of the *ts* determinants of PV Sabin strains. (a) *ts* determinants of Sabin 1 introduced into the EV71(BrCr-TR) genome. The corresponding sites of the EV71(BrCr-TR) genome were substituted to those of Sabin 1. Numbers in parentheses represent the nucleotide position of the mutations on the genomes (5′ NTR and 3′ NTR) or the position of amino acids on the $3D^{pol}$ protein. (b) Schematic diagram of the genomes of EV71 mutants. Sequences derived from the parental EV71(BrCr-TR) genome are represented as closed boxes and mutations derived from the Sabin 1 genome are represented as open boxes with open circles. EV71 mutants carrying mutations of Sabin 2 or 3 in the 5′ NTR [mutants EV71(S2) and EV71(S3), respectively] are not shown. (c) RNA secondary-structure model of domain V in the IRES of PV1(Mahoney) and EV71(BrCr-TR), proposed by Pilipenko *et al.* (1989). (d) RNA structural models of the 3′ NTR of PV1(Mahoney) and that of EV71(BrCr-TR), obtained by the MFOLD 3.1 program (http://www.bioinfo.rpi.edu/applications/mfold/). The position of a *ts* determinant of Sabin 1 (G7441) and the corresponding site of the EV71(BrCr-TR) genome (A7409) are shown in closed circles.

site (IRES) activity, like that of the PV genome, and its structure model revealed an RNA secondary structure that was typical of the enterovirus IRES (Thompson & Sarnow, 2003). In a predicted secondary-structure model of domain V of the EV71(BrCr) genome, nt 485, 486 and 474 corresponded to the mutations of Sabin 1, 2 and 3, respectively (Fig. 3c).

Secondly, mutations corresponding to the major *ts* determinants of Sabin 1 in the 3D polymerase ($3D^{pol}$)-coding region and 3′ NTR were introduced into the infectious clone of EV71(BrCr-TR). The locations of the triple mutations in the EV71(BrCr-TR) genome (Tyr-73 and Cys-363 in $3D^{pol}$ and 7409A in the 3′ NTR) were predicted from the amino acid or nucleotide sequence alignment between EV71(BrCr-TR) and Sabin 1 (Fig. 3a).

As shown in Fig. 2(b), all mutants except the EV71(S3) mutant formed medium-sized plaques, which were smaller than those of the parental strain, but larger than those of the EV71(S3) mutant. The EV71(S3) mutant showed small-sized plaques, similar to those of the BrCr-ts variant. A PV1 mutant carrying a mutation of the 5′ NTR of the Sabin 3 genome showed a significant reduction in virus growth (Malnou *et al.*, 2003).

Next, we examined the temperature sensitivity of EV71 mutants by measuring the virus titre in Vero cells at 36 or 39 °C (Table 3). The results indicated that mutations in the 5′ NTR [EV71(S1) mutant] were involved in a slight *ts* phenotype (Δ36/39 °C, 2·0 log) and that triple mutations in the $3D^{pol}$-coding region and the 3′ NTR [EV71(3′) mutant] conferred a strong *ts* phenotype to EV71(BrCr-TR) (Δ36/39 °C, >3·5 log) (Table 3). Therefore, the triple mutations in the $3D^{pol}$-coding region and 3′ NTR could serve as the strong *ts* determinants in the genetic context of EV71(BrCr-TR), as well as in the Sabin 1 genome.

In vitro growth kinetics of three EV71 mutants that were

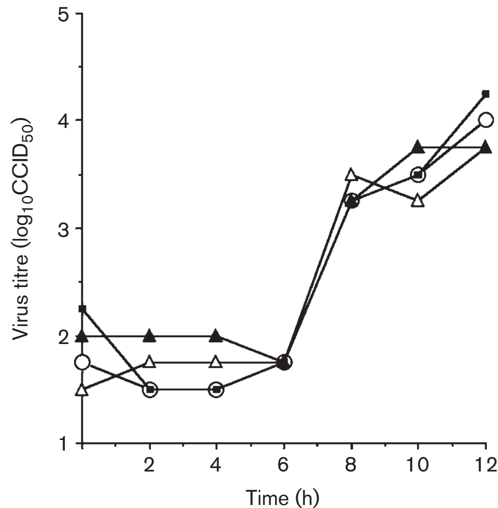

Fig. 4. Growth kinetics of EV71 mutants (BrCr-TR, ○; TR2784, ■; 3′, △; S1-3′, ▲).

used for the neurovirulence test in monkeys (see below) were measured in Vero cells at 36 °C (Fig. 4). All of the mutants showed growth kinetics similar to those of the parental EV71(BrCr-TR) strain.

The above results indicated that the introduction of *ts* mutations of the Sabin 1 genome into the 5′ NTR, $3D^{pol}$ coding region and 3′ NTR of the EV71(BrCr-TR) genome effectively generated *ts* mutants that retained *in vitro* growth kinetics comparable with those of the parental strain.

Neurovirulence of EV71 mutants in cynomolgus monkeys

We determined the neurovirulence of cDNA-derived EV71 mutants in cynomolgus monkeys by intravenous inoculation. Two monkeys inoculated with 10^7 $CCID_{50}$ of the cDNA-derived EV71(BrCr-TR) clone became moribund within 6 days p.i., similar to those inoculated with the parental EV71(BrCr-TR) strain (Nagata *et al.*, 2004). The cDNA-derived EV71(BrCr-TR) induced characteristic neurological manifestations, such as tremor and ataxia, from days 4 and 5 p.i., respectively (Table 4). In contrast, monkeys inoculated with the same dose of cDNA-derived mutants [EV71(TR2784), EV71(3′) and EV71(S1-3′)] showed mild neurological manifestations and histological changes. None of the six monkeys that were inoculated with EV71 mutants became moribund or showed ataxia within 10 days p.i. Moreover, mutant viruses in the CNS were detected in the spinal cord and in the brainstem, whilst the parental strain showed disseminated distribution (Nagata *et al.*, 2002). The total lesion scores of mutants were decreased (Table 4). For the infection of EV71(S1-3′), no viable virus was recovered from the CNS of an inoculated monkey on day 10 p.i. (monkey 4513) and the virus was only isolated from the spinal cord in another inoculated monkey (4514). Thus, the infection of EV71(S1-3′) resulted in the most limited clinical manifestations and in restricted distribution of the virus in the CNS.

To examine the selection pressure in the CNS of monkeys against the temperature sensitivity of EV71, we examined the *ts* phenotype of EV71(3′) and EV71(S1-3′) viruses recovered from the CNS of inoculated monkeys. All of the recovered viruses retained a strong *ts* phenotype, similar to that of the original EV71(3′) and EV71(S1-3′) viruses (Table 3). This suggests that the temperature sensitivity of EV71 is not the critical factor to achieve infection in the CNS of monkeys.

DISCUSSION

The BrCr strain was isolated from an aseptic meningitis patient as the prototype strain of EV71 (Schmidt *et al.*, 1974). Its entire genome sequence is far from that of PV (Brown & Pallansch, 1995). Epidemiological analyses of

Table 4. Summary of the clinical manifestations of monkeys and virus isolation

Virus	Monkey no.	Clinical manifestation*			Virus isolation				Lesion score
		Tremor	Ataxia	Moribund	Spinal cord	Brainstem	Cerebellum	Cerebrum	
EV71(BrCr-TR)	4507	Day 4	Day 5	Day 6	+	+	+	+	2·29
	4508	Day 4	Day 5	Day 6	+	+	+	+	2·51
EV71(TR2784)	4509	Day 7	−	−	+	+	−	−	0·78
	4510	Day 6	−	−	+	−	−	+	0·72
EV71(3′)	4511	Day 9	−	−	+	+	−	−	1·07
	4512	Day 8	−	−	−	+	−	−	0·59
EV71(S1-3′)	4513	Day 9	−	−	−	−	−	−	0·0
	4514	Day 9	−	−	+	−	−	−	1·09

*Time post-inoculation when the monkey started to show the clinical manifestation is indicated. Monkeys were sacrificed at day 6 (for 4507 and 4508) or day 10 (for 4509, 4510, 4511, 4512, 4513 and 4514) post-inoculation.

EV71 revealed that the BrCr strain is not related closely to two major genogroups of EV71 (B and C); thus, it is the sole member of genogroup A (Brown *et al.*, 1999). Previous studies showed that cynomolgus monkeys inoculated with the BrCr strain exhibited typical neurological manifestations and histopathological legions after intraspinal or subcutaneous inoculation (Hashimoto & Hagiwara, 1983; Hashimoto *et al.*, 1978). Furthermore, a cell culture-selected *ts* variant of the BrCr strain [EV71(BrCr-ts)] had been generated (Hagiwara *et al.*, 1983; Hashimoto & Hagiwara, 1983) and was one of the initial candidates of attenuated EV71 strains. Therefore, we first examined this laboratory variant of the BrCr strain to generate attenuated EV71 strains.

To generate attenuated strains of EV71, we focused on temperature sensitivity as an *in vitro* marker. In general, temperature sensitivity of PV vaccine strains serves as an *in vitro* phenotypic marker of attenuation. However, the extent of temperature sensitivity does not necessarily correlate with the extent of attenuation of PV (Bouchard *et al.*, 1995; Christodoulou *et al.*, 1990; Georgescu *et al.*, 1995; Macadam *et al.*, 1989, 1991; Minor, 1992; Omata *et al.*, 1986). Moreover, the *ts* revertant could retain its attenuated phenotype, suggesting that there is no direct link between the arbitrary *ts* phenotype and attenuation (Rowe *et al.*, 2000). Thus, among the *ts* determinants, only some could serve as the attenuation determinants. We examined the genome of EV71(BrCr-ts) to identify the *ts* determinant that could be an initial candidate of the attenuation mutation. We identified a mutation at nt 2784 (VP1-Tyr116 to His) as the *ts* determinant of EV71(BrCr-ts). However, this mutation also conferred a small-plaque phenotype (Fig. 2a). Alignment of the amino acid sequences of EV71(BrCr-TR) and PV1(Mahoney) in the VP1 region suggests that the corresponding amino acids of PV1(Mahoney) would be Thr115, which is located near an interface of protomers between the adjoining VP1, via Gln233 of VP3 (Hogle *et al.*, 1985). Tyr116 of the BrCr-ts strain is located in a region of VP1 that is highly conserved among different EV71 strains (data not shown). One of the attenuation determinants of Sabin 3 is located in the capsid protein (Phe91 of VP3) near an interface between protomers (Minor *et al.*, 1989; Westrop *et al.*, 1989) and affects the virus-assembly process in a temperature-dependent manner (Minor *et al.*, 1989). Therefore, we could not obtain *ts* mutants with a growth activity comparable with that of the parental strain by utilizing the *ts* determinant of EV71(BrCr-ts).

We examined the effect of the *ts* determinants of Sabin strains on the temperature sensitivity of EV71. We focused on *ts* determinants of Sabin 1 that are located in structurally and functionally conserved regions among enteroviruses, i.e. the 5′ NTR, $3D^{pol}$ and 3′ NTR (Kawamura *et al.*, 1989; Omata *et al.*, 1986). Between PV and EV71, the predicted secondary structures of 5′ NTR and 3′ NTR are highly conserved (Fig. 3). Mutations in the 5′ NTR of Sabin strains cause a reduction in the IRES activity (Muzychenko *et al.*, 1991; Svitkin *et al.*, 1985, 1988, 1990) and act as a *ts* determinant, as well as the major attenuation determinant (Bouchard *et al.*, 1995; Christodoulou *et al.*, 1990; Evans *et al.*, 1985; Georgescu *et al.*, 1995; Macadam *et al.*, 1989, 1991; Minor, 1992; Omata *et al.*, 1986). The attenuation determinant in the 5′ NTR of Sabin 3 leads to translation defects in neuronal and non-neuronal organs *in vivo* (Kauder & Racaniello, 2004). However, in PV1 infection, the reduced level of translation was not the main determinant of attenuation (Arita *et al.*, 2004). Therefore, the mechanism of the attenuation effect of the mutations in the 5′ NTR of Sabin strains remained to be further elucidated.

The mutation of nt 6203 (His73 of the $3D^{pol}$ protein) affects the oligomerization and uridylylation of viral protein $3B^{VPg}$ in a temperature-sensitive manner (Paul *et al.*, 2000). Another mutation of nt 7071(Ile362 of the $3D^{pol}$ protein) is required for the *ts* phenotype of Sabin 1 (Georgescu *et al.*, 1995). The amino acid residue at 362 of the $3D^{pol}$ protein is located at interface I of $3D^{pol}$ (Hansen *et al.*, 1997); together with the mutation of nt 6203, this amino acid residue may also affect the oligomerization of the $3D^{pol}$ protein. The mutation in the 3′ NTR, which is located in a stem–loop structure (Fig. 3d), has been suggested to have an effect on the *ts* phenotype, along with other mutations of $3D^{pol}$ (Georgescu *et al.*, 1995) or the mutation in the 5′ NTR (Christodoulou *et al.*, 1990), by an unknown mechanism. EV71 mutants with mutations in the $3D^{pol}$-coding region and 3′ NTR showed a strong *ts* phenotype, in contrast to a slight *ts* phenotype that was caused by mutations in the 5′ NTR (Table 3). These results indicate that EV71 and PV1 share common genetic determinants of temperature sensitivity, based at least in part on the conserved replication machinery.

We examined the neurovirulence of EV71 mutants by intravenous inoculation into cynomolgus monkeys. The inoculation route is a critical factor for neurological disorders of EV71 infection in the monkey model. After intraspinal inoculation of the BrCr strain, monkeys showed flaccid paralysis, but not tremor or ataxia, due to intraspinal spread of the virus (Nagata *et al.*, 2004). Therefore, we applied an intravenous inoculation model to avoid direct involvement of the inoculated virus in the CNS, which would not occur in the natural course of EV71 infection.

All three EV71 mutants caused mild neurological symptoms in monkeys after intravenous inoculation, but did not cause lethal neurological disorders that were observed in infection with EV71(BrCr-TR) (Table 4). Interestingly, mutant EV71(TR2784) had only a minor *ts* phenotype, but showed a slightly attenuated phenotype. Infections caused by EV71(TR2784) or EV71(3′), which showed a strong *ts* phenotype, resulted in a similar lesion score, despite their different temperature sensitivity. Therefore, temperature sensitivity could not serve as an absolute indicator of the attenuated neurovirulence of EV71. The distribution of EV71 mutants in the CNS was also restricted, compared

with that of the parental strain. Among the mutants examined, EV71(S1-3′) resulted in the most limited clinical manifestations and in a restricted distribution of the virus in the CNS. A cumulative effect of the mutations in the 5′ NTR and $3D^{pol}$-coding region on the attenuation of PV has been reported (Tardy-Panit *et al.*, 1993). However, infection by EV71(S1-3′) was still associated with minor neurological symptoms in inoculated monkeys and virus was isolated from the spinal cord. Thus, mutation of Sabin 1 in the 5′ NTR could not completely suppress infection by EV71 in the spinal cord of monkeys inoculated by the intravenous route. This observation might suggest that the spinal cord serves as a preferred site of infection for both PV and EV71 (Arita *et al.*, 2004). However, because of the different mechanisms of pathogenesis, the effect of mutations of Sabin 1 on the infection of EV71 remain to be further elucidated.

EV71 antigen was detected in the early phase of infection, followed by clinical manifestations, in monkeys (Nagata *et al.*, 2002). It was possible that EV71 mutants replicated in the CNS in a disseminated manner, as well as the parental virulent strain, in the early phase of infection. Therefore, the tissue specificity of EV71 mutants in the CNS remains to be further studied.

In conclusion, we have generated a cDNA-derived virulent strain of EV71 that maintained the *in vitro* and *in vivo* phenotypes of a neurovirulent strain, EV71(BrCr-TR). Based on this infectious cDNA clone of EV71, we identified several molecular determinants conferring *ts* and attenuated phenotypes to EV71. The cDNA-derived virulent and attenuated strains of EV71 should serve as a valuable tool for the elucidation of EV71-induced severe neurological disorders and development of a vaccine strain.

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