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**Supplementary Data**

**Development of stable, cold-adapted, temperature-sensitive/conditional lethal chimeric enterovirus A71 and coxsackievirus A16**

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**Supplementary Information**

**Materials and Methods**

***1. Construction of infectious cDNA clone of EV-A71 vector (EV71:eTLLβP20)***

*1.1 RNA extraction and synthesis of proximal and distal fragments of single-stranded cDNA of EV71:TLLβP20*

Viral RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, CA, Germany). Single strand cDNA synthesis was performed using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Briefly, 5 µg of extracted RNA, 100 pmol of specific primer (EV71-9R \_2011B for generation of 5’-proximal fragment, or ACYC-TLLb-D-R for generation of 3′-distal fragment) (Table S1) and 5 µL of 10 mmol/L dNTP mix were added into nuclease-free water in a reaction volume of 60 µL. The reaction mixture was incubated at 65 °C for 10 minutes and quenched on ice (4 °C) for 5 minutes. Subsequently, 20 µL of 5X First-Strand Buffer, 10 µL of 0.1mol/L DTT and 5 µL of RNase Inhibitor (40 U/µL) were added to the reaction mix and incubated at 42 °C for 5 minutes. Following which, 5 µL of SuperScript II RT (200 U/µL) was added, and the resultant mix was incubated at 46 °C for 30 minutes, then 30 minutes at 50 °C. The reaction was terminated by incubating at 72 °C for 15 minutes. RNA complementary to cDNA strand was subsequently removed by addition of 6.5 µL of RNaseH (1.5 U/µL) and incubation at 37 °C for 25 minutes. The synthesised single strand cDNA was extracted and purified using standard phenol-chloroform extraction.

*1.2 Synthesis of proximal and distal fragments of double-stranded cDNA of EV71:TLLβP20*

Primers (Table S1), ACYC-TLLb-Pf-F (forward) and ACYC-TLLb-Pf-R (reverse) were used to generate the proximal fragment (~3500 bp) by PCR of the single-stranded cDNA synthesised by reverse transcription of the virus genome using primer EV71-9R\_2011B. Primers (Table S1), ACYC-TLLb-D-F (forward) and ACYC-TLLb-D-R (reverse) were used to generate the distal fragment (~4000 bp) by PCR of the single-stranded cDNA synthesized using primer ACYC-TLLb-D-R. The PCR reaction was performed using iProof High-Fidelity Master Mix (Bio-Rad, Hercules, CA, USA) with initial denaturation at 98 °C for 2 minutes followed by 10 cycles of 98 °C for 10 seconds, 65 °C for 30 seconds and 72 °C for 2 minutes. This was followed by another 35 cycles of 98 °C for 10 seconds and 72 °C for 2.5 minutes and a final extension of 72 °C for 5 minutes.

*1.3 Cloning of proximal and distal fragments of EV71:TLLβP20 to pACYC177*

Both the PCR generated proximal and distal fragments were cloned into plasmid pACYC177 with *Bam*HI and *Aat*II. Briefly, 4 µg of pACYC177 (which has unique cutting sites for *Bam*HI and *Aat*II) and approximately 1 µg of proximal and distal PCR products were separately double digested with 50 U of *Bam*HI and *Aat*II at 37 °C for 2 hours, followed by inactivation at 65 °C for 30 mins. The digested DNA fragments were separately gel purified and then PCR products were ligated into pACYC177 using 5 U T4 DNA ligase (Fermentas, Vilnius, Lithuania) in a 20 µL reaction. This was incubated at 22 °C for 1 hour followed by inactivation at 70 °C for 5 minutes. 10 µL of ligation mixture was then transformed into 90 µL of XL10 electro-competent *E. coli* cells, followed by incubation in 400 µL of SOC medium at 30 °C for 2 hours. 150 µL of the mixture was then plated onto LB plate containing 100 µg/mL ampicillin and 35 µg/mL kanamycin. Positive clones were screened for correct inserts and selected for large scale plasmid preparation. Sequencing was routinely performed to confirm that the full-length sequence of the insert was successfully ligated to the vector. The pACYC177 plasmids carrying the cDNA copy of the proximal and distal portion of EV71:TLLβP20genome were designated as pACYC177(TLLβP20proximal) and pACYC177(TLLβP20distal) respectively.

*1.4 Site-directed mutagenesis (SDM) of pACYC177(TLLβP20proximal)*

PCR-based SDM was performed on the plasmid containing the proximal fragment of the virus genome to introduce a unique *Mlu*I recognition site at the 5' end of *VP4* gene. The *Mlu*I and *Eag*I restriction enzymes cutting sites were created in the EV71:TLLβP20genome to facilitate the subsequent cloning of the P1 region of CA16 or EV-A71 subgenogroup C5 for generation of the respective chimeric viruses. The reaction was performed using iProof High-Fidelity DNA polymerase (Bio-Rad) with primers SDM-pACYC-Pf-F and SDM-pACYC-Pf-R (Table S1) and 100 ng of plasmid. After initial denaturation at 98 °C for 2 minutes, the reaction underwent 35 cycles of 98 °C for 10 seconds, 65 °C for 30 seconds and 72 °C for 4 minutes, followed by a final extension of 72 °C for 5 minutes. Methylated template plasmid was removed by treatment with 40 U *Dpn*I (New England Biolabs, Ipswich, MA, USA) at 37 °C for 2 hours followed by inactivation at 80 °C for 20 minutes. The reaction mixture was then spin column purified and transformed into XL10 electro-competent *E. coli* cells as described above. Screening was subsequently carried out to select for clones which had the required nucleotide changes. Plasmid maxi kit (Qiagen) was used to extract large quantities of plasmid DNA from these selected clones.

*1.5 Construction of full-length infectious cDNA clone of EV71:eTLLβP20*

The plasmids containing the proximal and distal fragments were separately digested with 40 U of *Bam*HI and *Eag*I (New England Biolabs). The purified proximal fragment with the required SDM changes was ligated to the digested plasmid pACYC177(TLLβP20distal), carrying the distal fragment of EV71:TLLβP20, using 2 U of T4 DNA ligase (Fermentas). Ligated plasmid was transformed into XL10 electro-competent *E. coli* cells that were then plated on 100 µg/mL ampicillin and 35 µg/mL kanamycin LB plates and incubated at 30 °C. Colonies were screened for plasmids containing the full-length cDNA of the virus genome, which were then purified in large quantities. Sequencing was carried out using the complete set of TLLβP20 primers (Table S2) to confirm that the full-length genome of EV71:TLLβP20, with the engineered restriction enzymes (*Mlu*I and *Eag*I) sites, was successfully cloned into the vector. The pACYC177 plasmid carrying the modified full-length cDNA copy of EV71:TLLβP20 was designated as pACYC177(EV71:eTLLβP20).

*1.6 Recovering genetic engineered EV71:eTLLβP20 virus*

Transfection was performed using Lipofectamine 2000 (Invitrogen) with a total of 800 ng pACYC177(EV71:eTLLβP20) plasmid and T7 polymerase plasmid in a ratio of 1:2 onto 3 × 104 Vero-81 cells in a 24 well plate. Briefly, the mixture was incubated for 20 minutes at room temperature with 2 µL Lipofectamine diluted in OPTI-MEM. After which, the mixture complex was added onto Vero cells and incubated at 37 °C for 5 hours. The mixture complex was then removed; cells were washed and replaced with DMEM 1% FCS and incubated at 28 °C. After 10 days post transfection, cells and supernatant were passed onto fresh Vero cells seeded on a 6-well plate. Upon reaching full CPE, cells and supernatant were subsequently passed onto fresh Vero cells cultured in a T-25 flask. The virus was further passed in Vero cells incubated at 28 °C for 20 passages (EV71:eTLLβP20) to confirmed its genetic and phenotypic stability.

***2. Construction of an infectious full-length cDNA clone of the chimeric virus EV71:TLLeC5***

*2.1 Virus and PCR amplification of capsid protein gene (P1)*

Enterovirus A71 strain belonging to subgenogroup C5 (C5/3437/SIN-06) was subjected to 15 passages in Vero cells maintained in DMEM 1% FCS at 30 °C. Viral RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions.

The complete capsid protein gene (P1) of EV-A71 subgenogroup C5 was RT-PCR amplified using primers pACYC-C5-P1-F and pACYC-C5-P1-R (Table S1). The pACYC-C5-P1-F primer containing engineered *Cla*I and *Mlu*I restriction sites, and the primer pACYC-C5-P1-R containing engineered *Xma*I and *Eag*I restriction sites were used to facilitate the cloning of capsid protein gene of EV-A71 subgenogroup C5; initially into pACYC177 vector, and subsequently into the full-length cDNA clone of EV71:eTLLβP20. Amplification of the 2.6 kb capsid protein gene (P1) was performed using iProof High-Fidelity Master Mix (Bio-Rad).

*2.2 Cloning of C5-P1 fragment into pACYC177*

Both pACYC177 and C5 RT-PCR products were separately digested with 20 U of *Cla*I and *Xma*I at 37 °C for 2 hours. The reaction was then inactivated at 65°C for 30 minutes. The digested pACYC177 was gel purified. Spin column purification was performed to obtain the digested C5-P1 PCR product. Purified C5 DNA was then ligated to the digested pACYC177 using 2 U of T4 DNA ligase (Fermentas) in a 20 µL reaction, incubated at 22 °C for 1 hour followed by inactivation at 70 °C for 5 minutes. 10 µL of purified pACYC177 carrying C5-P1 DNA, now designated as pACYC177(C5-P1), was transformed into XL10 electro-competent *E. coli* cells as described above.

*2.3 SDM of EV71:C5 P1 gene*

SDM was performed on the plasmid carrying the P1 region of C5 to change alanine 858 (A) to threonine (T). PCR SDM was performed using primers pACYC-C5P1-TITTL-F and pACYC-C5P1-TITTL-R (Table S1), together with 50 ng of plasmid pACYC177(C5-P1). The reaction was carried out using iProof High-Fidelity DNA polymerase (Bio-Rad) with an initial denaturation of 98 °C for 2 minutes followed by 15 cycles of 98 °C for 10 seconds, 66°C for 30 seconds and 72 °C for 4 minutes, continued by 35 cycles of 98 °C for 10 seconds, 69 °C for 30 seconds and 72 °C for 4 minutes, and final extension of 72 °C for 5 minutes. The PCR reaction was then spin column purified before the template plasmid was removed using 40 U *Dpn*I (New England Biolabs) at 37 °C for 6 hours followed by inactivation at 80 °C for 20 minutes. The reaction mixture was then spin column purified and transformed into XL10 electro-competent *E. coli* cells as described above. Screening was performed to select for clones which have the required nucleotide changes. Plasmid maxi kit (Qiagen) was used to extract large quantities of plasmid from these clones. The plasmid carrying the A858T mutation is designated as pACYC177(eC5-P1).

*2.4 Construction of an infectious full-length cDNA clone of EV71:TLLeC5*

Both the purified plasmids, pACYC177(EV71:eTLLβP20) and pACYC177(eC5-P1) were separately digested with 20 U *Mlu*I and *Eag*I at 37 °C for 6 hours, followed by inactivation at 65 °C for 30 minutes. The digested products were then gel purified. The fragment containing the P1 region of eC5 derived from pACYC177(eC5-P1) was ligated to the pACYC177(EV71:eTLLβP20), devoid of the original P1 region, using 2 U T4 DNA ligase (Fermentas) as described above. The ligated product was transformed into XL10 electro-competent *E. coli* cells in a 100 µL reaction, followed by incubation in 400 µL of SOC medium at 30 °C for 2 hours. 150 µL of the electro-transformed cells were then plated onto LB plate containing 100 µg/µL ampicillin and 35 µg/µL kanamycin. Screening for positive clones and sequencing of the full genome were subsequently performed accordingly. The plasmid pACYC177 carrying the full-length cDNA genome was designated as pACYC177(EV71:TLLeC5).

*2.5 Recovering genetic engineered chimeric EV71:TLLeC5 virus*

The engineered chimeric EV71:TLLeC5 virus was recovered by transfecting pACYC177(EV71:TLLeC5) plasmid into Vero cells as described above. The virus was further passed in Vero cells incubated at 28°C for another 20 passages to confirm its genetic and phenotypic stability.

***3. Construction of an infectious full-length cDNA clone of chimeric virus TLLeCA16***

*3.1 PCR amplification of the capsid protein gene (P1) of CA16 (CA16-P1)*

The CA16 virus RNA was extracted in a similar manner as described for the extraction of RNA from EV-A71 subgenogroup C5. Amplification of the P1 region of CA16 was carried out using primers pACYC-CA16-P1-F and pACYC-CA16-P1-R (Table S1). The pACYC-CA16-P1-F primer contained engineered *Nhe*I and *Mlu*I restriction sites, and primer pACYC-CA16-P1-R contained engineered *Xho*I and *Eag*I restriction sites to facilitate cloning the capsid protein gene of CA16 into the pACYC177 vector, and subsequently into the full-length cDNA of EV71:eTLLβP20. The amplification of the 2.6 kb P1 region was performed using iProof High-Fidelity Master Mix (Bio-Rad).

*3.2 Cloning of PCR amplified CA16-P1 into* *pACYC177*

Both pACYC177 and the PCR amplified CA16-P1 region were separately digested with 50 U of *Nhe*I and *XhoI* at 37 °C for 2 hours, before inactivation at 65 °C for 30 minutes. Both digested pACYC177 and CA16-P1 were gel purified. The digested CA16-P1 DNA was then ligated to the digested pACYC177 vector using 5 U T4 DNA ligase (Fermentas) in a 20 µL reaction at 22 °C for 1hour, followed by inactivation at 70 °C for 5 minutes. 10 µL of the ligation was transformed into XL10 electro-competent *E. coli* cells, and the transformed *E. coli* cells were recovered as described above.

*3.3 SDM of the CA16 P1 gene*

SDM was performed on the plasmid carrying the P1 region of CA16 to change lysine 858 (K) to threonine (T). Primers pACYC-CA16P1-TITTL-F and pACYC-CA16P1-TITTL-R (Table S1) were used with 50 ng of plasmid pACYC177(CA16-P1). SDM was carried out as described above for pACYC177(C5-P1). The plasmid carrying the K858T mutation is designated as pACYC177(eCA16-P1).

*3.4 Construction of infectious full-length cDNA clone of TLLeCA16*

Both purified plasmids, pACYC177(EV71:eTLLβP20) and pACYC177(eCA16-P1) were separately digested with 20 U *Mlu*I and *Eag*I at 37 °C for 6 hours, followed by inactivation at 65 °C for 30 minutes. Digested products were gel purified and the infectious cDNA clone of TLLeCA16 was constructed in the manner as described above for construction of theinfectious cDNA clone of EV71:TLLeC5. The plasmid pACYC177 carrying the infectious full-length cDNA genome was designated as pACYC177(TLLeCA16).

*3.5 Recovering the genetically engineered chimeric TLLeCA16 virus*

The genetically engineered, chimeric, TLLeCA16 virus was recovered by transfecting pACYC177(TLLeCA16) plasmids into Vero cells as described earlier for recovering EV71:eTLLβP20. The virus was further passaged in Vero cells incubated at 28 °C for 20 passages to confirm its genetic and phenotypic stability.

***4. Viruses and Cells.***

A laboratory-established cold-adapted, temperature-sensitive/conditional lethal enterovirus A71 strain (EV71:TLLβP20) and its original wild-type strain (ST) (Chua *et al*. 2021), clinical isolates of EV-A71 subgenogroup C5 and CA16 of genogroup B (lineage 2), were propagated in Vero cells and used to generate the chimeric enteroviruses.

The Vero cell-line (CCL81) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Gaithersburg, MD, USA) supplemented with 10% (v/v) foetal calf serum (FCS, i-DNA® Singapore) and 0.22% (w/v) sodium bicarbonate (NaHCO3, Sigma Aldrich, St. Louis, MO, USA). For virus propagation, attenuation, titration, and assessment of temperature sensitivity phenotype, the cells were cultured and maintained at an incubation temperature of 37 °C, unless otherwise stated. Prior to infection, DMEM 10% FCS was replaced with DMEM 1% FCS, and the infected cells were subsequently incubated at the respective experimental temperature in a humidified environment of 5% CO2.

***5. Virus titration.***

Virus titre was determined by micro-titration assay in Vero cells in accordance with the method from the 2004 Polio Laboratory Manual of the World Health Organization, with minor modification as described previously (Chua *et al*. 2021).

***6. Virus antigen detection by indirect immunofluorescent assay (IFA)*.**

Cells suspended in the culture supernatant were pelleted by centrifugation at 800 ×*g* for 10 minutes. After two washes with sterile PBS, aliquots of re-suspended cells were seeded onto wells of a Teflon-coated slide, allowed to air dry in a biosafety Class II cabinet, fixed in cold acetone for 10 minutes prior to detection of the EV-A71 antigen by IFA using a commercial monoclonal antibody (Cat. No. 3324, Sigma Aldrich, St. Louis, MO, USA).

***7. Temperature-sensitivity phenotype assay.***

Two approaches were adopted to assess the growth characteristics of the virus strains in Vero cells at incubation temperatures of 28 °C, 37 °C and 39.5 °C. The first approach assessed the number of days taken for the virus strain to cause full CPE in infected cells (cell death kinetic), and the second approach assessed the titre of the virus at full CPE in cells incubated at each specific temperature tested as described previously (Chua *et al*. 2021). The assays were performed in duplicate. The stated 1+, 2+. 3+ and 4+ CPE is equivalent to approximately 25%, 50%, 75% and 100% of the infected cells that have undergone morphological changes/deaths.

***8. Virus growth kinetics, Genetic stability, and Temperature sensitivity reversion assays.***

Assessment of virus growth kinetics by total viral RNA quantity (qRT-PCR) in the culture supernatant, the genetic stability and temperature sensitivity reversion of the generated enterovirus vector, chimeric EV-A71 and chimeric CA16 was performed as described in Chua *et al.* (2021).

***9. General Molecular Work.***

Viral RNA extraction, RT-PCR, molecular cloning of PCR products and sequencing of ambiguous sequences was performed as described previously (Chua *et al*. 2021).

***10. Statistical analysis***

Two independent experiments and subsequent qRT-PCR were performed in duplicates for each virus strain to assay viral growth kinetics by viral RNA load. Their values were compared using Student’s *t*-test, ANOVA test or nonparametric Mann-Whitney test in Excel (Microsoft) and/or GraphPad Prism version 8.0.1 (GraphPad software, USA). Two-tailed *P* values < 0.05 were considered statistically significant.

Table S1: Primers used for construction of the infectious cDNA clones of EV71:eTLLβP20, EV71:TLLeC5 and TLLeCA16, inclusive the ones for performing site-directed mutagenesis.

|  |  |  |
| --- | --- | --- |
| **Primer Name** | **Primer Sequence** | **Remarks** |
| EV71-9R \_2011B | 5'- GGG ACT ACC RTG RCA MCC KAT CAG -3' | cDNA synthesis for infectious clone and cDNA synthesis for EV71:eTLLβP20. |
| ACYC-TLLb-Pf-F | 5’- GCT A**GG ATC C***TA ATA CGA CTC ACT ATA* GGT TAA AAC AGC CTG TGG GTT GCA CCC AC -3’ | PCR of eTLLβ |
| ACYC-TLLb-Pf-R | 5’- CGA T**GA CGT C***CG GCC G*AA CTT TCC AAG GGT AGT AAT GG – 3’ | PCR of eTLLβ |
| ACYC-TLLb-D-F | 5’ – TCG A**GG ATC** ***C****GG CCG* GCA ATC TGG GGC CAT GTA CG - 3’ | PCR of eTLLβ |
| ACYC-TLLb-D-R | 5’- GAT C**GA CGT C**(T)25G CTA TTC TGG TAA TAA CAA ATT TAC CC - 3’ | cDNA synthesis for infectious clone & PCR of eTLLβ |
| SDM-pACYC-Pf-F | 5’ – CA**A CGC GT**C GGC TCC CAC GAG AAC TCC – 3’ | SDM of eTLLβ |
| SDM-pACYC-Pf-R | 5’ – GCC G**AC GCG T**TG AGT AGA CAC TTG TGA GCC – 3’ | SDM of eTLLβ |
| pACYC-C5-P1-F | 5'- ATC G**AT CGA T**CA CAC A*AC GCG T*CG GCT CGC ATG AAA ACT CTA AC -3' | PCR of eC5 | |
| pACYC-C5-P1-R | 5'- TAT A**CC CGG G**TT GT*C GGC CG*A ATT TCC CAA GAG TGG TGA TCG CCG TG -3' | PCR of eC5 | |
| pACYC-C5P1-TITTL-F | 5'- CAG TCG CAC G**AC G**AT CAC CAC TCT TGG GAA ATT CGG CCG AC -3' | SDM of eC5 | |
| pACYC-C5P1-TITTL-R | 5'- TGG TGA T**CG T**CG TGC GAC TGG CAC CGG TTG GCT TAA TAG AAT CAC C -3' | SDM of eC5 | |
| pACYC-CA16-P1-F | 5’- TCT C**GC TAG C***AC GCG T*CG GGT CAC ATG AGA ACT CAA ACT CTG – 3’ | PCR of eCA16 | |
| ACYC-CA16-P1-R | 5’ - TGC A**CT CGA G**TG C*CG GCC G*AA CTC TCC CAA TGT TGT TAT CTT G -3’ | PCR of eCA16 | |
| pACYC-CA16P1-TITTL-F | 5’- CTA GTA GAG AC**A CG**A TAA CAA CAT TGG GAG AGT TCG GCC GGC ACT C – 3’ | SDM of eCA16 | |
| pACYC-CA16P1-TITTL-R | 5'- AAC TCT CCC AAT GTT GTT AT**C GT**G TCT CTA CTA GTG CTA GTG CAC TTA ATA TCA TTT C -3' | SDM of eCA16 | |

Note: Letters in bold and underlined indicate restriction enzyme sites. Letters in italic and underlined indicate another restriction enzyme site. Letters in italic indicate T7 RNA polymerase sequence. Letters highlighted in yellow indicate nucleotide change in the position to accommodate the restriction site. Letters highlighted in green indicate nucleotide changes in position which results in amino acid change.

Table S2: Common oligonucleotide primers used for RT-PCR and sequencing of the virus strains: EV71:TLLβP20, EV71:eTLLβP20, EV71:TLLeC5 and TLLeCA16.

|  |  |  |
| --- | --- | --- |
| **Primer Name** | **Primer Sequence** | **Remarks** |
| Race-3R | 5'- CCG GGG AAA CAG AAG TGC TTG ATC -3' | Sequencing |
| Race-2R | 5'- ATT CAG GGG CCG GAG GAC TAC -3’ | Sequencing |
| TLLb-1R | 5'- GAC ACC CAA AGT AGT CGG TTC CG -3' | Sequencing |
| EV71-1F | 5'- AAC AGC CTG TGG GTT GCA CCC AC-3' | Sequencing |
| TLLb-2F | 5'- TAG TCC TCC GGC CCC TGA ATG C -3' | Sequencing |
| TLLb-9F | 5'- GAA TTC CGT CTG GGA GGA CAG CT -3' | Sequencing |
| TLLb-9R | 5'- CTA CCG TGG CAC CCT ATC AGA GCT -3' | cDNA synthesis of EV71:TLLeC5 and TLLeCA16, Sequencing |
| TLLb-10F | 5'- AGG AGT GAT TAT GAC ATG GTC ACT CTC AC -3' | Sequencing |
| TLLb-10R | 5'- TAT CCA TCA AAG TGG TCC GGG TCT G -3' | Sequencing |
| TLLb-11F | 5'- AGA GCA AAC ACC GAA TTG AAC CTG TAT GTC -3' | Sequencing |
| TLLb-11R | 5'- GCT GAA TGG CCT TCC CAC ACA C -3' | Sequencing |
| TLLb-12F | 5'- CAA TGG CTT CCC TAG AAG AGA AAG GAG T -3' | Sequencing |
| TLLb-12R | 5'- CAA CCT TGA TCT CTA CAG TAC TGG CGT -3' | Sequencing |
| TLLb-13F | 5'- ACA CAA TTG AAG CAC TAT TCC AAG GTC CG -3' | Sequencing |
| TLLb-13R | 5'- CCA GAT TGT TTT GCC GGG CTG G -3' | Sequencing |
| TLLb-14F | 5'- AAC AGT GCA GGG TCC AAG TCT CG -3' | Sequencing |
| TLLb-14R | 5'- TAT TGG GCT TGA CCC ACT GGA TCT C -3' | Sequencing |
| TLLb-15F | 5'- CAA CCC ACC GTA CCA TGA TGT ACA AC -3' | Sequencing |
| TLLb-15R | 5'- AAT GGC CTC AAG GTT CTC AGT GCC -3' | Sequencing |
| TLLb-16F | 5'- GAC ACC TCC CAG ATG AGC ATG GAG -3' | Sequencing |
| TLLb-16R | 5'- CCA GGG AGT AAG ATG GGT AGT TTA CTC C -3' | Sequencing |
| TLLb-17F | 5'- TGA AAC CTT CCA TGC AAA CCC TGG -3' | Sequencing |
| TLLb-17R | 5'- ATT TGT CTG CAG GAG TCA TGG TTA AAC CG -3' | Sequencing |
| TLLb-18F | 5'- GAT GAT GTG CTT GCC AGT TAC CCT -3' | Sequencing |
| TLLb-18R | 5'- TTT TTT TTT TTT TTT TTT TTT TTT TGC TAT TCT GGT AAT AAC AAA TTT ACC C -3' | Sequencing |

Table S3: The number of nucleotide (NT), and corresponding amino acid (AA), mutations that occurred in each of the genomic segments of virus strains derived from temperature sensitivity reversion study compared with the genome of EV71:eTLLβP20. (R): reversion to wild-type

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Viral Gene Region/Protein** | | **EV71:eTLLβP20 (37**°C**-P3)** | | **EV71:eTLLβP20 (37**°C**-P6)** | |
| NT | AA | NT | AA |
| 5′-UTR | “Cloverleaf” | - | - | - | - |
| (1–746) | IRES | - | - | - | - |
|  |  |  |  |  |  |
| P1 | VP4 | - | - | - | - |
| (747–3332) | VP2 | - | - | - | - |
|  | VP3 | - | - | - | - |
|  | VP1 | - | - | Mixed populations of 2 strains (12 clones screened):  1) Without deletion (5 clones)  2) Deletion of 15nt (7 clones) (2731–2745) | Mixed populations of 2 strains:  1)Without deletion  2)Deletion of 5aa (662–666) and  1 aa change-  N667H |
|  |  |  |  |  |  |
| P2 | 2A | 1(1R)  G 3346 A | 1(1R)  R 867 Q | 1(1R)  G 3346 A | 1(1R)  R 867 Q |
| (3333–5066) | 2B | - | - | - | - |
|  | 2C | - | - | - | - |
|  |  |  |  |  |  |
| P3 | 3A | - | - | - | - |
| (5067–7325) | 3B | - | - | - | - |
|  | 3C | - | - | - | - |
|  | 3D | - | - | - | - |
|  |  |  |  |  |  |
| 3′-UTR |  | - | - | - | - |
| (7326–7411) |  |  |  |  |  |
|  |  |  |  |  |  |
| Total | | 1(1R) | 1(1R) | 1(1R) | 1(1R) |

Table S4: The number of nucleotide (NT), and corresponding amino acid (AA), mutations that occurred in each of the genomic segments of virus strains derived from temperature-sensitivity reversion study in comparison with the genome of EV71:TLLeC5. (R): reversion to wild-type

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|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Viral Gene Region/Protein** | | **TLLeC5 (37**°C**-P3)** | | **TLLeC5 (37**°C**-P6)** | |
| NT | AA | NT | AA |
| 5′-UTR | “Cloverleaf” | - | - | - | - |
| (1–746) | IRES | - | - | - | - |
|  |  |  |  |  |  |
| P1 | VP4 | - | - | - | - |
| (747–3332) | VP2 | - | - | - | - |
|  | VP3 | - | - | - | - |
|  | VP1 | - | - | - | - |
|  |  |  |  |  |  |
| P2 | 2A | 1(1R)  G 3346 A | 1(1R)  R 867 Q | 1(1R)  G 3346 A | 1(1R)  R 867 Q |
| (3333–5066) | 2B | - | - | - | - |
|  | 2C | 1  C 4566 T | 1  H 1274 Y | 1  C 4566 T | 1  H 1274 Y |
|  |  |  |  |  |  |
| P3 | 3A | - | - | - | - |
| (5067–7325) | 3B | - | - | - | - |
|  | 3C | - | - | - | - |
|  | 3D | - | - | - | - |
|  |  |  |  |  |  |
| 3′-UTR |  | - | - | - | - |
| (7326–7411) |  |  |  |  |  |
|  |  |  |  |  |  |
| Total | | 2(1R) | 2(1R) | 2(1R) | 2(1R) |