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Construction of an infectious cDNA clone of *Enterovirus 71*: Insights into the factors ensuring experimental success



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ABSTRACT

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Enterovirus 71 (EV 71) is a causative agent of mild Hand Foot and Mouth Disease but is capable of causing severe complications in the CNS in young children. Reverse genetics technology is currently widely used to study the pathogenesis of the virus. The aim of this work was to determine and evaluate the factors which can contribute to infectivity of EV 71 RNA transcripts *in vitro*. Two strategies, overlapping RT-PCR and long distance RT-PCR, were employed to obtain the full-length genome cDNA clones of the virus. The length of the poly(A) tail and the presence of non-viral 3'-terminal sequences were studied in regard to their effects on infectivity of the *in vitro* RNA transcripts of EV 71 in cell culture. The data revealed that only cDNA clones obtained after long distance RT-PCR were infectious. No differences were observed in virus titres after transfection with *in vitro* RNA harbouring a poly(A) tail of 18 or 30 adenines in length, irrespective of the non-viral sequences at the 3'-terminus.

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1. Introduction

Enterovirus 71 (EV 71) is a small nonenveloped RNA virus, which is classified as a member of the *Enterovirus* genus within the *Picornaviridae* family (Schmidt et al., 1974; Brown and Pallansch, 1995). It is a human pathogen associated both with sporadic cases and large-scale outbreaks of hand, foot and mouth disease (HFMD) observed predominantly in young children throughout the world (Brown et al., 1999; Bible et al., 2007; Chen et al., 2007; van der Sanden et al., 2009; Zhang et al., 2009; Wu et al., 2010). The clinical manifestations of EV 71 infection vary from mild and self-limited conditions to severe neurological complications sometimes with a fatal outcome (Wang et al., 1999; McMinn et al., 2001; Lin et al., 2002).

The lack of antiviral therapies against EV 71 or vaccines has led to major research activity into the pathogen's molecular biology, its interaction with the host and development of strategies to prevent new infections. Many of these studies involve genetic manipulations of EV 71 and cannot be conducted without a DNA clone of the viral genome or the targeted genes. Construction of a DNA clone of RNA viruses, including EV 71, utilizes reverse genetics

and recombinant DNA techniques, when viral RNA genome or its parts are converted into a complementary DNA (cDNA) followed by cloning of the cDNA molecules into a suitable DNA vector (Boyer and Haenni, 1994; Lai, 2000).

The EV 71 genome is a single-stranded positive RNA of approximately 7.4 kb in length, and comprises a 5' untranslated region (UTR), a single open reading frame (ORF) and a short 3' UTR followed by a polyadenylated (poly(A)) tail (Brown and Pallansch, 1995). To generate an infectious EV 71 cDNA clone, the entire viral genome including its 5' and 3' terminus must be amplified and inserted into a plasmid. To date, several research groups have succeeded in production of the full-length EV 71 cDNA clones by assembling several cDNA fragments of the viral genome into a single DNA vector *via* specific cleavage sites, and by using conventional step-by-step cloning procedures (Arita et al., 2005; Chua et al., 2008; Shang et al., 2013a). Additional steps such as screening of the sub-genomic cDNA clones, restriction digestion and purification of the cDNA fragments make the process tedious and time-consuming. In some cases, traditional multi-step cloning strategies could be simplified by the utilization of overlapping/fusion PCR which yields full-length viral cDNA, and eliminates the necessity of construction of the sub-genomic cDNA clones. Although successfully employed with a number of viruses (Ohsugi et al., 2004; Desbiez et al., 2012), this method has not been applied to EV 71. Another approach used in cDNA production of the full length viral genomes is long distance RT-PCR. EV 71 RNA transcripts synthesized from RT-PCR amplicons

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Table 1
Primers used in construction of EV 71 cDNA clones, colony PCR and cycle sequencing.

Primer name ^a	Sequence (5'–3') ^b	Application(s)
Ft7	<i>ggtaatacgcactcactataggggtaaaacagcctgtgggtgc</i>	PCR amplification (Strategies 1 and 2)
RMLul	<i>ggacgcgtttttttttttttttgctattctggtataacaaatttac</i>	PCR amplification (Strategy 1)
RMLul-30	<i>ggatctacgcgtttttttttttttttttttttttttttttttt</i>	PCR amplification (Strategies 1 and 2)
F416	<i>gggtggaagagcctattgag</i>	Sequencing
R644	<i>caccggatggccaatcca</i>	Sequencing, colony PCR
F795	<i>ctcagctaccgagggttc</i>	Sequencing
F1632	<i>tgcgacgccagtgatc</i>	Sequencing
R1779	<i>tgctgagaccatcgtc</i>	Sequencing
F2386	<i>accatgaagttgtgcaagg</i>	Sequencing
R2551	<i>gatggctacttacctgctga</i>	Sequencing
R3411	<i>gtcgttatgagtagcaagatgg</i>	Sequencing
F3821	<i>gcatggggtttacagacgcag</i>	PCR amplification (Strategy 1), sequencing
R3991	<i>caagtgttccggttaatgtgacca</i>	PCR amplification (Strategy 1), sequencing
F4556	<i>accagaccactttgacg</i>	Sequencing
F5222	<i>gccacttaaacagagctgt</i>	Sequencing
F5851	<i>ggtaaggtgattgggac</i>	Sequencing
F5987	<i>tcaacggactactcgcactaagc</i>	PCR, sequencing
F6013	<i>gaaccaagtgtcttcacgatgtg</i>	Sequencing
R6050	<i>ttagtccctcgaacaca</i>	Sequencing
R6251	<i>tctgtaccgtaacaagcatcctc</i>	PCR, sequencing
F6536	<i>ccaatccaggtacagtca</i>	Sequencing, colony PCR
R6739	<i>cttctatgagagacagtgc</i>	Sequencing
R7380	<i>accagcattaacacgacc</i>	Sequencing

^a The Ft7 and RMLul/RMLul-30 are forward (F) and reverse (R) primers containing sequences which are identical or reverse-complimentary to the 5' and 3'-terminus of the EV 71 genome, respectively. Names of the remaining primers indicate position of the 5'-terminal nucleotide of the primer within the EV 71 genome (numbering in accordance to the 6F/AUS/6/99 strain).

^b Mlul recognition site is in italics; T7 promoter sequence is underlined.

bearing a SP6 promoter were shown to be infectious both in cell culture and mice (Han et al., 2010). The performance of long distance PCR might vary with different viral strains and, therefore, would require optimization in order to amplify the entire viral genome in a single reaction (Boot et al., 2004). To date, long distance PCR used in the production of the full-length EV 71 cDNA was based on 35–40 PCR cycles and utilized DNA polymerases exhibiting various error rates (Han et al., 2010; Meng et al., 2012). Unfortunately, there is no information available on genetic diversity of the cDNA clones produced by those methods when compared to the original viral stocks. In general, it could be anticipated that an amplification strategy with a higher fidelity DNA polymerase and a lower number of PCR cycles will be advantageous when preservation of the viral genome sequences is essential.

Success in the generation of full-length cDNA viral clones does not necessary guarantee infectivity of those constructs (Arita et al., 2005; Desbiez et al., 2012). A number of factors might account for this problem, including additional nucleotides incorporated at the 5' and 3' ends of viral genome, length of the poly(A) tail, artificial mutations introduced into viral genome during RT-PCR, cell culture transfection conditions, proportion of infectious and non-infectious genomes in the original viral population (Moormann et al., 1996; Yanagi et al., 1997; Feuer et al., 2002; Kusov et al., 2005; Silvestri et al., 2006).

Thus, considering the number of possible factors involved, production of infectious viral clones represents a technical challenge. Even though several experimental approaches have been successful, the information regarding the proportion of the infectious and non-infectious viral clones generated by those approaches is often missing. In this context, the main objective of this study was to compare the efficacy of two experimental strategies, namely overlapping and long distance RT-PCR, when used in the production of full-length cDNA of EV 71 genome. Our results demonstrated the crucial importance of the chosen amplification method on rescuing the infectious virus progeny from the cDNA clones. Additionally, other factors such as a length of the poly(A) tail and presence of non-viral sequences at the 3' terminus of the viral genome were examined in relation to infectivity of the cDNA constructs. The

present work emphasized a number of factors which should be considered in order to succeed in the production of an infectious cDNA clone of EV 71. Practically, this study demonstrated that some steps can be eliminated from the procedure to make it rapid and less labour-intensive with no adverse effects on infectivity. Although the described method was applied to EV 71, it can be potentially used in a rapid production of infectious cDNA clones of other enteroviruses with genome organization similar to that of EV 71.

2. Materials and methods

2.1. Cell culture and virus

EV 71 stock used in this study was obtained after propagation of the 6F/AUS/6/99 strain (GenBank ID: DQ381846) in the African Green Monkey kidney (Vero) cell line.

Vero cells were maintained in Minimum Essential Medium (MEM) with Earle's salts, L-glutamine and sodium bicarbonate (Sigma–Aldrich®, St. Louis, MO, USA), and supplemented with 5% foetal bovine serum (FBS) (Gibco®, Life Technologies, Carlsbad, CA, USA). Cells and viruses were grown in 5% CO₂ at 37 °C.

2.2. Primers

All primers (Table 1) used in this study were designed based on the sequence of the 6F/AUS/6/99 strain. Primers Ft7, RMLul and RMLul-30 were used in genome amplification of EV 71 prior to cloning. The sense primer Ft7 contained the T7 promoter sequence upstream of the first 21 nucleotides of the EV 71 genome. The anti-sense primer RMLul consisted of a Mlul recognition site followed by a stretch of 17 thymidine residues and 25 nucleotides corresponding to the sequence of EV 71 immediately before the poly(A) tail. The anti-sense primer RMLul-30 contained a Mlul recognition site at its 5' end followed by a stretch of 29 thymidine residues and 3 nucleotides corresponding to the sequence of EV 71 immediately before the poly(A) tail. In order to stabilize the amplicons,

both sense and anti-sense primers had two additional guanosine residues at their 5' ends (Boyer and Haenni, 1994).

Primers used for cycle sequencing were designed with FastPCR v5.2 software (Kalendar et al., 2011).

2.3. Construction of EV 71 cDNA clones and production of *in vitro* RNA transcripts

Total RNA was extracted from EV 71-infected Vero cell culture supernatant with the QIAamp Viral RNA Mini Kit (QIAGEN, Limburg, The Netherlands). Two approaches, designated as Strategy 1 and 2, were employed in order to obtain cDNA clones of EV 71.

2.3.1. Strategy 1

Strategy 1 was based on PCR of two overlapping fragments constituting the 5' and 3' halves of the EV 71 genome.

The cDNA synthesis was performed with the Transcriptor High Fidelity cDNA Synthesis Kit and 2.5 μ M of the anchored-oligo (dT)₁₈ primer (both from Roche Applied Science, Upper Bavaria, Germany) at 45 °C for 60 min.

Prior to PCR, the RNA template was removed from the reverse transcription (RT) reaction with Ribonuclease H (Promega, Madison, WI, USA). The endonuclease was added directly to the RT product at a final concentration of 1 U per 10 μ l of reaction volume and the mixture was incubated at 37 °C for 20 min.

The PCR consisted of two rounds. The 5' and 3' half-genome fragments of EV 71 were amplified with Expand Long Range dNTPack (Roche Applied Science, Upper Bavaria, Germany) using Ft7/R3991 and F3821/RMLul primers, respectively. The 5' and 3' halves were gel-purified and used as templates in full-length genome amplification with Ft7 and RMLul primers. In the 1st round, the PCR mixture contained 1 \times PCR buffer, 0.3 μ M of each primer, 0.4 mM dNTPs mix and 3.15 U of Expand Long Template enzyme mix. The cDNA product was added up to make a total reaction volume of 45 μ l. The PCR was carried out under the following conditions: the initial denaturation at 95 °C (2 min), followed by 10 cycles of 95 °C (10 s), 60 °C (15 s), 68 °C (3 min 50 s), followed by 15 cycles of 95 °C (10 s), 60 °C (15 s), 68 °C (3 min 50 s with a 10 s increment in each cycle). Final elongation was carried out at 68 °C (7 min). The 2nd round PCR mixture contained 1 \times PCR buffer, 0.3 μ M of Ft7 and RMLul primers, 0.4 mM dNTPs mix and 3.75 U of Expand Long Template enzyme mix in a total reaction volume of 50 μ l. Approximately 10 ng of both 5' and 3' DNA were used as templates. The 2nd round included initial denaturation at 95 °C (2 min), then 1 cycle at 95 °C (10 s), 56 °C (15 s), 68 °C (3 min 50 s), followed by 10 cycles of 95 °C (10 s), 56 °C (15 s), 68 °C (7 min 30 s), followed by 20 cycles of 95 °C (10 s), 56 °C (15 s), 68 °C (7 min 30 s with a 10 s increment in each cycle). Final elongation was carried out at 68 °C (7 min).

The full-length EV 71 cDNA was purified from an 0.7% agarose gel stained with crystal violet and ligated to the pCR[®]-XL-TOPO[®] vector by following the protocol supplied with the TOPO[®] XL PCR Cloning Kit (Invitrogen[™], Life Technologies, Carlsbad, CA, USA). The EV 71 cDNA construct was transformed into One Shot[®] Chemically Competent *Escherichia coli* (Invitrogen[™], Life Technologies, Carlsbad, CA, USA) and bacterial transformants were grown on LB agar supplemented with 50 μ g/ml of kanamycin sulphate (Gibco[®], Life Technologies, Carlsbad, CA, USA) at 37 °C overnight.

2.3.2. Strategy 2

Strategy 2 employed long distance PCR with a highly processive DNA polymerase (iProof[™] High-Fidelity, Bio-Rad, Berkeley, CA, USA) in order to amplify the entire EV 71 genome in a single round. The extremely low error rate (4.4×10^{-7}) of the enzyme and PCR of only 20 amplification cycles were aimed at preserving the viral sequences from unwanted mutations.

The first strand cDNA was synthesized with the anchored-oligo (dT)₁₈ primer (Roche Applied Science, Upper Bavaria, Germany) at a final concentration of 2.5 μ M and SuperScript[™] II Reverse Transcriptase (Invitrogen[™], Life Technologies, Carlsbad, CA, USA), at 42 °C for 50 min. After the RT reaction, the RNA template was removed as described in Section 2.3.1.

The PCR was conducted in 1 \times High-Fidelity PCR buffer (Bio-Rad, Berkeley, CA, USA), with 0.5 μ M of Ft7 and RMLul-30 primers, 0.2 mM dNTP mix (Promega, Madison, WI, USA), 1 U of iProof High-Fidelity DNA Pol (Bio-Rad, Berkeley, CA, USA) and 2 μ l of the cDNA template in a total reaction volume of 50 μ l. The PCR conditions were set up at 98 °C (30 s) of initial denaturation, followed by 1 cycle at 98 °C (7 s), 56 °C (20 s), 72 °C (3 min 30 s), followed by 1 cycle at 98 °C (7 s), 46 °C (20 s), 72 °C (3 min 30 s), followed by 18 cycles at 98 °C (7 s), 61 °C (20 s) and 72 °C (3 min 30 s) with final elongation at 72 °C (5 min).

The amplified EV 71 cDNA was gel-purified and ligated to the pCR[®]-XL-TOPO[®] vector, which was transformed into One Shot[®] Chemically Competent *E. coli* (Section 2.3.1). Prior to ligation the cDNA was incubated with GoTaq[®] Flexi DNA Polymerase (Promega, Madison, WI, USA) at 72 °C (30 min) in order to add 3' A-overhangs. The reaction mixture contained 1 \times Colorless GoTaq[®] Flexi Buffer, 1.5 mM of MgCl₂, 200 μ M of dATP, 0.5 μ g of DNA and 10 U of the enzyme in 20 μ l of total volume.

2.3.3. *In vitro* transcription

Plasmid DNA containing the full length EV 71 genome was linearized with MluI and NotI (New England BioLabs, Ipswich, MA, USA). The DNA fragment containing the EV 71 insert was gel-purified with the Wizard[®] SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and used as a template in *in vitro* transcription carried out with the RiboMAX[™] Large Scale RNA Production System-T7 (Promega, Madison, WI, USA). Integrity, size and concentration of the synthesized RNA were evaluated by 1.5% agarose gel electrophoresis against the RiboRuler[™] High Range RNA Ladder (Thermo Fisher Scientific, Waltham, MA, USA).

2.4. Production of *in vitro* RNA transcripts of the EV 71 genome harbouring different 3' termini

The EV 71 genome was reverse transcribed and then amplified as described in Section 2.3.2. The Ft7/RMLul and Ft7/RMLul-30 primers were used in order to obtain cDNA with 18 and 30 thymidines downstream of the 3' UTR, respectively. PCR products were digested with MluI and 5' extensions were removed with Mung Bean Nuclease (New England BioLabs, Ipswich, MA, USA). The endonuclease was inactivated by the addition of SDS to 0.01% of final concentration. Ethanol precipitation with sodium acetate (pH 5.2, final concentration of 0.3 M) was carried out after the PCR and MluI digestion in order to de-salt the DNA from the reaction buffers. Ethanol precipitation with sodium chloride (final concentration of 0.2 M) was used to precipitate DNA samples containing SDS. In total, six DNA templates (Table 2) of EV 71 genome, different in their 3' ends, were obtained. These DNAs were gel-purified with the Wizard[®] SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and used for *in vitro* RNA synthesis with the RiboMAX[™] Large Scale RNA Production System-T7 (Promega, Madison, WI, USA). The *in vitro* produced RNA was purified with the SV Total RNA Isolation System (Promega, Madison, WI, USA) in accordance with the modified protocol recommended for this sample type (Rayner and Butler, 2004). The concentration and purity of the *in vitro* RNA were determined both spectrophotometrically using a Biowave II instrument (Biochrom, Cambridge, UK) and by agarose gel electrophoresis.

Table 2
Double-stranded (ds) cDNA templates of the EV 71 genome carrying different 3'-terminus.

DNA name	Schematic representation of the dsDNA sequence	Description
DNA 18++	GGT7-----A ₍₁₇₎ <u>A</u> CGCG TCC GGT7-----T ₍₁₇₎ <u>T</u> GCGC <u>AGG</u>	PCR product obtained with Ft7 and RmluI primers
DNA 18+	GGT7-----A ₍₁₈₎ GGT7-----T ₍₁₈₎ GCGC	MluI digestion of DNA 18++
DNA 18	GGT7-----A ₍₁₈₎ GGT7-----T ₍₁₈₎	Mung Bean Nuclease polished DNA 18+
DNA 30++	GGT7-----A ₍₂₉₎ <u>A</u> CGCG TAGATCC GGT7-----T ₍₂₉₎ <u>T</u> GCGC <u>ATCTAGG</u>	PCR product obtained with Ft7 and RmluI-30 primers
DNA 30+	GGT7-----A ₍₃₀₎ GGT7-----T ₍₃₀₎ GCGC	MluI digestion of DNA 30++
DNA 30	GGT7-----A ₍₃₀₎ GGT7-----T ₍₃₀₎	Mung Bean Nuclease polished DNA 30+

All DNA templates contained the T7 promoter followed by the full-length EV 71 genome, which is represented by dashed lines. The 3' ends of the DNA molecules differed in their sequences as indicated. The MluI recognition site is underlined. Positions at which MluI digests the DNA strands are marked with ▼ and ▲.

2.5. Transfection of cell culture with EV 71 *in vitro* RNA

The protocol used in transfection of Vero cells with the *in vitro* RNA (Section 2.3.3) was designed in accordance with the manufacturer's recommendations for Lipofectamine™ 2000 (Invitrogen™, Life Technologies, Carlsbad, CA, USA). Vero cells seeded at 2.5×10^5 cells per well into a 24 well plate were grown in MEM + 5% FBS without antibiotics for 24 h, and then in Opti-MEM® I reduced serum medium (Gibco®, Life Technologies, Carlsbad, CA, USA) for another 24 h. The transfection mixture containing the entire *in vitro* transcription reaction and Lipofectamine™ 2000 was inoculated into Vero cells monolayers (90–100% confluence) and incubated for 5 h at 37 °C. The inoculum was then discarded and cells were grown in MEM supplemented with 5% FBS for 6–7 days.

Slight changes were made in transfection protocol with *in vitro* RNA transcripts harbouring different 3' termini (Section 2.4): (a) transfection was carried out with 1 µg of the purified *in vitro* RNA; (b) after 5 h incubation at 37 °C, the transfection mixture was removed from the wells, cells were washed with PBS, treated with Trypsin-0.05% EDTA (Gibco®, Life Technologies, Carlsbad, CA, USA) and split into two wells in MEM supplemented with 10% FBS. Cell cultures were incubated at 37 °C for 6 days.

At the end of the incubation period, supernatant and cells were collected and stored at –80 °C. Centrifugation was used to clear samples from cell debris after thawing. Both infection of new Vero cells with the cleared supernatant and RT-PCR with EV 71 specific primers was used to confirm the presence of EV 71.

2.6. Virus titration

Virus titre was determined by the TCID₅₀ assay conducted in Vero cells. Ninety-six well plates were seeded with 2×10^4 cells per well in MEM supplemented with 5% FBS and incubated for 24 h prior to infection with serial dilutions of the virus. The inoculated plates were cultured at 37 °C for 8–10 days, and observed for cytopathic effect (CPE). The TCID₅₀ values were calculated according to the Reed–Muench method (Reed and Muench, 1938).

2.7. DNA automated cycle sequencing and sequence analysis

Nucleotide sequences of the viral stock and constructed EV 71 cDNA clones were determined by automated DNA sequencing using

the Big Dye Terminator method (Applied Biosystems®, Life Technologies, Foster City, CA, USA). Primers used in sequencing are listed in Table 1. Sequencing was performed at the Department of Pathology, The University of Melbourne, Australia. The viral stock used in cloning was separately amplified by RT-PCR and sequenced within the VP1 gene. Two viral clones, one of each from Strategy 1 and 2 (Section 2.3), were sequenced in full. Additionally, a few clones from each strategy were sequenced within the 5' UTR, VP4, VP1, 3D and 3' UTR genome regions. Sequences were aligned using the BioEdit v7.0.5.3 software (Hall, 1999). Strain 6F/AUS/6/99 (GenBank ID: DQ381846) served as a reference. In order to compare genetic similarity of the generated viral clones to the original viral stock, average distances were calculated between those two groups (viral stock versus viral clones) by using nucleotide sequences of VP1 region and the Kimura 2-Parameter model incorporated in MEGA3 software (Kumar et al., 2004).

3. Results

3.1. Amplification of the full-length genome of EV 71

The full-length cDNA of the EV 71 genome was successfully obtained by both overlapping PCR (Strategy 1) and long distance PCR (Strategy 2) (Fig. 1). In the first round of the overlapping PCR the 5' half of the EV 71 genome was produced at a lower amount compared to the 3' fragment (data not shown). That could be due to incomplete extension of the cDNA during reverse transcription or to inefficient annealing of the Ft7/R3991 primers whose individual melting temperatures differed to a greater degree when compared to the F3821/RmluI primers used for amplification of the 3' half. However, combining an equal amount of the 5' and 3' DNA fragments in the second round of the overlapping PCR resulted in an abundant DNA product comprising the full-length EV 71 genome (Fig. 1A). In contrast to the overlapping PCR, the long distance PCR approach required optimization of PCR conditions in order to obtain sufficient amounts of DNA from a single round for downstream analyses, gel purification and cloning. Additionally, a number of DNA polymerases recommended for long template amplification were used unsuccessfully (LongAmp™ Taq from New England BioLabs, Ipswich, MA, USA; Expand Long Template PCR System from Roche Applied Science, Upper Bavaria, Germany; and an in-house

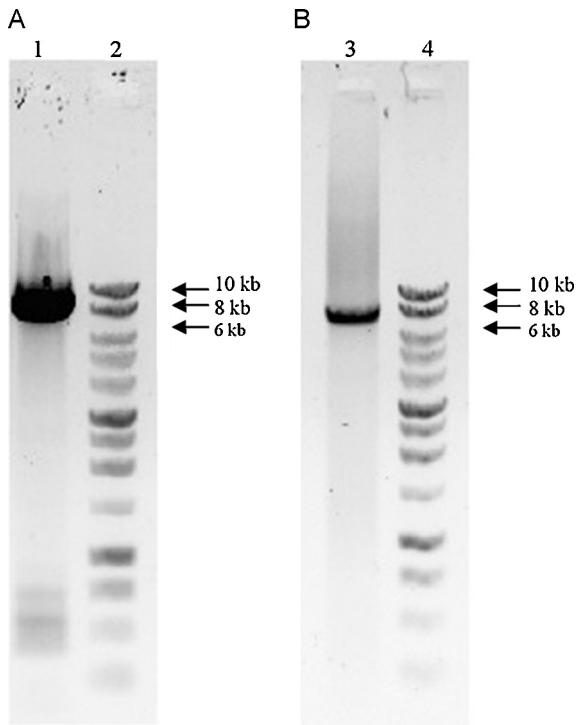


Fig. 1. Detection of the full-length genomic cDNA of EV 71 by agarose gel electrophoresis. (A) Amplification of the EV 71 genome by overlapping PCR consisted of two rounds. The 3' and 5'-halves of the EV 71 genome were amplified in the first round, and these were used as DNA templates in the second round of the overlapping PCR. (B) Amplification of the EV 71 genome by a single round long distance PCR. Lanes 1 and 3: cDNA of EV 71 genome, 7456 bp in length; lanes 2 and 4: 1 kb DNA ladder (Promega, Madison, WI, USA).

mix of *Pfu* DNA Pol and GoTaq[®] DNA Pol which were supplied from Promega, Madison, WI, USA) prior to obtaining positive results with iProof High-Fidelity DNA Pol. When performed under conditions described above (Section 2.3.2), the long-PCR amplification resulted in an adequate yield of the PCR product of expected length, and with no additional non-specific bands observed by agarose gel electrophoresis (Fig. 1B).

3.2. Infectivity of EV 71 cDNA clones derived from Strategy 1 and 2

Ligation into plasmid of the full-length EV 71 cDNA obtained by Strategy 1 and 2 followed by transformation of *E. coli* resulted

in approximately 80% and 95% of the clones carrying the EV 71 genome, respectively (data not shown). Due to TA cloning, two random orientations of the EV 71 genome within plasmid DNA were possible (Fig. 2A and B) and were observed after screening the *E. coli* colonies from both strategies. Orientation of the EV 71 genome with negative cDNA strand cloned downstream of the M13 Reverse priming site (Fig. 2B) was observed more often, and constituted 83.3% and 64.4% of the EV 71 clones produced in Strategy 1 and 2, respectively. Whether that particular orientation of the EV 71 genome was more stable within the plasmid was not determined. Also, it should be noted that the contrasting results with 66.6% of the colonies carrying plasmid DNA with EV 71 positive cDNA strand cloned downstream of the M13 Reverse priming site (Fig. 2A) were obtained in another cloning experiment which was not part of this study.

Four viral clones from Strategy 1 and eight from Strategy 2, including clones with both orientations, were used for *in vitro* RNA synthesis followed by transfection of Vero cell monolayers. Monitoring the cell cultures by microscopy showed increasing signs of CPE only after transfection with the RNA transcripts derived from cDNA clones obtained in Strategy 2 (Fig. 3). Similar results were obtained with the supernatants collected at the end of the incubation period and used in the infection of new Vero cells (Fig. 4A–D). In addition, the presence of the rescued EV 71 in cell culture supernatant derived from Strategy 2 was confirmed by RT-PCR amplification conducted with EV 71 specific primers, F5987 and R6251 (Fig. 4F). The proportion of the infectious clones produced in Strategy 2 was 87.5%. In contrast, no CPE and negative RT-PCR results were obtained with supernatants originated from Strategy 1 (Fig. 4A and E).

The *E. coli* recombinants produced in Strategy 2 seemed to be stable throughout the study and maintained plasmid constructs with the full-length EV 71 genomes over several passages or after reviving the recombinant *E. coli* cells from frozen stocks. However, further experiments are required to establish the long term stability of the cDNA constructs in highly passaged *E. coli*.

3.3. Genetic variation within EV 71 cDNA clones derived from Strategy 1 and 2

In order to determine the degree of genetic variation within EV 71 cDNA clones produced in Strategy 1 and 2, two viral clones randomly selected from each strategy were sequenced and compared within the 5' UTR, VP4, VP1, 3D and 3' UTR genome regions. The overall number of nucleotide (nt) and amino acid (aa) differences within cDNA clones derived from Strategy 1 was greater

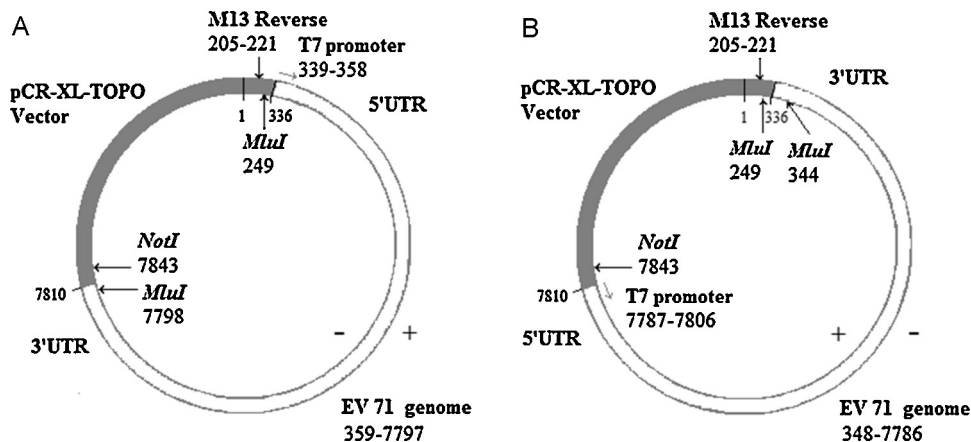


Fig. 2. Schematic diagram of the full-length cDNA clone of EV 71. Due to TA cloning, two orientations of the EV 71 genome within the pCR[®]-XL-TOPO[®] vector were possible, with either positive (A) or negative (B) cDNA strand cloned downstream of the M13 reverse priming site. The numbers indicate nucleotide positions of both the EV 71 genome and important recognition sites within the construct.

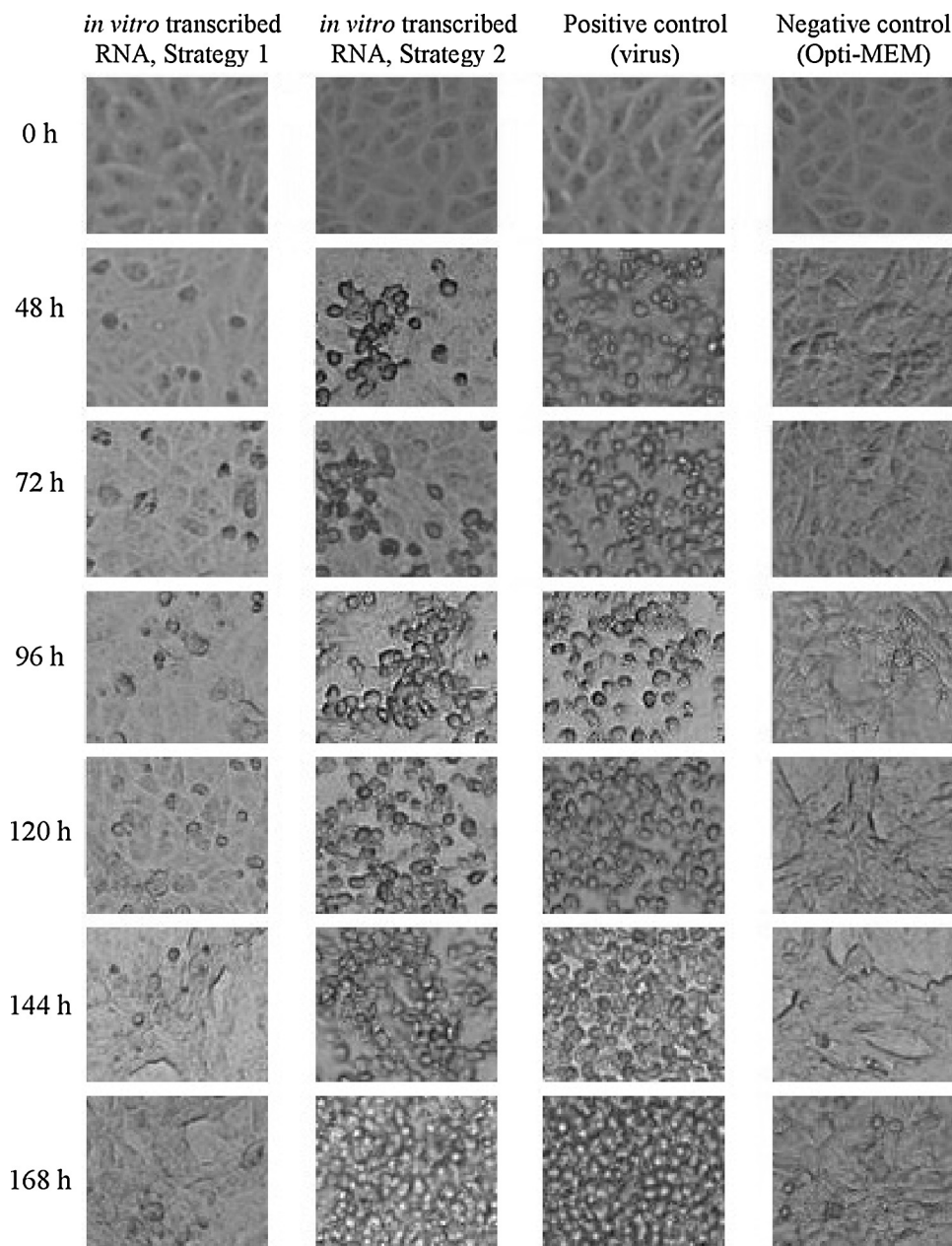


Fig. 3. CPE observed in Vero cell culture after transfection with the *in vitro* transcribed RNA of EV 71. The *in vitro* RNA transcripts obtained from EV 71 cDNA clones derived from Strategy 1 and 2 were transfected into Vero cells in presence of Lipofectamine™ 2000. Transfection mixture without *in vitro* RNA and virus stock were used as negative and positive controls, respectively. Signs of CPE developed both in the positive control and experimental wells, which were transfected with the *in vitro* RNA transcripts obtained with Strategy 2. The negative control cell culture and experimental wells transfected with the RNA transcripts obtained with Strategy 1 did not produce CPE but showed overgrowth after 96 h of incubation.

(Table 3). The complete list of mutations observed in each strategy is presented in Table 4. Among all mutations, only one, 3D-T1119C (Table 4), was present in both strategies. This indicates that this mutation was highly unlikely to be an RT-PCR error and existed prior to RT-PCR amplification. Additionally, several VP1 mutations (A275G, A292G, A293C and C848T) observed in viral clones corresponded to double peaks (R275, R292, M293 and Y848) within the VP1 sequence of an RT-PCR product amplified independently from the cDNA used in cloning. Therefore, those mutations most likely existed in the original viral population. For all other mutations, it was not possible to determine if they represented quasi-species diversity of the virus or had resulted from nucleotide mis-incorporations during RT-PCR. However, assuming that RNA was isolated from the same virus stock, a similar degree of sequence

Table 3
Genetic variation within cDNA clones of EV 71.

Genome region	Overall number of differences, nt(aa)	
	Strategy 1	Strategy 2
5' UTR	5 (N/A)	1 (N/A)
VP4	0 (0)	0 (0)
VP1	6 (4)	2 (2)
3D	3 (0)	1 (1)
3' UTR	0 (N/A)	0 (N/A)

Nucleotide sequences of two EV 71 cDNA clones randomly selected from each strategy were determined by BigDye Terminator Cycle Sequencing. Calculations were performed at nucleotide and amino acid levels, nt(aa), except the 5' UTR and 3' UTR, where amino acid sequences were not applicable (N/A). The complete list of the mutations used to calculate nt(aa) differences is presented in Table 4.

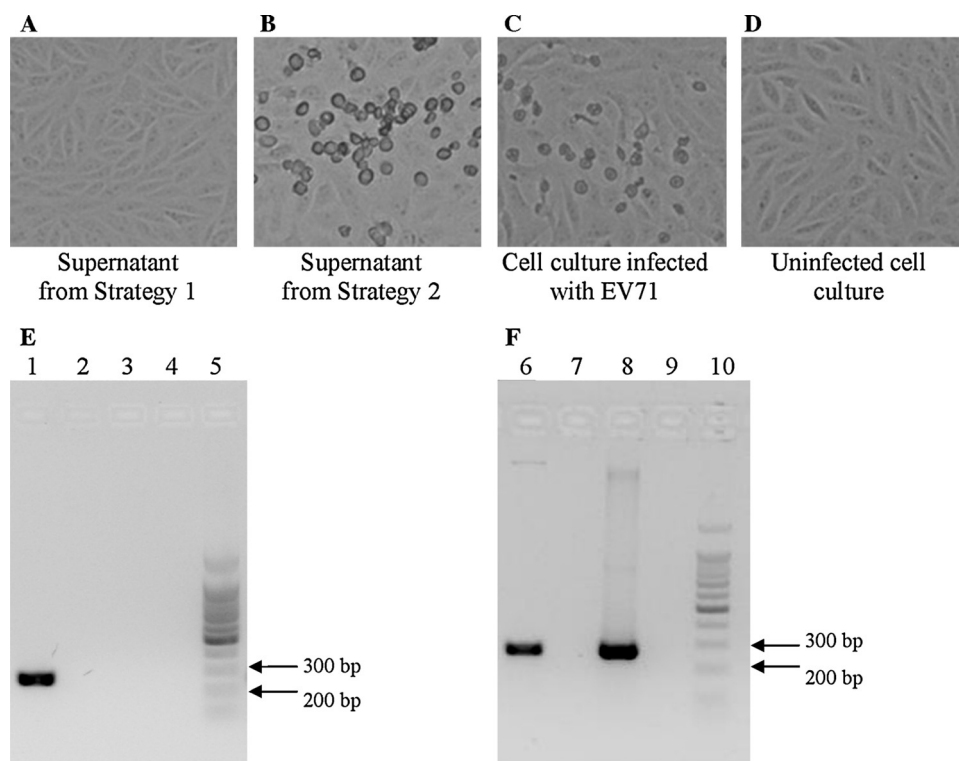


Fig. 4. Detection of EV 71 in Vero cells after second round infection. (A and B) Vero cell cultures are shown at 48 h after infection with supernatants collected after transfection with *in vitro* RNA derived from Strategy 1 and 2, respectively. (C) Cell culture infected with virus stock served as a positive control. (D) Uninfected cell culture served as a negative control. (E and F) RT-PCR detection of the rescued EV 71. Total RNA/DNA was isolated from the cell culture supernatants after the second round infection. Reverse transcription was performed with random hexamers. EV 71 specific primers, F5987 and R6251, were used in the following PCR amplification of a 265 bp DNA fragment of the EV 71 genome. Lane 1: RT-PCR of the positive control cell culture (C). Lane 2: RT-PCR of the cell culture infected with the supernatant derived from Strategy 1 (A). Lane 3: RT-PCR of the negative control cell culture (D). Lanes 4 and 9: negative controls of PCR with H₂O used instead of cDNA template. Lanes 5 and 10: 100 bp DNA ladder (Promega, Madison, WI, USA). Lane 6: RT-PCR of the cell culture infected with the supernatant obtained with Strategy 2 (B). Lane 7: PCR (no RT) of the cell culture (B) was performed in order to exclude a false positive PCR result due to possible DNA template carryover from the *in vitro* transcription reaction. Lane 8: positive control of PCR with full-length genomic cDNA of EV 71 as a template.

diversity could be expected for all EV 71 cDNA clones produced. When VP1 sequences of viral clones were compared to that of the original viral stock, the average number of nucleotide substitutions per site was 0.003 and 0.001 for Strategy 1 and 2, respectively. Thus, the greater number of mutations and the higher average distance calculated for Strategy 1 were likely as a result of more nucleotide mis-incorporations introduced during the RT-PCR when compared to Strategy 2. It was impossible to conclude, at least without conducting additional site-directed mutagenesis experiments, if some of the mutations produced by Strategy 1 resulted in non-infectious EV 71 clones. Additionally, even if single mutations did not affect infectivity, the recombination occurring during the second round of the overlapping PCR could have a negative effect on infectivity of the full-length EV 71 cDNA. It is most likely that the vast majority of the full-length cDNA molecules produced in Strategy 1 were the result of recombination between 5' and 3' halves of the viral genome derived from different viral particles. However, definitive conclusions regarding the effect of this event on infectivity of EV 71 clones would require additional experiments beyond the scope of in this study.

3.4. Effect of the poly(A) tail length and non-viral 3' terminal sequences on infectivity of the *in vitro* RNA of EV 71

It is known that shortened poly(A) tail can reduce infectivity of some viruses (Spector and Baltimore, 1974; Spagnolo and Hogue, 2000; Silvestri et al., 2006). In this study, we could not rescue EV 71 from *in vitro* RNA harbouring an A₁₈-tail (Strategy 1), whereas *in vitro* RNA with an A₃₀-tail (Strategy 2) could

produce infectious virus upon transfection of cell culture. To examine if poly(A) tail length, but not the RT-PCR amplification procedure, abolished infectivity of the EV 71 clones from Strategy 1, RNA transcripts harbouring A₁₈- or A₃₀-tails were obtained *in vitro* from ds cDNA templates amplified by the RT-PCR protocol used in Strategy 2. Additionally, there was experimental evidence that non-viral sequences downstream of the poly(A) tail had a negative effect on infectivity of poliovirus RNA molecules (Sarnow, 1989). To investigate if extra nucleotides at the end of the poly(A) tract reduced infectivity of EV 71 RNA in our study, we obtained *in vitro* RNA transcripts from ds cDNA templates with or without 3'-terminal extensions following the A₁₈- or A₃₀-tails (Table 2). Comparing the infectivity of *in vitro* RNA transcripts derived from those ds cDNA molecules would allow us to determine if extra steps, including treatment of the full-length PCR product with endonucleases, are necessary. As a result, six ds cDNA templates containing different 3' termini were created. Although the 3'-terminal sequences of those cDNAs have not been determined by sequencing, the size of the DNA fragments (approximately 100 nt in length) excised from the 3'-end of the cDNA templates by DraI endonuclease was estimated by the polyacrylamide gel electrophoresis (data not shown). Clear difference in size was observed between 3'-DNA fragments derived from DNA 18++ [poly(A)₁₈ followed by 7 nt], 18+ [poly(A)₁₈ followed by 4 nt], 30++ [poly(A)₃₀ followed by 11 nt] and 30+ [poly(A)₃₀ followed by 4 nt]. However, there was no measurable difference between 3'-DNA fragments of DNA 18+ and 18 [poly(A)₁₈ with no extra nt], and DNA 30+ and 30 [poly(A)₃₀ with no extra nt] (data not shown).

Table 4
Nucleotide and amino acid substitutions observed within the EV 71 cDNA clones.

Genome region	Mutations ^a		Mutation frequencies (<i>f</i>) ^b		
	nt	aa	Strategy 1	Strategy 2	
5' UTR	T1 deletion	n/a	1/2	0/2	
	A149G	n/a	1/2	0/2	
	A282G	n/a	1/2	0/2	
	A388G	n/a	1/2	0/2	
	C640T	n/a	1/2	0/2	
	T682C	n/a	0/2	1/2	
	VP4	–	–	0/2	0/2
VP2	–	–	0/1	0/1	
VP3	A680G	K227R	1/1	0/1	
VP1	A138G	–	1/2	0/2	
	T209C	L70P	1/2	0/2	
	A275G	E92G	0/2	2/2	
	<u>A292G</u>	K98E	0/2	1/2	
	<u>A293C</u>	K98T	2/2	0/2	
	G314A	G105E	1/2	0/2	
	T368C	V123A	1/2	0/2	
	C658T	L220F	0/2	2/2	
	A724C	K242Q	0/2	1/2	
	T789C	–	1/2	0/2	
	<u>C848T</u>	S283F	1/2	0/2	
	2A	A234G	–	1/1	0/1
	2B	–	–	0/1	0/1
	2C	A245G	Y82C	1/1	0/1
		G857A	C286Y	1/1	0/1
	3A	–	–	0/1	0/1
	3B	A35G	K12R	1/2	0/1
3C	A93G	–	1/2	0/1	
3D	G6A	–	1/2	0/2	
	T43G	L15V	0/2	1/2	
	A342G	–	1/2	0/2	
	<u>T1119C</u>	–	2/2	2/2	
	G1125A	–	1/2	0/2	
3' UTR	–	n/a	0/2	0/2	

The mutation highlighted in 3D-T1119C, was observed in viral clones from both strategies. The underlined mutations within VP1 showed double peaks (both nt were present) when cDNA separately produced from the cDNA used in cloning was sequenced. The highlighted and underlined mutations probably existed in the viral population prior to RT-PCR amplification.

^a Mutations are depicted according to their nt and aa positions in specified genes when compared to the 6F/AUS/6/99 reference strain (GenBank ID: DQ381846).

^b The mutation frequency (*f*) is presented as a number of clones, which contain the mutation, per total number of clones sequenced within the specified region.

All six cDNA templates were used in *in vitro* transcription with T7 RNA polymerase. The same amount of the *in vitro* synthesized RNA transcripts was transfected into Vero cells and titres of rescued EV 71 were compared in TCID₅₀ assays. Our results indicated that there was no significant difference in TCID₅₀ titres of the rescued viruses (Fig. 5). From this finding, we could conclude that the difference in poly(A) tail length and the presence of the 3'-terminal sequences within the EV 71 clones obtained by Strategy 1 and 2 did not have any effect on infectivity of the EV 71 RNA transcripts in cell culture.

4. Discussion

During the last two decades, the public health threat associated with increases in EV 71 incidence and death rates in the Asia-Pacific region has prioritized the development of preventive and antiviral treatment strategies for EV 71 infection. Although, several antiviral agents showed a potent anti-EV 71 activity in cell culture or animal models, they are yet to be evaluated in human clinical trials (Tsai et al., 2011; Shang et al., 2013b). Promising results were recently obtained in the phase 2 clinical trials with an EV 71 inactivated vaccine candidate (Zhu et al., 2013). However, it might take several years in order to complete the phase 3 trials and resolve the usual practical issues associated with a vaccine production (Lee et al., 2012; Pallansch and Oberste, 2013). Additionally, it can be

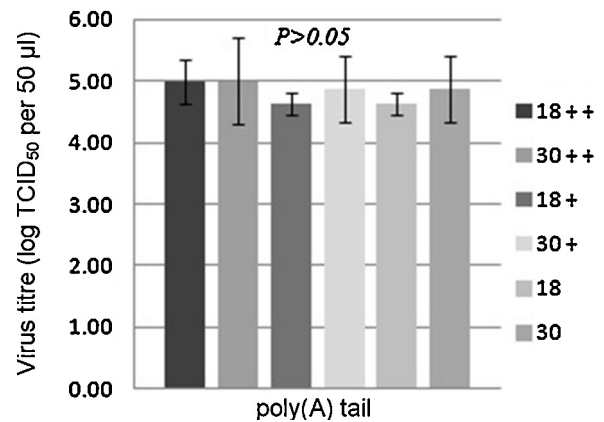


Fig. 5. Cell culture titres of EV 71 rescued from *in vitro* RNA transcripts harbouring different 3'-termini. Vero cells were transfected with EV 71 RNAs which were obtained by *in vitro* transcription from ds cDNA templates of the EV 71 genome harbouring different 3'-termini (Table 2). At the end of incubation, the cell cultures were collected and cleared supernatants were titrated by TCID₅₀ assay. Results represent means of two experiments; error bars represent standard deviation. $P > 0.05$ of the "unpaired" Student's *t*-test indicates there were no statistically significant differences found between the TCID₅₀ titres of viruses rescued from different RNA transcripts.

anticipated that, due to the high genetic diversity of the circulating EV 71 strains and ability of the virus to evolve rapidly, some of the genetic variants of EV 71 might escape from the immunity generated by a vaccine, and result in new outbreaks.

In spite of evident progress made in understanding EV 71 infection, some of the epidemiological characteristics of the virus are still poorly understood. EV 71 is one of the most common enteroviruses detected both from samples of clinical cases and healthy children around the world and has been associated with large scale outbreaks predominantly in the Western Pacific Region (Khetsuriani et al., 2006; Witso et al., 2006; WHO, 2011). As a result, beside the development of antiviral treatment strategies, the major research interest is also focused on revealing the mechanisms behind EV 71 pathogenicity, and functional analysis of viral genes. The availability of full-length genome cDNA clones of EV 71 is often an absolute requirement for such experiments; however, none are commercially available.

Several research groups have succeeded in obtaining infectious EV 71 clones by designing their own experimental approaches and, in some cases, troubleshooting the reasons for failure in infectivity of the original EV 71 cDNA constructs (Arita et al., 2005). In most cases, construction of EV 71 cDNA clones has been based on subcloning techniques which are typically laborious and time-consuming (Arita et al., 2005; Chua et al., 2008; Shang et al., 2013a). Recent advances in reverse genetics and availability of long template DNA polymerases allowed amplification of the full length viral genome in a single PCR (Han et al., 2010; Meng et al., 2012). Unfortunately, there is little or no information available on the proportion of infectious and non-infectious cDNA viral clones produced by those methods. In this study, we compared two amplification strategies which theoretically were expected to be successful in the production of infectious EV 71 cDNA clones. First, overlapping PCR which was conducted in two rounds, allowing the generation of an abundant amount of the full-length viral cDNA. The PCR enzyme blend utilized offered 3-times higher fidelity than *Thermus aquaticus* DNA polymerase (*Taq* DNA Pol) alone and, at the same time, could produce a PCR product with 3' A-overhangs, ready for TA-cloning. The second strategy was based on a long distance PCR with iProof High-Fidelity DNA Pol which possesses a rapid extension capacity and a 52-fold lower error rate than *Taq* DNA Pol. The cDNA amplified in this strategy was blunt-ended and 3' A-overhangs had

to be added in an additional step prior to cloning. Although both amplification methods allowed generation of the full-length cDNA of EV 71 genome, the subsequent cloning and cell culture transfection experiments revealed that only viral clones containing the cDNA amplified by the long distance PCR were infectious.

The next task was to investigate possible factors which could lead to the loss in infectivity of the cDNA clones produced in Strategy 1 (overlapping PCR). One of the factors could be deleterious mutations introduced into the viral genome during the RT-PCR step. Even though both experimental strategies used DNA polymerases with proof-reading activity, the overall fidelity of a chosen amplification method depends both on enzyme error rate and the number of PCR cycles. The overlapping PCR performed in two rounds resulted in an increase of the total number of the PCR cycles required for amplification of the full-length viral genome. With the long distance PCR and, additionally, utilizing a highly processive DNA polymerase, the number of required PCR cycles could be minimized to 20. As a result, when the overall number of mutations was compared, it was found to be lower in the infectious clones produced in Strategy 2 (Table 3).

On the other hand, the two strategies differed not only in their RT-PCR amplification methods but also in their reverse PCR primers used to anneal to the 3' end of EV 71 genome. Those primers, RMLu1 or RMLu1-30, allowed production of the cDNA harbouring the T₁₈ or T₃₀-tail, respectively, with various non-viral sequences at the 3' terminus. It has been previously shown that inhibition of infectivity by extra non-viral nucleotides at the 3' end depends on the length and nature of the sequence (Sarnow, 1989). Long heteropolymeric 3' end extensions decreased or abolished infectivity whereas short extra sequences were usually tolerated. With some viruses, the biological activity of the *in vitro* RNA transcripts was affected by the length of the poly(A) tail as well. A shortened poly(A) tail was shown to decrease virus replication for a number of viruses (e.g. poliovirus, hepatitis A virus and coronavirus) (Sarnow, 1989; Kusov et al., 2005; Silvestri et al., 2006). With poliovirus, a stretch of at least 20A residues was essential in order to approach the level of the negative strand RNA synthesis similar to that of the wild-type RNA transcripts. It was suggested that the poly(A) tail should be long enough in order to interact with the PABP and initiate the VPg uridylation (Silvestri et al., 2006). Additionally, the poly(A) tail probably plays some role in the protection of the RNA transcripts *in vivo* against host cell ribonucleases (Kusov et al., 2005). Interestingly, with some other viruses, an absence of the poly(A) tail or an addition of long non-viral sequence downstream of the viral genome did not have any effect on infectivity of the RNA transcripts (Eggen et al., 1989; Liu et al., 2008).

Obtaining the RNA transcripts carrying a poly(A) tail with a minimum or no extra nucleotides downstream is possible by incorporating a unique restriction site into a reverse primer used during amplification. Subsequently, this site can be used to linearize the plasmid DNA downstream of the poly(A) tail rather than using the multiple restriction sites existing in a vector. In this study, the MluI restriction enzyme was optimal for this purpose as it does not have a recognition site within the EV 71 genome and leaves an extension of only 4 nucleotides at the 5' end of the digested DNA. Those extra bases (5'-CGCG) can be removed by a single-strand nuclease in order to produce a template with no extraneous sequence downstream of the poly(A) tail. However, introduction of this enzymatic reaction would require an additional DNA purification step which, in the end, makes the approach more laborious. To understand if non-viral sequences and/or length of the poly(A) tract plays a certain role in infectivity of the cloned EV 71, six different cDNA templates of the EV 71 genome were constructed and used in the production of the *in vitro* RNA transcripts (Table 2). The cDNA templates had the identical 5' ends and differed only in their sequences downstream of the 3' UTR. Vero cell cultures were transfected with

the same amount of the *in vitro* RNA transcripts and viral titres of the rescued viruses were estimated in TCID₅₀ assay. Our results demonstrated insignificant variations among the viral titres (Fig. 5). Thus, it could be concluded that under experimental conditions the length of the poly(A) tail or the presence of the non-viral 3'-terminal sequences did not account for the loss in infectivity of the EV 71 RNA transcripts produced in Strategy 1.

In summary, we have compared two reverse genetics approaches for production of EV 71 infectious cDNA clones. Our results highlight the importance of the amplification method on the overall success. Using a DNA polymerase with high fidelity and reducing the number of PCR cycles are absolutely essential in order to minimize the number of undesirable deleterious mutations and to ensure infectivity of the cloned viruses. This approach can be useful if the genetic diversity of the viral population is to be preserved. To the best of our knowledge, and for the first time, a sufficient amount of the full length 7.4 kb cDNA of EV 71 was amplified in 20 PCR cycles. Additionally, when designing the PCR reverse primer, the length of the poly-thymidine stretch can be shortened to 18 residues, as a longer poly(A) tail did not result in higher infectivity. Incorporation of the unique restriction site into the reverse PCR primer can allow the linearization of a cDNA clone and, finally, non-viral sequences remaining at the end of the poly(A) tail after restriction digestion do not interfere with infectivity. In conclusion, an experimental approach described in this paper as Strategy 2 represents a convenient and reliable method allowing production of infectious EV 71 clones and will enable further molecular genetic studies of the virus.

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