Virologica Sinica 37 (2022) 769–773

Contents lists available at ScienceDirect

Virologica Sinica

journal homepage: www.keaipublishing.com/en/journals/virologica-sinica
www.virosin.org

Letter

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

Kaw Bing Chua *, Qimei Ng, Tao Meng, Qiang Jia

Temasek Life Sciences Laboratory, 1 Research Link, National University of Singapore, 117604, Singapore

Dear Editor,

Coxsackievirus A16 (CA16) and enterovirus A71 (EV-A71) are the main causative agents of hand foot and mouth disease (HFMD) (Alexander et al., 1994). Besides HFMD, EV-A71 infection in young children can lead to a spectrum of other clinical diseases and associated neurological complications and mortality (Melnick JL., 1996). In recent decades, EV-A71 infections have become a major public health concern throughout the world, following the frequent occurrence of epidemics and outbreaks of HFMD associated with neurological complications and high mortality in young children (Ishimaru et al., 1980; Tagaya et al., 1981; Chomnaitree et al., 1982; Samuda et al., 1987; Gilbert et al., 1988; Hayward et al., 1989; Lum et al., 1998; Chang et al., 1998; Liu et al., 2000; Yang et al., 2011; Zeng et al., 2012).

Infection by EV-A71 does not cross-protect children against HFMD by CA16 and vice-versa. In addition, our earlier study of monkeys infected with a stable, cold-adapted, temperature-sensitive conditional lethal EV-A71 (EV71:TLLβP20) showed induction of a high neutralizing antibody titre against EV-A71 within the same genogroup, but of lower neutralizing antibody titre against heterologous genogroups (Chua et al., 2021). The finding concurred with a previously published monkey study undertaken in Japan (Arita et al., 2007).

In this study, we generated an equally stable, cold-adapted temperature-sensitive/conditional lethal EV-A71 virus vector (EV71:eTLLβP20) based on the availability of the EV71:TLLβP20 strain and currently known reverse genetic bio-molecular technology. This virus vector was subsequently used to generate equally stable, cold-adapted, temperature-sensitive/conditional lethal strains of EV-A71 carrying the capsid protein gene of EV-A71 subgenogroup C5 (EV71:TLLeC5), and the capsid protein gene of CA16 (TLLeCA16). The materials and methods adopted to generate such virus vector and specified virus strains were fully described in the Supplementary Information.

Schematic diagrams representing the genome structure and respective translated proteins of engineered enteroviruses (EV71:eTLLβP20, EV71:TLLeC5 and TLLeCA16) are shown in Fig. 1. The representative genomic structure and translated proteins of EV71:eTLLβP20 is similar to that of EV71:TLLβP20, except at the nucleotide level where two specific restriction sites (*Mlu*I, *ACGCGT)* and *Eag*I, *CGGCCG)* (red arrows) were engineered into the genome, one in the *VP4* gene near the beginning of protein coding region (nt 770 to 775), and the other in the *2A* gene sequence near the junction with VP1 (nt 3341 to 3346). The nucleotide changes (in lower case, g*CG*atc) at the introduced *Mlu*I site (*ACGCGT*) lead to a serine > valine, (S10V) substitution. Nucleotide changes (in lower case, t*GGCC*a) at the introduced *Eag*I site (*CGGCCG*) lead to a glutamine > arginine, (Q867R) substitution in the viral polyprotein. The introduction of restriction enzyme sites does not lead to a change in the phenotypic characteristics change of the virus as shown in detail below. Both EV71:TLLeC5 and TLLeCA16 retain the same sequence changes at the two introduced specific restriction sites (*Mlu*I and *Eag*I) as EV71:eTLLβP20. The nucleotide sequence of the capsid protein genes (*P1*) of EV71:TLLeC5 and TLLeCA16 were derived entirely from an equivalent region of EV-A71 subgenogroup C5 and CA16 (genogroup B, lineage 2) respectively, as indicated in Fig. 1B and C. The complete genomes of engineered enteroviruses (EV71:eTLLβP20, EV71:TLLeC5 and TLLeCA16), passaged 20 times in Vero cells incubated at 28 °C, were sequenced (GenBank Accession numbers MT241237, MT241238, MT241239).

The genome of EV71:eTLLβP20 has nine nucleotide differences (six were purposefully introduced to create sites for specific restriction endonucleases) from its original source sequence, resulting in three amino acids changes (two due to introduction) (Fig. 1A). EV71:eTLLβP20 has two spontaneous synonymous mutations, one in the *VP1* gene (A2966G) and the other in *2A* gene (C3362A). A non-synonymous spontaneous mutation occurred in the *VP4* gene (C754T) that resulted in serine > leucine (S3L) substitution. The genome of EV71:TLLeC5 differs from its original source sequence by eight nucleotides, resulting in five amino acid substitutions (Fig. 1B). Only two of the nucleotides (C3362A, A5044T) and one of the amino acid changes (asparagine > isoleucine, N1433I) were due to spontaneous mutations. The genome of TLLeCA16 differs from its original source sequence by 14 nucleotides that result in 5 amino acid substitutions (Fig. 1C). Seven

* Corresponding author.
E-mail address: chuakb@tll.org.sg (K.B. Chua).

https://doi.org/10.1016/j.virs.2022.08.001
Received 17 August 2021; Accepted 2 August 2022
Available online 11 August 2022
1995-820X/© 2022 The Authors. Publishing services by Elsevier B.V. on behalf of KeAi Communications Co. Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

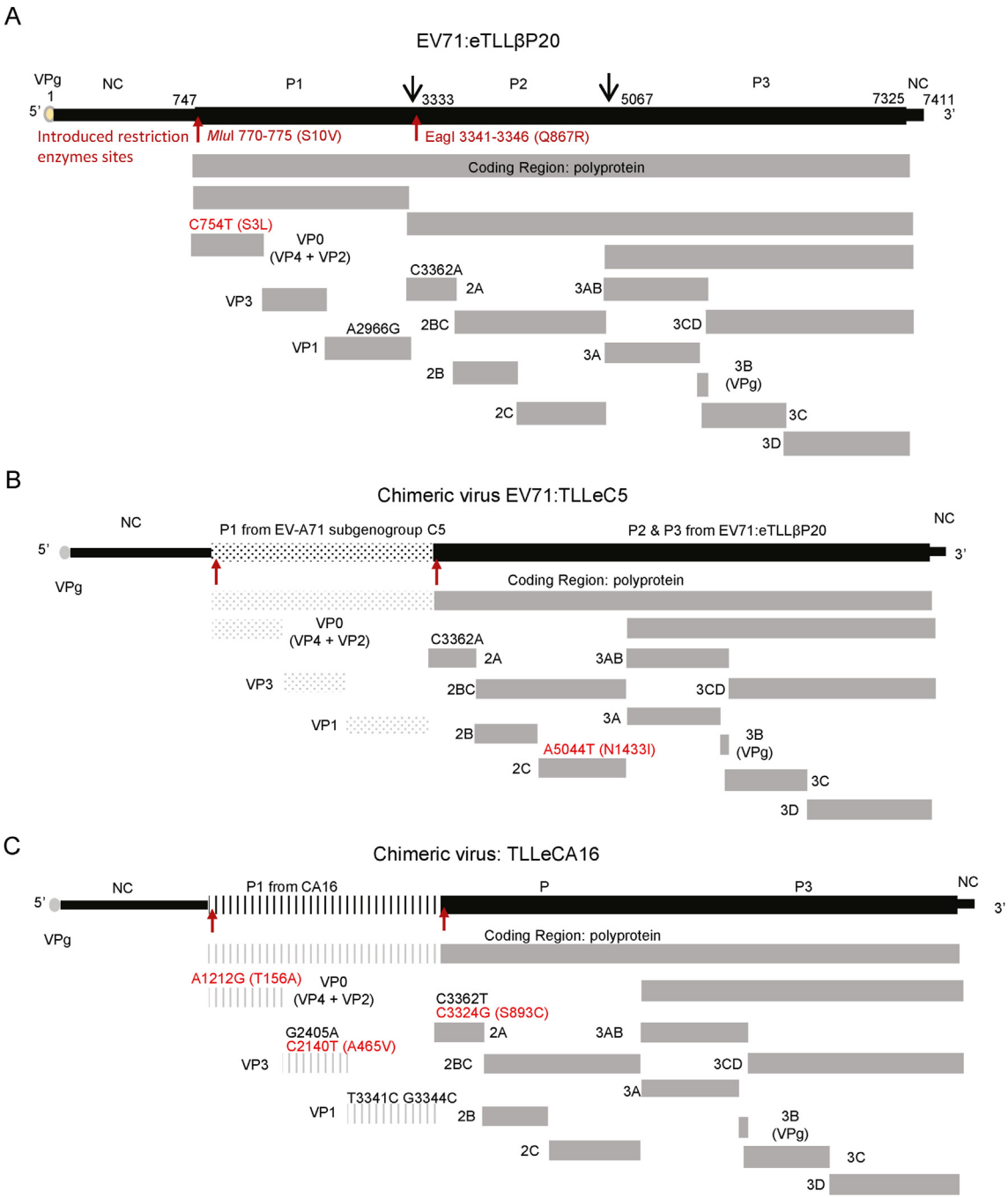


Fig. 1. Schematic diagrams representing the genome structures and respective translated proteins of EV71:eTLLβP20 (**A**), EV71:TLLeC5 (**B**) and TLLeCA16 (**C**). The thicker bars denote the coding regions (P1, P2 and P3) and thinner bars denote the 5′ and 3′ non-coding (NC) regions of the virus genomes. The lighter colour denotes translated polyproteins and respective viral proteins after cleavage. The black arrows indicate the positions of cleavage sites separating P1, P2 and P3 component of the viral polyproteins. The red arrows indicate the sites of introduction of the restriction enzymes (*Mlu*I and *Eag*I). The red lettering and black lettering respectively indicate the type and position of non-synonymous and synonymous mutations that occurred after 20 successive passages in Vero cells incubated at 28 °C.

nucleotide changes were due to spontaneous mutations, resulting in three amino acid changes. One spontaneous nucleotide mutation (A1212G) resulted in a threonine to alanine (T156A) substitution in the *VP2* gene. Two spontaneous mutations (C2140T, G2405A) with C2140T mutation resulting in an amino acid change from alanine to valine (A465V) occurred in the *VP3* gene. Two synonymous spontaneous mutations (T3341C, G3344C) occurred in the *VP1* gene. Two spontaneous mutations (C3362T, C3424G) occurred in the *2A* gene with C3424G mutation resulting in an amino acid change from serine to cysteine (S893C).

The genetically modified (EV71:eTLLβP20) and engineered chimeric (EV71:TLLeC5 and TLLeCA16) enteroviruses retained the same cold-adapted, temperature-sensitive/conditional lethal growth characteristics in Vero cells as their parental strain (EV71:TLLβP20). After 20 passages in Vero cells incubated at 28 °C, EV71:eTLLβP20 took 3 and 7 days to induce full CPE in Vero cells incubated at 28 °C and 37 °C respectively. EV71:TLLeC5 took 3 and 7 days to induce full CPE in Vero cells incubated at 28 °C and 37 °C respectively. TLLeCA16 took 3 and 4 days to induce full CPE in Vero cells incubated at 28 °C and 37 °C respectively. The virus titres of the respective engineered strains in the culture supernatant of

infected Vero cells at full CPE, at culture temperatures of 28 °C, 37 °C and 39.5 °C are shown in Table 1. In summary, all the three engineered cold-adapted strains took fewer days to cause full CPE and achieved higher virus titres in the culture supernatants of infected Vero cells incubated at 28 °C. As with EV71:TLLβP20 (Chua et al., 2021), all the strains were unable to replicate in Vero cells incubated at 39.5 °C, as indicated by the absence of CPE and negative detection of virus antigen in suspended cells by IFA. The absence of viable virus progeny in culture supernatants was confirmed by a lack of CPE and absence of virus antigen after a blind passage into fresh Vero cells incubated at 28 °C, 37 °C and 39.5 °C.

Virus growth kinetics of EV71:eTLLβP20, EV71:TLLeC5 and TLLeCA16 compared to the original wild-type ST and parental EV71:TLLβP20 strains by total viral RNA quantity in the culture supernatant of infected Vero cells incubated at 28 °C, 37 °C and 39.5 °C are shown in Fig. 2A. The results corroborated with earlier findings of virus growth kinetics by cell death leading to full CPE at respective culture temperatures. As with the parental EV71:TLLβP20 strain, viral growth kinetics by RNA load of these engineered cold-adapted strains were significantly different from that of their original wild-type ST strain at culture temperature of 28 °C and 37 °C but not at 39.5 °C (Fig. 2).

Assessment of temperature sensitivity phenotype reversion was performed by six successive passages of the three engineered enteroviruses in Vero cells incubated at 37 °C. The growth characteristics of the virus strains derived from each respective passage in Vero cells incubated at 37 °C, are shown in terms of kinetics of cell death (Fig. 2D) and virus titre (Fig. 2E) at full CPE on incubation at 28 °C and 37 °C. Similar to their parental strain, all the engineered virus strains derived from fourth passage onward were able to cause some cell death at 39.5 °C and some of the suspended cells gave positive immunofluorescence staining (Fig. 2F–d, F–e and F–f), however, none of their culture supernatants were able to cause cell death on re-passaging into fresh Vero cells incubated at 39.5 °C. In addition, the fluorescein signal generated by the infected cells was not as brilliant green in comparison to the positive control (Fig. 2F–a). These findings imply the engineered virus strains were able to infect the Vero cells at incubation temperature of 39.5 °C, leading to some degree of virus translation and cell death, but remained unable to produce viable virus progeny.

Following successive passages at 37 °C, the complete genomes of EV71:eTLLβP20, EV71:TLLeC5 and TLLeCA16 at passage three and six were sequenced and analyzed. The number of nucleotide and corresponding amino acid changes at each respective gene of EV71:eTLLβP20 and EV71:TLLeC5 are shown in Supplementary Tables S3 and S4. At passage three, reversion to the wild-type virus genomic sequence occurred in the EV71:eTLLβP20 *2A* gene at nucleotide position 3346 (G3346A), leading to substitution from arginine to glutamine (R867Q). This reversion was maintained at passage six. In addition, a deletion of 15 nucleotides, leading to deletion of five amino acids and a change of asparagine to histidine at position 667 (N667H), occurred in 58% (7/12) of the *VP1* genes (Supplementary Table S3). Interestingly, the same reversion at nucleotide position 3346 (G3346A, R867Q) of the *2A* gene occurred in the consensus genomic sequence of EV71:TLLeC5 at both passage three and six. In addition to this reversion, the genomic sequence of EV71:TLLeC5, at both passage three and six, had a spontaneous mutation in the viral *2C* gene (C4566T) leading to a histidine to tyrosine substitution (H1274Y) (Supplementary Table S4). No mutation was detected in the consensus genomic sequence of TLLeCA16 at both passage three and six, although a mixed population of either guanine (G) or adenine (A) occurred at nucleotide position 1212 on sequencing its complete genome. The nucleotide substitution is synonymous.

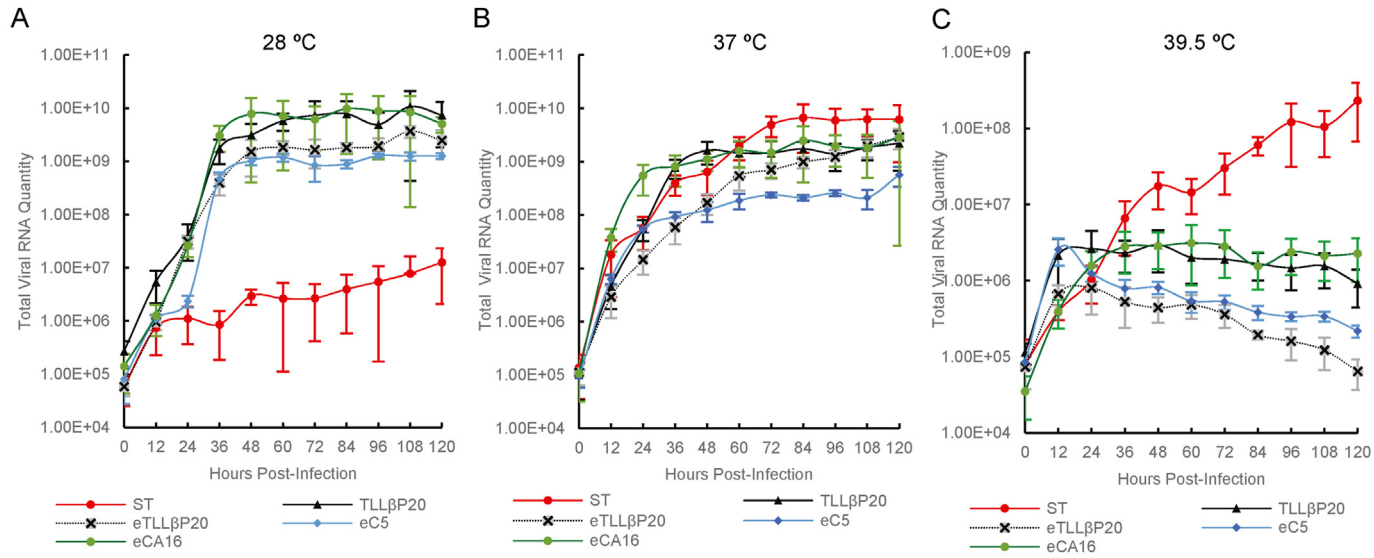
Table 1
The virus titres of the respective engineered virus strains in the culture supernatant of infected Vero cells at full CPE, at culture temperatures of 28 °C, 37 °C and 39.5 °C.

Engineered virus strain	Virus titre ($CCID_{50}$) attained at culture temperature		
	28 °C	37 °C	39.5 °C
EV71:eTLLP20	2.15×10^7	4.65×10^6	No virus titre
EV71:TLLeC5	2.15×10^8	2.15×10^7	No virus titre
TLLeCA16	4.64×10^7	2.15×10^6	No virus titre

Viral vectors allow the development of chimeric strains that can help to improve our understanding of viral infection and rapid investigation of any new variants that may arise. Based on extensive previous work on polioviruses and other enteroviruses, the process of enterovirus protein translation and genome replication is known to be closely coupled. The viral polyprotein is co-translationally cleaved into multiple individual structural and non-structural proteins of various distinct functions. This continuum process is regulated, co-ordinated and executed by the viral 5′ (Cloverleaf and IRES) and 3′ non-coding sequences, as well as non-structural proteins, to bring about the simultaneous recruitment of specific host cell proteins to form the virus replication machinery (Hellen and Wimmer, 2005; Johnson and Sirnow, 2005; Haller and Semler, 2005). Armed with this understanding, and existing powerful biomolecular techniques, we created the enterovirus vector containing restriction enzymes sites (*Mlu*I and *Eag*I) sequences engineered into the EV71:TLLβP20 genome flanking the viral capsid region (P1). EV71:eTLLβP20 behaves phenotypically as EV71:TLLβP20, suggesting the stability of the virus replication machinery was not perturbed in the process. The same virus vector was used to generate chimeric enteroviruses separately expressing the capsid proteins of EV-A71 sub-genogroup C5 and CA16. However, the initial generated chimeric enteroviruses were noted to be unstable, which progressively adapted with better ability to replicate at 28 °C and reduced ability to replicate at 37 °C (data not shown), akin to a cold-adapted EV71:TLLα strain described in Chua et al. (2021). The stability was restored by converting the P1-2A motif sequences of EV-A71 subgenogroup C5 (**A**ITTLG) and CA16 (**K**ITTLG) to that of EV71:TLLβP20 (**T**ITTLG) through site-directed mutagenesis as described in the respective subsections of Materials and Method. It is interesting to note that a mere amino acid difference at crucial point of viral genome can lead to destabilization of viral function. Thus, the change of alanine (A) or lysine (K) to threonine (T) returns the genetic and phenotypic stability of the chimeric enteroviruses. The "TITTL" amino acid sequence motif is situated and formed part of the cleavage site between VP1 (structural protein) and 2A (non-structural protein) which is self-cleaved during the process of translation.

In the temperature reversion study, although all the engineered virus strains derived from the fourth passage onward were able to cause some cell death at 39.5 °C but none of their culture supernatants were able to cause cell death on re-passaging into fresh Vero cells incubated at 39.5 °C. This finding implies the three engineered enteroviruses were still unable to produce viable virus progeny in Vero cells incubated at 39.5 °C following six successive passaging in Vero cells incubated at 37 °C. Six unique mutations that are highly conserved that may have contributed to the phenotypic and genetic stability and temperature sensitive/conditional lethal characteristics of the parental virus strain was previously discussed (Chua et al., 2021). The six unique highly conserved mutations were also retained in the three genetic-engineered chimeric enteroviruses. In comparison to polioviruses of Sabin oral vaccine (GlaxoSmithKline, London, UK; A0PVB326BC), having only 1 to 3 unique conserved mutations (depending on the serotypes) which contributed to their attenuated phenotypes but also ease of reversion to their wild-types (Hellen and Wimmer, 2005; Sutter et al., 2012), the presence of a combination of the six unique highly conserved mutations of these three engineered chimeric enteroviruses may have contributed their difficulty of reversion to wild-type (ST) strain.

Numerous attempts were also undertaken to generate a chimeric enterovirus expressing the capsid protein of an attenuated poliovirus 1, derived from an oral Sabin poliovirus vaccine (Mcgoldrick et al., 1995). Although the plasmids carrying the chimeric cDNA clone of EV71:eTLLβP20, expressing the P1 region of poliovirus 1, gave positive immunofluorescent staining of poliovirus 1 antigen, following



Culture Temperature	EV71:TLLβP20		EV71:eTLLβP20		EV71:TLLeC5		TLLeCA16	
	t-value	*P*-value	t-value	*P*-value	t-value	*P*-value	t-value	*P*-value
28 °C	-13.177	0.000	-10.503	0.000	-20.698	0.000	-15.906	0.000
37 °C	2.985	0.031	2.778	0.027	3.989	0.010	2.573	0.042
39.5 °C	1.600	0.171	1.617	0.167	1.615	0.167	1.584	0.174

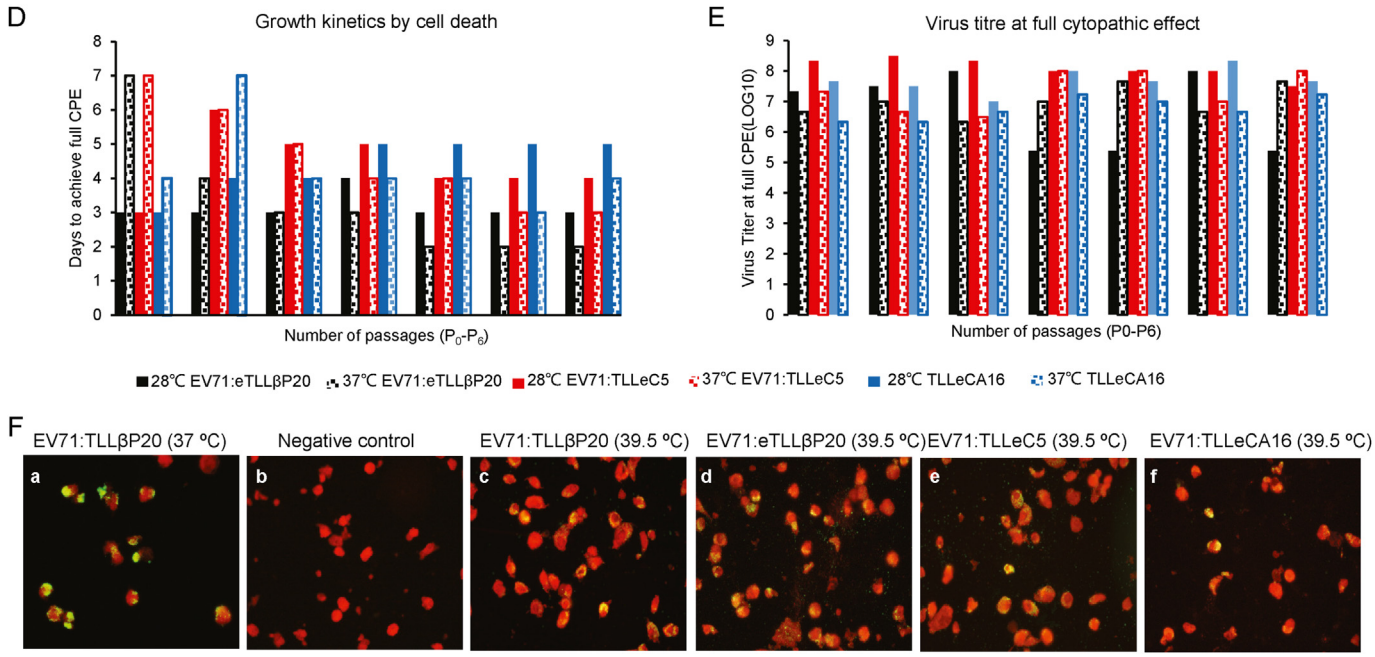


Fig. 2. **A–C** Virus growth kinetics of EV71:TLLβP20, EV71:eTLLβP20, EV71:TLLeC5 and TLLeCA16 in comparison with the original wild-type ST strain by total viral RNA quantity in the culture supernatant of infected Vero cells incubated at 28 °C (**A**), 37 °C (**B**) and 39.5 °C (**C**). **D–E** Assessment of temperature reversion growth characteristics of EV71:eTLLβP20 (black bar), EV71:TLLeC5 (red bar) and TLLeCA16 (blue bar) following six successive passages in Vero cells incubated at 37 °C. Growth characteristics of the virus derived from each successive culture at 37 °C in terms of virus growth kinetics by cell death (**D**) and virus titre in $CCID_{50}$/mL at full cytopathic effect (CPE) (**E**) at incubation temperature of 28 °C (solid bar) and 37 °C (dotted bar). **F** A composite photograph showing Vero cells incubated at 39.5 °C and infected with EV71:TLLβP20 (**F-c**), EV71:eTLLβP20 (**F-d**), EV71:TLLeC5 (**F-e**) and TLLeCA16 (**F-f**) after six successive passages at incubation temperature of 37 °C. EV71:TLLβP20-infected Vero cells incubated at 37 °C served as positive control (**F-a**). Non-infected Vero cells served as negative control (**F-b**). The infected cells were stained with fluorescein-tagged monoclonal antibody specific for EV-A71 and examined under UV-microscopy (×40).

transfection into both Vero and RD cells, no infectious virus progeny were recovered. The failure to recover viable virus progeny is most likely due to the incompatibility of interaction between the EV-A71 (*Enterovirus* A species) 2C and the poliovirus 1 (*Enterovirus* C species) VP3 protein (Liu et al., 2010). It will be of great interest to explore whether EV71:eTLLβP20 can be successfully utilised to generate chimeric enteroviruses expressing capsid proteins of all known enteroviruses of *Enterovirus A* species.

Footnotes

We thank Dr. Peter McMinn (University of Sydney, New South Wales) for his generosity in providing the pCMV-T7pol plasmid that was used for production of clone-derived viruses. We are grateful to Dr. Greig Jamie Alan (Temasek Lifesciences Laboratory) for his contribution in proof-reading and editing the manuscript. This research was funded by the Temasek Lifesciences Laboratory core funding. The virus strains as mentioned in the manuscript and use of the strains, including usage as a vector are the subject matter of several patent applications by Temasek Lifesciences Laboratory. The funder had no role in the design and execution of the experiment. The authors declare that they have no conflict of interest.

The data on viral RNA load by qRT-PCR of EV71:TLLβP20 and wild-type ST strains previously published (Chua et al., 2021) were reused for comparison.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2022.08.001.

References

Alexander, J.P., Baden, L., Pallansch, M.A., Anderson, L.J., 1994. Enterovirus 71 infection and neurologic disease – United States, 1977-1991. J. Infect. Dis. 169, 905–908.

Arita, M., Nagata, N., Iwata, N., Ami, Y., Suzaki, Y., Mizuta, K., Iwasaki, T., Sata, T., Wakita, T., Shimizu, H., 2007. An attenuated strain of enterovirus 71 belonging to genotype A showed a broad spectrum of antigenicity with attenuated neurovirulence in Cynomolgus monkeys. J. Virol. 81, 9386–9395.

Chang, L.Y., Huang, Y.C., Lin, T.Y., 1998. Fulminant neurogenic pulmonary oedema with hand, foot and mouth disease. Lancet 352, 367–368.

Chomnaitree, T., Menegus, M.A., Schervish-swierkosz, E.M., Schwalenstocker, E., 1982. Enterovirus 71 infection: a report of an outbreak with two cases of paralysis and a review of the literature. Pediatrics 671, 489–493.

Chua, K.B., Ng, Q., Meng, T., Qiang, J., 2021. Safety and immunogenicity of a stable, cold-adapted, temperature-sensitive/conditional lethal enterovirus A71 in monkey study. Viruses 13, 438.

Gilbert, G.L., Dickson, K.E., Waters, M.J., Kennett, M.L., Land, S.A., Sneddon, M., 1988. Outbreak of enterovirus 71 infection in Victoria, Australia, with a high incidence of neurologic involvement. Pediatr. Infect. Dis. 7, 484–488.

Haller, A.A., Semler, B.L., 2005. Translation and host cell shutoff. In: Rotbart, H.A. (Ed.), Human Enterovirus Infections. ASM Press, Washington DC, pp. 113–133 (Chapter 5).

Hayward, J.C., Gillespie, S.M., Kaplan, K.M., Packer, R., Pallansch, M., Plotkin, S., Schonberger, L.B., 1989. Outbreak of poliomyelitis-like paralysis associated with enterovirus 71. Pediatr. Infect. Dis. 8, 611–616.

Hellen, C.U.T., Wimmer, E., 2005. Enterovirus genetics. In: Rotbart, H.A. (Ed.), Human Enterovirus Infections. ASM Press, Washington DC, pp. 25–72 (Chapter 2).

Ishimaru, Y., Nakano, S., Yamaoka, K., Takami, S., 1980. Outbreaks of hand, foot, mouth disease by enterovirus 71: high incidence of complication disorders of central nervous system. Arch. Dis. Child. 55, 583–588.

Johnson, K.L., Sarnow, P., 2005. Viral RNA synthesis. In: Rotbart, H.A. (Ed.), Human Enterovirus Infections. ASM Press, Washington DC, pp. 95–112 (Chapter 4).

Liu, C.C., Tseng, H.W., Wang, S.M., Wang, J.R., Su, I.J., 2000. An outbreak of enteovirus 71 infection in Taiwan, 1998: epidemiologic and clinical manifestations. J. Clin. Virol. 17, 23–30.

Liu, Y., Wang, C., Mueller, S., Paul, A.V., Wimmer, E., Jiang, P., 2010. Direct interaction between two viral proteins, the nonstructural protein $2C^{ATPase}$ and the capsid protein VP3, is required for enterovirus morphogenesis. PLoS Pathog. 6, e1001066, 1–14.

Lum, L.C.S., Wong, K.T., Lam, S.K., Chua, K.B., Goh, A.Y.T., Lim, W.L., Ong, B.B., Paul, G., AbuBakar, S., Lambert, M., 1998. Fatal enterovirus 71 encephalitis. J. Pediatr. 133, 795–798.

Mcgoldrick, A., Macadam, A.J., Dunn, G., Rowe, A., Burlison, J., Minor, P.D., Meredith, J., Evans, D.J., Almond, J.W., 1995. Role of mutations G-480 and C-6203 in the attenuation phenotype of Sabin type 1 poliovirus. J. Virol. 69, 7601–7605.

Melnick, J.L., 1996. Enteroviruses: polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses. In: Fields, B.N., Knipe, D.M., Howley, P.M., et al. (Eds.), Fields Virology, third ed. Lippincott-Raven, Philadelphia, pp. 655–712.

Samuda, G.M., Chang, W.K., Yeung, C.Y., Tang, P.S., 1987. Monoplegia caused by enterovirus 71: an outbreak in Hong Kong. Pediatr. Infect. Dis. 6, 206–208.

Sutter, R.W., Kew, O.M., Cochi, S.L., Aylward, R.B., 2012. In: Plotkin, S.A., Orenstein, W., Offit, P.A. (Eds.), Poliovirus Vaccine-Live, Vaccines sixth ed. Elsevier Health Sciences, pp. 598–645. Section 2, (Chapter 28).

Tagaya, I., Takayama, R., Hagiwara, A., 1981. A large scale epidemic of hand, foot, mouth disease associated with enterovirus 71 infection in Japan in 1978. Jpn. J. Med. Sci. Biol. 34, 191–196.

Yang, F., Zhang, T., Hu, Y., Wang, X., Du, J., Li, Y., Sun, S., Sun, X., Li, Z., Jin, Q., 2011. Survey of enterovirus infections from hand, foot and mouth disease outbreak in China, 2009. Virol. J. 8, 508.

Zeng, M., El Khatib, N.F., Tu, S., Ren, P., Xu, S., Zhu, Q., Mo, X., Pu, D., Wang, X., Altmeyer, R., 2012. Seroepidemiology of enterovirus 71 infection prior to the 2011 season in children in Shanghai. J. Clin. Virol. 53, 285–289.