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., 1991; Choamnaitre 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 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

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

Contents lists available at ScienceDirect

Virologica Sinica

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

© 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/).
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:TLLiP20 (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:eTLLiP20, EV71:TLLeCS and TLLeCA16 compared to the original wild-type ST and parental EV71:TLLiP20 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:TLLiP20 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:eTLLiP20, EV71:TLLeCS 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:eTLLiP20 and EV71:TLLeCS are shown in Supplementary Tables S3 and S4. At passage three, reversion to the wild-type virus genomic sequence occurred in the EV71:eTLLiP20 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:TLLeCS at both passage three and six. In addition to this reversion, the genomic sequence of EV71:TLLeCS, 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).

Table 1

| Engineered virus strain | Virus titre (CCID50) attained at culture temperature | 28 °C | 37 °C | 39.5 °C |
|-------------------------|-----------------------------------------------|-------|-------|--------|
| EV71:eTLLiP20          | 2.15 × 10⁶                                  | 4.65 × 10⁶ | No virus titre |
| EV71:TLLeCS            | 2.15 × 10⁶                                  | 2.15 × 10⁶ | No virus titre |
| TLLeCA16               | 4.64 × 10⁶                                  | 2.15 × 10⁶ | No virus titre |

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:eTLLiP20, expressing the P1 region of poliovirus 1, gave positive immunofluorescent staining of poliovirus 1 antigen, following
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.

**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 CCID50/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 (x40).
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., Iwasa, 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-swiwiorza, 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 67, 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. 6, 611–616.
Heilen, C.U.T., Wimmer, E., 2005. Enterovirus genotypes. 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 enterovirus 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 2CNS 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., 1996. Fatal enterovirus 71 encephalitis. J. Pediatr. 135, 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, T., 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., Xu, S., Ren, P., Xu, S., Zhu, Q., Mo, X., Pu, D., Wang, X., Altmeyer, R., 2012. Seroprevalence of enterovirus 71 infection prior to the 2011 season in children in Shanghai. J. Clin. Virol. 53, 285–289.