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Short communication

Construction and characterization of an infectious clone of coxsackievirus A6 that showed high virulence in neonatal mice

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ABSTRACT

Atypical hand, foot, and mouth disease (aHFMD) outbreaks have been frequently reported worldwide in recent years. It is believed that coxsackievirus A6 (CA6) is the major pathogen for aHFMD. Studies regarding CA6 infection are limited and the genetic mechanism for the high pathogenicity of some new CA6 variants is still unclear. Infectious clones are powerful tools for studying the genetic mechanisms of RNA viruses. In this study, we describe the construction of a full-length cDNA clone of CA6 strain TW-2007-00141. The whole genome of CA6 was amplified in a single step and ligated into a plasmid vector through an efficient cloning method, Gibson assembly. The whole genome sequence of CA6 strain TW-2007-00141 was determined and phylogenetic analysis indicated that it shared a high degree of similarity (≥94%) with the CA6 strains found in Taiwan in 2009. The infectious clone of CA6 viruses were recovered by transfection into 293FT cells and showed similar biological properties to the parental virus. Viral particles were purified by CsCl isopycnic centrifugation, and two types of viral particles were observed under transmission electron microscopy. The rescued virus showed high virulence in one-day-old suckling mice. This clone may be useful for establishing animal models for the evaluation of CA6 vaccine efficiency in future.

Hand, foot, and mouth disease (HFMD) is a common contagious childhood illness around the world. Coxsackievirus A16 (CA16) and enterovirus 71 (EV71) are the most common HFMD pathogens (Chen et al., 2007; McMinn et al., 2001). Other enteroviruses, such as coxsackievirus A6 (CA6) and coxsackievirus A10 (CA10), can also be associated with the disease (Blomqvist et al., 2010; Lu et al., 2012). Recently, some CA6-related HFMD outbreaks have been reported in Asia, Europe and North America (reviewed by Feder et al. (2014)). Clinical data indicating that CA6 infections can affect adults and tend to cause a more severe course of the disease when compared to classic HFMD cases (Feder et al., 2014; Ramirez-Fort et al., 2014). Additionally, CA6 infections can also cause herpangina and onychomadesis during convalescence (Guimbao et al., 2010; Wei et al., 2011).

CA6 belongs to the genus *Enterovirus* in the family Picornaviridae. Its genome is a positive single-stranded RNA approximately 7400 nucleotides long and has a single open reading frame (ORF) that is flanked by untranslated regions (UTRs) at the 5′ and 3′ ends. Infectious cDNA clone is a typical tool for genetic manipulation of the genome of RNA viruses. Infectious cDNA clones of many enteroviruses have been constructed (reviewed by Hou et al. (2015)), including EV71 and CA16 (Arita et al., 2005; Liu et al., 2011). The infectious cDNA clones of EV71 have been widely used to study the functions of viral genes (Kung et al., 2010; Shih et al., 2004), viral infection (Chen et al., 2012; Cordey et al., 2012; Yamayoshi et al., 2009), viral replication (Sadeghipour et al., 2013; Tang et al., 2007), and vaccine development (Meng et al., 2011; Wang et al., 2013). Due to the significant role of infectious clones, it is necessary to

Abbreviations: HFMD, Hand, foot, and mouth disease; FITC, Fluorescein isothiocyanate; ORF, Open reading frame; RD, Rhabdomyosarcoma cell; $TCID_{50}$, 50% tissue culture infectious doses; TEM, Transmission electron microscopy; UTR, Untranslated region.

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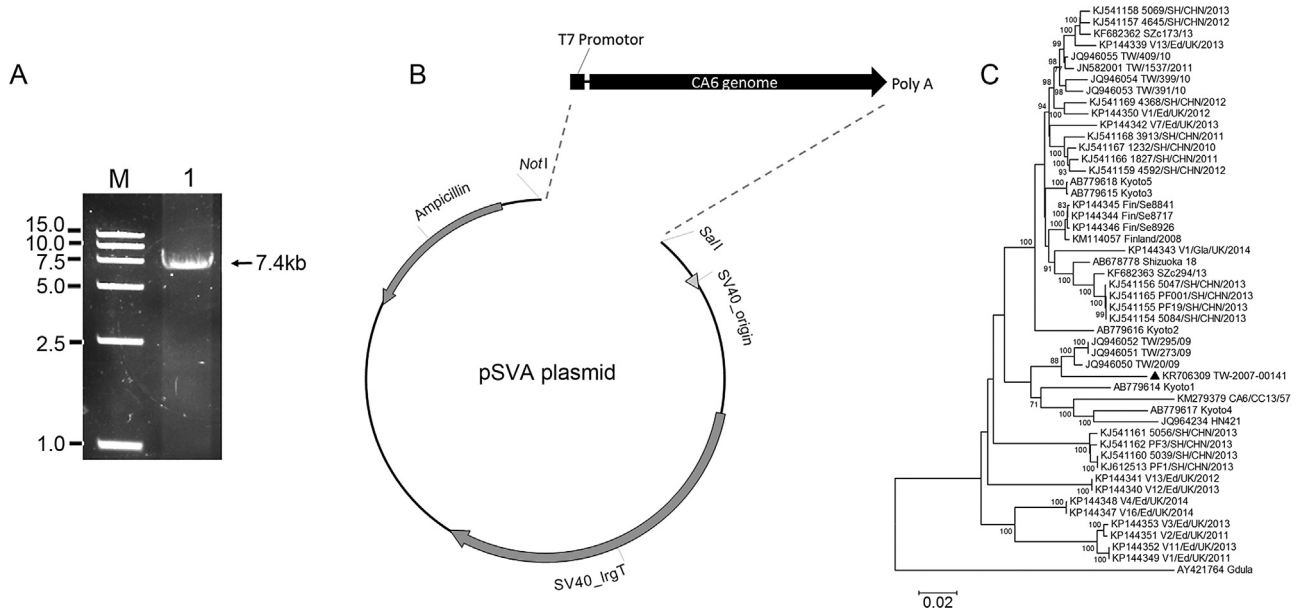

Fig. 1. Construction of a full-length infectious clone of CA6. (A) Amplification of the whole genome of CA6 strain TW-2007-00141. Lane M is the DL15,000 DNA marker (TaKaRa Biotechnology, Dalian, China); Lane 1 shows the PCR amplification products of the CA6 genome with the Not1-T7-5UTR and Sal1-PloyT-3UTR primers. (B) Schematic representation of construction of the infectious clone of CA6. (C) Phylogenetic analysis of 50 whole genome sequences of CA6. The phylogenetic tree of CA6 was constructed using a neighbor-joining method using the MEGA version 5 software with 1000 replications of the bootstrap analyses. Bootstrap values over 70% are shown at the branch nodes. Genbank accession numbers and the name of strain are included. The scale bar indicates branch distances; the sequence of CA6 strain TW-2007-00141 is labeled with a triangle.

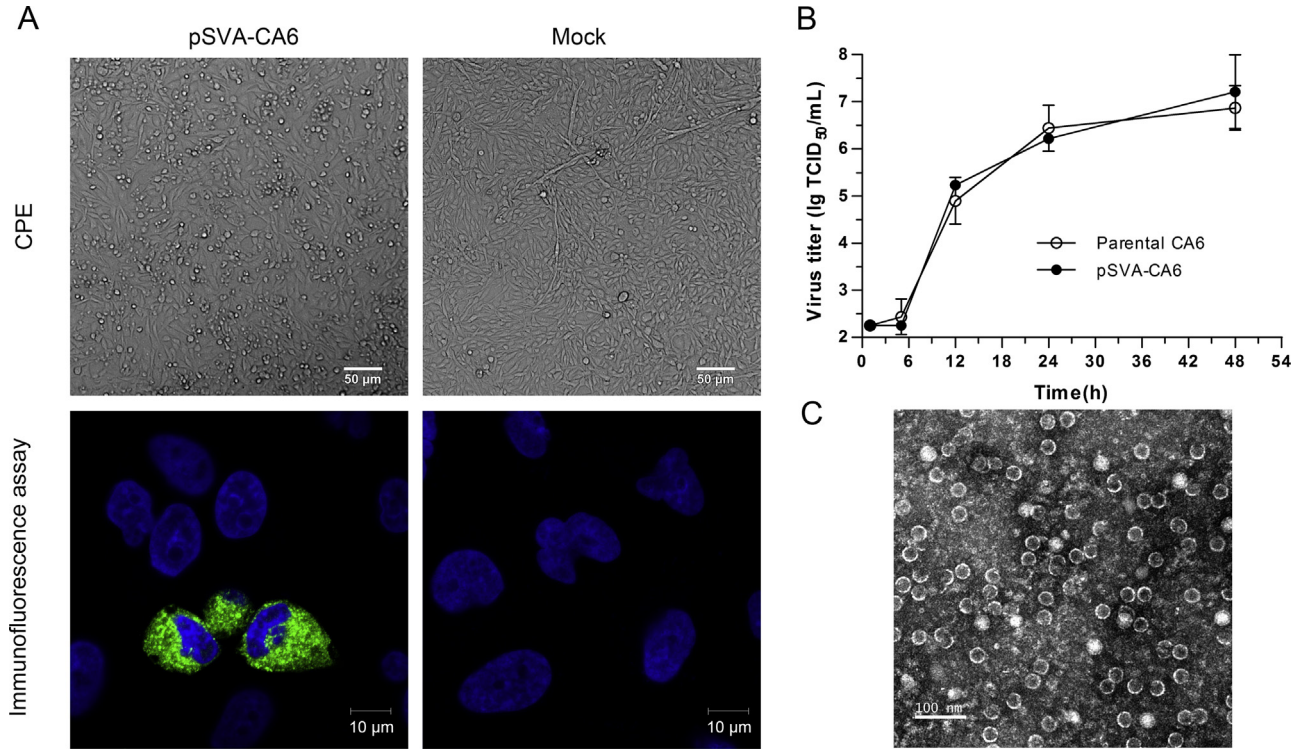

Fig. 2. Rescue and characterization of infectious CA6 from the cDNA clone. (A) Cytopathic effects displayed in RD cells infected with the rescued viruses and immunofluorescence assay. (B) One-step growth curves of the rescued and parental CA6 viruses on RD cells. RD cells were seeded in 24-well plates and infected with CA6 virus at 2000 $TCID_{50}$ per well. The cells and the supernatants were collected at different time points and titrated using a $TCID_{50}$ assay. At each time point, titer values are means of the six samples; error bars represent the standard deviation. (C) Electron microscopy examination of the CA6 particles. The scale bar represents 100 nm.

construct an infectious clone of CA6 for relevant basic research; however, there is still no relevant report about an infectious clone for CA6.

In this study, we describe the construction of a full-length cDNA clone of CA6 strain TW-2007-00141 (GenBank accession number: KR706309). The overall strategy to construct the infectious clone of CA6 strain TW-2007-00141 is shown in Fig. 1. Viral RNA was extracted from the infected RD cells using the TRIzol Reagent (Invitrogen, NY, USA) and subjected to reverse transcription using oligo(dT) primers and the highly efficient reverse