

DEVELOPMENT OF STABLE COLD-ADAPTED TEMPERATURE SENSITIVE CHIMERIC ENTEROVIRUSES

CROSS-REFERENCE TO RELATED APPLICATION

[0001] The present application is related to and claims priority to U.S. provisional patent application Serial No. 62/011,406 filed 12 June 2014. This application is incorporated herein by reference.

SEQUENCE SUBMISSION

[0002] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is entitled 2577236PCTSequenceListing.txt, created on 23 April 2015 and is 62 kb in size. The information in the electronic format of the Sequence Listing is incorporated herein by reference in their entirety.

BACKGROUND OF THE INVENTION

[0003] The present invention relates to chimeric virus strains, particularly to the stable cold-adapted temperature sensitive Enterovirus 71 strains EV71:eTLL β P20 and EV71:TLL ϵ C5 and to the stable chimeric enterovirus strain TLL ϵ CA16. The present invention also relates to vectors containing the nucleotide sequences of these strains and vaccines containing these strains.

[0004] The publications and other materials used herein to illuminate the background of the invention or provide additional details respecting the practice, are incorporated by reference, and for convenience are respectively grouped in the Bibliography.

[0005] Hand foot and mouth disease (HFMD) is a febrile sickness complex characterized by cutaneous eruption (exanthem) on the palms and soles of hands and feet with simultaneous occurrence of muco-cutaneous vesiculo-ulcerative lesions (enanthem) affecting the mouth. The illness is caused by a number of enteroviruses with coxsackievirus A16 (CA16) and enterovirus 71 (EV71) as the main causative agents (1). Besides HFMD, EV71 has been associated with a spectrum of other clinical diseases including non-specific febrile illness, acute infantile respiratory infections, aseptic meningitis, encephalitis and poliomyelitis-like acute flaccid paralysis (1-3). As with other enteroviruses, EV71 and CA16 are small non-enveloped viruses of 28-30 nm in diameter. The viral capsid is of icosahedral symmetry and composes of 60 identical units (protomers), with each unit consisting of one copy of the four viral structural proteins VP1-VP4. The capsid surrounds a core of a single-stranded positive-sense RNA genome of about 7,450 nucleotides (nts). The virus genome consists of a single open reading frame which

encodes a polyprotein of about 2200 amino acids and is flanked by a long non-translated region of about 750 nts at its 5' end and a short non-translated region of about 85 nts at its 3' end with a variable length of poly-A tract at its 3' terminus. Human enterovirus 71 (HEV71) is classified as *Human Enterovirus A* species under the genus *Enterovirus* within the family *Picornaviridae* (2, 3). EV71 is divided into three major genogroups (A, B, and C), and genogroups B and C are further subdivided into genotypes B1 to B5 and C1 to C5 respectively, based on phylogenetic analysis of its major capsid protein (VP1) gene (4).

[0006] There is an increase in activity and virulence of EV71 in the last 15 years, especially within the Asia-Pacific region, causing severe outbreaks of HFMD and affecting central nervous system with neurological sequelae and mortalities especially in young children. Currently, there is no specific anti-viral drug for treatment of diseases due to EV71 nor is there any effective vaccine for the control and prevention of infections. The success of live attenuated oral and inactivated parental poliovirus vaccine in preventing poliomyelitis indicates the potential for preventing illnesses due to EV71 by vaccination (28). Many previous attempts to develop EV71 vaccine have been reported and some have even reached various stages of clinical trials (29-49). However, several challenges need to be addressed for an efficacious vaccine to control and prevent outbreak of HFMD (50-52). Firstly, HFMD is caused by a number of enteroviruses with EV71 and CA16 as the main causative agents especially in outbreak situations (5-27). A recent published study by Chou et al (2012) has confirmed that antibodies raised against EV71 will not cross protect against infections by CA16 and vice versa (50). A polyvalent vaccine targeted against both EV71 and CA16 is highly needed to prevent outbreak of HFMD due to these two main causative agents. Secondly, EV71 is found to evolve quickly in the past 15 years and more than one genotypes of EV71 are known to co-circulate and cause outbreaks in various part of the world. Cross-protective immunity between various genotypes after infection with a specific genotype has been found to be non-homogenous and candidate EV71 vaccine needs to address this issue in order to provide sufficient cross-protection against all known circulating genotypes.

[0007] Recently a stable cold-adapted temperature sensitive attenuated strain of EV71, EV71:TLL β P20 has been developed. See international application number PCT/SG2013/000027 filed on 18 January 2013, incorporated herein by reference in its entirety. The virus will not replicate in culture cells incubated at temperature of 39.5° C (human body high grade fever temperature) even when the culture cells were inoculated with virus inoculum of high multiplicity of infection (m.o.i). EV71:TLL β P20 is both phenotypically and genetically stable under defined culture conditions. The stability was ascertained by multiple passages in

temperature reversion study. The safety, immunogenicity and effectiveness (neutralization antibodies) of this potential candidate EV71 vaccine has recently been confirmed in monkey's study. However, the neutralizing antibodies induced by EV71:TLL β P20 in the monkey study showed high neutralizing antibody titre against genotypes of EV71 within similar genogroup but of low neutralizing titre against genotypes belonging to a different genogroup. The finding confirmed previous published findings of monkey study carried out in National Institute of Infectious Disease (NIID), Japan which showed that neutralizing antibodies induced by one genotype conferred high neutralizing antibody titre against EV71 strains of homologous genogroup but of lower neutralizing titre against strains belonging to heterologous genogroup (32).

[0008] Thus, there is a need to develop chimeric virus strains that will be useful for the development of vaccines to HFMD due to EV71 and/or CA16.

SUMMARY OF THE INVENTION

[0009] The present invention relates to chimeric virus strains, particularly to the stable cold-adapted temperature sensitive Enterovirus 71 strains EV71:eTLL β P20 and EV71:TLLcC5 and to the stable chimeric enterovirus strain TLLcCA16, as well as inactivated forms thereof. The present invention also relates to vectors containing the nucleotide sequences of these strains and vaccines containing these strains.

[0010] Thus, in one aspect, the present invention provides stable cold-adapted temperature sensitive Enterovirus 71 strains and stable chimeric enterovirus strains, and inactivated forms thereof. In one embodiment, the stable cold-adapted temperature sensitive Enterovirus 71 strain is EV71:eTLL β P20 as described herein. In another embodiment, the stable cold-adapted temperature sensitive Enterovirus 71 strain is EV71:TLLcC5 as described herein, which carries the capsid protein of EV71 genotype C5. In an additional embodiment, the stable chimeric enterovirus strain is TLLcCA16 as described herein, which carries the capsid protein of CA16.

[0011] In a second aspect, the present invention provides a composition comprising one or more of the Enterovirus 71 strains and chimeric enterovirus strains described herein, alone or in combination with the parent stable cold-adapted temperature sensitive Enterovirus 71 strain EVL:TLL β P20 described in international application number PCT/SG2013/000027. As used in this context, "alone" means that the composition comprises one or more of the virus strains EV71:eTLL β P20, EV71:TLLcC5 and TLLcCA16. Also as used in this context, "in combination with" means that the composition comprises one or more of the virus strains EV71:eTLL β P20,

EV71:TLLcC5 and TLLcCA16 and the parent EV71:eTLL β P20. In one embodiment, the composition comprises an effective amount of one or more of the virus strains described herein, alone or in combination with the parent EVL:TLL β P20. In some embodiments, one, some or all of the strains may be inactivated forms of the virus strain. In another embodiment, the composition comprises one or more physiologically or pharmaceutically acceptable carriers. In a further embodiment, the composition is a vaccine. Vaccines containing one or more of the virus strains described herein, alone or in combination with the parent EVL:TLL β P20, are prepared using techniques well known to the skilled artisan. As used in this context, "alone" means that the vaccine comprises one or more of the virus strains EV71:eTLL β P20, EV71:TLLcC5 and TLLcCA16. Also as used in this context, "in combination with" means that the vaccine comprises one or more of the virus strains EV71:eTLL β P20, EV71:TLLcC5 and TLLcCA16 and the parent EV71:eTLL β P20. In one embodiment, the vaccine contains live strain(s). In another embodiment, the live strain(s) are attenuated. In an additional embodiment, the vaccine contains inactivated strain(s). In a further embodiment, the vaccine may be an oral vaccine. Such vaccines are useful for providing immunity against the parent virus strains by administering the vaccine to a subject, such as a human subject, using techniques well known to the skilled artisan.

[0012] In a third aspect, the present invention provides a method of eliciting a protective immune response in a subject, such as a human subject, which comprising administering to a subject a prophylactically or therapeutically or immunologically effective amount of one or more of the virus strains described herein, alone or in combination with the parent EVL:TLL β P20. In one embodiment, the protective immune response protects the subject against a disease caused by Enterovirus 71 and/or Coxsackievirus CA16. In one embodiment, the disease is hand, foot and mouth disease. In another embodiment, the disease is aseptic meningitis. In an additional embodiment, the disease is encephalitis. In a further embodiment, the disease is poliomyelitis-like paralysis. In one embodiment, one or more of the virus strains described herein, alone or in combination with the parent EVL:TLL β P20, is administered as a vaccine. In an additional embodiment, the subject has been exposed to wild-type Enterovirus 71 and/or Coxsackievirus CA16. In another embodiment, the administration of one or more of the virus strains described herein, alone or in combination with the parent EVL:TLL β P20, prevents a subject, such as a human subject, from becoming afflicted with an Enterovirus 71-associated disease and/or Coxsackievirus CA16-associated disease. In an additional embodiment, the subject has been exposed to wild-type Enterovirus 71 and/or Coxsackievirus CA16. In a further

embodiment, the administration of one or more of the virus strains described herein, alone or in combination with the parent EVL:TLL β P20, delays the onset of or slows the rate of progression of an Enterovirus 71-associated disease and/or Coxsackievirus CA16-associated disease in a virus-infected subject, such as a human subject. In some embodiments, one, some or all of the strains are inactivated.

[0013] In a fourth aspect, the present invention provides vaccine technology associated with the virus strains described herein. In one embodiment, the virus strains described herein are used in a method of making a vaccine. In another embodiment, a nucleic acid comprising the nucleotide sequence set forth in SEQ ID NO:84, SEQ ID NO:86, SEQ ID NO:88 or SEQ ID NO:83 are used in a method of making a vaccine. In an additional embodiment, the Enterovirus 71 strains described herein, or inactivated forms thereof, are used for vaccine development. In a further embodiment, a nucleic acid comprising the nucleotide sequence set forth in SEQ ID NO:84, SEQ ID NO:86, SEQ ID NO:88 or SEQ ID NO:83 are used for vaccine development.

BRIEF DESCRIPTION OF THE FIGURES

[0014] Figure 1 shows a schematic diagram representing the genome structure and respective encoded proteins of engineered stable cold-adapted temperature sensitive EV71:eTLL β P20. The thick black bar denotes the coding regions (P1, P2, and P3) of the virus genome and thinner bars denote the 5' and 3' non-coding (NC) regions of the virus genome. The grey color denotes the translated polyprotein and the respective viral proteins after cleavage.

[0015] Figures 2a and 2b show a schematic diagram representing the genome structure of EV71:eTLL β P20 and EV71 of genotype C. Figure 2a represents the genome structure of EV71:eTLL β P20 (black color) and translated polyprotein (grey color). Figure 2b represents the genome structure of EV71 of genotype C5 (black color with dotted pattern).

[0016] Figure 2c shows a schematic diagram representing the genome structure and respective encoded proteins of engineered stable cold-adapted temperature sensitive chimeric enterovirus 71, EV71:TLLcC5. The capsid protein genes (P1) (black color with dotted pattern) and translated proteins (VP1,2,3,4) (grey color with dotted pattern) of EV71:TLLcC5 are derived from EV71 of genotype C5.

[0017] Figures 3a and 3b show a schematic diagram representing the genome structure of EV71:eTLL β P20 and coxsackievirus CA16. Figure 3a represents the genome structure of EV71:eTLL β P20 (black color) and translated polyprotein (grey color). Figure 3b represents the genome structure of coxsackievirus CA16 (black color with vertical-line pattern).

[0018] Figure 3c shows a schematic diagram representing the genome structure and respective encoded proteins of engineered stable cold-adapted temperature sensitive chimeric enterovirus, TLLeCA16. The capsid protein genes (P1) (black color with vertical-line pattern) and translated proteins (VP1,2,3,4) (grey color with vertical-line pattern) of TLLeCA16 are derived from coxsackievirus CA16.

DETAILED DESCRIPTION OF THE INVENTION

[0019] The present invention relates to chimeric virus strains, particularly to the stable cold-adapted temperature sensitive Enterovirus 71 strains EV71:eTLL β P20 and EV71:TLLeC5 and to the stable chimeric strain TLLeCA16. The present invention also relates to vectors containing the nucleotide sequences of these strains and vaccines containing these strains.

[0020] Thus, in one aspect, the present invention provides Enterovirus 71 strains and chimeric strains. In one embodiment, a stable cold-adapted temperature sensitive Enterovirus 71 strain is EV71:eTLL β P20 as described herein. In another embodiment, a stable cold-adapted temperature sensitive Enterovirus 71 strain is EV71:TLLeC5 as described herein, which carries the capsid protein gene of EV71 genotype C5. In an additional embodiment, a stable chimeric enterovirus strain is TLLeCA16 as described herein, which carries the capsid protein gene of CA16. Parent strain EV71:TLL β P20, described in international application number PCT/SG2013/000027, was deposited on 25 October 2012 under terms of the Budapest Treaty with the American Type Culture Collection located at 10801 University Boulevard, Manassas, Virginia 20110, USA and assigned Accession Number PTA-13285. EV71:eTLL β P20 was deposited on 30 May 2014 under terms of the Budapest Treaty with China Center for Type Culture Collection located at Wuhan University, Wuhan 430072 Peoples Republic of China, and assigned Accession Number CCTCC V201414. EV71:TLLeC5 was deposited on 30 May 2014 under terms of the Budapest Treaty with China Center for Type Culture Collection, and assigned Accession Number CCTCC V201415. TLLeCA16 was deposited on 30 May 2014 under terms of the Budapest Treaty with China Center for Type Culture Collection, and assigned Accession Number CCTCC V201416.

[0021] In a second aspect, the present invention provides a composition comprising one or more of the virus strains described herein, alone or in combination with the parent EV71 strain EVL:TLL β P20 described in international application number PCT/SG2013/000027. As used in this context, "alone" means that the composition comprises one or more of the virus strains EV71:eTLL β P20, EV71:TLLeC5 and TLLeCA16. Also as used in this context, "in combination

with” means that the composition comprises one or more of the EV71 strains EV71:eTLL β P20, EV71:TLLeC5 and TLLeCA16 and the parent EV71:eTLL β P20. In one embodiment, the composition comprises an effective amount of one or more of the virus strains described herein, alone or in combination with the parent EV71:TLL β P20. In some embodiments, one, some or all of the strains may be inactivated forms of the virus strain. In another embodiment, the composition comprises one or more physiologically or pharmaceutically acceptable carriers. In a further embodiment, the composition is a vaccine. Vaccines containing one or more of the virus strains described herein, alone or in combination with the parent EVL:TLL β P20, are prepared using techniques well known to the skilled artisan. As used in this context, “alone” means that the vaccine comprises one or more of the virus strains EV71:eTLL β P20, EV71:TLLeC5 and TLLeCA16. Also as used in this context, “in combination with” means that the vaccine comprises one or more of the virus strains EV71:eTLL β P20, EV71:TLLeC5 and TLLeCA16 and the parent EV71:eTLL β P20. In one embodiment, the vaccine contains live strain(s). In another embodiment, the live strain(s) are attenuated. In an additional embodiment, the vaccine contains inactivated strain(s). In a further embodiment, the vaccine may be an oral vaccine. Such vaccines are useful for providing immunity against the parent virus strains by administering the vaccine to a subject, such as a human subject, using techniques well known to the skilled artisan.

[0022] It should be understood that a virus strain described herein, where used to elicit a protective immune response in a subject or to prevent a subject from becoming afflicted with a virus-associated disease or to delay the onset of or slow the rate of progression of a virus-associated disease, is administered to the subject in the form of a composition additionally comprising one or more a physiologically or pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are well known to the skilled artisan and include, but are not limited to, one or more of 0.01 M - 0.1 M and preferably 0.05 M phosphate buffer, phosphate-buffered saline (PBS), or 0.9% saline. Such carriers also include aqueous or non-aqueous solutions, suspensions, and emulsions. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, saline and buffered media. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's and fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Solid compositions may comprise nontoxic solid

carriers such as, for example, glucose, sucrose, mannitol, sorbitol, lactose, starch, magnesium stearate, cellulose or cellulose derivatives, sodium carbonate and magnesium carbonate. For administration in an aerosol, such as for pulmonary and/or intranasal delivery, an agent or composition is preferably formulated with a nontoxic surfactant, for example, esters or partial esters of C6 to C22 fatty acids or natural glycerides, and a propellant. Additional carriers such as lecithin may be included to facilitate intranasal delivery. Pharmaceutically acceptable carriers can further comprise minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives and other additives, such as, for example, antimicrobials, antioxidants and chelating agents, which enhance the shelf life and/or effectiveness of the active ingredients. The instant compositions can, as is well known in the art, be formulated so as to provide quick, sustained or delayed release of the active ingredient after administration to a subject.

[0023] In a third aspect, the present invention provides a method of eliciting a protective immune response in a subject, such as a human subject, which comprising administering to a subject a prophylactically or therapeutically or immunologically effective amount of one or more of the virus strains described herein, alone or in combination with the parent EVL:TLL β P20, or inactivated strains thereof. In one embodiment, the protective immune response protects the subject against a disease caused by Enterovirus 71 and/or Coxsackievirus CA16. In one embodiment, the disease is hand, foot and mouth disease. In another embodiment, the disease is aseptic meningitis. In an additional embodiment, the disease is encephalitis. In a further embodiment, the disease is poliomyelitis-like paralysis. In one embodiment, one or more of the virus strains described herein, alone or in combination with the parent EVL:TLL β P20, is administered as a vaccine. In an additional embodiment, the subject has been exposed to wild-type Enterovirus 71 and/or Coxsackievirus CA16. In another embodiment, the administration of one or more of the virus strains described herein, alone or in combination with the parent EVL:TLL β P20, prevents a subject, such as a human subject, from becoming afflicted with an Enterovirus 71-associated disease and/or Coxsackievirus CA16-associated disease. In an additional embodiment, the subject has been exposed to wild-type Enterovirus 71 and/or and/or Coxsackievirus CA16. In a further embodiment, the administration of one or more of the virus strains described herein, alone or in combination with the parent EVL:TLL β P20, delays the onset of or slows the rate of progression of an Enterovirus 71-associated disease and/or Coxsackievirus CA16-associated disease in a virus-infected subject, such as a human subject.

[0024] As used herein, "administering" means delivering using any of the various methods and delivery systems known to those skilled in the art. Administering can be performed, for example, intraperitoneally, intracerebrally, intravenously, orally, transmucosally, subcutaneously, transdermally, intradermally, intramuscularly, topically, parenterally, via implant, intrathecally, intralymphatically, intralesionally, pericardially, or epidurally. An agent or composition may also be administered in an aerosol, such as for pulmonary and/or intranasal delivery. Administering may be performed, for example, once, a plurality of times, and/or over one or more extended periods.

[0025] Eliciting a protective immune response in a subject can be accomplished, for example, by administering a primary dose of a vaccine to a subject, followed after a suitable period of time by one or more subsequent administrations of the vaccine. A suitable period of time between administrations of the vaccine may readily be determined by one skilled in the art, and is usually on the order of several weeks to months. The present invention is not limited, however, to any particular method, route or frequency of administration.

[0026] A "prophylactically effective dose" or "a immunologically effective dose" is any amount of a vaccine that, when administered to a subject prone to viral infection or prone to affliction with a virus-associated disorder, induces in the subject an immune response that protects the subject from becoming infected by the virus or afflicted with the disorder. "Protecting" the subject means either reducing the likelihood of the subject's becoming infected with the virus, or lessening the likelihood of the disorder's onset in the subject, by at least two-fold, preferably at least ten-fold. For example, if a subject has a 1% chance of becoming infected with a virus, a two-fold reduction in the likelihood of the subject becoming infected with the virus would result in the subject having a 0.5% chance of becoming infected with the virus. Most preferably, a "prophylactically effective dose" induces in the subject an immune response that completely prevents the subject from becoming infected by the virus or prevents the onset of the disorder in the subject entirely.

[0027] Certain embodiments of any of the instant immunization and therapeutic methods may further comprise administering to the subject at least one adjuvant. An "adjuvant" shall mean any agent suitable for enhancing the immunogenicity of an antigen and boosting an immune response in a subject. Numerous adjuvants, including particulate adjuvants, suitable for use with both protein- and nucleic acid-based vaccines, and methods of combining adjuvants with antigens, are well known to the skilled artisan. Adjuvants suitable for use with protein immunization include, but are not limited to, alum, Freund's complete adjuvant (FCA), Freund's

incomplete adjuvant (FIA), alum adjuvants, saponin-based adjuvants, such as Quil A, and QS-21, and the like.

[0028] In a fourth aspect, the present invention provides vaccine technology associated with the virus strains described herein. In one embodiment, the virus strains described herein are used in a method of making a vaccine. In another embodiment, a nucleic acid comprising the nucleotide sequence set forth in SEQ ID NO:84, SEQ ID NO:86, SEQ ID NO:88 or SEQ ID NO:83 are used in a method of making a vaccine. In an additional embodiment, the virus strains described herein, or inactivated forms thereof, are used for vaccine development. In a further embodiment, a nucleic acid comprising the nucleotide sequence set forth in SEQ ID NO:84, SEQ ID NO:86, SEQ ID NO:88 or SEQ ID NO:83 are used for vaccine development.

[0029] The invention also provides a kit for immunization of a subject with a stable cold-adapted temperature sensitive Enterovirus 71 strain described herein, or an inactivated form thereof, and/or a stable chimeric enterovirus strain described herein or an inactivated form thereof. In some embodiments, the kit comprises two or more stable cold-adapted temperature sensitive Enterovirus 71 strains described herein, or inactivated forms thereof, with or without a stable chimeric enterovirus strain described herein. The kit comprises a stable cold-adapted temperature sensitive virus strain described herein, or inactivated form thereof, and/or a stable chimeric enterovirus strain described herein, or inactivated form thereof, a pharmaceutically acceptable carrier, an applicator, and an instructional material for the use thereof. The invention includes other embodiments of kits that are known to the skilled artisan. The instructions can provide any information that is useful for directing the administration of the of a stable cold-adapted temperature sensitive virus strain described herein, or inactivated form thereof.

[0030] The practice of the present invention employs, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA, genetics, immunology, cell biology, cell culture and transgenic biology, which are within the skill of the art. See, e.g., Maniatis *et al.*, 1982, *Molecular Cloning* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Sambrook *et al.*, 1989, *Molecular Cloning*, 2nd Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Sambrook and Russell, 2001, *Molecular Cloning*, 3rd Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Green and Sambrook, 2012, *Molecular Cloning*, 4th Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Ausubel *et al.*, 1992, *Current Protocols in Molecular Biology* (John Wiley & Sons, including periodic updates); Glover, 1985,

DNA Cloning (IRL Press, Oxford); Russell, 1984, *Molecular biology of plants: a laboratory course manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.); Anand, *Techniques for the Analysis of Complex Genomes*, (Academic Press, New York, 1992); Guthrie and Fink, *Guide to Yeast Genetics and Molecular Biology* (Academic Press, New York, 1991); Harlow and Lane, 1988, *Antibodies*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Methods In Enzymology*, Vols. 154 and 155 (Wu et al. eds.), *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Riott, *Essential Immunology*, 6th Edition, Blackwell Scientific Publications, Oxford, 1988; Fire et al., *RNA Interference Technology: From Basic Science to Drug Development*, Cambridge University Press, Cambridge, 2005; Schepers, *RNA Interference in Practice*, Wiley-VCH, 2005; Engelke, *RNA Interference (RNAi): The Nuts & Bolts of siRNA Technology*, DNA Press, 2003; Gott, *RNA Interference, Editing, and Modification: Methods and Protocols (Methods in Molecular Biology)*, Human Press, Totowa, NJ, 2004; Sohail, *Gene Silencing by RNA Interference: Technology and Application*, CRC, 2004.

EXAMPLES

[0031] The present invention is described by reference to the following Examples, which is offered by way of illustration and is not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described below were utilized.

EXAMPLE 1

Materials and Methods

[0032] *Cell lines and Virus:* All cell lines used in this study were obtained from the American Tissue Type Culture Collection (ATCC®). The cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco®, USA) supplemented with 10% (v/v) Fetal Bovine Serum (FBS, i-DNA® Singapore) and 0.22% (w/v) sodium bicarbonate (NaHCO₃, Sigma Aldrich®, USA). Vero cells (ATCC, CCL81) were cultured and maintained at an incubation temperature of 37°C, unless otherwise stated. Prior to infection, the culture medium of 10%

DMEM was replaced with 1% DMEM and the infected cells were subsequently incubated at the respective experimental temperature of incubation in a humidified 5% CO₂ environment. A laboratory-established stable cold-adapted temperature sensitive strain of enterovirus 71 (EV71:TLLβP20), a clinical enterovirus 71 isolate of genotype C5 and a clinical isolate of coxsackievirus A16 which belongs to genogroup B (lineage 2) propagated in Vero cells were used to generate stable cold-adapted temperature sensitive strains of chimeric enteroviruses (EV71:TLLcC5 and TLLcCA16).

[0033] Full length viral RNA genome derived from the laboratory-established stable cold-adapted temperature sensitive strain of enterovirus 71 (EV71:TLLβP20) was used to generate the infectious cDNA clone of enterovirus 71 (EV71), named EV71:eTLLβP20, which contains engineered nucleotide changes at two specific sites of the virus genome that are recognised by tailored specific restriction enzymes. The infectious cDNA clone of EV71:eTLLβP20 was subsequently used to generate infectious cDNA clones of chimeric virus EV71:TLLcC5 and chimeric virus TLLcCA16.

[0034] *Virus Titration:* Virus titre was determined by micro-titration assay in Vero cells in accordance with the method described in Poliovirus Laboratory Manual 2004 of World Health Organization with minor modification and virus titre was calculated as 50% cell culture infectious dose (CCID₅₀) per millilitre following the method of Reed & Munch (1938) (53-55). Briefly, following treatment with equal volume of chloroform to disperse virus aggregates, a 10-fold serial dilution of the clarified virus supernatant was made in DMEM containing 1% FCS. Vero cell monolayers (10⁴ cells per well) in 96-well flat-bottom tissue culture plate were inoculated with 100 µl the serially diluted virus stock and incubated at each respective incubation temperature in an ambient of 5% CO₂. The inoculated culture plates were observed daily for 5 days for the presence of CPE.

[0035] *Temperature-sensitivity phenotype assay:* Two approaches were used to assess the growth characteristic of the virus strains in Vero cells at incubation temperature of 28° C, 37° C and 39.5° C. The first approach assessed the number of days taken for the virus strain to cause full CPE in inoculated monolayer cells (kinetic of infected cell death) and second approach assessed the titre of the virus strain in the supernatant of inoculated cells that were incubated at each specific test temperature. Briefly, in the first approach, the growth medium of three T-25 tissue culture flasks containing confluent monolayer Vero cells of similar age and cell density were replaced with maintenance medium (DMEM with 1% FCS). The medium in each flask was then allowed to equilibrate to the specified temperature to be tested by placing in respective

incubators for 1 hour and subsequently inoculated with the virus strain at a dose of 10 multiplicity of infection (m.o.i). If no CPE was noted at the end of 10-day culture, the supernatant was passed into a new flask of freshly prepared monolayer Vero cells and similarly incubated for another 10 days. It was taken as no virus replication, if no CPE was noted after second passage. In the second approach, Vero cell suspension of density 10^4 cells per 100 μ l was seeded into each well of three 96-well cell-culture plates and incubated at 37°C in an ambient of 5% CO₂. After 10 hours of incubation, each cell-culture plate was then allowed to equilibrate to the specific temperature to be tested by placing in respective incubators for 1 hour. The cells in each well were subsequently inoculated with 100 μ l of 10-fold serial dilutions of the virus strain before being transferred to incubators of respective temperature of incubation. The inoculated culture plates were observed daily for 5 days for the presence of CPE and virus titre was calculated as 50% cell culture infectious dose (CCID₅₀) per millilitre following the method of Reed & Munch (1938) (40).

[0036] *Genetic Stability and Temperature Sensitivity Reversion Assay:* To assess the genetic stability of the virus strains cultured at the specified culture environment and cell-type, the virus strains were further passaged twenty times in Vero cells at virus inoculum of 5 m.o.i and incubated at an incubation temperature of 28°C. At the end of twenty passages, the virus strains were assessed for their temperature sensitivity phenotypic characteristics by culturing at incubation temperature of 28°C, 37°C and 39.5°C as described above. The complete nucleotide sequences of their respective genomes were subsequently sequenced and analyzed with respect to original source genomic sequences of their respective parental viruses.

[0037] Temperature sensitivity reversion phenotype assay was carried out on stable cold-adapted temperature sensitive chimeric virus strains to assess how rapidly they would revert to the phenotypic characteristics of their original wild-type. Briefly, the selected strain was successively passaged 5 times in monolayer of Vero cells cultured in T-25 flask and incubated at temperature of 37°C in an ambient of 5% CO₂. A virus inoculum of 10 m.o.i was used at each passage. The derived virus strain at each passage was assessed for its growth characteristics in Vero cells at incubation temperature of 28°C, 37°C and 39.5°C by similar method as described above for temperature sensitivity phenotype assay. After 6 successive passages in Vero cells incubated at temperature of 37°C, the complete genomes of the virus strains of passages 3 and 6 were sequenced by Sanger method and analysed for mutations that may have reverted to genetic sequences of their respective parental strains.

EXAMPLE 2

Reagents, Vectors (Plasmids) and General Methods of Molecular Cloning

[0038] *Escherichia coli* (*E. coli*) strain TOP10 (Invitrogen) was used for preparation of pZEROTM-2 plasmids (Invitrogen) that were used as holding vectors to clone fragments of DNA derived from RT-PCR or 5' Rapid Amplification of cDNA end (5'-RACE) of viral genomes. XL10-Gold ultracompetent *E. coli* strain (Agilent Technologies) was used for preparation of plasmids (pACYC177) (New England Biolabs) that were used for construction of infectious cDNA clones of the respective engineered chimeric enteroviruses. Restriction enzymes *Bam*HI and *Aat*II, (New England Biolabs) were used for sites-specific digestion in the cloning of proximal and distal fragments of EV71:TLLβP20 into pACYC177 plasmids respectively which were subsequently used in the construction of infectious cDNA clone of EV71:eTLLβ. Restriction enzyme pair, *Cla*I and *Xma*I (New England Biolabs), was used for sites-specific digestion of PCR amplified P1 genetic region of EV71 genotype C5 into pACYC177 plasmids. Restriction enzyme pair, *Nhe*I and *Xho*I (New England Biolabs), was used for sites-specific digestion of PCR amplified P1 genetic region of CA16 into pACYC177 plasmids. Restriction enzyme pair, *Mlu*I and *Eag*I (New England Biolabs), was used for sites-specific digestion of P1 genetic region of C5 and CA16 from pACYC177 plasmids carrying the respective inserts for subsequent cloning into pACYC177 plasmids carrying full length cDNA genome of EV71:eTLLβ to produce the respective chimeric virus.

[0039] All ligation of DNA fragments were performed using T4 DNA ligase (Fermentas). Site-directed mutagenesis (SDM) was performed using iProof High-Fidelity DNA polymerase (Bio-Rad). Following SDM, PCR reaction was purified using spin column (Geneaid Biotech, Taiwan) before being subjected to *Dpn*I digestion. Methylated plasmid was removed by treatment with 40 U *Dpn*I (New England Biolabs) enzyme incubating at 37° C for 6 hours followed by inactivation at 80° C for 20 minutes. The reaction product was then purified using spin column (Geneaid Biotech, Taiwan) and transformed into XL10-Gold ultracompetent *E. coli* cells. The transformed bacterial cells were plated on LB plate containing 100 ug/ml Ampicillin and 35ug/ml Kanamycin to screen and select for clones which have the required changes at SDM site. QIAGEN Plasmid Maxi Kit (Qiagen, Germany) was used to extract the plasmids in large quantities from the selected clones. DNA sequencing was performed using BigDye Terminator v3.0 cycle sequencing reaction kit (Applied Biosystems, USA) and results were analysed using BioEdit programme (56, 57).

[0040] *General molecular method of RNA extraction, RT-PCR and Sequencing:* Virus RNA was extracted from culture supernatant of Vero cells respectively infected with EV71:eTLL β P20, EV71:TLLcC5 or TLLcCA16 using QIAamp Viral RNA Mini Kit (Qiagen, Germany). First strand cDNA synthesis was performed by reverse transcription using SuperScript II reverse transcriptase (Invitrogen) and random hexamers or respective downstream specific primers. The first strand cDNA subsequently served as template for PCR amplification of the targeted fragments of the virus genome using GoTaq Green PCR mix (Promega, USA) and respective specific primer pairs. The amplified fragments generated were either sequenced directly or cloned into holding plasmid vector pZerO™-2 and the inserts in the purified plasmids were sequenced using BigDye Terminator sequencing kit (Applied Biosystems, USA). The 5'-RACE was performed to determine the nucleotide sequences of 5'- end of the viral genome (non-translated region) using primer Race-2R to synthesise the single strand cDNA. The single stranded cDNA was phenol-chloroform extracted, purified and ligated to oligonucleotide RACE-DT88, a 3' end cordecypin-blocked adaptor (58). The ligated product was amplified using primer RACE-DT89, complementary to oligonucleotide RACE-DT88, and primer Race-3R. 3'-RACE was also performed to determine the 3'-UTR viral sequence using the primer pair EV71-19F and oligo-(dT)₁₅. The PCR amplified product was cloned into pZerO™-2 vector (Invitrogen) and transformed into *E. coli* strain TOP10 (Invitrogen). The insert within the extracted plasmid was sequenced using BigDye Terminator sequencing kit (Applied Biosystems, USA).

[0041] *Transfection of plasmids carrying insert of full length cDNA of virus genome:* Transfection of plasmid carrying the full length cDNA genome of respective engineered/chimeric virus was performed using Lipofectamine 2000 Transfection Reagent (Invitrogen). A mixture, consisting of transfection reagent, plasmid carrying the full length cDNA of the virus genome of respective engineered/chimeric virus (EV71:eTLL β , EV71:TLLcC5 or TLLcCA16) and plasmid expressing T7 polymerase, was transferred onto freshly seeded Vero cells in a 24-well plate. Following incubation at 37° C for 5 hours, the mixture was removed and cells were washed twice with sterile PBS. After the last wash, it was replaced with 1% DMEM and incubated at 28° C in an ambient of 5% carbon dioxide. After 10 days of incubation, cells and supernatant in wells of a 24-well plate were passed onto new Vero cells freshly seeded on a 6-well plate. As soon as the inoculated Vero cells had reached full cytopathic effect (CPE), culture supernatant containing the virus was subsequently passed onto freshly seeded Vero cells cultured in T25 flask.

[0042] *Primers:* Primers used in the Examples described herein are set forth in Tables 1-4.

TABLE 1

Primers in Common Used for Running PCR and Sequencing Work
of the Virus Strains: eTLLβP20, EV71:eTLLβP20, EV71:TLLeC5 and TLLeCA16

Primer Name	Primer Sequence (SEQ ID NO:)	Remarks
Race-3R	5'- CCG GGG AAA CAG AAG TGC TTG ATC -3' (1)	Sequencing
Race-2R	5'- ATT CAG GGG CCG GAG GAC TAC -3' (2)	Sequencing
TLLb-1R	5'- GAC ACC CAA AGT AGT CGG TTC CG -3' (3)	Sequencing
EV71-1F	5'- AAC AGC CTG TGG GTT GCA CCC AC-3' (4)	Sequencing
TLLb-2F	5'- TAG TCC TCC GGC CCC TGA ATG C -3' (5)	Sequencing
TLLb-9F	5'- GAA TTC CGT CTG GGA GGA CAG CT -3' (6)	Sequencing
TLLb-9R	5'- CTA CCG TGG CAC CCT ATC AGA GCT -3' (7)	cDNA synthesis of EV71:TLLeC5 and TLLeCA16, Sequencing
TLLb-10F	5'- AGG AGT GAT TAT GAC ATG GTC ACT CTC AC -3' (8)	Sequencing
TLLb-10R	5'- TAT CCA TCA AAG TGG TCC GGG TCT G -3' (9)	Sequencing
TLLb-11F	5'- AGA GCA AAC ACC GAA TTG AAC CTG TAT GTC -3' (10)	Sequencing
TLLb-11R	5'- GCT GAA TGG CCT TCC CAC ACA C -3' (11)	Sequencing
TLLb-12F	5'- CAA TGG CTT CCC TAG AAG AGA AAG GAG T -3' (12)	Sequencing
TLLb-12R	5'- CAA CCT TGA TCT CTA CAG TAC TGG CGT -3' (13)	Sequencing
TLLb-13F	5'- ACA CAA TTG AAG CAC TAT TCC AAG GTC CG -3' (14)	Sequencing
TLLb-13R	5'- CCA GAT TGT TTT GCC GGG CTG G -3' (15)	Sequencing
TLLb-14F	5'- AAC AGT GCA GGG TCC AAG TCT CG -3' (16)	Sequencing
TLLb-14R	5'- TAT TGG GCT TGA CCC ACT GGA TCT C -3' (17)	Sequencing
TLLb-15F	5'- CAA CCC ACC GTA CCA TGA TGT ACA AC -3' (18)	Sequencing
TLLb-15R	5'- AAT GGC CTC AAG GTT CTC AGT GCC -3' (19)	Sequencing
TLLb-16F	5'- GAC ACC TCC CAG ATG AGC ATG GAG -3' (20)	Sequencing
TLLb-16R	5'- CCA GGG AGT AAG ATG GGT AGT TTA CTC C -3' (21)	Sequencing
TLLb-17F	5'- TGA AAC CTT CCA TGC AAA CCC TGG -3' (22)	Sequencing
TLLb-17R	5'- ATT TGT CTG CAG GAG TCA TGG TTA AAC CG -3' (23)	Sequencing
TLLb-18F	5'- GAT GAT GTG CTT GCC AGT TAC CCT -3' (24)	Sequencing
TLLb-18R	5'- TTT TTT TTT TTT TTT TTT TTT TTT TGC TAT TCT GGT AAT AAC AAA TTT ACC C -3' (25)	Sequencing

TABLE 2

Primers Used for Running PCR and Sequencing Work Related to P1
Region of the Viruses: eTLL β , EV71:eTLL β P20, EV71:TLLeC5 and TLLeCA16

Primer Name	Primer Sequence (SEQ ID NO:)	Remarks
TLLb-3R	5'- CCA AAA TTG CAA TGG TTC AGG GCA G -3' (26)	cDNA synthesis of EV71: eTLL β and EV71:eTLL β P20. Sequencing of EV71: eTLL β , EV71:eTLL β P20 and EV71: TLLeC5.
TLLb-CR	5'- GAG CTG TCC TCC CAG ACG GAA -3' (27)	cDNA synthesis of EV71: eTLL β and EV71:eTLL β P20. Sequencing of EV71: eTLL β , EV71:eTLL β P20 and EV71: TLLeC5.
TLLb-2R	5'- CTC ACC ATA ACC GAC TAT GAT GTT TGC C -3' (28)	Sequencing of EV71: eTLL β and EV71:eTLL β P20
TLLb-3F	5'- GCG TGG CAC AAC TCA CCA TTG G -3' (29)	Sequencing of EV71: eTLL β and EV71:eTLL β P20
TLLb-4F	5'- TAC GTG CTT GAT GCT GGG ATT CC -3' (30)	Sequencing of EV71: eTLL β and EV71:eTLL β P20
TLLb-4R	5'- ACA CGG CAC ACA ATT CAC CTT TCC C -3' (31)	Sequencing of EV71: eTLL β and EV71:eTLL β P20
TLLb-5F	5'- ACC ACC GAT GAC GGC GTC TCA G -3' (32)	Sequencing of EV71: eTLL β and EV71:eTLL β P20
TLLb-5R	5'- GGT AAA ATT CTT CTG GGC TGC CGC -3' (33)	Sequencing of EV71: eTLL β , EV71:eTLL β P20 and EV71: TLLeC5
TLLb-AF	5'- TGT CAC CCT TGT GAT ACC ATG GAT CAG -3' (34)	Sequencing of EV71: eTLL β and EV71:eTLL β P20
TLLb-AR	5'- GTG TGA GTT AAG AAC GCA CCG TGT TTC -3' (35)	Sequencing of EV71: eTLL β and EV71:eTLL β P20
TLLb-BF	5'- TGA TGA GAG TAT GAT TGA AAC ACG GTG CG -3' (36)	Sequencing of EV71: eTLL β and EV71:eTLL β P20
TLLb-BR	5'- AAA ACC ACT GGT AAG CGC TCG C -3' (37)	Sequencing of EV71: eTLL β and EV71:eTLL β P20
TLLb-CF	5'- CAC AAA CCC CTC AAT TTT TGT CAA GTT GAC -3' (38)	Sequencing of EV71: eTLL β and EV71:eTLL β P20
TLLb-8F	5'- GCA CTT TCT CGG TGC GGA CTG -3' (39)	Sequencing of EV71: eTLL β and EV71:eTLL β P20
TLLb-8R	5'- TAG AGA AGC TGA CTG GAT AGT GCT TTC TC -3' (40)	Sequencing of EV71: eTLL β , EV71:eTLL β P20, EV71: TLLeC5 and TLLeCA16
E71-2R	5'- CCA TAG CCA ACT ATG ATG TTT GC -3' (41)	Sequencing of EV71: TLLeC5
E71-3F	5'- CGG GTG GCR CAA TTA ACT ATT GG -3' (42)	Sequencing of EV71: TLLeC5

EV71-4F 2011	5'- TAC GTG CTC GAT GCT GGS ATY CC -3' (43)	Sequencing of EV71: TLLcC5
EV71-4R 2011	5'- ACA CGG CAC ACA RYT CAC CTT TCC C -3' (44)	Sequencing of EV71: TLLcC5
AF	5'- CAC CCT TGT AAT ACC ATG GAT CAG - 3' (45)	Sequencing of EV71: TLLcC5
AR	5'- GTG AAT TAA GAA CRC AYC GTG TYT - 3' (46)	Sequencing of EV71: TLLcC5
BF	5'- TCG TCA AAT RCT AGT GAT GAG AGT - 3' (47)	Sequencing of EV71: TLLcC5
BR	5'- AAC CAY TGR TAR GCG CTC GCR GGT - 3' (48)	Sequencing of EV71: TLLcC5
CF	5'- GCC ACW AAY CCC TCA GTT TTT G -3' (49)	Sequencing of EV71: TLLcC5
E71-8F	5'- GAT GGG TAC RTT CTC AGT GCG GAC - 3' (50)	Sequencing of EV71: TLLcC5
CA16P1-1F	5'- CCG GTG ACC AAC AGA GCT ATT GTT TAC C -3' (51)	Sequencing of TLLcCA16
CA16P1-1R	5'- ATA CCA TCC CTT TGA ATC CTT GGC CC -3' (52)	Sequencing of TLLcCA16
CA16P1-2F	5'- AGT ACT GCC CAG ACA CAG ATG CAA -3' (53)	Sequencing of TLLcCA16
CA16P1-2R	5'- TTC TCA GGT TGA TCC ACT GAT GTG GG -3' (54)	Sequencing of TLLcCA16
CA16P1-3F	5'- ACT ACA CAG CCT GGT CAG GTT GG - 3' (55)	Sequencing of TLLcCA16
CA16P1-3R	5'- ATC TCT TCC AGG GTC TGC TCT AAA GGC -3' (56)	Sequencing of TLLcCA16
CA16P1-4F	5'- ACA ACC TGA AGA CCA ATG AGA CCA CC -3' (57)	Sequencing of TLLcCA16
CA16P1-4R	5'- ACT TGC TTC AGT ATT GGC AGC TGT AGG -3' (58)	Sequencing of TLLcCA16
CA16P1-5F	5'- GAC ACA GAG GAC ATT GAG CAA ACA GC -3' (59)	Sequencing of TLLcCA16
CA16P1-5R	5'- TTT CTC AAA GGC CTT GGG ATC CAT GCC -3' (60)	Sequencing of TLLcCA16
CA16-P1-6F	5'- TGA TGG TTA TCC CAC CTT YGG WGA GC -3' (61)	Sequencing of TLLcCA16

TABLE 3

Primers in Common Used to Run 5'RACE and 3'RACE in Sequencing
the 5' and 3' Termini Sequences of EV71:eTLL β P20, EV71:TLLcC5 and TLLcCA16

Primer Name	Primer Sequence (SEQ ID NO:)	Remarks
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Race-2R	5'- ATT CAG GGG CCG GAG GAC TAC -3' (62)	5'RACE cDNA synthesis
RACE-DT88	5'- GAA GAG AAG GTG GAA ATG GCG TTT TGG -3' (63)	5'RACE adaptor
RACE-DT89	5'- CCA AAA CGC CAT TTC CAC CTT CTC TTC -3' (64)	5'RACE adaptor-primer
Race-3R	5'- CCG GGG AAA CAG AAG TGC TTG ATC -3' (65)	5'RACE PCR
EV71-19F	5'- GAG AAT CTG AGA MGM AAT TGG CTC G -3' (66)	3' RACE PCR
Oligo (dT) ₁₅	5'- TTT TTT TTT TTT TTT -3' (67)	3'RACE PCR and cDNA synthesis of EV71:eTLL β , EV71:eTLL β P20, EV71:TLLeC5 and TLLeCA16

TABLE 4

Primers Used in the Construction of Infectious cDNA
Clones of EV71:eTLL β P20, EV71:TLLeC5 and TLLeCA16

Primer Name	Primer Sequence (SEQ ID NO:)	Remarks
EV71-9R_2011B	5'- GGG ACT ACC RTG RCA MCC KAT CAG -3' (68)	cDNA synthesis for infectious clone and cDNA synthesis for EV71:eTLL β P20.
ACYC-TLLb-Pf-F	5'- GCT <u>AGG ATC CTA ATA CGA CTC ACT ATA</u> GGT TAA AAC AGC CTG TGG GTT GCA CCC AC -3' (69)	PCR of eTLL β
ACYC-TLLb-Pf-R	5'- CGA <u>TGA CGT CCG GCC</u> GAA CTT TCC AAG GGT AGT AAT GG -3' (70)	PCR of eTLL β
ACYC-TLLb-D-F	5' - TCG <u>AGG ATC CGG CCG</u> GCA ATC TGG GGC CAT GTA CG -3' (71)	PCR of eTLL β
ACYC-TLLb-D-R	5'- GAT <u>CGA CGT C</u> (T) ₂₅ G CTA TTC TGG TAA TAA CAA ATT TAC CC -3' (72)	cDNA synthesis for infectious clone & PCR of eTLL β
SDM-pACYC-Pf-F	5' - CAA <u>CGC GTC</u> GGC TCC CAC GAG AAC TCC -3' (73) [yellow highlight: nucleotides 3 and 6-8]	SDM of eTLL β
SDM-pACYC-Pf-R	5' - GCC <u>GAC GCG TTG</u> AGT AGA CAC TTG TGA GCC -3' (74) [yellow highlight: nucleotides 5 and 8-10]	SDM of eTLL β
pACYC-C5-P1-F	5'- ATC <u>GAT CGA TCA</u> CAC <u>AAC GCG TCG</u> GCT CGC ATG AAA ACT CTA AC -3' (75)	PCR of eC5
pACYC-C5-P1-R	5'- TAT <u>ACC CGG GTT GTC GGC CGA</u> ATT TCC CAA GAG TGG TGA TCG CCG TG -3' (76)	PCR of eC5
pACYC-C5P1-TITTL-F	5'- CAG TCG CAC <u>GAC GAT</u> CAC CAC TCT TGG GAA ATT CGG CCG AC -3' (77) [green highlight: nucleotide 11]	SDM of eC5
pACYC-C5P1-	5'- TGG TGA <u>TCG TCG</u> TGC GAC TGG CAC CGG TTG	SDM of eC5

TITTL-R	GCT TAA TAG AAT CAC C -3' (78) [green highlight: nucleotide 10]	
pACYC-CA16b_F	5'- TCT <u>CGC</u> <u>TAG</u> <u>CAC</u> <u>GCG</u> TCG GGT CAC ATG AGA ACT CAA ACT CTG - 3' (79)	PCR of eCA16
ACYC-CA16-P1-R	5' - TGC <u>ACT</u> <u>CGA</u> <u>GTG</u> <u>CCG</u> <u>GCC</u> <u>GAA</u> CTC TCC CAA TGT TGT TAT CTT G -3' (80)	PCR of eCA16
pACYC-CA16P1-TITTL-F	5'- CTA GTA GAG ACA <u>CGA</u> TAA CAA CAT TGG GAG AGT TCG GCC GGC ACT C - 3' (81) [green highlight: nucleotide 13]	SDM of eCA16
pACYC-CA16P1-TITTL-R	5'- AAC TCT CCC AAT GTT GTT ATC <u>CGT</u> GTG TCT CTA CTA GTG CTA GTG CAC TTA ATA TCA TTT C -3' (82) [green highlight: nucleotide 22]	SDM of eCA16

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

EXAMPLE 3

Construction of Infectious cDNA Clone of EV71:eTLLβP20

[0043] *RNA extraction and cDNA synthesis:* Viral RNA (EV71:TLLβP20) was extracted using QIAamp Viral RNA Mini Kit (Qiagen, Germany) and performed according to the manufacturer's instructions. Single strand cDNA synthesis was performed using SuperScript II reverse transcriptase (Invitrogen). Briefly, 5ug of extracted RNA, 100pmol specific primer (EV71-9R_2011B for generation of 5'-proximal fragment, or ACYC-TLLb-D-R for generation of 3'-distal fragment) and 5ul of 10mM dNTP mix were added into nuclease-free water with reaction volume adjusted to 60ul. The reaction mixture was incubated at 65° C for 10mins and quenched in ice (4° C) for 5min. Subsequently, 20ul of 5X First-Strand Buffer, 10ul of 0.1M DTT and 5ul of RNase Inhibitor (40U/ul) was added to the reaction mix and allowed to be incubated at 42° C for 5mins. Following which, 5ul of SuperScript II RT (200U/ul) was added and the resultant mix was incubated at 46° C for 30mins, followed by another 30mins at 50° C. The reaction was terminated by incubating the reaction mix at 72° C for 15mins. The RNA which was complementary to cDNA strand was then removed by addition of 6.5ul of RNaseH (1.5U/ul) and incubated at 37° C for 25mins. The synthesized single strand cDNA was extracted and purified using the standard phenol-chloroform extraction protocol.

[0044] *PCR Amplification of fragments for construction of full length cDNA copy of viral genome:* The full length cDNA copy of the viral genome (EV71:eTLLβP20) of approximately 7500 bp was generated by RT-PCR amplification of the viral RNA genome in two fragments, followed by ligation of the proximal (5') cDNA fragment (approximately 3500 bp) of the virus

genome to its amplified distal (3') cDNA fragment (approximately 4000 bp). Primer pair, ACYC-TLLb-Pf-F (forward primer) and ACYC-TLLb-Pf-R (reverse primer) was used to generate the proximal fragment by PCR amplification of the single-stranded cDNA which was synthesised by reverse transcription of virus genome using primer EV71-9R_2011B. Primer pair, ACYC-TLLb-D-F (forward primer) and ACYC-TLLb-D-R (reverse primer) was used to generate the distal (3') fragment by PCR amplification of the single-stranded cDNA which was synthesized using primer ACYC-TLLb-D-R. The PCR reaction was performed using iProof High-Fidelity Master Mix (Bio-Rad) with initial denaturation temperature of 98° C for 2 mins followed by 10 cycles of 98° C for 10 secs, 65° C for 30 secs and 72° C for 2 mins, which subsequently continued by another 35 cycles of 98° C for 10 secs and 72° C for 2.5 mins and final extension of 72° C for 5 mins. The nucleotide sequence for EV71:TLLβP20 as disclosed in international patent application number PCT/SG2013/000027 is set forth in SEQ ID NO:83.

[0045] *Cloning of Proximal and Distal fragments of EV71:TLLβP20 to pACYC177:* Both the PCR generated proximal and distal fragments were separately digested with restriction enzymes *Bam*HI and *Aat*I and cloned into plasmid pACYC177 which was similarly digested with the same set of restriction enzymes. Briefly, 4 ug of pACYC177 (which have unique cutting sites for *Bam*HI and *Aat*II) and approximately 1 ug of proximal and distal PCR products were separately double digested with 50 U of *Bam*HI and *Aat*II by incubating at 37° C for 2hrs, followed by inactivation at 65° C for 30 mins. The digested DNA fragments were separately gel purified. The digested PCR products were then ligated to the digested pACYC177 using 5 U T4 DNA ligase (Fermentas) in a 20 ul reaction mix which was incubated at 22° C for 1 hr, and followed by inactivation at 70° C for 5 mins. Ten microliter (10 ul) of ligation mixture was then transformed into XL10 electro-competent *E. coli* cells by electroporation in 100 ul reaction followed by incubation in 400 ul of SOC medium at 30° C for 2 hrs. Following which, 150 ul of the electro-transformed cells were then plated onto LB plate containing 100 ug/ml Ampicillin and 35 ug/ml Kanamycin. Positive clones were then screened for presence of correct inserts and selected for preparation of large quantities of plasmids. Sequencing was routinely performed to confirm full length sequence of the insert which was successfully ligated to the vector. The pACYC177 plasmids carrying the cDNA copy of the proximal and distal portion of EV71:TLLβP20 genome were designated as pACYC177(TLLβP20proximal) and pACYC177(TLLβP20distal) respectively.

[0046] *Site-directed mutagenesis (SDM) of pACYC177(TLLβP20proximal):* PCR-based site-directed mutagenesis was performed on the plasmid containing the proximal fragment of the

virus genome to introduce a unique restriction enzyme (*Mlu*I) recognition site located at the 5' end of VP4 gene region. The *Mlu*I and *Eag*I restriction enzymes cutting sites were created to facilitate the subsequent cloning the P1 region of Coxsackievirus A16 or Enterovirus 71 genotype C5 for generation of the respective chimeric viruses. The reaction was performed using iProof High-Fidelity DNA polymerase (Bio-Rad) containing primers SDM-pACYC-Pf-F and SDM-pACYC-Pf-R together with 100 ng of the plasmid. After an initial denaturation of 98° C for 2mins, the reaction underwent 35 cycles of 98° C for 10 secs, 65° C for 30 secs and 72° C for 4 mins, followed by final extension of 72° C for 5 mins. The methylated plasmid was then removed by treatment with 40U *Dpn*I (New England Biolabs) at 37° C for 2 hrs followed by inactivation at 80° C for 20 mins. The reaction mixture was then spin column purified and transformed into XL10 electro-competent *E. coli* cells by electroporation and the transformed *E. coli* cells were recovered as described above. Screening was subsequently carried out to select for clones which had the required nucleotides changes at the SDM site. QIAGEN plasmid maxi kit (Qiagen, Germany) was used to extract large quantities of plasmids from these selected clones having the required nucleotides changes.

[0047] *Construction of full-length infectious cDNA clone of EV71:eTLLβP20:* The plasmids holding the proximal and distal fragments were separately digested with 40 U of restriction enzymes, *Bam*HI and *Eag*I (New England Biolabs) and gel purified followed by extraction using spin column. The “freed” proximal fragment having the required SDM site was ligated to the digested plasmid pACYC177(TLLβP20distal) carrying the distal fragment of EV71:TLLβP20 by using 2 U of T4 DNA ligase (Fermentas). The ligated plasmid was transformed into XL10 electro-competent *E. coli* cells, plated onto 100 ug/ml Ampicillin and 35 ug/ml Kanamycin LB plates and incubated at 30° C. Colonies were then screened and selected for preparation of plasmids containing full length cDNA copy of the virus genome in large quantities. Sequencing was carried out using the complete set of TLLbP20 primers to confirm full length genome of EV71:TLLβP20 having the engineered restriction enzymes (*Mlu*I and *Eag*I) sites (designated as EV71:eTLLβP20) was successfully cloned into the vector. The pACYC177 plasmid carrying the modified full length cDNA copy of EV71:TLLβP20 was designated as pACYC177(EV71:eTLLβP20). The nucleotide sequence for EV71:eTLLβP20 is set forth in SEQ ID NO:84.

[0048] *Recovering engineered EV71:eTLLβP20 virus:* Transfection was performed using Lipofectamine 2000 Transfection Reagent (Invitrogen) containing a total of 800 ng pACYC177(EV71:eTLLβ) plasmid and T7 polymerase plasmid in a ratio of 1:2 onto an

overnight seeded Vero-81 cells at a seeding density of 3×10^4 cells per well in a 24 well plate. Briefly, The mixture was incubated for 20 minutes at room temperature with 2 ul Lipofectamine diluted in OPTI-MEM. After which, the mixture complex was added onto the cells and incubated at 37° C for 5 hrs. The mixture complex was then removed; cells were washed and replaced with 1% DMEM and incubated at 28° C. After 10 days post transfection, cells and supernatant were passed onto fresh Vero cells seeded on a 6-well plate. Upon reaching full cytopathic effects, cells and supernatant were subsequently passed onto fresh Vero cells cultured in T25 flask. The virus was further passed in Vero cells incubated at 28° C for 20 passages (EV71:eTLLβP20) to confirmed its genetic and phenotypic stability.

EXAMPLE 4

Construction of Infectious Full Length cDNA Clone of Chimeric Virus EV71:TLLc5

[0049] *Virus and RNA:* Enterovirus 71 strain belonging to genotype C5 (EV71:C5) (C5/3437/SIN-06) was subjected to 15 passages in Vero cells grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% fetal bovine serum at incubation temperature of 30°C in a humidified environment containing 5% CO₂. Viral RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen, Germany) and performed according to the manufacturer's instructions.

[0050] *PCR amplification of capsid protein genes (P1):* The whole of capsid protein genes (P1) region of EV71 genotype C5 was RT-PCR amplified using primers pACYC-C5-P1-F and pACYC-C5-P1-R. The pACYC-C5-P1-F primer which contained engineered *Cla*I and *Mlu*I restriction enzyme recognition sequences and the primer pACYC-C5-P1-R which contained engineered *Xma*I and *Eag*I restriction enzyme recognition sequences were used to facilitate the cloning of capsid protein genes of EV71 genotype C5 initially into pACYC177 vector and subsequently into full length cDNA clone of EV71:eTLLβP20. The amplification of this 2.6kbp capsid protein genes (P1) region was performed using iProof High-Fidelity Master Mix (Bio-Rad). The nucleotide sequence for the unmodified P1 gene of EV71 C5 is set forth in SEQ ID NO:85.

[0051] *Digestion and Cloning of C5-P1 fragment into pACYC177:* Both pACYC177 and C5 RT-PCR amplified product were separately digested with 20 U *Cla*I and *Xma*I at 37° C for 2 hours. Enzymatic reaction was terminated by inactivating the reaction mixture at 65° C for 20 minutes. The digested pACYC177 was gel purified. Spin column purification was performed to obtain the C5-P1 PCR amplified product containing the over-hanging 'sticky ends' following

digestion with the restriction endonucleases. The purified C5 DNA was then ligated to the digested pACYC177 by 2 U T4 DNA ligase (Fermentas) in a 20 ul reaction mixed which was incubated at 22° C for 1 hr followed by inactivation at 70° C for 5 mins. Ten microlitre of purified pACYC177 carrying C5-P1 DNA, now designated as pACYC177(C5-P1), was transformed into XL10 electro-competent *E. coli* cells by electroporation and the transformed *E. coli* cells were recovered as described above. Positive clones carrying the plasmids with correct inserts were then screened and selected for preparation of large quantities of the plasmids. Sequencing also was performed to confirm that the full length insert was successfully cloned to the vector.

[0052] *Site-directed mutagenesis EV71:C5 P1 gene:* Site-directed mutagenesis was performed on the plasmid carrying the P1 region of C5 to change the 858th amino acid from Alanine to Threonine. The PCR site-directed mutagenesis was performed using primers pACYC-C5P1-TITTL-F and pACYC-C5P1-TITTL-R together with 50 ng of the plasmid, pACYC177(C5-P1). The reaction was carried using iProof High-Fidelity DNA polymerase (Bio-Rad) with initial denaturation of 98° C for 2 mins followed by 15 cycles of 98° C for 10 secs, 66° C for 30 secs and 72° C for 4 mins, continued by 35 cycles of 98° C for 10 secs, 69° C for 30 secs and 72° C for 4 mins, and final extension of 72° C for 5 mins. The PCR reaction was then spin column purified before undergoing *DpnI* digestion. The methylated plasmid was removed by 40 U *DpnI* (New England Biolabs) incubated at 37° C for 6 hrs followed by inactivation at 80° C for 20mins. The reaction mixture was then spin column purified and transformed into XL10 electro-competent *E. coli* cells as described above. Screening was performed to select for clones which have the required SDM site. QIAGEN plasmid maxi kit (Qiagen, Germany) was used to extract large quantities of plasmids from these clones. The plasmid carrying the required nucleotide changes at the site directed mutagenesis is designated as pACYC177(eC5-P1).

[0053] Construction of infectious full-length cDNA clone of EV71:TLLC5: Both purified plasmids, pACYC177(EV71:eTLL β P20) and pACYC177(eC5-P1) were separately digested with 20 U *MluI* and *EagI* incubated at 37° C for 6 hours followed by inactivation at 65° C for 30 minutes to terminate the digestion. The digested products were gel purified. The digested fragment containing P1 region of eC5 derived from pACYC177(eC5-P1) was ligated to digested pACYC177(EV71:eTLL β P20) devoid of original P1 region using 2 U T4 DNA ligase (Fermentas) in a 20 ul reaction mixed which was incubated at 22° C for 1 hr followed by inactivation at 70° C for 5 mins. The ligated product was transformed into XL10 electro-

competent *E. coli* cells by electroporation in 100 ul reaction followed by incubation in 400 ul of SOC medium at 30° C for 2 hrs. Following which, 150 ul of the electro-transformed cells were then plated onto LB plate containing 100 ug/ml Ampicillin and 35 ug/ml Kanamycin. Screening for positive clones and sequencing of the full genome were subsequently performed accordingly. The plasmid pACYC177 carrying the full length cDNA genome was designated as pACYC177(EV71:TLLc5). The nucleotide sequence for EV71:TLLc5 is set forth in SEQ ID NO:86.

[0054] *Recovering engineered chimeric EV71:TLLc5 virus:* The engineered chimeric EV71:TLLc5 virus was recovered by transfecting pACYC177(EV71:TLLc5) plasmid onto Vero cells by similar laboratory process as described earlier for recovering engineered EV71:eTLLβP20 virus. The virus was further passed in Vero cells incubated at 28° C for 20 passages to confirmed its genetic and phenotypic stability.

EXAMPLE 5

Construction of Infectious Full Length cDNA Clone of Chimeric Virus TLLcA16

[0055] *PCR amplification of capsid protein genes (P1) of CA16 (CA16-P1):* The CA16 virus RNA was extracted in a similar manner as described for the extraction of RNA of EV71 genotype C5. Amplification of P1 (capsid protein genes) region of CA16 was carried out using primers pACYC-CA16P1-F and pACYC-CA16-P1-R. The pACYC-CA16-P1-F primer contained engineered *NheI* and *MluI* restriction enzyme recognition sequences and primer pACYC-CA16-P1-R contained engineered *XhoI* and *EagI* restriction enzyme recognition sequences to facilitate the cloning of capsid protein genes of CA16 into pACYC177 vector and subsequently into full length cDNA of EV71:eTLLβP20 “backbone”. The amplification of this 2.6kbp capsid protein genes (P1) region was performed using iProof High-Fidelity Master Mix (Bio-Rad). The nucleotide sequence for the unmodified P1 gene of CA16 is set forth in SEQ ID NO:87.

[0056] *Cloning of PCR amplified fragment CA16-P1 region into pACYC177:* Both pACYC177 and PCR amplified product of CA16-P1 region were separately digested with 50 U *NheI* and *XhoI* at reaction temperature of 37° C for 2 hours. Enzymatic reaction was inactivated at 65° C for 30 minutes. Both digested pACYC177 and CA16-P1 were gel purified. The restriction endonuclease (RE) digested CA16-P1 DNA was then ligated to the digested pACYC177 using 5U T4 DNA ligase (Fermentas) in a 20 ul reaction mix incubated at 22° C for 1 hr followed by inactivation at 70° C for 5 mins. Ten microlitre of purified pACYC177

carrying CA16-P1 DNA, now designated as pACYC177(CA16-P1), was transformed into XL10 electro-competent *E. coli* cells by electroporation and the transformed *E. coli* cells were recovered as described above.

[0057] *Site-directed mutagenesis CA16 P1 gene:* Site-directed mutagenesis was performed on the plasmid carrying the P1 region of CA16 to change the 858th amino acid from Lysine to Threonine. Primers pACYC-CA16P1-TITTTL-F and pACYC-CA16P1-TITTTL-R were run together with 50 ng of the plasmid, pACYC177(CA16-P1), using iProof High-Fidelity DNA polymerase (Bio-Rad) with initial denaturation of 98° C for 2 mins followed by 15 cycles of 98° C for 10 secs, 66° C for 30 secs and 72° C for 4 mins, continued by 35 cycles of 98° C for 10 secs, 69° C for 30 secs and 72° C for 4 mins, and final extension of 72° C for 5 mins. The PCR reaction was then spin column purified before undergoing *DpnI* digestion. The methylated plasmid was removed by 40 U *DpnI* (New England Biolabs) incubated at 37° C for 6 hrs followed by inactivation at 80° C for 20 mins. The reaction mixture was then spin column purified and transformed into XL10 electro-competent *E. coli* cells as described above. Screening was performed to select for clones which have the required SDM site. QIAGEN plasmid maxi kit (Qiagen, Germany) was used to extract large quantities of plasmids from these clones. The plasmid carrying the required nucleotide changes at the site directed mutagenesis is designated as pACYC177(eCA16-P1).

[0058] *Construction of infectious full-length cDNA clone of TLLeCA16:* Both purified plasmids, pACYC177(EV71:eTLLβP20) and pACYC177(eCA16-P1) were separately digested with 20U *MluI* and *EagI* incubated at 37° C for 6 hours. Following which, the digestion was terminated by inactivation at 65° C for 30 minutes. The digested products were gel purified. The digested fragment containing P1 region of CA16 derived from pACYC177(eCA16-P1) was ligated to digested pACYC177(EV71:eTLLβP20) which is devoid of original P1 region using 2 U T4 DNA ligase (Fermentas) in a 20 ul reaction mixed. The reaction mix was incubated at 22°C for 1hr followed by inactivation at 70° C for 5 mins. The ligated product was transformed into XL10 electro-competent *E. coli* cells by electroporation in 100 ul reaction followed by incubation in 400 ul of SOC medium at 30° C for 2 hrs. Following which, 150 ul of the electro-transformed cells were then plated onto LB plate containing 100 ug/ml Ampicillin and 35 ug/ml Kanamycin. Screening for positive clones and sequencing of the full genome were subsequently performed accordingly. The plasmid pACYC177 carrying the infectious full length cDNA genome was designated as pACYC177(TLLeCA16). The nucleotide sequence for TLLeCA16 is set forth in SEQ ID NO:88.

[0059] *Recovering engineered chimeric TLLeCA16 virus:* The engineered chimeric TLLeCA16 virus was recovered by transfecting pACYC177(TLLeCA16) plasmids into Vero cells by the similar laboratory method as described earlier for recovering engineered EV71:eTLL β P20 virus by transfection using Lipofectamine 2000 Transfection Reagent (Invitrogen). The virus was further passed in Vero cells incubated at 28° C for 20 passages to confirm its genetic and phenotypic stability.

EXAMPLE 6

cDNA Copies and Recovered Viruses of EV71:eTLL β P20, EV71:TLLeC5 and TLLeCA16

[0060] The pACYC177(EV71:eTLL β P20) plasmid carries the infectious cDNA copy of the complete genome of EV71:eTLL β P20. The virus strain was recovered by transfection of the pACYC177(EV71:eTLL β) plasmids in Vero cells as described earlier. The recovered virus was subsequently passed in Vero cells incubated at 28° C for another 20 passages (EV71:eTLL β P20) to confirm its genetic and phenotypic stability.

[0061] The pACYC177(EV71:TLLeC5) plasmid carries the infectious cDNA copy of the complete genome of EV71:TLLeC5. The engineered chimeric EV71:TLLeC5 virus was recovered by transfecting the pACYC177(EV71:TLLeC5) plasmids into Vero cells by the similar laboratory process as described earlier for recovering engineered EV71:eTLL β virus. The recovered virus was similarly passage in Vero cells incubated at 28° C for another 20 passages to confirm its genetic and phenotypic stability.

[0062] The pACYC177(TLLeCA16) plasmids carries the infectious cDNA copy of the complete genome of the TLLeCA16 virus. The engineered chimeric TLLeCA16 virus was recovered by transfecting pACYC177(TLLeCA16) plasmids into Vero cells by the similar laboratory method as described earlier for recovering engineered EV71:eTLL β virus using Lipofectamine 2000 Transfection Reagent (Invitrogen). The recovered virus was similarly passage in Vero cells incubated at 28° C for another 20 passages to confirm its genetic and phenotypic stability.

EXAMPLE 7

Genome Structure of Engineered Enteroviruses

[0063] The schematic diagrams representing the genome structure and respective encoded proteins of engineered stable cold-adapted temperature sensitive enteroviruses (EV71:eTLL β P20, EV71:TLLeC5 and TLLeCA16) are shown in Figures 1, 2 and 3.

Schematically, the representative genomic structure and encoded proteins of EV71:eTLL β P20 is no different from that of EV71:TLL β P20 except at the nucleotide level where two specific restriction endonuclease (*Mlu*I and *Eag*I) sequences (*ACGCGT* and *CGGCCG*) were engineered into the genome, one in VP4 gene sequence at around the beginning of protein coding genes region (nt 770 to 775) and the other in 2A gene sequence at around the VP1 and 2A genes junction (nt 3341 to 3346). The nucleotide changes (in lower case, gCGatc) at the introduced *Mlu*I site (*ACGCGT*) lead to an amino acid change (serine > valine, S10V) and the nucleotide changes (in lower case, tGCCa) at the introduced *Eag*I site (*CGGCCG*) lead to an amino acid change (glutamine > arginine, Q867R) at amino acid position 867 of the viral polyprotein. Both EV71:TLLcC5 and TLLcCA16 retain the same nucleotide sequence changes at the two introduced specific restriction endonuclease (*Mlu*I and *Eag*I) sites as EV71:eTLL β P20. In addition, the nucleotide sequences of capsid protein genes (P1) region of EV71:TLLcC5 were derived entirely from the equivalent region of the genome of an isolate of EV71 that belongs to genotype C5 and the nucleotide sequences of capsid protein genes (P1) region of TLLcCA16 were derived from equivalent nucleotide sequences of a coxsackievirus A16 (genogroup B, lineage 2), as indicated by different pattern scheme (dotted bar and vertical-line bar, respectively) in Figures 2 and 3.

EXAMPLE 8

Genetic Changes of Engineered Enteroviruses

[0064] The complete genomes of genetically engineered stable cold-adapted temperature sensitive enteroviruses (EV71:eTLL β P20, EV71:TLLcC5 and TLLcCA16) after 20 passages in Vero cells incubated at 28⁰C were completely sequenced and their respective nucleotide and amino acid differences from the original source genome sequences (EV71:TLL β P20 except for the P1 region which were replaced with the respective P1 regions of EV71 genotype C5 and CA16) in each respective gene are shown in Tables 5, 6 and 7. The numbering in bold and within the parenthesis are changes which is due to intentional introduction for specific purposes. The genome of EV71:eTLL β P20 has 9 nucleotide differences (6 was purposefully introduced to create sites for specific restriction endonucleases) from its original genomic source sequence and resulted in 3 amino acids changes (2 was introduced) (Table 5). One spontaneous mutation (A 2966 G) in the genome of EV71:eTLL β P20 which does not result in amino acid change occurred in the VP1 gene. The other 2 spontaneous mutations (C 754 T, C 3362 A) that resulted in an amino acid change (serine > leucine, S3L) occurred in the VP4 gene.

TABLE 5
 Number of Nucleotides (NT) and Corresponding Amino
 Acids (AA) Mutations Occurred in Each of the Genomic Segments
 of EV71:eTLLP20 in comparison with the genome of EV71:TLLβP20

Viral Gene Region/Protein		Number of Mutations	
		NT	AA
5'-UTR	"Cloverleaf"	-	-
(1-746)	IRES	-	-
P1	VP4	5 *(4)	2 (1)
(747-3332)	VP2	-	-
	VP3	-	-
	VP1	1	-
P2	2A	3 (2)	1 (1)
(3333-5066)	2B	-	-
	2C	-	-
P3	3A	-	-
(5067-7325)	3B	-	-
	3C	-	-
	3D	-	-
3'-UTR		-	-
(7326-7411)			
Total		9 (6)	3 (2)

*The figure numbers in bold and within the parenthesis are changes which were purposefully introduced mutations.

TABLE 6

Number of Nucleotides (NT) and Corresponding Amino Acids (AA)
Mutations Occurred in Each of the Genomic Segments of EV71:TLLcC5
in Comparison with the Genome of EV71:TLL β P20 (Except for P1 Region
Which is Compared with Respect to Original P1 Region of EV71 Genotype C5)

Viral Gene Region/Protein		Number of Mutations	
		NT	AA
5'-UTR	"Cloverleaf"	-	NA
(1-746)	IRES	-	NA
P1	VP4	1*(1)	1(1)
(747-3332)	VP2	-	-
	VP3	-	-
	VP1	1(1)	1(1)
P2	2A	5(4)	2(2)
(3333-5066)	2B	-	-
	2C	1	1
P3	3A	-	-
(5067-7325)	3B	-	-
	3C	-	-
	3D	-	-
3'-UTR		-	NA
(7326-7411)			
Total		8(6)	5(4)

*The figure numbers in bold and within the parenthesis are changes which were purposefully introduced mutations.

TABLE 7

Number of Nucleotides (NT) and Corresponding Amino Acids (AA)
Mutations Occurred in Each of the Genomic Segments of TLLeCA16 in
Comparison with the Genome of EV71:TLL β P20 (Except for P1 Region Which
is Compared with Respect to Original P1 Region of Parental Cocksackievirus CA16)

Viral Gene Region/Protein		Number of Mutations	
		NT	AA
5'-UTR	"Cloverleaf"	-	-
(1-746)	IRES	-	-
P1	VP4	4*(4)	1(1)
(747-3332)	VP2	1	1
	VP3	2	1
	VP1	3(1)	1(1)
P2	2A	4(2)	1
(3333-5066)	2B	-	-
	2C	-	-
P3	3A	-	-
(5067-7325)	3B	-	-
	3C	-	-
	3D	-	-
3'-UTR		-	-
(7326-7411)			
Total		14(7)	5(2)

*The figure numbers in bold and within the parenthesis are changes which were purposefully introduced mutations.

[0065] The genome of EV71:TLLeC5 differs from its original genomic source sequence by 8 nucleotides and 5 amino acids (Table 6). Of which, only 2 nucleotides (C 3362 A, A 5044 T) and 1 amino acids (asparagine > isoleucine, N1433I) were due to spontaneous mutations. The genome of TLLeCA16 differs from its original genomic source sequence by 14 nucleotides and

5 amino acids (Table 7). Of which, 7 nucleotides were due to spontaneous mutations, resulting in 3 changes in amino acids. One spontaneous nucleotide mutation (A 1212 G) resulting in amino acid change from threonine to alanine (T156A) occurred in the VP2 gene. Two spontaneous mutations (C 2140 T, G 2405 A) resulting in an amino acid change from alanine to valine (A465V) occurred in VP3 gene. Two spontaneous mutations (T 3341 C, G3344 C) occurred in VP1 gene do not lead to any amino acid change. However, two spontaneous mutations (C 3362 T, C 3424 G) occurred in 2A gene resulted in an amino acid change from serine to cysteine (S893C).

EXAMPLE 9

Phenotypic Characteristics of Engineered Enteroviruses

[0066] The genetically modified (EV71:eTLLβP20) and engineered chimeric (EV71:TLLcC5 and TLLcCA16) enteroviruses retained the cold-adapted temperature sensitive growth characteristics in Vero cells as their original parental virus strain (EV71:TLLβP20). All the virus strains demonstrate more efficient replication (infected cells achieving full CPE at a faster rate and producing a higher virus titre in the culture supernatant) in Vero cells incubated at 28°C in comparison to the cells incubated at 37° C. Similar to their original parental virus, 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 inoculated cells following 10 days of culture. The absence of virus progenies in culture supernatants was confirmed by lack of CPE and negative detection of virus antigen in inoculated cells after a blind passage of the respective resultant culture supernatants into fresh culture of Vero cells incubated at 28° C, 37° C and 39.5° C.

[0067] Incubation temperature of 28° C and 37° C was used to assess the virus replication characteristics of these three genetically engineered cold-adapted strains and the assays were repeated at least 4 times. After 20 passages in Vero cells incubated at the incubation temperature of 28° C, EV71:eTLLβP20 took 3 and 7 days to induce full CPE in monolayer of cultured Vero cells incubated at 28° C and 37° C respectively. The titres of EV71:eTLLβP20 were 2.15×10^7 CCID₅₀/ml and 4.64×10^6 CCID₅₀/ml when the titrated cultures were incubated at 28° C and 37° C respectively (Table 8). EV71:TLLcC5 also took 3 and 7 days to induced full CPE in monolayer of cultured Vero cells incubated at 28° C and 37° C respectively. EV71:TLLcC5 achieved a virus titre of 2.15×10^8 CCID₅₀/ml in the culture supernatant of infected Vero cells at an incubation temperature of 28° C and a titre of 2.15×10^7 CCID₅₀/ml at 37° C (Table 9). TLLcCA16 took 3 and 4 days to induced full CPE in monolayer of cultured Vero cells incubated

at 28° C and 37° C respectively. TLLeCA16 attained a virus titre of 4.64×10^7 CCID₅₀/ml at an incubation temperature of 28° C and a titre of 2.15×10^6 CCID₅₀/ml at 37° C (Table 10). In summary, all the three genetically engineered cold-adapted strains took less number of days to cause full cell deaths and achieved virus titres in the culture supernatants of approximately one log higher for the inoculated Vero cells that were incubated at 28° C.

TABLE 8

Assessment of Reversion from Cold-Adapted Temperature Sensitive Phenotype of EV71:eTLLβP20 after 6 Successive Passaging in Vero Cells Incubated at 37° C*

Virus Inoculum	Days to achieve Full Cytopathic Effect (CPE)			Virus Titre in CCID ₅₀ /ml	
	28° C	37° C	39.5° C	28° C	37° C
EV71:eTLLβP20	3	7	Day 10: 1+ CPE (IFA: -ve) Control cell : 1+ CPE	2.15×10^7	4.64×10^6
eTLLβP20 (37° C-P1)	3	4	Day 10: 1+ CPE (IFA: -ve) Control cell : 1+ CPE	3.16×10^7	1×10^7
eTLLβP20 (37° C-P2)	3	3	Day 10: 2+ CPE (IFA: -ve) Control cell : 1+ CPE	1×10^8	2.15×10^6
eTLLβP20 (37° C-P3)	4	3	Day 9: 4+ CPE (IFA: -ve) Control cell : 2+ CPE	5.87×10^7	1×10^7
eTLLβP20 (37° C-P4)	3	2	Day 5: 4+ CPE (IFA: +ve) Control cell : 1+ CPE	5.87×10^7	4.64×10^7
eTLLβP20 (37° C-P5)	3	2	Day 4: 3+ CPE (IFA: +ve) Control cell : 1+ CPE	1×10^8	4.64×10^6
eTLLβP20 (37° C-P6)	3	2	Day 4: 4+ CPE (IFA: +ve) Control cell : 1+ CPE	-	-

* The growth characteristic and titre of the virus at each passage was cultured or titrated in Vero cells at incubation temperature of 28° C, 37° C and 39.5° C.

P1: Passage 1, IFA: Indirect Immunofluorescence Assay. CPE in this context refers to cell death.

TABLE 9

Assessment of Reversion from Cold-Adapted Temperature Sensitive Phenotype of EV71:TLLcC5 after 6 Successive Passaging in Vero Cells Incubated at 37° C*

Virus Inoculum	Days to achieve Full Cytopathic Effect (CPE)			Virus Titre in CCID ₅₀ /ml	
	28° C	37° C	39.5° C	28° C	37° C
EV71:TLLcC5	3	7	Day10: 1+ CPE(IFA: -ve) Control cell 1+ CPE	2.15 X10 ⁸	2.15 X10 ⁷
TLLcC5 (37° C-P1)	6	6	Day 10: 2+ CPE(IFA: -ve) Control cell 2 CPE	3.16 X10 ⁸	4.64 X10 ⁶
TLLcC5 (37° C-P2)	5	5	Day 10: 2+ CPE (IFA: -ve) Control cell 1+ CPE	2.15 X10 ⁸	3.16 X10 ⁶
TLLcC5 (37° C-P3)	5	4	Day 10: 2+ CPE (IFA: -ve) Control cell 1+ CPE	1 X10 ⁸	1 X10 ⁸
TLLcC5 (37° C-P4)	4	4	Day 10: 3 CPE (IFA: +ve) Control cell 2 CPE	1 X10 ⁸	1 X10 ⁸
TLLcC5 (37° C-P5)	4	3	Day 10: 4+ CPE (IFA: +ve) Control cell 2 CPE	1 X10 ⁸	1 X10 ⁷
TLLcC5 (37° C-P6)	4	3	Day 10: 4 CPE (IFA: +ve) Control cell 2 CPE	-	-

* The growth characteristic and titre of the virus at each passage was cultured or titrated in Vero cells at incubation temperature of 28° C, 37° C and 39.5° C.

P1: Passage 1, IFA: Indirect Immunofluorescence Assay. CPE in this context refers to cell death.

TABLE 10

Assessment of Reversion from Cold-Adapted Temperature Sensitive Phenotype of TLLcC5A16 after 6 Successive Passaging in Vero Cells Incubated at 37° C*

Virus Inoculum	Days to achieve Full Cytopathic Effect (CPE)			Virus Titre in CCID ₅₀ /ml	
	28° C	37° C	39.5° C	28° C	37° C
TLLcCA16	3	4	Day10: 1+ CPE(IFA: -ve) Control cell 1+ CPE	4.64 X 10 ⁷	2.15 X 10 ⁶
TLLcCA16 (37° C-P1)	4	7	Day 10: 2+ CPE(IFA: -ve) Control cell 2 CPE	3.16X 10 ⁷	2.15 X 10 ⁶
TLLcCA16 (37° C-P2)	4	4	Day 10: 4+ CPE (IFA: +ve) Control cell 2 CPE	1 X10 ⁷	4.64 X10 ⁶

TLLeCA16 (37°C-P3)	5	4	Day 10: 4+ CPE (IFA: +ve) Control cell 1+ CPE	1 X10 ⁸	1.7 X10 ⁷
TLLeCA16 (37°C-P4)	5	4	Day 8: 4+ CPE (IFA: +ve) Control cell 1+ CPE	4.64 X10 ⁷	1 X10 ⁷
TLLeCA16 (37°C-P5)	5	3	Day 8: 4+ CPE (IFA: +ve) Control cell 1+ CPE	2.15 X10 ⁸	4.64 X10 ⁶
TLLeCA16 (37°C-P6)	5	4	Day 7: 4+ CPE (IFA: +ve) Control cell 1+ CPE	-	-

* The growth characteristic and titre of the virus at each passage was cultured or titrated in Vero cells at incubation temperature of 28° C, 37° C and 39.5° C.

P1: Passage 1, IFA: Indirect Immunofluorescence Assay. CPE in this context refers to cell death.

EXAMPLE 10

Assay for Reversion of Temperature Dependent Growth Sensitivity

[0068] Assessment for reversion of virus phenotypic characteristics basing on temperature dependent growth sensitivity was performed by 6 successive passaging of these three genetically engineered cold-adapted temperature sensitive strains in Vero cells incubated at 37° C. The clarified culture supernatant of each successive passage at 37° C was re-inoculated into fresh culture of Vero cells as soon as the inoculated cells achieved full CPE. The growth characteristics, in term of kinetics of cell deaths and virus titre at incubation temperature of 28° C, 37° C and 39.5° C, of the virus strains derived from each respective passage in Vero cells incubated at 37° C are shown in Tables 8, 9, and 10. EV71:eTLLβP20 was unable to produce viable infectious particles (lack of positive immunofluorescent stained cells) in Vero cells at incubation temperature of 39.5° C after 3 successive passages in cells incubated at 37° C. EV71:TLLeC5 was also unable to produce viable infectious particles in Vero cells at incubation temperature of 39.5° C after 3 successive passages in cells incubated at 37° C. TLLeCA16 was unable to produce viable infectious particles (lack of positive immunofluorescent stained cells) in Vero cells at incubation temperature of 39.5° C after 2 successive passages in cells incubated at 37° C.

EXAMPLE 11

Mutations in the Genomes of EV71:eTLLβP20, EV71:TLLeC5 and TLLeCA16 in Temperature Reversion Assay

[0069] The complete genomes of EV71:eTLLβP20, EV71:TLLeC5 and TLLeCA16 at passage 3 and 6 after 6 successive passages in Vero cells incubated at 37° C were sequenced and analysed for genetic mutations. The number of nucleotide and corresponding amino acids

mutations at each respective segment of the genes of EV71:eTLL β P20, and EV71:TLLcC5 at passage 3 and 6 as a result of 6 successive passaging in Vero cells at incubation temperature of 37° C are shown in Tables 11 and 12. At passage 3, reversion mutation to its wild-type virus genomic sequence occurred in viral 2A gene at nucleotide position 3346 (G 3346 A) lead to an amino acid change from arginine to glutamine (R867Q) at amino acid position 867 of EV71:eTLL β P20 polyprotein. The same reversion mutation to its wild-type virus genomic sequence was maintained in EV71:eTLL β P20 at passage 6. In addition, a deletion of 15 nucleotides leading to deletion of 5 amino acids and a change of amino acid asparagine to histidine (N667H) occurred in the VP1 gene of about 58% (7/12) of the genomes of EV71:eTLL β P20 sequenced at passage 6 (Table 11). Interestingly, the same reversion mutation to its wild-type virus genomic sequence occurred in viral 2A gene at nucleotide position 3346 (G 3346 A) lead to an amino acid change from arginine to glutamine (R867Q) at amino acid position 867 noted in EV71:eTLL β P20 polyprotein also occurred in the consensus genomic sequence of EV71:TLLcC5 at both passage 3 and 6. In addition to the reversion mutation, the genomic sequence of EV71:TLLcC5 at both passage 3 and 6 in the temperature reversion study had a spontaneous mutation in the viral 2C gene (C 4566 T) leading to an amino acids change from histidine to lysine (H1274Y). No mutation was detected in the consensus genomic sequence of TLLcCA16 at both passage 3 and 6 though a mix population of either nucleotide guanine (G) or adenine (A) occurred at the nucleotide position 1212 on sequencing its complete genome. The PCR amplified product containing the mix population of nucleotide sequences was cloned into plasmid pZErO™-2 and transformed into *E. coli* strain TOP10 (Invitrogen). The plasmids of twenty-three selected colonies of *E. coli* were screened for the mutation. Thirteen of the 23 colonies retained the guanine residue and 10 colonies having the plasmids carried adenine residue but the nucleotide mutation does not lead to any amino acids change of the virus consensus genome.

TABLE 11

Number of Nucleotides (NT) and Corresponding Amino Acids (AA)
Mutations Occurred in Each of the Genomic Segments of Virus Strains Derived from
Temperature Sensitive Reversion Study in Comparison with the Genome of EV71:eTLL β P20

Viral Gene Region/Protein	EV71:eTLL β P20 (37° C-P3)		EV71:eTLL β P20 (37° C-P6)	
	NT	AA	NT	AA
5'-UTR "Cloverleaf"	-	-	-	-
(1-746) IRES	-	-	-	-

P1 (747-3332)	VP4	-	-	-	-
	VP2	-	-	-	-
	VP3	-	-	-	-
	VP1	-	-	Mixed populations of 2 strains (12 clones screened): 1) Without deletion (5 clones) 2) Deletion of 15nt (7 clones)(2731-2745)	Mixed populations of 2 strains: 1) Without deletion 2) Deletion of 5aa(662-666) and 1 aa change- N 667 H 1(-)
P2	2A	1(1R) G 3346 A	1(1R) R 867 Q	1(1R) G 3346 A	1(1R) R 867 Q
(3333-5066)	2B	-	-	-	-
	2C	-	-	-	-
P3	3A	-	-	-	-
(5067-7325)	3B	-	-	-	-
	3C	-	-	-	-
	3D	-	-	-	-
3'-UTR (7326-7411)		-	-	-	-
Total		1(1R)	1(1R)	1(1R)	1(1R)

TABLE 12

Number of Nucleotides (NT) and Corresponding Amino Acids (AA)
Mutations Occurred in Each of the Genomic Segments of Virus Strains Derived from
Temperature Sensitive Reversion Study in Comparison with the Genome of EV71:TLLc5

Viral Gene Region/Protein	TLLc5 (37° C-P3)		TLLc5 (37° C-P6)	
	NT	AA	NT	AA
5'-UTR "Cloverleaf"	-	-	-	-
(1-746) IRES	-	-	-	-
P1	-	-	-	-
(747-3332)	-	-	-	-
VP4	-	-	-	-
VP2	-	-	-	-
VP3	-	-	-	-
VP1	-	-	-	-
P2	1(1R) G 3346 A	1(1R) R 867 Q	1(1R) G 3346 A	1(1R) R 867 Q
(3333-5066)	2B	-	-	-
	2C	1 C 4566 T	1 C 4566 T	1 H 1274 Y

P3 (5067-7325)	3A	-	-	-	-
	3B	-	-	-	-
	3C	-	-	-	-
	3D	-	-	-	-
3'-UTR (7326-7411)		-	-	-	-
Total		2(1R)	2(1R)	2(1R)	2(1R)

[0070] The use of the terms “a” and “an” and “the” and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms “comprising,” “having,” “including,” and “containing” are to be construed as open-ended terms (i.e., meaning “including, but not limited to,”) unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. For example, if the range 10-15 is disclosed, then 11, 12, 13, and 14 are also disclosed. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”) provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0071] It will be appreciated that the methods and compositions of the instant invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. Embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

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WHAT IS CLAIMED IS:

1. A cold-adapted temperature sensitive virus strain EV71:eTLL β P20, EV71:TLLeC5 or TLLeCA16.
2. The cold-adapted temperature sensitive virus strain of claim 1, wherein the strain is EV71:eTLL β P20.
3. The cold-adapted temperature sensitive virus strain of claim 1, wherein the strain is EV71:TLLeC5.
4. The cold-adapted temperature sensitive virus strain of claim 1, wherein the strain is TLLeCA16.
5. A composition comprising one or more of the cold-adapted temperature sensitive virus strain of any one of claims 1 to 4.
6. The composition of claim 5 further comprising a cold-adapted temperature sensitive Enterovirus 71 strain EV71:TLL β P20.
7. The composition of claim 5 or 6 which further comprises a pharmaceutically acceptable carrier.
8. The composition of claim 5, 6 or 7 which further comprises an adjuvant.
9. The composition of any one of claims 5 to 8 which is a vaccine.
10. The composition of claim 9, wherein the vaccine is an oral live attenuated vaccine.
11. The composition of claim 9, wherein the vaccine is an inactivated vaccine.
12. A kit for immunization of a subject with of a cold-adapted temperature sensitive virus strain comprising the cold-adapted temperature sensitive virus strain of any one of claims

- 1 to 4 or an inactivated form thereof, or a composition of any one of claims 5 to 11, a pharmaceutically acceptable carrier, and an instructional material for the use thereof.
13. The kit of claim 12 which further comprises an applicator.
 14. A method of making a vaccine comprising using the cold-adapted temperature sensitive virus strain of any one of claims 1 to 4 or an inactivated form thereof.
 15. A method of making a vaccine comprising using one or more nucleic acids comprising the nucleotide sequence set forth in SEQ ID NO:84, SEQ ID NO:86 or SEQ ID NO:88.
 16. The method of claim 15 further comprising using a nucleic acid comprising the nucleotide sequence set forth in SEQ ID NO:83.
 17. Use of the cold-adapted temperature sensitive virus strain of any one of claims 1 to 4 or an inactivated form thereof for vaccine development.
 18. Use of one or more nucleic acids comprising the nucleotide sequence set forth in SEQ ID NO:84, SEQ ID NO:86 or SEQ ID NO:88 for vaccine development.
 19. The use of claim 18 further comprising a nucleic acid comprising the nucleotide sequence set forth in SEQ ID NO:83.
 20. A cold-adapted temperature sensitive virus strain of any one of claims 1 to 4 or an inactivated form thereof for use in vaccine development.
 21. A nucleic acid comprising the nucleotide sequence set forth in SEQ ID NO:84, SEQ ID NO:86 or SEQ ID NO:88 for use in vaccine development.
 22. An isolated nucleic acid comprising the nucleotide sequence set forth in SEQ ID NO:84, SEQ ID NO:86 or SEQ ID NO:88.

23. A virus vector comprising the nucleotide sequence set forth in SEQ ID NO:84, SEQ ID NO:86 or SEQ ID NO:88.

ABSTRACT

DEVELOPMENT OF STABLE COLD-ADAPTED
TEMPERATURE SENSITIVE CHIMERIC ENTEROVIRUSES

[0130] The present invention relates to chimeric virus strains, particularly to the stable cold-adapted temperature sensitive Enterovirus 71 strains EV71:eTLL β P20 and EV71:TLLeC5 and to the stable chimeric enterovirus strain TLLeCA16. The present invention also relates to vectors containing the nucleotide sequences of these strains and vaccines containing these strains.

Figure 1