

COLD-ADAPTED TEMPERATURE SENSITIVE STRAINS
OF ENTEROVIRUS 71 AND PROCESSES OF DEVELOPING
COLD-ADAPTED TEMPERATURE SENSITIVE VIRUS STRAINS

SEQUENCE SUBMISSION

[0001] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is entitled 2577224PCTSequenceListing.txt, created on 11 January 2013 and is 21 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

[0002] The present invention relates to cold-adapted temperature sensitive Enterovirus 71 strains, particularly to the cold-adapted temperature sensitive Enterovirus 71 strains EV71:TLL β P20 and EV71:TLL α P20. The present invention also relates to processes of developing cold-adapted temperature sensitive virus strains, particularly RNA virus strains.

[0003] 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.

[0004] Hand foot and mouth disease (HFMD) is caused by a group of human enteroviruses which essentially belong to the enterovirus species A. Of these, enterovirus 71 (EV71) and coxsackievirus A16 (CA16) are responsible for causing more than 90% of HFMD cases. Besides causing HFMD, EV71 has been known to cause severe neurological diseases with deaths especially in young children.

[0005] Human enterovirus 71 (EV71) is a small non-enveloped virus of approximately 30 nm in size with a single-stranded positive RNA genome of about 7,450 nucleotides. The virus is classified as *Human enterovirus A* species under the genus *Enterovirus* within the family *Picornaviridae* (Alexander et al., 1994; Melnick, 1996). EV71 is divided into three major genogroups (denoted A, B, and C), and genogroups B and C are further subdivided into subgenogroups B1 to B5 and C1 to C5, based on phylogenetic analysis of its major capsid protein (VP1) gene (Bible et al., 2008). EV71 has been associated with an array of clinical diseases including hand, foot and mouth disease (HFMD), aseptic meningitis, encephalitis and poliomyelitis-like paralysis mainly in infants and young children (Alexander et al., 1994; Melnick, 1996). The virus was first isolated from a child with aseptic meningitis in California, USA and subsequently characterized as a new serotype of the genus *Enterovirus* (Schmidt et al.,

1974). In the years following its initial isolation, outbreaks of HFMD with complications due to the virus were reported in various parts of the world (Blomberg et al., 1974; Kennett et al., 1974; Deibel et al., 1975; Hagiwara et al., 1978).

[0006] In recent years, EV71 infections have become a major public health burden and concern throughout the world especially in the Asia-Pacific Region following the occurrence of epidemic and sporadic outbreaks of neurovirulent EV71. Neurovirulence of EV71 first gained prominent global attention during an outbreak in Bulgaria in 1975 which caused 705 cases of poliomyelitis-like disease with 44 deaths (Chumakov et al., 1979; Shindarov et al., 1979). Outbreak of similar nature occurred in Hungary in 1978 and resulted in many cases of poliomyelitis-like disease and 47 deaths (Nagy et al., 1982). Subsequently, several milder epidemics of CNS disease associated with EV71 have been reported in New York, Hong Kong, Australia and Philadelphia (Chomnaitree et al., 1958; Samuda et al., 1987; Gilbert et al., 1988; Hayward et al., 1989). In Japan two epidemics of EV71 occurred with most of the cases characterized by HFMD and a low incidence of CNS disease (Tagaya et al., 1981; Ishimaru et al., 1980; Hagiwara et al., 1983). In 1997, a large outbreak of HFMD due to highly neurovirulent EV71 emerged in Malaysia and caused 48 deaths. A larger outbreak occurred in Taiwan in 1998 with more than 100,000 cases of HFMD, 405 severe infections and 78 deaths due to acute brainstem encephalomyelitis with neurogenic cardiac failure and pulmonary oedema (Lum et al., 1998a; Lum et al., 1998b; Chang et al., 1998; Yan et al., 200; Liu et al., 2000; Wang et al., 2000). A total of 488,955 HFMD cases with 126 deaths was recorded in the mainland of People's Republic of China in 2008 (Chinese Center, 2008). The number of HFMD cases was reported to increase to 1,155,525 with 353 fatalities in 2009 (Yang et al., 2011). In 2010, the country experienced the largest ever outbreak with more than 1.7 million HFMD cases, 27,000 patients with severe neurological complications and 905 deaths. In all the three outbreaks, almost all the severe cases with neurological complications and deaths were due to EV71 (Zeng et al., 2012).

[0007] Currently, the molecular determinants of virus virulence and pathogenesis of EV71 infections are still not fully understood. Neither is there any anti-viral drug that has been approved for clinical treatment of severe infections and associated neurological complications nor any vaccine available for use to control and prevent recurrent outbreaks. In terms of control and preventive strategies, the development of cost effective vaccines especially for use in developing countries is of top priority and great urgency. Various types of vaccines against EV71 under investigation and development appear to elicit an immune response in rodents or

monkeys (Wu et al., 2001; Arita et al., 2005; Chiu et al., 2006; Arita et al., 2007; Tung et al., 2007; Chung et al., 2008; Chen et al., 2008; Ong et al., 2010; Chen et al., 2011; Lee and Chang, 2010; Xhang et al., 2010). Though virus-like particle vaccine and subunit peptide vaccine basing on VP1 capsid remain viable potential vaccine strategies worthy of further study and development, the injectable inactivated and oral attenuated EV71 vaccines remain the most promising candidates based on vast past experience of the development and usage of inactivated injectable Salk and live attenuated oral Sabin poliovirus vaccines in the control and near eradication of wild-type poliovirus infections (Zhang et al., 2010).

[0008] Currently, there is no vaccine available in the market to protect children against infections and hand foot and mouth disease due to EV71. Based on recent information, there are two centers in People Republic of China, a center in Singapore and a center in Taiwan which are in the process of developing injectable inactivated EV71 vaccine (press releases and personal communication). The Enterovirus Unit in the National Institute of Infectious Diseases, Tokyo, Japan headed by Dr H. Shimizu had been undertaking intensive study on the pathogenesis of EV71 in monkeys and temperature sensitive strains of EV71 as potential candidate oral live attenuated EV71 vaccine since the 1980s (Arita et al., 2005; Arita et al., 2007). All the potential vaccine candidates of temperature sensitive strains of EV71 in his monkey study were still able to cause neuro-invasion and histopathological lesions of CNS though to a lesser degree in comparison to the wild-type virus after intravenous inoculation.

[0009] Live attenuated vaccines represent one of the first successful methods of vaccination, dated back to the 18th century when the British doctor Edward Jenner began using cowpox virus to vaccinate children against the devastating disease of smallpox. Live attenuated vaccines use live viruses or microorganisms that have been weakened so that they are unable to cause disease, yet induce protective immune response. Traditional, classical and genetic methods have been successful to some extent in attenuating viruses and microorganism for use as live attenuated vaccine.⁴⁴⁻⁴⁸ Traditional method uses naturally occurring related organisms that are avirulent in humans, such as, use of cowpox or vaccinia viruses. Classical method involves rounds of growth of virulent viruses or microorganism under conditions that attenuated them, such as, in tissue culture or harsh liquid media. Genetic method utilizes modern molecular biotechnology to manipulate the genomes to reduce their virulence (Huygelen, 1997; Robinson, 2008; Coleman et al., 2008; Lauring et al., 2010; Kenney et al., 2011). In classical method of obtaining temperature sensitive phenotype viruses as marker of attenuation, the wild-type virus is plaque-selected via plaque assay technique by culturing the virus in suitable cells incubated at lower

temperature. The selected virus strain is subsequently passaged repeatedly at the targeted lower incubation temperature (Hagiwara et al., 1982; Hashimoto and Hagiwara, 1983; Richman and Murphy, 1997).

[0010] It is desired to develop virus strains which can be used to treat viral diseases which retain phenotypic and genetic stability in specific *in vitro* cell culture conditions and do not exhibit neuro-virulence in monkeys following intravenous inoculation. It is also desired to develop cold-adapted temperature sensitive strains of viruses, including RNA viruses, that are derived following serial passages in cell-culture.

SUMMARY OF THE INVENTION

[0011] The present invention relates to cold-adapted temperature sensitive Enterovirus 71 strains, particularly to the cold-adapted temperature sensitive Enterovirus 71 strains EV71:TLL β P20 and EV71:TLL α P20. The present invention also relates to processes of developing the cold-adapted temperature sensitive virus strains, particularly RNA virus strains.

[0012] Thus, in one aspect, the present invention provides the cold-adapted temperature sensitive Enterovirus 71 strains. In one embodiment, the cold-adapted temperature sensitive Enterovirus 71 strain is EV71:TLL β P20 as described herein. In another embodiment, the cold-adapted temperature sensitive Enterovirus 71 strain is EV71:TLL α P20 as described herein.

[0013] The Enterovirus 71 strains of the present invention are prepared by a method to attenuate Enterovirus 71 using temperature sensitivity as a phenotypic marker. The method is an *in vitro* laboratory process to change the biologic growth characteristic of the virus to adapt for optimal replication at incubation temperature of below 30° C. The adaption process, which is described in detail below, is carried out in a systematic stepwise manner of incremental lower incubation temperature for culturing the virus until the targeted temperature chosen for optimal replication of the virus is achieved.

[0014] In a second aspect, the present invention provides a composition comprising the cold-adapted temperature sensitive Enterovirus 71 strains described herein. In one embodiment, the composition comprises an effective amount of the virus strains described herein. 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 a cold-adapted temperature sensitive Enterovirus 71 strain described herein are prepared using techniques well known to the skilled artisan. Such vaccines are useful for providing immunity

against the parent virus strain by administering the vaccine to a subject, such as a human subject, using techniques well known to the skilled artisan.

[0015] 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 a cold-adapted temperature sensitive Enterovirus 71 strain described herein. In one embodiment, the protective immune response protects the subject against a disease caused by Enterovirus 71. 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, a cold-adapted temperature sensitive Enterovirus 71 strain described herein is administered as a vaccine. In an additional embodiment, the subject has been exposed to wild-type Enterovirus 71. In another embodiment, the administration of a cold-adapted temperature sensitive Enterovirus 71 strain described herein prevents a subject, such as a human subject, from becoming afflicted with an Enterovirus 71-associated disease. In an additional embodiment, the subject has been exposed to wild-type Enterovirus 71. In a further embodiment, the administration of a cold-adapted temperature sensitive Enterovirus 71 strain described herein delays the onset of or slows the rate of progression of an Enterovirus 71-associated disease in a virus-infected subject, such as a human subject.

[0016] In fourth aspect, the present invention provides a method to attenuate a virus using temperature sensitivity as a phenotypic marker. In accordance with this aspect, the method develops cold-adapted temperature sensitive virus strains. The method of the present invention is an *in vitro* laboratory process to change the biologic growth characteristic of the virus to adapt for optimal replication at incubation temperature of below 30° C. The adaption process is carried out in a systematic stepwise manner of incremental lower incubation temperature for culturing the virus until the targeted temperature chosen for optimal replication of the virus is achieved. Thus, in accordance with the present invention, the method comprises the following steps: (i) preparing a reference stock of parental wild-type virus, (ii) incubating a culture of cells infected with the reference stock of parental wild-type virus at higher multiplicity of infection (MOI) and an incubation temperature of about 34° C to about 36° C, preferably about 34° C, for five or more passages until a full cytopathic effect (CPE) is obtained at each passage using an inoculum of a lower MOI and a shorter period of incubation for each passage to obtain full CPE, (iii) incubating a culture of cells infected with the resultant virus of the previous step at higher

MOI and an incubation temperature of about 1° C to about 3° C lower than in the previous step for five or more passages until a full cytopathic effect (CPE) is obtained at each passage using an inoculum of a lower MOI and a shorter period of incubation for each passage to obtain full CPE, and (iv) repeating step (iii) in a systematic stepwise manner of incremental lower incubation temperature until the targeted temperature chosen for optimal replication of the virus is achieved. In one embodiment, the targeted temperature is about 26° C to about 29° C, preferably about 28° C. In one embodiment, the incrementally lower incubation temperature is a temperature lowered about 1° C to about 2° C.

[0017] In one embodiment the reference stock of parental wild-type virus is prepared by incubating a culture of cells infected with a wild-type virus at a temperature of about 36° C to about 38° C, preferably about 37° C for one or two passages until a full cytopathic effect (CPE) is obtained. Aliquots of culture supernatant containing the produced virus are placed in vials or other suitable storage devices. This culture supernatant serves as a reference stock of parental wild-type virus. The reference parental wild-type virus is used for subsequent attenuation process. In another embodiment, aliquots of the reference stock of parental wild-type virus are stored at a suitable temperature, such as at -80° C.

[0018] In one embodiment, the virus is any virus. In another embodiment, the virus is an RNA virus. In an additional embodiment, the RNA virus is a plus strand RNA virus. In a further embodiment, the virus is a member of the Picornaviridae family. In another embodiment, the virus is a member of the Enterovirus genus. In one embodiment, the virus is Enterovirus 71 (EV71). In another embodiment, the virus is cocksackievirus A16 (CA16). The method of the present invention is useful in producing cold-adapted temperature sensitive virus strains of any of these viruses, including but not limited to cold-adapted temperature sensitive strains of EV71 and CA16.

[0019] In one embodiment, the cell to be infected by the virus is any cell which is permissive for the growth of the virus. In a preferred embodiment, the cells are Vero cells (ATCC CCL-81). In one embodiment, the cells are maintained by regular passage in a medium suitable for growth of the cells. In an embodiment in which the cells are Vero cells, the Vero cells are cultured in Dulbecco's modified Eagles's medium (DMEM) supplemented with 10% fetal calf serum (FCS). In one embodiment, Vero cells maintained in DMEM supplemented with 1% FCS is used for production of the parental wild-type virus, virus culture, attenuation, titration and assessment of temperature sensitive phenotype. In another embodiment, the DMEM is

supplemented with 1% FCS for adapting the virus strains for replication in successively lowered temperature of incubation.

[0020] The present invention also relates to the cold-adapted temperature strains of virus produced by the method described herein. The cold-adapted temperature viruses produced by the method described herein are useful in the production of vaccines using techniques well known to the skilled artisan. Such vaccines are useful for providing immunity against the parent virus strain by administering the vaccine to a subject using techniques well known to the skilled artisan.

BRIEF DESCRIPTION OF THE FIGURES

[0021] Figure 1a shows a peripheral blood mononuclear cell (arrow), derived from the blood of a monkey on day 4 after given an intravenous dose of enterovirus 71 (EV71:TLLBP20), staining positive by indirect immunofluorescence assay using a commercial monoclonal antibody specific for the virus.

[0022] Figure 1b shows photograph of GelRed-stained electrophoresed agarose gels showing One-step RT-PCR amplified products of tissues derived from Day 4 post-immunized monkeys (2202F, 2891F) using an oligonucleotide primer pair specific for detection of enterovirus 71. Expected size of RT-PCR amplified product is 427 bp. The lanes in the two gels are as follows. *Gel 1*: Lane 1: 100 bp DNA ladder; Lane 2: 2202F-Heart; Lane 3: 2202F-Spleen; Lane 4: 2202F-Lymph Node; Lane 5: 2202F-Kidney; Lane 6: 2202F-Liver; Lane 7: 2891F-Heart; Lane 8: 2891F-Spleen; Lane 9: 2891F-Lymph Node; Lane 10: 2891F-Kidney; Lane 11: 2891F-Liver; Lane 12: 2202F-Brain stem (Pons); Lane 13: 2202F-Brain stem (medulla oblongata); Lane 14: 2202F-Cortex (Gyrus); Lane 15: 2202F-Spinal Cord (Cervical); Lane 16: 2202F-Spinal Cord (Lumbar); Lane 17: 2202F-Spinal Cord (Thoracic); Lane 18: 2891F- Brain stem (medulla oblongata); Lane 19: 2891F- Brain stem (Pons); Lane 20: 2891F-Cortex (Left Cerebellum). *Gel 2*: Lane 21: 100 bp DNA ladder; Lane 22: 2891F-Cortex (Right Cerebellum); Lane 23: 2891F-Spinal Cord (Cervical); Lane 24: 2891F- Spinal Cord (Lumbar); Lane 25: 2891F- Spinal Cord (Thoracic); Lane 26: No Template Control; Lane 27: 100 bp DNA ladder.

DETAILED DESCRIPTION OF THE INVENTION

[0023] The present invention relates to cold-adapted temperature sensitive Enterovirus 71 strains, particularly to the cold-adapted temperature sensitive Enterovirus 71 strains

EV71:TLL β P20 and EV71:TLL α P20. The present invention also relates to processes of developing the cold-adapted temperature sensitive virus strains, particularly RNA virus strains.

[0024] Thus, in one aspect, the present invention provides the cold-adapted temperature sensitive. In one embodiment, the cold-adapted temperature sensitive Enterovirus 71 strain is EV71:TLL β P20 as described herein. In another embodiment, the cold-adapted temperature sensitive Enterovirus 71 strain is EV71:TLL α P20 as described herein. EV71:TLL β P20 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:TLL α P20 was deposited on 25 October 2012 under terms of the Budapest Treaty with American Type Culture Collection, and assigned Accession Number PTA-13284.

[0025] The Enterovirus 71 strains of the present invention are prepared by a method to attenuate Enterovirus 71 using temperature sensitivity as a phenotypic marker. The method is an *in vitro* laboratory process to change the biologic growth characteristic of the virus to adapt for optimal replication at incubation temperature of below 30° C. The adaption process is carried out in a systematic stepwise manner of incremental lower incubation temperature for culturing the virus until the targeted temperature chosen for optimal replication of the virus is achieved. As disclosed herein, (i) preparing a reference stock of parental wild-type virus, (ii) incubating a culture of cells infected with the reference stock of parental wild-type virus at higher multiplicity of infection (MOI) and an incubation temperature of about 34° C for five or more passages until a full cytopathic effect (CPE) is obtained at each passage using an inoculum of a lower MOI and a shorter period of incubation for each passage to obtain full CPE, (iii) incubating a culture of cells infected with the resultant virus of the previous step at higher MOI and an incubation temperature of about 1° C to about 3° C lower than in the previous step for five or more passages until a full cytopathic effect (CPE) is obtained at each passage using an inoculum of a lower MOI and a shorter period of incubation for each passage to obtain full CPE, and (iv) repeating step (iii) in a systematic stepwise manner of incremental lower incubation temperature until the targeted temperature chosen for optimal replication of the virus is achieved. In one embodiment, the targeted temperature is about 26° C to about 29° C, preferably about 28° C.

[0026] In one embodiment the reference stock of parental wild-type virus is prepared by incubating a culture of cells infected with a wild-type virus at a temperature of about 36° C to about 38° C, preferably about 37° C for one or two passages until a full cytopathic effect (CPE)

is obtained. Aliquots of culture supernatant containing the produced virus are placed in vials or other suitable storage devices. This culture supernatant serves as a reference stock of parental wild-type virus. The reference parental wild-type virus is used for subsequent attenuation process. In another embodiment, aliquots of the reference stock of parental wild-type virus are stored at a suitable temperature, such as at -80° C.

[0027] In one embodiment, the cell to be infected by the virus is any cell which is permissive for the growth of the virus. In a preferred embodiment, the cells are Vero cells (ATCC CCL-81). In one embodiment, the cells are maintained by regular passage in a medium suitable for growth of the cells. In an embodiment in which the cells are Vero cells, the Vero cells are cultured in Dulbecco's modified Eagles's medium (DMEM) supplemented with 10% fetal calf serum (FCS). In one embodiment, Vero cells maintained in DMEM supplemented with 1% FCS is used for production of the parental wild-type virus, virus culture, attenuation, titration and assessment of temperature sensitive phenotype. In another embodiment, the DMEM is supplemented with 1% FCS for adapting the virus strains for replication in successively lowered temperature of incubation.

[0028] In one embodiment, the virus is passaged in each step of the process by obtaining a clarified culture supernatant containing the virus and passing this supernatant into each successive fresh new culture flask of cells, e.g., Vero cells, as soon as the virus obtains a full cytopathic effect (CPE). In another embodiment, the culture supernatant containing the virus is passed at a higher multiplicity of infection (MOI) of 20 at the beginning of each successive change to a lower temperature of incubation. After it is noted to be able to cause full CPE in Vero cells within 3 days after inoculation, the new culture flask containing freshly confluent monolayer Vero cells is inoculated with the same MOI of virus for at least three more passages before subsequently reduced to a lower MOI of 5 to 10. Once it is noted to be able to cause full CPE within 3 days after inoculation with a lower MOI of 5 to 10, the attenuation process is then moved on to the next phase of successive lower incubation temperature after passaging for at least three more times at a MOI of 5 to 10. The number of days required for each passage depends on how fast the virus is adapted at each phase of incrementally lower incubation temperature. The skilled artisan will readily know when a full CPE is reached. Further details of the method for preparing the cold-adapted temperature sensitive Enterovirus 71 strains of the present invention are described below.

[0029] In a second aspect, the present invention provides a composition comprising the cold-adapted temperature sensitive Enterovirus 71 strains described herein. In one embodiment, the

composition comprises an effective amount of the virus strains described herein. 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 a cold-adapted temperature sensitive Enterovirus 71 strain described herein are prepared using techniques well known to the skilled artisan. Such vaccines are useful for providing immunity against the parent virus strain by administering the vaccine to a subject, such as a human subject, using techniques well known to the skilled artisan.

[0030] It should be understood that a cold-adapted temperature sensitive Enterovirus 71 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.

[0031] 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 a cold-adapted temperature sensitive Enterovirus 71 strain described herein. Accordingly, the present invention also provides a cold-adapted temperature sensitive Enterovirus 71 strain or a composition comprising the cold-adapted temperature sensitive Enterovirus 71 strain for use in eliciting a protective immune response in a subject. The present invention also provides the use of cold-adapted temperature sensitive Enterovirus 71 strain or a composition comprising the cold-adapted temperature sensitive Enterovirus 71 strain for the manufacture of a medicament for eliciting a protective immune response in a subject. In one embodiment, the protective immune response protects the subject against a disease caused by Enterovirus 71. 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, a cold-adapted temperature sensitive Enterovirus 71 strain described herein is administered as a vaccine. In an additional embodiment, the subject has been exposed to wild-type Enterovirus 71. "Exposed" to an Enterovirus 71 means contact with the Enterovirus 71 such that an infection could result. In another embodiment, the administration of a cold-adapted temperature sensitive Enterovirus 71 strain described herein prevents a subject, such as a human subject, from becoming afflicted with an Enterovirus 71-associated disease. Accordingly, the present invention also provides a cold-adapted temperature sensitive Enterovirus 71 strain or a composition comprising the cold-adapted temperature sensitive Enterovirus 71 strain for use in preventing a subject, such as a human subject, from becoming afflicted with an Enterovirus 71-associated disease. The present invention also provides the use of cold-adapted temperature sensitive Enterovirus 71 strain or a composition comprising the cold-adapted temperature sensitive Enterovirus 71 strain for the manufacture of a medicament for preventing a subject, such as a human subject, from becoming afflicted with an Enterovirus 71-associated disease. In an additional embodiment, the subject has been exposed to wild-type Enterovirus 71. In a further embodiment, the administration of a cold-adapted temperature sensitive Enterovirus 71 strain described herein delays the onset of or slows the rate of progression of an Enterovirus 71-associated disease in a virus-infected subject, such as a human subject. Accordingly, the present invention also provides a cold-adapted

temperature sensitive Enterovirus 71 strain or a composition comprising the cold-adapted temperature sensitive Enterovirus 71 strain for use in delaying the onset of or slows the rate of progression of an Enterovirus 71-associated disease in a virus-infected subject, such as a human subject.. The present invention also provides the use of cold-adapted temperature sensitive Enterovirus 71 strain or a composition comprising the cold-adapted temperature sensitive Enterovirus 71 strain for the manufacture of a medicament for delaying the onset of or slows the rate of progression of an Enterovirus 71-associated disease in a virus-infected subject, such as a human subject.

[0032] 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.

[0033] 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.

[0034] 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.

[0035] 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.

[0036] The present invention also provides a method to attenuate a virus using temperature sensitivity as a phenotypic marker. In accordance with this aspect, the method develops cold-adapted temperature sensitive virus strains. The method of the present invention is an *in vitro* laboratory process to change the biologic growth characteristic of the virus to adapt for optimal replication at incubation temperature of below 30° C. The adaption process is carried out in a systematic stepwise manner of incremental lower incubation temperature for culturing the virus until the targeted temperature chosen for optimal replication of the virus is achieved.

[0037] Thus, in accordance with the present invention, the method comprises the following steps: (i) preparing a reference stock of parental wild-type virus, (ii) incubating a culture of cells infected with the reference stock of parental wild-type virus at higher multiplicity of infection (MOI) and an incubation temperature of about 34° C to about 36° C, preferably about 34° C, for five or more passages until a full cytopathic effect (CPE) is obtained at each passage using an inoculum of a lower MOI and a shorter period of incubation for each passage to obtain full CPE, (iii) incubating a culture of cells infected with the resultant virus of the previous step at higher MOI and an incubation temperature of about 1° C to about 3° C lower than in the previous step for five or more passages until a full cytopathic effect (CPE) is obtained at each passage using an inoculum of a lower MOI and a shorter period of incubation for each passage to obtain full CPE, and (iv) repeating step (iii) in a systematic stepwise manner of incremental lower incubation temperature until the targeted temperature chosen for optimal replication of the virus is achieved. In one embodiment, the targeted temperature is about 26° C to about 29° C, preferably about 28° C. In one embodiment, the incrementally lower incubation temperature is a temperature lowered about 1° C to about 2° C.

[0038] In one embodiment the reference stock of parental wild-type virus is prepared by incubating a culture of cells infected with a wild-type virus at a temperature of about 36° C to about 38° C, preferably about 37° C for one or two passages until a full cytopathic effect (CPE) is obtained. Aliquots of culture supernatant containing the produced virus are placed in vials or other suitable storage devices. This culture supernatant serves as a reference stock of parental wild-type virus. The reference parental wild-type virus is used for subsequent attenuation process. In another embodiment, aliquots of the reference stock of parental wild-type virus are stored at a suitable temperature, such as at -80° C.

[0039] In one embodiment, the virus is passaged in each step of the process by obtaining a clarified culture supernatant containing the virus and passing this supernatant into each successive fresh new culture flask of cells, e.g., Vero cells, as soon as the virus obtains a full cytopathic effect (CPE). In another embodiment, the culture supernatant containing the virus is passed at a higher multiplicity of infection (MOI) of 20 at the beginning of each successive change to a lower temperature of incubation. After it is noted to be able to cause full CPE in Vero cells within 3 days after inoculation, the new culture flask containing freshly confluent monolayer Vero cells is inoculated with the same MOI of virus for at least three more passages before subsequently reduced to a lower MOI of 5 to 10. Once it is noted to be able to cause full CPE within 3 days after inoculation with a lower MOI of 5 to 10, the attenuation process is then moved on to the next phase of successive lower incubation temperature after passaging for at least three more times at a MOI of 5 to 10. The number of days required for each passage depends on how fast the virus is adapted at each phase of incrementally lower incubation temperature. The skilled artisan will readily know when a full CPE is reached.

[0040] In one embodiment, the virus is any virus. In another embodiment, the virus is an RNA virus. In an additional embodiment, the RNA virus is a plus strand RNA virus. In a further embodiment, the virus is a member of the Picornaviridae family. In another embodiment, the virus is a member of the Enterovirus genus. In one embodiment, the virus is Enterovirus 71 (EV71). In another embodiment, the virus is cocksackievirus A16 (CA16). The method of the present invention is useful in producing cold-adapted temperature sensitive virus strains of any of these viruses, including but not limited to cold-adapted temperature sensitive strains of EV71 and CA16.

[0041] In one embodiment, the cell to be infected by the virus is any cell which is permissive for the growth of the virus. In a preferred embodiment, the cells are Vero cells (ATCC CCL-81). In one embodiment, the cells are maintained by regular passage in a medium suitable for

growth of the cells. In an embodiment in which the cells are Vero cells, the Vero cells are cultured in Dulbecco's modified Eagles's medium (DMEM) supplemented with 10% fetal calf serum (FCS). In one embodiment, Vero cells maintained in DMEM supplemented with 1% FCS is used for production of the parental wild-type virus, virus culture, attenuation, titration and assessment of temperature sensitive phenotype. In another embodiment, the DMEM is supplemented with 1% FCS for adapting the virus strains for replication in successively lowered temperature of incubation.

[0042] The present invention also relates to the cold-adapted temperature strains of virus produced by the method described herein. The cold-adapted temperature viruses produced by the method described herein are useful in the production of vaccines using techniques well known to the skilled artisan. Such vaccines are useful for providing immunity against the parent virus strain by administering the vaccine to a subject using techniques well known to the skilled artisan.

[0043] The present method is applicable to the production of cold-adapted temperature sensitive viruses of the Picornaviridae family and of the Enterovirus genus. The applicability has been demonstrated herein by the production of a cold-adapted temperature sensitive strains of EV71 (TLL α) and EV71 (TLL β) which are derived following serial passages in cell-culture at incremental lower temperature of incubation. The EV71 (TLL β) strain retains phenotypic and genetic stability in specific *in vitro* cell culture conditions and does not exhibit neuro-virulence in monkeys following intravenous inoculation. The applicability has also been demonstrated herein by the production of a cold-adapted temperature sensitive CA16 which is derived following serial passages in cell-culture at incremental lower temperature of incubation.

[0044] The invention also provides a kit for immunization of a subject with of a cold-adapted temperature sensitive Enterovirus 71 strain described herein. The kit comprises a cold-adapted temperature sensitive Enterovirus 71 strain described herein, 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 cold-adapted temperature sensitive Enterovirus 71 strain described herein.

[0045] 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.), *Immunóchemical 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

[0046] 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 for Developing Cold-Adapted
Temperature Sensitive Strains of Human Enterovirus 71 (EV71)

[0047] *Cell, Virus and Cold-Adaption Process:* Vero cells (ATCC CCL-81) maintained by regular passage in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) were used for virus culture, attenuation, titration and assessment of temperature sensitive phenotype. Three EV71 isolates belong to genotype C1, B3 and B4 isolated in Vero cells from respective oral secretion, stool and brain-stem specimens of patients presented with hand foot and mouth disease (HFMD) were plaque purified once in accordance with the technique described previously (Dougherty, 1964). After plaque purification, the virus stock designated as the respective parental wild-type strains was prepared following two passages in Vero cells at 37° C. All virus stock and respective passaged strains were stored in minus 80° C freezer.

[0048] Young freshly confluent monolayer Vero cells in DMEM containing 1% FCS were used to adapt the virus strains for replication in successively lower temperature of incubation, starting from the initial low incubation temperature of 34° C. The clarified culture supernatant containing the virus was passed into each successive fresh new culture flask of Vero cells as soon as it attained full cytopathic effect (CPE). The culture supernatant containing the virus was passed at a higher multiplicity of infection (MOI) of 20 at the beginning of each successive change to a lower temperature of incubation. After it was noted to be able to cause full CPE in Vero cells within 3 days after inoculation, the new culture flask containing freshly confluent monolayer Vero cells was inoculated with the same MOI of virus for three more passages before subsequently reduced to a lower MOI of 5 to 10. Once it was noted to be able to cause full CPE within 3 days after inoculation with a lower MOI of 5 to 10, the attenuation process was then moved on to the next successive lower incubation temperature after passaging for at least three more times. The succeeding incremental lower temperatures of incubations for developing cold-adapted temperature sensitive strains of enterovirus 71 in this example were 34° C, 32° C, 30° C, 29° C, and 28° C.

[0049] *Virus Titration:* Virus titer was determined by microtitration assay in Vero cells in accordance with the method described in Polio Laboratory Manual 2004 of World Health Organization with minor modification and virus titer was calculated as 50% cell culture infectious dose (CCID₅₀) per milliliter following the method of Reed and Muench (1938). 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 (10^4 cells per well) in 96-well flat-bottom tissue culture plate were inoculated with 100 μ l the serially diluted of each virus stock and incubated in an ambient of 5% CO₂ at each respective incubation temperature for 5 days prior to observation for the presence of CPE.

[0050] *Temperature-sensitivity 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 infected cells (replication kinetic) and second approach assessed the titer of the virus strain in cells incubated at each specific tested temperature. Briefly, in the first approach, the growth medium of three T-25 tissue culture flasks containing confluent monolayer Vero cells of similar age 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 (MOI). If no CPE was noted at the end of 10-day culture, the supernatant was passed into a new of flask of 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 and incubated for 5 days prior to observation for the presence of CPE.

[0051] *Genetic Stability and Temperature Sensitivity 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 cell culture at viral inoculum of 5 MOI 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 complete genomes of their respective parental wild-type viruses.

[0052] Reversion of temperature sensitivity assay was carried out on a stable cold-adapted temperature sensitive virus strain. The selected strain was passaged 5 times in monolayer Vero cells incubated at temperature of 37° C in an ambient of 5% CO₂. At each passage, a virus inoculum of 10 MOI was used. 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 assay. The complete genomes of virus strains at each successive passage at culture temperature of 37° C were sequenced and analyses.

[0053] *RNA Extraction, RT-PCR and Sequencing:* Viral genomic RNA was extracted from the culture fluid of infected cells at full CPE using a commercially available Viral RNA Extraction Kit (Qiagen, Germany). First-strand synthesis was performed with EV71-specific primers using Superscript II RNA polymerase (Invitrogen, USA), and subsequent PCR with 18 degenerate primer pairs was done using GoTaq Green PCR mix (Promega, USA). Fragments generated were sequenced using BigDye Terminator sequencing kit (Applied Biosystems, USA). 5'-RACE was performed to determine the 5'-UTR viral sequence by ligating the 5'-cordycepin-blocked adaptor DT88 (5'-GAA GAG AAG GTG GAA ATG GCG TTT TTG G-cordycepin-3'; SEQ ID NO:1) to the 5'-end of the EV71 cDNA using standard T4 DNA Ligase (Fermentas, USA), and standard PCR was performed afterwards using a DT88-complementary primer (5'-CCA AAA CGC CAT TTC CAC CTT CTC TTC 3'; SEQ ID NO:2) and an EV71-specific primer (5'-ATT CAG GGG CCG GAG GAC TAC-3'; SEQ ID NO:3). 3'-RACE was also performed to determine the 3'-UTR viral sequence using an oligo-dT primer (Li et al., 2005).

[0054] *Molecular Cloning and Plasmid Purification:* Fragments with ambiguous sequences were cloned into pZero-2 plasmid (Invitrogen, USA) and transformed into TOP10 *E. coli* cells (Invitrogen, USA). Plasmids containing the cloned EV71 fragments were extracted from at least 10 colonies from each transformant using commercially available Plasmid Miniprep Kit (Qiagen, Germany) and the sequence of the cloned fragment was subsequently determined.

[0055] Fragment sequences obtained were merged using the European Molecular Biology Open Software Suite (EMBOSS; <http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::merger>) (Rice et al. 2000). Merged sequences were aligned with a reference sequence of EV71 strain 3799-SIN-98 (GenBank Accession No. DQ341354.1) using the program BioEdit Sequence Alignment Editor v. 7.0.9.0 (Hall, 1997).

[0056] *Monkey Study:* The monkey study on the safety and immunogenicity the selected cold-adapted temperature sensitive strain of EV71 (EV71:TLL β P20) was contracted to investigators based in the Animal Facility of DUKE-NUS, Singapore. Seven cynomolgus monkeys (*Macaca fascicularis*) free of *Mycobacterium tuberculosis* and simian immunodeficiency virus, three females (2202F, 2207F, 2891F) and four males (0791M, 2247M, 2889M, 2890M) with a mean weight of 3.23 Kg (range 2.44 to 4.11, SD = 0.7) were used to study the safety and immunogenicity of the stable cold-adapted temperature sensitive EV71:TLL β P20. All seven monkeys were pre-screened for the absence of binding (indirect immunofluorescence) and neutralizing antibodies against EV71. The study and all animal procedures were approved by the Committee for Biosafety and Animal Handling and the Ethical Committee of DUKE-NUS, Singapore. Virus inoculation and observation, animal care and necropsy were performed in accordance with guidelines of the committees.

[0057] Under light anesthesia with ketamine, 1 ml of virus inoculum was intravenously inoculated into the right saphenous vein. Three monkeys (2889M, 0791M and 2891F) were given an intravenous dose of EV71:TLL β P20 at 10^7 CCID₅₀ per monkey, another three (2890M, 2202F and 2247M) were given 10^8 CCID₅₀ each and the seventh monkey served as negative control. The monkeys were observed twice daily for clinical illness especially neurological manifestations and their body temperature were recorded by an in-planted temperature sensor. The stool from each monkey was collected daily and stored in minus 80° C freezer for virus isolation at a later date. Two monkeys, one from each different virus dose of inoculum, were sacrificed under deep anesthesia on day 4 post-inoculation (PI). At autopsy, various parts of central nervous system (CNS), non-neural tissues and blood were collected for histopathological and virological analysis. On day 8 PI, another similar set of two monkeys were sacrificed and same types of tissues and blood were collected for histopathological and virological study at autopsy. The remaining two monkeys were given respective equivalent booster dose of EV71:TLL β P20 at day 14 PI after blood samples were collected for assessment of anti-EV71 antibodies. They were put to sleep 16 days after receiving the booster dose and CNS tissues were collected for histopathological study at autopsy.

[0058] *Histology and Immunohistochemistry:* CNS tissue specimens (cerebrum, cerebellum, basal ganglia, brain-stem and spinal cord) and non-neural tissues (lymph node, spleen, liver, kidney, lung and heart) were fixed in 10% formalin in phosphate buffered saline (PBS) and embedded in paraffin after fixation. The spinal cord was sectioned horizontally 10 times at cervical, 8 times at thoracic and 10 times at lumbar levels respectively. Paraffin sections, 6 μ m

in thickness, were stained with haematoxylin and eosin (H&E) and with Luxol-fast blue/cresyl violet (Kluver-Barrera method) after paraffin removal and rehydration process.

[0059] *Antigen Detection and Virus Isolation from Monkeys:* Blood specimen was collected in both plain and heparinized tubes. Peripheral blood mononuclear cells (PBMC) were harvested from heparinized blood using Ficoll-Paque™ PLUS (GE Healthcare, Sweden). After two washes with sterile PBS, aliquots of PBMC suspension were seeded onto wells of Teflon-coated slide for detection of EV71 antigen by indirect immunofluorescence assay using commercial detecting monoclonal antibodies (Cat. No. 3360, Light Diagnostics, USA). Virus isolation was carried out by inoculating PBMC suspension into wells of 24-well cell culture plate containing monolayer Vero cells. Virus isolation on each serum specimen was performed by inoculating 50 µl and 100 µl of serum into respective wells of 24-well culture plate containing monolayer Vero cells. Monkey tissues were lightly washed with two exchanges of sterile PBS and homogenized by grinding using mortar and pestle inside a class II biosafety cabinet. Tissue homogenates (10%, w/v) prepared in DMEM was clarified by centrifugation at 1000 g for 10 minutes. The clarified supernatant was filtered through a 0.22 micron syringe filter and virus isolation was performed by inoculating 100 µl and 200 µl of the filtrate. A 10% stool suspension was made in PBS and clarified by centrifugation at 1000 g for 10 minutes. After filtration through a 0.22 micron syringe filter, virus isolation was performed by inoculating 100 µl and 200 µl of stool filtrate. All virus isolation work on monkeys' samples was performed in duplicate with one set of the inoculated cell culture incubated at 28° C and the other at 37° C.

[0060] *Molecular Detection and Complete Genome Sequencing:* A commercial viral RNA extraction and purification kit (Qiagen, Germany) was used to extract viral genomic RNA from serum, PBMCs and clarified tissue homogenates. A commercial one-step RT-PCR kit (Qiagen, Germany) and a consensus oligonucleotide primer-pair (Sense: 5'-CACCTTGTGATACCATGGATCAG-3' (SEQ ID NO:4); Anti-sense: 5'-GTGAATTAAGAACRCAYCGTGTYT-3' (SEQ ID NO:5)) that amplified the proximal third of VP1 gene of all EV71 genotypes were used for molecular amplification and detection of EV71-specific viral RNA after extraction from tissues. Eighteen pairs of sequence-specific primers basing on complete genomic sequence of EV71:TLLβ were used to amplify and sequence the complete genome of EV71 present in the sera of two monkeys obtained on Day 4 and Day 8 PI. Any PCR-amplified fragment that failed to give a good sequence read by direct sequencing was cloned into pZero-2 (Invitrogen, USA) and transformed into TOP10 *E. coli*. At least ten colonies were selected from each transformant and sequencing was performed on the purified plasmids carrying the inserts.

[0061] *Serum Binding and Neutralizing Antibodies Assay:* Vero cells infected with the EV71 of genotype B3 were harvested at near full CPE and washed five times with sterile PBS. After the last wash, suspension of infected cells was mixed with washed non-infected Vero cell suspension in the ratio of approximately 4 infected cells to one non-infected cell. Ten micro-litre of the mixed cell suspension containing 250 cells was carefully layered onto each well of the 12-well Teflon coated slide and allowed to dry over a warm plate. The dried slide was fixed for 10 minutes in cold acetone and used as in-house antigen to assay for EV71 binding antibody starting from an initial dilution of 1:10 for IgM and 1:20 for IgG by indirect immunofluorescence assay. In the assay for anti-EV71 IgM titer, the monkeys' sera were treated with appropriate concentration of protein A (Invitrogen, USA) to remove IgG prior carry out serial 2-fold dilution with sterile PBS.

[0062] The neutralizing antibodies titer of monkeys was determined by micro-neutralizing assay using Vero cells in accordance with the method described in Polio Laboratory Manual 2004 of World Health Organization with minor modification. The concentration of each genotype of EV71 used for neutralization was 100 CCID₅₀ per 100 µl. The virus neutralization assay was performed using 96-well flat bottom culture plate. A serial 2-fold dilution of each serum sample was prepared in duplicate at a volume of 100 µl DMEM (1% FCS) starting at 1:10 dilution. An equal volume (100 µl) of virus working stock in DMEM (1% FCS) containing 100 CCID₅₀ EV71 was added into each well of the diluted sera and incubated for two hours at 37° C. After incubation, 100 µl of Vero cell suspension in DMEM (10% FCS) containing 250 cells was added into each well. The 96-well culture plate was carefully sealed and incubated at 37° C in an ambient of 5% CO₂. The plate was read daily up to 8 days for presence of CPE affecting Vero cells in each well. The titer of neutralizing antibodies of each serum sample was determined by the well with the highest dilution that did not show CPE.

EXAMPLE 2

Results on the Phenotypic and Genotypic Characteristics of the Cold-Adapted Temperature Sensitive Strains of Enterovirus 71 (EV71:TLL α , EV71:TLL α P20, EV71:TLL β , EV71:TLL β P20 and EV71:TLL β P40)

[0063] The original three parental EV71 viruses used to derive the cold-adapted strains caused full CPE in Vero cells within 3 days after inoculation at a virus inoculum of 10 MOI and incubation temperature of 37.5° C. At the same virus inoculum, the original parental EV71 viruses caused full CPE in Vero cells within 5 days at incubation temperature of 39.5° C.

However, all the three original parental EV71 viruses derived from first two passages in Vero cells cultured at 37.5° C did not cause CPE in Vero cells at a virus inoculum of 10 MOI and incubation temperature of 28° C. No CPE was also noted after a blind passage at the end of 10 days culture at 28° C. The absence of virus replication in the inoculated Vero cells was further supported by negative staining of suspended cells presence in the culture supernatant fluid at the end of 10 days culture using commercial detecting monoclonal antibodies against EV71 by indirect immunofluorescence assay.

[0064] After more than 90 passages in successively lower incubation temperature, all the three passaged EV71 strains were able to cause full CPE in Vero cells within 3 days of inoculation at a virus inoculum of ≤ 5 MOI and incubation temperature of 28° C. The virus strains were passaged further at an incubation temperature of 28° C until 100th passage. At 100th passage, virus strains derived from original EV71 isolated from oral fluid, brain-stem tissue and stool specimens of patients were respectively designated as EV71:TLL, EV71:TLL α and EV71:TLL β . In temperature sensitive assay, all the three cold-adapted strains caused full CPE in Vero cells within 2 days at an incubation temperature of 28° C using a virus inoculum of 10 MOI but took 4 days to achieve full CPE at an incubation temperature of 37° C. At an incubation temperature of 39.5° C, no CPE was noted for all the three strains after 10 days of culture. However, EV71:TLL gave 2+ CPE at 10th day of culture after a blind passage into a fresh flask of Vero cells incubated at 39.5° C. No CPE was noted for EV71:TLL α and EV71:TLL β even after two further blind passages into fresh flasks of Vero cells incubated at 39.5° C. No suspended cells harvested from culture supernatant, inclusive those derived from blind passages, inoculated with either EV71:TLL α or EV71:TLL β at the end of each 10 days culture gave positive immunofluorescent staining with EV71 detecting monoclonal antibodies. Approximately 1% of suspended cells harvested from culture supernatant inoculated with EV71:TLL gave positive staining though no CPE was noted after 10 days of culture.

[0065] Incubation temperature of 28° C and 37° C was used to assay the virus replication titer of three cold-adapted strains and the assay was repeated at least 4 times. The titer of EV71:TLL was 1×10^8 CCID₅₀/ml and 2 to 3×10^7 CCID₅₀/ml when the titrated cultures were incubated at 28° C and 37° C respectively. EV71:TLL α gave a virus titer of 1×10^8 CCID₅₀/ml at an incubation temperature of 28° C and a titer of 1 to $2 \times 10^{5.5-6}$ CCID₅₀/ml at 37° C. EV71:TLL β gave a titer of 2 to 5×10^8 CCID₅₀/ml at an incubation temperature of 28° C and a titer of 1×10^7 CCID₅₀/ml at 37° C.

[0066] The stability of cold-adaptation of EV71:TLL α and EV71:TLL β was assessed by passaging both virus strains for another 20 passages in Vero cells at an incubation temperature of 28° C and a virus inoculum of 10 MOI. After an additional 20 passages, EV71:TLL α caused full CPE in cells incubated at 28° C within 2 days PI but failed to cause CPE in cells incubated at 37° C even after 2 blind passages. It remained the ability to achieve a virus titer of 1×10^8 CCID₅₀/ml at an incubation temperature of 28° C. EV71:TLL β maintained the same cold-adapted phenotype in term of growth kinetic and virus titer at both incubation temperature of 28° C and 37° C after an additional 20 passages. The stability of cold-adaptation of EV71:TLL β was assessed further by passaging another 20 more passages (40 additional passages from the 100th passage) under the same culture conditions and it was found to remain similar cold-adapted temperature sensitive phenotype.

[0067] Assessment of reversion from cold-adapted temperature sensitive phenotype was performed on EV71:TLL β P20 by 6 successive passaging of the virus in Vero cells incubated at 37° C at each full CPE. The growth characteristics of the virus derived from each respective culture incubated at 37° C in term of growth kinetic and virus titer at incubation temperature of 28° C, 37° C and 39.5° C is shown in Table 1. The virus remained inability to produce viable infectious particles (lack of positive immunofluorescent staining cells) in Vero cells at incubation temperature of 39.5° C after 3 successive passages in cells incubated at 37° C and inability to cause full CPE in cell culture at incubation temperature of 39.5° C at 6th repeated passage.

TABLE 1

Assessment of reversion from cold-adapted temperature sensitive phenotype of EV71:TLL β P20 after 6 successive passaging Vero cells incubated at 37° C.

Virus Inoculum	Days to achieve Full Cytopathic Effect (CPE)			Virus Titer in CCID ₅₀ /ml	
	28° C	37° C	39.5° C	28° C	37° C
EV71:TLL β P20	2	4	Day10: No CPE	2.1×10^8	1×10^7
TLL β P20 (37° C-P1)	4	4	Day 10: No CPE	1.7×10^8	3.2×10^5
TLL β P20(37° C-P2)	3	4	Some cells dying at day 8 (IFA: -ve)	4.6×10^7	2.1×10^7
TLL β P20 (37° C-P3)	3	2	Some cells dying at day 5 (IFA: -ve)	1.7×10^8	3.1×10^7
TLL β P20 (37° C-P4)	3	2	Day 10: 1+ CPE (IFA: +ve)	3.1×10^7	4.6×10^7

TLL β P20(37°C-P5)	3	2	Day 10: 2+ CPE (IFA: +ve)	1 X10 ⁷	3.1 X10 ⁷
TLL β P20 (37°C-P6)	3	2	Day 10: 2+ CPE (IFA: +ve)		

The growth characteristic and titer 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

[0068] The complete genomes of EV71:TLL α and EV71:TLL β , their respective strains after an additional 20 passages (EV71:TLL α P20 and EV71:TLL β P20) and original parental wild-types were sequenced and analyzed. The nucleotide sequence for EV71:TLL α P20 is set forth in SEQ ID NO:6. The nucleotide sequence for EV71:TLL β P20 is set forth in SEQ ID NO:7. The number of nucleotide changes at each segment of their genes between EV71:TLL α , EV71:TLL α P20 and original parental wild-type is shown in Table 2. The number of nucleotide changes between original parental wild-type, EV71:TLL β , EV71:TLL β P20 and EV71:TLL β P40 (additional 40 passages) is shown in Table 3. The complete genomes of virus strains derived from temperature sensitive reversion study by 6 successive passaging at incubation temperature of 37° C were also sequenced and number of nucleotide changes with respect to that of EV71:TLL β P20 is shown in Table 4.

TABLE 2

The number of nucleotide (NT) and corresponding amino acids (AA) mutations that occurred in each of the genomic segments of EV71:TLL α and EV71:TLL α P20 in comparison with the complete genome of their original parental wild-type virus.

Viral Gene Region/Protein		EV71:TLL α		EV71:TLL α P20	
		NT	AA	NT	AA
5'-UTR	"Cloverleaf"				
(1-747)	IRES	1		2	
P1	VP4	2	1	3	1
(748-3333)	VP2	2	2	2	2
	VP3	2	2	3	3
	VP1	2	2	4	3
P2	2A	2	2	4	3
(3334-5067)	2B	1		1	

	2C	2	1	2	1
P3	3A			1	1
(5068-7326)	3B				
	3C	1	1	1	1
	3D	3	1	3	1
3'-UTR					
(7327-7412)					
Total		18	12	26	16

TABLE 3

The number of nucleotides (NT) and corresponding amino acids (AA) mutations that occurred in each of the genomic segments of EV71:TLL β , EV71:TLL β P20 and EV71:TLL β P40 in comparison with the genome of their original parental wild-type virus.

Viral Gene Region/Protein		EV71:TLL β		EV71:TLL β P20		EV71:TLL β P40	
		NT	AA	NT	AA	NT	AA
5'-UTR	"Cloverleaf"	2		1		2	
(1-746)	IRES	2		2		3	
P1	VP4					1	
(747-3332)	VP2	4		4		4	
	VP3	2	2	2	2	2	2
	VP1	9	8	8	8	8	8
P2	2A	4	2	4	2	4	2
(3333-5066)	2B	1	1	1	1	1	1
	2C	2		3		3	
P3	3A	1	1	1	1	1	1
(5067-7325)	3B						
	3C	2	2	2	2	2	2
	3D	2	2	2	2	2	2
3'-UTR		1		1		1	
(7326-7411)							

27

Total	32	18	31	18	34	18
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TABLE 4

The 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:TLLβP20.

Viral Gene Region/Protein	TLLβ (37 ⁰ C-P1)		TLLβ (37 ⁰ C-P2)		TLLβ (37 ⁰ C-P3)		TLLβ (37 ⁰ C-P4)		TLLβ (37 ⁰ C-P5)		TLLβ (37 ⁰ C-P6)	
	NT	AA	NT	AA	NT	AA	NT	AA	NT	AA	NT	AA
5'-UTR (1-746)			1(R)		1(R)				1(R)			
“Cloverleaf” IRES												
P1 VP4 (747-3332)												
VP2												
VP3												
VP1							1	1	2	1		
P2 2A (3333-5066)									1(R)		1(R)	
2B												
2C												
P3 3A (5067-7325)												
3B												
3C												
3D							1(R)	1(R)	3(1R)	2(1R)	2(1R)	
3'-UTR (7326-7411)												
Total			1(R)		2(R)	1(R)	5(2R)	3(1R)	6(2R)	4(2R)		

EXAMPLE 3

Results on Monkey Study of Cold-Adapted Temperature
Sensitive Strains of Human Enterovirus 71 (EV71:TLL β P20)

[0069] *Clinical Findings:* General observation on the clinical status and measurement of body temperature of the studied monkeys was performed twice daily, morning and evening. Throughout, all the monkeys were noted to be active and feeding normally. None of the monkeys developed any subtle focal neurological deficit, such as limb weakness, tremor or abnormal movement. None except one monkey (2247M) that was given an intravenous dose of 1×10^8 CCID₅₀/ml developed a spike of low grade fever (39.3° C) on day 3 post-inoculation. None of the monkeys had weight loss on reweighing at the time they were sacrificed under deep anesthesia.

[0070] *Autopsy and Histological Findings:* No gross post-mortem lesion was noted for all monkeys at autopsy. No abnormal histological finding was found on all monkeys tissues collected for histopathological examinations.

[0071] *Virological Investigations:* Virus isolation was performed on monkeys' sera, PBMC, stool samples and all autopsied tissues using Vero cells at incubation temperature of 28° C and 37° C. No virus was isolated from any of the monkeys' samples despite a blind passage after 10 days of culture. One additional blind passaging in Vero cells were carried out for sera, PBMC samples and those tissue homogenates that were tested positive for EV71 by RT-PCR. Despite failure to isolate the virus, EV71 antigen was detected in a few PBMC derived from heparinised blood of two monkeys, 2891F (received 1×10^7 CCID₅₀ of EV71:TLL β P20) and 2890M (received 1×10^8 CCID₅₀ of EV71:TLL β P20), collected on Day 4 PI by indirect immunofluorescence assay (Figure 1a) using commercial monoclonal antibodies. No virus antigen was detected in PBMC harvested from heparinized blood of monkeys collected on Day 8 PI.

[0072] EV71 specific genomic sequence was detected in serum samples of all monkeys collected on both Day 4 and day 8 PI by RT-PCR. Of the non-neuronal tissues, only EV71 genomic sequence was detected in spleen homogenate of 2 monkeys (2202F and 2891F) (Figure 1b). No EV71 genomic sequence was detected in any of the neuronal tissues homogenates. The genome of EV71:TLL β P20 present in the serum samples of two monkeys (2889M and 2890M) collected on both Day 4 and Day 8 PI were extracted and completely sequenced. The number of nucleotides (NT) and corresponding amino acids (AA) mutations or reversion that occurred in

each of the genomic segments of virus strains present in the sera in comparison with the genome of EV71:TLL β P20 is shown in Table 5.

TABLE 5

The number of nucleotide (NT) and corresponding amino acids (AA) mutations that occurred in each of the genomic segments of EV71 present in the serum samples of two monkeys (2889M and 2890M) collected on Day 4 and Day 8 PI in comparison with the complete genome of EV71:TLL β P20.

Viral Gene Region/Protein		Monkey (2889M)				Monkey (2890M)			
		Day 4		Day 8		Day 4		Day8	
		NT	AA	NT	AA	NT	AA	NT	AA
5'-UTR	Cloverleaf								
(1-746)	IRES								
P1	VP4								
(747-3332)	VP2								
	VP3								
	VP1	1				5	1(R)	6	1(R)
P2	2A	3	2	3	2	3	2	3	2
(3333-5066)	2B								
	2C	2(1R)	1(R)	1		1(R)		1(R)	
P3	3A								
(5067-7325)	3B								
	3C								
	3D	1(R)	1(R)	1(R)	1(R)	1(R)	1(R)	1(R)	1(R)
3'-UTR									
(7326-7411)									
Total		7(3R)	4(2R)	5(1R)	3(1R)	10(2R)	4(2R)	11(2R)	4(2R)

[0073] *Monkey Humoral Immune Response:* Assay for the presence of binding antibodies (IgM and IgG) in the sera of monkeys given intravenous EV71:TLL β P20 was performed by indirect immunofluorescence assay using in-house prepared infected Vero cells as antigen. The

titers of anti-EV71 IgM and IgG present in the blood of the two remaining monkeys (2889M and 2890M) collected on Day 14 PI, prior given an equivalent intravenous booster dose, and Day 30 PI (16 days post-booster) are shown in Table 6.

TABLE 6

The titer of binding antibodies (IgM and IgG) of monkeys after given intravenous EV71:TLL β P20 as determined by indirect immunofluorescence assay using in-house prepared infected Vero cells as antigen.

Monkey	IgM titre		IgG titre	
	Day 14 PI	Day 30 PI	Day 14 PI	Day 30 PI
2889M	1:20	1:10	1:160	1:320
2890M	1:40	1:40	1:160	1:640

[0074] The neutralizing antibodies titer in the serum samples of the two monkeys (2889M and 2890M) collected on day 14 and Day 30 PI against EV71 genotypes A (BrCr), B3, B4, B5, C1 and C5 was determined by micro-neutralization assay. The respective titer of neutralizing antibodies of the monkeys' sera against each of the EV71 genotypes is shown in Table 7.

TABLE 7

The titer of neutralizing antibodies against a number of genotypes of EV71 (A, B3, B4, B5, C1 and C5) presence in sera of two monkeys after given intravenous EV71:TLL β P20 as determined by micro-neutralization assay.

EV71 Genotype	Monkey			
	2889M		2890M	
	Day 14 PI	Day 30 PI	Day 14 PI	Day 30 PI
A (BrCr)	1:20	1:40	1:40	1:80
B3	1:160	1:640	1:160	1:640
B4	1:160	1:640	1:160	1:320
B5	1:80	1:320	1:160	1:320-640
C1	1:40	1:160	1:40	1:160
C5	1:20	1:40	1:20	1:40

EXAMPLE 4

Materials and Methods for Developing Cold-Adapted
Temperature Sensitive Strains of Human Coxsackievirus A16

[0075] *Materials and Methods:* Vero cells (ATCC CCL-81) maintained by regular passages in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) were used for virus culture, attenuation, titration and assessment of temperature sensitive of coxsackievirus A16. The parental wild-type coxsackievirus A16 (CA16) for the attenuation process is derived from oral secretion of a patient having hand foot and mouth disease (HFMD). The virus was plaque purified once in accordance with the technique described previously. After plaque purification, the virus stock designated as the respective parental wild-type strain was prepared following two passages in Vero cells at 37° C. All virus stock and respective passaged strains were stored in minus 80° C freezer.

[0076] Young freshly confluent monolayer Vero cells in DMEM containing 1% FCS were used to adapt the original wild-type CA16 for replication in successively lower temperature starting from the initial lower incubation temperature of 34° C. Passaging of virus into each successive new culture flask of Vero cells was carried out at full cytopathic effect (CPE). The virus was passaged at a higher multiplicity of infection (MOI) of 20 at the change to a successive lower incubation temperature and was reduced to a lower MOI of 5 to 10 following at least three more passages after noting it was able to cause full CPE within 3 days after inoculation. It was moved to the next successive lower incubation temperature following at least three more passages after noting it was able to cause full CPE within 3 days after inoculation with a MOI of 5 to 10. The culture supernatant of each successive passage was stored in minus 80° C freezer for analysis at later date.

[0077] *Virus Titration:* Virus titer was determined by microtitration assay in Vero cells in accordance with the method described in Polio Laboratory Manual 2004 of World Health Organization with minor modification and virus titer was calculated as 50% cell culture infectious dose (CCID₅₀) per milliliter following the method of Reed & Munch (1938). 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 of each virus stock and incubated in an ambient of 5% CO₂ at each respective incubation temperature for 5 days prior to observation for the presence of CPE.

[0078] *Temperature-Sensitive Assay*: Two approaches were used to assess the growth characteristic of the cold-adapted virus strain 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 infected cells (replication kinetic) and second approach assessed the titre of the virus strain in cells incubated at each specific tested temperature. Briefly, in the first approach, the growth medium of three T-25 tissue culture flasks containing confluent monolayer Vero cells of similar age 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 (MOI). If no CPE was noted at the end of 10-day culture, the supernatant was passed into a new flask of 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 and incubated for 5 days prior to observation for the presence of CPE.

EXAMPLE 5

Results on Virus Characteristics in Cells of Cold-Adapted Temperature Sensitive Strains of Human Coxsackievirus A16

[0079] At the 100th passage through successively lower incubation temperature, cold-adapted CA16 strain was able to cause full CPE in Vero cells within 3 days of inoculation at a virus inoculum of ≤ 5 MOI and incubation temperature of 28° C but took 4 days to achieve full CPE at an incubation temperature of 37° C. At an incubation temperature of 39.5° C, no CPE was noted.

[0080] Incubation temperature of 28° C and 37° C was used to assay the virus replication titer of cold-adapted temperature sensitive strain of CA16. The titre of cold-adapted temperature sensitive strain of CA16 was 1×10^8 CCID₅₀/ml and 1×10^7 CCID₅₀/ml when the titrated culture plates were incubated at 28° C and 37° C respectively.

[0081] 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.

[0082] 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 Enterovirus 71 strain which is the strain EV71:TLL β P20 as deposited with the American Type Culture Collection under Accession No. PTA-13285 or the strain EV71:TLL α P20 as deposited with the American Type Culture Collection under Accession No. PTA-13284.
2. A composition comprising the cold-adapted temperature sensitive Enterovirus 71 strain of claim 1.
3. The composition of claim 2 which further comprises a pharmaceutically acceptable carrier.
4. The composition of claim 2 or 3 which further comprises an adjuvant.
5. The composition of any one of claims 2 to 4 which is a vaccine.
6. A cold-adapted temperature sensitive Enterovirus 71 strain of claim 1 or a composition of any one of claims 2 to 5 for use in eliciting a protective immune response in a subject.
7. A cold-adapted temperature sensitive Enterovirus 71 strain of claim 1 or a composition of any one of claims 2 to 5 for use in preventing a subject from becoming afflicted with an Enterovirus 71-associated disease.
8. A cold-adapted temperature sensitive Enterovirus 71 strain of claim 1 or a composition of any one of claims 2 to 5 for use in delaying the onset of or slowing the rate of an Enterovirus 71-associated disease in an Enterovirus 71-infected subject.
9. Use of cold-adapted temperature sensitive Enterovirus 71 strain of claim 1 or a composition of any one of claims 2 to 5 for the manufacture of a medicament for eliciting a protective immune response in a subject against an Enterovirus 71-associated disease.

10. Use of cold-adapted temperature sensitive Enterovirus 71 strain of claim 1 or a composition of any one of claims 2 to 5 for the manufacture of a medicament for preventing a subject from becoming afflicted with an Enterovirus 71-associated disease.
11. Use of cold-adapted temperature sensitive Enterovirus 71 strain of any claim 1 or a composition of any one of claims 2 to 5 for the manufacture of a medicament for delaying the onset of or slowing the rate of an Enterovirus 71-associated disease in an Enterovirus 71-infected subject.
12. The Enterovirus strain according to claim 1 or the composition according to any one of claims 2 to 5 for eliciting a protective immune response in a subject against Enterovirus 71.
13. The Enterovirus strain or composition of claim 12, wherein the subject has been exposed to wild-type Enterovirus 71.
14. The Enterovirus strain according to claim 1 or the composition according to any one of claims 2 to 5 for preventing a subject from becoming afflicted with an Enterovirus 71-associated disease.
15. The Enterovirus strain or composition of claim 14, wherein the subject has been exposed to wild-type Enterovirus 71.
16. The Enterovirus strain according to claim 1 or the composition according to any one of claims 2 to 5 for slowing the onset of an Enterovirus 71-associated disease in an Enterovirus 71-infected subject.
17. The Enterovirus strain or composition of any one of claims 12 to 16, wherein the subject is a human subject.
18. A kit for immunization of a subject with of a cold-adapted temperature sensitive Enterovirus 71 strain comprising the cold-adapted temperature sensitive Enterovirus 71 strain of claim 1 or a composition of any one of claims 2 to 5, a pharmaceutically acceptable carrier, and an instructional material for the use thereof.
19. The kit of claim 18 which further comprises an applicator.

20. A method of making a vaccine comprising using the cold-adapted temperature sensitive Enterovirus 71 strain of claim 1.
21. Use of the cold-adapted temperature sensitive Enterovirus 71 strain of claim 1 for vaccine development.
22. A cold-adapted temperature sensitive Enterovirus 71 strain of claim 1 for use in vaccine development.
23. A strain according to claim 1 or a composition of any one of claims 2 to 5 for use as a medicament.
24. Use of the strain according to claim 1 or a composition of any one of claims 2 to 5 for the manufacture of a medicament for the treatment of hand foot and mouth disease.