ENTEROVIRUS 71 ANIMAL MODEL

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application is related to and claims priority to U.S. provisional patent application Serial No. 62/114,880 filed on 11 February 2015 and to U.S. provisional patent application Serial No. 62/108,828 filed on 28 January 2015. Each application is incorporated herein in its entirety.

SUBMISSION OF SEQUENCE LISTING

[0002] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is entitled 2577243PCTSequenceListing.txt, created on 29 December 2015 and is 32 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 Enterovirus 71 (EV71), the development of an animal model and screening of candidate anti-EV71 compounds.

[0004] The publications and other materials used herein to illuminate the background of the invention, and in particular, cases to provide additional details respecting the practice, are incorporated by reference, and for convenience are referenced in the following text by author and date and are listed alphabetically by author in the appended bibliography.

[0005] Enterovirus 71 (EV71) is a small non-enveloped virus approximately 30 nm in diameter. The viral capsid exhibits icosahedral symmetry and is comprised of 60 identical units (protomers), with each consisting of four viral structural proteins VP1–VP4. The capsid surrounds a core of a single-stranded positive-sense RNA genome of 7,450 nucleotides (nt) long. The genome contains a single open reading frame which encodes a polyprotein of 2193 amino acids (aa) and is flanked by a long 5′ untranslated region (UTR) of 745 nt and a shorter 3′ UTR of 85 nt with a poly-A tract of variable length at its 3′ terminus. The polyprotein is divided into three regions, i.e., P1, P2 and P3. P1 encodes four viral structural proteins 1A-1D (VP4, VP2, VP3 and VP1); P2 and P3 encode seven non-structural proteins 2A-2C and 3A-3D [1–3]. Phylogenetic analysis of the major capsid protein (VP1) gene divided EV71 into six genotypes (denoted A to F) [66], and genotypes B and C are further subdivided into subgenotypes B1 to B5 and C1 to C5 [3].

[0006] EV71 causes 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 [4, 5]. The virus was first isolated from a child with acute encephalitis in California, USA in 1969, and subsequently characterized as a new serotype of the genus *Enterovirus* in 1974 [6]. Outbreaks of HFMD with or without neurologic complications and deaths were reported in various parts of the world [7–20]. Since 1997, EV71 infections have been a major public health burden and of constant epidemiologic concern in the Asia-Pacific Region. An HFMD outbreak due to highly neurovirulent EV71 emerged in Malaysia resulting in 48 deaths in 1997 [21, 22], followed by a larger outbreak that occurred in Taiwan in 1998 with more than 129,000 cases of HFMD, 405 severe infections and 78 deaths due to acute brainstem encephalomyelitis with neurogenic cardiac failure and pulmonary edema [23–26]. In People's Republic of China, 488,955 HFMD cases with 126 deaths were recorded in 2008 [27] and increased to 1,155,525 cases with 353 fatalities in 2009 [28]. In 2010, China experienced the largest ever HFMD outbreak with more than 1.7 million cases, 27,000 patients with severe neurologic complications, and 905 deaths [29].

[0007] Similar to other human enteroviruses, EV71 is unable to infect animals other than humans, although rhesus and cynomolgous monkeys can be experimentally infected [30–32]. This is primarily due to the inefficient binding of EV71 to its receptor for uncoating and entry into mouse cells. which had been recently identified as Scavenger Receptor Class B Member-2 (SCARB2) protein [47]. The human and murine SCARB2 proteins exhibit only 84% amino acid sequence identity and therefore display significant structural divergence [49, 67], hence precipitating the virus-receptor incompatibility underlying the natural resistance of non-human cells to EV71 infection. Once the virus successfully engages the SCARB2 receptor on the cellular surface and uncoats into the cytoplasm, the viral RNA is translated, resulting in the expression of various viral non-structural proteins. The viral RNA is subsequently replicated, packaged into the capsid, and released into the environment free to re-infect healthy cells.

[0008] Understanding its pathogenesis and development of specific therapeutics against the virus are hampered by the lack of suitable small animal models, because EV71 is unable to naturally infect small rodents. Attempts to establish mouse models of EV71 infection and disease have been made, mostly through virus adaptation by serial passages in young suckling mice [33–39]. Although some models were able to recapitulate symptoms of clinical illness, none has been reported to cause disease in immuno-competent mice aged 2 weeks old or older. Moreover, clinical features of disease and pathology of EV71 infections in humans and

experimental monkeys could not be replicated in mice, with the exception of the immunocompromised interferon receptor-deficient AG129 mice [35]. More recently, transgenic mice expressing human PSGL-1 [68] and human SCARB2 [69, 70] proteins have been available, but these exhibit only marginal improvements in susceptibility to EV71 infection.

[0009] RNA viruses, by virtue of their error-prone replication and high mutation rates [40–42], replicate as a swarm of related variant sequences known as *quasispecies* [43, 44]. It is comprised of a master species exhibiting the highest fitness in a certain environment, and of a mutant spectrum composed of a collection of closely related mutant sequences with a certain probability distribution [44, 45]. These endow RNA viruses with genome plasticity, which is reflected in their ability to quickly adapt to changing environments.

[0010] The mechanisms by which EV71 infection causes fatal neurological disease are not fully understood, hence several research groups have attempted to reproduce the pathology of human infection in experimental animals including rhesus and cynomolgous monkeys [114-120], laboratory mice [112, 121-129], and other mammals [130-133]. Unfortunately, none of these models exhibits the full spectrum of neurological features observed in human cases, especially those attributable to acute brainstem encephalitis with fulminant neurogenic pulmonary edema (NPE) [21, 22, 134-137]. Indeed, even in experimental systems that more accurately replicate the signs and symptoms of EV71 infection in humans, the underlying mechanisms differ substantially from those that confer disease in human patients. To date, no single animal model has conclusively replicated EV71-induced NPE. A key distinction is that EV71 is restricted to CNS tissues in human patients [92-94, 111], whereas in animal models the virus can also be detected in non-nervous tissues including the skeletal muscles [33-36, 38, 98] and liver [119]. While there have also been efforts to create transgenic mice that express the human EV71 receptor proteins PSGL-1 (P-selectin glycoprotein ligand-1) and SCARB2 (S 66 cavenger Receptor Class B, member 2) [68-70, 47, 138], none of these models exhibits NPE, hence their utility for identifying novel interventions for human patients is limited.

[0011] No suitable animal models exist to study infection and disease progression in EV71 infected animals or that could be used to screen anti-viral compounds or anti-viral vaccines. It is desired to develop an animal model for these purposes.

SUMMARY OF THE INVENTION

[0012] The present invention relates to Enterovirus 71 (EV71), the development of an animal model and screening of candidate anti-EV71 compounds. More specifically, the present

invention relates to the discovery that Enterovirus 71 (EV71) strains that have been adapted to infect rodent cell lines or cloned derived virus containing mutations in VP1 can cause disease in immuno-competent rodents and immuno-compromised rodents.

[0013] In addition, the present invention relates to the development of a clinically authentic model of EV71-induced neurological disease by infecting BALB/c mice with a modified strain (e.g., *EV71:TLLmv*) adapted to infect NIH/3T3 mouse fibroblasts. Using this approach, the modified EV71 is used to induce acute encephalomyelitis associated with neurogenic pulmonary edema in mice, characterized by lung swelling and increased organ weight compared with mock-infected lungs. Despite the absence of lung or cardiac tissue inflammation, focal hemorrhage and proteinaceous fluid in the alveoli, high serum levels of catecholamines, and extensive tissue damage in the brainstem, particularly the medulla oblongata were observed. These data demonstrate that the model accurately reproduces the signs and symptoms of human EV71-induced neurogenic pulmonary edema.

[0014] Thus, in one aspect, the present invention relates to an animal model that comprises a rodent infected with an Enterovirus 71 capable of infecting the rodent, sometimes referred to herein as a modified Enterovirus 71. In one embodiment, such an Enterovirus 71 is a rodent cell line adapted Enterovirus 71. In another embodiment, such an Enterovirus 71 is a clone derived virus (CDV) containing mutations in VP1. In some embodiments, the mutations in VP1 enable the CDV to use rodent SCARB2 proteins to infect rodent cells. In one embodiment, the rodent is an immuno-competent rodent. In another embodiment, the rodent is an immuno-compromised rodent. Suitable animals for use as models are preferably mammalian animals, most preferably convenient laboratory animals such as rabbits, rats, mice, and the like. In one embodiment, the animal is a mouse. In some embodiments, the mouse is a BALB/c mouse. In another embodiment, the rodent cell line is a mouse cell line. In a further embodiment, the mouse cell line is a mouse NIH/3T3 cell line. In another embodiment, the mouse cell line is a mouse Neuro-2a cell line. In one embodiment, the rodent cell line adapted Enterovirus 71is *EV71:TLLm*. In another embodiment, the rodent cell line adapted Enterovirus 71 is *EV71:TLLmv*. In one embodiment, the clone derived virus containing mutations in VP1 is *CDV:BS$_{VP1}$[K98E/E145A/L169F]*. The animal model is useful for studying systemic spread of the virus and human disease spectrum in animal models. The animal model is also useful for screening antiviral drugs and vaccines.

[0015] In another aspect, the present invention provides a method for preparing an animal model with the full-spectrum of EV71-induced neurological infection, disease and pathology

observed in humans. In some embodiments, the method comprises infecting a rodent described herein with a modified Enterovirus 71 described herein and raising the infected rodent for up to about 4 weeks. In some embodiments, the age of the rodent to be infected is between about 1 week and about 4 weeks. In other embodiments, the infected rodent is raised for about 1 week to about 4 weeks. In some embodiments, the rodent is a mouse as described herein. In other embodiments, the rodent is infected by inoculating the rodent with the modified Enterovirus 71. In one embodiment, the inoculation is intraperitoneal (I.P.). In another embodiment, the inoculation is intramuscular (I.M.). In some embodiments, the virus dose inoculated into the rodent is a median cell culture infectious dose ($CCID_{50}$) between about between about 10^3 and about 10^7.

[0016] In an additional aspect, the present invention provides a method to screen antiviral drugs. In accordance with this aspect, the method comprises the following steps: providing a test group of animals and a control group of animals in which the animals of each group are animals of the animal model described herein; administering to the test group an antiviral drug candidate; monitoring disease progression in the test group and the control group; comparing the disease progression in the test group to the disease progression in the control group; and selecting the antiviral drug candidate that reduces disease progression in the test group relative to the control group. In one embodiment, the antiviral drug is first screened in a test rodent cell line infected with a rodent cell line adapted Enterovirus 71 before screening in the animals. In another embodiment, the antiviral drug is first screened in a test rodent cell line infected with a clone derived virus (CDV) containing mutations in VP1 before screening in the animals.

[0017] In a further aspect, the present invention provides a method to screen effective antiviral vaccines. According to this aspect, the method comprises the following steps: providing a test group of animals and a control group of animals in which the animals of each group are animals of the animal model described herein; administering to the test group an antiviral vaccine candidate; monitoring disease progression in the test group and the control group; comparing the disease progression in the test group to disease progression in the control group; and selecting the antiviral vaccine candidate that reduces disease progression in the test group relative to the control group. In one embodiment, the antiviral vaccine candidate is first screened in a test rodent cell line infected with a rodent cell line adapted Enterovirus 71 before screening in the animals. In another embodiment, the antiviral vaccine candidate is first screened in a test rodent cell line infected with clone derived virus (CDV) containing mutations in VP1 before screening in the animals.

BRIEF DESCRIPTION OF THE FIGURES

[0018] Figures 1A-1O show cytopathic effects (CPE) observed following virus infection of various primate cell lines. Primate cells: RD cells (Figures A1–1C), HeLa cells (Figures 1D-1F), HEp-2 cells (Figures 1G–1I), Vero cells (Figures 1J–1L), and COS-7 cells (Figures 1M–1O) infected with 1 MOI of either EV71:BS (Figures 1A, 1D, 1G, 1J and 1M), EV71:TLLm (Figures 1B, 1E, 1H, 1K and 1N), or EV71:TLLmv (Figures 1C, 1F, 1I, 1L and 1O) virus were observed at 48 hpi for cytopathic effects or death of the cell monolayer. Images are representative of results in three independent experiments.

[0019] Figures 2A-2O show virus antigen detection in cell lines infected with EV71:BS, EV71:TLLm and EV71:TLLmv. Overnight seeded mammalian cell lines: HeLa (Figures 2A–2C), HEp-2 (Figures 2D–2F), CHO-K1 (Figures 2G–2LI, NRK (Figures 2J–2L), and TCMK (Figures 2M–2O), were infected with 1 MOI of respective virus. Cells were harvested at 48 hpi, coated onto Teflon slides and fixed in cold acetone. Cells were probed with pan-enterovirus antibody and stained with FITC-conjugated anti-mouse IgG. Images are representative of two independent experiments.

[0020] Figures 3A-3D show growth kinetics of *EV71:BS*, *EV71:TLLm*, and *EV71:TLLmv* determined in NIH/3T3 and Vero cells. Supernatants from various mammalian cells infected with 1 MOI of respective virus were harvested at various time points and subjected to titration and enumerated using the Reed and Muench method. Figure 3A: EV71:BS virus titer determined in Vero cells. Figure 3B: *EV71:TLLmv* virus titer determined in NIH/3T3 cells. Figures 3C and 3D: *EV71:TLLm* virus titer determined in NIH/3T3 cells. Growth curves from cell lines that did not exhibit productive infection are not shown.

[0021] Figures 4A-4O show cytopathic effects (CPE) observed following virus infection of various rodent cell lines. Rodent cells: NIH/3T3 cells (Figures 4A–4C), Neuro-2A cells (Figures 4D–4F), TCMK cells (Figures 4G–4I), CHO-K1 cells Figures (4J–4L), and NRK cells (Figures 4M–4O) infected with 1 MOI of either *EV71:BS* (Figures 4A, 4D, 4G, 4J and 4M), *EV71:TLLm* (Figures 4B, 4E, 4H, 4K and 4N), or *EV71:TLLmv* (Figures 4C, 4F, 4I, 4L and 4O) viruses were observed at 48 hpi for cytopathic effects or death of the cell monolayer. Images are representative of results from three independent experiments.

[0022] Figures 5A-5D show virus fitness assessment of *EV71:BS*, *EV71:TLLm*, and *EV71:TLLmv* in NIH/3T3 determined by the titer ratio. Virus titer determined separately in NIH/3T3 and Vero cells were used to calculate the virus fitness as log[(titer in NIH/3T3

cells)/(titer in Vero cells)]. Virus fitness of (Figure 5A) *EV71:BS*, (Figure 5B) *EV71:TLLmv*, and (Figures 5C and 5D) *EV71:TLLm* were calculated from the virus titer values shown in Figures 3A-3D. Virus fitness assays obtained from cell lines that did not exhibit productive infection are not shown.

[0023] Figures 6A and 6B show transfection of NIH/3T3 with *EV71:BS* viral RNA induces productive infection. Overnight seeded NIH/3T3 and Vero cells were inoculated with virus supernatant harvested from NIH/3T3 cells previously transfected viral RNA extracted from *EV71:BS*, *EV71:TLLm*, and *EV71:TLLmv*. Figure 6A: Cells were imaged using inverted light microscope at 24 hpi to observe induced CPE. Figure 6B: Cells were harvested at 7 dpi, coated onto Teflon slides, probed with pan-enterovirus antibody, and stained with anti-mouse FITC-conjugated antibody.

[0024] Figures 7A and 7B show virus fitness assessment of *EV71:BS*, *EV71:TLLm*, and *EV71:TLLmv* in NIH/3T3 and Vero cells at 30°C. Overnight seeded (Figure 7A) NIH/3T3 and (Figure 7B) Vero cells infected with *EV71:BS* (panels a, d, g), *EV71:TLLm* (panels b, e, h), or *EV71:TLLmv* (panels c, f, i) were incubated at 30°C and observed under the light microscope with phase-contrast at 24 hpi (panels a–c), 48 hpi (panels d–f), and 72 hpi (panels g–i). Images taken are representative of two independent experiments.

[0025] Figures 8A and 8B show virus fitness assessment of *EV71:BS*, *EV71:TLLm*, and *EV71:TLLmv* in NIH/3T3 and Vero cells at 37°C. Overnight seeded (Figure 8A) NIH/3T3 and (Figure 8B) Vero cells infected with *EV71:BS* (panels a, d, g), *EV71:TLLm* (panels b, e, h), or *EV71:TLLmv* (panels c, f, i) were incubated at 37°C and observed under the light microscope with phase-contrast at 24 hpi (panels a–c), 48 hpi (panels d–f), and 72 hpi (panels g–i). Images taken are representative of two independent experiments.

[0026] Figures 9A and 9B show virus fitness assessment of *EV71:BS*, *EV71:TLLm*, and *EV71:TLLmv* in NIH/3T3 and Vero cells at 39°C. Overnight seeded (Figure 9A) NIH/3T3 and (Figure 9B) Vero cells infected with *EV71:BS* (panels a, d, g), *EV71:TLLm* (panels b, e, h), or *EV71:TLLmv* (panels c, f, i) were incubated at 39°C and observed under the light microscope with phase-contrast at 24 hpi (panels a–c), 48 hpi (panels d–f), and 72 hpi (panels g–i). Images taken are representative of two independent experiments.

[0027] Figures 10A-10L show transfection of murine cell lines NIH/3T3, Neuro-2A, and TCMK with *EV71:BS* viral RNA for evidence of virus replication. Overnight seeded NIH/3T3, Neuro-2A, and TCMK cells were either infected with 1000 $CCID_{50}$ of *EV71:BS* virus (Figures 10A, 10C, and 10E) or transfected with equivalent amounts of viral RNA (Figures 10B, 10D,

and 10F). and harvested at 48 hpi for viral antigen detection. Virus in the supernatants were harvested at 7 dpi and passaged onto fresh Vero (Figures 10G, 10I, and 10K) and NIH/3T3 cells (Figures 10H, 10J, and 10L). Cells were harvested and stained for viral antigens at 48 hpi.

[0028] Figures 11A-11D show localization in VP1 and VP2 of adaptive mutations in the genomes of *EV71:TLLm* and *EV71:TLLmv*. Adaptive mutations observed in the VP1 (Figures 11A and 11B) and VP2 (Figures 11C and 11D) regions of *EV71:TLLm* (Figures 11A and 11C) and *EV71:TLLmv* (Figures 11B and 11D) were modelled using DeepView/SwissPDBviewer v3.7 and the 3D structure of EV71 capsid P1 region (PDB ID 4AED). The mutations were observed to be mostly localized to the surface-exposed loops of the protein as shown.

[0029] Figure 12 shows titer ratio in NIH/3T3 cells relative to titer in Vero cells of virus supernatant harvested from cells either transfected with EV71:BS viral RNA or infected with live virus. Supernatants from NIH/3T3, Neuro-2A, Vero, and TCMK either transfected with viral RNA or infected with live virus were harvested and subjected to virus enumeration by Reed-and Muench method. The ratio of the log(titer) determined in NIH/3T3 cells relative to the titer determined in Vero cells is shown. *3T3-TRANS:* RNA transfected NIH/3T3 cells; *3T3-INF:* virus infected NIH/3T3 cells. Asterisks indicate Student's t-test with p-value <0.05.

[0030] Figures 13A and 13B show survival analysis of infected animals. Infected animals were observed and weighed daily. Figure 13A: Kaplan-Meier plot of infected animals showing number of deaths at various days post-infection. Figure 13B: Changes in body weight were plotted to determine the general health of the animals.

[0031] Figures 14A-14D show symptoms and pathology of infected animals. Majority of the infected animals displayed symptoms of disease. Figure 14A: Paralysis of the hind limbs (arrow). Figure 14B: Gross anatomy of the inflated lungs following necropsy (arrows). Tissue sections were also stained with Hematoxylin and Eosin staining (Figure 14C at 10x and Figure 14D at 20x). Black arrows point to the mucous substance infiltrating the alveolar spaces.

[0032] Figures 15A-15E show that transfection of viral genomic RNA into both primate and rodent cells yields viable virus. Figure 15A: Genomic RNA extracted from either *EV71:BS*, *EV71:TLLm*, or *EV71:TLLmv* were individually transfected into Vero, NIH/3T3, and Neuro-2a cells (P0). Transfection supernatants were harvested and inoculated onto either Vero or NIH/3T3 cells (P1) to assess for viability of virus progeny. Infection of P0 cells was assessed by observation of cytopathic effects (CPE) (Figure 15B) and immunofluoresence detection of viral antigens (Figure 15C). Similarly, infection of P1 cells from *EV71:BS* RNA-transfected cells was

assessed by CPE induction (Figure 15D) and immunofluorescence detection of expressed viral antigens (Figure 15E).

[0033] Figures 16A-16F show that the capsid-encoding region of mouse cell-adapted *EV71:TLLm* drives productive infection of mouse cells with *EV71:BS*. Figure 16A: Infectious cDNA clones of the full genome of EV71:BS were generated, and the P1 region replaced with sequences from *EV71:TLLm* capsid to generate chimeric virus, *EV71:BS[M-P1]*. Figure 16B: Cells were infected with clone-derived virus (CDV) from either *EV71:BS* or *EV71:BS[M-P1]*, and infection was assessed by induction of lytic cytopathic effects (CPE) (Figure 16C) and viral antigen expression (Figure 16D). Supernatants were re-inoculated onto fresh cells, and virus titers were measured from passages (P1 and P2) obtained from infected Vero (Figure 16E), as well as passage P1 of NIH/3T3 (3T3) and Neuro-2A (N2a) cells (Figure 16F). Error bars indicate SD. * $p<0.05$.

[0034] Figures 17A-17G show that the VP1-L169F amino acid substitution in the capsid is sufficient to enable *EV71:BS* entry into murine cells. Figure 17A: Various mutant cDNA clones were generated by incorporating amino acid substitutions in VP1: K98E, E145A, and L169F; and VP2: S144T and K149I; into the full-length *EV71:BS* genome. Mutations corresponding to amino acid substitutions are written in parentheses. Infection of various cell lines with clone-derived virus (CDV) was monitored by assessing induction of lytic cytopathic effects (CPE) (Figures 17B and 17C) and expression of viral antigens (Figures 17D and 17E). Virus titers from infected Vero cells (Figure 17F) and NIH/3T3 and Neuro-2a cells (Figure 17G) were determined to assess generation of viable virus progeny. Error bars indicate SD.

[0035] Figures18A-18E show that *EV71:BS* virus with combined VP1 amino acid substitutions in the capsid exhibit improved infection of mouse cells. Figure 18A: Various mutant cDNA clones were generated by incorporating combinations of amino acid substitutions in VP1 and VP2 into the full-length *EV71:BS* genome. Mutations corresponding to amino acid substitutions are written in parentheses. Infection of various cell lines with clone-derived virus (CDV) was monitored by assessing cytopathic effects (Figure 18B), viral antigen expression (Figure 18C), and virus yield from infected Vero (Figure 18D), and NIH/3T3 (3T3) and Neuro-2a (N2A) cells (Figure 18E). Other clones with no virus yield are not shown. Error bars indicate SD.

[0036] Figurers 19A-19C show that EV71:BS virus with combined VP1-K98E, E145A, L169F amino acid substitutions in the capsid could be stably passaged in mouse neuronal cell line Neuro-2a. Clone-derived viruses were passaged twice in Neuro-2a and NIH/3T3 cells. At

each passage, infection was monitored by assessment of viral antigen expression (Figure 19A) and virus titer measured in Vero cells (Figure 19B). Error bars indicate SD. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$. Figure 19C: Genome sequences of representative *CDV:BS$_{VP1}$[K98E/E145A/L169F]* were determined to assess evidence of amino acid substitutions K98E (A2734G), E145A (A2876C), and L169F (C2947T). The mutation site is marked with an asterisk

[0037] Figures 20A-20 F show that *EV71:TLLmv* utilize SCARB2 to infect both primate and murine cells. Pre-incubation of NIH/3T3 cells (Figure 20A) and Vero cells (Figure 20B) fixed onto Teflon slides with murine SCARB2 (mSCARB2) antiserum inhibits *EV71:TLLmv* binding, as determined by reduced fluorescence signals. Fluorescence intensity on membranes was measured using Imaris imaging software (n = 100). *NSP* (non-specific rabbit serum). Pre-incubation of *EV71:TLLmv* with recombinant soluble protein of either mSCARB2 (Figure 20C) or human SCARB2 (hSCARB2) (Figure 20D) prior to inoculation onto NIH/3T3 cells reduces virus infection severity, as assessed by immunofluorescence assay. Pre-incubation of live NIH/3T3 cells with either hSCARB2 (Figure 20E) or mSCARB2 (Figure 20F) antiserum prior to infection with *EV71:TLLmv* reduces the virus titer in culture supernatant. * $p<0.05$; ** $p<0.005$; *** $p<0.0005$.

[0038] Figures 21A-21D show that incubation of Neuro-2A cells with murine SCARB2 rabbit antiserum reduced severity of infection with CDV mutants. Infection severity in cells pre-incubated with rabbit mSCARB2 antiserum prior to infection with *CDV:BS$_{VP1}$[K98E/E145A/L169F]* (Figures 21A and 21B or *CDV:BS[M-P1]* (Figures 21C and 21D) was monitored by assessing induction of cytopathic effects (CPE) (Figures 21A and 21C) and virus yield at 7 days post-infection (Figures 21B and 21D). Error bars indicate SD; * $p < 0.05$; ** $p < 0.005$. ***Degree of CPE***: 1 (0-25% cell death), 2 (25-50%), 3 (50-75%), 4 (75-100%).

[0039] Figures 22a-22c show that *EV71:TLLmv* is the most virulent of the three modified viral strains as evidenced by induction of severe disease in 1-week old BALB/c mice. Figure 22a: Neutralizing antibody titers in sera collected from mice infected (intraperitoneal; I.P.) with either EV71:BS (n=6), *EV71:TLLm* (n=5) or *EV71:TLLmv* (n=7) as assessed at the end of the observation period. Figures 22b and 22c: Kaplan-Meier survival curves of mice inoculated with 10^6 $CCID_{50}$ (median cell culture infective dose) of *EV71:BS*, *EV71:TLLm*, or *EV71:TLLmv* either via I.P. route (Figure 22b) or intramuscular (I.M.) route (Figure 22c). Statistical significance was determined using t-test with Welch's correction for unequal variance (Figure 22a) or the Mantel-Cox log-rank test (Figures 22b and 22c). *$p<0.05$, **$p<0.005$, ***$p<0.0005$.

[0040] Figures 23a-23h show that *EV71:TLLmv* infection in mice is characterized by acute severe disease resembling the human disease spectrum. Figure 23a and 23b: Dose-dependent lethality of *EV71:TLLmv* infection in 1 week-old mice. Figure 23a: Kaplan-Meier survival curve of 6 day-old pups I.P. injected with various doses of *EV71:TLLmv.* Figure 23b: The median humane endpoint (HD50) was equivalent to a virus dose of 3.98 x 10^3 $CCID_{50}$. Figure 23c: Kaplan-Meier survival curves of 1 week-old mice inoculated with *EV71:TLLmv* via I.P. or I.M. route. Figure 23d: Age- and route-dependent lethality induced by *EV71:TLLmv* infection in mice inoculated with a virus dose of 10^6 $CCID_{50}$. Figures 23e and 23f: Clinical signs observed in terminally-ill mice, some of which presented with paralysis of the hind limbs (*gray arrow*) and/or forelimbs. Others also exhibited small hairless lesions on the torso (*black arrow*). Figures 23g and 23h: Disease classification of 1 week-old mice inoculated with *EV71:TLLmv* via the I.P. route (Figure 23g), or I.M. route (Figure 23h).

[0041] Figures 24a-24e show that Severity of *EV71:TLLmv* infection in BALB/c mice depends on host age, virus dose, and route of administration. Figures 24a and 24b: Groups of 8-10 mice were inoculated with 10^6 $CCID_{50}$ of virus by I.P. route (Figure 24a) or I.M. route (Figure 24b) and the Kaplan-Meier survival curves determined for animals of different age groups. Figures 24c and 24d: Neutralizing antibody titers in sera collected at the end of the observation period from mice of various ages inoculated via either I.P. route (Figure 24c) or I.M. route (Figure 24d); 1 week (I.P. n=7; I.M. n=4), 2 weeks (n=5, n=7), 3 weeks (n=4; n=8), and 4 weeks (n=4; n=6). Figure 24e: Neutralizing antibody titers in sera collected from mice inoculated (I.P.) with various doses of *EV71:TLLmv*; CCID50 102 (n=5), 103 (n=4), 104 (n=4), 105 (n=5), or 106 (n=7). Statistical significance was determined by Mantel-Cox log-rank test (Figures 24a and 24b) or t-test with Welch's correction for unequal variance (Figures 24c, 24d and 24e). *$p<0.05$, **$p<0.005$, ***$p<0.0005$.

[0042] Figures 25a-25k show Signs of EV71-induced neurogenic pulmonary edema (NPE) in *Class IA* mice. Figures 25a-25d: Representative gross pathology of the lungs obtained from mock-infected mice (Figure 25a), or *EV71:TLLmv* infected mice presenting with signs of disease *Class IA* (Figure 25b), *Class IB* (Figure 25c), or *Class II* (Figure 25d). Images show top- and side-views. Note the incomplete collapse of the lungs apparent in Figure 25b (*white arrows*). Figure 25e: Wet weight comparison of lungs harvested from mock-infected mice (n=4), or *EV71:TLLmv*-infected mice presenting with signs of disease *Class IA* (n=8), *Class IB* (n=9), or *Class II* (n=4). (Figures 25f-25i: Representative images of lung tissue sections (5μm) stained with hematoxylin & eosin (H&E). Shown are low- and high magnification images of lungs

obtained from mock-infected mice (Figure 25f) or *EV71:TLLmv*-infected mice presenting with signs of disease *Class IA* (Figure 25g), *Class IB* (Figure 25h), or *Class II* (Figure 25i). Note the presence of pink proteinaceous fluid in the alveolar spaces (*asterisks* in Figure 25g, high mag.), some of which were also filled with erythrocytes (*gray arrows* in Figure 25g, high mag.). Figures 25j and 25k: Serum levels of adrenaline/epinephrine (Figure 25j) and noradrenaline/norepinephrine (Figure 25k) as determined in mock-infected mice (n=9), or *EV71:TLLmv*-infected mice presenting with signs of disease *Class IA* (n=8), *Class IB* (n=9), or *Class II* (n=3). Error bars indicate *SEM.* Statistical significance was determined by Mann-Whitney test (Figure 25e) or *t*-test with Welch's correction for unequal variance (Figures 25j and 25k). *p<0.05, **p<0.005.

[0043] Figures 26a and 26b show the absence of viral replication or inflammation in the lung and heart tissues of *Class IA* mice. Figure 26a: Representative images of lung tissue sections (5 μm) derived from various groups of mice infected with *EV71:TLLmv* and stained with either Hematoxylin & Eosin (H&E) for histopathological examination, or labelled using rabbit serum against EV71 antigens (EV71 IHC) for virus antigen localization. Figure 26b: Representative images of heart tissue sections (5 μm) processed for H&E and EV71 IHC.

[0044] Figures 27a-27d show representative maps depicting the localization and distribution of EV71 antigens and virus-induced lesions in different regions of *Class IA* and *Class IB* mouse brains. The cerebellar cortex (CTX) (Figures 27a, 27b and 27c); hypothalamus (HY) (Figures 27a and 27b); hippocampus (HP) (Figures 27b and 27c); thalamus (TH) (Figure 27b); midbrain (MB) and pons (P) (Figure 27c); and cerebellum (CBX) and medulla oblongata (MY) (Figure 27d) are indicated. Areas where viral antigens and pathologic lesions were detected are marked accordingly. Larger dots indicate stronger signals/lesion size. Template images were downloaded from brainstars.org1 and licensed under the Creative Commons of Japan. The brain tissue coronal section maps were obtained from the interactive Mouse Brain Atlas (http colon slash slash mouse dot brain-map dot org slash static slash atlas) [113].

[0045] Figures 28a-28n show that *EV71:TLLmv* infection in mice is associated with nervous tissue destruction and extensive viral replication. Figures 28a-28l: Representative images of brain tissue sections (5μm) stained with hematoxylin and eosin (H&E) or immunostained with rabbit serum against EV71 antigens (EV71 IHC). Sections were derived from mice presenting with signs of disease *Class IA* (left panels), or *Class IB* (right panels). Pathologic lesions in the brain included edema (*dashed boxes*), infiltrating cells (diagonal area in upper left quadrant of left panel and diagonal area in lower right quadrant of Figure 28k), neuronophagia (in left panels

of Figures 28a-28c), neurodegeneration (*black asterisks*), and degeneration of Purkinje cells (*gray asterisks*). (Figures 28m and 28n: Representative maps of spinal cord coronal sections from mice in disease *Class IA* (Figure 28m) or *Class IB* (Figure 28n). Highlighted are areas where viral antigens and pathologic lesions were detected. Larger dot sizes indicate more extensive signals/lesions. *V*, ventral side; *D*, dorsal side.

[0046] Figures 29a-29c show EV71 antigens and virus-induced lesions in other areas of the hindbrain of *Class IA* mice. Figure 29a: Representative images of the dentate nucleus stained with either Hematoxylin and Eosin (H&E) for histopathological examination or rabbit serum against EV71 antigens (EV71 IHC) for virus antigen localization. Boxed areas are shown magnified in the inset. Figure 29b: Representative images of the caudal brainstem from *Class I* mice. Images depict the cerebellar cortex (CBX) and medulla oblongata (MY). The area prostrema (AP; asterisk) and nucleus of the solitary tract (NTS; dashed circle) are also labelled for reference. Areas where viral antigens and pathologic lesions were detected are indicated. Larger dots represent stronger signals/lesion size. Template images were downloaded from brainstars.org1 and licensed under the Creative Commons of Japan. The brain tissue coronal section maps were obtained from the Mouse Brain Atlas (http colon slash slash mouse dot .brain-map dot org slash static slash atlas) [113]. Figure 29c: Representative images of the medulla from Class IA mice depicting the H&E and EV71 IHC staining patterns in the AP and NTS.

[0047] Figures 30a-30f show histological sections of nervous tissues from mock-infected mice (healthy controls). Representative images of normal mouse tissue sections (5 μm) stained with either Hematoxylin & Eosin (H&E) for histopathological examination or rabbit serum against EV71 antigens (EV71 IHC) for virus antigen localization. Brain sections show CA3 pyramidal neurons in the hippocampus (Figure 30a); reticular neurons in the hypothalamus (Figure 30b) and thalamus (Figure 30c); neurons in the periaqueductal gray matter (Figure 30d); Purkinje cell layer in the cerebellar cortex (Figure 30e). Note the normal Purkinje cell morphology (black asterisks in left panel of Figure 30e); and reticular neurons in the medulla oblongata (Figure 30f).

[0048] Figures 31a-31e show *EV71:TLLmv*-induced pathology and viral antigen distribution in other nervous tissues. Representative images of mouse tissue sections (5 μm) stained with either Hematoxylin & Eosin (H&E) for histopathological examination or rabbit serum against EV71 antigens for immunohistochemical analysis (EV71 IHC). Brain tissues were obtained from *EV71:TLLmv*-infected or mock-infected mice. Brain coronal sections depict motor cortex

pyramidal neurons (Figure 31a); pontine gray neurons (Figure 31b); and spinal cord coronal sections from the cervical (Figure 31c), thoracic (Figure 31d), and lumbar columns (Figure 31e). Note the cellular infiltrate (lower right quadrant of left panel in Figure 31a) and neuronal necrosis (black asterisks in second panel of Figure 31a) apparent in the motor cortex. Boxed areas in Figures 31c-31e are shown magnified in the respective insets.

DETAILED DESCRIPTION OF THE INVENTION

[0049] The present invention relates to Enterovirus 71 (EV71), the development of an animal model and screening of candidate anti-EV71 compounds. More specifically, the present invention relates to the discovery that Enterovirus 71 (EV71) strains that have been adapted to infect rodent cell lines or cloned derived virus containing mutations in VP1 can cause disease in immuno-competent rodents and immuno-compromised rodents. These EV71 strains are sometimes referred to as modified Enterovirus 71 herein.

[0050] In addition, the present invention relates to the development of a clinically authentic model of EV71-induced neurological disease by infecting BALB/c mice with a modified strain (e.g., *EV71:TLLmv*) adapted to infect NIH/3T3 mouse fibroblasts. Using this approach, the modified EV71 is used to induce acute encephalomyelitis associated with neurogenic pulmonary edema in mice, characterized by lung swelling and increased organ weight compared with mock-infected lungs. Despite the absence of lung or cardiac tissue inflammation, focal hemorrhage and proteinaceous fluid in the alveoli, high serum levels of catecholamines, and extensive tissue damage in the brainstem, particularly the medulla oblongata were observed. These data demonstrate that the model accurately reproduces the signs and symptoms of human EV71-induced neurogenic pulmonary edema.

[0051] Thus, in one aspect, the present invention relates to an animal model that comprises a rodent infected with an Enterovirus 71 capable of infecting the rodent, sometimes referred to herein as a modified Enterovirus 71.. In one embodiment, such a modified Enterovirus 71 is a rodent cell line adapted Enterovirus 71. In another embodiment, such a modified Enterovirus 71 is a clone derived virus (CDV) containing mutations in VP1. In some embodiments, the mutations in VP1 enable the CDV to use rodent SCARB2 proteins to infect rodent cells. In one embodiment, the rodent is an immuno-competent rodent. In another embodiment, the rodent is an immuno-compromised rodent. Suitable animals for use as models are preferably mammalian animals, most preferably convenient laboratory animals such as rabbits, rats, mice, and the like. In one embodiment, the animal is a mouse. In another embodiment, the rodent cell line is a

mouse cell line. In a further embodiment, the mouse cell line is a mouse NIH/3T3 cell line. In another embodiment, the mouse cell line is a mouse Neuro-2a cell line. In one embodiment, the rodent cell line adapted Enterovirus 71is *EV71:TLLm*. In another embodiment, the rodent cell line adapted Enterovirus 71 is *EV71:TLLmv*. In one embodiment, the clone derived virus containing mutations in VP1 is *CDV:BS$_{VP1}$[K98E/E145A/L169F]*. The animal model is useful for studying systemic spread of the virus and human disease spectrum in animal models. The animal model is also useful for screening antiviral drugs and vaccines.

[0052] The animal model is prepared on an as needed basis. A large standardized stock of rodent cell line-adapted EV71 strains is prepared, titrated and kept in a deep freezer (minus 80°C). A "standardized" (based on statistical calculation) number of rodents, such as BALB/c mice or NSG mice, are infected with a standardized titer of the virus strains to produce the animal model. The animal model of the present invention develops neurological symptoms (similar to those that can develop in humans) upon infection. As shown herein, the modified Enterovirus 71, such as rodent cell line-adapted EV71 virus strains, affect brain and a variety of neurological diseases that are manifested in mice.

[0053] In some embodiments, the rodent cell line adapted Enterovirus 71is *EV71:TLLm*. *EV71:TLLm* was derived following serial passage of the human EV71 BS strain in NIH/3T3 mouse cell line for a minimum of 60 cycles. In one embodiment, *EV71:TLLm* was deposited on 12 January 2015 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 V201437. In another embodiment, *EV71:TLLm* can be recovered using advanced reverse genetics if the viral RNA is synthesized using the viral RNA sequence (GenBank Accession No. KF514879; SEQ ID NO:1). Techniques for advanced reverse genetics are well known in the art [84-87].

[0054] In another embodiment, the rodent cell line adapted Enterovirus 71 is *EV71:TLLmv*. *EV71:TLLmv* was derived from further passage of *EV71:TLLm* in NIH/3T3 mouse cell line for another 40 cycles. In one embodiment, *EV71:TLLmv* was deposited on 12 January 2015 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 V201438. In another embodiment, *EV71:TLLmv* can be recovered using advanced reverse genetics if the viral RNA is synthesized using the viral RNA sequence (GenBank Accession No. KF514880; SEQ ID NO:2). Techniques for advanced reverse genetics are well known in the art.

[0055] In a further embodiment, the modified Enterovirus 71 is a clone derived virus (CDV) having mutations in the capsid protein VP1 which enables the modified Enterovirus 71 to use rodent SCARB2 proteins to infect rodent cells. A modified Enterovirus 71 having mutations in VP1 is made by preparing a full length genomic cDNA clone using techniques known to the skilled artisan or as described herein. Mutations in VP1 or other proteins of Enterovirus 71 are made using site-directed mutagenesis or CRISPR technology (see, e.g., PCT Publication No. WO2014/127287). Live virus (clone derived virus (CDV)) is prepared from the cDNA clones clone using techniques known to the skilled artisan or as described herein. CDVs having different mutations or collections of mutations are then tested for their ability to infect rodent cells. Alternatively, CDVs having different mutations or collections of mutations are then tested for their ability to bind to rodent SCARB2 proteins as an initial screening. Any suitable Enterovirus 71 strains can be used to develop CDVs having mutations in VP1. The number of mutations may and specific mutations vary for each strain in order to produce CDVs that are sufficient to produce a full blown infection in the target rodent cell. Thus, multiple rodent virulent EV71 strains can be produced that can infect several, many or all types of rodent, e.g., mouse, cell lines. In embodiment, the EV71 strain used to produce a CDV having mutations in VP1 is Enterovirus 71 BS strain. In one embodiment, the modified Enterovirus 71 is *CDV:BS$_{VP1}$[K98E/E145A/L169F]*.

[0056] In another aspect, the present invention provides a method for preparing an animal model with the full-spectrum of EV71-induced neurological infection, disease and pathology observed in humans. In some embodiments, the method comprises infecting a rodent described herein with a modified Enterovirus 71 described herein and raising the infected rodent for up to about 4 weeks. In one embodiment, the modified Enterovirus 71 is *EV71:TLLmv*. In another embodiment, the modified Enterovirus 71 is *EV71:TLLm*. In a further embodiment, the modified Enterovirus 71 is *CDV:BS$_{VP1}$[K98E/E145A/L169F]*. In some embodiments, the age of the rodent to be infected is between about 1 week and about 4 weeks. In other embodiments, the age of the rodent to be infected is between about 1 week and about 3 weeks. In other embodiments, the age of the rodent to be infected is between about 1 week and about 2 weeks. In one embodiment, the age of the rodent to be infected is about 1 week. In another embodiment, the age of the rodent to be infected is about 2 weeks. In a further embodiment, the age of the rodent to be infected is about 3 weeks. In some embodiments, the infected rodent is raised for about 1 week to about 4 weeks. In other embodiments, the infected rodent is raised for about 1 week to about 3 weeks. In other embodiments, the infected rodent is raised for about

1 week to about 2 weeks. In one embodiment, the infected rodent is raised for about 1 week. In another embodiment, the infected rodent is raised for about 2 weeks. In an additional embodiment, the infected rodent is raised for about 3 weeks. In a further embodiment, the infected rodent is raised for about 4 weeks. In some embodiments, the rodent is an immune-compromised rodent. In some embodiments, the rodent is a mouse as described herein. In one embodiment, the immune-compromised mouse is a BALB/c mouse. In other embodiments, the rodent is infected by inoculating the rodent with the modified Enterovirus 71. In one embodiment, the inoculation is intraperitoneal (I.P.). In another embodiment, the inoculation is intramuscular (I.M.). In some embodiments, the virus dose inoculated into the rodent is a median cell culture infectious dose ($CCID_{50}$) between about between about 10^3 and about 10^7. In other embodiments, the virus dose inoculated into the rodent is a $CCID_{50}$ between about between about 10^3 and about 10^6. In one embodiment, the virus dose inoculated into the rodent is a $CCID_{50}$ between about 4 x 10^3 and about 10^6. In another embodiment, the virus dose inoculated into the rodent is a $CCID_{50}$ between about 10^4 and about 10^6. In an additional embodiment, the virus dose inoculated into the rodent is a $CCID_{50}$ between about 10^5 and about 10^6. In one embodiment, the virus dose inoculated into the rodent is a $CCID_{50}$ of about 10^6.

[0057] The animal model prepared in this manner is an authentic mouse model of EV71 neuro-infection that exhibits face validity, *i.e.*, these animals display the entire range of clinical signs that can be observed across the full spectrum of neurological disease induced by EV71 infection in human patients, including NPE. This animal model also displays construct validity with respect to the gross and histopathological features of disease, which closely resemble those reported in fatal human cases. This new *in vivo* model represents a powerful tool for identifying the key events in EV71 neuro-pathogenesis, for dissecting the mechanism of EV71-induced NPE, developing novel treatment modalities and potential antiviral therapies, and for conducting pre-clinical evaluation of novel vaccines.

[0058] In an additional aspect, the present invention provides a method to screen antiviral drugs. In accordance with this aspect, the method comprises the following steps: providing a test group of animals and a control group of animals in which the animals of each group are animals of the animal model described herein; administering to the test group an antiviral drug candidate; monitoring disease progression in the test group and the control group; comparing the disease progression in the test group to the disease progression in the control group; and selecting the antiviral drug candidate that reduces disease progression in the test group relative to the control group. In one embodiment, the antiviral drug is first screened in a test rodent cell

line infected with the rodent cell line adapted Enterovirus 71 before screening in the animals. In another embodiment, the antiviral drug is first screened in a test rodent cell line infected with a clone derived virus (CDV) containing mutations in VP1 before screening in the animals.

[0059] In a further aspect, the present invention provides a method to screen effective antiviral vaccines. According to this aspect, the method comprises the following steps: providing a test group of animals and a control group of animals in which the animals of each group are animals of the animal model described herein; administering to the test group an antiviral vaccine candidate; monitoring disease progression in the test group and the control group; comparing the disease progression in the test group to disease progression in the control group; and selecting the antiviral vaccine candidate that reduces disease progression in the test group relative to the control group. In one embodiment, the antiviral vaccine candidate is first screened in a test rodent cell line infected with the rodent cell line adapted Enterovirus 71 before screening in the animals. In another embodiment, the antiviral vaccine candidate is first screened in a test rodent cell line infected with clone derived virus (CDV) containing mutations in VP1 before screening in the animals.

[0060] In accordance with the methods of the present invention, a large standardized stock of rodent cell line-adapted EV71 strains is prepared, titrated and kept in a deep freezer (minus 80°C). Alternatively, a large standardized stock of clone derived virus (CDV) containing mutations in VP1 strains is prepared, titrated and kept in a deep freezer (minus 80°C). A "standardized" (based on statistical calculation) number of animals, such as Balb/c mice or NSG mice, are infected with a standardized titer of the virus strains. The candidate antiviral drug or antiviral vaccine is administered to the infected rodents at various standardized dosages at before appearance of illness (for assay of preventive effect) or at onset of illness (for assay of therapeutic effect of the drug). In one embodiment, high through-put *in vitro* screening of anti-EV71 compounds is performed using tissue culture cell lines susceptible to cytolytic infection by the rodent cell lined adapted EV71 virus strains, such as those described herein, including those described in the Examples. In another embodiment, high through-put *in vitro* screening of anti-EV71 compounds is performed using tissue culture cell lines susceptible to cytolytic infection by clone derived virus (CDV) containing mutations in VP1 strains, such as those described herein, including the Examples. The in vitro screening is performed using techniques well known in the art. The selected promising compounds from the *in vitro* screening are then screened *in vivo* in the animal model described herein.

[0061] As shown in Examples 2-8, sequential passage of the human EV71 isolate (*EV71:BS*) generated virus strains that gained the ability to infect *in vitro* cultured rodent cell lines. The mouse adherent fibroblast cell line NIH/3T3, which was derived from the NIH/Swiss mouse embryo [46], was used to adapt the human EV71 strain to infect rodent cells. Two such NIH/3T3-adapted strains are described - *EV71:TLLm* and *EV71:TLLmv*, where *EV71:TLLm* represents the early stage (passage number 60) and *EV71:TLLmv* represents the late stage (passage number 100) of the adaptation process. Based on the appearance of virus-induced CPE, measurement of high titer values, and positive detection of viral antigen through immunostaining, we categorized the virus-induced infection in cells as either productive or non-productive. Productive infection exhibits positive viral antigen detection as well as high virus titers, regardless of observation of CPE. On the other hand, non-productive infection is characterized by immeasurable virus titer at cut-off assay limit despite viral antigen detection and/or observation of CPE.

[0062] Whereas the clinical isolate *EV71:BS* infects only primate cell lines, *EV71:TLLm* productively infects both primate and rodent cell lines. It is worth noting that although *EV71:TLLm* virus has successfully achieved the ability to infect rodent cells, the degree of adaptation to NIH/3T3 cells is less pronounced. The virus titer determined using Vero cells is much higher than that determined in NIH/3T3 cells, as indicated by negative values in relative replication rates (RRR) assay (Figures 5C and 5D). In addition, *EV71:TLLm* successfully infects Vero cells leading to full CPE at various incubation temperatures whereas it can only achieve full CPE in infected NIH/3T3 at 37°C (Table 1). Further adaptation in mouse cells, which yielded the *EV71:TLLmv* virus, resulted in a virus strain that displays a higher degree of adaptation to mouse cells (Figure 5B) but at the cost of narrowing down the spectrum of permissible host cells. *EV71:TLLmv* does not infect primate cell lines as effectively as mouse cells although it exhibits successful infection leading to full CPE of NIH/3T3 cells incubated at a broader range of temperatures (Table 1). However, compared to the predecessor *EV71:TLLm*, *EV71:TLLmv* seemed to have lost the ability to enter and replicate in monkey kidney COS-7 cells, as well as human HeLa and Hep-2 cells (Figures 3B-3C; Figures 2B-2C, 2E-2F, and 2H-2I). It also lost the ability to replicate efficiently within hamster CHO-K1 and rat NRK cells (Figures 3B–3D; Figures 2K–2L and 2N–2O). These observations indicate that further passage of the virus in NIH/3T3 cells increases the degree of adaptation in mouse cells at the cost of losing infective ability in cell lines of other origin.

[0063] Although it is not possible to pinpoint which amino acid substitutions are adaptive to the mouse NIH/3T3 host cell, viral whole genome sequencing may shed light to potential adaptive mechanisms. Most of the amino acid substitutions identified reside in the P1 (capsid) and RNA polymerase (3D region) proteins (Table 2, 3), suggesting possible altered virus protein activity in host cell entry and replication. The accumulation of mutations in the P1 region is expected from the acquired ability *of EV71:TLLm* and *EV71:TLLmv* strains to infect new host cells. Capsid proteins form the structural context with which the virus initiates interaction with the permissive host cell through the virus receptor, which had been identified recently as Scavenger Receptor Class B Member 2 (SCARB2) [47] and later characterized as the main virus uncoating receptor of EV71 [48] and which is also utilized by some members of Human Enterovirus A (HEV-A) species. The human SCARB2 protein shares approximately 99% sequence identity with that of other primates. On the other hand, mouse SCARB2 protein exhibits 15% sequence dissimilarity compared to the primate protein [49], implying significant structural deviations from primate SCARB2 and perhaps contributing to the recalcitrance of rodent cells to native EV71 infection. It is plausible that adaptive mutations in the virus capsid may render the virus competent to bind the mouse cell receptor and result in successful entry and infection of novel hosts.

[0064] Mapping of the capsid protein mutations indicate that majority of the identified amino acid substitutions in the viral P1 region reside in exposed regions of the protein (Figures 11A-11D), specifically in the B–C, D–E, E–F, and G-H loops on the surface of VP1. The VP1 residues 150–180 harbour the viral capsid canyon that engages SCARB2 protein. This region centred at Gln-172 contains a major VP1 neutralization epitope at amino acids 163–177 [32]. Both EV71:TLLm and EV71:TLLmv exhibited substitutions E167D and L169F in the E-F loop of the VP1 canyon (Figures 11A–11B), loci which have not been previously reported. Other significant amino acid substitutions near the SCARB2 docking site include N104D within the B–C loop and S241L in the G-H loop, which are located within a 20 Å radius from Gln-172. The VP1 S241L mutation, in association with the K244E, had been previously reported arising from mouse passage of a CHO cell line-adapted EV71 [50]. This mutation, in combination with a VP2 K149I, was found to be associated with a non-virulent phenotype in 5-day mouse pups. However, a reverse mutation at VP1 241 from Leu to Ser [51] was reported arising from adaptation in NOD/SCID mouse brain tissues and found to be associated with a mouse virulent phenotype. The VP1 E145A mutation, which is far from the SCARB2 docking site and located in the D–E loop, is another candidate for conferring the ability to infect murine cells. The VP1

145 mutation had been previously reported [34, 37] and a single E145A mutation leads to virulence in NOD/SCID mice [51]. Another mutation in VP1 of a C4 genotype EV71, Q145E, was associated with virulence in 5-day old mice [52]. The mouse cell-adaptive mutations in VP2, particularly those within the neutralizing epitope of residues 136–150 [53], may also contribute to the virus ability to infect rodent cells. Two substitutions in VP2 E-F loop were observed in *EV71:TLLm* (Figure 11C), while three substitutions were present in *EV71:TLLmv* (Figure 11D). None of these mutations have been previously described, although a nearby locus at VP2 149 in the E-F loop had been mentioned in the literature [34, 50, 54] and described as an adaptive mutation to passage in PSGL-1 overexpressing cells [55].

[0065] To explore the possible role of the P1 region mutations in binding the virus receptor for host cell entry, *EV71:BS* viral RNA was transfected into murine cells. Direct introduction of *EV71:BS* RNA into the mouse cell cytoplasm results to productive infection in NIH/3T3 cells, as suggested by the observation of virus-induced CPE and measurable virus titers in the culture supernatant as assayed in Vero cells (Figure 12). Re-inoculation of the virus supernatant onto fresh NIH/3T3 cells, however, fails to induce productive infection (Figure 6A) and no viral antigens were detected (Figure S4H). Similarly, transfection of *EV71:BS* RNA into Neuro-2A cells, but not direct virus infection, resulted in positive antigen staining in Neuro-2A cells (Figures 10C and, 10D) and measurable virus titers (Figure 12). Virus supernatants passaged onto fresh Vero and NIH/3T3 cells resulted to positive antigen staining in Vero (Figure 10I) but not in NIH/3T3 cells (Figure 10J). These data indicate that circumventing the requirement of receptor engagement for host cell entry led to successful infection and production of virus progenies in NIH3T3 and Neuro-2A cells. These data also support that human *EV71:BS* cannot successfully enter mouse NIH/3T3 and Neuro-2A cells through murine cellular receptor. Moreover, these data suggest that mutations within the P1 region of *EV71:TLLm* and *EV71:TLLmv* genomes confer the virus with the ability for efficient receptor engagement, and consequently host cell entry.

[0066] Several amino acid mutations were also observed in the P2 and P3 regions, which encode virus proteins that are crucial for virus replication and hijacking the host cell protein translation machinery [56]. These adaptive mutations may function in optimizing EV71 genome replication and translation within mouse cells. The viral 3D protein alone accumulated 8 amino acid substitutions in *EV71:TLLmv* and 4 in *EV71:TLLm*, and both strains exhibited 1 mutation each in 3B and 2 mutations each in 3C. Direct inoculation of viral RNA into TCMK cells give insight into the possible role of the adaptive mutations in the nonstructural proteins of the virus.

Although TCMK cells have been shown to be permissible to *EV71:TLLm* and *EV71:TLLmv* infection (Figures 3B and 3D; Figures 4Q and 4R), transfection of EV71:BS viral RNA into TCMK cells did not result to successful infection. Viral antigen signals were not detected in infected and transfected cells (Figures 10E–10F), and passage of virus supernatant onto fresh NIH/3T3 and Vero cells did not yield positive viral antigen detection (Figures 10K and 10L). Moreover, there was no assayable virus titer in both infected and transfected cells (Figure 12). These data suggest that apart from mutations in the capsid region, mutations within the P2 and P3 regions, which are found in *EV71:TLLm* and *EV71:TLLmv*, are required to successfully infect TCMK cells.

[0067] It is believed that this is the first report of EV71 strains originally derived from human clinical sample that have successfully gained the ability to productively infect several rodent cell lines following successive passages in a mouse cell line. The relatively high mutation rates during RNA replication results in variant genomes that serve as genetic reservoirs of phenotypic traits for future adaptive potential, and the consequential replication as a quasispecies distribution [57, 58] leads to the dynamic plasticity of RNA viral genomes conferring adaptability to changing environments [59, 60]. Although EV71 infecting suckling mice [34, 37, 38, 51] and other rodents (e.g., gerbils) [39] have been reported previously, none was reported to productively infect *in vitro* cultured rodent cells. This may be due to the high genetic barrier associated with major changes in phenotype and host range [58]. Interestingly, this is the first report of a large number of adaptive mutations within the consensus full genome sequence of EV71. Whereas previously documented mouse-adapted EV71 reported less than 10 amino acid substitutions in the genome [34, 38, 51], we report 21 in *EV71:TLLm* and 36 in *EV71:TLLmv*, still more than the most number of adaptive mutations previously identified [37]. These suggest that few passages of the virus in mouse tissues [34, 36, 37] may not be sufficient to break the genetic barrier and successfully adapt the virus to infect cultured mouse cells. Instead, hundreds of successive passages might be necessary, as is observed in this study.

[0068] The host cell restriction of EV71 had recently been explained with the demonstration that EV71 utilizes Scavenger Receptor Class B Member-2 (SCARB2) as its functional receptor for host cell entry [47] and virus uncoating in the endosome [72]. The human and murine SCARB2 proteins exhibit only 84% amino acid sequence identity [67] suggesting structural differences between the two proteins. This scenario explains the general inability of EV71 clinical isolates to infect mouse-derived cells, as well as the general resistance of mice and other

rodents to EV71 experimental infections, which complicates efforts in developing small animal models of EV71 infection. Despite this virus-receptor incompatibility, however, we have shown that some murine cells support EV71 infection when the initial stage of infection, *i.e.* receptor-mediated cell entry, is bypassed through viral RNA transfection into cells. *EV71:BS* does not infect Neuro-2a and NIH/3T3 cells [71], but transfection of viral genomic RNA results to expression of EV71 proteins, induction of lytic cytopathic effects (CPE), and production of viable virus progeny. Similarly, live virus had been previously generated following transfection of Poliovirus RNA into mammalian cells [73-75], though the cells used (HeLa) were known to be permissive to Poliovirus infection. In our experiments, non-permissive mouse neuronal Neuro-2a and fibroblast NIH/3T3 cells were demonstrated to support viral replication and generate live virus progeny upon transfection of *EV71:BS* RNA into the cytoplasm, suggesting that the internal environment of murine cells contain host factors required for EV71 infection and support completion of the virus infection cycle, and that EV71 proteins are functional in murine cytosol. These findings also imply that the absence of NIH/3T3 and Neuro-2a cellular infection upon virus inoculation may be due to a defect in receptor-mediated host cell entry and uncoating, which is mainly the function of the capsid protein. These results were similar to our previous observations (above or [71]) and led us to the hypothesis that certain amino acid substitutions in the *EV71:BS* capsid protein may enable it to bind its receptor, thus leading to virus entry and uncoating, and subsequently infect mouse cells. Two unique tools - the mouse cell line (NIH/3T3)-adapted EV71 strains (*EV71:TLLm* and *EV71:TLLmv*) and the two mouse cell lines (Neuro-2a and NIH/3T3), provided the opportunity to investigate this hypothesis.

[0069] The infection phenotype of chimeric clone-derived virus exhibiting *EV71:TLLm* capsid proteins but expressing *EV71:BS* non-structural proteins (*CDV:BS[M-P1]*) confirmed that the "mouse cell-entry phenotype" could be conferred by the capsid protein derived from mouse cell line-adapted EV71 strains.. These chimeric CDV behaved similar to mouse cell-adapted EV71 strains and induced productive infection in Vero, as well as NIH/3T3 and Neuro-2a cells, with direct evidence for completion of virus infection cycle, *i.e.* generation of viable virus progeny. Further, mutagenesis of *EV71:BS* VP1 to introduce amino acid substitutions K98E, E145A, and L169F and in VP2 S144T and K149I led to limited infection of murine cells. Only CDV with *EV71:TLLm P1* region replacement (*CDV:BS[M-P1]*) and CDV having the combined amino acid substitutions VP1-K98E/E145A/L169F (*CDV:BS$_{VP1}$[K98E/E145A/L169F]*) could be successfully passaged in Neuro-2a and NIH/3T3 cells to produce viable virus progenies in the resultant culture supernatant. The rest of the CDVs

containing other amino acid substitutions, either individually or in various combinations, exhibited limited entry into murine cells leading to one cycle of replication, as supported by the observation of CPE and detection of viral antigens in the inoculated cells. Live virus progeny were absent in the infected cell culture supernatants, as its re-inoculation onto healthy mouse cells failed to induce infection. The reason precipitating the failure of viable virus progeny production in the culture supernatant of cells infected with these CDV is unclear, but one possibility may be due to structural instability of the resultant capsid following the mutations. We suspect that the introduced amino acids may be incompatible with other amino acids comprising the protein, thus affecting the entire capsid protein fold and altering the capsid structure. This assumption highlights the complexity of capsid protein assembly, where four viral proteins (VP1-4) cooperatively interact to generate a functional capsid. Thus, amino acid substitutions that may enable the virus to bind new receptors could also have catastrophic consequences, especially if it inadvertently destabilizes the capsid complex because of incompatibility with other amino acid residues in the protein.

[0070] The results shown in Examples 10-17 reveal that three VP1 substitutions: K98E, E145A, and L169F are necessary and sufficient to bind its receptor on mouse cells and enable EV71 entry. Although the VP1-169 residue had not been previously mentioned in the literature, the VP1-98 and VP1-145 residues had been previously implicated as markers for binding human PSGL-1 receptor proteins on leukocytes [76]. Analysis of 1,702 VP1 sequences of EV71 clinical isolates published in Genbank confirmed the viability, albeit rarity, of the mutant combination VP1 98E/145A, which appeared in only 5 isolates (0.3% of the database). On the other hand, the VP1-169F variant was not observed in the surveyed database of EV71 VP1 sequences, demonstrating its extreme rarity. The *CDV:BS$_{VP1}$[K98E/E145A/L169F]* could be stably passaged onto Neuro-2a cells and consistently produced live virus progeny while retaining the introduced mutations into the virus genome for three passages, suggesting that this virus is viable and stable at least in Neuro-2a cells. Thus, it is possible that introducing these three residues: VP1 98E, 145A, and 169F, into other EV71 clinical isolates could enable it to infect murine cells, although this remains to be demonstrated.

[0071] Similar to previous findings that EV71 utilizes Scavenger Receptor Class B Member-2 (SCARB2) protein as its receptor for host cell entry and uncoating into the cytosol [47, 72, 77], our data also demonstrate that the mouse cell-adapted *EV71:TLLmv* utilize mouse SCARB2 (mSCARB2) to infect murine cells. The virus directly bound recombinant soluble mSCARB2 *in vitro*, and pre-incubation of live virus with the protein prior to inoculation onto healthy cells

reduced the severity of cellular infection. Further, blocking the free surfaces of mSCARB2 on host cells by binding with polyclonal antibodies against mSCARB2 reduced both virus binding on fixed cells and inhibited the infection of live cells. The results also exhibit *EV71:TLLmv* binding to human SCARB2 (hSCARB2) protein, which the virus use to infect primate cells. Binding to and infection of primate cells with *EV71:TLLmv* is also blocked by antibodies against hSCARB2. While the data presented here only showed EV71:TLLmv, the results are also extended to *EV71:TLLm*. *EV71:TLLmv* was derived from further passage of *EV71:TLLm* in NIH/3T3c cells, and only a few amino acid substitutions were observed between the two mouse cell line-adapted EV71 strains. Therefore, these indicate that both *EV71:TLLm* and *EV71:TLLmv* utilize cellular mSCARB2 for infection of rodent cells.

[0072] Similarly, both *CDV:BS[M-P1]* and *CDV:BS$_{VP1}$[K98E/E145A/L169F]* utilize mSCARB2 to infect murine cells. Pre-incubation of Neuro-2a cells with mSCARB2 antiserum blocked cellular infection with the virus, as supported by reduced CPE induction and lower virus titers compared to control. Recent data showed SCARB2 binding to EV71 canyon triggers the release of the "pocket factor," a lipid within the capsid canyon that stabilizes the mature virion and is presumed to be sphingosine in EV71, [78], precipitating a series of events in virus uncoating: capsid expansion (to form the 135-S or A-particle), extrusion of the VP1 N-terminus and VP4 from the five-fold axis capsid junction into the endosome membrane, and release of the viral genomic RNA into the host cell cytoplasm [78-81]. The recent human SCARB2 crystal structure data also reveals a lipid tunnel traversing the entire protein [67], which in the context of SCARB2 function of delivering β-glucocerebrosidase to the lysosome has no relevance, but to which Dang et al. [72] proposed that it serves as a conduit for removal and transport of sphingosine from the capsid canyon during SCARB2 binding. The SCARB2 amino acid residues 140-151, whose sequences are highly divergent between the human and murine proteins, are the main binding site for EV71 [49]. This same region acts as a gate controlling the opening and closing of the SCARB2 lipid tunnel, an event triggered by acidic pH during virus uncoating. The VP1-169 residue lies within the capsid canyon, and probably has a direct function in SCARB2 binding. The drastic change from Leucine to Phenylalanine in this position may have altered the canyon structure resulting to a better fit with murine SCARB2 protein. The VP1 98 and 145 residues, on the other hand, lie on the fringe surrounding the capsid canyon and may have another function aside from SCARB2 binding. These are simply hypotheses, and it would be necessary to solve the structure of murine SCARB2 protein and map the interactions

with the virus capsid in order to elucidate the exact mechanism by which the three VP1 amino acids substitutions could confer the "mouse cell-entry phenotype."

[0073] It is believed that this is the first scientific exploration of EV71 capsid site-specific mutations that induce productive infection of naturally non-permissible murine cells. Previous studies attempted to overcome virus host restriction through either selective virus adaptation, as in the case of passage in live animals to generate mutants with improved infection profile in mice [34, 37, 38, 82], or cellular alteration by ectopic expression of human SCARB2 protein [47, 69, 70, 83]. The incompatibility of EV71 to murine SCARB2 generally accounts for the resistance of mice to EV71 infection, thus impeding the development of mouse model of EV71 infections. This probably also explains why most mouse models of EV71 infection that use either wild type or mouse-adapted virus strains fail to recapitulate the spectrum of diseases observed in severe human infections [34, 37, 38]. On the other hand, transgenic mice expressing human SCARB2 exhibited features of more extensive EV71 infection without the need for mouse adaptation of the virus, but the full spectrum of human diseases still could not be clearly reproduced [69, 70]. The demonstration herein that certain amino acid substitutions in EV71 enable it to infect murine cells provides the first step in understanding the molecular mechanisms that enable the mouse cell line-adapted *EV71:TLLm* and *EV71:TLLmv* to efficiently infect cultured rodent cells. In addition, it may provide another approach to generating a mouse model of EV71 infection to study detailed pathogenesis and reproduce the full spectrum of neurological diseases seen in human infections.

[0074] In Examples 22-25, it is demonstrated for the first time that young BALB/c mice inoculated with a mouse cell-adapted enterovirus 71 (EV71) exhibit an acute encephalomyelitis associated with neurogenic pulmonary edema (NPE) that closely resembles the pathology observed in infected human patients. Animals challenged with the adapted viral strain *EV71:TLLmv* displayed varying levels of virus-induced tissue damage in both the pyramidal and extrapyramidal regions of the brain, presenting as paralysis, ataxia and tremors, and consistent with the CNS-localized pathology identified in fatal cases of EV71 infection [91-95]. Furthermore, some mice displayed respiratory distress compatible with autonomic nervous dysfunction. Based on disease presentation at the time of euthanasia, animals could be readily classified into four groups: *Class IA, Class IB, Class II* and *Survivors.* While *Survivors* did not present signs of disease, mice in *Class II* exhibited persistent flaccid paralysis and severe weight loss, whereas *Class IA* and *Class IB* mice additionally suffered from acute neurologic disease

that was universally lethal within 3-7 DPI. *Class IA* mice also exhibited patent severe respiratory distress that was not due to either congestive heart failure or pneumonitis. Instead, animals in *Class IA* exhibited extensive tissue damage in the caudal brainstem, particularly the medulla, along with high serum levels of catecholamines, strongly suggesting that the respiratory signs observed in these mice were a consequence of neurogenic pulmonary edema (NPE). Gross pathological comparison of lungs from *Class IA* mice with those from other groups revealed incomplete lung collapse at necropsy, as well as significantly increased lung wet weight, likely due to hemorrhage and fluid leakage into the alveolar spaces. These features closely resembled those observed in experimental animal models of non-virally-induced NPE and fatal human cases with fulminant NPE [96, 97]. Indeed, histopathological analyses of *Class IA* animals further revealed focal areas of alveolar spaces filled with proteinaceous and erythrocyte-filled transudate consistent with observations in fatal human cases [21, 98-100].

[0075] Pulmonary edema (PE) is typically defined as an extravascular increase in the water content of the lungs,48 and can be subcategorized on the basis of cardiogenic or neurogenic origin. Since *Class IA* mouse heart tissues exhibited normal histology and lacked overt signs of disease we were able to exclude cardiogenic PE, and the absence of viral replication or inflammation in the lung parenchyma excluded direct virus-induced tissue injury. Instead, we detected high serum levels of catecholamines in *Class IA* mice, strongly indicating that this group exhibited neurogenic PE (NPE), which has previously been demonstrated as a consequence of catecholamine storm induced by severe sympathetic discharge [96, 101]. In this scenario, autonomic nervous system dysfunction triggers a catecholamine storm, resulting in systemic and pulmonary vasoconstriction. This leads to a shift in blood volume from systemic to pulmonary circulation, which culminates in plasma leakage and hemorrhage into the alveolar spaces either through a hydrostatic mechanism or due to direct pulmonary endothelial injury [101-104]. While several experimental animal models of non-virally-induced NPE have been developed in recent years (see Sedy [103] and Davison [104] for review), ours is the first to successfully induce the classical signs of pulmonary edema using EV71 infection alone. Thus, our new model constitutes a significant advance in the pursuit of antiviral therapies and treatment regimens that can limit EV71 infection and prevent the onset of fulminant NPE in human patients. Although it is possible that pulmonary edema can also be induced by host cytokine storm in response to EV71 infection [105-107], the data strongly suggest that a major mechanism of EV71-induced NPE does not involve a massive inflammatory response and is instead associated with tissue damage in specific regions of the brain.

[0076] Brain regions associated with NPE induction have been designated as trigger zones, which encompass the hypothalamic paraventricular and dorsomedial nuclei [101, 103], and the ventrolateral and dorsal medulla, including the NTS and AP regions [96, 104, 108-110]. EV71-induced NPE has previously been attributed to extensive damage of brainstem tissue [21, 22, 93, 111], and in our novel murine model we detected both viral antigens and extensive damage in the brainstem and spinal cord. While *Class IA* and *Class IB* mice exhibited similar distributions of lesions and viral antigens in the brainstems and spinal cords, as well as comparable up-regulation of serum catecholamines, the absence of NPE in *Class IB* animals might be explained by the reduced number and severity of brain lesions and/or the lower viral antigen intensity in the NTS, medullary reticular nuclei (MdRN), and AP regions of the medulla, the dentate nucleus in the cerebellum, and the dorsomedial nuclei of the anterior hypothalamus. We therefore propose that acute, severe destruction of brainstem tissue, particularly those associated with the vasomotor areas, leads to a catecholamine storm in EV71-infected hosts, and that this can progress to NPE if known trigger zones are sufficiently damaged.

[0077] In summary, the present invention is an authentic mouse model of EV71 neuro-infection that exhibits face validity [112], i.e., these animals display the entire range of clinical signs that can be observed across the full spectrum of neurological disease induced by EV71 infection in human patients, including NPE. Hallmark observations in *EV71:TLLmv*-infected mice presenting *Class IA* signs of disease were made by video comprising two video clips of two different *Class IA* mice. Both animals were unable to self-right and were in a state of coma. Severe respiratory distress presenting as tachypnea with subcostal recession was evident in the first mouse. Gasping, subcostal recession and a frothy fluid emanating from the nostrils were seen in the second mouse. Hallmark observations in *EV71:TLLmv*-infected mice presenting *Class IB* signs of disease were made by a video of one *Class 1B* mouse. The animal was unable to self-right and was in a state of stupor. Ipsilateral paralysis of the right limbs and persistent tremor of the left hind-limb were also observed. This model also displays construct validity[112] with respect to the gross and histopathological features of disease, which closely resemble those reported in fatal human cases. This new *in vivo* model represents a powerful tool for identifying the key events in EV71 neuro-pathogenesis, for dissecting the mechanism of EV71-induced NPE, developing novel treatment modalities and potential antiviral therapies, and for conducting pre-clinical evaluation of novel vaccines.

[0078] 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

[0079] The present invention is described by reference to the following Examples, which are offered by way of illustration and are 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 Examples 2-8

[0080] *Cell lines and virus strains*: All cell lines used in this study were purchased from the American Tissue Type Culture Collection (ATCC, USA). Studies were performed using various mammalian cell lines; human adenocarcinoma cell lines HeLa (CCL-2) and HEp-2 (CCL-23), and rhabdomyosarcoma RD (CCL-136); African green monkey kidney Vero (CCL-81), and Vervet monkey kidney fibroblast COS-7 (CRL-1651); mouse neuroblastoma Neuro2A (CCL-131), embryonic fibroblast NIH/3T3 (CRL-1658), and kidney epithelial TCMK (CCL-139); hamster ovarian epithelial-like CHO-K1 (CCL-61), and normal rat kidney epithelial NRK (CRL-6509).

[0081] The human *EV71 BS* strain (*EV71:BS*) was previously isolated from the brainstem of a deceased patient infected with EV71. The virus was passaged in Vero cells for four cycles prior to storage at −80°C until further use. The mouse cell (NIH/3T3)-adapted *EV71:TLLm* strain was derived from the EV71:BS strain via continuous serial passage (>60 cycles) in mouse NIH/3T3 cells. The *EV71:TLLm* strain was further passaged (40 cycles) in NIH/3T3 cells to generate the mouse cell-adapted virulent strain (*EV71:TLLmv*).

[0082] *Maintenance of cell lines and infection with virus*: All cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) supplemented with 10% ($^{v}/_{v}$) of fetal bovine serum (FBS, i-DNA Singapore) and 0.22% ($^{w}/_{v}$) sodium bicarbonate ($NaHCO_3$, Sigma Aldrich, USA) and incubated at 37°C and 5% CO_2, unless otherwise stated. All infected cells were incubated in maintenance medium (DMEM supplemented with 1% FBS and 0.22% $NaHCO_3$).

[0083] Cells ($2.5–5.0\times10^5$ cells per well) were seeded in tissue culture-treated six-well plates (Nunc, Fisher Scientific) overnight, infected with 500 μl of virus suspension (MOI 1), and incubated at 30°C, 37°C, or 39°C for 2 hours. Cells were washed twice in sterile Phosphate-Buffered Saline (PBS, pH 7.4) solution before addition of fresh maintenance medium (DMEM, 1% FBS). Infected cells were observed daily for appearance of distinct lytic cytopathic effects (CPE).

[0084] For virus growth kinetic studies, plates containing the infected cells were frozen at −80°C at various time-points: 0, 6, 12, 24, 36, 48, and 54 hours post-infection (hpi). Plates were

subjected to three cycles of freezing and thawing, and lysates were harvested and cleared by vigorous vortexing followed by centrifugation at 1,500×g for 10 minutes. Cleared supernatants were stored in cryovials (Nunc, Fisher Scientific) at −80°C until further use.

[0085] For temperature adaptation assays, inoculated Vero and NIH/3T3 cells were incubated at 30°C, 37°C, and 39°C and observed daily for appearance of CPE. Respective culture supernatants were harvested at 48 hpi and stored in cryovials at −80°C until further use.

[0086] Various mammalian cell lines, i.e. RD, HeLa, and HEp-2 (human), Vero and COS-7 (monkey), NIH/3T3, Neuro-2A, and TCMK (mouse), CHO-K1 (hamster), and NRK cells (rat), were infected with either parental *EV71:BS* or derived NIH/3T3-adapted EV71 strains at MOI (multiplicity of infection) of 1 and incubated at 37°C for 10 days. Cultures were observed daily for appearance of CPE.

[0087] *Determination of virus titer and relative replication rates (RRR)*: Virus supernatants were subjected to endpoint titration and assayed in both NIH/3T3 and Vero cells. The virus titer was enumerated using the Reed and Muench method [61] and the Reed and Muench calculator [62]. Briefly, NIH/3T3 (1×10^4cells per well) and Vero cells (4×10^3 cells per well) were seeded overnight in a 96-well plate. Frozen virus thawed to room temperature were diluted (10^{-1}) in sterile 1% aqueous sodium deoxycholate (Sigma Aldrich, USA), and vigorously mixed for 15 minutes to disaggregate virus. Disaggregated virus was subjected to ten-fold serial dilution in maintenance medium, and 100 µl diluted virus from 10^{-3} dilution onwards was added onto each well of cells. Plates were incubated at 37°C and observed daily under inverted light microscopy for the appearance of distinct CPE. Virus titer was reported as 50% cell culture-infectious doses per volume ($CCID_{50}$/ml).

[0088] To assess the degree of adaptation of *EV71:TLLm* and *EV71:TLLmv* in NIH/3T3 cells, virus supernatants harvested from previously infected primate and rodent cell lines were subjected to virus titer assay using both NIH/3T3 and Vero cells. The titer ratio used to measure *relative replication rates* (RRR) in NIH/3T3 and Vero cells was calculated using the following formula:

$$RRR = log\ (A/B)$$

where *A* is the virus titer assayed in NIH/3T3 cells, and *B* is the virus titer assayed in Vero cells.

[0089] *Virus antigen detection by immunofluorescence assay*: For infected cells that did not exhibit significant CPE, immunofluorescence (IF) staining was performed to verify infection. Cells were trypsinized at 72 hpi, washed twice in sterile PBS, and coated onto Teflon slides (Erie, USA). Slides were air-dried inside the biosafety cabinet and UV-treated for 15 minutes to

inactivate live virus prior to fixation in cold acetone at 4°C for 10 minutes. Slides were probed with pan-Enterovirus antibody (Merck Millipore, USA) and subsequently with FITC-conjugated mouse IgG (DAKO Cytomation, Denmark) mixed with 0.01% ($^{w}/_{v}$) Evan's blue counter stain.

[0090] *Transfection of cells with EV71 viral RNA:* Vero and NIH/3T3 cells (3×10^4 cells per well) seeded overnight in 24-well plates were transfected with viral RNA extracted from 4×10^6 $CCID_{50}$ virus using Lipofectamine 2000 (Life technologies, USA) following the manufacturer's protocol. RNA from *EV71:BS*, *EV71:TLLm*, and *EV71:TLLmv* was extracted using Viral RNA kit (Qiagen, Germany) and incubated with Lipofectamine 2000 on cells for 6 hours at 37°C. Transfected cells were observed daily for appearance of CPE. At 7 dpi, supernatant was harvested from infected cells and passaged onto freshly seeded Vero and NIH/3T3 cells. Cells were observed daily for appearance of CPE, and at 7 dpi, cells were trypsinized and processed for immunofluorescence viral antigen detection.

[0091] *Full genome sequencing and genetic mapping of EV71 strains*: Viral RNA of *EV71:BS*, *EV71:TLLm*, and *EV71:TLLmv* strains was extracted using Viral RNA kit (Qiagen, Germany) and reverse-transcribed using Superscript II (SII-RT, Life Technologies, USA). The cDNA obtained was amplified with GoTaq Green (Promega, USA) and degenerate EV71 primers (primers' sequences are available upon request). The amplicon was purified using PCR clean up kit (Geneaid Biotech, Taiwan) and cloned into pZero2 vector (Lifetech, USA). Clones were selected from Kanamycin plates, inoculated into LB broth (50 μg/ml Kanamycin) and allowed to grow overnight at 37°C for plasmid extraction with QiaSpin Miniprep kit (Qiagen, Germany). Plasmids were subsequently sequenced by the BigDye terminator method (Applied Biosystems, USA) using the same primers. The 500-bp fragment sequences obtained were aligned using BioEdit v7.0.9.0 [63] against the whole genome sequence of an EV71 Singapore isolate 3799-SIN-98 (GenBank accession no. DQ341354.1) to reconstruct the full genome sequences of *EV71:BS*, *EV71:TLLm*, and *EV71:TLLmv*. Molecular modelling of the protomers of *EV71:TLLm*, and *EV71:TLLmv* was performed using Deepview/Swiss pdbviewer ver. 3.7 (http colon slash slash expasy dot org slash spdbv slash) and the SWISS-MODEL server [64, 65].

EXAMPLE 2

Primate Cell Lines but not Rodent Cell Lines Permissible to Infection by *EV71:BS*

[0092] All primate cell lines used in this study were permissible to infection *by EV71:BS* virus. Human RD cells, as well as monkey Vero and COS-7 cells, exhibited full lytic cytopathic effects (CPE) within 48 hours post-infection (hpi) (Figures 1A, 1J, and 1M), and viral antigens were detected in fixed infected cells (Figures 2A-2L; data not shown for RD, COS-7 and Vero cells). Growth kinetic curves of the virus harvested from supernatant of various infected cell lines confirmed productive infection in RD, Vero, and COS-7 cells (Figure 3A). Virus harvested from RD and Vero cells reached an endpoint titer of 3×10^9 $CCID_{50}$/ml, while virus titer from COS-7 was 10^6 $CCID_{50}$/ml (Figure 3A). *EV71:BS* did not induce full CPE in HeLa and Hep-2 cells (Figures 1D and 1G), and the resulting viral titer was not measurable within the assay cut-off limit. However, viral antigen was detected by indirect immunofluorescent staining in both HeLa cells (Figure 2a) and Hep-2 cells (Figure 2D) indicating successful virus entry into the cells but inefficient or defective replication may have resulted in immeasurable virus titer.

[0093] All the rodent cell lines tested were determined to be non-permissive to *EV71:BS* infection. Lytic CPE of cells was absent following infection (Figures 4A, 4D, 4G, 4J and 4M), virus titer from supernatants was not measurable, and viral antigens could not be detected in inoculated cells (Figures 2G, 2J and 2M; Figures 10A, 10C and 10E).

EXAMPLE 3

Mouse Cell (NIH/3T3)-adapted *EV71:TLLm* Virus Productively Infected both Primate and Rodent Cell Lines

[0094] *EV71:TLLm* was derived following serial passage of *EV71:BS* in NIH/3T3 mouse cell line for a minimum of 60 cycles. All primate and rodent cell lines tested, with the exception of NRK cells, were permissible to productive infection by *EV71:TLLm*. Full CPE was observed in RD, Vero, and COS-7 (Figures 1B, 1K, and 1N), as well as NIH/3T3 and Neuro-2A cells (Figures 4B, E) at 48 hpi. High virus titer was measurable in supernatants harvested from all infected cell lines except NRK (Figures 3C and 3D), and the infected cells were tested positive for viral antigen by indirect immunofluorescent assay (Figures 2B, 2E, 2H, 2K and 2N). Full CPE and measurable viral titer were not observed in NRK cells (Figure 4N; Figure 3D), but viral antigens could be detected (Figure 2K), indicating successful virus entry into NRK cells by *EV71:TLLm* but inefficient virus replication could have resulted in non-measurable virus titer.

EXAMPLE 4

Mouse Cell (NIH/3T3)-adapted *EV71:TLLmv* Virus Productively Infected Rodent Cell Lines but not All Primate Lines

[0095] The *EV71:TLLmv* virus strain was derived from further passage of EV71:TLLm in NIH/3T3 cells for another 40 cycles. EV71:TLLmv caused lytic CPE in fewer number of cell lines - RD, Vero, NIH/3T3, Neuro-2A, and TCMK cells (Figure 3B), and full CPE was only observed in RD, NIH/3T3, and Neuro-2A cells (Figure 1C; 4C, F). TCMK, CHO-K1 and NRK cells were also noted to be permissible to infection without progressing to full CPE (Figure 4I, L, O), as shown by positive viral antigen detection in the infected cells (Figures 2L, 2O, and 2R).

[0096] On the other hand, the primate cell lines HeLa, Hep-2, and COS-7 were observed to be non-permissible to *EV71:TLLmv* infection, as shown by the absence of CPE (Figures 1F, 1I, and 1O), immeasurable virus titers (Figure 3B), and negative viral antigen detection (Figures 2C, 2F, and data not shown).

EXAMPLE 5

EV71:TLLmv Virus Exhibited a Higher Degree of Adaptation to NIH/3T3 Cells, While *EV71:TLLm* was More Adapted to Replicate in Vero Cells

[0097] The amount of viable virus in supernatants harvested from infected cells at various time points was determined by enumerating the virus titer in both Vero and NIH/3T3 cells. The relative reproductive ratio (*RRR*), calculated by taking the ratio of virus titer values assayed in NIH/3T3 to the titer values assayed in Vero, was used as a surrogate measure of the degree of virus adaptation to NIH/3T3 cells. The parental *EV71:BS* virus displayed highly negative *RRR* values for RD, Vero, and COS-7 (Figure 5A), indicating that the virus titer assayed in Vero cells far exceeds the titer assayed in NIH/3T3 cells. The relative reproductive ratio values for other cell lines could not be determined since the virus titers could not be measured. On the other hand, *EV71:TLLmv* virus exhibited positive *RRR* values, with the exception of virus propagated in Vero cells (Figure 5B). The positive *RRR* values were indicative of more efficient replication, and therefore higher titer values, in NIH/3T3 cells compared to Vero cells. The negative *RRR* value determined for *EV71:TLLmv* harvested from Vero cells was consistent with the observed slow growth kinetics (Figure 3B) and lower virus titer. *EV71:TLLm* exhibited negative *RRR* values (Figures 5C and 5D), although of lesser degree than the *RRR* values for *EV71:BS*. This suggested that although *EV71:TLLm* could productively infect a few rodent cell lines, it was still more adapted to replicate in Vero than NIH/3T3 cells.

EXAMPLE 6

EV71:TLLm Exhibited Better Adaptability to Changing Temperatures than *EV71:TLLmv*

[0098] Vero and NIH/3T3 cells infected with the parental EV71:BS and the derived NIH/3T3-adapted *EV71:TLLm* and *EV71:TLLmv* strains were incubated at various temperatures –30°C, 37°C, and 39°C, to determine virus adaptability to temperature variation or changes. EV71:BS displayed the most limited adaptability, with full CPE observed only in Vero cells incubated at 37°C (Figure 8B; Table 1). *EV71:TLLmv* displayed moderate adaptability, based on the observed full CPE induction in Vero cells at 37°C (Figure S2B) and in NIH/3T3 cells in both 37°C and 39°C (Figure 8A, Figure 9A; Table 1*). EV71:TLLm*, on the other hand, displayed the greatest adaptability, where it induced full CPE in Vero cells for all incubation temperatures (Figure 7B, Figure 8B, Figure 9B) though only at 37°C in NIH/3T3 cells (Figure 8A; Table 1).

TABLE 1

Assessment of Virus Adaptability *of EV71:BS*, *EV71:TLLm* and *EV71:TLLmv* Grown in NIH/3T3 and Vero Cells to Various Incubation Temperatures

	Induction of Full Cytopathic Effect (CPE) within 48 Hours Following Infection[1]					
Incubation Temperature (°C)	*EV71:BS*		*EV71:TLLm*		*EV71:TLLmv*	
	Vero	NIH/3T3	Vero	NIH/3&3	Vero	NIH/3T3
30	-[2]	-	+	(30%)[3]	-	-
37	+[3]	-	+	+	+	+
39	(20%)[4]	-	+	(<10%)[3]	-	+

[1] Infected cells were observed daily for signs of lytic cell death and time of appearance of full CPE.
[2] Indicates absence of full CPE in infected cells.
[3] Indicates observation of full CPE.
[4] Indicates absence of full CPE, while number in parenthesis indicates the maximum degree of CPE observed in cells.

EXAMPLE 7

Viral Genomes of *EV71:TLLm* and *EV71:TLLmv* Accumulated Multiple Missense Mutations as a Result of Adaptation to NIH/3T3 Cells

[0099] Viral RNA of *EV71:BS*, *EV71:TLLm*, *EV71:TLLmv* were subjected to Sanger sequencing to determine the consensus genome sequence and identify possible adaptive mutations arising from the adaptation process in NIH/3T3 cells. The consensus sequences of the genomes representing dominant population of the quasi-species have been deposited in the GenBank, NCBI (National Center for Biotechnology Information). Alignment of the full genome sequences of *EV71:TLLm* (GenBank Accession No. KF514879; SEQ ID NO:1) against *EV71:BS* (GenBank Accession No. KF514878; SEQ ID NO:3) revealed 60 nucleotide mutations, 21 of which resulted in amino acid substitutions (Table 2). On the other hand, 83 mutations with 36 amino acid substitutions, were noted between the genomes of *EV71:TLLmv* (Genbank Accession No. KF514880; SEQ ID NO:2) *and EV71:BS*. Majority of the missense mutations were located in the P1 (capsid protein genes) region (Table 2), particularly within the VP1 protein gene (Table 3).

[00100] Amino acid changes were also observed within the P2 and P3 regions, most notably in the RNA-dependent RNA polymerase (3D region). *EV71:TLLm* acquired four amino acid changes, mostly in the palm and thumb domains of the enzyme (Table 4). *EV71:TLLmv*, on the other hand, accumulated eight amino acid changes, mostly also in the palm and thumb domains of the polymerase. Nucleotide mutations were also observed in the 5′ untranslated region (UTR) of the genome. Apart from base changes, a 1-base insertion was found in *EV71:TLLm*, while a 4-bp insertion and a 20-bp deletion were observed in *EV71:TLLmv* 5′UTR (Tables 2 and 3). On the other hand, no amino acid substitutions were observed in the VP3 and 3A regions of *EV71:TLLm*, as well as in the 3′UTR of both *EV71:TLLm* and *EV71:TLLmv*.

TABLE 2

Nucleotide and Amino Acid Changes in the Genomes of *EV71:TLLm* and *EV71:TLLmv* Compared to *EV71:BS*

	EV71:BS vs *EV71:TLLm*		*EV71:BS* vs *EV71:TLLmv*	
Genomic Region	No. Nucleotide Changes	No. Amino Acid Changes	No. Nucleotide Changes	No. Amino Acid Changes
5’ UTR	11	NA	11	NA
	1 bp Insertion		4 bp Insertion	

			20 bp Deletion	
P1	22	12	39	22
P2	11	2	11	2
P3	16	7	22	12
3' UTR	0	NA	0	NA
Total	60	21	83	36

TABLE 3

Adaptive Mutations Observed in the 5'UTR and
P1 Regions of *EV71:TTLm* and *EV71:TLLmv* Viral Genomes

	EV71:BS vs *EV71:TLLm*			*EV71:BS* vs *EV71:TLLmv*		
		Amino Acid Changes			Amino Acid Changes	
Genome Region	Nucleotide Changes	Polyprotein[1]	Mature Protein[2]	Nucleotide Changes	Polyprotein	Mature Protein
Cloverleaf				C140G		
IRES[3]	A141G			A141C		
	G195C			T209C		
	T209C			G258A		
	G258A			C370T		
	C370T			G448A		
	G448A			A502C		
	T502C			G675T		
	C709T			T677C		
	A671T			C687T		
	G675T			C709T		
	T678Insertion			678-681 Insertion		
				726-745 Deletion		

VP4	A809G	E21G	E21G	A809C	E21G	E21G
VP2	G1359A	V204I	V135I	G1359A	V204I	V135I
	G1385C	S213T	S144T	G1385C	S213T	S144T
	A1400T	K218I	K149I	A1400T	K218I	K149I
				T1428C	E228P	E159P
				G1429C	E228P	E159P
VP3				G1900C	A385P	A62P
				A2287G	7514A	T191A
				A2421G	I558M	I235M
VP1	T2462C	V572A	V7A	T2462C	V572A	V7A
	C2725T	L660F	L95F	C2530A	Q595K	Q30K
	A2734G	K663E	K98E	A2719G	I658V	I93V
	A2752G	N669D	N104D	T2724A	D659E	D64E
	A2876G	E710A	E145A	C2725T	L660F	L95F
	A2943T	E732D	E167D	A2734G	K663E	K98E
	C2947T	L734F	L169F	A2752G		
	C3165	S806L	S241L	A2753G	N669G	N104G
				A2876C	E710A	E145A
				A2943T	E732D	E167D
				C2947T	L734F	L169F
				C3165T	S806L	S241L
				T3175C	Y810H	Y245H
				G3148A	V813I	V248I
				G3319T	A858S	A293S

[1] Numbering of amino acids in the uncleaved polyprotein prior to maturation.
[2] Numbering of amino acids in the mature protein.
[3] Internal Ribosome Entry Site.
Mutations are based on alignment with reference *EV71:BS* genome.

TABLE 4

Adaptive Mutations Observed in the P2 and
P3 Regions of *EV71:TTLm* and *EV71:TLLmv* Viral Genomes

	EV71:BS vs *EV71:TLLm*			*EV71:BS vs EV71:TLLmv*		
		Amino Acid Changes			Amino Acid Changes	
Genome Region	Nucleotide Changes	Polyprotein[1]	Mature Protein[2]	Nucleotide Changes	Polyprotein	Mature Protein
2A	G3772A	G992E	G130E	G3772A	G992E	G130E
2C	A4366G	T1207A	T96A	A4366G	T1207A	T96A
3A				C5117T	A1457V	A17V
3B	T5357C	L1537P	L119	T5357C	L1537P	L119
3C	A557G	I1604V	I56V	A557G	I1604V	I56V
	A5569G	I1608V	I60V	A5569G	I1608V	I60V
3D	A6211G	N1822D	N91D	A6070T	T1775S	T44S[3]
	T6835A	S2030T	S299T[3]	T6137C	V1797A	V66A
	A7128G	R2127S	R396S	A6211G	N1822D	N91D
	T7247C	V2167A	V436A	C6404G	S1886C	S155C
				T6592G	W1949G	W218G[5]
				T6835A	S2030T	S299T
				A7128G	R2127S	R396S[6]
				T7247C	V2167A	V436A

[1] Numbering of amino acids in the uncleaved polyprotein prior to maturation.
[2] Numbering of amino acids in the mature protein.
[3] Mutation located in the Ring finger domain of the RNA-dependent RNA polymerase.
[4] Mutation located in the Palm domain of the RNA-dependent RNA polymerase.
[5] Mutation located in the Thumb domain of the RNA-dependent RNA polymerase.
Mutations are based on alignment with reference *EV71:BS* genome.

EXAMPLE 8

Transfection of *EV71:BS* Viral RNA into Murine Cells Resulted in Productive Infection but the Virus Progeny could not Re-infect the Same Mouse Cells

[0100] Vero and NIH/3T3 cells transfected with viral RNA exhibited full CPE at 7 days post-transfection (dpt) (data not shown). Viral antigens were detected in NIH/3T3 cells transfected with viral RNA of *EV71:BS* (Figure 10B), but not in NIH/3T3 cells subjected to infection with the virus (Figure 10A). Virus supernatants re-inoculated onto fresh Vero and NIH/3T3 cells resulted in productive infection (100% CPE) only in Vero but not in NIH/3T3 cells (Figure 6A), and viral antigen detection confirmed infection in Vero cells, but not NIH/3T3 (Figure 6B).

EXAMPLE 9

Materials and Methods for Examples 10-17

[0101] *Plasmids, viruses, bacteria, and cell lines*: The plasmid encoding murine SCARB2 cDNA (pMD18-mSCARB2) (Genbank accession no. NP_031670.1) was purchased from Sino Biological, Inc. (Beijing, China). The pQE30 vector (Qiagen, Germany) for recombinant expression of soluble mSCARB2 protein in *E. coli* cells was a generous gift from Dr. Kian Hong Ng (Temasek Lifesciences Laboratory, Singapore). Plasmids encoding the full-length cDNA of EV71 were generated using the low-copy no. plasmid pACYC177 (New England Biolabs, Singapore). A plasmid construct expressing T7 polymerase (pCMV-T7pol) was a generous gift from Dr. Peter McMinn of University of Sydney, New South Wales. The plasmid pZero-2 used for fragment sequencing of clone-derived viruses was purchased from Invitrogen (Life Technologies, USA).

[0102] The clinical isolate *EV71:BS* (Genbank accession no. KF514878), *EV71:TLLm* (Genbank Accession No. KF514879.1), and *EV71:TLLmv* (Genbank Accession No. KF514880.1) used in this study were described above or previously [71].

[0103] All cell lines used in this study - African green monkey kidney Vero (CCL-81); mouse neuroblastoma Neuro-2a (CCL-131), and fibroblast NIH/3T3 (CRL-1658) cells were purchased from the American Tissue Culture Collection (ATCC®, USA). Cells were grown and maintained as described above or previously [71].

[0104] *E. coli* cells *BL21* strain (New England Biolabs, Singapore) was used for high-level protein expression, *TOP10* strain (Life Technologies, USA) for fragment sequencing of individual clones, and XL-10 Gold ultracompetent strain (Stratagene, USA) for generation of full-length genomic cDNA clones.

[0105] *Construction of EV71:BS full-length genomic cDNA clones, capsid-chimeric clones, and VP1/VP2 mutant clones*: *EV71:BS* cDNA clones were generated by two-step cloning. Viral RNA extraction (Qiagen Viral RNA kit, Germany) and conversion to cDNA (Life Technologies Superscript-II RT, USA) have been described above or previously [71]. The genome proximal fragment encoding the *5'UTR* and *P1* regions was amplified using the primer pair: ***EV71_BamHI-PfF*** and ***EV71_Pf-AatIIR*** (Table 5), which contains *BamHI* and *AatII* restriction sites for cloning into the plasmid pACYC177. The distal fragment encoding the *P2*, *P3*, and *3'UTR* was amplified with the primer pair ***EV71_HindIII-DF*** and ***EV71_D-BamHIR***, which also contains *HindIII* and *BamHI* restriction sites for cloning. The proximal fragment contains a T7 polymerase promoter region upstream of the *5'UTR* to facilitate transcription. The proximal fragment was ligated to the distal fragment following digestion with *EagI* and *AatII*, and the full-length EV71:BS clone was produced.

TABLE 5

Primers

Primer name	Sequence (SEQ ID NO:)	Remarks
EV71_BamHI-PfF	CTAGGGATCCTAATACGACTCACTATAGGTTCAAA CAGCCTGTGGGTTGCACCCACTCACAGG (4)	For cloning EV71:BS proximal fragment (*5'UTR-P1* regions) into the pACYC177 plasmid
EV71_Pf-AatIIR	CTAGGACGTCCGGCCGAACTTTCCAAGGGTAGTAA TGGCAGTACGACTAGTGCC (5)	
EV71_HindIII-DF	TAATAAGCTTCGGCCGGCAGTCTGGGGCCATCTAC GTG (6)	For cloning EV71:BS distal fragment (*P2-3'UTR*) into the pACYC177 plasmid
EV71_D-BamHIR	GCGCGGATCCTTTTTTTTTTTTTTTTTTTGCTATTCT GGTTATAACAAATTTACCCCCAC (7)	
SDM_MluIF	GGTGTCCACTCAACGCGTCGGCTCCCACGAGAACT CCAATTCAGCTACAGAAGGCTCC (8)	For introducing *MluI* restriction site into the proximal fragment clone
SDM_MluIR	CAAGCATGGGCTCACAGGTGTCCACTCAACGCGTC GGCTCCCACG (9)	
MluI-TLLm-P1F	ACTCAACGCGTCGGCTCCCACGAGAACTCCAATTC AGCTACAGAAGGC (10)	For amplifying the *P1* gene sequences of EV71:TLLm flanked by *MluI* and *EagI* restriction sites
EagI-TLLm-P1R	ACTGCCGGCCGAACTTTCCAAGGGTAGTAATGGCA GTACGACTAGTGCC (11)	

VP2_G1385C-F	CAGAGGACA***C***CCACCCTCCTTACAAACAAACACAA CCTGGCGCC (12)	For introducing the G1385C mutation (VP2 S144T substitution)
VP2_G1385C-R	GGAGGGT***G***GGTGTCCTCTGTTCCTGTACCGCCTG (13)	
VP2_A1400T-F	CTCCTTACA***T***ACAAACACAACCTGGCGCCGACG (14)	For introducing the A1400T mutation (VP2 K149I substitution)
VP2_A1400T-R	TGTGTTTGT***A***TGTAAGGAGGGTGGCTGTCCTCTGTT CC (15)	
VP1_A2734G-F	CTCCCTCTT***G***AGGGTACCACCAATCCAAATGGTTAT GCCAACTGGG (16)	For introducing the A2734G mutation (VP1 K98E substitution)
VP1_A2734G-F	TGGTACCCT***C***AAGAGGGAGATCTATCTCTCCTACCA AACCTGCCC (17)	
VP1_A2876C-F	CTACTGGTG***C***GGTTGTTCCACAATTACTCCAGTATA TGTTTGTTCCCCCTGG (18)	For introducing the A2876C mutation (VP1 E145A substitution)
VP1_A2876C-R	GGAACAACC***G***CACCAGTAGGAGTGCACGCAACAA AAGTGAATT (19)	
VP1_C2947T-F	AGAGAATCA***T***TTGCTTGGCAGACAGCCACAAACCC C (20)	For introducing the C2947T mutation (VP1 L169F substitution)
VP1_C2947T-R	GCCAAGCAA***A***TGATTCTCTAGACTCTGGTTTGGGA GCACC (21)	
pQE-mSCARB2F	ATTAGGATCCGTCTTTCAGAAGGCGGTAGACCAG (22)	For cloning mouse SCARB2 gene into pQE30 protein expression plasmid
pQE-mSCARB2R	TATAAAGCTTCGACACGCCAGCCACGTGAAAACCA AGCCAAAG (23)	

[0106] To replace the *P1* region of *EV71:BS* with the *P1* of EV71:TLLm, an *MluI* restriction site was engineered within the boundary between *5'UTR* and *P1* (primer pair ***SDM_MluIF*** and ***SDM_MluI-R***). The *P1* cDNA sequence of EV71:TLLm was amplified (primer pair ***MluI-TLLm-P1F*** and ***EagI-TLLm-P1R***), digested with *MluI* and *EagI*, and cloned into the construct harbouring the proximal fragment. This modified proximal fragment was subsequently ligated to the distal fragment as described.

[0107] To generate clones with amino acid substitutions in the VP2 and VP1 proteins, site-directed mutagenesis was performed in the proximal fragment prior to ligation to the distal

fragment. The VP2 S144T (nt G1385C) amino substitution was introduced into the proximal fragment with the specific primer pair ***VP2_G1385C-F*** and ***VP2_G1385C-R*.** The VP2 S144T (nt G1385C), VP1 K98E (nt A2734G), E145A (nt A2876C), and E167D (nt C2947T) were also generated in a similar manner using different primer pairs (Table 5).

[0108] *Assessment of "mouse cell-entry phenotype"*: To generate live virus, the cDNA clones were co-transfected with another construct expressing the T7 RNA polymerase (pCMV-T7pol) into Vero cells using Lipofectamine 2000 (Life Technologies, USA) following the manufacturer's recommended protocol. Transfection supernatants were harvested 7-10 days post-transfection and inoculated onto overnight seeded cell lines at 1 MOI as described above or previously [71]. Cells were incubated with virus supernatant at 37° C for 1 hour and washed twice with PBS prior to replacement of media with fresh DMEM, 1% FBS.

[0109] In order to assess the infection phenotype, progression of lytic cytopathic effects (CPE) induction was recorded, and images were taken at various time points. Infected cells were also harvested and processed for immunofluorescence detection of viral proteins as described above or previously [71]. Briefly, adherent cells were trypsinized and combined with pelleted cells from culture supernatant, washed twice in sterile phosphate buffered saline (PBS), and fixed onto Teflon slides. Fixed cells were incubated with pan-Enterovirus monoclonal antibodies (Merck Millipore, USA) and detected with standard FITC-conjugated anti-mouse IgG antibodies. Infected cell culture supernatants were also harvested, cleared, and subjected to serial dilutions for virus titer determination using the Reed and Muench method [61]. Once the titer is known, the supernatants were passaged onto freshly seeded NIH/3T3 and Neuro-2a cells at 1 MOI, and the infection phenotype was again assessed using the method described here.

[0110] *Recombinant protein expression of soluble SCARB2 proteins and production of SCARB2 rabbit anti-sera*: The plasmid pMD18-mSCARB2 encoding the extracellular domain of mouse SCARB2 (aa Arg27 - Thr 432) was amplified with the primer pair ***pQE-mSCARB2F*** and ***pQE-mSCARB2R*** to introduce *BamHI* and *HindIII* restriction sites to facilitate cloning into the pQE30 protein expression vector. The clones were transformed into BL21 *E. coli* cells, and protein expression was induced with 1mM IPTG overnight. Harvested cells were lysozyme (1 mg/ml) digested, and the crude extract was purified using a Ni-NTA column (Qiagen®, Germany). Cleared lysate was incubated overnight in 1 ml of 50% Ni-NTA slurry at 4º C with gentle shaking. The protein was washed 5 times in Wash Buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20mM imidazole, pH 8.0) and eluted with Elution Buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250mM imidazole, pH 8.0.

[0111] Two healthy male rabbits were immunized with 1.4 µg purified mouse SCARB2 protein mixed with Freund's complete adjuvant (Sigma-Aldrich®) at day 0. Booster containing 0.8 µg antigen mixed with Freund's incomplete adjuvant (Sigma-Aldrich®) was injected at days 21, 42, 63, 84, and 105. Terminal bleed by cardiac puncture was performed at day 117, and collected blood was incubated overnight at 4° C prior to centrifugation at 3,000 rpm for 30 minutes. Cleared serum was collected and stored at -20° C until further use. Production of SCARB2 rabbit antiserum was approved by the Temasek Lifesciences Laboratory Institution Animal Care and Use Committee (TLL-IACUC) [Approval No. 047/12].

[0112] *Virus competition assay with murine SCARB2 protein*: *In vitro* binding assays were performed to confirm the interaction of *EV71:TLLmv* with mouse and human SCARB2 proteins. NIH/3T3 cells (6000 per well) were seeded overnight onto sterile Teflon coated slides (Erie, USA). Prior to virus inoculation, 100 MOI EV71:TLLmv was incubated with various concentrations of recombinant mouse SCARB2 (mSCARB2) or human SCARB2 (hSCARB2) proteins (4.0 µg, 2.0 µg, 1.0 µg, 0.5 µg, 0.25 µg, 0.125 µg, and 0 µg) for 2 hours at 37° C in a shaking platform. Infected cells were observed daily for signs of CPE and fixed at 48 hours post-infection in absolute acetone (4° C, 10 mins). Fixed cells were immunofluorescently assayed with pan-Enterovirus antibody (Merck Millipore®, USA). Slides were imaged with an upright fluorescence microscope (Nikon, Japan).

[0113] *Virus-SCARB2 binding assays*: Antibody-mediated SCARB2 blocking assays were performed on fixed cells to assess whether masking cell surface SCARB2 proteins affects binding virus binding. NIH/3T3 and Vero cells cultured on Teflon slides were fixed (4% PFA, 25 minutes, room temp.), and blocked with 5% BSA in PBS for 1 hour at 37° C. Slides were incubated in polyclonal rabbit sera raised against mSCARB2 (1:100) for 1 hour at 37° C. For negative controls, cells were incubated with polyclonal rabbit sera raised against Saffold Virus L protein. Slides were washed in PBS prior to incubation with live *EV71:TLLmv* (1000 MOI) for 1 hour at 37° C and probed with pan-Enterovirus antibody and detected with FITC-conjugated Ab. Slides were imaged with Zeiss LSM 510 Meta inverted confocal laser microscope (Zeiss, Germany), and fluorescence intensities were measured using the Imaris (BitPlane Scientific Software, Germany) imaging software. Statistical analyses of fluorescence intensity differences were performed using Prism GraphPad ver. 6.01 (GraphPad Software, Inc., USA).

[0114] *Assay of cell protection from virus infection using rabbit anti- SCARB2 polyclonal sera*: Antibody-mediated SCARB2 blocking assays were also performed on live cells to assess its effect on cellular infection. Overnight seeded NIH/3T3 cells ($1x10^4$ cells per well in 96-well

plates) were incubated with two-fold serial dilutions (1:20 to 1:640) of rabbit polyclonal sera raised against either mouse or human SCARB2 proteins for 1 hour at 37^{o} C. Cells were subsequently inoculated with 100 MOI EV71:TLLmv or clone-derived virus mutants *CDV:BS[M-P1]* and *CDV:BS$_{VP1}$[K98E/E145A/L169F]* for 1 hour at 37^{o} C. Cells were washed twice in PBS prior to replacement with fresh DMEM (1% FBS). Cells were observed daily for signs of CPE, and infected cell culture supernatants were harvested at 3 days post-infection (dpi). Supernatants were subjected to virus titration with prior virus disaggregation process by vigorous vortexing for 15 minutes at room temperature in 1% sodium deoxycholate, as previously [6, 71]. Virus titers were enumerated with the Reed and Muench method [61] and reported as $CCID_{50}$/ ml with the Infectivity Calculator [62].

EXAMPLE 10

Transfection of *EV71:BS* Genomic RNA into Mouse Neuronal Neuro-2a and Fibroblast NIH/3T3 Cells Generate Viable Virus Progeny

[0110] Murine fibroblast NIH/3T3 and neuroblastoma Neuro-2a cells were previously demonstrated as non-permissible to *EV71:BS* infection, while Vero cells are (above or [71]). Two strains, *EV71:TLLm* and *EV71:TLLmv*, both derived from *EV71:BS* successfully entered and replicated within these murine cells. To determine whether the *EV71:BS* genome can replicate in these non-permissible cells, genomic RNA from *EV71:BS* was extracted and transfected into Vero, NIH/3T3, and Neuro-2a cells. Similarly, genomic RNA from *EV71:TLLm* and *EV71:TLLmv* were transfected into these three cell lines for comparison. To assess the viability of the virus progeny generated, transfection supernatants were subsequently re-inoculated onto fresh cells (Figure 15A).

[0111] Genomic RNA transfection of all three virus strains into Vero cells resulted in lytic cytopathic effects (CPE) in the transfected cell monolayer (Figure 15B). Viral antigen expression was also observed in dead cells (Figure 15C), indicating successful virus replication. Similar results were observed from transfection of either *EV71:TLLm* or *EV71:TLLmv* viral RNA into NIH/3T3 and Neuro-2a cell monolayers (Figures 15B and 15C). On the other hand, transfection of EV71:BS RNA into NIH/3T3 and Neuro-2a cells led to death of some cells that exhibited viral antigen expression, but did not result in full CPE of the cell monolayer. Further passage of *EV71:BS* transfection supernatants from both NIH/3T3 and Neuro-2a cells onto fresh Vero cells led to induction of full lysis of the cell monolayer (Figure 15D) and detection of viral

antigens in dead cells (Figure 15E). However, passage of the transfection supernatants onto fresh NIH/3T3 cells yielded neither CPE (Figure 15D) nor viral antigen expression (Figure 15E).

EXAMPLE 11

The Capsid-Encoding P1 Region of Mouse Cell Line-Adapted *EV71:TLLm* Is Responsible for Successful Virus Entry into Murine NIH/3T3 and Neuro-2a Cells

[0112] The previous analysis suggests that the capsid protein of *EV71:TLLm* and *EV71:TLLmv* may be responsible for successful entry into NIH/3T3 and Neuro-2a cells)above or [71]). To confirm this, full-length cDNA clones of *EV71:BS* genome were generated by standard reverse genetics. The *P1* (capsid) region of the full-length cDNA clone of *EV71:BS* was subsequently replaced with the genetic sequence of EV71:TLLm *P1* to generate a chimeric plasmid clone (Figure 16A). The *EV71:BS* cDNA clone was transfected into Vero cells to generated clone-derived virus (CDV:BS). Similarly, the chimeric clone was transfected to generate *CDV:BS[M-P1]* that exhibits the capsid protein of EV71:TLLm and expresses the non-structural proteins of *EV71:BS*. These CDV were re-inoculated onto various cell lines to assess the infection phenotype (Figure 16B).

[0113] *EV71:BS* clone-derived virus (*CDV:BS*) induced CPE in Vero cells but not NIH/3T3 and Neuro-2a cells, while *CDV:BS[M-P1]* induced CPE in all three cell lines at 48 hours post-inoculation (hpi) (Figure 16C). Murine cells infected with *CDV:BS[M-P1*], but not with CDV:BS, resulted in detection of viral antigens in dead cells (Figure 16D). To assess the production and release of viable virus progeny in the first passage (*P1*), clarified culture supernatants from infected cells were re-inoculated onto fresh monolayers of the same cell line, and viral yields were measured at 72 hpi. Passage of both *CDV:BS* and *CDV:BS[M-P1]* onto Vero cells exhibited high virus titers, and a significant titer increase was observed after further passage (*P2*) (Figure 16E). Meanwhile, passage (*P1*) of *CDV:BS[M-P1]*, but not *CDV:BS*, produced high virus yield in both NIH/3T3 and Neuro-2a cells (Figure 16F).

EXAMPLE 12

The VP1-L169F Amino Acid Substitution in *EV71:BS* Capsid Enables the Virus to Enter and Induce Limited Infection in Murine Cells

[0114] The capsid protein of mouse cell-adapted *EV71:TLLm* enables entry of *EV71:BS* into murine cells, and we are interested in the identity of specific residues that confer this novel phenotype. Previous data on the comparison of polyprotein sequence alignments of *EV71:BS* and mouse cell-adapted EV71 strains showed multiple amino acid substitutions in VP1 and VP2

proteins that may be involved in virus receptor engagement on host cells (above or [71]). These residues include VP1 K98E, E145A, and L169F, as well as VP2 S144T and K149I. To determine which of these amino acid substitutions is/are necessary for mouse cell entry, individual mutations resulting in these amino acid substitutions were introduced into the *EV71:BS* full-length cDNA clone via standard site-directed mutagenesis (Figure 17A). These modified cDNA clones were independently transfected into Vero cells to generate clone-derived virus (CDV), and harvested supernatants were inoculated onto freshly-seeded Vero, NIH/3T3, and Neuro-2a cells to assess the infection phenotype.

[0115] Vero cells infected with all the mutant clone-derived viruses (CDV) exhibited 100% CPE, but only those CDV harbouring VP1 amino acid substitutions - *CDV:BS$_{VP1}$[K98E]*, *CDV:BS$_{VP1}$[E145A]*, and *CDV:BS$_{VP1}$[L169F]* - resulted in 100% CPE in Neuro-2a cells (Figures 17B and 17 C). Viral antigen expression was detected in Vero and Neuro-2a cells infected with CDV containing amino acid substitutions in VP1 (*CDV:BS$_{VP1}$*) and VP2 (*CDV:BS$_{VP2}$*), but only the NIH/3T3 cells infected with *CDV:BS$_{VP2}$* exhibited viral antigen expression (Figures 17D and 17E). Furthermore, Vero cells infected with all the mutant CDV yielded measurable virus titers (Figure 17F), suggesting virus viability, but only *CDV:BS$_{VP1}$[L169F]* generated measurable virus titer in the culture supernatant of infected NIH/3T3 and Neuro-2a cells (Figure 17G) as assayed in Vero cells. However, further passage onto healthy murine cells, of culture supernatants from NIH/3T3 and Neuro-2a cells infected with *CDV:BS$_{VP1}$[L169F]*, failed to induce infection.

EXAMPLE 13

Efficient Productive Infection in Murine Cells Requires the Combined Amino Acid Substitutions at VP1

[0116] To assess whether combining the amino acid substitutions in VP1 and VP2 could also enable *EV71:BS* to enter mouse cells, full-length genomic cDNA clones of *EV71:BS* with various combinations of amino acid substitutions (*BS$_{VP2}$[S144T/K149I]*, *BS$_{VP1}$[K98E/E145A]*, *BS$_{VP1}$[K98E/E145A/L169F]*, and *BS[VP1/VP2]*) were generated (Figure 18A). The plasmid clones were independently transfected onto Vero cells, and the resulting supernatant was used to inoculate Vero, NIH/3T3, and Neuro-2a cells to assess the infection phenotype.

[0117] All the assayed CDV, except *CDV:BS$_{VP2}$[S144T/K149I]*, induced CPE in Vero and Neuro-2a cells (Figure 18B). Viral antigens were also detected in all cells infected with the various CDV, although immunostaining was more prominent in Neuro-2a than NIH/3T3 cells

when comparing the murine cell lines (Figure 18C). High virus titer was measurable in the culture supernatants of Vero cells infected with all the CDV (Figure 18D), but only *CDV:BS$_{VP1}$[K98E/E145A]* and *CDV:BS$_{VP1}$[K98E/E145A/L169F]* yielded measurable viral titers in the culture supernatant of infected NIH/3T3 and Neuro-2a cells (Figure 18E), as assayed in Vero cells.

EXAMPLE 14

EV71:BS Virus with the Combined Amino Acid Substitutions at VP1 K98E, E145A, and L169F Could Be Successfully Passaged in Mouse Neuro-2a Cells

[0118] Four of the clone-derived virus isolates have so far enabled *EV71:BS* to enter and infect cultured mouse cell lines: *CDV:BS[M-P1]*, *CDV:BS$_{VP1}$[L169F]*, *CDV:BS$_{VP1}$[K98E/E145A]*, and *CDV:BS$_{VP1}$[K98E/E145A/L169F]*. To identify which of these CDV could stably infect mouse cells for multiple cycles, virus supernatants were passaged twice in the same cell line, i.e. from Neuro-2a to fresh Neuro-2a cells. Infection was monitored by assessment of CPE induction and viral antigen expression, and production of viable virus progeny.

[0119] Only *CDV:BS$_{VP1}$[K98E/E145A/L169F]* and *CDV:BS[M-P1]* could be successfully passaged consecutively in Neuro-2a cells as demonstrated by detection of viral antigens (Figure 19A) and measurable virus titers (Figure 19B). Positive staining was observed in both passage No. 2 and 3, and an increase in virus titer was recorded in passage No. 2 compared to the first passage. On the other hand, only *CDV:BS[M-P1]* was able to induce expression of viral antigens in NIH/3T3 cells, (Figure 19A), but no viable virus progeny was detected. Genomic sequencing of *CDV:BS$_{VP1}$[K98E/E145A/L169F]* derived from the third passage in Neuro-2a cells exhibited no change in the introduced amino acid mutations (Figure 19C).

EXAMPLE 15

The Mouse Cell Line-Adapted Strain *EV71:TLLmv* Binds SCARB2 Protein Both *In Vivo* and *In Vitro*

[0120] EV71 was recently demonstrated to utilize Scavenger Receptor Class B Member-2 (SCARB2) protein as its receptor for host cell entry [47]. To confirm whether the mouse cell line-adapted EV71 strains also utilize SCARB2 during infection of mouse cells, competitive virus binding assays were performed. Firstly, NIH/3T3 and Vero cells grown overnight in Teflon-coated slides and fixed gently with 4% paraformaldehyde were incubated with rabbit sera against mouse SCARB2 protein (mSCARB2) prior to *in vitro* binding with live *EV71:TLLmv*.

Bound cells were fluorescently detected using pan-Enterovirus monoclonal antibodies, and the fluorescence intensity was quantified. Pre-incubation of NIH/3T3 cells with the anti-mSCARB2 sera resulted in significant reduction of *EV71:TLLmv* binding (Figure 20A), which was not observed when cells were pre-incubated with nonspecific serum (NSP). Similar results were observed using Vero cells (Figure 20B).

[0121] In the second experiment, live *EV71:TLLmv* was incubated with recombinant soluble SCARB2 proteins prior to inoculation onto seeded NIH/3T3 cells, and infection was assessed by fluorescence tagging of bound pan-Enterovirus monoclonal antibodies. Pre-incubation of virus with soluble mSCARB2 reduced the severity of cellular infection in a dose-dependent manner (Figure 20C). Similar results were obtained when using human SCARB2 (hSCARB2) protein in the pre-incubation (Figure 20D).

EXAMPLE 16

Cellular Infection with *EV71:TLLmv* Is Blocked by Pre-Incubation of Mouse Cells with Serum Raised Against SCARB2 Protein

[0122] In order to assess whether cellular infection is reduced by blocking the interaction of *EV71:TLLmv* with SCARB2, seeded NIH/3T3 cells were incubated with various dilutions of SCARB2 protein antiserum prior to inoculation with live *EV71:TLLmv*, and infection was assessed by measuring the titer of live virus progeny. Pre-incubation of cells with low dilutions of hSCARB2 antiserum (1:20 to 1:80) resulted in significant dose-dependent reduction of virus titer compared with control (Figure 20E). Similar results were obtained when cells were pre-incubated with mSCARB2 antiserum (Figure 20F).

EXAMPLE 17

The CDV:BS[M-P1] and *CDV:BS$_{VP1}$[K98E/E145A/L169F]* Utilize Murine SCARB2 Protein as Their Functional Receptor for Entry into Murine Cells

[0123] Both *CDV:BS[M-P1]* and *CDV:BS$_{VP1}$[K98E/E145A/L169F]* stably infect mouse Neuro-2a cells. In order to determine whether these CDV, like the mouse cell-adapted EV71:TLLmv strain, also utilize mSCARB2 for virus entry and uncoating, Neuro-2a cells were incubated with mSCARB2 antiserum prior to infection with the CDV mutants. Dose-dependent reduction of lytic CPE was observed in cells infected with either *CDV:BS$_{VP1}$[K98E/E145A/L169F]* (Figure 21A) or *CDV:BS[M-P1]* (Figure 21B). Titration of culture supernatants at 7 days post-infection (dpi) reveal no significant difference in *CDV:BS$_{VP1}$[K98E/E145A/L169F]* virus titer in cells pre-incubated with SCARB2 antiserum

compared to controls (Figure 21C). On other hand, significant virus titer reduction was detected in *CDV:BS[M-P1]*-infected cells pre-incubated with sera with respect to controls (Figure 21D).

EXAMPLE 18

Materials and Methods for Example 19

[0124] *Animal model*: To determine the animal infection phenotype of the mouse cell-adapted strains (*EV71:TLLm* and *EV71:TLLmv*), 5-6-day old Balb/c mice were infected with 10^6 $CCID_{50}$ of the virus and observed for symptoms of disease and neurological complications. The animals were followed up for a maximum of 28 days, after which the animals were sacrificed and sera were collected for detection of EV71-specific antibodies.

EXAMPLE 19

Neuro-Virulence Study of Mouse Cell Line-Adapted EV71 (*EV71:TLLm* and *EV71:TLLmv*) In Balb/c Mice

[0125] Of the immuno-competent animals infected with *EV71:TLLm* (n = 7), two died (29%) of severe and persistent (> 24 hours) paralysis at 8 days post-infection (Figure 13A). Of the other surviving mice, 5 (5/7, 71.4%) exhibited tremors and ataxia, 5 exhibited paresis in either one or both hind limbs, and 4 (4/7, 57.1%) exhibited temporary paralysis in either one or both hind limbs. On the other hand, animals infected with *EV71:TLLmv* exhibited a more severe clinical manifestation of the disease. Nine out of ten (90%) infected animals succumbed to the disease within 8 days of infection (Figure 13A), with 6 of the 9 (66.7%) deaths occurring within the fourth day post-infection. Other symptoms presented include tremors (5/10, 50%), paresis in either one or both hind limbs (6/10, 60%), and paralysis in either one or both hind limbs (5/10, 50). While there seems to be no difference in the body weights of mice infected with *EV71:TLLm* compared to mock-infected animals, mice infected with *EV71:TLLmv* exhibited a drastic reduction in body weight within the first 10 days of infection (Figure 13B). More interestingly, we observed a novel symptom in EV71-infected mice, whereby the paralyzed animals (Figure 14A, arrow) presented with tachypnea with prominent subcostal recession. Further, 6 of the 9 (66.7%) fatalities presented this symptom prior to euthanasia. Upon necropsy, we also observed failure of the lungs to collapse upon opening of thoracic cavity (Figure 14B, arrows), a normal procedure in necropsy when collecting lung and heart tissues, suggesting the existence of pulmonary edema. Histological examination of the tissues revealed features of pulmonary edema and hemorrhage in the alveolar spaces of the lungs (Figure 14C), and higher

magnification images show the present of homogenous proteinaceous material (Figure 14D, arrows).

[0126] This Example is also performed using immuno-compromised mice, such as NSG mice. Similar results are obtained except that severity of disease is greater and the mortality rate is higher.

EXAMPLE 20

Screening Candidate Anti-EV71 Compounds

[0127] High through-put *in vitro* screening of candidate anti-EV71 compounds is performed using the mouse NIH/3T3 cell line which has been shown to be susceptible to cytolytic infection by *EV71:TLLm* or *EV71:TLLmv* virus strains. The selected promising compounds from the *in vitro* screening are then *in vivo* tested in the animal model. To accomplish the *in vivo* screening, a standardized (based on statistical calculation) number of BALB/c mice are infected with a standardized titer of the virus strains taken from a standardized stock of mouse cell line-adapted EV71 strains (*EV71:TLLm* and *EV71:TLLmv*) that is prepared, titrated and kept in a deep freezer (-80°C). The candidate anti-EV71 compound is administered to the infected mice at various standardized dosages either before appearance of illness for assaying a potential preventive effect of the candidate compound or after onset of illness for assaying a potential therapeutic effect of the candidate compound.

EXAMPLE 21

Materials and Methods for Examples 22-25

[0128] *Mouse and virus strains*: Adult BALB/c mice were purchased from InVivos (Singapore), and mated to obtain pups. EV71 strains used for inoculation included *EV71:BS*, *EV71:TLLm*, and *EV71:TLLmv*, whose details and characteristics have been described herein.

[0129] *Ethics statement*: The animal procedures were approved by the Institutional Care and Use of Animal Committee (IACUC) of Temasek Lifesciences Laboratory (approval no. TLL-14-023). Infected animals that became moribund were euthanized by injection with 90 mg/kg pentobarbitone via the I.P. route. Neurologic examination was performed following the guidelines and standard procedures set by The Institutional Care and Use of Animals Committee (IACUC) of University of California San Francisco. Criteria for euthanasia included previously set guidelines [34]: (1) loss of > 20% maximum recorded body weight, (2) paralysis lasting >48 h, (3) absence of feeding or inability to feed, (4) inability to self-right, and (5) altered state of

consciousness presenting as either stupor or coma. Pups were observed for 28 days total, and animals that survived throughout this period were euthanized by I.P. injection of pentobarbitone.

[0130] *Animal handling and infection*: Groups of eight mice of varying age (6, 14, 21, or 28 days old) were inoculated with *EV71:TLLmv* (dose 10^6 $CCID_{50}$) either by I.P. or I.M. injection. To determine the optimum dose of *EV71:TLLmv*, groups of 6-day old pups (n=8 per group) were challenged with varying doses of virus (10^6, 10^5, 10^4, 10^3, or 10^2 $CCID_{50}$) via the I.P. route. Infected animals were observed twice daily for disease presentation during the first week post-infection. Both moribund animals and those that survived the observation period were euthanized as described above. Terminal blood collection was performed via cardiac puncture using a 26G needle.

[0131] *Necropsy, gross pathological observations, and tissue collection*: Euthanized animals were necropsied using standard protocols to harvest organs. Gross pathologic examination was also performed and photographs were taken with IACUC approval. Lungs were superficially flushed twice with sterile PBS, and then blotted dry on filter paper prior to measuring the wet weight. Harvested organs for histological studies were stored in 10% neutral buffered formalin (NBF) for 1 week at 4° C.

[0132] *Determination*: of tissue viral load. Frozen tissues were macerated using a Teflon pestle and reconstituted in 1ml DMEM (1% FBS). Samples were then mixed for 1h and centrifuged twice (20 *g*, 30 min, 4° C) to remove tissue debris and obtain clarified virus. The virus sample was disaggregated in 1% sodium deoxycholate [88] prior to ten-fold serial dilution and transfer onto NIH/3T3 cells. Infected cells were observed daily for cytopathic effects (CPE), and cells were stained with pan-enterovirus monoclonal antibody (Merck Millipore, USA) [88]. Virus titers were enumerated and reported as $CCID_{50}$ per g tissue.

[0133] *Tissue processing for histological analyses*: Fixed tissues were dehydrated in a series of increasing concentrations of 70%, 95% and 100% ethanol. Tissues were incubated in two changes of alcohol and three changes of Histoclear II (Electron Microscopy Sciences, USA), and finally infiltrated with four changes of melted paraffin wax. All incubations were performed for 1 h at room temperature with gentle rocking at 100 rpm. Paraffin infiltrations were performed in an oven set at 65° C. Paraffin-embedded tissue blocks were sectioned (5 μm) using a microtome, loaded onto poly-lysine-coated glass slides, dried overnight at 42° C, and then stored at room temperature until further use.

[0134] *Staining of tissue sections*: Tissue sections were de-waxed by incubation in two changes of Histoclear II and then slowly rehydrated in decreasing alcohol concentrations of

100%, 95%, 70%, and 50%. Slides were incubated in PBS for 10min prior to staining. Hematoxylin and eosin (H &E) staining was performed by first flooding the slides with Harris' hematoxylin (Sigma Aldrich, USA) and incubating at room temperature (RT) for 15 min. The slides were then rinsed in water, de-stained in 1% acid alcohol (95% ethanol, 1% HCl), dipped in 0.2% NH4OH, and rinsed in water for 10 min prior to counterstaining in eosin solution. The slides were next de-stained in 95% ethanol, dehydrated by three changes of absolute alcohol and two changes of Histoclear II. Tissues were finally set in DPX mounting fluid (Sigma Aldrich, USA).

[0135] *Immunohistochemistry*: Following de-waxing and rehydration, slides were subjected to heat-induced antigen retrieval by incubation in a histology-grade microwave oven and citrate buffer (pH 6.0) for 30 min at 96° C. Slides were allowed to cool to RT over 3h and were subsequently blocked with 5% normal pig serum for 1 h at RT. Without further washing, the slides were then incubated at 4° C overnight in rabbit serum containing polyclonal antibodies against EV71 (a generous gift from Dr. Hiroyuki Shimizu of NIID, Japan). The slides were then washed 5 times in Tris-buffered saline (pH 7.4), 0.05% Tween-20 (TBS-T), and rinsed twice in TBS prior to quenching endogenous peroxidases by addition of 3% H_2O_2 for 1 h at RT. Slides were subsequently washed twice in TBS prior to incubation with swine-anti rabbit Ig-HRP (Dako Cytomation, Denmark) for 1h at RT. After washing, slides were incubated in diaminobenzidine (DAB) substrate, and counterstained with hematoxylin.

[0136] *Mapping viral antigens and virus-induced lesions in tissue sections*: Template images of representative coronal sections of the mouse brain were downloaded from www dot brainstars dot org [89]. These images are free to be used and modified under license from the Creative Commons of Japan. The observed lesions and viral antigens were marked onto template images. Affected brain regions were identified using the mouse brain atlas of coronal sections (www dot mouse dot brain-map dot org slash static slash atlas) [90]. Similarly, a representative coronal section of the thoracic spinal cord was used as the template for spinal cord maps. Areas depicting the presence of viral antigens and virus-induced lesions were then marked onto the template images.

[0137] *Measurement of serum catecholamine levels*: Blood samples were collected by cardiac puncture of moribund animals during necropsy. Serum was obtained by centrifuging coagulated blood samples at 3000 *g*, 4° C, for 30 min, then stored at -20° C until determination of catecholamine levels using the 2-CAT (A-N) ELISA kit (Labor Diagnostika Nord, Germany) according to the manufacturer's protocols.

[0138] *Statistical analyses*: All graphs were created and statistical analyses performed using GraphPad Prism (version 6.01) for Windows (GraphPad Software, USA, www dot graphpad dot com).

[0139] *Comparison of the infection phenotypes induced by viral strains EV71:TLLm* and *EV71:TLLmv*: Groups of 6-day old BALB/c pups were inoculated with a 106 CCID50 dose of *EV71:BS*, *EV71:TLLm*, or *EV71:TLLmv* via the intraperitoneal (I.P.) or intramuscular (I.M.) route (n=10 mice per group). Animals were observed twice daily for signs of disease during the first week post-inoculation and euthanized when critical signs were detected as already described.

[0140] *Measurement of neutralizing antibody levels in sera from infected mice*: Blood samples were collected by cardiac puncture at necropsy before being clotted at room temperature and the serum obtained by centrifugation for 20min at 3000g, 4° C. Samples were stored at -20° C until further analysis. Random samples of the frozen stocks were assayed for neutralizing antibody titers. Two-fold serial dilutions of serum (1:20 to 1:1280) were prepared in 96-well plates and mixed with 100 $CCID_{50}$ virus. The mixture was incubated for 1 h at 37° C prior to addition of NIH/3T3 cells (6,000 cells/well). Plates were incubated at 37° C for several days and observed for CPE between days 4-10. Neutralizing antibody titers were determined using the Reed and Muench method (reported as units per ml sera).

EXAMPLE 22

Infection Dynamics of Modified EV71 Strains in Murine Hosts

[0141] Current animal models of enterovirus 71 (EV71)-induced neurological disease and pathology only partially replicate the human disease. We therefore aimed to develop a clinically authentic model of disease pathology using the mouse cell (NIH/3T3)-adapted virus strains *EV71:TLLm* and *EV71:TLLmv*, which can productively infect both rodent and primate cell lines [88]. First, the relative virulence of *EV71:TLLm* and *EV71:TLLmv* compared with the parental strain *EV71:BS* was assessed by assessing disease pathology in 1-week old BALB/c mice infected with virus via the intraperitoneal (I.P.) route. While seroconversion was observed in all the inoculated animals, only mice infected with either *EV71:TLLm* or *EV71:TLLmv* progressed to lethal disease (Figures 22a and 22b). Similarly, when the adapted strains were administered via the alternative intramuscular (I.M.) route, these were again associated with reduced host survival relative to the parental strain *EV71:BS* (57% survival; Figure 22c). When the I.P. and I.M. infection routes were considered together, the median survival time after inoculation was 4

days post-infection (DPI) for *EV71:TLLmv* infection and 7 DPI for *EV71:TLLm* infection (x^2 = 6.840; p = 0.0089). Together, these data indicated that mouse cell-adapted viral strain *EV71:TLLmv* was associated with the greatest levels of lethality, and was therefore used in all subsequent experiments.

[0142] The optimal virus dose, inoculation route, and mouse age to use for further model development were next assessed. First, it was confirmed that disease severity in *EV71:TLLmv*-infected mice was dependent on virus dose; minimum survival rate was observed in animals inoculated with a median cell culture infectious dose ($CCID_{50}$) of 10^6 (Figure 23a) and the median humane endpoint (HD50) was equivalent to 3.98 x 10^3 $CCID_{50}$ (Figure 23b). The influence of animal age and infection route on host survival was then evaluated; the survival curves of 1 week-old mice injected with *EV71:TLLmv* were not significantly affected by inoculation route (Figure 23c), but young animals exhibited consistently poorer survival than did older animals, irrespective of virus injection site (Figure 23d). Only in older animals was it possible to discern any effect of infection route on disease severity; while 3 week-old mice were completely resistant to I.P. infection, some animals did not survive virus injection via the I.M. route (Figures 24a and 24b). Seroconversion was detected in all mice that survived the infection, including those that did not exhibit any signs of disease (Figures 24c, 24d and 24e). These data demonstrated that *EV71:TLLmv* induces acute and severe infection that is lethal in mice aged 1-3 weeks old. Of the conditions tested here, disease severity was greatest in 1 week-old mice injected with a virus dose of 10^6 $CCID_{50}$ either into the peritoneal cavity or muscle tissue.

EXAMPLE 23

Disease Progression in *EV71:TLLmv*-Infected Mice

[0143] The majority of 1-week old mice inoculated with *EV71:TLLmv* succumbed to disease and exhibited myriad clinical signs of neurological illness. Infected animals exhibited ataxia, localized or whole-body tremors, unsteady gait, and limb paresis and paralysis either transiently or persisting until the time of euthanasia. Based on clinical presentation, the sick animals could be readily categorized into four groups (Table 6). *Survivors* included mice that did not appear moribund at any point during the observation period of 28 days. *Class I* animals presented after just 3-7 DPI with severe signs including an inability to self-right and either stupor or coma. All mice in this group exhibited spastic limb paresis and/or paralysis (fore-limbs, hind-limbs, or both), but while some animals were devoid of respiratory symptoms (*Class IB)*, others were additionally characterized by signs of respiratory distress, including tachypnea, hiccupping,

gasping, and subcostal recession (*Class IA*). Hallmark observations in *EV71:TLLmv*-infected mice presenting *Class IA* signs of disease were made by video comprising two video clips of two different *Class IA* mice. Both animals were unable to self-right and were in a state of coma. Severe respiratory distress presenting as tachypnea with subcostal recession was evident in the first mouse. Gasping, subcostal recession and a frothy fluid emanating from the nostrils were seen in the second mouse. Hallmark observations in *EV71:TLLmv*-infected mice presenting *Class IB* signs of disease were made by a video of one *Class 1B* mouse. The animal was unable to self-right and was in a state of stupor. Ipsilateral paralysis of the right limbs and persistent tremor of the left hind-limb were also observed. Finally, *Class II* mice presented after 7 DPI with signs of infection including persistent flaccid paralysis of the limbs (>48h duration) (Figure 23e) together with severe weight loss (>20% max. body weight). In all disease classes, some of the infected animals displayed hairless lesions or bald spots on their backs that persisted for a few days (Figure 23f). The majority of pups inoculated I.P. with *EV71:TLLmv* were categorized into *Class I* (Figure 23g); *Class IA* animals comprised 19.3% (n=11; patent respiratory signs), while *Class IB* animals comprised 43.9% (n=25; no overt respiratory signs). *Class II*4 animals represented just 12.3% (n=7) of the infected pups, and *Survivors* constituted 24.5% (n=14) of all infected mice. A similar pattern of distribution between disease categories was observed in pups inoculated via the I.M. route (Figure 23h). Together, these data indicated that mice infected with *EV71:TLLmv* exhibit variable incidence and severity of both neurological and respiratory symptoms that reflect the full spectrum of disease observed in human patients.

TABLE 6

Clinical Features of *EV71:TLLmv*-Infected
BALB/c Mice at the Time of Euthanasia

	Class IA	*Class IB*	*Class II*	*Survivors*[a]
Time to onset of signs [days post-infection (DPI)]	Within 3-5	Within 3-7	> 7	N/A
Level of consciousness	Stupor[b] / Coma[c]	Stupor[b]	Active	Active
Weight loss	< 20% body weight	< 20% body weight	> 20% body weight	None or < 20% body weight
Limb function (>48 h)	Spastic limb paresis / paralysis	Spastic limb paresis / paralysis	Flaccid limb paralysis	Normal
Breathing function	Tachpnea / gasping /	Regular	Regular	Regular

	hiccupping			
Cardiac function	Tachycadia	Tachycardia[d] / Regular	Regular	Regular
Limb tremors	May be present	May be present	Absent	Absent

[a] Animals were assessed at the end of the observation period (28 DPI)
[b] Animal was unable to self-right, but responded to physical stimulation of the toes.
[c] Animal was unable to self-right and did not respond to physical stimulation of the toes.
[d] Tachycardia was observed in 35% of *Class IB* mice

EXAMPLE 24

Neurogenic Pulmonary Edema in Mice Presenting *Class IA* Signs

[0144] Whether the respiratory distress observed in *Class IA* mice was a sign of virus-induced pulmonary edema (PE) was further investigated. Comparison of gross lung pathology between *Class IA, Class IB,* and *Class II* mice revealed that *Class IA* lungs were swollen, incompletely collapsed at necropsy (Figures 25a-25d), and displayed increased wet weight relative to lungs from other groups (Figure 22E). Comparison of lung tissue sections from sham-inoculated (Figure 25f) and *Class IA* lungs revealed focal areas of hemorrhage and accumulation of proteinaceous and erythrocyte-filled fluid in the alveolar spaces of the infected animals only (Figure 25g). These pathological features were absent from the lungs of *Class IB* and *Class II* mice (Figures 25h and 25i). Furthermore, we found no evidence of inflammatory infiltrate or viral antigens in the lungs collected from any group of mice (Figure 26a). Similarly, histochemical analyses of the cardiac muscles in each class of infected mice were also unable to uncover any evidence of an inflammatory infiltrate, cardiac muscle necrosis, or viral antigens (Figure 26b). Together, these data demonstrated that the respiratory distress evident in *Class IA* mice could not be attributed to either pneumonitis or congestive heart failure. We therefore sought to determine whether the PE observed in this group was of neurogenic origin, so we next measured serum levels of catecholamines to determine whether neurotransmitter concentrations were modulated in *EV71:TLLmv*-infected mice with respiratory signs (*Class IA*). Using this approach, we observed that blood concentrations of both adrenaline (epinephrine) and noradrenaline (norepinephrine) were significantly higher in *Class IA* mice than those detected in either *Class II* mice or mock-infected animals (Figures 25j and 25k). These data strongly indicated that *Class IA* mice exhibited EV71-induced neurogenic pulmonary edema (NPE) prior to death.

EXAMPLE 25

EV71:TLLmv Viral Neurotropism in *Class IA*, *Class IB*, and *Class II* Mice

[0145] The distribution of *EV71:TLLmv* and virus-induced lesions in the brains and spinal cords of animals from each disease group were next assessed in order to identify factors that might contribute to the selective development of NPE in *Class IA* mice only. The majority of animals in *Class IA* and *Class IB* exhibited ubiquitous staining of viral antigens (>10 positive neurons detected) and mild pathological lesions (>5 lesions observed) in all of the CNS regions assessed (Table 7). In contrast, only 1 of 5 mice with *Class II* disease exhibited viral antigens and/or lesions in the sensory cortex, hippocampus, diencephalon, mesencephalon, medulla oblongata, or lumbar spinal cord. We were unable to detect viral antigens in any tissues tested outside of the CNS (except for the skeletal muscles after inoculation via the I.M. route; data not shown).

TABLE 7

CNS Distribution of EV71 Antigens and
Virus-Induced Lesions in Terminally-Infected BALB/c Mice

		Class IA				*ClassIB*				*Class II*			
		EV71 Antigens		Pathology		EV71 Antigens		Pathology		EV71 Antigens		Pathology	
Cerebral Cortex	Motor cortex	+++[a]	100%[b] (5/5)	+++[c]	80%[d] (4/5)	+++	80% (4/5)	+++	80% (4/5)		0% (0/5)		0% (0/5)
Cerebral Cortex	Sensory cortex	++	60% (3/5)	++	80% (4/5)	++	80% (4/5)	++	80% (4/5)	+	20% (1/5)	+	20% (1/5)
Hippocampus		++	60% (3/5)	+	60% (3/5)	+	40% (2/5)	+	20% (1/5)	+	20% (1/5)	+	20% (1/5)
Diencephalon	Thalamus	+++	100% (5/5)	+++	60% (3/5)	++	60% (3/5)	++	20% (1/5)	+	20% (1/5)	+	20% (1/5)
Diencephalon	Hypothalamus	+++	80% (4/5)	+++	80% (4/5)	++	60% (3/5)	++	60% (3/5)	+	20% (1/5)	+	20% (1/5)
Mesencephalon		+++	100% (5/5)	+++	100% (5/5)	+	60% (3/5)	++	60% (3/5)	+	20% (1/5)	+	20% (1/5)
Metencephalon	Cerebellum	++	80% (4/5)	++	80% (4/5)	+	40% (2/5)	+	60% (3/5)		0% (0/5)		0% (0/5)
Metencephalon	Pons	++	80% (4/5)	++	80% (4/5)	++	20% (1/5)	++	60% (3/5)		0% (0/5)		0% (0/5)
Myelencephalon	Medulla	+++	100% (5/5)	+++	100% (5/5)	++	40% (2/5)	++	60% (3/5)	+	20% (1/5)	+	20% (1/5)
Spinal Cord	Cervical	++	100% (5/5)	++	100% (5/5)	++	80% (4/5)	++	60% (3/5)		0% (0/5)		0% (0/5)
Spinal Cord	Thoracic	++	100% (5/5)	++	100% (5/5)	++	80% (4/5)	++	80% (4/5)		0% (0/5)		0% (0/5)
Spinal Cord	Lumbar	++	100% (5/5)	++	100% (5/5)	++	80% (4/5)	++	60% (3/5)	+	20% (1/5)	+	20% (1/5)

[a] Density of viral antigens detected per slide: +, < 10 positive neurons; ++, 10-20 positive neurons; +++, > 20 positive neurons
[b] Percentage of animals exhibiting viral antigens in the specified brain region (n = 5)
[c] Distribution of pathologic lesions in nervous tissues: +, < 5 lesions; ++, 5-10 lesions; +++, >10 lesions
[d] Percentage of animals exhibiting lesions in the specified brain region (n = 5)

[0146] When CNS pathology between *Class IA* and *Class IB* mice was compared, it was observed that both tissue lesions and viral antigens were localized to the same areas within the hippocampus, diencephalon, mesencephalon, cerebellum, and medulla, but pathology was more severe in animals with *Class IA* disease (Table 7 and Figures 27a-27d). Indeed, when compared with *Class IB* mice, animals in *Class IA* displayed more extensive neuronal degeneration, phagocytosis, and necrosis in CA3 neurons of the hippocampus (Figures 28a and 28b). *Class IA* mice also exhibited intense viral antigen staining in hypothalamus, accompanied by marked tissue inflammation and neuronal necrosis, whereas these pathological features were limited in *Class IB* animals (Figures 28c and 28d and Figure 27b). Similarly, *Class IA* mice also presented features of more severe virus-induced pathology and viral antigen intensity in the ventro-posterior complex of the thalamus (Figures 28e and 28f and Figure 27b), the mesencephalon-associated tissues including the peri-aqueductal gray (PAG) matter, midbrain reticular area, and motor-related superior colliculus (Figures 28g and 28h and Figure 27c), as well as in the Purkinje cells and dentate nucleus of the cerebellum (Figures 28i and 28j, Figure 27d and Figure 29a.

[0147] In both disease groups (*Class IA* and *Class IB*), the most extensive distribution of viral antigens and pathological lesions involving neuronal damage and tissue inflammation were detected in the medulla oblongata (Figures 28k and 28l), particularly in the motor-related areas of the intermediate reticular nuclei (IRN), parvicellular reticular nuclei (PARN), and spinal nucleus of the trigeminal nerve (sptV) (Figure 27d and Figure 29b). However, only *Class 1A* mice exhibited viral antigens and tissue lesions in the ventral and dorsal regions of the medullary reticular nucleus (MdRN), the nucleus of the solitary tract (NTS) and area prostrema (AP) (Figures 29b and 29c). For comparison, representative images of the hippocampus, hypothalamus, thalamus, midbrain, cerebellum, and medulla from mock-infected mice are also shown (Figures 30a-30f). In contrast, *Class IA* and *Class IB* mice did not differ with respect to the distribution, localization or extent of tissue lesions or viral antigen staining within the motor cortex, somatosensory cortex, pons or ventral horns of the spinal cord gray matter (Figures 28m and 28n, Figures 27a-27c and Figures 31a-31e), consistent with the concept that NPE is caused by virally triggered damage to specific brain regions rather than a uniform increase in EV71-induced pathology across all tissues.

[0148] 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. 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.

[0149] 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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1. An animal model of Enterovirus 71 (EV71) neuro-infection comprising a mouse that is up to four weeks in age having been infected at between one week and two weeks in age with an EV71 modified for infecting the mouse,

wherein the mouse is infected by injecting a mean cell culture infectious dose ($CCID_{50}$) of between 10^5 and 10^6 of the modified EV71 into the peritoneal cavity or into muscle tissue,

wherein the modified EV71 is *EV71:TLLmv*, which

(a) has been deposited with China Center for Type Culture Collection and assigned accession number CCTCC V201438 or

(b) has been recovered using advanced reverse genetics to synthesize the viral RNA sequence from SEQ ID NO:2 and

wherein the animal model displays acute encephalomyelitis associated with neurogenic pulmonary edema characterized by focal hemorrhage and proteinaceous fluid in the lung alveoli, high serum levels of catecholamines and extensive tissue damage in the brainstem.

2. The animal model of claim 1, wherein the $CCID_{50}$ is 10^6.

3. The animal model of claim 1, wherein the rodent is immuno-competent.

4. The animal model of claim 3, wherein the mouse is a BALB/c mouse.

5. The animal model of claim 1, wherein the mouse is immuno-compromised.

6. The animal model of claim 5, wherein the mouse is a NSG mouse.

7. A method to screen antiviral drugs, comprising: providing a test group of animals and a control group of animals, wherein the animals of each group comprise the animal model of any one of claims 1-6; administering to the test group an antiviral drug candidate; monitoring disease progression in the test group and the control group; comparing the disease progression in the test group to the disease progression in the control group; and

selecting the antiviral drug candidate that reduces disease progression in the test group relative to the control group.

8. The method of claim 7, wherein the antiviral drug is first screened in a test mouse cell line infected with the modified Enterovirus 71 before screening in the animals.

9. A method to screen effective antiviral vaccines, comprising: providing a test group of animals and a control group of animals, wherein the animals of each group comprise the animal model of any one of claims 1-6; administering to the test group an antiviral vaccine candidate; monitoring disease progression in the test group and the control group; comparing the disease progression in the test group to disease progression in the control group; and selecting the antiviral vaccine candidate that reduces disease progression in the test group relative to the control group.

10. A method of preparing an animal model of Enterovirus 71 (EV71) neuro-infection comprising

(i) infecting a mouse at between one week and two weeks in age with an EV71 modified for infecting the mouse and

(ii) raising the infected mouse for up to four weeks in age,

whereby an animal model of EV71 neuro-infection is prepared,

wherein the mouse is infected by injecting a mean cell culture infectious dose of between 10^5 and 10^6 of into the peritoneal cavity or into muscle tissue,

wherein the modified EV71 is *EV71:TLLmv*, which

(a) has been deposited with China Center for Type Culture Collection and assigned accession number CCTCC V201438 or

(b) has been recovered using advanced reverse genetics to synthesize the viral RNA sequence from SEQ ID NO:2, and

wherein the animal model displays acute encephalomyelitis associated with neurogenic pulmonary edema characterized by focal hemorrhage and proteinaceous fluid in the lung alveoli, high serum levels of catecholamines and extensive tissue damage in the brainstem.

11. The method of claim 10, wherein the $CCID_{50}$ is 10^6.

12. The method of claim 10 or 11, wherein the mouse is immuno-competent.

13. The method of claim 12, wherein the mouse is a BALB/c mouse.

14. The method of claim 10 or 11, wherein the rodent is immuno-compromised.

15. The method of claim 14, wherein the mouse is a NSG mouse.