

STABLE FORMULATIONS OF LOW ABUNDANCE ENTEROVIRUSES VACCINES AND PROCESS OF MANUFACTURE THEREOF

RELATED PATENT APPLICATION:

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This application claims the priority to and benefit of Indian Provisional Patent Application No. 202041042411 filed on September 29, 2020; the disclosure of which are incorporated herein by reference.

10 **FIELD OF THE INVENTION:**

The invention relates to enteroviruses vaccines and process of manufacture thereof. Specifically, the invention relates to stable formulations of low abundance enteroviruses vaccines and process of manufacture thereof. The invention also relates to a process of
15 isolation of low abundance enteroviruses from clinical source of suspected hand, foot, and mouth disease case/s by adapting and propagating in cell substrate and utilizing the isolated virus for thermostable formulation, studying pathogenicity in animal model, for preparing vaccine formulations, process of vaccine manufacturing thereof and for diagnostic tools.

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BACKGROUND OF THE INVENTION:

Hand, foot, and mouth disease (HFMD) is an infectious disease that mainly affects children below 5 years of age, although, children up to 15 years are also affected. HFMD
25 has distinct clinical manifestations with clinical features like hand, foot, and mouth rashes and clinical symptoms like fever, sore throat, and malaise. The two most common causative agents of this disease are Enterovirus A71 (EV71) and Coxsackievirus A16 (CVA16), both belonging to genus Enterovirus. Neurological complications and severity are more common among HFMD patients caused by Enterovirus 71. HFMD caused by
30 CVA16 are often self-limiting, but occasionally HFMD cases caused by some strains of Coxsackievirus A16 can lead to neurological complications and even fatality (Legay et al., Emerg Infect Dis, 2007; Goto et al., Emerg Infect Dis, 2009). HFMD can also be caused by other members of enteroviruses like CA4-6, CA10, echoviruses and some members of Coxsackievirus B (Xu et al., PLoS One, 2015). HFMD or neurological disease associated
35 epidemics outbreaks or outbreaks sporadic cases caused by Enterovirus 71 and

Coxsackievirus A16 occurs worldwide (Ferson and Bell., Am J Public Health, 1991; Ljubin-Sternak et al., Euro Surveill, 2011; Zander et al., Med J Aust, 2014; Gonzalez-Sanz et al., Euro Surveill, 2019), especially the frequent epidemics makes it a major health related problem in Asian countries (Chen et al., Pediatrics 2007; Chang., Pediatr Neonatol, 5 2008; Ang et al., Ann Acad Med Singapore 2009; Chua et al., Virol Sin 2011; Xing et al., Lancet Infect Dis 2014). HFMD outbreaks are also occurring in India in recent years (Sasidharan et al., Indian j Pediatr 2005; Gopalkrishna et al., J Med Microb 2012; Sarma et al., Indian J Dermatol Venereol Leprol 2013; Kumar et al., Indian J Public Health, 2015; Palani et al., Indian Pediatr, 2018) and is potentially an emerging public health problem in 10 the country. Inactivated vaccine against Coxsackievirus A16 and the bivalent formulation of inactivated CVA16 and EV71 are warranted to provide protection against these two viruses and will reduce the global burden of Hand foot and mouth disease and other associated neurological complications. Thus, the present invention discloses the genome wide cDNA sequence derived from Vero cell adapted Coxsackievirus A16 (CVA16) 15 strains isolated from India and also describes the possible usage thereafter including the preparation of thermostable live virus formulation, the methods of inactivation for making inactivated CVA16 vaccine and its formulation with inactivated EV71 or EVD68 virus vaccine to make bivalent vaccines or its formulation with both inactivated EV71 and EVD68 virus vaccines to make trivalent vaccines, usage of the isolated CVA16 for 20 establishing the animal model and for diagnostic purposes.

OBJECTIVES OF THE INVENTION:

The main objective of the invention is to disclose and provide stable formulations of low 25 abundance enteroviruses vaccines and process of manufacture thereof.

Another main objective of the invention is to disclose and provide a method of isolating the enteroviruses from the clinical sample by propagating in mammalian cell substrate.

30 In one objective, the invention provides Coxsackievirus A16 strain characterized by DNA sequences denoted by SEQ ID No. 1 and SEQ ID No. 3.

In one objective, the invention provides the amino acid sequences of the polyprotein of Coxsackievirus A16 denoted by SEQ ID No. 2 and SEQ ID No. 4 which have been 35 encoded from the cDNA sequence denoted by SEQ ID No. 1 and SEQ ID No. 3

respectively.

Another objective of the invention is to utilize the isolated virus as challenge strain in mice model to study the *in vivo* pathogenicity with the intention of establishment of suitable
5 animal model to be used for vaccine and antiviral drug discovery.

In one objective, the invention provides a method for inactivation of viruses including CVA16 of present invention and its use in the manufacture of inactivated vaccine formulation.

10 Yet in another objective, the invention provides a method of using Coxsackievirus A16 with sequence mentioned above and stored in formulations mentioned above to make inactivated vaccine.

15 It is the objective of the invention to provide a stable formulation of the isolated virus and other enteroviruses for long term storage at different temperatures. The viruses used in the stable formulation may be live virus (for live virus vaccine formulation) or inactivated virus (for inactivated virus vaccine formulation).

20 In one objective, the invention provides stable, live Enterovirus vaccine composition and formulation comprising one or more live Enterovirus strain(s) including the CVA16 virus strain described in the present invention, and method of preparation of such live vaccine.

One another objective of the invention is to provide vaccine formulation for inactivated
25 Coxsackievirus A16 (CVA16) vaccine utilizing the CVA16 virus strain described in the present invention, and method of preparation of such inactivated vaccine.

In a further objective, the invention provides immunogenic composition comprising stable formulation of inactivated Coxsackievirus A16 virus antigen as stated above and
30 optionally one or more pharmaceutically acceptable excipients selected from adjuvants, stabilizers, or preservatives.

Yet another objective of the present invention is to describe the usage of recombinant viral protein derived from the present invention for raising antibodies that can be utilized for
35 detection and diagnosis of the virus or respective individual proteins.

SUMMARY OF THE INVENTION:

Accordingly, the present invention discloses and provides stable formulations of low abundance enteroviruses vaccines and process of manufacture thereof.

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In one embodiment the present invention discloses the genome wide cDNA sequence derived from Vero cell adapted Coxsackievirus A16 (CVA16) strains isolated from India and also describes the possible usage thereafter including the preparation of thermostable live virus formulation, the methods of inactivation for making inactivated CVA16 vaccine and its formulation with inactivated EV71 or EVD68 virus vaccine to make bivalent vaccines or its formulation with both inactivated EV71 and EVD68 virus vaccines to make trivalent vaccines, usage of the isolated CVA16 for establishing the animal model and for diagnostic purposes.

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15 In one embodiment the present invention discloses a thermostable formulation for enteroviruses wherein the formulation composition comprises:

- a) one or more Enterovirus antigen(s);
- b) carbohydrate in a concentration range between 5 to 50%;
- c) serum protein in a concentration upto 10%;
- 20 d) cation in a concentration upto 1M;
- e) amino acid; and
- f) M199 media in a concentration between 0 to 9.4 g/L with pH between 7.0 to 7.5.

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In one embodiment, the above formulation is a liquid formulation, wherein one or more Enterovirus antigen is/are live virus antigen(s) selected from Enterovirus 71 (EV71), Coxsackievirus A16 (CVA16) and Enterovirus D68 (EVD68) present in a concentration range between 10^6 to 10^7 PFU/ml.

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In one embodiment, the above the formulation is a monovalent formulation comprising a live Enterovirus antigen selected from Enterovirus 71 (EV71), Coxsackievirus A16 (CVA16) and Enterovirus D68 (EVD68); or a multivalent formulation comprising combination of two or more live Enterovirus antigens selected from Enterovirus 71 (EV71), Coxsackievirus A16 (CVA16) and Enterovirus D68 (EVD68).

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In one embodiment, the above said thermostable formulation comprises Coxsackievirus A16 (CVA16) strain which is characterized by SEQ. ID. Nos. 1-4.

5 In one embodiment, the above said (b) carbohydrate(s) comprises sugar which includes sucrose, trehalose, maltose, or combination thereof present in a concentration ranging from 5% to 50%, preferably 5% to 20%, more preferably at a concentration ranging between 10% to 20%.

10 In one embodiment, the above said (c) serum protein(s) comprises fetal calf or bovine serum, bovine serum albumin, human serum albumin or any combination thereof present at a concentration upto 10%, preferably between 2% to 10%.

In one embodiment, the above said (d) cation(s) comprises Mg^{+2} , Ca^{+2} , or salt thereof or combination thereof at a concentration upto 1M.

15 In one embodiment, the above said (e) amino acid(s) can be one or multiple selected from the group consisting of Glutamic acid, Glycine, Arginine and Lysine or combination thereof. In one embodiment, the above said amino acid comprises two amino acids specifically comprising Glycine (upto 2%, preferably 0.5-2%) and Glutamate (upto 2mM, 20 preferably between 1-2 mM).

In one embodiment, the above said (f) media M199 with pH between 7.0 to 7.5 is present in an amount of 9.4g/L.

25 In one embodiment, the above said formulation is a liquid formulation which comprises:
a) one or more Live Enteroviruses antigen(s) in a concentration range between 10^6 to 10^7 PFU/ml;
b) sucrose (carbohydrate) in a concentration range between 5 to 20%, preferably 10 to 20%;
30 c) fetal calf (Serum protein) in a concentration upto 10%, preferably 2 to 10%;
d) Mg^{+2} or salt thereof (cation) in a concentration upto 1M;
e) amino acids comprising Glycine (upto 2%, preferably 1%) and Glutamate (upto 2mM, preferably 1.5mM); and
f) M199 media in a concentration of 9.4 g/L with phosphate buffer pH 7 to 7.5.

35 In one embodiment, the invention provides a method for isolation of low abundance

enteroviruses specifically coxsackievirus A16 (CVA16) by the following steps:

- a) Passage of swab having suspected viral sample through 0.22 micron filter and addition to the cells in 12 or 24 well tissue culture well as neat or diluted in cell culture media (DMEM +1% serum);
- 5 b) centrifugation of cells to remove the cells after appearance of significant cytopathic effect (CPE);
- c) in absence of visible CPE, supernatant was collected and added to the fresh Vero cells and the cycle was repeated upto four times;
- d) viruses were harvested by freeze thawing thrice for complete cell lysis; and
- 10 e) pre-clariication filtration steps or centrifugation to remove host cells.

The above said method for isolation, wherein the CVA16 isolated and obtained comprises nucleotide sequence of SEQ ID No. 1 and SEQ ID No. 3; and amino acid sequence of SEQ ID No. 2 and SEQ ID No. 4.

- 15 A Coxsackievirus A16 (CVA16) adapted to propagate in Vero cells encoded by a cDNA molecule having the nucleotide sequence of SEQ ID No. 1 and SEQ ID No. 3.

- 20 An amino acid sequence of Coxsackievirus A16 (CVA16) polyprotein disclosed in SEQ ID No. 2 and SEQ ID No. 4.

A lethal animal model of Coxsackievirus A16 (CVA16) comprising a rodent infected with CVA16.

- 25 An aqueous transport media capable of maintaining the viability of viruses with the composition as described above with pH ranging between 7.0-7.5.

- 30 In one embodiment, the invention provides a method of using Coxsackievirus A16 (CVA16) with sequence having SEQ. ID No. 1-4 and stored in formulations mentioned as described above to use in and make inactivated virus vaccine which consists of the following steps:

- a) propagation in cells and harvesting;
- b) concentrated by 100kD cut off membrane or by PEG precipitation;
- c) removal of host nucleic acids by nuclease;
- 35 d) purification using ion exchange chromatography with DEAE resins or by size exclusion chromatography using Sepharose CL-6B resin or by mixed mode

resins or two steps chromatography or multi-steps chromatography using more than one chromatography methods described;

- 5 e) inactivation of virus using 1:2000 or 1:4000 formalin and incubating the suspension for upto two weeks at 37°C or using with 1:2000 or 1:3000 or 1:3500 Beta-propiolactone (BPL) at 4°C for upto 120 hrs and then incubation for 2-3 hrs at 37°C for hydrolysis of BPL.

A method of in inactivation of CVA16 virus using the inactivation method as described in step (e) above.

- 10 In one embodiment, the invention provides a stable formulation for inactivated Coxsackievirus A16 (CVA16) vaccine which comprises the inactivated CVA16 antigen in a physiologically acceptable vehicle; 5 to 50%, preferably 5 to 20% sucrose or trehalose; and upto 2mM Glutamate or upto 2% Glycine with pH 7 to 8.

- 15 The stable formulation for inactivated CVA16 as described above, wherein the formulation comprises formalin or BPL inactivated CVA16 having phosphate buffer as vehicle, 10-20% sucrose, and 0.5-2mM Glutamate or 0.5 to 2% Glycine.

- 20 In one embodiment, the invention provides an immunogenic composition comprising stable formulation of inactivated Coxsackievirus A16 virus antigen of SEQ. ID No. 1-4 and *optionally* one or more pharmaceutically acceptable excipients selected from buffer as vehicle, adjuvants, stabilizers, or preservatives.

- 25 In one embodiment, the invention provides an immunogenic composition comprising of combination of different enterovirus antigens including but not limited to Enterovirus 71, Coxsackievirus A16 and Enterovirus D68 in equal amounts in a physiologically acceptable buffer as vehicle and optionally one or more pharmaceutically acceptable excipients selected from adjuvants, stabilizers, preservatives, and other excipients selected from the
30 group consisting of salt such as NaCl, and Polysorbate 80.

The immunogenic composition as described above, wherein the said composition is:

- a bivalent vaccine composition comprising combination of two different enterovirus antigens selected from Enterovirus 71, Coxsackievirus A16 and Enterovirus D68; or
35 - a trivalent vaccine composition comprising combination of three different enterovirus antigens Enterovirus 71, Coxsackievirus A16 and Enterovirus D68.

The immunogenic composition as described above, wherein

- the vehicle comprises phosphate buffer 10-30mM;
 - the adjuvant that may be optionally used for vaccine formulation includes but is not
5 limited to alum adjuvant which may be aluminium phosphate or aluminium hydroxide, calcium phosphate, chitosan and complex carbohydrates, biodegradable polymers like poly PLGA, squalene based adjuvant like MF59, any oil in water emulsions, any water in oil emulsions, lipid based delivery system like liposomes, RIBI adjuvant systems, saponins including but not limited to QS-21, ISCOMs, ISCOMATRIX etc, muramyl
10 dipeptides, any TLR ligands based adjuvants, any cytokines or other immunomodulants, vitamins, any bacterial cell components based adjuvants like Monophosphoryl lipid A and derivatives, CpG and non-CpG containing oligonucleotides etc;
 - the stabilizer that may be optionally used for vaccine formulation comprises the group
15 selected from sugars or polyols, or amino acids or different combinations of sugars, polyols, and amino acids;
 - the preservative that may be optionally used for vaccine formulation comprises includes Thiomersal, 2-phenoxyethanol or combination thereof.
- 20 The immunogenic composition as described above, wherein
- the sugar includes sucrose, trehalose, maltose, and the polyol includes sorbitol, mannitol, glycerol, wherein the sugar and/or polyols is/are present in a concentration ranging from 5-50%, preferably 10-20%;
 - the amino acid includes salts of glutamic acid, Glycine, Arginine, Lysine, or
25 combination thereof, preferably Glutamate upto 2mM and/or Glycine upto 2%.

The immunogenic composition as described above, wherein the said composition is a bivalent or trivalent vaccine composition comprising:

- combination of different enterovirus antigens selected from Enterovirus 71,
30 Coxsackievirus A16 and Enterovirus D68;
- Phosphate buffer in a concentration of 10-30mM;
- Alum in a concentration of ≤ 0.9 mg/dose;
- Glycine in a concentration of 0.5-2%; and
- Optionally, Polysorbate 80 in a concentration of 0.01-0.1%.

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The immunogenic composition as described above, wherein the said composition is a bivalent or trivalent vaccine composition comprising:

- combination of different enterovirus antigens selected from Enterovirus 71, Coxsackievirus A16 and Enterovirus D68;
- 5 - Phosphate buffer in a concentration of 10-30mM;
- Alum in a concentration of ≤ 0.9 mg/dose;
- NaCl in a concentration of 137-154mM;
- Sucrose in a concentration of 10-20%;
- Glycine in a concentration of 0.5-2%; and
- 10 - Optionally, Polysorbate 80 in a concentration of 0.01-0.1%.

The immunogenic composition as described above, wherein the compositions are stable composition and the vaccine formulations prepared therefrom are stable vaccine formulation. In one embodiment the immunogenic composition is stable for at least 6
15 months at 2-8°C and for at least 7 days at 37°C.

Use of Coxsackievirus A16 strain (CVA16 virus strain) having nucleotide sequences of SEQ ID No. 1 and SEQ ID No. 3; and/or its amino acid sequences of SEQ ID No. 2 and SEQ ID No. 4 as vaccine antigen; in the preparation of vaccine, vaccine and/or
20 immunogenic composition and formulation for prophylaxis and/or treatment of infectious disease caused by Enteroviruses; and/or for raising antibodies that can be utilized for detection and diagnosis of the virus or respective individual proteins.

A method for preparation of immunogenic vaccine composition and formulation as
25 described in the description, as exemplified in examples and Figures 1-6.

BRIEF DESCRIPTION OF DRAWINGS/FIGURES OF THE INVENTION:

Figure-1: Detection and confirmation of Vero cell adapted Coxsackievirus A16 (CVA16)
30 by RT-PCR. RNA was isolated from the Vero cell adapted CVA16 followed by cDNA synthesis and detection using pan-enterovirus specific primers (Lane 1) and CVA16 specific primers (Lane 3).

- Lane 1: CVA16 with pan-enterovirus primers,
- Lane 2: Negative control with pan-enterovirus primers,
- 35 Lane 3: CVA16 with CVA16 specific primers,

Lane 4: Negative control with CVA16 specific primers,

Lane 5: 100 bp marker (NEB).

Figure-2: Expression of Coxsackievirus A16 (CVA16) proteins in supernatant of infected

5 Vero cells checked by Western blot using specific antibodies:

Figure-2A: Expression of VP1 proteins of CVA16.

Figure-2B: Expression of VP2 proteins of CVA16.

Figure-3: Post-infection results of neonatal Balb/c mice after challenged with CVA16:

10 Figure-3A: Graph showing Survival curve.

Figure-3B: Graph showing Clinical score.

Figure-4: Total IgG titer in the sera of the Balb/C mice immunized with different amount of Formalin and BPL inactivated CVA16 vaccine formulated with or without alum

15 adjuvant:

Figure-4A: Graph shows the total IgG titer values (Formalin inactivated).

Figure-4B: Graph shows IgG the total titer values (BPL inactivated).

Figure-5: Graph shows the ratio of IgG1/IgG2a in the sera of the Balb/C mice immunized
20 with Formalin and BPL inactivated CVA16 vaccine formulated with or without alum adjuvant.

Figure-6: Graph shows the total virus specific IgG titer in the sera of the Balb/C mice immunized with BPL inactivated CVA16 vaccine formulated with or without adjuvants.

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DETAILED DESCRIPTION OF THE INVENTION:

Accordingly, the present invention discloses enteroviruses vaccines, process of manufacture thereof and method of isolating the enteroviruses from the clinical sample by
30 propagating in mammalian cell substrate. In one embodiment, the invention discloses and provides stable formulations of low abundance enteroviruses vaccines and process of manufacture thereof.

In one embodiment the invention further discloses a process of isolation of low abundance
35 enteroviruses from clinical source of suspected hand, foot, and mouth disease case/s by adapting and propagating in cell substrate and utilizing the isolated virus for thermostable

formulation, studying pathogenicity in animal model, for preparing vaccine formulations, process of vaccine manufacturing thereof and for diagnostic tools.

5 In one embodiment, in the above said vaccines, process of manufacture thereof and method of isolation, said enteroviruses comprises Coxsackievirus A16 (CVA16), Enterovirus 71 (EV71), polioviruses, and Enterovirus D68 (EVD68). The vaccine may be a monovalent vaccine comprising any one virus or may be a multivalent vaccine comprising combination of two or more Enteroviruses described above.

10 In one embodiment, the invention provides a stable formulation of the isolated virus and other enteroviruses for long term storage at different temperatures. In one particular embodiment, the CVA16 virus and other enteroviruses including but not limited to Enterovirus 71, polioviruses, Enterovirus D68 are described.

15 In one particular embodiment, CVA16 virus was isolated and was used in the manufacture of vaccine (**live** and/or **inactivated**) and immunogenic vaccine composition/formulation. The isolated CVA16 virus alone may be used in the vaccine formulation (**monovalent**) or may also be used in the vaccine formulation comprising said isolated CVA16 virus in combination with one or more other enteroviruses as described above (**multivalent**).

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BIOLOGICAL RESOURCES USED:

In one embodiment, the Enterovirus 71 (EV71) used in the present invention and the vaccine formulations described is EV71 H strain (ATCC).

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In one embodiment, the Enterovirus D68 (EVD68) used in the present invention and the vaccine formulations described is EVD68 strain US/KY/14-18953 adapted in Vero (PCT/IN2019/050960 – incorporated herein for reference to virus strain).

30 In one embodiment, the Coxsackievirus A16 (CVA16) used in the present invention and the vaccine formulations is CVA16, locally collected in India (SEQ. ID No. 1-4).

The biological resources (CVA16) used in the invention are collected from swab samples from patients from private practitioner located at Krishnanagar, Nadia district of West

35 Bengal, India and Hyderabad, Telangana, India.

ISOLATION AND CVA16 STRAIN:

In one embodiment, the invention discloses and provides a method of isolating the low abundance enteroviruses specifically coxsackievirus A16 (CVA16) from the clinical sample by propagating in mammalian cell substrate, wherein the isolation method
5 comprises the steps as described in Example-1 and following steps:

- a) passaging the swab having suspected viral sample through 0.22 micron filter and addition to the cells in 12 or 24 well tissue culture well as neat or diluted in cell culture media (DMEM +1% serum);
- 10 b) centrifugating the cells to remove the cells after appearance of significant cytopathic effect (CPE);
- c) in absence of visible CPE, collecting supernatant and adding to the fresh Vero cells and repeating the cycle upto four times;
- d) harvesting virus by freeze thawing thrice for complete cell lysis; and
- 15 e) subjecting to pre-clarification filtration steps or centrifugation to remove host cells.

In one embodiment the above method of isolation is applied to obtain the isolated CVA16 strain of present invention, wherein the isolated CVA16 strain is identified and/or characterized by following sequences 1-4:

- 20
- SEQ ID No. 1: Nucleotide sequence encoding polyprotein of CVA16/KR/Hyd.
 - SEQ ID No. 2: Amino acid sequence of polyprotein of CVA16/KR/Hyd.
 - SEQ ID No. 3: Nucleotide sequence encoding polyprotein of CVA16/VS/WB.
 - SEQ ID No. 4: Amino acid sequence of polyprotein of CVA16/VS/WB.

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In one embodiment the present invention provides the **DNA sequences** comprising of the cDNA sequence derived from Coxsackievirus A16, which are denoted by **SEQ ID No. 1** and **SEQ ID No. 3**.

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In one embodiment the present invention provides the **amino acid sequences** of the polyprotein denoted by **SEQ ID No. 2** and **SEQ ID No. 4** which have been encoded from the cDNA sequence denoted by **SEQ ID No. 1** and **SEQ ID No. 3** respectively.

The virus with the RNA that is reversed transcribe to cDNA denoted by SEQ ID No. 1 and
35 SEQ. ID No. 3 as mentioned in the present invention is adapted to propagate in mammalian cells.

The nucleic acid sequences of the invention can be used for making infectious cDNA clone of the virus.

5 PATHOGENICITY:

In one embodiment, the present invention utilizes the above isolated virus as challenge strain in mice model to study the *in vivo* pathogenicity with the intention of establishment of suitable animal model to be used for vaccine and antiviral drug discovery.

- 10 The information of the cDNA sequence from the present invention and amino acid sequences derived thereafter can be used to manipulate the virus to study the function of the particular amino acids in pathogenicity. The particular sequence or sequences included in the whole cDNA sequence of the invention coding for the particular amino acids can be mutated by site directed mutagenesis and can be used to make mutated virus strain. The
- 15 mutated virus and the wild type virus can be used to infect neonatal mouse or interferon knockout mice or immunocompromised mice to observe the difference in pathogenicity.

- In one particular embodiment, the CVA16 virus and other enteroviruses including but not limited to Enterovirus 71, polioviruses, Enterovirus D68 have been formulated to increase
- 20 the stability of the viruses. Virus strains isolated from clinical source or produced in laboratory by using molecular tools can often experiences reduction of infectivity due to thermal degradation and other stress factors. This is a huge problem for successful product development or any future applications of the virus strains. The applicability of the stable formulation is unlimited including the enhancement of thermal stability of both wild type
- 25 and mutated nonenveloped viruses. The mutated virus includes the attenuated viruses, rescued virus generated by reverse genetics and chimeric viruses. The stable formulation described in the present invention can be also used for storing the clinical samples predicted to contain viruses during the transport from the source of sample collection to the testing laboratory.

- 30 In one embodiment, the viruses described in the present invention is pathogenic and lethal in mice particularly Balb/C mice and can be used as challenge strain in mice model to study the *in vivo* pathogenicity, efficacy of vaccine and antiviral drugs against Cocksackievirus A16 viruses.

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INACTIVATION:

In one embodiment the invention discloses and provides a method for inactivation of viruses including CVA16 of present invention and its use in the manufacture of inactivated vaccine formulation.

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Thus, in an embodiment, the isolated virus/viruses from the present invention was/were inactivated for producing inactivated vaccine in Vero cells or any mammalian cells or for diagnostic purpose using either physical methods or chemical methods to make stable vaccine formulation for human use. Chemical methods of inactivation comprise of inactivation by formalin, β -propiolactone, ethyleneimine monomer (EI or BEI), ascorbic acids, ascorbate, detergents (non-ionic and ionic). Physical methods of inactivation comprise of ultraviolet light or heat or gamma irradiation. Inactivation method may comprise of combination of two or more physical or chemical methods.

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In one embodiment, the inactivation of virus comprises the inactivation methods as described above and as exemplified in Example-7. In one embodiment the inactivation of virus comprises:

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- inactivating virus using 1:2000 or 1:4000 formalin and incubating the suspension for upto two weeks at 37°C or using with 1:2000 or 1:3000 or 1:3500 Beta-propiolactone (BPL) at 4°C for upto 120 hrs and
- then incubation for 2-3 hrs at 37°C for hydrolysis of BPL.

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Virus may or may not be concentrated before inactivation. Coxsackievirus A16 strains disclosed in the present invention have been be concentrated either by PEG precipitation or ultrafiltration. Purification of the virus have been done by ultrafiltration or chromatography or ultracentrifugation or combination of more than one of the methods prior to inactivation. The chromatography process consists of Size exclusion chromatography or Ion exchange chromatography or chromatography using mixed mode resins. Two or more chromatography steps can be combined as purification strategy.

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Alternatively, purification was done following inactivation process. In one embodiment, the CVA16 virus strain of the present invention has been purified by the method as described in Example-6.

VACCINE FORMULATIONS:

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In one embodiment the invention discloses and provides a stable formulation of low

abundances Enteroviruses for long term storage at different temperatures. The virus may be the isolated virus of present invention and/or other enteroviruses. In one embodiment the virus may be the isolated CVA16 virus and/or other enteroviruses such as EV71, EVD68, and Poliovirus for long term storage at different temperatures. The viruses used in
5 the stable formulation may be **live virus** (for **live virus vaccine formulation**) or **inactivated virus** (for **inactivated virus vaccine formulation**), wherein the viruses are inactivated by the virus inactivation method as described in the present invention.

Thus, the invention discloses and provides a **live Enterovirus vaccine** composition and
10 formulation comprising one or more live Enterovirus strain(s) such as and/or other enteroviruses such as EV71, EVD68, and Poliovirus including the CVA16 virus strain described in the present invention.

Thus, the invention discloses and provides a **vaccine formulation for inactivated**
15 **Coxsackievirus A16 (CVA16)** utilizing the CVA16 virus strain described in the present invention (monovalent) and also multivalent vaccine comprising combination of CVA16 and one or more other live Enterovirus strain(s) such as EV71, EVD68, and poliovirus.

The inactivated vaccine may be formulated after adsorption of the antigen in alum
20 adjuvant. Alum may be aluminium phosphate or aluminium hydroxide. Other adjuvant that may be used for vaccine formulation includes but is not limited to calcium phosphate, chitosan and complex carbohydrates, biodegradable polymers like poly PLGA, squalene based adjuvant like MF59, any oil in water emulsions, any water in oil emulsions, lipid based delivery system like liposomes, RIBI adjuvant systems, saponins including but not
25 limited to QS-21, ISCOMs, ISCOMATRIX etc, muramyl dipeptides, any TLR ligands based adjuvants, any cytokines or other immunomodulants, vitamins, any bacterial cell components based adjuvants like Monophosphoryl lipid A and derivatives, CpG and non-CpG containing oligonucleotides etc.

30 In one more embodiment, the inactivated coxsackievirus A16 antigen was stabilized in formulations comprising of sugars or polyols or amino acids or different combinations of sugars, polyols and amino acids. The sugar includes sucrose, trehalose, maltose ranging from 5-50%. The polyols include sorbitol, mannitol, glycerol. The amino acids include salts of glutamic acid, Glycine, Arginine, and Lysine.

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In yet another embodiment, the inactivated CVA16 vaccine/s disclosed in the present invention can be combined with other enteroviruses that includes but is not limited to inactivated EV71 virus or inactivated polio virus or inactivated EVD68 virus to make bivalent or trivalent or multivalent vaccine.

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In one embodiment, the above said vaccine of said isolated CVA16 is provided as a **monovalent vaccine** comprising **inactivated CVA16** as isolated and obtained by the method of the present invention disclosed and described.

10 In one embodiment, the above said vaccine of said isolated CVA16 is provided as a multivalent vaccine such as **bivalent** or **trivalent vaccine** comprising **inactivated CVA16** as isolated and obtained by the method of the present invention in combination with one or more other enterovirus selected from but not limited to inactivated EV71, inactivated polioviruses, and inactivated EVD68.

15

In one embodiment the said multivalent inactivated vaccine is a bivalent vaccine which comprises combination of inactivated CVA16 and EV71 virus antigens.

In one embodiment the said multivalent inactivated vaccine is a bivalent vaccine which
20 comprises combination of inactivated CVA16 and EVD68 virus antigens.

In one embodiment the said multivalent inactivated vaccine is a trivalent vaccine which comprises combination of inactivated CVA16, EV71 and EVD68 virus antigens.

25 In another embodiment, the cDNA of the individual structural (VP1, VP2, VP3 and VP4) and non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C and 3D) of Coxsackievirus A16 described in this invention or cDNA of the polyprotein disclosed in this invention can be cloned in expression plasmids and can be expressed in Escherichia coli, yeast, insect, or mammalian cells with or without after proper codon optimization. Furthermore, the
30 expressed viral proteins can be used to make antibodies against the individual proteins or polyprotein which can be used for virus specific detection and diagnosis.

The expression vector suitable for expressing the nucleic acid sequence of the invention can be a bacterial, yeast, insect, mammalian cell or virus. The vector may be plasmid or
35 may be virus. The nucleic acid expressed can be the whole virus or any protein or peptide sequences which is part of the whole sequence that can be fused to separate amino acid

sequence, e.g. glutathione S-transferase or poly-histidine, any other tag, signal sequences, immunomodulatory sequences or carrier proteins.

Isolation and Harvesting:

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EXAMPLE 1: Isolation of virus, Adaptation of the virus in Vero cells and harvesting

Swab sample predicted to have enteroviruses (Coxsackievirus A16) were passed through 0.22 micron filter and added to the Vero cells in 12 or 24 well tissue culture well as neat or diluted in cell culture media (DMEM +1% serum) and waited up to 7 days for cytopathic effect (CPE). If CPE was visible, the cells were centrifuged to remove the cells and cell free supernatant was collected, otherwise, supernatant was collected and added to the fresh Vero cells. Next, the supernatant was passed through higher to lower micron filter units. Clinical samples were passaged upto four cycles in Vero cells to detect the presence of viruses in the form of CPE. Next, CPE showing cell free supernatant were propagated in T75 cm² or T175 cm² tissue culture flask containing Vero cells. Coxsackievirus A16 were harvested when $\geq 90\%$ CPE was visible by freeze thawing thrice for complete cell lysis. This was followed by pre-clarification filtration steps or centrifugation to remove host cells. For large scale production, Coxsackievirus A16 was propagated in Roller bottle or cell stacks. The virus supernatant was subsequently passed through 0.2 micron filter for filtration.

SEQ ID No. 1: Nucleotide sequence encoding polyprotein of CVA16/KR/Hyd.

SEQ ID No. 2: Amino acid sequence of polyprotein of CVA16/KR/Hyd.

SEQ ID No. 3: Nucleotide sequence encoding polyprotein of CVA16/VS/WB.

25 SEQ ID No. 4: Amino acid sequence of polyprotein of CVA16/VS/WB.

Stable virus formulations (Live Virus Antigen)

Stable virus formulations were prepared wherein Viruses in cell culture media or in physiological buffer were mixed with different additives for long term storage of the viruses in different temperatures. The formulated viruses were stored in different temperatures: 4°C to 37°C and sub-zero (-10°C to -80°C) for different durations.

Later at the end of the study time points, virus titer was quantified using TCID₅₀ or number of plaque forming units per ml (PFU/ml). The titer of the virus at the beginning of the study (T=0) and the titer of the viruses with additive stabilizers (Formulations coded as

F2, F3, F4, F5, F6, F7) or without stabilizers (F1) stored in different temperatures for different time periods were measured and calculated such as at $36\pm 2^{\circ}\text{C}$, $25\pm 2^{\circ}\text{C}$, $2-8^{\circ}\text{C}$ and also at conditions when subjected to Freeze thaw cycle(s).

- 5 The stabilizers used for the study includes carbohydrates, serum protein, M199 media, Cations, and amino acids; and different combination of carbohydrates, serum protein, M199 media, cations, and amino acids.

Thus, in one embodiment, the present invention provides thermostable formulations for
10 enteroviruses wherein the formulation comprises:

- a. One or more live Virus Antigen(s);
- b. Carbohydrate in a concentration range between 5 to 50%;
- c. Serum protein in a concentration upto 10%;
- d. Cation in a concentration upto 1M;
- 15 e. Amino acid; and
- f. M199 media in a concentration between 0 to 9.4 g/L with pH between 7.0 to 7.5.

a. One or more Virus Antigen:

- 20 The formulation of the present invention may comprise one or more live enteroviruses as antigen including but not limiting to Enterovirus 71 (EV71), Coxsackievirus A16 (CVA16) and Enterovirus D68 (EVD68) or combination thereof in a concentration range of 10^6 to 10^7 PFU/ml or 10^6 to 10^7 TCID₅₀/ml.

25 Monovalent:

In one embodiment the Virus in the vaccine formulation is EV71.

In one embodiment the Virus in the vaccine formulation is EVD68.

In one embodiment the Virus in the vaccine formulation is CVA16.

Divalent:

- 30 In one embodiment the Virus in the vaccine formulation comprises EV71 and CVA16.

In one embodiment the Virus in the vaccine formulation comprises EVD68 and CVA16.

In one embodiment the Virus in the vaccine formulation comprises EV71 and EVD68.

Trivalent:

- In one embodiment the Virus in the vaccine formulation comprises EV71, EVD68 and
35 CVA16.

b. Carbohydrate:

The carbohydrate stabilizer(s) can be sugar which includes sucrose, trehalose, maltose, or combination thereof which can be used at a concentration ranging from **5%** to **50%**. In one embodiment sucrose or trehalose at concentration ranging between 5% to 20% is used. In one preferred embodiment sucrose is used for stable virus formulation at a concentration ranging between **10%** to **20%**. In one example embodiment 10% sucrose is used. In another example embodiment 20% sucrose is used.

10 c. Serum Protein:

Serum protein that can be used as stabilizers for providing the stable virus formulation of present invention can be fetal calf or bovine serum, bovine serum albumin, human serum albumin or any combination thereof, or preferably fetal calf serum protein is used at a concentration upto **10%**. In one embodiment 0.1 % to 10 % serum protein can be used. More preferably, in one embodiment the concentration of serum protein (fetal calf serum) used for stable virus formulation is **2%** to **10%**. In one example embodiment 2% fetal calf serum is used. In another example embodiment 10% fetal calf serum is used.

d. Cations:

20 Cations such as Mg^{+2} , Ca^{+2} or salt thereof or combination thereof at a concentration upto **1M** may be included in the stable virus formulation of present invention. More precisely Mg^{+2} is used at a concentration upto **1M**. In one preferred embodiment the cation used is chloride salt of Mg^{+2} cation and preferably 1M $MgCl_2$ cationic salt is used.

25 e. Amino Acid:

Amino acid(s) can be one or multiple selected from the group consisting of glutamic acid, Glycine, Arginine and lysine or combination thereof. Salts of these amino acid can also be used. More precisely two amino acids have been used in one formulation. In one embodiment the combination of Amino acids specifically comprising Glycine (upto 2%) and Glutamate (upto 2mM) is used. In one preferred embodiment Amino acid in the formulation comprises Glycine in a concentration between 0.5-2% + Glutamate in a concentration between 1-2 mM. In one most preferred embodiment amino acids combination comprising 1% Glycine + 1.5 mM Glutamate is used in the example provided.

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f. Media component:

The stable virus formulations of the present invention may comprise media component such as M199 with pH between 7.0 to 7.5. In one preferred embodiment 0 to 9.4 g/L M199 media component can be used. In one most preferred embodiment 9.4g/L M199 media is used.

One type of stable virus formulation comprises of virus and (a) 10%-20% sucrose, (b) 2% fetal calf as serum protein, (c) 1M MgCl₂, (d) 1-2mM Glutamate + 0.5-2% Glycine as Amino acid, and (e) 9.4g/L M199 as media.

Alternatively, stable formulation comprises of virus and (a) 10% sucrose, (b) 2% fetal calf serum as protein, (c) 1M MgCl₂, (d) 0.5-2% Glycine as Amino Acid, and (e) 9.4g/L M199.

Another stable virus formulation comprises of virus and (a) 10-20% sucrose, (b) 10% fetal calf as serum protein, (c) 1M MgCl₂, (d) 1-2mM Glutamate + 0.5-2% Glycine and (e) 9.4g/L M199.

The scope of the formulations is not limited to the ones cited here.

TABLE-A						
(a) Antigen	Additive components					
	(b)	(c)	(d)	(e)		(f)
Live Virus EV71 or EVD68 or CVA16 or combinations (10 ⁶ to 10 ⁷ PFU/ml or 10 ⁶ to 10 ⁷ TCID50/ml.)	Carbohydrate	Serum Protein	Cation	Amino Acid		Media
10 ⁶ to 10 ⁷ PFU/ml or 10 ⁶ to 10 ⁷ TCID50/ml.	Sucrose 10%-20%	fetal calf serum 2%	MgCl ₂ 1M	Glutamate 1-2mM	Glycine 0.5-2%	M199 9.4g/L
10 ⁶ to 10 ⁷ PFU/ml or 10 ⁶ to 10 ⁷ TCID50/ml.	Sucrose 10%-20%	fetal calf serum 10%	MgCl ₂ 1M	Glutamate 1-2mM	Glycine 0.5-2%	M199 9.4g/L
10 ⁶ to 10 ⁷ PFU/ml or 10 ⁶ to 10 ⁷ TCID50/ml.	Sucrose 10%	fetal calf serum 2%	MgCl ₂ 1M	-	Glycine 0.5-2%	M199 9.4g/L

Table A: Combined live virus stable formulations

Better thermostability is observed for the viruses with stabilizers in comparison to the viruses without stabilizers. The formulations consisting of 2% fetal calf serum, 1M MgCl₂, 9.4g/L M199 media, 10%-20% sucrose, 1-2mM Glutamate, 0.5-2% Glycine provides very
 5 good virus stability when stored at different temperatures (2-8°C, 25°C and 36±2°C) or when the virus undergoes multiple freeze thaw cycles.

Thus, in one preferred embodiment, the present invention provides thermostable formulations for enteroviruses including but not limiting to Enterovirus 71,
 10 Coxsackievirus A16 and Enterovirus D68, wherein the formulation comprises:

- a) One or more Live Enteroviruses antigen (10⁶ to 10⁷ PFU/ml);
- b) Carbohydrate(s) in a concentration range between 5 to 20%, preferably 10 to 20%;
- 15 c) Serum protein(s) in a concentration upto 10%, preferably 2 to 10%;
- d) Cation(s) in a concentration upto 1M;
- e) Amino acid(s) comprising Glycine (upto 2%, preferably 1%) and Glutamate (upto 2mM, preferably 1.5mM); and
- 20 f) M199 media in a concentration between 0 to 9.4 g/L, preferably 9.4 g/L with pH between 7.0 to 7.5.

EXAMPLE 2: Examples of Stable virus formulations (Live)

In an exemplary embodiment, test live virus formulations F1 to F7 were prepared and
 25 stability was tested wherein Viruses in cell culture media or in physiological buffer were mixed with different additives (including stabilizers) for long term storage of the viruses in different temperatures. The formulated viruses in formulations F1 to F7 were stored in different temperatures: 4°C to 37°C and sub-zero (-10°C to -80°C) for different durations. Later at the end of the study time points, virus titer was quantified using TCID₅₀ or
 30 number of plaque forming units per ml (PFU/ml). The titer of the virus at the beginning of the study (T=0) and the titer of the viruses with stabilizers (Formulations coded as F2, F3, F4, F5, F6, F7) or without stabilizers (F1) stored in different temperatures for different time periods were calculated such as at 36±2°C, 25±2°C, 2-8°C and also at conditions when subjected to Freeze thaw cycle(s).

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TABLE-B							
Formulations	Virus and Concentration	Serum fetal-calf	Sucrose	MgCl ₂	M199	Glycine	Glutamate
F1	CVA16/EV71/EVD68 10 ⁶ to 10 ⁷ PFU/ml or 10 ⁶ to 10 ⁷ TCID50/ml.	-	-	-	-	-	-
F2	CVA16/EV71/EVD68 10 ⁶ to 10 ⁷ PFU/ml or 10 ⁶ to 10 ⁷ TCID50/ml.	2 %	-	-	-	-	-
F3	CVA16/EV71/EVD68 10 ⁶ to 10 ⁷ PFU/ml or 10 ⁶ to 10 ⁷ TCID50/ml.	2 %	-	1M	-	-	-
F4	CVA16/EV71/EVD68 10 ⁶ to 10 ⁷ PFU/ml or 10 ⁶ to 10 ⁷ TCID50/ml.	2 %	10 %	1M	9.4g/L	1 %	1.5 mM
F5	CVA16/EV71/EVD68 10 ⁶ to 10 ⁷ PFU/ml or 10 ⁶ to 10 ⁷ TCID50/ml.	10 %	10 %	1M	9.4g/L	1 %	1.5 mM
F6	CVA16/EV71/EVD68 10 ⁶ to 10 ⁷ PFU/ml or 10 ⁶ to 10 ⁷ TCID50/ml.	-	10 %	1M	9.4g/L	1 %	1.5 mM
F7	CVA16/EV71/EVD68 10 ⁶ to 10 ⁷ PFU/ml or 10 ⁶ to 10 ⁷ TCID50/ml.	-	20 %	1M	9.4g/L	1 %	1.5 mM

Table B: Summary table of live virus formulations F1-F7

METHOD OF PREPARATION OF VACCINES F1 TO F7:5 **Preparation of Formulation F1:**

A liquid formulation of a virus or different combinations of viruses CVA16, EV71 and EVD68 was mixed in concentration ranging between 10⁶ to 10⁷ PFU/ml or 10⁶ to 10⁷ TCID50/ml in a mixing vessel. The vehicle is 10mM phosphate buffer pH 7 to 7.5.

10 **Preparation of Formulation F2:**

A liquid formulation of a virus or different combinations of viruses CVA16, EV71 and EVD68 was mixed in concentration ranging between 10⁶ to 10⁷ PFU/ml or 10⁶ to 10⁷ TCID50/ml. The serum of 2% concentration was added as stabilizer and mixed in a mixing vessel. The vehicle is 10mM phosphate buffer pH 7 to 7.5.

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Preparation of Formulation F3:

A liquid formulation of a virus or different combinations of viruses CVA16, EV71 and EVD68 was mixed in concentration ranging between 10⁶ to 10⁷ PFU/ml or 10⁶ to 10⁷ TCID50/ml. The serum of 2% concentration and 1M MgCl₂ were added as stabilizer and mixed in a mixing vessel. The vehicle is 10mM phosphate buffer pH 7 to 7.5.

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Preparation of Formulation F4:

- A liquid formulation of a virus or different combinations of viruses CVA16, EV71 and EVD68 was mixed in concentration ranging between 10^6 to 10^7 PFU/ml or 10^6 to 10^7 TCID₅₀/ml. The serum of 2% concentration, 1M MgCl₂, 10% Sucrose, 9.4g/l of M199 powder media, 1% Glycine and 1.5mM Glutamate were added as stabilizer and mixed in a mixing vessel. The vehicle is 10mM phosphate buffer pH 7 to 7.5.

Preparation of Formulation F5:

- A liquid formulation of a virus or different combinations of viruses CVA16, EV71 and EVD68 was mixed in concentration ranging between 10^6 to 10^7 PFU/ml or 10^6 to 10^7 TCID₅₀/ml. The serum of 10% concentration, 1M MgCl₂, 10% Sucrose, 9.4g/l of M199 powder media, 1% Glycine and 1.5mM Glutamate were added as stabilizer and mixed in a mixing vessel. The vehicle is 10mM phosphate buffer pH 7 to 7.5.

Preparation of Formulation F6:

- A liquid formulation of a virus or different combinations of viruses CVA16, EV71 and EVD68 was mixed in concentration ranging between 10^6 to 10^7 PFU/ml or 10^6 to 10^7 TCID₅₀/ml. 1M MgCl₂, 10% Sucrose, 9.4g/l of M199 powder media, 1% Glycine and 1.5mM Glutamate were added as stabilizer and mixed in a mixing vessel. The vehicle is 10mM phosphate buffer pH 7 to 7.5.

Preparation of Formulation F7:

- A liquid formulation of a virus or different combinations of viruses CVA16, EV71 and EVD68 was mixed in concentration ranging between 10^6 to 10^7 PFU/ml or 10^6 to 10^7 TCID₅₀/ml. 1M MgCl₂, 20% Sucrose, 9.4g/l of M199 powder media, 1% Glycine and 1.5mM Glutamate were added as stabilizer and mixed in a mixing vessel. The vehicle is 10mM phosphate buffer pH 7 to 7.5.
- Better thermostability was observed for the viruses with stabilizers in comparison to the viruses without stabilizers. The formulation consisting of 2% fetal calf serum, 1M MgCl₂, 9.4g/L M199 media, 10%-20% sucrose, 1-2mM Glutamate, 0.5-2% Glycine provides very good virus stability when stored at different temperatures (2-8°C, 25°C and 36±2°C) or when the virus undergoes multiple freeze thaw cycles. The tables 1-3 provided below show the Virus titer values (PFU/ml) and illustrates the stability of different enteroviruses

EV71 (Table-1), EVA16 (Table-2), and EVD68 (Table-3) with or without stabilizers.

TABLE-1 Enterovirus 71: Stability in presence and absence of stabilizers								
Formulations	F1	F2	F3	F4	F5	F6	F7	
Temp./Freeze thaw cycles	Time points	No stabilizer	2% serum	2% serum + 1M MgCl ₂	2% serum + 10% sucrose + 1M MgCl ₂ + 9.4 g/L M199 + 1% Glycine + 1.5mM Glutamate	10% serum + 10% sucrose + 1M MgCl ₂ + 9.4 g/L M199 + 1% Glycine + 1.5mM Glutamate	10% sucrose + 1M MgCl ₂ + 9.4 g/L M199 + 1% Glycine + 1.5mM Glutamate	20% sucrose + 1M MgCl ₂ + 9.4 g/L M199 + 1% Glycine + 1.5mM Glutamate
Virus titer (PFU/ml)								
	T=0	1.2X10 ⁶	10 ⁶	1.05X10 ⁶	1.2X10 ⁶	1.1X10 ⁶	1.1X10 ⁶	1.2X10 ⁶
36±2°C	T=3 days	6.5X10 ⁴	7X10 ⁴	7X10 ⁴	7X10 ⁴	10 ⁵	9X10 ⁴	8.5X10 ⁴
	T=5 days	5X10 ³	6.5X10 ⁴	5X10 ⁴	6.5X10 ⁴	5X10 ⁴	5X10 ⁴	6X10 ⁴
	T=7 days	3X10 ³	1.5X10 ⁴	4X10 ⁴	4X10 ⁴	4X10 ⁴	4.5X10 ⁴	4X10 ⁴
	T=10 days	<10 ²	5X10 ³	10 ⁴	2X10 ⁴	10 ⁴	2X10 ⁴	1.75X10 ⁴
25±2°C	T=1 week	5X10 ³	1.25X10 ⁴	1.25X10 ⁵	1.2X10 ⁴	10 ⁵	1.25X10 ⁵	1.1X10 ⁵
	T=2 weeks	<10 ²	<10 ²	1.5X10 ⁴	10 ⁴	2.5X10 ⁴	1.75X10 ⁴	1.5X10 ⁴
	T=4 weeks	<10 ²	<10 ²	3X10 ³	3.5X10 ³	2X10 ³	2X10 ³	1.25X10 ³
2-8°C	T=2 weeks	5.5X10 ⁵	5.5X10 ⁵	7X10 ⁵	8.5X10 ⁵	8.5X10 ⁵	7.5X10 ⁵	8.5X10 ⁵
	T=1 month	3.5X10 ⁵	3X10 ⁵	5X10 ⁵	5.5X10 ⁵	4.5X10 ⁵	6.5X10 ⁵	5.5X10 ⁵
	T=2 months	4.5X10 ⁴	1.5X10 ⁵	3.5X10 ⁵	5X10 ⁵	3.5X10 ⁵	4X10 ⁵	3X10 ⁵
	T=3 months	<10 ²	3X10 ³	2X10 ⁵	3X10 ⁵	2X10 ⁵	5X10 ⁴	1.25X10 ⁵
Freeze thaw cycles	1 cycle	1.1X10 ⁶	10 ⁶	1.1X10 ⁶	1.1X10 ⁶	1.2X10 ⁶	1.1X10 ⁶	1.1X10 ⁶
	2 cycles	8.5X10 ⁵	10 ⁶	1.1X10 ⁶	1.2X10 ⁶	1.05X10 ⁶	1.1X10 ⁶	1.2X10 ⁶
	3 cycles	5X10 ⁵	4.5X10 ⁵	9.5X10 ⁵	9.5X10 ⁵	8.5X10 ⁵	10 ⁶	9.5X10 ⁵
	4 cycles	1.25X10 ⁵	2.25X10 ⁵	7.5X10 ⁵	6.5X10 ⁵	7X10 ⁵	6X10 ⁵	6.5X10 ⁵
	5 cycles	<10 ²	4.5X10 ⁴	5X10 ⁵	6.5X10 ⁵	3.75X10 ⁵	3.75X10 ⁵	4X10 ⁵

Table 1: Enterovirus 71 stability in presence and absence of stabilizers

TABLE-2								
Coxsackievirus A16: Stability in presence and absence of stabilizers								
	Formulations	F1	F2	F3	F4	F5	F6	F7
Temp./Freeze thaw cycles	Time points	No stabilizer	2% serum	2% serum + 1M MgCl ₂	2% serum + 10% sucrose + 1M MgCl ₂ + 9.4g/L M199 + 1% Glycine + 1.5mM Glutamate	10% serum + 10% sucrose + 1M MgCl ₂ + 9.4 g/L M199 + 1% Glycine + 1.5mM Glutamate	10% sucrose + 1M MgCl ₂ + 9.4 g/L M199 + 1% Glycine + 1.5mM Glutamate	20% sucrose + 1M MgCl ₂ + 9.4 g/L M199 + 1% Glycine + 1.5mM Glutamate
	Virus titer (PFU/ml)							
36±2°C	T=0	1.6X10 ⁶	1.5X10 ⁶	1.5X10 ⁶	1.5X10 ⁶	1.5X10 ⁶	1.5X10 ⁶	1.7X10 ⁶
	T=3 days	6.5X10 ⁴	9.5X10 ⁴	1.5X10 ⁵	1.5X10 ⁵	1.75X10 ⁵	1.25X10 ⁵	1.4X10 ⁵
	T=5 days	5X10 ³	3.5X10 ⁴	8X10 ⁴	9X10 ⁴	1.1X10 ⁵	9X10 ⁴	9X10 ⁴
	T=7 days	<10 ²	3X10 ³	1.25X10 ⁴	4.5X10 ⁴	9X10 ⁴	1.75X10 ⁴	3.5X10 ⁴
	T=10 days	<10 ²	<10 ²	3X10 ³	1.25X10 ⁴	3X10 ⁴	4.5X10 ³	5X10 ³
25±2°C	T=1 week	2X10 ³	2X10 ⁵	3X10 ⁵	3.5X10 ⁵	2.5X10 ⁵	3.5X10 ⁵	1.2X10 ⁵
	T=2 weeks	5X10 ²	3X10 ³	5X10 ⁴	2.5X10 ⁵	2.5X10 ⁵	8X10 ⁴	9X10 ⁴
	T=1 month	<10 ²	<10 ²	5X10 ³	1.2X10 ⁴	1.5X10 ⁴	4X10 ³	1.1X10 ⁴
2-8°C	T=2 weeks	2.5X10 ⁵	5X10 ⁵	5X10 ⁵	8X10 ⁵	8X10 ⁵	6.6X10 ⁵	6X10 ⁵
	T=1 month	5X10 ⁴	4.5X10 ⁴	7X10 ⁴	2X10 ⁵	2.5X10 ⁵	2.5X10 ⁵	3X10 ⁵
	T=2 months	<10 ²	1.5X10 ⁴	4X10 ⁴	8X10 ⁴	5X10 ⁴	5X10 ⁴	10 ⁵
	T=3 months	<10 ²	<10 ²	1.5X10 ⁴	6X10 ⁴	3.5X10 ⁴	3X10 ⁴	8X10 ⁴
Freeze thaw cycles	1 cycle	10 ⁶	1.25X10 ⁶	1.1X10 ⁶	1.2X10 ⁶	1.05X10 ⁶	1.1X10 ⁶	1.05X10 ⁶
	2 cycles	8.5X10 ⁵	9.5X10 ⁵	10 ⁶	9X10 ⁵	9.5X10 ⁵	1.1X10 ⁶	9.5X10 ⁵
	3 cycles	5X10 ⁵	5X10 ⁵	8.5X10 ⁵	9.5X10 ⁵	8.5X10 ⁵	10 ⁶	8X10 ⁵
	4 cycles	7X10 ⁴	10 ⁵	6.5X10 ⁵	6.5X10 ⁵	6.5X10 ⁵	6.5X10 ⁵	6.5X10 ⁵
	5 cycles	<10 ²	7.5X10 ⁴	5X10 ⁵	5X10 ⁵	5.5X10 ⁵	5.5X10 ⁵	5.5X10 ⁵

Table 2: Coxsackievirus A16 stability in presence and absence of stabilizers

TABLE-3 Enterovirus D68: Stability in presence and absence of stabilizers								
	Formulations	F1	F2	F3	F4	F5	F6	F7
Temp./Freeze thaw cycles	Time points	No stabilizer	2% serum	2% serum + 1M MgCl ₂	2% serum + 10% sucrose + 1M MgCl ₂ + 9.4 g/L M199 + 1% Glycine + 1.5mM Glutamate	10% serum + 10% sucrose + 1M MgCl ₂ + 9.4 g/L M199 + 1% Glycine + 1.5mM Glutamate	10% sucrose + 1M MgCl ₂ + 9.4 g/L M199 + 1% Glycine + 1.5mM Glutamate	20% sucrose + 1M MgCl ₂ + 9.4 g/L M199 + 1% Glycine + 1.5mM Glutamate
		Virus titer (TCID ₅₀ /ml)						
36±2°C	T=0	3.5X10 ⁶	3.5X10 ⁶	3.8X10 ⁶	3.8X10 ⁶	3.5X10 ⁶	3.8X10 ⁶	3.6X10 ⁶
	T=3 days	1.2X10 ⁶	1.6X10 ⁶	2.1X10 ⁶	2.8X10 ⁶	2.4X10 ⁶	2.1X10 ⁶	2.4X10 ⁶
	T=5 days	6.8X10 ⁴	2.16X10 ⁵	5.62X10 ⁵	5.21X10 ⁵	6.28X10 ⁵	6.12X10 ⁵	4.48X10 ⁵
	T=7 days	1.5X10 ⁴	2.5X10 ⁴	1.5X10 ⁵	3.56X10 ⁵	2.91X10 ⁵	2.1X10 ⁵	3X10 ⁵
	T=10 days	1.9X10 ³	3.9X10 ³	1.4X10 ⁴	1.28X10 ⁴	9.4X10 ³	1.8X10 ⁴	1.5X10 ⁴
25±2°C	T=1 week	2.1X10 ⁵	9.5X10 ⁴	7.8X10 ⁵	8.6X10 ⁵	4.8X10 ⁵	6.4X10 ⁵	4.2X10 ⁵
	T=2 weeks	6X10 ³	1.8X10 ⁴	3.1X10 ⁴	6.3X10 ⁴	7.8X10 ⁴	2.94X10 ⁴	2.89X10 ⁴
	T=1 month	<10 ²	<10 ²	1.2X10 ²	2.1X10 ²	1.8X10 ²	1.1X10 ²	<10 ²
2-8°C	T=2 weeks	1.6X10 ⁶	2.1X10 ⁶	2.8X10 ⁶	2.9X10 ⁶	2.31X10 ⁶	2.18X10 ⁶	2.09X10 ⁶
	T=1 month	8.6X10 ³	2.94X10 ⁴	1.58X10 ⁶	1.9X10 ⁶	1.4X10 ⁶	1.64X10 ⁶	1.78X10 ⁶
	T=2 months	1.2X10 ²	4.8X10 ²	6.2X10 ⁵	5.8X10 ⁵	3.84X10 ⁵	2.1X10 ⁵	1.8X10 ⁵
Freeze thaw cycles	1 cycle	2.31X10 ⁶	2.2X10 ⁶	2.61X10 ⁶	2.46X10 ⁶	2.5X10 ⁶	2.16X10 ⁶	2.28X10 ⁶
	2 cycles	1.18X10 ⁶	1.9X10 ⁶	2.06X10 ⁶	2.21X10 ⁶	1.98X10 ⁶	2.08X10 ⁶	1.78X10 ⁶
	3 cycles	4.24X10 ⁵	6.8X10 ⁵	1.78X10 ⁶	1.81X10 ⁶	1.64X10 ⁶	1.28X10 ⁶	1.14X10 ⁶
	4 cycles	4.8X10 ⁴	9.8X10 ⁴	1.1X10 ⁶	1.08X10 ⁶	10 ⁶	9.8X10 ⁵	1.18X10 ⁶
	5 cycles	6.8X10 ³	7.8X10 ⁴	3.08X10 ⁵	5.1X10 ⁵	2.9X10 ⁵	1.89X10 ⁵	1.09X10 ⁵

Table 3: Enterovirus D68 stability in presence and absence of stabilizers

Discussion of Virus Stability Result:

Table-1 stability data: In the case of EV71 virus, formulations F4 and F5 has shown better stability when the virus was stored at $36\pm 2^{\circ}\text{C}$, formulations F4-F7 worked better when the virus was stored at $25\pm 2^{\circ}\text{C}$ and formulations F3 to F7 has shown good stability for the virus when the virus was subjected to repeated freeze thaw cycles.

Table-2 stability data: In the case of CVA16 virus, the formulation F4 and F5 has worked better when CVA16 virus was stored at $36\pm 2^{\circ}\text{C}$, while the formulation F4 and F5 showed very good stability for CVA16 virus when stored at $25\pm 2^{\circ}\text{C}$. All the formulations from F3 to F7 has worked well as stabilizer for the virus when the virus was subjected to repeated freeze thaw cycles.

Table-3 stability data: In the case of EVD68 virus, the formulations F3, F4, F6 and F7 has worked well as stabilizer when EVD68 virus was stored at $36\pm 2^{\circ}\text{C}$, while the formulations F4 and F5 has been found to be better stabilizers for the virus stored at $25\pm 2^{\circ}\text{C}$. The formulations F3, F4, F5 has worked well as stabilizers for the EVD68 virus that has been subjected to multiple freeze thaw cycles.

EXAMPLE 3: Measurement of virus titer

Virus titer was measured by determining TCID₅₀ (Tissue culture infectious dose). Distinct cytopathic effect was noted and TCID₅₀ was calculated by standard protocols. Plaque counting method was also used to check the virus titer. Plaques will be ready within 4-5 days after infection. Plaque purification was performed to get homogenous virus population.

25

EXAMPLE 4: RNA isolation, cDNA synthesis and sequencing

Cell free supernatant was collected for concentrating upto 1/100th of the starting volume either by 100kD cut off membrane or by PEG precipitation. Concentrated sample is used for RNA isolation either by Guanidium isothiocyanate or by silica membrane based method. Isolated RNA was reverse transcribed to cDNA. The presence of viral RNA is confirmed by rt-PCR using virus specific or Pan-enterovirus primers (**Figure 1**). The primer sequences are provided below:

Pan-enterovirus primers (Thao et al., J. Virol. Methods 2010, 170:134-139)

(F): 5'-CAAGCACTTCTGTTTCCCCGG-3'

(R): 5'-ATTGTCACCATAAGCAGCCA-3'

Coxsackievirus A16 (CVA16) specific primers (Thao et al., J. Virol. Methods 2010, 170:134-139):

5 (F): 5' - AGGGTAATGGARTGTGGTGAYT-3'

(R): 5'- TGTGTGTTGAACCATCACTC-3'

For genome wide sequencing, RNA sequencing libraries were prepared with Illumina-compatible SureSelect Strand-Specific RNA Library Prep kit (Agilent, Santa Clara, CA, U.S.A.). The qPCR quantified libraries were pooled in equimolar amounts to create a final multiplexed library pool for sequencing on an Illumina sequencer for 75 bp paired-end chemistry.

The isolated virus was confirmed to be Coxsackievirus A16 by sequencing.

15 SEQ ID No. 1: Nucleotide sequence encoding polyprotein of CVA16/KR/Hyd.

SEQ ID No. 2: Amino acid sequence of polyprotein of CVA16/KR/Hyd.

SEQ ID No. 3: Nucleotide sequence encoding polyprotein of CVA16/VS/WB.

SEQ ID No. 4: Amino acid sequence of polyprotein of CVA16/VS/WB.

20 **EXAMPLE 5: Expression of Coxsackievirus A16**

The isolated and Vero cell adapted Coxsackievirus A16 antigens were checked for the expression of Coxsackievirus A16 VP1 and VP2 proteins by Western Blot using Coxsackievirus A16-VP1 and VP2 antibodies as shown in **Figure 2**. The whole virus antigen was separated by 12% SDS-PAGE gel followed by transfer to PVDF membrane. The blot was blocked with 1% BSA and subsequently incubated with VP1 and VP2 antibodies for 90 mins.

Subsequently, the blot was washed four times with phosphate buffered saline with Tween 20 (PBST) followed by incubation with Horseradish peroxidase (HRP) conjugated secondary antibody. The signal for expression was detected using chromogenic substrate-metal enhanced DAB (Thermo Scientific).

EXAMPLE 6: Virus purification

35 Cell free supernatant containing Coxsackievirus A16 (CVA16) was concentrated by

100kD cut off membrane or by PEG precipitation, followed by deactivation of host nucleic acids by nuclease and then proceeded for further purification using ion exchange chromatography with DEAE resins or by size exclusion chromatography using Sepharose CL-6B resin or by mixed mode resins or two steps chromatography or multi-steps
5 chromatography using more than one chromatography methods described. The concentrated and/or purified virus was used for inactivation. Alternatively, the clarified virus was inactivated by formalin or BPL and subsequently purified. The virus could be also purified by ultracentrifugation on a 10-50% sucrose gradient after centrifugation at 28000 rpm for 4-5 hrs using P28S swinging bucket rotors in Hitachi HIMAC
10 ultracentrifuge. Upto 97% of pure virus obtained from the purification methods described above.

EXAMPLE 7: Inactivation of CVA16:

15 Inactivation of Virus suspension was carried by adding 1:2000 or 1:4000 formalin and incubating the suspension for upto two weeks at 37°C. Inactivated virus was checked for the presence of live virus after repeated Vero cell culture for upto 3 weeks.

Inactivation of Virus suspension was also carried with 1:2000 or 1:3000 or 1:3500 Beta-propiolactone (BPL) at 4°C for upto 120 hrs and then incubation for 2-3 hrs at 37°C for
20 hydrolysis of BPL.

Inactivation of virus suspension was also done by using oxidative agent like H₂O₂ (3%) for 2-12 hrs, more precisely for 4 hrs or in another embodiment for 8 hrs. H₂O₂ activity was stopped by using Catalase.

25 No live virus was detected after repeated Vero cell culture for upto 3 weeks confirming the complete inactivation.

EXAMPLE 8: Pathogenicity in mice:

30 Different dilutions of the CVA16 virus described in the present invention were used to inoculate in suckling mice (<48 hrs of age) intraperitoneally.

The mice infected with CVA16 displayed either limb weakness or limb paralysis or limb weakness followed by paralysis. The virus strain was found to be highly lethal in suckling
35 or neonatal mice as 100% lethality of the mice was observed (Figure 3).

TABLE-4					
Days post infection	% of survival of mice infected with 50000 PFU of CVA16 virus	% of survival of mice infected with 5000 PFU of CVA16 virus	% of survival of mice infected with 500 PFU of CVA16 virus	% of survival of mice infected with 50 PFU of CVA16 virus	% of survival of mice without CVA16 virus infection
1	100	100	100	100	100
2	100	100	100	100	100
3	100	100	100	100	100
4	100	100	100	100	100
5	0	91.66	81.81	100	100
6	0	16.66	72.72	100	100
7	0	0	72.72	100	100
8	0	0	54.54	100	100
9	0	0	27.27	100	100
10	0	0	0	100	100
11	0	0	0	90	100
12	0	0	0	90	100
13	0	0	0	70	100
14	0	0	0	70	100

Table 4: Percent of survival of neonatal mice infected or uninfected with CVA16 virus (Tabular Data for **Figure 3A**)

TABLE-5					
Days post infection	Average clinical score of mice infected with 50000 PFU of CVA16 virus	Average clinical score of mice infected with 5000 PFU of CVA16 virus	Average clinical score of mice infected with 500 PFU of CVA16 virus	Average clinical score of mice infected with 50 PFU of CVA16 virus	Average clinical score of mice without CVA16 virus infection
1	0	0	0	0	0
2	1	0.54	0	0	0
3	2.1	1.45	0.63	0	0
4	3.7	2.27	1.81	0	0
5	5	3.72	2.09	0	0
6		4.81	2.45	0	0
7		5	2.9	0	0
8			3.63	0	0
9			4.54	0.7	0
10			5	0.9	0
11				1.1	0
12				1.3	0
13				1.5	0
14				1.5	0

5

Table 5: Average clinical score of neonatal mice infected or uninfected with CVA16 virus (Tabular Data for **Figure 3B**)

EXAMPLE 9: Vaccine formulations:

Formulation of Formalin and BPL inactivated whole virus antigen was prepared by adding sugars or polyols or amino acids. In alternative formulations, the inactivated whole virus
5 antigens have been formulated with different combinations of sugars, polyols and amino acids. The inactivated vaccine formulation may comprise 4-8 µg/dose of each of inactivated virus antigen.

In one set of formulations, inactivated Coxsackievirus A16 (CVA16) virus antigens along
10 with sugars, amino acids and detergent are present. The inactivated antigen was further adsorbed in alum adjuvant.

In another formulation, formalin or BPL inactivated viral antigens having phosphate buffer, 10-20% sucrose, 0.5-2mM Glutamate are adsorbed in alum adjuvant. The pH of the
15 inactivated enterovirus vaccine formulation ranges from 7.0 to 7.5.

In yet another formulation, the inactivated antigen is not formulated with adjuvant. The vaccine formulation also includes Thiomersal or 2-phenoxyethanol as preservatives.

20 In alternative formulations, the vaccine formulation is devoid of any preservatives. Alternatively, the inactivated antigens were formulated with toll like receptors or squalene based adjuvants.

In one more embodiment, the inactivated coxsackievirus A16 antigen was stabilized in
25 formulations comprising of stabilizer consisting of the group selected from sugars or polyols, or amino acids or different combinations of sugars, polyols, and amino acids. The sugar includes sucrose, trehalose, maltose, and the polyols include sorbitol, mannitol, glycerol, wherein the sugar and/or polyols is/are present in a concentration ranging from 5-50%; the amino acid includes salts of glutamic acid, Glycine, Arginine, lysine or
30 combination thereof; and the preservative includes Thiomersal, 2-phenoxyethanol or combination thereof.

In one embodiment, the stabilizer that may be optionally used for vaccine formulation comprises the group selected from sugars or polyols, or amino acids or different
35 combinations of sugars, polyols, and amino acids. the sugar includes sucrose, trehalose, maltose, and the polyols include sorbitol, mannitol, glycerol, wherein the sugar and/or

polyols is/are present in a concentration ranging from 5-50%; the amino acid includes salts of glutamic acid, Glycine, Arginine, Lysine, or combination thereof.

COMBINED VACCINE FORMULATION

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EXAMPLE-10:

For combination vaccine formulations, inactivated Cocksackievirus A16 antigens along with one or more other inactivated Enterovirus antigens are formulated with different combination of sugars, amino acids, and detergents. The combination vaccines optionally include adjuvants. The combination vaccines for enterovirus includes two or three enteroviruses including but not limited to Cocksackievirus A16, Enterovirus D68, Enterovirus 71. The mentioned combination of enterovirus vaccine is mixed in the ratio of 1:1 for bivalent vaccine or 1:1:1 for trivalent vaccine.

One formulation consists of 6 µg of BPL inactivated Cocksackievirus A16 antigen, 6 µg of inactivated Enterovirus 71 and 6 µg of inactivated Enterovirus D68 with or without adjuvants in 10-30 mM phosphate buffer. The adjuvants are selected from the alum (0.5 µg), squalene (1:1 v/v with antigens) or TLR agonists (2 to 10 µg). The combination antigens were mixed together in equal amount (1:1 w/w for bivalent vaccine or 1:1:1 w/w for trivalent vaccine) and then adsorbed in alum adjuvant. Alternatively, equal volume of combined antigens and squalene adjuvants were mixed together. Formulations F8 to F25 as provided in Tables 6-7 are prepared by this method wherein the compositions ingredients as specified in examples are mixed at specified amounts and combined vaccine formulations are prepared.

25

In another formulation, combined antigens were mixed with 2 to 10 µg of TLR agonists. No significant interference of virus specific antigenicity by one viral antigen to the other viral antigen/s was observed. The combination vaccine was formulated with or without 2-phenoxyethanol (2.5 mg) in Phosphate buffer or normal saline.

30

TABLE-6			
	Formulation 8	Formulation 9	Formulation 10
EVD68 antigen	4-8 µg/dose of formalin inactivated antigen	4-6 µg/dose of formalin inactivated antigen	4-6 µg/dose of formalin inactivated antigen
EV71 antigen	4-8 µg/dose of formalin inactivated antigen	4-6 µg/dose of formalin inactivated antigen	4-6 µg/dose of formalin inactivated antigen

CVA16 antigen	4-8 µg/dose formalin inactivated antigen	4-6 µg/dose formalin inactivated antigen	4-6 µg/dose formalin inactivated antigen
Phosphate buffer	10-30mM	10-30mM	10-30mM
Alum	≤0.9mg/dose	≤0.9mg/dose	≤0.9mg/dose
NaCl	None	None	137-154mM
Sucrose	None	10-20%	10-20%
Glycine	0.5-2%	0.5-2%	0.5-2%
Polysorbate 80	0.01-0.1%	0.01-0.1%	None
	Formulation 11	Formulation 12	Formulation 13
EVD68 antigen	4-8 µg/dose of formalin inactivated antigen	4-8 µg/dose of formalin inactivated antigen	4-8 µg/dose of formalin inactivated antigen
EV71 antigen	4-8 µg/dose of formalin inactivated antigen	4-8 µg/dose of formalin inactivated antigen	4-8 µg/dose of formalin inactivated antigen
CVA16 antigen	4-8 µg/dose of BPL inactivated antigen	4-8 µg/dose of BPL inactivated antigen	4-8 µg/dose of BPL inactivated antigen
Phosphate buffer	10-30mM	10-30mM	10-30mM
Alum	≤0.9mg/dose	≤0.9mg/dose	≤0.9mg/dose
NaCl	None	None	137-154mM
Sucrose	None	10-20%	10-20%
Glycine	0.5-2%	0.5-2%	0.5-2%
Polysorbate 80	0.01-0.1%	0.01-0.1%	None

Table 6: Trivalent vaccines formulations 8-13 (Inactivated)

TABLE-7			
	Formulation 14	Formulation 15	Formulation 16
EVD68 antigen	4-8 µg/dose of formalin inactivated antigen	4-6 µg/dose of formalin inactivated antigen	4-6 µg/dose of formalin inactivated antigen
CVA16 antigen	4-8 µg/dose of formalin inactivated antigen	4-6 µg/dose of formalin inactivated antigen	4-6 µg/dose of formalin inactivated antigen
Phosphate buffer	10-30mM	10-30mM	10-30mM
Alum	≤0.9mg/dose	≤0.9mg/dose	≤0.9mg/dose
NaCl	None	None	137-154mM
Sucrose	None	10-20%	10-20%
Glycine	0.5-2%	0.5-2%	0.5-2%
Polysorbate 80	0.01-0.1%	0.01-0.1%	None
	Formulation 17	Formulation 18	Formulation 19
EVD68 antigen	4-8 µg/dose of formalin inactivated antigen	4-6 µg/dose of formalin inactivated antigen	4-6 µg/dose of formalin inactivated antigen
CVA16 antigen	4-8 µg/dose of BPL inactivated antigen	4-6 µg/dose of formalin inactivated antigen	4-6 µg/dose of formalin inactivated antigen

Phosphate buffer	10-30mM	10-30mM	10-30mM
Alum	≤0.9mg/dose	≤0.9mg/dose	≤0.9mg/dose
NaCl	None	None	137-154mM
Sucrose	None	10-20%	10-20%
Glycine	0.5-2%	0.5-2%	0.5-2%
Polysorbate 80	0.01-0.1%	0.01-0.1%	None
	Formulation 20	Formulation 21	Formulation 22
EV71 antigen	4-8 µg/dose of formalin inactivated antigen	4-6 µg/dose of formalin inactivated antigen	4-6 µg/dose of formalin inactivated antigen
CVA16 antigen	4-8 µg/dose of formalin inactivated antigen	4-6 µg/dose of formalin inactivated antigen	4-6 µg/dose of formalin inactivated antigen
Phosphate buffer	10-30mM	10-30mM	10-30mM
Alum	≤0.9mg/dose	≤0.9mg/dose	≤0.9mg/dose
NaCl	None	None	137-154mM
Sucrose	None	10-20%	10-20%
Glycine	0.5-2%	0.5-2%	0.5-2%
Polysorbate 80	0.01-0.1%	0.01-0.1%	None
	Formulation 23	Formulation 24	Formulation 25
EV71 antigen	4-8 µg/dose of formalin inactivated antigen	4-6 µg/dose of formalin inactivated antigen	4-6 µg/dose of formalin inactivated antigen
CVA16 antigen	4-8 µg/dose of BPL inactivated antigen	4-6 µg/dose of BPL inactivated antigen	4-6 µg/dose of BPL inactivated antigen
Phosphate buffer	10-30mM	10-30mM	10-30mM
Alum	≤0.9mg/dose	≤0.9mg/dose	≤0.9mg/dose
NaCl	None	None	137-154mM
Sucrose	None	10-20%	10-20%
Glycine	0.5-2%	0.5-2%	0.5-2%
Polysorbate 80	0.01-0.1%	0.01-0.1%	None

Table 7: Bivalent vaccines formulations 14-25 (Inactivated)**EXAMPLE 11: Vaccine stability:**

5

Stability of CVA16 inactivated antigen was tested with or without stabilizer at 2-8°C.

Accelerated stability was also tested at $36\pm 2^{\circ}\text{C}$ for upto 15 days. In presence of 10% sucrose, the antigen has maintained >60% stability after 6 months storage at $2-8^{\circ}\text{C}$. When stored at $36\pm 2^{\circ}\text{C}$, more than 40% of the antigen was found to be stable after 7 days and more than 20% of the antigen was stable after 15 days. Presence of 1-2 mM Glutamate additionally to 10% sucrose, has maintained more than 60% stability of the inactivated CVA16 antigen when stored at $36\pm 2^{\circ}\text{C}$ for 7 days and more than 40% stability when stored at $36\pm 2^{\circ}\text{C}$ for 15 days.

The table provided below displayed the stability of the inactivated CVA16 antigen with or without stabilizers. The pH was maintained between 7 to 8 more specifically at 7.4 or 7.5.

TABLE-8						
Stability study of inactivated Coxsackievirus A16 antigen						
		Stability at $2-8^{\circ}\text{C}$	Stability at $36\pm 2^{\circ}\text{C}$			
1 month	Sample	3 months	6 months	3 days	7 days	15 days
CVA16 inactivated antigen; pH:7.0-7.5	70%	60%	50%	41%	10%	1%
CVA16 inactivated antigen + 10% sucrose; pH:7.0-7.5	81%	71%	62%	80%	42%	22%
CVA16 inactivated antigen + 10% sucrose + 1-2mM Glutamate; pH:7.0-7.5	90%	60%	50%	91%	62%	41%
CVA16 inactivated antigen + 20% sucrose; pH:7.0-7.5	95%	70%	60%	85%	60%	35%
CVA16 inactivated antigen + 20% sucrose + 1-2mM Glutamate; pH:7.0-7.5	95%	50%	40%	85%	65%	50%

Table 8: Stability study of inactivated Coxsackievirus A16 antigen

15 EXAMPLE 12: Immunogenicity and efficacy

Balb/C mice was immunized with formalin or BPL inactivated antigens with or without alum adjuvant. Mice were bleed at 0 day to collect pre-immunized sera, and after immunization to collect post immunization sera after each immunization.

Coxsackievirus A16 specific immune response was checked by ELISA. Briefly, ELISA plate was coated with inactivated CVA16 antigen followed by blocking with 1% BSA. Subsequently, the ELISA plate was incubated with immunized mice serum for 1 hr followed by incubation with diluted anti-mouse IgG HRP conjugated secondary antibody (Thermo Scientific). Coxsackievirus A16 virus specific IgG antibody titer was detected in sera collected from mice immunized with formalin or BPL inactivated coxsackievirus A16 virus antigen. (**Figure-4**)

TABLE-9			
Antigen formulation	Virus specific IgG antibody titer at different time points post 1st vaccination		
	Week 3	Week 6	Week 8
Formalin inactivated CVA16 antigen	400	10000	50000
Formalin inactivated CVA16 antigen formulated with alum adjuvant	400	10000	100000

Table 9: CVA16 virus specific IgG antibody titer at different time points post vaccination with Formalin inactivated CVA16 antigen formulated with or without alum adjuvant (Tabular data for **Figure 4A**).

TABLE-10			
Antigen formulation	Virus specific IgG antibody titer at different time points post 1st vaccination		
	Week 3	Week 6	Week 8
BPL inactivated CVA16 antigen	1000	25000	50000
BPL inactivated CVA16 antigen formulated with alum adjuvant	1000	100000	100000

Table 10: CVA16 virus specific IgG antibody titer at different time points post vaccination with BPL inactivated CVA16 antigen formulated with or without alum adjuvant (Tabular data for **Figure 4B**).

IgG isotype specific ELISA was performed to check the ratio of different IgG isotypes in the immunized mice sera using anti-mouse IgG1 HRP conjugated antibody (Abcam) or IgG2a HRP conjugated antibody (Abcam) as secondary antibody. Subsequently, after incubation with HRP-conjugated secondary antibody, the ELISA plate was washed with

PBS followed by the addition of TMB (3,3',5,5'-Tetramethylbenzidine) substrate for ELISA(Amresco) for color development and finally the reaction was stopped with strong acid.

- 5 For detecting the neutralization titer of the serum generated from mice immunized with Formalin or BPL inactivated antigen, serially diluted immunized serum was incubated with CVA16 virus for 2 hrs in 1:1 ratio. Later, the serum and virus mixture was added to Vero cells and incubated for 2 hrs followed by addition of 0.8% carboxymethylcellulose overlay. After 5 days of incubation, the cells were fixed with 10% formalin for 1 hr and
- 10 stained with 0.8% crystal violet solution. PRNT50 was calculated as the highest dilution of the sera displaying 50% reduction of number of plaques. Sera from mice immunized with both formalin or BPL inactivated Cocksackievirus A16 antigen induces significantly high neutralization antibody titer against Cocksackievirus A16 viruses (≥ 32).
- 15 The ratio of IgG1/IgG2a in the sera of the Balb/C mice immunized with Formalin and BPL inactivated CVA16 vaccine formulated with or without alum adjuvant was measured and the result is shown in graph of **Figure-5**.

TABLE-11	
Antigen formulation	IgG1: IgG2a
Formalin Inactivated CVA16 antigen	1.06
Formalin inactivated CVA16 antigen formulated with alum adjuvant	1.12
BPL inactivated CVA16 antigen	1.02
BPL inactivated CVA16 antigen formulated with alum adjuvant	1.02

- 20 **Table 11:** Ratio of IgG1/ IgG2a in the sera of Balb/C mice immunized with Formalin and BPL inactivated CVA16 vaccine formulated with or without alum adjuvant (Tabular data for **Figure 5**).

- 25 In a separate experiment, inactivated CVA16 antigen formulated with or without adjuvants-alum, MPLA or AddaVax (squalene based, MF59 equivalent) induces high virus specific total IgG titer (**Figure 6**) and significant neutralization titer (≥ 32).

30

TABLE-12	
Antigen formulation	Virus specific IgG titer
BPL inactivated CVA16 antigen +Alum adjuvant	100000
BPL inactivated CVA16 antigen +MPLA adjuvant	50000
BPL inactivated CVA16 antigen +AddaVax adjuvant	100000
BPL inactivated CVA16 antigen +AddaVax adjuvant+MPLA adjuvant	100000
BPL inactivated CVA16 antigen	100000

Table 12: Virus specific IgG titer in the sera of the Balb/C mice immunized with BPL inactivated CVA16 vaccine formulated with or without adjuvants (Tabular data for **Figure 6**)

5

For detecting the in-vivo efficacy of the vaccine, female Balb/c mice immunized with inactivated CVA16 antigen with or without adjuvants were mated with male mice. The pups which were born from the immunized female mice were challenged with lethal dose of CVA16 virus. Mice were observed for 2 to 3 weeks.

10

Neonatal mice born from the antigen immunized mice with or without adjuvants survived 100% while all the neonatal mice which were born from the PBS or mock immunized female mice died within 1 week of the CVA16 virus challenge.

15 **EXAMPLE 13: Antibody generation**

In house polyclonal antibody was generated against Vero adapted and purified Coxsackievirus A16 (CVA16) inactivated antigen using Coxsackievirus strain mentioned in the present invention in mice and rabbit by immunizing three to four times. The antigen for immunization was formulated with complete Freund's adjuvant for first immunization and incomplete freund's adjuvant for subsequent immunization. Alternatively, the antigen was formulated with alum adjuvant. Good virus specific IgG titer (>10,000) was achieved.

25

We claim:

1. A thermostable formulation for enteroviruses wherein the formulation composition comprises:
 - a) one or more Enterovirus antigen(s);
 - b) carbohydrate in a concentration range between 5 to 50%;
 - c) serum protein in a concentration upto 10%;
 - d) cation in a concentration upto 1M;
 - e) amino acid; and
 - f) M199 media in a concentration between 0 to 9.4 g/L with pH between 7.0 to 7.5.
2. The thermostable formulation as claimed in claim 1, wherein the formulation is a liquid formulation, wherein one or more Enterovirus antigen is/are live virus antigen(s) selected from Enterovirus 71 (EV71), Coxsackievirus A16 (CVA16) and Enterovirus D68 (EVD68) present in a concentration range between 10^6 to 10^7 PFU/ml.
3. The thermostable formulation as claimed in claims 1 to 2, wherein the formulation is
 - a monovalent formulation comprising a live Enterovirus antigen selected from Enterovirus 71 (EV71), Coxsackievirus A16 (CVA16) and Enterovirus D68 (EVD68); or
 - a multivalent formulation comprising combination of two or more live Enterovirus antigens selected from Enterovirus 71 (EV71), Coxsackievirus A16 (CVA16) and Enterovirus D68 (EVD68).
4. The thermostable formulation as claimed in claims 1 to 3, wherein said Coxsackievirus A16 (CVA16) strain is characterized by SEQ. ID. Nos. 1-4.
5. The thermostable formulation as claimed in claims 1 to 4, wherein (b) carbohydrate(s) comprises sugar which includes sucrose, trehalose, maltose, or combination thereof present in a concentration ranging from 5% to 50%, preferably 5% to 20%, more preferably at a concentration ranging between 10% to 20%.
6. The thermostable formulation as claimed in claims 1 to 4, wherein the (c) serum protein(s) comprises fetal calf or bovine serum, bovine serum albumin, human serum albumin or any combination thereof present at a concentration upto 10%, preferably

between 2% to 10%.

7. The thermostable formulation as claimed in claims 1 to 4, wherein the (d) cation(s) comprises Mg^{+2} , Ca^{+2} , or salt thereof or combination thereof at a concentration upto 1M.
8. The thermostable formulation as claimed in claims 1 to 4, wherein the (e) amino acid(s) can be one or multiple selected from the group consisting of Glutamic acid, Glycine, Arginine and Lysine or combination thereof.
9. The thermostable formulation as claimed in claim 8, wherein the amino acid comprises two amino acids specifically comprising Glycine (upto 2%, preferably 0.5-2%) and Glutamate (upto 2mM, preferably between 1-2 mM).
10. The thermostable formulation as claimed in claims 1 to 4, wherein the (f) media M199 with pH between 7.0 to 7.5 is present in an amount of 9.4g/L.
11. The thermostable formulation as claimed in claims 1-10, wherein the formulation is a liquid formulation which comprises:
 - a) one or more Live Enteroviruses antigen(s) in a concentration range between 10^6 to 10^7 PFU/ml;
 - b) sucrose (carbohydrate) in a concentration range between 5 to 20%, preferably 10 to 20%;
 - c) fetal calf (Serum protein) in a concentration upto 10%, preferably 2 to 10%;
 - d) Mg^{+2} or salt thereof (cation) in a concentration upto 1M;
 - e) amino acids comprising Glycine (upto 2%, preferably 1%) and Glutamate (upto 2mM, preferably 1.5mM); and
 - f) M199 media in a concentration of 9.4 g/L with phosphate buffer pH 7 to 7.5.
12. A method for isolation of low abundance enteroviruses specifically coxsackievirus A16 (CVA16) by the following steps:
 - a) Passage of swab having suspected viral sample through 0.22 micron filter and addition to the cells in 12 or 24 well tissue culture well as neat or diluted in cell culture media (DMEM +1% serum);
 - b) centrifugation of cells to remove the cells after appearance of significant cytopathic effect (CPE);

- c) in absence of visible CPE, supernatant was collected and added to the fresh Vero cells and the cycle was repeated upto four times;
 - d) viruses were harvested by freeze thawing thrice for complete cell lysis; and
 - e) pre-clarification filtration steps or centrifugation to remove host cells.
13. The method for isolation as claimed in claim 12, wherein the CVA16 isolated and obtained comprises nucleotide sequence of SEQ ID No. 1 and SEQ ID No. 3; and amino acid sequence of SEQ ID No. 2 and SEQ ID No. 4.
14. A Coxsackievirus A16 (CVA16) adapted to propagate in Vero cells encoded by a cDNA molecule having the nucleotide sequence of SEQ ID No. 1 and SEQ ID No. 3.
15. An amino acid sequence of Coxsackievirus A16 (CVA16) polypeptide disclosed in SEQ ID No. 2 and SEQ ID No. 4.
16. A lethal animal model of Coxsackievirus A16 (CVA16) comprising a rodent infected with CVA16.
17. An aqueous transport media capable of maintaining the viability of viruses with the composition as claimed in claim 1 with pH ranging between 7.0-7.5.
18. A method of using Coxsackievirus A16 (CVA16) with sequence having SEQ. ID No. 1-4 and stored in formulations mentioned in claim 1 to use in and make inactivated virus vaccine which consists of the following steps:
- a) propagation in cells and harvesting;
 - b) concentrated by 100kD cut off membrane or by PEG precipitation;
 - c) removal of host nucleic acids by nuclease;
 - d) purification using ion exchange chromatography with DEAE resins or by size exclusion chromatography using Sepharose CL-6B resin or by mixed mode resins or two steps chromatography or multi-steps chromatography using more than one chromatography methods described;
 - e) inactivation of virus using 1:2000 or 1:4000 formalin and incubating the suspension for upto two weeks at 37°C or using with 1:2000 or 1:3000 or 1:3500 Beta-propiolactone (BPL) at 4°C for upto 120 hrs and then incubation for 2-3 hrs at 37°C for hydrolysis of BPL.

19. A stable formulation for inactivated Coxsackievirus A16 (CVA16) vaccine which comprises the inactivated CVA16 antigen in a physiologically acceptable vehicle; 5 to 50%, preferably 5 to 20% sucrose or trehalose; and upto 2mM Glutamate or upto 2% Glycine with pH 7 to 8.
20. The stable formulation for inactivated CVA16 as claimed in claim 19, wherein the formulation comprises formalin or BPL inactivated CVA16 having phosphate buffer as vehicle, 10-20% sucrose, and 0.5-2mM Glutamate or 0.5 to 2% Glycine.
21. An immunogenic composition comprising stable formulation of inactivated Coxsackievirus A16 virus antigen of SEQ. ID No. 1-4 and *optionally* one or more pharmaceutically acceptable excipients selected from buffer as vehicle, adjuvants, stabilizers, or preservatives.
22. An immunogenic composition comprising of combination of different enterovirus antigens including but not limited to Enterovirus 71, Coxsackievirus A16 and Enterovirus D68 in equal amounts in a physiologically acceptable buffer as vehicle and optionally one or more pharmaceutically acceptable excipients selected from adjuvants, stabilizers, preservatives, and other excipients selected from the group consisting of salt such as NaCl, and Polysorbate 80.
23. The immunogenic composition as claimed in claim 22, wherein the said composition is:
- a bivalent vaccine composition comprising combination of two different enterovirus antigens selected from Enterovirus 71, Coxsackievirus A16 and Enterovirus D68; or
 - a trivalent vaccine composition comprising combination of three different enterovirus antigens Enterovirus 71, Coxsackievirus A16 and Enterovirus D68.
24. The immunogenic composition as claimed in claims 21-22, wherein
- the vehicle comprises phosphate buffer 10-30mM;
 - the adjuvant that may be optionally used for vaccine formulation includes but is not limited to alum adjuvant which may be aluminium phosphate or aluminium hydroxide, calcium phosphate, chitosan and complex carbohydrates, biodegradable polymers like poly PLGA, squalene based adjuvant like MF59, any oil in water emulsions, any water in oil emulsions, lipid based delivery

system like liposomes, RIBI adjuvant systems, saponins including but not limited to QS-21, ISCOMs, ISCOMATRIX etc, muramyl dipeptides, any TLR ligands based adjuvants, any cytokines or other immunomodulants, vitamins, any bacterial cell components based adjuvants like Monophosphoryl lipid A and derivatives, CpG and non-CpG containing oligonucleotides etc;

- the stabilizer that may be optionally used for vaccine formulation comprises the group selected from sugars or polyols, or amino acids or different combinations of sugars, polyols, and amino acids;
- the preservative that may be optionally used for vaccine formulation comprises includes Thiomersal, 2-phenoxyethanol or combination thereof.

25. The immunogenic composition as claimed in claim 24, wherein

- the sugar includes sucrose, trehalose, maltose, and the polyol includes sorbitol, mannitol, glycerol, wherein the sugar and/or polyols is/are present in a concentration ranging from 5-50%, preferably 10-20%;
- the amino acid includes salts of glutamic acid, Glycine, Arginine, Lysine, or combination thereof, preferably Glutamate upto 2mM and/or Glycine upto 2%.

26. The immunogenic composition as claimed in claim 22 or claim 23, wherein the said composition is a bivalent or trivalent vaccine composition comprising:

- combination of different enterovirus antigens selected from Enterovirus 71, Coxsackievirus A16 and Enterovirus D68;
- Phosphate buffer in a concentration of 10-30mM;
- Alum in a concentration of ≤ 0.9 mg/dose;
- Glycine in a concentration of 0.5-2%; and
- Optionally, Polysorbate 80 in a concentration of 0.01-0.1%.

27. The immunogenic composition as claimed in claim 22 or claim 23, wherein the said composition is a bivalent or trivalent vaccine composition comprising:

- combination of different enterovirus antigens selected from Enterovirus 71, Coxsackievirus A16 and Enterovirus D68;
- Phosphate buffer in a concentration of 10-30mM;
- Alum in a concentration of ≤ 0.9 mg/dose;

- NaCl in a concentration of 137-154mM;
- Sucrose in a concentration of 10-20%;
- Glycine in a concentration of 0.5-2%; and
- Optionally, Polysorbate 80 in a concentration of 0.01-0.1%.

28. The immunogenic composition as claimed in claims 19-22, wherein the composition is stable for at least 6 months at 2-8°C and for at least 7 days at 37°C.

29. Use of Coxsackievirus A16 strain (CVA16 virus strain) having nucleotide sequences of SEQ ID No. 1 and SEQ ID No. 3; and/or its amino acid sequences of SEQ ID No. 2 and SEQ ID No. 4 as vaccine antigen; in the preparation of vaccine, vaccine and/or immunogenic composition and formulation for prophylaxis and/or treatment of infectious disease caused by Enteroviruses; and/or for raising antibodies that can be utilized for detection and diagnosis of the virus or respective individual proteins.