VACCINE COMPOSITION COMPRISING AN INACTIVATED CHIKUNGUNYA VIRUS STRAIN

FIELD OF THE INVENTION

The invention relates to stable immunogenic compositions for prophylaxis and treatment against any infections caused by Chikungunya Virus. The present invention particularly relates to compositions of Chikungunya virus (henceforth termed as CHIKV) strains and use of the subunit antigens of the virus thereof, for prophylaxis, therapeutic treatment and diagnosis of Chikungunya infections in humans. More particularly, the invention relates to stable immunogenic vaccine compositions for prophylaxis and treatment against any genotypes or antigenic variants or mutants of Chikungunya virus conferring an antibody titer sufficient for the seroprotection for any genotypic variant or mutant for the Chikungunya virus. The invention also relates to vaccine compositions for immunization against Chikungunya virus in combination with other bacterial and viral infections selected from the following list that include but is not limited to vaccines for Japanese encephalitis virus, dengue vaccines, West Nile virus vaccine and Chandipura virus vaccine and rabies vaccines. Combinations with other viral vaccines are also within the scope of the invention.

BACKGROUND OF THE INVENTION

Chikungunya virus (CHIKV) is an alphavirus of the family *Togaviridae*. It is a positive strand RNA virus that causes a generally non-fatal infection characterized by high fever and sudden onset of polyarthralgia. Hemorrhagic and neurological manifestations including seizures, lymphadenopathy, fulminant hepatitis and conjunctivitis not hitherto associated with CHIKV infections were reported since the re-surgent infection in 2005 (Sourisseau et al., 2007; Kannan et al., 2007). Phylogenetic analyses based on the partial E1 structural glycoprotein sequences have identified three CHIKV lineages, the West African, Asian and the East, Central and South African (ECSA) (Powers et al., 2000). Asian lineage circulated in India and Southeast Asia until it was replaced by the ECSA genotype, which emerged during the 2005–2006 outbreak in the Indian Ocean islands (Yergolkar et al., 2006). Sub-lineages of ECSA strains that had established locally were spread by travellers from endemic areas to Africa, Asia and Europe and caused local outbreaks (Powers and Logue, 2007).

Nearly 1.39 million suspected cases of Chikungunya virus infection occurred in India in 2006. (National Vector Borne Disease Control Programme (NVBDCP), 2007) which was caused by the ECSA strain carrying the E1-226A (Arankalle et al., 2007). The E1-A226V adaptive mutation that increases transmissibility by *Aedes albopictus* is responsible for the wide geographical spread of the virus since then (de Lamballerie et al., 2008). Host immune pressure and resultant site specific mutations in the human leukocyte antigen (HLA) class-1 restricting elements of CHIKV genome are implicated in the explosive Chikungunya virus outbreaks since 2005 (Tong et al., 2010). Prior art known in the field do not include any vaccine candidate derived from the ECSA strain. Bharat Biotech International Limited has earlier developed (disclosed in WO 2008/026225) the 2006 ECSA strain with E1-226A and its use in the development of potential vaccines against Chikungunya virus infections.

Chikungunya virus strains of the urban (epidemic) transmission cycles show a higher evolutionary rate than that of the enzootic (sylvatic) cycle, and the difference in the evolutionary dynamics between the two transmission cycles are influenced by several factors that determine virus-host interactions such as vector diversity and abundance, vector larval habitats and herd immunity in the population (Volk et al., 2010). Arboviruses like Chikungunya interacts with both the arthropod and the vertebrate hosts, and the selection pressure on the envelope glycoproteins are driven by preferences for vector adaptation and by vertebrate host immune defense mechanisms. Viral evolution tends to select for mutations in the antigenic determinants involved in neutralization as well as those residues involved in vector/host adaptation.

The vaccines under development such as that disclosed in WO 2008030220 and in Akahata et al. 2010 make use of the West African genotype and the E1-A226V isolates. Another CHIKV vaccine development is a DNA vaccine (Mallilankaraman et al., 2011) which is different in scope from that disclosed in this invention. An earlier prototype vaccine which is a live attenuated vaccine used the Asian genotype of the virus (Edelman et al., 2001). DNA vaccines have not been successful in human prophylactic vaccination so far, and live attenuated CHIKV vaccine caused side effects in human subjects (Edelman et al., 2001) who received the vaccine. The CHIKV strain used in the earlier vaccine development (WO 2008/026225) was the 2006 ECSA strain with E1-226A. The strains isolated in 2009-2010

from India as disclosed in this invention belong to a distinct sub-lineage within the ECSA lineage and carry novel mutations in the E2 and E1 envelope glycoproteins. One of the mutations in the E1 glycoprotein in all the isolates reported in the study maps to a region that determines host vector specificity and is under significant positive selection for enhanced adaptation to *Adis. aegypti*, which is the most abundant mosquito vector in the region and indeed in the tropical countries where prevalence of Chikungunya virus infection is now endemic. Other novel mutations hitherto unreported are also disclosed. Thus it is desirable to make a vaccine composition which would confer immunity to the newly developed and distinct sublineages of the ECSA strain of the Chikungunya virus which would also confer immune protection to the other mutated strains of the ECSA strain propagated by the vector *Aedis aegypti.* Inventors in this application after prolonged research disclose such an effective vaccine in this application including other additional advantages over the earlier vaccine (WO 2008/026225) such as new methods of inactivation of the virus and improved formulations with novel adjuvants that enhance the immunogenicity of the inactivated viral vaccine and the recombinant subunit vaccines and virosomes which are also included herein this invention.

OBJECT OF THE INVENTION

One object of the present invention is to provide a stable vaccine composition that is capable to prevent as well as provide treatment from infections caused by Chikungunya virus. The said vaccine composition is applicable to any genotypic variants of the Chikungunya virus for prophylaxis and treatment thereof.

Another object of the invention is to provide for a stable vaccine composition that is capable to prevent as well as provide treatment from infections caused by Chikungunya virus propagated by any suitable vector which includes prevention and treatment of Chikungunya infections propagated by the vectors *Aedis albopictus* and *Aedis aegypit* which happens to be the most commonly adaptable vectors of the Chikungunya virus.

Yet another object of the invention is to provide for a stable vaccine composition which is effective against any genotypic variants of the Chikungunya virus particularly of the ECSA strain and its particular distinct and unique sublineages as applicable thereof.

One more object of the invention is to provide for a stable vaccine composition wherein the antigenic component of the vaccine includes the whole inactivated virion or the subunit antigens of the recombinant CHIKV viral strains that can be expressed as Virus Like particles (henceforth termed as VLPs) in combination of suitable pharmaceutically acceptable carriers, stabilizers, and adjuvants.

Yet another object of the invention is to provide a method for preparation of a stable vaccine composition that is capable to elicit an immune response sufficient to prevent as well as provide treatment from infections caused by any genotypic mutants or variants of Chikungunya virus including inactivation of the CHIKV virus and mixing with adjuvants in appropriate amounts.

Another object of the invention is to provide antibodies so generated against the Chikungunya virus strains or its subunit antigens useful for diagnosis of Chikungunya virus infections in humans.

One more object of the invention relates to provide major antigenic determinants of the Chikungunya virus which are suitable as effective vaccine candidates and nucleotide and protein sequences disclosed thereof.

Yet another object of the invention includes combined vaccine compositions which are effective for prophylaxis and treatment of infections caused by Chikungunya virus and other other bacterial and viral infections selected from the following list that includes but is not limited to vaccines for Japanese encephalitis virus vaccines, dengue vaccines, West Nile virus vaccine and Chandipura virus vaccine and rabies vaccines.

SUMMARY OF THE INVENTION

According to one aspect of the invention, the invention includes vaccine compositions which specifically contain the whole inactivated virion or the subunit antigens of the CHIKV virus strains. The compositions of the present invention more particularly relate to vaccine capable of eliciting protective antibody and strong T cell responses against Chikungunya virus infection.

Another aspect of the invention is to provide inactivated recombinant CHIKV vaccines along with appropriate adjuvants that offer high protective efficacy.

Yet another aspect of the invention of the present invention more particularly relate to vaccine capable of eliciting protective antibody and strong T cell responses against Chikungunya virus infections.

One another aspect of the invention relate to methods of preparing and using Chikungunya virus (CHIKV) antigens of defined sequences expressed as recombinant proteins, virus like particles and as virosomes which are used to elicit protective immune response. The potency of such subunit vaccines are comparable to that elicited by the vaccine consisting of whole inactivated virion of CHIKV that are inactivated with reagents under conditions that confer high immunogenicity to the vaccine.

Another aspect of the invention relates to methods of inactivation of the virus which comprises heat, gamma irradiation, ultraviolet light or chemically inactivated whole virion of Chikungunya virus isolates in a stable formulation. A combination of two or more inactivating agents has also been used with similar effect. The virus isolates disclosed in the invention are used in vaccine development, and all the methods are applicable to any genotypes or genotypic variants/serotypes/strains/mutants of Chikungunya virus.

One another aspect of the invention is to provide vaccine compositions against Chikungunya virus that elicit strong immunological response when administered parenterally, preferably intradermally, intramuscularly or sub-cutaneously in mammals preferably in humans, and are effective when administered mucosally and by other routes such as oral routes.

Yet another object of the invention is to provide antibodies against Chikungunya virus or the subunit antigens thereof to be used for treatment and diagnosis of Chikungunya virus infections in mammals, preferably humans.

One another aspect of the invention is to provide a composition for eliciting protective antibody and strong T cell responses either singly or in combination with other vaccines included within the scope of the invention. The other vaccines in combination are but not limited to vaccines for Japanese encephalitis virus vaccines, dengue vaccines, West Nile virus vaccine and Chandipura virus vaccine and rabies vaccines.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1:

Immunogenicity of CHIKV whole virion antigen inactivated by several inactivation methods were tested for potency. The details of inactivation procedures are provided in Example 2. Potency of the 15 μg of the inactivated viral vaccine was tested in three intramuscular injections in 4-6 week old Balb/c mice (8 nos per group) at intervals of 0, 7 and 21 days and bled 7 days after the last dose administration. Only a single dose of the live virus was administered for comparison. The potency of the vaccine preparations were tested by estimating the titer of neutralizing antibodies by $PRNT_{50}$.

Figure 2:

Immunogenicity of the CHIKV vaccine preparation with and without adjuvants was tested in three intramuscular injections in 4-6 week old Balb/c mice (8 nos per group) at intervals of 0, 7 and 21 days and bled 7 days after the last dose administration. The composition of the adjuvanted vaccine formulations are provided in Example 5. The potency of the vaccine preparations were tested by estimating the titer of neutralizing antibodies by $PRNT_{50}$.

DETAILED DESCRIPTION OF THE INVENTION

No detailed study on evolution of CHIKV serotypes due to sequence diversity has been reported. We report for the first time the adaptive evolution of ECSA strains of CHIKV to *Ae.aegypti* as found in the 2009-2010 virus isolates from India. Incidentally, *Ae.aegypti* is the most prevalent vector in India and indeed in several tropical countries. Despite unique mutations in isolates reported in the current invention, the virus strains cross neutralize the Asian genotypes and various ECSA sub-lineages of CHIKV indicating that they are good candidates for vaccine development. Using virus strains or antigens derived from such strains thereof, that are better adapted to the most prevalent vector in the region is important for vaccine development rather than using strains of West African or Asian genotype which are not so widely prevalent now than the ECSA genotype. Even among the ECSA genotype, using candidates such as LR2006 isolates from Reunion Island that carry E1-A226V mutation which is an adaptive mutation to increase transmissibility in *Ae.albopictus* is less advantageous as *Ae.albopictus* vector in India is prevalent widely only along the West coast of India such as in the states of Kerala and South coastal Karnataka, whereas the mosquito vector that is most abundant in the rest of the country is *Ae.aegypti*. The virus strains isolated and reported in this invention are unique in that they show adaptive evolution to *Ae.aegypti* and at the same time also infect *Ae.albopictus*. Apart from the unique mutations that increase adaptation to *Aedes aegypti*, the advantage of the invention is that the virus isolates cross neutralize the Asian genotypes and various ECSA variant strains and hence are good candidate vaccines. Hence, a subunit vaccine derived from the virus antigens or recombinant antigens of these isolates are good vaccine candidates as well, as the recombinant vaccine antisera also cross neutralizes the different genotypes and genotypic variants.

Hence, using the Indian virus strains that show unique adaptation to *Ae.aegypti* and also infects *Ae.albopictus* is advantageous than using the West African, Asian or ECSA E1-226A and other variant strains as *Ae.aegypti* is the most widely prevalent vector in the India which has the highest incidence of CHIKV infection in the world.

The Chikungunya virus isolates within the scope of the invention are those that belong to the ECSA (East, Central and South African) genotype whose structural polyprotein sequence comprises of the capsid, E3, E2, 6K and E1 (C-E3-E2-6K-E1) proteins. The

isolates obtained from the Indian epidemic of 2009-2010 are unique in the sequence reported so far. The structural polyprotein sequence comprising the C-E3-E2-6K-E1 proteins have been deposited in the public sequence repository (GenBank) on 27th April 2010 and have been assigned the accession numbers HM159385 to HM159390. The sequences were published in March 2012 after the date of filing the provisional patent. The unique nucleotide sequences reported in this invention are SEQ ID NO.1 (isolate TN01610), SEQ ID NO.2 (isolate TN15110) SEQ ID NO.3 (isolate TN06210), SEQ ID NO. 4 (TN06310), SEQ ID NO. 5 (TN06410) and SEQ ID NO.6 (AP0109), whose corresponding protein sequences when translated are SEQ ID NO.8, SEQ ID NO.9, SEQ ID NO.10, SEQ ID NO.11, SEQ ID NO.12 and SEQ ID NO.13 respectively. The CHIKV strain CHIKV/03/06 has structural polyprotein of SEQ ID NO.7 and was isolated during the 2006 Indian epidemic, the corresponding protein sequence is SEQ ID NO.14. The names of the virus isolates are provided in the brackets. The full length genomic RNA sequences of the above mentioned virus isolates of the current invention are provided in SEQ ID NO.15 to SEQ ID NO.20.

The sequence of the isolates disclosed in the invention have unique genetic signatures such as the combination of T1766C (E2-V264A) + A3058G (E1-K211E) + 3104C (E1-226A) in the structural polyprotein sequence in addition to other amino acid changes when compared to the S27 African prototype (Gen Bank Acc No. AF369024). The position of nucleotide substitution in the structural polyprotein and the corresponding amino acid change in the individual proteins within the polyprotein is indicated in brackets. Other unique mutations that are being reported are Capsid-A232V in TN06310, E3-D40N in TN15110, E2-K47N in TN06210, E2-G55R in TN01610 and AP0109, E2-K66E in TN064110, E1-P58L in AP0109, and E1-G195R in TN15110 and TN06310. Codon by codon analyses by maximum likelihood estimates of 'ω' (the ratio of non-synonymous to synonymous substitutions) of the ECSA strains show that the amino acid mutation E1-K211E in the isolates reported in the invention (of SEQ ID NO. 8 to SEQ ID NO.13) is under significant positive selection (posterior probability of ≥ 0.97; $p<0.05$) and is suggestive of adaptive mutation to increase infectivity in the *Aedes* mosquito vectors, particularly in *Aedes aegypti*. The amino acid residue E1-211E is conserved in the Asian genotypes of CHIKV which are circulated by *Ae.aegypti*. Additional mutations disclosed in this invention such as the three

novel mutations E2-K47N, E2-G55R and E2-K66E also cluster in the same region of the E2 protein that are reported to increase the infectivity of the Sindbis virus in *Aedes aegypti.* The E2 aa 52 – 82 region is exposed at the top of the spike, which is the point of contact with cellular receptors. Codon by codon maximum likelihood estimates of 'ω' by SLAC (Single Likelihood Ancestor Counting), eFEL (Fixed Effects Likelihood), iFEL (internal Fixed Effects Likelihood) and REL (Random Effects Likelihood) identified amino acid sites across the capsid and the structural glycoproteins under significant purifying selection. Among the amino acid sites that were negatively selected, the E2-199Y residue was selected as the genetic loci under most significant purifying selection by all the four likelihood estimates (posterior probability >0.99 by REL, $p<0.01$ by iFEL, $p=0.001$ by SLAC and $p=0.00$ by eFEL). E2-199Y is an important residue in Chikungunya virus determining virus fitness in mosquitoes.

Viral evolution tends to select for mutations in the antigenic determinants involved in neutralization as well as those residues involved in vector/host adaptation. Because of its high immunological specificity, the serum neutralization test is often the gold standard against which the specificity of the other serological techniques is evaluated. The antisera raised against the virus isolates reported in the invention neutralized the virus isolates of Asian and ECSA lineages and several variant strains of ECSA genotype including the E1-A226V ECSA variant strain, indicating that they are good vaccine candidates as they have broad neutralizing activity.

The properties of Chikungunya virus particles as an immunogen, adaptation and propagation of the virus in host cell lines to a high titer, determination of the identity of the virus by RT-PCR, methods of purification and inactivation of the virus, preparation of stable vaccine formulation in a pharmaceutically acceptable carrier suitable for administration in humans, the viral assays and tests for vaccine potency in animal models are also within the scope of the invention. The virus particles obtained from infected patients or isolated from the vectors of the virus where the virus resides, are adapted in cell lines and propagated *in vitro* in cell culture in several passages.

The use of the CHIKV strains in the development of an inactivated whole virion vaccine is one aspect of the invention. The Chikungunya virus strains were infected in mammalian cell lines for production of the virions. The mammalian cells include but are not limited to Vero cells (ATCC CCL-81), MRC-5 or any other cell line suitable for vaccine production for human use.

The whole virions obtained from cell culture were inactivated with different inactivating agents. The optimum time, temperature and use of stabilizers such as sugars like sucrose, lactose, trehalose and other sugars and sugar combinations, and the addition of sugar alcohols such as mannitol or sorbitol either alone or in combination with different sugars, addition of human serum albumin either alone or in combination with sugars, amino acids and sugar alcohols during the inactivation process are within the scope of the invention. The virus was rendered non-infectious by inactivating either by heat, gamma irradiation or ultra violet light or by chemical means with formalin and beta-propiolactone (BPL) among others under conditions that retained high immunogenicity of the vaccine preparation. The conditions of virus inactivation were optimized and are presented in Example 2. Chemical inactivating agents are selected from the following list which includes but is not limited to: formalin, beta-propiolactone, glutaraldehyde, N-acetylethyleneimine, binary ethyleneimine, tertiary ethyleneimine, ascorbic acid, caprylic acid, psolarens, detergents including non-ionic detergents etc. is added to a virus suspension to inactivate the virus. The concentration of the sugars, sugar alcohols, human serum albumin and amino acids either when used alone or in various combinations were in the concentration range of 0.01% to 20%, preferably 0.1% to 10% and most preferably 0.1% to 5%. Time and temperature of inactivation in the presence of the stabilizers were optimized from 2-8^{0}C to 37^{0}C for varying period of time such as 30 min to 20 days. Such vaccine formulations were highly immunogenic and elicited protective neutralizing antibodies.

The structural glycoproteins C-E3-E2-6K-E1 of the Chikungunya virus are the major antigenic determinants. Hence, the structural glycoproteins are excellent vaccine candidates for subunit vaccine for prophylaxis of CHIKV infections. The sequence of the structural proteins as defined in SEQ ID NO.8 to SEQ ID NO. 14. The recombinant non-structural proteins are also immunogenic and are good candidate vaccines. The eukaryotic expression system of choice includes mammalian cells, baculovirus in insect cells, and yeast cells of

any species, most preferably *Pichia pastoris* or *Saccharomyces cerevisiae.* Genes encoding the subunit antigens were also expressed in prokaryotic cells such as *E.coli* using any of the suitable prokaryotic expression vectors. *Pichia pastoris* as recombinant expression host is advantageous at industrial scale as it is cost effective for large scale manufacture compared to other eukaryotic expression systems. Recombinant proteins derived from *Pichia pastoris* have been successfully commercialized and have been found safe for human use. The structural proteins such as C-E3-E2-6K-E1 of the sequences disclosed in this application are capable of assembling into 'virus like particles' (VLPs). Alternatively, the VLPs contain only the E3-E2-6K-E1 or E2-6K-E1 or only E2-E1 proteins and are immunogenic and elicited protective immune response when administered in animals. The subunit antigens comprising E3-E2-6K-E1 or E2-6K-E1 are also capable of assembling into virosomes as CHIKV is an enveloped virus. Virosomes comprising E3-E2-6K-E1 or E2-6K-E1 or only E2-E1 are also immunogenic. The liposomes and virosomes can contain different combination of lipid soluble substances which include but are not limited to cholecalciferol, cholesterol, phospholipids etc. and the viral envelope proteins. The methods for virosomes preparation such as solubilization of the virus particles with detergents or with short chain phospholipids and reconstitution of the envelope proteins after removal of the chaotropic agents and the non-envelope proteins and RNA that are applicable to any enveloped virus are also applicable to CHIKV.

Purification of the virus was achieved by physical or chemical means and preferably by a combination of both. Physical methods utilize the physical properties of the virus such as density, size, mass, sedimentation coefficient etc. and include any of the following techniques but are not limited to: ultracentrifugation, density gradient centrifugation, ultrafiltration etc. Purification through chemical means employs methods such as adsorption/desorption through chemical or physiochemical reactions such as ion exchange chromatography, affinity chromatography, hydrophobic interaction chromatography, gel filtration chromatography, hydroxyapatite matrix, salting with inorganic salts one such example being ammonium sulphate, and by the use of proprietary Himax^{TM} technology, organic salts and organic compounds such as polyethylene glycol. Purification of the virus or the recombinant virus antigens was achieved by either one or a combination of two or more of the above mentioned methods.

The antigenic compositions of the above mentioned CHIKV candidate vaccines, such as the inactivated whole virion vaccines or the recombinant vaccines were formulated in pharmaceutically acceptable carrier for immunization in mammals, preferably humans. The Chikungunya virus vaccine formulation was adjuvanted and adjuvants were selected from the following list, which includes but is not limited to: alum; calcium phosphate; inulin of any polymorphic form, preferably gamma inulin; adjuvants containing inulin in combination with other organic and inorganic compounds such as aluminum hydroxide, aluminum phosphate, aluminum sulphate phosphate and calcium phosphate; liposomes, chitosan and complex carbobhydrates such as dextran, dextrins, starch, inulin, mannans and glucomannans, galactomannans, beta-glucans, heparin, cellulose, pectins and pectinates, lectins and any other carbohydrates either synthetic or derived from any source, any biodegradable and biocompatible polymers, such as poly lactide and poly(lactide co-glycolides; PLG) or PLGA; any emulsions including but not limited to oil in water emulsions one such example being ASO3, other squalene based adjuvants such as MF59 etc., any water in oil emulsion; liposomes prepared with cholecalciferol as one of the ingredients along with other lipid soluble compounds; liposomes of other compositions; RIBI adjuvant systems, saponins including but not limited to QS-21, QuilA, tomatine, ISCOMs, ISCOMATRIX etc, lipopeptides, glycopeptides, lipopolysaccharides, muramyl dipeptides and any peptide based adjuvants, oligonucleotides, any TLR ligands as adjuvants, any cytokine, vitamins and non-toxic bacterial toxins etc. The most compatible and cost effective adjuvant was selected in the final vaccine formulation after testing for immunogenicity which was enhanced by the addition of adjuvants. In addition to the above, any other organic and inorganic substances that have good immunopotentiating activity can also be used as adjuvants either singly or in combinations to enhance the immunogenicity of Chikungunya virus vaccines. In addition to the inactivated whole virion vaccine, the aforementioned adjuvants or adjuvant combinations are also effective with recombinant Chikungunya virus vaccine using recombinant subunit antigens either when presented as virosome, virus like particles (VLPs) or when expressed, purified and formulated as individual recombinant proteins. The use of suitable adjuvants in the vaccine formulations reduces the amount of antigen required and helps in the manufacture of low-cost vaccines thus conferring economic advantage.

The buffer used in the formulations is phosphate or phosphate-citrate buffer or any other pharmaceutically acceptable buffer. The vaccines optionally contain preservative(s), stabilizer(s) etc. The excipients were selected from a list that includes but is not limited to reducing and non-reducing sugars, sugar alcohols such sorbitol and mannitol, glycerol, amino acids, human serum albumin, inulin, thiomerosol and a choice of adjuvant from the aforementioned list of adjuvants. The excipients are added in the range of 0.01% to 20% for the liquid formulation and upto 60% of the total solids for a lyophilized formulation. The vaccine formulations were also presented as emulsions, either as water in oil emulsion or as oil in water emulsion. Such emulsions of vaccine antigens contain preservatives and stabilizers and other adjuvants. Such a stable formulation of the immunogen either in a liquid or in a lyophilized form and after reconstitution in a pharmaceutically acceptable buffer or water is suitable for administration parenterally in human host and is also formulated for mucosal administration. The vaccine formulations were highly immunogenic and neutralized homologous and heterologous CHIKV strains.

For potency testing of the vaccine, the vaccine formulations were tested in Balb/c mice and rabbits. The resultant serum is assayed by *in vitro* neutralization tests and the antibody titer is determined by ELISA. Seroconversion was observed in the animals immunized with the vaccine formulations described in the present invention. Efficacy of the recombinant vaccine in offering a protective immune response was comparable with the whole virion vaccine and the titers of the neutralizing antibody responses were determined by either serum neutralization test (SNT), plaque reduction neutralization test ($PRNT_{50}$) and ELISA among other methods. Passive immunization of the vaccine antibody offered good protection against virus infection indicating therapeutic use of CHIKV antibodies. The presence of virus in infected patients samples were accurately determined using CHIKV antibodies. Chikungunya virus vaccine obtained by the methods included in the scope of the current invention elicits strong neutralizing antibodies in combination with other vaccines. The vaccines that can be included in the combination are selected from the following list that includes but is not limited to vaccines for Japanese encephalitis virus, Dengue vaccines, West Nile virus vaccine and Chandipura virus vaccine and rabies vaccines. Combinations with other viral vaccines are also within the scope of the invention. As known to those skilled in the art, a bivalent or polyvalent vaccine can be prepared by mixing vaccines

produced from two or more CHIKV strains, and is mixed in a suitable ratio based on the antigen content. Such mixing provides a vaccine preparation having a broad antigenic spectrum for protection against the infection.

According to the present invention, the methods and compositions of CHIKV strains of the current invention is applicable to any CHIKV strain. The vaccines of this invention offered good immune protection against plural strains of CHIKV in addition to the virus strains used in production of the vaccine. The CHIKV isolates reported in the study have broad neutralizing activity as they cross neutralize different genotypes /genotypic variants / strains of CHIKV and are ideal vaccine candidates for development of whole inactivated virion vaccine or recombinant vaccines comprising the antigens derived from these virus isolates. The methods disclosed in the invention are applicable to any genotype/genotypic variants/serotype/strain of Chikungunya virus and as demonstrated offer good cross protection against multiple gentotypes/genotypic variants of the virus.

The invention is further described in the following examples. It should be noted that features, integers, characteristics, ranges, compounds, and/or groups described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example described herein unless incompatible therewith and should be considered within the scope of the invention.

Example 1: Isolation of virus strains

The virus strains were isolated from blood samples collected from febrile patients with their informed consent during an epidemic outbreak in India in 2009-2010. The blood samples were collected during the acute phase of Chikungunya virus infection when patients reported high fever, acute polyarthralgia and painful swelling in joints and rashes. The patients' sera samples were transported on dry ice to the laboratory. About 0.05 ml of the serum was used for infection of Vero cells (ATCC No. CCL-81) in 25^2cm flask in medium containing DMEM (Dulbecco's Modified Eagle Medium; Sigma- Aldrich Catalog # D5523) containing 1% fetal bovine serum (FBS). The flasks were incubated at 34^0C to 37^0C. The virus was harvested 48 hours after infection. Scaled up cultures of the virus were

made in cell stacks or in cell factories or in bioreactors in liquid culture All the blood samples were negative for Dengue infection by specific IgM ELISA (National Institute of Virology, Pune). The infectious titer of the virus increased more than 10 fold after the virus particles were passaged once in suckling mice brain or after passage in mosquito cell lines such as C6/36 cells, and also after repeated passage of the virus in cell culture *in vitro*.

Example 2: Purification and inactivation of CHIKV virus

The two virus isolates TN01610 and TN15110 were purified from the infected Vero cell monolayers from scaled up cultures by initial ultrafiltration to remove cellular debris, and by filtration and concentration through a 300 kD membrane followed by purification by ion exchange and gel filtration column chromatography. Heat inactivation of the virus was carried out at different temperatures ranging from 45^0C to 60^0C for 30 min to 4 hrs and optimally at 56^0C for 30 min. Inactivation by ultraviolet (UV) light was done at 254 nm for varying period of time from 30 – 120 min on ice, and optimally for 40 min. Chikungunya virus was inactivated effectively by formalin at ratios upto 1:3000 for formalin:virus at 2^0C -8^0C upto 7 days, and with beta propiolactone at 1:1000 to 1:2500 (beta propiolactone:virus) for upto 7 days at 2^0C -8^0C. In both the cases, the time of inactivation was reduced to 24-48 hrs when carried out at ambient temperatures of +20 - 25^0C. Formalin and beta propiolactone were removed by dialysis. During inactivation, use of additives such as glycine, mannitol, sorbitol and sugars and sugar combinations increased the stability of the vaccine preparation. The sugars used may be selected from sucrose, lactose, trehalose, maltose at varying concentrations from 0.5% to 5%. Inactivation of the virus by gamma irradiation was carried out by exposure of the virus samples to a dose of 10 kGy (Kilo Gray) to 25 kGy from a ^{60}Co source (Ms.Gamma Agro-Medical Processings Pvt.Ltd. Hyderabad) and optimally to 20 kGy. Complete inactivation of the virus samples by all of the above methods were confirmed by three serial passages in Vero cells for absence of virus cytopathic effect, and additionally by the absence of growth abnormalities and death when inoculated by intracerebral route in the brain of 2-day old mice. The inactivated virus antigens were tested for potency as candidate vaccines..

Example 3: Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR) and Sequencing:

Viral RNA was isolated using Absolutely RNA Miniprep kit (Stratagene, La Jolla, CA) from infected Vero cells (ATCC CCL-81), after a single passage. RT-PCR was carried out using the AccuScript High Fidelity 1st Strand cDNA Synthesis Kit (Stratagene) as per the kit protocols, and the 3,747 bp structural polyprotein gene was amplified with the PfuUltra High-Fidelity DNA polymerase (Stratagene). PCR primers were designed based on the consensus sequence of the S27-African prototype (AF369024) and the Indian 2006 isolate (HM159384), and used to amplify overlapping sequences of the structural polyprotein gene. PCR reaction consisted of initial denaturation at 95^{0}C for 1 min, followed by 32 thermal cycling steps at 94^{0}C for 40 sec, annealing at 52–65^{0}C (depending on the primer sets) for 30 sec and extension at 70^{0}C for 3 min, followed by final extension at 70^{0}C for 10min. PCR products were purified by QIAquick gel extraction kit (QIAGEN, Hilden, Germany) after separation on 1% agarose gel and used for DNA sequencing. Nucleotide sequencing of CHIKV structural polyprotein gene gel purified PCR products were sequenced on both strands of DNA by BigDye terminator v3.1 reaction (Applied Biosystems, Foster City, CA) and the sequence data was analyzed using Sequencher v4.7 (GeneCodes, Ann Arbor, MI). The sequences were deposited in GenBank on 27th April 2010 before filing the provisional patent and published by GenBank on 02 March 2012. The unique nucleotide sequences reported in this invention are SEQ ID NO.1 (isolate TN01610), SEQ ID NO.2 (isolate TN15110) SEQ ID NO.3 (isolate TN06210), SEQ ID NO. 4 (TN06310), SEQ ID NO.5 (TN06410) and SEQ ID NO.6 (AP0109), whose corresponding protein sequences when translated are SEQ ID NO.8, SEQ ID NO.9, SEQ ID NO.10, SEQ ID NO.11, SEQ ID NO.12 and SEQ ID NO.13 respectively. The CHIKV strain CHIKV/03/06 has structural polyprotein gene of sequence SEQ ID NO.7 and was isolated during the 2006 Indian epidemic and its corresponding protein sequence is SEQ ID NO.14. The names of the virus isolates are provided in the brackets. For complete genomic RNA sequences, the sequencing reactions were performed using sequencing by synthesis (SBS) technology on the Illumina GAIIx (Genotypic Technology Pvt. Ltd. Bangalore). The complete nucleotide sequences (in the form of cDNA) of the virus genomic RNA of the above mentioned virus strains are provided in SEQ ID NO.15 to SEQ ID NO.20. Mutations identified with

reference to strain S27-African prototype (AF369024) were mapped to the individual structural proteins and are presented in Table 1.

TABLE I. Unique mutations in the Chikungunya virus structural genes reported in this study.

Amino acid position		Nucleotide change in polypeptide	strain S27-African prototype	CHIKV/03/06	TN01610	TN151100	TN06210	TN06310	TN06410	AP0109
Poly-peptide	Protein									
232	C-232	c695t	A	.	.	.	.	V	.	.
301	E3-40	g901a	D	.	.	N	.	.	.	.
372	E2-47	a1116t	K	.	.	.	N	.	.	.
380	E2-55	g1138a	G	.	R	.	.	.	.	R
391	E2-66	a1171g	K	.	.	.	.	.	E	.
589	E2-264	t1766c	V	.	A	A	A	A	A	.
867	E1-58	c2600t	P	.	.	.	.	.	.	L
1004	E1-195	g3010c	G	.	.	R	.	R	.	.
1020	E1-211	a3058g	K	.	E	E	E	E	E	E

Unique mutations identified in the capsid, E1, E2 and the E3 structural glycoproteins in the 2009-2010 CHIKV isolates from the States of Tamil Nadu and Andhra Pradesh.

"." Amino acids identical to the reference strain S27-African prototype (AF369024). The GenBank accession numbers of the isolates from Tamil Nadu are HM159385 (TN01610), HM159386 (TN15110), HM159387 (TN06210), HM159388 (TN06310), HM159389 (TN06410), and from Hyderabad, Andhra Pradesh are HM159384 (CHIKV/03/06) and HM159390 (AP0109).

Example 4: Phylogenetic Analyses and Inference of Selection Pressure

The sequences reported in this study and those retrieved from GenBank were screened for recombination by the Genetic Algorithm Recombination Detection (GARD) (Kosakovsky Pond et al. 2006) prior to phylogenetic analysis. Evolutionary analyses were performed in MEGA5 (Tamura et al. 2007) using Kimura-2 parameter model of nucleotide substitution with 1000 bootstrap replicates. Multiple sequence alignment was performed using ClustalW2.0.3. The ECSA structural polyprotein sequences from 2005-2010 retrieved from GenBank and those reported in the study were used in the inference of selection pressure on the ECSA lineage. About 52 unique sequences were short listed by Hyphy (Pond et al. 2005) from 58 sequences retrieved from GenBank for the analyses. Codon-based Maximum Likelihood estimates of ω or the dN/dS (the ratio of non-synonymous to synonymous substitutions) were inferred by Random Effects Likelihood (REL), Fixed Effects Likelihood (eFEL) and selection along the internal branches of phylogeny was tested using Internal Fixed Effects Likelihood (iFEL) method in HyPhy. In the likelihood methods, positive selection was inferred as significant if the p value of the likelihood ratio test (LRT) was less than 0.05 or when the Bayes factor was equal to or larger than 100 for a site. Statistical testing of positive selection operating on the entire protein was inferred by Single Likelihood Ancestor Counting (SLAC) method in HyPhy. Inference of ω by empirical Bayesian method using LRT (Likelihood Ratio Test) with the MEC (Mechanistic Empirical Combination) model for positive selection, and M8a model for purifying and neutral selection was carried out using Selecton v2.2 (Stern et al. 2007). The amino acid sites of CHIKV structural proteins under significant positive and purifying selection is provided in accompanying Table II.

TABLE II. Amino acid sites of CHIKV structural proteins under significant positive and purifying selection

Method	Codon no. in structural polyprotein	Positively selected amino acid	Negatively selected amino acid	p-value	Posterior probability	Bayes factor†
REL	523	E2-198R			0.87	111.55
	524		**E2-199Y**		0.999	505.10
	645	E2-320T			0.86	100.71
	711	E2-386A			0.87	108.86

	1020	**E1-211K**			0.97	532.15
	1035	**E1-226A**			0.98	773.33
	1078	E1-269V			0.86	100.10
	1113	E1-304 P			0.88	120.58
iFEL	28		C-28I	0.034		
	273		E3-12N	0.008		
	326		E2-1S	0.036		
	397		E2-72N	0.008		
	524		**E2-199Y**	**0.003**		
	834		E1-25S	0.036		
	909		E1-100N	0.005		
	916		E1-107H	0.016		
	1020	**E1-211K**		0.040		
	1035	**E1-226A**		0.006		
	1120		E1-311D	0.042		
	1245		E1-436F	0.009		

The amino acids under positive selection in the capsid (C) and in the E1, E2 and E3 glycoproteins in the 2009-2010 Indian CHIKV isolates were inferred by Random Effects Likelihood (REL) and by Internal Fixed Effects Likelihood (iFEL) methods using the HyPhy package. The amino acid sites under significant positive and purifying selection in the E1 and E2 proteins respectively (Bayes factor >500, posterior probability ≥0.97 and $p<0.05$) are indicated in boldface. †Bayes factor is statistical estimation of posterior odds/prior odds for positive selection (dN>dS) at the site.

Example 5: Cloning and Expression of the Structural Polyprotein Sequences

The virus isolates reported in this patent was used as the source for cloning and expression of all viral antigens. The complete open reading frame of the Chikungunya virus structural polyprotein encoded by the SEQ ID NO.1 was amplified by RT-PCR of the viral genomic RNA using the primers CHKVCPFP as the forward primer and CHKVE1RP as the reverse

primer to obtain a ~ 3747 bp PCR fragment. The sequence of the PCR primers used for PCR amplification is:

CHKVCPFP:

55' ACAGAATTCATATGGAGTTCATCCCAACCCAAAC 3'

CHKVE1RP:

5' AATTGGATCCGCGGCCGCTTAGTGCCTGCTGAACGACACGC 3'

The PCR fragment was digested with Nde1 and BamH1 and cloned into the Nde1 and BamH1 sites of the prokaryotic expression vector, pET-11B and the recombinant plasmid containing the insert was transformed in *E.coli* DH5a. The recombinant plasmid DNA isolated from DH5a was used to transform the *E.coli* strain BL21(DE3). The PCR gene fragment was digested with EcoR1 and Not 1, gel purified by standard protocols and cloned into EcoR1 and Not1 sites of the yeast expression vector pPIC3.5K (Invitrogen Corporation, Carlsbad, USA) and transformed in *E.coli* DH5a. Recombinant plasmid DNA isolated from *E.coli* clone was linearized with BglII and was transformed into *Pichia Pastoris* GS115 as per the protocol from manufacturers (Invitrogen). The gene has been cloned into the *AOX1* locus and expressed under the *AOX1* promoter by methanol induction. The cloning, screening, isolation of the recombinant *Pichia* strains and induction of the cloned gene with methanol were carried out as per the User's manual "A Manual of Methods for Expression of Recombinant Proteins in *Pichia pastoris*" Version M Jan 2002, of *Pichia* Expression Kit, Catalog # K1710-01, Invitrogen Corporation, Carlsbad, USA).

Example 6: *In vivo* potency testing of the vaccine formulations:

The inactivated virus sample in vaccine formulations was tested with different adjuvants for potency. The adjuvants tested (at concentrations per single human dose) include a) aluminum hydroxide (0.5mg aluminum content) b) aluminum phosphate (0.5 mg aluminum content) c) gamma inulin (10 mg), d) algammulin (a combination of aluminum hydroxide and gamma inulin) at 10 mg, e) cholecalciferol in oil at 0.75 mg per dose, f) an oil in water emulsion OWEM1, containing 4.3% squalene, 0.5% tween-80, and 0.5% Span-85 (Sigma Aldrich product # S7135) in 10 mM phosphate-citrate buffer, f) oil in water emulsion OWEM2

containing 9.5mg squalene, 1 mg tween-80, 1 mg Span-85, 11 mg alpha tocopherol in phosphate-citrate buffer, g) an oil in water emulsion OWEM3 containing at the same concentration of excipients as in OWEM2 except that alpha tocopherol is replaced with 1-10 mg cholecalciferol. The formulated and adjuvanted vaccine preparations were injected intramuscularly in mice and booster doses were administered on day 7 and day 21 after administration of the first dose. Blood was collected at 28 days after the first dose was administered. Pooled sera from each test group were complement inactivated at 56^0C for about 30 min. All the formulations contained 15 μg viral antigen in 40 mM phosphate buffer, pH 6.8 – 7.2 containing 150 mM NaCl. Sera samples were used for estimation of neutralizing antibodies and for the estimation antibody titer by ELISA. Vaccinated animals offered complete protection against viremia with a virus challenge dose of $10^{4.5}$ pfu/ml when monitored over a period of 72 hours after intravenous/intraperitoneal administration of the challenge virus. In another experiment, passive immunization with rabbit antisera with $PRNT_{50}$ titer of 640 when administered intravenously in 4-6 week old Balb/c mice offered complete protection against viremia when challenged with $10^{4.5}$ pfu/ml of the challenge virus. For serotype analyses, antisera against CHIKV/03/06 neutralized heterotypic virus isolates of the Asian genotype (GenBank Acc No. EF027140, isolated in Kolkata in 1963), ECSA, (E1-A226V, E1-211K; GenBank Acc No. FJ000069, isolated in Kerala in 2007) and ECSA (E1-226A, E1-K211E, GenBank Acc No. HM159386, obtained from Tamil Nadu in 2010 with neutralizing antibody titer ≥ 40 indicating heterotypic protection against genotypic variants, and also indicating that no distinct serotypes have evolved.

Example 7: Plaque Reduction Neutralization Assay

One day prior to the assay 6-well plates were seeded with 2.5 x 10^3 Vero cells (ATCC CCL-81) per well and the plates were incubated at 37°C in a 5% CO_2 incubator. To 4-fold dilutions of the sera samples in MEM containing 2% fetal bovine serum, equal volume of the standardized virus (10^5 pfu/ml) was added and incubated at 37°C with 5% CO_2 for 90 min. The cells were washed twice with 1 x PBS pH 7.4 (10 mM phosphate with 150 mM NaCl) and 0.3 ml of each dilution of the serum-virus mixture was added to the corresponding well and incubated for 90 min at 37°C in a 5% CO_2 incubator. Each assay was carried out in triplicates. The cells were overlaid with 2 ml of 0.85% methyl cellulose in MEM containing 10% fetal bovine serum, 1% penicillin-streptomycin and 1% L-

glutamine. The plates were incubated at 37°C in a 5% CO_2 incubator for 5 days. At the end of incubation, the plaques were fixed with 10% formalin, washed with 1 x PBS, pH 7.4 and were visualized with 0.1% crystal violet. The highest dilution of serum causing 50% reduction in plaques formed by the control virus sample was estimated as the $PRNT_{50}$ titer. $PRNT_{50}$ assays were carried out to test the potency of the vaccine preparations by various inactivation methods, as well as for adjuvanted CHIKV vaccines and vaccine combination with JEV vaccine..

Example 8: Vaccine combinations

A combination of CHIKV vaccine inactivated by beta-propiolactone was tested in combination with formalin inactivated vaccine for Japanese encephalitis virus (JEV). 15 μg of CHIKV vaccine antigen formulated in alum (0.5mg aluminum/ dose) was tested in combination with inactivated JE (JEV) virus vaccine containing 6 μg of Japanese encephalitis virus whole virion antigen also formulated in alum. The vaccine combination was injected in 8 nos of Balb/c mice with appropriate controls that included either of the antigens alone, and also control animals that received equivalent amount of alum. The animals were boosted at 7 and at 21 days after the first immunization. Blood was collected at 7 days after the last booster injection. Pooled sera from each group were complement inactivated at 56^0C for about 30 min. The sera samples were used for estimation of neutralizing antibody by $PRNT_{50}$ for both CHIKV and JEV. The buffer used in all the formulations was 40 mM phosphate buffer, pH 6.8 – 7.2 containing 150 mM NaCl. All the methods disclosed above are applicable to any genotype/genotypic variants/serotypes and strains of Chikungunya virus.

References:

1. Kannan M, Rajendran R, Sunish IP, Balasubramaniam R, Arunachalam N, Paramsivan R, Tewari SC, Samuel PP, Tyagi BK. 2007. A study on Chikungunya outbreak during 2007 in Kerala, south India. Indian J Med Res 129:311-315

2. Sourisseau M, Schilte C, Casartelli N, Trouillet C, Guivel-Benhassine F, Rudnicka D, Sol-Foulon N, Le Roux K, Prevost MC, Fsihi H, Frenkiel MP, Blanchet F, Afonso PV, Ceccaldi PE, Ozden S, Gessain A, Schuffenecker I, Verhasselt B, Zamborlini A, Saïb A, Rey FA, Arenzana-Seisdedos F, Desprès P, Michault A, Albert ML, Schwartz O. 2007. Characterization of reemerging Chikungunya virus. PLoS Pathog 3:e89.

3. Powers AM, Brault AC, Tesh RB, Weaver SC. 2000. Re-emergence of Chikungunya and o'nyong-nyong viruses: evidence for distinct geographical lineages and distant evolutionary relationships. J Gen Virol 81:471-479.

4. Yergolkar PN, Tandale BV, Arankalle VA, Sathe PS, Sudeep AB, Gandhe SS, Gokhle MD, Jacob GP, Hundekar SL, Mishra AC. 2006. Chikungunya outbreaks caused by African genotype, India. Emerg Infect Dis 12:1580-1583.

5. Powers AM, Logue CH. 2007. Changing patterns of Chikungunya virus: re-emergence of a zoonotic arbovirus. J Gen Virol 88:2363–2377.

6. Arankalle VA, Shrivastava S, Cherian S, Gunjikar RS, Walimbe, AM, Jadhav, SM, Sudeep AB, Mishra AC. 2007. Genetic divergence of Chikungunya viruses in India (1963-2006) with special reference to the 2005-2006 explosive epidemic. J Gen Virol 88:1967-1976.

7. de Lamballerie X, Leroy E, Charrel RN, Ttsetsarkin K, Higgs S, Gould EA. 2008. Chikungunya virus adapts to tiger mosquito via evolutionary convergence: a sign of things to come? Virol J 5:33.

8. Tong JC, Simarmata D, Lin RT, Rénia L, Ng LF. 2010. HLA Class I restriction as a possible driving force for Chikungunya evolution. PLoS One 5:e9291.

9. Volk SM, Chen R, Tsetsarkin KA, Adams AP, Garcia TI, Sall AA, Nasar F, Schuh AJ, Holmes EC, Higgs S, Maharaj PD, Brault AC, Weaver SC. 2010. Genome-scale phylogenetic analyses of Chikungunya virus reveal independent emergences of recent epidemics and various evolutionary rates. J Virol 84:6497-6504.

10. Akahata W, Yang ZY, Andersen H, Sun S, Holdaway HA, Kong WP, Lewis MG, Higgs S, Rossmann MG, Rao S, Nabel GJ. 2010. A virus-like particle vaccine for epidemic Chikungunya virus protects nonhuman primates against infection. Nat Med. 2010; 16(3):334-8.

11. Mallilankaraman K, Shedlock DJ, Bao H, Kawalekar OU, Fagone P, Ramanathan AA, Ferraro B, Stabenow J, Vijayachari P, Sundaram SG, Muruganandam N, Sarangan G, Srikanth P, Khan AS, Lewis MG, Kim JJ, Sardesai NY, Muthumani K, Weiner DB. 2011: A DNA

vaccine against Chikungunya virus is protective in mice and induces neutralizing antibodies in mice and nonhuman primates. PLoS Negl Trop Dis.; 5(1):e928.

12. Edelman R, Tacket CO, Wasserman SS, Bodison SA, Perry JG, Mangiafico JA. 2001: Phase II safety and immunogenicity study of live Chikungunya virus vaccine TSI-GSD-218. Am J Trop Med Hyg. 2000; 62(6):681-5.

We claim :

1. A stable vaccine composition of inactivated Chikungunya virus strain with one or more mutations that enhance the adaptation of the virus to *Aedes aegypti* and capable of infecting *Aedes albopictus* or purified virus antigens derived from the strains thereof, as the therapeutically active ingredient for prophylaxis, treatment and diagnosis of infections caused by Chikungunya virus thereby making it capable of conferring immunity against any genotypic variants or mutants of Chikungunya virus.

2. The vaccine composition of claim 1, wherein the strains of the Chikungunya virus isolates are selected from TN01610, TN015110, TN06210, TN06310, TN06410, and AP109 containing the nucleotide sequences disclosed in SEQ. ID. NO. 1 to SEQ ID NO.7 and SEQ ID NO.15 to SEQ ID NO.20 for recombinant cloning and expression of structural antigens in eukaryotic or prokaryotic expression vectors to be used as potential vaccine candidates against Chikungunya virus infections.

3. The vaccine composition of claim 1, wherein the Chikungunya virus strain has a non-synonymous mutation K1020E in the structural polyprotein corresponding to E1-K211E in the E1 structural glycoprotein either singly or in combination with other mutations selected from A232V, D301N, K327N, G380R, K391E, V589A, P867L, G1004R, and A1035V of the structural polyprotein sequence of the Chikungunya virus.

4. The vaccine composition of claim 1, wherein the Chikungunya virus isolates having the structural polyprotein sequences as disclosed in SEQ. ID No. 8 to SEQ. ID No. 14 corresponding to nucleotide SEQ ID. NO.1 to SEQ ID NO.7 respectively, for use as vaccine antigens against Chikungunya virus infections and any genotypes or mutants thereof.

5. The vaccine composition of claim 1, wherein the therapeutically active antigen is a combination of the capsid protein and the structural glycoproteins of the

Chikungunya virus strains as disclosed, comprising of C-E3-E2-6K-E1, C-E2-E1 and E2-E1 proteins for expression as Virus Like Particles.

6. The vaccine composition of claim 1, wherein the prokaryotic expression system for expression of recombinant proteins of the Chikungunya virus strains as disclosed is *E.coli* and the eukaryotic expression system is yeast *Pichia pastoris*.

7. The vaccine composition of claim 1 wherein the Chikungunya virus is inactivated by any of the following methods:

   i) heat inactivation at 45°C to 60°C for 30 min to 4 hrs;
   ii) Ultraviolet radiation at 254 nm for 30 min to 120 min;
   iii) Formalin treatment at ratios upto 1: 3000 (formalin:virus) at 2°C-8°C for 7 days or at ambient temperatures ranging from 20°C-25°C for 2 days;
   iv) Beta propiolactone (henceforth BPL) ratios upto 1: 1000 to 1:2500 (BPL:virus) at 2°C-8°C for 7 days or at ambient temperatures ranging from 20°C-25°C for 2 days wherein the additive is selected from glycine, mannitol, sorbitol, sucrose and trehalose;
   v) Gamma irradiation by exposing the virus samples to a dose of 10kGy (Kilo Gray) to 25 kGy from a ^{60}Co source.

8. The vaccine composition of claim 1 further comprising an adjuvant selected from (a) aluminum hydroxide (b) aluminum phosphate (c) gamma inulin, (d) algammulin (a combination of aluminum hydroxide and gamma inulin) (e) cholecalciferol in oil (f) an oil in water emulsion OWEM1, containing squalene, tween-80, Span-85 in 10 mM phosphate-citrate buffer, (f) oil in water emulsion OWEM2 containing squalene, tween-80, Span-85, alpha tocopherol in phosphate-citrate buffer, (g) an oil in water emulsion OWEM3 containing squalene, tween-80, Span-85, cholecalciferol in phosphate-citrate buffer.

9. The vaccine composition of claim 1, further comprising the viral antigen at doses ranging from 1 μg to 100 μg per human dose in 40 mM phosphate buffer and 150mM NaCl.

10. A method of eliciting a protective immune response in human individuals against Chikungunya virus infections comprising administering the vaccine composition of claim 1 to humans through any of the routes selected from intramascular, intradermal, subcutaneous, intravenous, oral or intranasal.

11. The use of Chikungunya virus isolates whose structural polyprotein gene sequence is disclosed in SEQ. ID. Nos.1 to 7 and proteins translated thereof, as disclosed in SEQ. ID. Nos.8 to 14 as an immunodiagnostic agent for detection of Chikungunya virus infections in humans.

12. A combined vaccine composition comprising inactivated Chikungunya virus antigen and inactivated Japanese Encephalitis whole virion antigen and an adjuvant selected from (a) aluminum hydroxide (b) aluminum phosphate (c) gamma inulin, (d) algammulin (a combination of aluminum hydroxide and gamma inulin) (e) cholecalciferol in oil (f) an oil in water emulsion OWEM1, containing squalene, tween-80, Span-85 in 10 mM phosphate-citrate buffer, (f) oil in water emulsion OWEM2 containing squalene, tween-80, Span-85, alpha tocopherol in phosphate-citrate buffer, (g) an oil in water emulsion OWEM3 containing squalene, tween-80, Span-85, cholecalciferol in phosphate-citrate buffer for eliciting a protective immune response against Chikungunya virus and Japanese Encephalitis virus infections in humans