CHIKUNGUNYA VACCINE FORMULATIONS

FIELD OF THE INVENTION

The present invention is related to novel liquid and lyophilized formulations of Chikungunya virus useful as vaccines and methods for their preparation.

BACKGROUND OF THE INVENTION

The Chikungunya virus (CHIKV) is currently regarded as one of the most-likely viruses to spread globally or at least regionally, and morbidity due to this virus is considered a serious threat to global and regional public health, raising an urgent demand for efficient prophylaxis. Eminently, due to climate change, the threat posed by Chikungunya could be amplified increasing the size of the human population at risk of infection. However, at present there is no treatment or vaccine available against this CHIKV-induced debilitating disease and its various symptoms. CHIKV has been reported in over 100 countries with more than 2.2 million suspected cases in the Americas alone (World Health Organization. Number of reported cases of chikungunya fever in the Americas by country or territory 2013-2016: http://www.paho.org/hq/index.php?option=com_topics).

CHIKV is a small spherical RNA virus and a member of the Alphavirus genus in the family Togaviridae. The arthropod-borne virus is closely related to other viruses in Africa, South America and Australia that cause similar symptoms such as Ross River Virus, Mayaro-virus or O'nyong-nyong-virus. The virus is vectored by the daytime-biting *Aedes aegypti* mosquito, which also transmits yellow fever, Zika and dengue viruses. CHIKV can also be transmitted by *Aedes albopictus* mosquitoes, a more cold-tolerant mosquito that could easily facilitate the spread of Chikungunya to more temperate areas of the world. An infection with CHIKV results in chronic and incapacitating arthralgia affecting all gender and age groups accompanied by an acute febrile disease with headache, muscle pain, and skin rashes. The severe, often debilitating joint pain in infected patients can persist for years, especially in adults. Individuals who are at higher risk of more serious complications include infants, the elderly and individuals with chronic medical conditions. Since neither a specific antiviral treatment nor a vaccine is available to prevent CHIKV infection, prevention against CHIKV infection is therefore limited to non-treatment interventions such as the employment of insecticides, wearing long sleeves and pants, and other means to restrict exposure to vector mosquitoes.

Recently, a live-attenuated Chikungunya virus vaccine candidate designed for active immunization for the prevention of disease caused by CHIKV has been developed (Hallengärd *et al.*, Novel Attenuated Chikungunya Vaccine Candidates Elicit Protective Immunity in C57BL/6 mice, Journal of Virology, 2014, Vol. 88(5) p. 2858–2866). The candidate vaccine was further developed with the aim to prevent CHIKV disease in the general population living in endemic regions, as well as to serve as a

prophylactic measure for travelers to epidemic areas or areas at risk for an upcoming outbreak (see also WO2019057793, WO2017109223, WO2017109224). The replicating CHIKV vaccine comprises a large deletion of 60 amino acids in the nsP3 gene encoding the non-structural replicase complex protein nsP3 leading to attenuation of the virus *in vivo*. The candidate vaccine is based on the La Reunion strain of the East Central South African genotype and is produced in Vero cells and purified by centrifugation, ultrafiltration, batch-chromatography and sucrose gradient centrifugation. In C57BL/6 mice, the vaccine elicited high titers of binding and neutralizing antibodies after a single immunization and mice were subsequently protected from a high dose CHIKV challenge (Hallengard D, Kakoulidou M, Lulla A, Kummerer BM, Johansson DX, Mutso M, *et al.* Novel Attenuated Chikungunya Vaccine Candidates Elicit Protective Immunity in C57BL/6 mice. 2014 J Virol 88:2858–66. doi:10.1128/JVI.03453-1311). Essentially, a single immunization in non-human primates protected against a wild-type CHIKV infection (Roques P, Ljungberg K, Kümmerer BM, Gosse L, Dereuddre-Bosquet N, Tchitchek N, *et al.* Attenuated and vectored vaccines protect nonhuman primates against Chikungunya virus. 2017 JCI Insight 2:e83527. doi:10.1172/jci.insight.83527). The novel vaccine is designed to protect against all circulating genotypes of CHIKV throughout the world.

For worldwide distribution of CHIKV vaccines, it is necessary to formulate vaccines such that they are stable under a variety of environmental conditions and over an extended period of time. Components used to stabilize vaccines are known. However, particular formulations of components useful to stabilize CHIKV vaccines must be determined and might not be achieve suitable stability of the vaccine, e.g. of the attenuated vaccine candidate described above. Unfortunately, the live-attenuated forms of CHIKV proposed for vaccine use may be rather unstable in various environments. Thus, one object of the present invention is to provide formulations which stabilize CHIKV vaccines, and in particular which stabilize the CHIKV vaccine at a particular dose.

SUMMARY OF THE INVENTION

The present invention provides novel formulations of CHIKV useful as vaccines and methods for their preparation.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings are not intended to be drawn to scale. The Figures are illustrative only and are not required for enablement of the disclosure. For purposes of clarity, not every component may be labeled in every drawing. In the drawings:

Figure 1 Schematic illustration of the CHIKV-Δ5nsP3 genome structure. The Chikungunya virus genome encodes two polyproteins: non-structural proteins 1-4 (nsP1-4) and structural proteins (C, E3, E2, 6K, E1). Compared with the wild-type genomic sequence, the CHIKV-Δ5nsP3 sequence contains a 183-bp deletion in the 3' part of the sequence encoding nsP3 (amino acids 1656 to 1717 in the nsP1-4 polyprotein), which results in a 60 amino acid deletion in the nsP3 replicase protein (indicated by Δ60aa). SP, subgenomic promoter; UTR, untranslated region. (Figure adapted from Hallengärd D, *et al.*, 2014, *supra.*)

Figure 2 Effects of sorbitol, magnesium chloride and L-methionine on the stability of freeze-dried CHIKV at 37°C. $TCID_{50}$ assay (error bars of ±0.3 log) performed directly after reconstitution of the lyophilized pellet in WFI.

Figure 3 Effects of sorbitol, magnesium chloride and L-methionine on the stability of freeze-dried CHIKV at room temperature. $TCID_{50}$ assay (error bars of ±0.3 log) performed directly after reconstitution of the lyophilized pellet in WFI.

Figure 4 Effects of sorbitol, magnesium chloride and L-methionine on the stability of freeze-dried CHIKV at 2-8°C. $TCID_{50}$ assay (error bars of ±0.3 log) performed directly after reconstitution of the lyophilized pellet in WFI.

Figure 5 Overlay of DLS signals obtained from High dose DS before lyophilization and after lyophilization/reconstitution.

Figure 6 RT-qPCR data of DP before lyophilization and after lyophilization/reconstitution in WFI. WT sequence in the respective region compared to E168K mutation.

Figure 7 Stability of lyophilized CHIKV DP formulations at lab and intermediate (tech transfer) scales at 2-8°C. Results of $TCID_{50}$ assay (error bars of ±0.5 log) performed directly after reconstitution in WFI.

Figure 8 Stability of lyophilized CHIKV DP formulations at lab and intermediate (tech transfer) scales at room temperature. $TCID_{50}$ assay (error bars of ±0.5 log) performed directly after reconstitution in WFI.

Figure 9 Stability of lyophilized CHIKV DP formulations at lab and intermediate (tech transfer) scales at 37°C. $TCID_{50}$ assay (error bars of ±0.5 log) performed directly after reconstitution in WFI.

Figure 10 Influence of increasing phosphate concentration on CHIKV size in the liquid frozen formulation as assessed by DLS.

Figure 11 Influence of increasing NaCl concentration on CHIKV size in the liquid (frozen) formulation as assessed by DLS.

Figure 12 Influence of increasing citrate concentration on CHIKV size in the liquid (frozen) formulation as assessed by DLS.

Figure 13 Stability of CHIKV DS in the liquid (frozen) formulation at various temperatures.

Figure 14 Stability of CHIKV DP High dose in the liquid (frozen) formulation at various temperatures. Material stored in glass vials.

Figure 15 Clinical trial design **(A)** and profile **(B)**.

Figure 16 Radar plot of solicited local and systemic symptoms after single vaccination and revaccination (challenge) including severity grading (Safety Population). Participants with solicited AEs within 14 days after single vaccination with **(A)** Low dose (Group L; $3.2x10^3$ $TCID_{50}$/0.1ml) **(B)** Medium dose (Group M; $3.2x10^4$ $TCID_{50}$/1ml) and **(C)** High dose (Groups H1 and H2; $3.2x10^5$ $TCID_{50}$/1ml); or after high dose revaccination at 6 months **(D)** group H2 at M6; or 12 months **(E)** group L at M12; **(F)** group M at M12; **(G)** group H1 at M12, by maximum severity. Solicited AEs were graded as mild (Grade 1), moderate (Grade 2) or severe (Grade 3).

Figure 17 Viremia in plasma at Days 0, 3, 7 and 14 after immunization **(A)** and any challenge **(B)**. Limit of Detection (LOD)=1087 GCE/mL, Lower Limit of Quantification (LLOQ)=3261 GCE/mL. Time points with no available results in the treatment group were graphed as 500. GCE/mL – Genome copy equivalents per mL determined by quantitative real-time PCR.

Figure 18 Shedding of viral particles in urine at Days 0, 3, 7 and 14 after vaccination **(A)** and any challenge **(B)**. Limit of Detection (LOD) = 1087 GCE/mL, Lower Limit of Quantification (LLOQ) = 3261 GCE/mL. Time points with no available results in the treatment group were graphed as 500 GCE/mL – Genome copy equivalents per mL determined by quantitative real-time PCR; * Single subject in Group L.

Figure 19 Assessment of neutralizing antibodies after single vaccination. Seroconversion rates **(A)** and Geometric Mean Titer **(B)** after single vaccination by study group. The seroconversion rate was defined for the purposes of the trial as the percentage of subjects reaching a CHIKV-specific antibody titer of at least 20 ($\mu NT_{50} \geq 20$). * Pairwise test p=0.0092.

Figure 20 Assessment of neutralizing antibodies after challenge at Day 180 (M6) or Month 12 (M12). Seroconversion rates **(A)** and Geometric Mean Titer **(B)** in Groups L, M, H1 and H2 before and after re-vaccination with the highest dose at Month 6 or Month 12. Seroconversion was defined for the purposes of the trial as the percentage of subjects reaching a CHIKV-specific antibody titer of at least 20 ($\mu NT_{50} \geq 20$).

Figure 21 Assessment of seroconversion rates using increasingly stringent cutoff values. Seroconversion rate defined as percentage of subjects reaching a CHIKV-specific antibody titer of at

least **(A)** 40 (μNT_{50} 1:40), **(B)** 80 (μNT_{50} 1:80) and **(C)** 160 (μNT_{50} 1:160) after single and re-vaccination by study day and treatment groups.

Figure 22 (A) Correlation of neutralization titers of a panel of 111 CHIKV-Δ5nsP3 phase 1 sera from different time points tested in μNT against the vaccine strain CHIKV-Δ5nsP3 (based on the La Reunion strain (LR2006-OPY1) of the East Central South African (ECSA) genotype) and in μPRNT against the attenuated CHIKV strain 181/clone 25 of the Asian genotype. Correlation of titers was calculated by Pearson correlation coefficient using all samples with titers ≥LLOQ in both assays (n=75) (Only samples with positive titers were shown.) **(B)** Correlation of neutralization titer of CHIKV-Δ5nsP3 sera tested in μPRNT against CHIKV strain 181/clone 25 of the Asian genotype and CHIKV total IgG ELISA titer (based on viral proteins C, E1 and E2 from West African strain 37997). Correlation of titers was calculated by Pearson correlation coefficient using all samples with titers ≥LLOQ in both assays (n=65) (Only positive titers were shown in the analysis.)

Figure 23 Pair-wise comparison of neutralization titers of CHIKV-Δ5nsP3 trial sera collected at different visits of individual subjects measured against the vaccine strain CHIKV-Δ5nsP3 (μNT_{50}) and **(A)** the wild-type La Reunion strain ($PRNT_{50}$ wt CHIKV-LR) and **(B)** the wild type West African strain 37997 ($PRNT_{50}$ wt CHIKV-WA 37997). Three samples having a titer >5,120 in the $PRNT_{50}$ wt CHIKV-LR assay are plotted as 5,120. Negative samples were imputed with half the LLOQ for each method (μNT_{50}=10, $PRNT_{50}$=5).

Figure 24 Stability comparison of lyophilized DP produced at intermediate (technical transfer) and lab scales at 2-8°C storage up to 19 and 21 months, respectively, as assessed by $TCID_{50}$ at each timepoint directly after reconstitution in WFI.

Figure 25 Stability of lyophilized DP produced at intermediate scale (technical transfer; TTR2) at 2-8°C, room temperature (RT) and 37°C, as assessed by $TCID_{50}$ at each timepoint directly after reconstitution in WFI.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides novel formulations of CHIKV useful as vaccines and methods for their preparation. More particularly, the invention relates to stabilizing formulations for liquid and lyophilized CHIKV vaccines.

Due to the worldwide distribution of vaccines, the wide variety of ambient temperatures and the requirement to produce vaccine at an affordable price, it is necessary to formulate vaccines such that they are stable under a variety of environmental conditions, incur reasonable costs and can be produced swiftly in accordance to the demand which may fluctuate substantially, e.g. during an

outbreak. A variety of stabilization methods and approaches in this regard have been used. These include the following:

a) Low temperatures (−10° C. to −70° C.). Most vaccines are stable during storage at extremely low temperatures. However, low temperature storage facilities are costly and are not always available, particularly in developing countries; this limits the utility and practicality of this approach.

b) Lyophilization. Freeze-dried vaccines are reasonably stable and can be stored at 2-8° C for a predefined length of time. Lyophilization may, however, result in a loss of viral titer during drying thereby reducing the yield of the manufacturing process or might alter sub-population of the virus in a heterogeneous virus population. A lyophilized virus vaccine is typically more stable than a liquid formulation; however, during long-term storage, the lyophilized vaccine may still deteriorate, sometimes to the point where it contains an insufficient titer to confer immunization. Furthermore, since a lyophilized vaccine requires reconstitution prior to use, a liquid reconstituted preparation may lose potency while standing at room temperature before use. This loss of titer during reconstitution may also result in insufficient titer to confer immunity.

c) Stabilizers. These are specific chemical compounds that interact with and stabilize biological molecules and/or general pharmaceutical excipients that are added to the vaccine and used in conjunction with either lower temperature storage or lyophilization methods. However their use and effectiveness is not known for a new virus and might be restricted.

These formulations can be prepared by either (1) dilution of bulk vaccine into the stabilizer, (2) dialysis/diafiltration into the stabilizer, or (3) concentration of bulk vaccine and dilution or diafiltration into the stabilizer, followed by lyophilization.

The amounts and concentrations of the components of the formulations described herein will be understood by those skilled in the art to refer to the weight/volume percentage when referring to lyophilized or liquid formulations. For example, a 10% concentration in a liquid formulation is 10 grams per 100 milliliters and a 10% concentration of a lyophilized formulation refers to 10 grams per 100 milliliters in the liquid form before lyophilization. The lyophilized formulations are reconstituted in WFI or another acceptable diluent to approximately the same volume as before lyophilisation, i.e., the concentrations in the lyophilized formulations as used herein generally refer to concentrations both before lyophilisation and after reconstitution. Other measures, such as the molarity of a compound, refer to a liquid formulation or to a lyophilized formulation both before lyophilization and after reconstitution.

The compositions of the present invention contain the respective ingredients in about the amounts indicated. For convenience, the amounts are stated in round numbers. However, one skilled in the art will recognize that amounts within 10 or 20 percent of the stated values can also be expected to be

appropriate, i.e., where 20% is stated, a range of from 16-18% to 22-24%, e.g., a range of about 16-24% is implicit and can be appropriate.

In the course of the current invention, it was found that certain buffer systems, such as e.g. histidine, under certain conditions, induced aggregation of CHIKV vaccine candidates, e.g. those described herein. A low (e.g. ~5 mM) phosphate-citrate buffer system, which was shown to be amenable to lyophilisation and promoted the stability and desired size of CHIKV particles, also containing other stabilizing components, was developed for the lyophilized CHIKV vaccine formulation. A similar formulation was developed for the liquid (frozen) CHIKV vaccine.

For liquid CHIKV formulations:

Sucrose: 1-50% (w/v)

Sodium or potassium phosphate: 1-20 mM

Sodium succinate or sodium citrate: 1-50 mM

Human Serum Albumin: 0.001 to 1%

Tissue culture medium, saline, or water: balance of remaining volume

For lyophilized CHIKV formulations:

Sucrose: 1-20% (w/v)

Sodium or potassium phosphate: 1-20 mM

Sodium succinate or sodium citrate: 1-50 mM

In addition, the following can also be present:

$MgCl_2$: 1-10 mM

D-sorbitol: 0.1-5 % (w/v)

L-methionine: 1-20 mM

Buffering agents in vaccine formulations

An important aspect of any vaccine formulation is maintenance of a steady pH value. A buffer for the composition of the CHIKV vaccines of the current invention must not only maintain pH during storage, but must also be compatible with the necessary processing steps of the CHIKV vaccine. For example, the CHIKV vaccine must be sterile filtered after formulation; therefore, the buffering system (and other components) must not promote the formation of virus aggregates which are too large to filter. The buffer must effectively maintain the pH under a broad range of temperatures during lyophilisation. In this regard, the use of phosphate at generally used concentrations, such as 10 mM or

above, may be problematic due to its concentration during freezing and subsequent possible precipitation leading to significant pH shifts, especially at higher phosphate concentrations. For this reason, alternative buffering agents were investigated, but did not result in improved stability or cake structure (data not shown). In a preferred embodiment, it was found that phosphate could be reduced to 1 to 5 mM, preferably to about 5 mM in the lyo formulation. Alternatively, HEPES buffer at a concentration of about 20 mM is also acceptable. It is preferred to use an additional buffering agent along with phosphate, preferably the additional buffering agent is a carboxylate, selected from the group consisting of succinate, citrate, fumarate, tartarate, maleate and lactate, preferably citrate. These particular carboxylates help to inhibit aggregation.

The following compounds can be used in place of sucrose, and at comparable osmolality: sucrose, mannitol, dextran, lactose, sorbitol, dextrose, fucose, and trehalose. In a preferred embodiment, sucrose and/or trehalose is used, most preferably sucrose Sucrose at 5% has been shown to protect CHIKV during freeze/thaw stress. Trehalose has the advantage of enabling the use of higher temperatures during the lyophilisation process.

The concentration of sugar relates to the viscosity of the formulation. In instances where reduced viscosity is desired, it is known in the art to be preferable to use lower concentrations of sugar, e.g., sucrose. It will also be appreciated by persons in the art that the upper limit for the concentration of sugar can be dictated by the ability of a formulation to undergo required filtration or processing steps.

Amino acids can be employed in the lyophilized formulations taught herein. It has been found that amino acids can improve the stability of a vaccine prepared in the lyophilized formulations. Preferred amino acids are L-methionine, arginine and glutamine. A concentration of about 1-20 mM is appropriate. A concentration of about 10 mM is preferred in lyophilized formulations. A combination of amino acids can be used but the overall concentration of the combined amino acids should be no more than 20 mM.

Another excipient useful in the lyophilized formulations is D-sorbitol which addition in small quantities can result in retention of protein native structure and improved stability as demonstrated for lyophilized antibodies (Chang et al. Liuquan (Lucy) Chang. Deanna Shepherd. Joanna Sun. Xiaolin (Charlie) Tang Michael J. Pikal; J Pharm Sci. 2005 Jul;94(7):1445-55. Effect of sorbitol and residual moisture on the stability of lyophilized antibodies: Implications for the mechanism of protein stabilization in the solid state) and for ribonuclease A (Xie Guifu and Timasheff Serge. Protein Science 1997. 6. 211-221: Mechanism of the stabilization of ribonuclease A by sorbitol: Preferential hydration is greater for the denatured than for the native protein).

Another excipient useful in the lyophilized formulations is magnesium chloride which is assumed to stabilize RNA structure of CHIKV and exhibited a positive effect on infectivity after storage. A particularly useful concentration for this purpose of the invention is about 5 mM.

Another excipient useful in both liquid and lyophilized formulations of vaccines as taught herein is recombinant human serum albumin (rHSA). Recombinant human serum albumin is produced using gene expression systems and therefore is safer to use than albumin isolated from the serum of human beings. The concentration of the albumin is typically in the range of about 0.1 to about 2%, preferably about 1.0%. However, we found to be able to reduce the albumin to 0.01% which can reduce the costs considerably.

Tissue culture medium, saline or water, such as WFI or milliQ water, preferably WFI, may be used as a diluent. The amount of diluent used to reconstitute the lyophilized cake may be from 0.25 to 2.5 times the volume of the formulation before lyophilisation, or 0.5 to 1.75 times, 0.75 to 1.25 times; however, there is a clear preference for reconstituting the lyophilized cake to about the same volume as before lyophilisation.

A preferred formulation of the liquid (frozen) CHIKV formulation of the present invention is as follows:

- 10 mM potassium phosphate
- 25 mM sodium citrate
- 5% sucrose
- 0.01% rHSA
- pH 7.3±0.2
- water

In these preferred formulations, it can be appropriate to use saline or tissue culture medium in place of water.

A preferred formulation of the lyophilized formulation (concentrations refer to the formulation before lyophilisation as well as after lyophilisation and reconstitution) of the present invention is as follows:

- 5 mM potassium phosphate
- 25 mM sodium citrate
- 5% sucrose
- 0.5% D-sorbitol
- 10 mM L-Methionine
- 5 mM $MgCl_2$
- 0.01% rHSA
- pH 7.3±0.2
- water

Any general method of lyophilization known in the art may be used. As a general example of a lyophilization process, freezing may be performed at -40°C, followed by primary drying in 2 steps

(e.g., -35°C for 3 hours, followed by -25°C for 30 hours, both at 100 µbar measured by Pirani). Secondary drying may be performed at 20°C followed by a second step at 25°C (both at 70 µbar vacuum). Particularly preferred is a lyophilisation method providing an adequate cake structure, i.e., a cake without cracks and with minimal shrinkage.

The dilution factor of DS to DP bulk depends on the viral concentration of DS (determined by $TCID_{50}$ assay). Formulation buffer for DS and DP formulation are identical: 25 mM sodium citrate. 5 mM potassium phosphate. 5% sucrose. 0.01% rHSA. 10 mM L-methionine. 5 mM MgCl2. 0.5% sorbitol. pH 7.3. The target concentrations after dilution to DP are 5.8 $\log_{10}$ $TCID_{50}$/mL (= 6.3 x 10^5 $TCID_{50}$/mL, DP high) and 4.8 $\log_{10}$ $TCID_{50}$/mL (= 6.3 x 10^4 $TCID_{50}$/mL, DP low). This accounts for possible losses during filtration and fill/lyophilization of the vaccine at larger scale and should guarantee a final virus dose per vial of ~10^5 (~10^4) $TCID_{50}$ after freeze drying.

This invention involves formulations of CHIKV and in particular for attenuated live CHIKV, preferably "CHIKV-Δ5nsP3" which is the CHIKV "LR2006_OPY1" lacking a 60 amino acid stretch in the nsP3 region, also referred to herein as CHIKV-Δ5nsP3 (see Hallengärd, *supra*, **Figure 1** and SEQ ID NO: 1) or any functional variant of CHIKV-Δ5nsP3 with 95%, preferably 96%, 97%, 98%, 99%, most preferred 99% sequence identity, still including the deletion of said 60 aa stretch. Variants of CHIKV-Δ5nsP3 include variants in E2 of CHIKV-Δ5nsP3 with one or more mutation(s) in E168K (SEQ ID NO: 3), G55R (SEQ ID NO: 4), E247K (SEQ ID NO: 5), G82R (SEQ ID NO: 6) and/or H232Y (SEQ ID NO: 7). Variants may also include silent mutations. SEQ ID NO: 2 is the E2 wild type sequence of LR2006_OPY1. In a preferred embodiment, the mutations (e.g. the mutation E168K) in the E2 structural protein are present at a frequency of 70% or less, e.g., less than 70% of the total CHIKV-Δ5nsP3 particles comprise one or more mutations (or the mutation E168K) and 30% or more of the total CHIKV-Δ5nsP3 particles express a non-mutated E2 structural protein or an E2 structural protein that does not comprise the mutation E168K. A particularly preferred formulation comprises a population of two or more variants as described herein. An even more preferred formulation comprises a population of substantially two strains encoding for E2 amino acids sequences SEQ ID NO: 2 (wild type) and SEQ ID NO: 3 (with the E168K mutation) and wherein the E1 structural protein is unchanged, i.e. the wild type E1 protein as encoded in the genomic sequence provided by SEQ ID NO: 1. Said formulation is also referred to as a formulation comprising the "CHIKV-Δ5nsP3-inv" population, wherein the frequency is about 10% to 90% of SEQ ID NO: 2. In a more preferred embodiment, the frequency of said SEQ ID NO: 2 may be around 30 to 70%, more preferably about 50%.

Said CHIKV-Δ5nsP3 constructs, including CHIKV-Δ5nsP3-inv, that are alone or in combination suitable for use as vaccines are characterized by safety to humans and the ability to confer immune protection against CHIKV infection in humans. Interestingly, it was found that small changes to the RNA genomic sequence corresponding to the DNA sequence provided by SEQ ID NO: 1 might render

certain formulations less useful for industrial use because of changes in charges that are exposed to the formulation solution.

In one embodiment, the pharmaceutical composition (herein also referred to simply as "formulation" or "vaccine", said terms can be used interchangeably in this invention) increases serum antibody titers in a vaccinated human subject by at least 1 log, relative to a control, within about 5 to 28 days. In a preferred embodiment, the pharmaceutical composition increases serum antibody titers in a vaccinated human subject by at least 1 log, relative to a control, within about 14 days. In a preferred embodiment, the pharmaceutical composition increases serum antibody titers in a vaccinated human subject by at least 1 log, relative to a control, within about 7 days. In one embodiment, said control is pre-immune sera from the same human subject; e.g., collected before vaccination. In one embodiment, said control is sera from a placebo-treated or non-vaccinated subject or subjects.

In one embodiment, the pharmaceutical composition of the invention stimulates seroconversion in at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, up to 100% of vaccinated subjects within 14 days of a single vaccination. In one embodiment, the pharmaceutical composition of the invention stimulates seroconversion in at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, up to 100% of vaccinated subjects within 7 days of a single vaccination. In one embodiment, seroconversion is defined as reaching a CHIKV-specific antibody titer, i.e., a neutralizing antibody titer, of at least 10. The neutralization of Chikungunya virus may be assessed in an *in vitro* assay, such as a $TCID_{50}$ assay and/or a neutralizing assay such as a PRNT, i.e. $PRNT_{50}$ or microtiter assay, i.e., μNT_{50} wherein a range of serum dilutions are tested for neutralization of CHIKV growth and calculating the dilution that neutralizes 50% of growth compared with a negative control. As used herein, a PRNT50 assay gives a readout in countable plaques and a µNT50 assay gives a colorimetric readout which is proportional to cytopathic effect. The 50% reduction of CHIKV virus growth in a $TCID_{50}$ assay (or a $PRNT_{50}$ or μNT_{50} assay) by a 1:10 or 1:20, preferably 1:20, or higher dilution of immune sera is defined herein as seroconversion. The value is reported as the reciprocal of the dilution factor, e.g., 50% CHIKV neutralization at a 1:10 immune serum dilution is referred to as a neutralizing titer of 10, or 50% CHIKV neutralization at a 1:20 immune serum dilution is referred to as a neutralizing titer of 20. As defined herein, neutralizing titer values of 10 or higher are all defined as seroconversion, with 10 being the minimum and 20 or higher being preferred.

In one embodiment, the pharmaceutical composition of the invention confers a protective immune response against CHIK virus disease, which protective immune response is long-lasting. In one embodiment, the pharmaceutical composition of the invention confers lifelong protection against CHIK virus disease. In one embodiment, the protective immune response is sustained from at least 6 months up to a lifetime, e.g., several decades, such as 10 to 70 years or beyond. In one embodiment,

the protective immune response is sustained up to at least 50 years, at least 40 years, at least 30 years, at least 25 years, at least 20 years, at least 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 years, at least 1 year. In a preferred embodiment, the protective immune response lasts at least 6 months, at least 12 months, at least 24 months. A protective immune response is an immune response in which neutralizing antibodies are produced which are sufficient for reducing or preventing signs or symptoms of Chikungunya virus disease.

In one embodiment, the pharmaceutical composition is suitable for use in a method of treating or preventing a Chikungunya virus infection but also include treating or preventing certain cancers. Particularly, the pharmaceutical composition is suitable for use in vaccinating a human subject and stimulating a protective immune response in said subject. In a preferred embodiment, the method of treating or preventing a Chikungunya virus infection according to the current invention comprises administering an effective amount of the pharmaceutical composition as defined herein to a subject in need thereof. A subject in need of vaccination against CHIKV according to the current disclosure can be any human subject in danger of exposure to the virus, such as a traveller to an endemic or outbreak country or an inhabitant of an endemic or outbreak country or a country in danger of an outbreak.

Unless otherwise defined herein, scientific and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. The methods and techniques of the present disclosure are generally performed according to conventional methods well known in the art. Generally, nomenclatures used in connection with, and techniques of biochemistry, enzymology, molecular and cellular biology, microbiology, virology, cell or tissue culture, genetics and protein and nucleic chemistry described herein are those well-known and commonly used in the art. The methods and techniques of the present disclosure are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated. The formulations, such as e.g. vaccines, of this invention are particularly useful for the prevention and/or therapy of any Chikungunya infection (induced by any CHIKV strain) in humans or other susceptible animals. However, said formulations might also be useful in other indications such as e.g. cancer of any form (again in a preventative and/or therapeutic setting).

Method of Vaccination

Therefore preferred, also included in the invention is a method of vaccinating humans against human CHIKV infection with the novel CHIKV vaccine compositions of this invention. The formulations, i.e. vaccine compositions, including one or more of the attenuated live CHIKV and excipients described herein are administered, preferably by the intramuscular or subcutaneous route, in a suitable dose as determined also herein, preferably in a lyophilized (reconstituted) form to a subject.

In some embodiments, the preparations or compositions may be administered via conventional routes, such as parenterally. As used herein, "parenteral" administration includes, without limitation, subcutaneous, intracutaneous, intradermal, intravenous, intramuscular, intraarticular, intraperitoneal, intrathecal or by infusion.

The dosage for the intramuscular or subcutaneous route is proposed to be between 1 x 10^3 $TCID_{50}$/dose and 2 x 10^4 $TCID_{50}$/dose with a target such as $5.01x10^3$ $TCID_{50}$/dose (dose, e.g. 1 ml in volume, then also $5.01x10^3$/ml) of the attenuated CHIKV candidates herein disclosed (mixture of strains with E2 of SEQ ID NOs: 2 and E2 of SEQ ID NO: 3) and data presented herein support a one shot dosage administration for being fully protected possibly as soon as 7 days after primary vaccination, but clearly after 14 days after primary vaccination. CHIKV infection in humans has been observed to occur in various geographical regions including in the United States.

The following examples illustrate methods for preparation, usage and administration of the CHIKV vaccine formulations of the invention. These examples are illustrative only and do not limit the scope of the invention.

EXAMPLES

Example 1. CHIKV Freeze Dried (Lyo) Product Formulation Development

Definitions & Abbreviations

CHIKV	Chikungunya virus
CHIKV-Δ5nsP3-inv	an immunogenic mixture comprising CHIKV-Δ5nsP3 particles comprising the RNA genome corresponding to the DNA sequence provided by SEQ ID NO: 1 and CHIKV-Δ5nsP3 variants with an RNA genome at least 99% identical to the corresponding DNA sequence provided by SEQ ID NO:1, but encoding a viral polyprotein having at least one amino acid difference, preferably in the region encoding envelope protein E2 (also referred to herein as CHIKV-Δ5nsP3)
CTMA	CTM Analyitics & Development department
CMO	Contract Manufacturing Organization
DLS	Differential Light Scattering
DP	Drug Product comprising CHIKV-Δ5nsP3-inv
DS	Drug Substance comprising CHIKV-Δ5nsP3-inv
DSP	Down Stream Process
mDSC	Modulated Differential Scanning Calorimetry
GCE	Genome copy equivalents
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
PC	Polycarbonate
PD	Process Development Department
PETG	Polyethylene terephthalate glycol-modified
PS	Particle Size
rHSA	Recombinant Human Serum Albumin
RT-qPCR	Reverse Transcription - quantitative Polymerase Chain Reaction
SGP	Sucrose gradient pool
$TCID_{50}$	Tissue culture infectious dose 50%
TRIS	Tris(hydroxymethyl)-aminomethane
TTR	Technical Transfer Run (non-GMP)

WFI Water for Injection

Materials and Methods

CHIKV Material

CHIKV-Δ5nsP3-inv with SEQ ID: 1 encoding for E2 (SEQ ID NO: 2) (including the other substantial variant CHIKV-Δ5nsP3 encoding for E2 (SEQ ID NO:3, with E1 and other expressed proteins unchanged) was produced in Vero cells and purified according to a processes described elsewhere (see WO2019057793, WO2017109223, WO2017109224). As used herein, CHIKV-Δ5nsP3-inv is also referred to herein as CHIKV-Δ5nsP3, CHIKV candidate.

Relevant experiments were conducted with representative virus material - produced in several lots - with regard to impurity profile and virus seed passage (P3).

Table 1. Sucrose Gradient Pool (SGP) material.

lot #	**Description**	**log_{10} $TCID_{50}$/mL**
1	SGP	9.0
2	SGP	8.8
3	SGP	8.0
4	SGP	8.4
5	SGP	10.0
6	SGP	9.8
7	SGP	10.0

$TCID_{50}$ Assay

Virus infectivity was determined by $TCID_{50}$ assay on vero cells. Virus titers were determined on Vero cells using the $TCID_{50}$ assay. Briefly, cells were seeded in microplates and infected with 10-fold serially diluted virus samples in EMEM supplemented with 0.5% FBS and 2 mM glutamine. After a one week incubation at 35°C / 5% CO2, virus-induced cytopathic effects were monitored and viral titers were calculated according to the Reed and Muench method (Reed, L.J.; Muench, H. A simple method of estimating fifty percent endpoints (1938) The American Journal of Hygiene 27:493–497). Assay control samples were included in each analysis. The assay variability between individual runs was estimated as ± 0.3 log_{10} $TCID_{50}$.

Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) is a technique that can be used to determine the size distribution profile of biopolymers including viral particles in solution at a size range from 1 nm to approx. 1000 nm. Since this method can be used with the native sample without any pre-treatment (e.g. no chromatography column that might filter out larger multimers/aggregates), a full picture of all particles in solution can be obtained. For DLS measurements a Malvern Zetasizer system was used. CHIKV-Δ5nsP3-inv sucrose gradient pools (~35% sucrose in Tris/NaCl) were analyzed without any pre-treatment (i.e. dilution) assuming a solution viscosity of 6.15 cP and refractive index 1.4. The virus particle refractive index was assumed as 1.45. For accurate measurement the particle concentration should not fall below a certain threshold which also depends on the size of the particles. For CHIKV-Δ5nsP3-inv the most accurate results are obtained for undiluted SGP. A comparison of representative SGP material (SGP pools in **Table 1**) showed a viral particle diameter of approximately 60 nm for all analyzed SGP samples (data not shown), which correlates to data referenced in literature.

Chemicals

Table 2. Chemicals and sources

Chemical	**Manufacturer**	**Order #**	**Quality**
$MgCl_2$ * 6 H_2O	Merck	105832	Multi-compendial
L-Methionine	AppliChem	A1340,0100	Ph.Eur., USP
K_2HPO_4 dibasic anhydrous	Sigma	RES20765-A7	Ph.Eur.
	MERCK	105101	Ph Eur,BP,E 340
KH_2PO_4 monobasic	Fluka	04243	Ph.Eur.
	MERCK	104871	Multi-compendial
Trisodium citrate dihydrate	Sigma	S1804	Ph.Eur.
	Citrique belge	04 12325	Multi-compendial
	MERCK	106432	Eur,BP,JP,USP
Sucrose	JT Baker	4005	Ph.Eur.
	MERCK	107653	Multi-compendial
rHSA (20% stock solution) Recombumin® Alpha (RF20-005)	Novozymes	n.a.	Ph.Eur.
D-Sorbitol	AppliChem	A2222,1000	Ph.Eur., USP-NF
WFI	HALIX B.V.	n.a.	Ph.Eur.

Preparation of Lyo formulation buffer

In short, for preparation of e.g. 5 liter formulation buffer the following procedure is applied:

- Fill approximately 4.5 L of WFI into a tared glass bottle with a stirring bar

Add all buffer components under stirring:

- 36.8 g - Trisodium citrate dihydrate

- 3.13 g di-Potassium Hydrogen Phosphate

- 0.975 g Potassium di-Hydrogen Phosphate

- 250 g Sucrose

- 25 g Sorbitol

- 7.46 g L-Methionine

- 5.08 g Magnesium Chloride hexahydrate

- 2.5 mL recombinant human Albumin (20% solution)

Stir until a clear solution is obtained.

Fill up to the final volume of 5000 mL with WFI.

The density (ρ) of the final solution is 1.025 g/mL (20°C). If 5000 mL are prepared the final resulting weight is 5125 g.

Freeze Dryers

Lab scale:

Lyophilization was performed on an AdVantage Pro bench top shelf tray dryer with Intellitronics Control from SP Scientific (USA):

Three shelves (total 2766 cm^2)

Shelf temperature: -60 to +60°C

Lowest condenser temperature: -70°C

Condenser capacity of 6 L

Stoppering: top-down pneumatic

Intermediate scale:

Lyophilization at an intermediate scale was performed with a Lyofast 7 freeze drier from IMA (Industria Macchine Automatiche S.p.A., Italy)

Six shelves (total 6.7 m^2)

Shelf temperature minimum: -55°C

Lowest condenser temperature: -75°C

Condenser Capacity: 148 Kg

Primary Packaging (Vials and Stoppers)

For phase I:

2R Type I plus® glass vials (Schott AG), FluroTec stoppers (West Pharmaceutical Services)

Intended primary packaging for further clinical phases (lyophilized DP):

2R Type I glass vials (Schott AG), bromobutyl stoppers (West Pharmaceutical Services)

Results

The liquid formulation buffer of Example 2 (herein, below) was chosen as a starting buffer composition for the further development of a lyophilized formulation which would ensure sterile filterability during DS and DP production:

10 mM potassium phosphate

25mM sodium citrate

5% sucrose

0.01% rHSA

pH 7.3

Most analytical data were generated by $TCID_{50}$ assay as this method not only indicates infectivity of the virus but is also used during release and stability testing. In addition, dynamic light scattering (DLS) for evaluation of the particle size and qPCR for total viral particle determination were used. Results shown in all of Figures 2-14 were generated using formulations of the high dose of the CHIKV vaccine.

Justification of Buffer Components

In general, the concentration of buffer ions used in freeze-dried and frozen systems must be low enough to prevent concentration effects during the freezing process, but still high enough to provide adequate buffering capacity at the desired pH. Phosphate ions are generally avoided for freeze drying purposes as concentration effects and precipitation during freezing can lead to significant pH shifts, especially at higher concentrations (Sek, D. Breaking old habits: moving away from commonly used buffers in pharmaceuticals 2012 European Pharmaceutical Review https://www.europeanpharmaceuticalreview.com/article/13699/breaking-old-habits-moving-away-from-commonly-used-buffers-in-pharmaceuticals/). Therefore Tris, HEPES and Histidine were tested alongside phosphate (4 mM, 5 mM and 10 mM) as alternative buffer components. The influence of

these buffering agents on the stability of the lyophilized product in the presence of 10 mM L-methionine and 25 mM sodium citrate was assessed at 37°C, room temperature and 4°C storage temperature. Overall, phosphate and HEPES buffer (20 mM) showed comparable stability profiles at all investigated temperatures and outperformed the other buffer compositions (data not shown). Therefore, additional experiments were performed for comparison of these two buffers: 5 mM phosphate buffer or 20 mM HEPES, both formulations including 4% Sucrose, 1% Trehalose, 10 mM L-Methionine, 2mM EDTA), showing no significant differences in stability of the lyo CHIKV formulation over time (data not shown).

Based on the overall results it was decided to keep phosphate as buffering agent (as in the liquid frozen formulation, also together with a citrate buffer), but at the lower concentration of 5 mM to minimize buffer concentration effects and possible pH shifts during freezing.

Phosphate-Citrate Buffer: Liquid formulation buffer development for early clinical phases was aimed at 0.2µm sterile filterability of CHIK virus during DS and DP manufacturing. A buffer system consisting of phosphate and citrate at pH 7.3 proved to stabilize the viral particle size of CHIKV and guarantees 0.2 µm filterability, which is crucial for aseptic manufacturing. To minimize possible ion concentration effects and to facilitate lyophilization, the final phosphate concentration in the lyo formulation was reduced to 5 mM.

Sucrose

During downstream processing, a sucrose gradient centrifugation is performed for final concentration and polishing of the CHIKV material, resulting in a sucrose concentration of approximately 35% in the sucrose gradient pool (SGP). As sucrose is a well-known stabilizer during freezing of biological material and also serves as a bulking material, it was kept in the formulation buffer for the freeze dried product. Sucrose at 5% has been shown to protect CHIKV during freeze/thaw stress. By subsequent dilution of SGP to DS (currently 1:60) and DP with formulation buffer a final sucrose concentration of 5% is obtained.

Recombinant Human Albumin

The concentration of rHSA was kept constant for the lyophilized product compared to the liquid formulation at a level of 0.01% (0.1 mg/mL). The incorporation of a minimal amount of rHSA is desired to prevent unspecific adsorption to surfaces of containers. Additionally rHSA at this concentration does not adversely affect the sterile filterability of the CHIKV nor the stability of the freeze dried product.

After these initial studies, the basic formulation of the lyophilized formulation ("basic lyo") was:

5 mM potassium phosphate

25mM sodium citrate

5% sucrose

0.01% rHSA

pH 7.3

Additional excipients tested for improved stability of the lyo formulation:

D-Sorbitol

Lyophilization of CHIKV-Δ5nsP3-inv in the basic lyo buffer showed insufficient stability in the freeze dried state (see Figs. 2-4). The addition of sorbitol alone exhibited a significant improvement of stability, especially under accelerated storage conditions (37°C, RT; Figs. 2 and 3, respectively). A positive stabilizing effect of sorbitol on CHIKV was observed for all exploratory formulations tested during development. As sucrose alone already exhibits a rather low collapse temperature (T_C) of -32°C during freeze drying, the concentration of sorbitol (T_C -45°C) was set to 0.5% to prevent an additional significant decrease of the overall T_C but providing significant stabilization of the freeze dried product.

Magnesium Chloride

Magnesium chloride is assumed to stabilize the RNA structure of CHIKV and exhibited a positive effect on infectivity after storage (Figs. 2-4). It is incorporated into the lyo formulation buffer at a concentration of 5 mM.

L-Methionine

L-Methionine is regarded as an oxidant scavenger applied in protein formulations. When added at a final concentration of 10 mM it increased stability during storage at 2-8°C (Fig. 4) and more pronounced at accelerated temperatures (37°C, RT; Figs. 2 and 3, respectively).

Effect of excipients on CHIKV stability in the freeze dried state

Positive effects the stability of freeze dried CHIKV-Δ5nsP3-inv of addition to the basic lyo formulation of sorbitol (0.5%), magnesium chloride (5 mM) and L-methionine (10 mM) and combinations thereof at various temperatures (37°C, RT and 2-8°C) are summarized in Figures 2-4, respectively (as assessed by $TCID_{50}$ over days of storage). The one-by-one addition of the respective additives to this basic buffer is indicated in the graphs. As starting point the theoretical $TCID_{50}$ value after dilution to DP concentration (5.7 $\log_{10}$ $TCID_{50}$/mL) was assumed for all formulations.

Compared to lyophilization of CHIKV-Δ5nsP3-inv in basic lyo formulation buffer, a significant stabilization by addition of sorbitol alone and especially in combination with L-methionine and magnesium chloride was observed under accelerated conditions.

At 37°C, the loss of infectivity improved from approximately 3 $\log_{10}$ to 1 $\log_{10}$ per month and at room temperature from approximately 1 $\log_{10}$ to 0.2 $\log_{10}$ per month. When CHIKV-Δ5nsP3-inv was

lyophilized in basic lyo formulation buffer without the addition of sorbitol, $MgCl_2$ or L-methionine and stored at 2-8°C (Fig. 4), a significant difference could be observed (approximately 1 log_{10} loss after half a year) compared to the other formulations.

Subsequent testing of lyophilized CHIKV was performed in lyophilization buffer (also referred to herein as freeze drying formulation buffer and lyo buffer) as follows:

5 mM potassium phosphate

25mM sodium citrate

5% sucrose

0.01% rHSA

5 mM $MgCl_2$

0.5% D-sorbitol

10 mM L-methionine

pH 7.3

Comparison of CHIKV before and after freeze drying

Dynamic Light Scattering (DLS)

The exact size of CHIKV can only be determined in concentrated samples (e.g. SGP) due to signal interference with buffer excipients (e.g. rHSA) at lower virus content. Comparative results are obtainable for samples diluted in lyo formulation buffer (containing rHSA) as long as the virus concentration is high enough. Therefore, Lot 1 SGP (9.0 log_{10} $TCID_{50}$/mL) was diluted 1:40 in freeze drying formulation buffer resulting in a virus concentration of approximately 7.4 log_{10} $TCID_{50}$/mL. This material was measured by DLS both before lyophilization and after lyophilization/reconstitution of the freeze dried virus (**Figure 5**). The determined virus size before freeze drying (135 nm) and after reconstitution (118 nm) were comparable.

Plaque Assay / RT-qPCR

When propagated in host cells, CHIKV show minor genetic heterogeneities at defined positions in the RNA genomic sequence, resulting in different populations of virus in any given preparation. Some of these defined heterogeneities are characterized by reduced immunogenicity of the virus (e.g., an E168K point mutation in the CHIKV E2 protein). Therefore, it was important to identify any potential change of virus composition due to different stability profiles of the individual viral genetic populations during lyophilization.

DP with a nominal concentration of 5.7 log_{10} $TCID_{50}$/mL was prepared from Lot 3 SGP by dilution in lyophilization buffer and subsequent freeze drying. Samples were taken before (5.69 log_{10} $TCID_{50}$/mL)

and after lyophilization (5.61 log_{10} $TCID_{50}$/mL) and analyzed in a plaque assay to determine plaque morphology (data not shown) and by RT-qPCR for quantification of E168K heterogeneity compared to the wild type sequence of the respective region (**Figure 6**). Lyophilization and reconstitution had no substantial effect on the ratio of the two populations.

CHIKV Stability Lyophilized DP

CHIKV Material

Relevant experiments (n=4) summarized in this report were conducted with representative DP material. **Table 3** below summarizes the investigated DP formulations and the CHIKV material used, which included both lab and intermediate scale (TTR) formulations.

Table 3. Residual moisture content of lab and intermediate (technical transfer) scale lyophilized DP.

Run	**Residual Moisture %**
Lab Scale Run F59B	1.7
Lab Scale Run F72	1.4
TTR2 intermediate scale	1.7
TTR3 intermediate scale	1.6

The standard storage condition of lyophilized CHIKV DP is 2-8°C. **Figure 7** shows stability data of different lots, i.e. TTR2, TTR3, F59B and F72, stored at 2-8°C. Data are shown as log_{10} $TCID_{50}$/mL values. Longer stability data are available for Lab sample F72 (21 months) and TTR2 (19 months) (see **Fig. 24**). As expected, the titer loss of the lyophilized formulation at refrigerated temperatures was minimal.

Accelerated stability studies conducted by the incubation of samples at elevated temperatures provides information with respect to stability differences within a shorter time frame. **Figure 8** shows stability data of TTR2, TTR3 and F59B stored at room temperature. The stability of TTR3 was also assess in parallel by a different internal department as a further control (TTR3-B). **Figure 9** depicts the stability data of the same formulations at 37°C. All data represent log_{10} $TCID_{50}$/mL values as assessed directly after reconstitution.

At both accelerated storage temperatures, no significant differences between lab and intermediate scale material were observed. At 37°C, the loss of infectivity was approximately 1 log_{10} per month and at room temperature approximately 1 log_{10} in 6 months. Results from a longer study with one of the intermediate scale samples (TTR2) comparing stability at all three temperatures indicated good stability at 2-8°C up to 19 months (**Fig. 25**).

To illustrate the influence of $TCID_{50}$ assay variability on the predictability of long term stability at 2-8°C a lab scale formulation is shown in **Table 4**.

Table 4. Stability results ($TCID_{50}$) of lyophilized DP at 2-8°C, RT and 37°C.

	Log_{10} $TCID_{50}$/mL										
Days	**0**	**14**	**21**	**28**	**60**	**90**	**120**	**150**	**180**	**270**	**365**
2-8°C	5.7	n.a.	n.a.	5.5	5.5	5.7	5.3	n.a.	5.5	5.7	5.3
RT (22°C)		5.9	n.a.	5.6	5.3	5.1	4.8	n.a.	n.a.	n.a.	n.a.
37°C		5.0	5.0	4.7	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

CHIKV-Δ5nsP3-inv showed excellent stability at 2-8°C and room temperature based on the currently available data for lab and intermediate scales. An acceptable loss of infectivity of approximately 1 log_{10} $TCID_{50}$/mL was observed when stored for 28 days at 37°C. Studies are currently ongoing to confirm long-term storage stability of CHIKV-Δ5nsP3-inv at 2-8°C with an anticipated stability profile of less than 1 log_{10} $TCID_{50}$/mL loss after two years storage.

It should also be noted that slight variations of lyophilization parameters (e.g., temperature and duration during primary and secondary drying) did not significantly alter the stability profile of CHIKV-Δ5nsP3-inv after lyophilization at various storage temperatures (data not shown).

Overall conclusion on stability of the CHIKV lyo formulation: Loss of infectivity over time when batches were stored at 2-8°C was minimal considering a potential $TCID_{50}$ assay variability of 0.3 log_{10}. In other words, significant differences at 2-8°C may be better assessed after long term storage. Extrapolation of stability up to two years based on the existing data estimates a loss in infectivity of up to 1 log_{10} $TCID_{50}$/mL at 2-8°C.

Stability data generated at accelerated temperatures provides information on stability differences between batches in a shorter time frame. In this regard, no significant differences between lyophilized DP derived from lab and intermediate scale were observed from studies carried out at 25°C and 37°C. Based on the data obtained, the loss of infectivity at 37°C is approximately 1 log_{10} $TCID_{50}$/mL per month and at 25°C approximately 1 log_{10} $TCID_{50}$/mL in 6 months.

Example 2. CHIKV Liquid Frozen Formulation Development

During the development of the Chikungunya vaccine candidate CHIKV-Δ5nsP3-inv, the generation of specific mutations in the virus genome could be observed in response to the adaption required for growing on Vero cells (see also WO2019057793, which is incorporated herein by reference in its entirety). One of these mutations is located in the structural E2 protein at position 168, changing a

glutamic acid residue to lysine (E168K). This mutation is the dominant phenotype in later passages on vero cells (P6 and higher) and correlated with a loss of immunogenicity of the attenuated CHIKV in the mouse model. To reduce the risk of producing non-immunogenic batches, a virus master bank was generated as P1 and a working bank as P2, resulting in production passage P3. Interestingly, it was found that different virus passages need different buffer compositions with regard to stability and degradation effects, apparently due to different surface charges introduced by mutations.

Most of the initial formulation development work was done using Passage 8 material. The following formulation was developed:

- The optimal pH range is 6.5 to 7.3 with higher stability at lower pH
- Human Serum Albumin (rHSA) is required at concentrations of between 0.01 – 1%
- Sucrose is needed to improve freeze/thaw stability (5% final concentration)
- Histidine is best buffering compound (20mM final concentration)

Composition of initial liquid formulation buffer:

20 mM Histidine pH 6.8, 5% sucrose, 0.1% rHSA

Additionally, during initial development, buffers were prepared with MilliQ water of high purity. However, when Passage 3 (P3) material was diluted using the same formulation buffer but prepared in water for injection (WFI), it was found that this virus passage was not compatible with the buffer any more. Upon dilution the virus size increased immediately (larger than 200nm in diameter) most probably because of aggregation. This virus solution was therefore not sterile filterable (0.2 µm filter) which is a prerequisite for vaccine production. Phosphate-citrate buffering systems have been reported to be compatible with CHIKV VLPs (Richard Schwartz, Formulation and Stability of a Chikungunya Virus-Like Particle (ChikV VLP) Based Vaccine" in "Vaccine Technology IV", B. Buckland, University College London, UK; J. Aunins, Janis Biologics, LLC; P. Alves , ITQB/IBET; K. Jansen, Wyeth Vaccine Research Eds, ECI Symposium Series, (2013). http://dc.engconfintl.org/vaccine_iv/17).

Therefore, the following phosphate-citrate buffered formulation was developed, guided by extensive previous experience with the histidine-buffered CHIKV formulation, and evaluated for its suitability to formulate CHIKV and to ensure sterile filterability during DS and DP production:

- 10 mM potassium phosphate
- 25 mM sodium citrate
- 5% sucrose
- 0.01% rHSA
- pH 7.3

The useful concentration range of its components was investigated and is summarized in the following sections. As the final rHSA concentration was not fixed in most experiments 0.02% rHSA was chosen

at the beginning to prevent unspecific adsorption at surfaces. Most analytical data were generated by DLS as this method provides a fast evaluation of the particle size, which is critical for filterability.

Influence of Phosphate Concentration

The buffering component - potassium phosphate - was tested in the range of 10 to 50 mM. As shown in **Figure 10**, the DLS data generated indicated that higher phosphate concentrations led to an increase in particle size of CHIKV-Δ5nsP3-inv (~90nm at 10 mM to ~140nm at 50 mM phosphate). A concentration of 10 mM was chosed for phosphate.

Influence of NaCl Concentration in Phosphate Buffered Solution

In order to evaluate if sodium chloride exhibits a similar effect as citrate with regard to virus size, it was investigate between 0 and 150 mM (no citrate present). All measurements were done within 15 minutes after addition of SGP to the buffer. **Figure 11** clearly shows that NaCl in phosphate buffered solution significantly increases the virus size from approximately 100nm (no NaCl) to ~200nm at 150 mM NaCl. Therefore, NaCl was not further investigated and was excluded as an additional excipient.

Influence of Citrate Concentration

Citrate has been reported to inhibit aggregation of CHIKV VLPs (Kramer RM, *et al*. Development of a Stable Virus-Like Particle Vaccine Formulation against Chikungunya Virus and Investigation of the Effects of Polyanions. 2013 J Pharm Sci. 102(12): 4305-4314. doi:10.1002/jps.23749). Reasoning that whole CHIK virus particles may behave similarly in solution to CHIKV VLPs, different concentrations of citrate were tested to evaluate its influence on virus size. Indeed, a significant and dose-dependent reduction in particle size was observed by addition of citrate (**Figure 12**). Virus in phosphate formulation buffer without citrate showed a size of ~300 nm compared to ~90nm with 25 mM citrate. Therefore, the citrate concentration was fixed at 25 mM in the formulation buffer to keep the virus size as small as possible to facilitate 0.2µm sterile filtration.

Influence of pH

Depending on the extent of mutations of viral proteins, the charges presented at the surface might change. With respect to this changed surface charge, also pH changes might significantly change the aggregation behavior of the virus. pH was investigated in the range of 7.0 to 7.6 (10mM potassium phosphate, 25mM sodium citrate, 5% sucrose, 0.02% rHSA). The influence of pH changes in this range seems not to be significant regarding the particle size with approximately 100 nm (data not shown).

Influence of other additives

Various additional buffer additives were investigated for potential stabilizing effects on CHIKV-Δ5nsP3-inv size:

- 10mM $CaCl_2$

- 10mM $MgCl_2$
- 5mM EDTA
- 25mM KCl
- 25mM alanine
- 2.5% sorbitol

Components were added at the indicated concentrations to the formulation buffer (10 mM PO_4, 25 mM citrate, 5% sucrose, 0.02% rHSA, pH 7.3). DLS measurement was performed within 30 minutes after virus addition. No major influences on CHIKV diameter caused by the different buffer additives were observed compared to the original buffer (data not shown). As CHIKV diameter was already stable in the basic formulation buffer, no further advantages of the incorporation of these additional excipients could be determined. Apart from that, also no negative effect was determined. Therefore, the buffer additives tested represent an opportunity if further components are needed within the formulation buffer system for later CHIKV formulation optimization.

Influence of rHSA

In order to evaluate which effect rHSA exerts on CHIKV-Δ5nsP3-inv size, different amounts of rHSA (0-0.1%) were added to the formulation buffer (10 mM potassium phosphate, 25 mM sodium citrate, 5% sucrose, pH 7.3) and measured by DLS immediately after virus addition (SGP, 1:40 in respective buffer). Increasing rHSA concentrations caused CHIKV aggregation from 79nm (without rHSA) to >250nm in diameter (0.1% rHSA). The same result was observed when analyzing DLS data for size distribution by volume. Observed CHIKV diameters at the respective rHSA concentrations are listed in **Table 5**. The effect of rHSA on CHIKV diameters over time is shown in **Table 6**.

Table 5. Influence of rHSA on CHIKV-Δ5nsP3-inv diameter (size distribution by intensity).

rHSA (%)	CHIKV Diameter (nm)
0	79
0.01	141
0.02	203
0.05	188
0.1	266

Based on these data, it was concluded that an rHSA concentration up to 0.01% is still suitable for 0.2 µm filtration, whereas rHSA concentrations ≥0.02% would lead to significant losses of virus during sterile filtration.

The incorporation of a minimal amount of rHSA is desired to prevent unspecific adsorption to surfaces of containers. Therefore, 0.01% rHSA in the formulation buffer is desirable andmay be present

without significantly reducing recovery during 0.2µm sterile filtration of DS or DP as virus diameter is still below 200nm.

Table 6. CHIKV-Δ5nsP3-inv diameter at different rHSA concentrations over time.

Time (h)	CHIKV Diameter (nm)		
	0% rHSA	0.01% rHSA	0.1% rHSA
0	79	141	266
2	87	149	434
3	99	153	499
22	94	175	459

Therefore, the rHSA concentration present in the formulation buffer was set to 0.01%, resulting in the following buffer composition for the liquid (frozen) formulation:

- 10 mM potassium phosphate
- 25 mM sodium citrate
- 5% sucrose
- 0.01% rHSA
- pH 7.3

Stability Studies of DS and DP

Buffer: 10 mM potassium phosphate (K_2HPO_4 and KH_2PO_4), 25 mM sodium citrate ($Na_3C_6H_5O_7$), 5% sucrose, 0.01% rHSA, pH 7.3 (conductivity 6.0 mS/cm). Before usage, formulation buffer was 0.2 µm sterile filtered. SGP-lot was diluted 1:40 in this buffer (195 mL buffer + 5 mL SGP lot) stirred for 3 minutes and left at RT for 15 minutes (to simulate later manufacturing process in larger scale). Thereafter, virus solution was 0.2µm filtered (PALL Mini Kleenpak, sterilized by gamma irradiation) into a 250 mL PETG bottle. DS after filtration was aliquoted in 60 mL PETG bottles (25 mL filling volume) when stored frozen at -80°C (stability study ongoing) or in 1.5 mL Eppendorf tubes when stored in liquid form (2-8°C, RT, 37°C).

As expected, DS in the liquid (frozen) formulation stored at -80°C remained stable at the day 60 timepoint (**Figure 13**). When stored at 2-8°C a decline of infectivity of approximately 0.5 log $TCID_{50}$/mL per week was observed. A complete loss of infectivity within a short time frame was observed when the material was stored at room temperature (after approx. two weeks) and at 37°C (less than one week).

This DS was further processed to DP by a 1:50 dilution (196mL formulation buffer + 4mL CHIKV DS). After mixing for 3 minutes and incubation for 15 minutes at room temperature, DP was filtered into a PETG bottle (Mini Kleenpak EKV membrane) and filled into glass vials (1 mL filling volume)

closed with Flurotec stoppers. Stability studies were undertaken on vials stored at -20°C (normal storage temperature) and under accelerated conditions (2-8°C and RT). The results after approx. 8 months storage are shown in the **Figure 14**.

As expected CHIKV-Δ5nsP3-inv presented in the liquid (frozen) formulation was unstable at 2-8°C (~0.5 $\log_{10}$ loss per week) and especially if stored at room temperature (complete loss of infectivity within two weeks).

Example 3. Safety and tolerability of the attenuated CHIKV-Δ5nsP3 vaccine

A randomized, observer-blinded, multicenter phase 1 trial to assess the safety, immunogenicity and antibody persistence of three escalating dosages of the live-attenuated Chikungunya virus vaccine candidate CHIKV-Δ5nsP3 (a.k.a. CHIKV-Δ5nsP3-inv; i.e., a mixture of CHIKV-Δ5nsP3 and variants) in healthy male and female volunteers was conducted. For the trial, the liquid frozen formulations as disclosed herein were used. Healthy volunteers aged 18 to 45 years were randomly assigned 1:1:2 to Low, Medium and High dose groups (L=$3.2x10^3$ $TCID_{50}$/0.1ml dose, M=$3.2x10^4$ $TCID_{50}$/1ml dose, H=$3.2x10^5$ $TCID_{50}$/1ml dose) and each received a single-shot immunization on Day 0. Half of the individuals in Group H (Group H2) were challenged with the High dose at Month 6 and followed up 28 days post-challenge until Month 12. Individuals in Groups L, M and H1 were challenged with the High dose vaccine at Month 12 and followed up to 28 days post-challenge. (See **Figs. 15A** and **B**.)

The study was conducted in compliance with the current International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use / Guideline for Good Clinical Practice and in accordance with the principles set forth in the Declaration of Helsinki. Throughout the study, an independent data safety monitoring board consisting of four external medical experts performed periodic reviews of accruing safety information. All enrolled subjects provided their written informed consent prior to any study-related procedure.

Healthy adults of both genders, aged 18 to 45 years, were eligible for inclusion in the trial. The baseline characteristics of the subjects are provided in **Table 13**. Female participants were eligible if they were of non-childbearing potential (i.e. surgically sterile or five years post-menopause). The main exclusion criteria included prior CHIKV infection, history of immune-mediated or chronic arthritis/arthralgia or immunization with an inactivated vaccine within 4 weeks or a live vaccine within 8 weeks prior to vaccination in the study. A full list of inclusion and exclusion criteria is provided in **Table 2**. One hundred and twenty participants were selected and randomly assigned to receive the single vaccination on Day 0 (**Fig. 15B**). Twenty-nine vaccinees terminated the study prior to Month 13, six were lost to follow-up and 20 withdrew their consent (none due to AEs). One subject was withdrawn from challenge due to an AE (syncope). Two other subjects were withdrawn due to

unknown reasons. Baseline characteristics across all dosage groups were similar, with the exception that the majority of volunteers were male **(Table 13)**. This gender disparity is reflected by the inclusion criterion allowing the enrollment of female subjects of non-childbearing potential only **(Table 8)**.

Table 13. Baseline Characteristics of Participants

	Group L (N=31)	Group M (N=30)	Group H (N=59)	All Participants N=120
Sex n (%)				
Male	28 (90·3)	23 (76·7)	55 (93·2)	106 (88·3)
Female	3 (9·7)	7 (23·3)	4 (6·8)	14 (11·7)
Ethnic origin (%)				
American Indian or Alaskan Native	0 (0·0)	0 (0·0)	1 (1·7)	1 (0·8)
Asian	2 (6·5)	1 (3·3)	2 (3·4)	5 (4·2)
African American	6 (19·4)	3 (10·0)	8 (13·6)	17 (14·2)
Native Hawaiian or Other Pacific Islander	0 (0·0)	0 (0·0)	0 (0·0)	0 (0·0)
Caucasian	22 (71·0)	26 (86·7)	48 (81·4)	96 (80·0)
Other	1 (3·2)	0 (0·0)	0 (0·0)	1 (0·8)
Age at screening [years] Mean	32·8	32·3	32·5	32·5
(Min/Max)	(21·0 / 43·0)	(21·0 / 45·0)	(19·0 / 45·0)	(19·0 / 45·0)
Height [cm] Mean	179·3	174·8	179·1	178·1
(Min/Max)	(157·5 / 195·.6)	(152·4 / 190·5)	(160·0 / 200·7)	(152·4 / 200·7)
Weight [kg] Mean	84·1	77·1	83·7	82·1
(Min/Max)	(63·0 / 104·0)	(45·7 / 100·7)	(57·6 / 118 0)	(45·7 / 118·0)
BMI [kg/m^2] Mean	26·2	25·1	26·0	25·8
(Min/Max)	(20·8 / 29·4)	(19·0 / 29·9)	(20·1 / 29·8)	(19·0 / 29·7)

Table 8. Inclusion and Exclusion Criteria for Participants

Inclusion Criteria	
Subjects who meet ALL of the following criteria are eligible for this study:	
1.	Subject is 18 to 45 years of age on the Day of screening (Visit 0);
2.	Subject has a BMI of ≥ 18.5 and < 30 kg/m2 on the Day of screening (Visit 0);
3.	Subject has an understanding of the study and its procedures, agrees to its provisions, and gives written informed consent prior to any study-related procedures;
4.	Subject is generally healthy as determined by the Investigator's clinical judgement based on medical history, physical examination and screening laboratory tests;

5.	If subject is of childbearing potential:
a)	Subject has practiced an adequate method of contraception (see below) during the 30 days before screening (Visit 0);
b)	Subject has a negative serum pregnancy test at screening (Visit 0);
c)	Subject agrees to employ adequate birth control measures for the duration of the study. This includes one of the following measures:
	· Hormonal contraceptives (e.g. implants, birth control pills, patches);
	· Intrauterine device;
	· Barrier type of birth control measure (e.g. condoms, diaphragms, cervical caps);
	· Vasectomy in the male sex partner ≥3 months prior to first vaccination.
Exclusion Criteria	
Subjects who meet ANY of the following criteria are NOT eligible for this study:	
1.	Subject has a history of known CHIKV infection;
2.	Subject has plans to travel to areas with active CHIKV transmission during the course of the study or has travelled to an endemic CHIKV area within 4 weeks prior to study enrollment;
3.	Subject has participated in a clinical study involving an investigational CHIKV vaccine;
4.	Subject has received an inactivated vaccine within 4 weeks or live vaccine within 8 weeks prior to vaccination in this study;
5.	Subject tests positive for human immunodeficiency virus (HIV), hepatitis B surface antigen (HBsAg) or hepatitis C virus (HCV);
6.	Subject has at screening (Visit 0): (1) abnormal laboratory liver function values (≥ grade 1), (2) any grade 1 abnormal lab values deemed clinically relevant by the Investigator, or (3) any ≥ grade 2 abnormal lab values irrespective of clinical significance;
7.	Subject has a clinically significant abnormal ECG at screening (Visit 0);
8.	Subject currently has or has a history of significant cardiovascular, respiratory (including asthma), metabolic, neurological, hepatic, heumatic, autoimmune, hematological, gastrointestinal or renal disorder ;
9.	Subject has a history of immune-mediated or clinically significant arthritis/arthralgia;
10.	Subject has a history of malignancy other than squamous cell or basal cell skin cancer, unless there has been surgical excision that is considered to have achieved a cure. A history of hematologic malignancy is a permanent exclusion. Subjects with a history of skin cancer must not be vaccinated at the previous tumor site;
11.	Subject has a disease or is undergoing a form of treatment or was undergoing a form of treatment that can be expected to influence immune response. Such treatment includes, but is not limited to, systemic or high dose inhaled (>800 µg/day of beclomethasone dipropionate or equivalent) corticosteroids within 4 weeks prior to study entry, radiation therapy or immunosuppressive cytotoxic drugs/ monoclonal antibodies in the previous 3 years;
12.	Subject has a history of severe hypersensitivity reactions or anaphylaxis;
13.	Subject has a history of any vaccine related contraindicating event (e.g., anaphylaxis, allergy to components of the candidate vaccine, other known contraindications);
14.	Subject had acute febrile infections within two weeks prior to vaccination;
15.	Subject has plans to become pregnant during the course of the study, or is pregnant (positive serum pregnancy test at screening) or lactating at the time of enrollment;
16.	Subject has donated blood within 30 days or received blood-derived products (e.g. plasma) within 90 days prior to vaccination in this study or plans to donate blood or use blood products during the course of the study;
17.	Subject has a rash, dermatological condition or tattoos that would, in the opinion of the Investigator, interfere with injection site reaction rating;
18.	Subject has a known or suspected problem with alcohol or drug abuse as determined by the Investigator;
19.	Subject has any condition that, in the opinion of the Investigator, may compromise the subjects well-being, might interfere with evaluation of study endpoints, or would limit the subject's ability to complete the study;
20.	Subject is committed to an institution (by virtue of an order issued either by the judicial or the administrative authorities);
21.	Subject has participated in another clinical study involving an investigational medicinal product (IMP) or device within 30 days prior to study enrollment or is scheduled to participate in another clinical study involving an IMP, or device during the course of this study;
22.	Subject is a member of the team conducting the study or in a dependent relationship with one of the study team members. Dependent relationships include close relatives (i.e., children, partner/spouse, siblings, parents) as well as employees of the Investigator or site personnel conducting the study.

As shown in **Fig. 15A**, individuals were randomized 1:1:2 to Low dose (Group L) $3.2x10^3$ $TCID_{50}$/0.1ml dose, Medium dose (Group M) $3.2x10^4$ $TCID_{50}$/ml dose or High dose (Group H) $3.2x10^5$ $TCID_{50}$/ml dose to receive a single i.m. vaccination on Day 0. Dosing was adjusted by injection volume. Participants in dose Group H were re-randomized 1:1 at Month 6 to receive either a challenge with the High dose at Month 6 or Month 12. Participants in the Low and Medium dose groups were challenged with the High dose vaccine at Month 12 only. Participants and investigators were blinded to the assignment into dose groups. Randomization was performed via randomization envelopes in ascending order. The vaccine was prepared by unblinded study staff, unobserved by blinded staff members and the participant. Syringe content was masked prior to administration. For safety and immunogenicity evaluations, blood was drawn before the vaccinations (Day 0), at Days three, seven, 14, 28 and 180 post-vaccination, as well as at 84 days and 12 months after the single vaccination.

The primary objective was to assess safety and tolerability of the vaccine after a single vaccination. Participant diaries were used for the collection of daily oral body temperature, solicited injection and systemic reactions up to 14 days post-vaccination, which are assessed using FDA's toxicity grading scale. In addition, participants were monitored for symptoms suggesting an acute stage of CHIKV-associated events manifested by systemic symptoms presenting with sudden onset of fever, myalgia, headache, back pain and macular to maculopapular rash, sometimes with cutaneous pruritus (foot arch) and edema of the face and extremities, polyadenopathies, acute (poly)arthritis most frequently in the extremities (wrists, ankles and phalanges), tenosynovitis, neurological symptoms or cardiac symptoms.

For determination of viremia and shedding after vaccination and challenge, plasma and urine from subjects were analyzed for the presence of CHIKV genomic RNA by Reverse Transcriptase quantitative PCR (RT-qPCR) (Panning, M. et al., 2008, Chikungunya Fever in Travelers Returning to Europe from the Indian Ocean Region, 2006. Emerging Infectious Diseases 14(3):416-422; Pastorino B. et al., 2015, Development of a TaqMan RT-PCR assay without RNA extraction step for detection and quantification of African Chikungunya viruses, Journal of Virological Methods, 65-71). In brief, total RNA was extracted from individual specimens and subjected to RT-qPCR using a hydrolysis probe and primers specific to the CHIKV nsP1 gene. The read-out was quantitative and reported as the number of CHIKV genome copy equivalents (GCE) per 1 mL of initial subject specimen. The assay was qualified for precision and specificity. The limits of detection and quantification were defined as 1087 GCE/mL (10 GCE/reaction) and 3261 GCE/mL (30 GCE/reaction), respectively. Time points with no available results in the treatment group were plotted at 500.

Statistical analysis. The sample size of 120 participants allowed for the detection of AEs, which commonly have a close relationship to vaccination, and with a true underlying prevalence of 2.5% with a probability of 95%. The study was not powered to detect uncommon or rare AEs, thus a

placebo group was not included. All participants who received a single vaccination at Day 0 were included in the safety dataset. The number and percentage of individuals with solicited injection site and systemic reactions up to 14 days after each vaccination, and with unsolicited AEs and SAEs were presented for each dose group overall and by body system / preferred term and were compared using Fisher's exact test for differences between groups; a significant overall test was amended by pair-wise tests between individual groups.

The primary outcome of the study was to assess the safety and tolerability of the vaccine. The live-attenuated CHIKV-Δ5nsP3 vaccine was generally safe and well-tolerated up to Month 12 after the single vaccination in the Low and Medium dosage groups and generally safe in all dosage groups. A summary of adverse events after the single vaccination is provided in **Table 9**. The Low and Medium dosages showed a superior reactogenicity profile compared to the High dosage group (p-value 0.0089; pairwise test M vs. H 0.0042). The vast majority of AEs across the dose groups were assessed as mild or moderate and the majority of AEs were reported after the single vaccination. No adverse event of special interest and no vaccine related serious adverse events were reported. Two unrelated serious adverse events occurred; one event of polytrauma following a car accident and one event of atrial ectopy 62 days following the 6 month re-vaccination **(Table 9)**. Following any challenge, rates of AEs were substantially diminished, only six participants reported related AEs occurring within 28 days after any challenge, indicating that participants were protected from challenge-induced AEs (summary provided in **Table 10)**.

Table 9. Summary of Adverse Events after Single Vaccination up to M12

	Statistics	Group L (N=31)	Group M (N=30)	Group H (N=59)	p-value (Overall)
Any AE	n (%) Obs	21 (67·7) 57	19 (63·3) 69	48 (81·4) 209	0·1349
	[95% CI]	[50·1, 81·4]	[45·5, 78·1]	[69·6, 89·3]	
Any related AE	n (%) Obs	18 (58·1) 43	14 (46·7) 49	46 (78·0) 168	**0·0089**
	[95% CI]	[40·8, 73·6]	[30·2, 63·9]	[65·9, 86·6]	
Pairwise test	vs. M	0·4462	..	..	
	vs. H	0·0550	**0·0042**	..	
Any severe AE	n (%) Obs	4 (12·9) 4	3 (10·0) 3	7 (11·9) 8	1·0000
	[95% CI]	[5·1, 28·9]	[3·5, 25·6]	[5·9, 22·5]	
Any related severe AE	n (%) Obs	4 (12·9) 4	2 (6·7) 2	7 (11·9) 8	0·7998
	[95% CI]	[5·1, 28·9]	[1·8, 21·3]	[5·9, 22·5]	
Any SAE	n (%) Obs	0 (0·0) 0	1 (3·3) 1	0 (0·0) 0	0·2500
	[95% CI]	[0·0, 11·0]	[0·6, 16·7]	[0·0, 6·1]	
Any related SAE	n (%) Obs	0 (0·0) 0	0 (0·0) 0	0 (0·0) 0	NC
	[95% CI]	[0·0, 11·0]	[0·0, 11·4]	[0·0, 6·1]	
Any medically attended AE	n (%) Obs	2 (6·5) 4	5 (16·7) 5	10 (16·9) 15	0·3856
	[95% CI]	[1·8, 20·7]	[7·3, 33·6]	[9·5, 28·5]	

	Statistics	Group L (N=31)	Group M (N=30)	Group H (N=59)	p-value (Overall)
Any related medically attended AE	n (%) Obs	1 (3·2) 2	0 (0·0) 0	0 (0·0) 0	0·5083
	[95% CI]	[0·6, 16·2]	[0·0, 11·4]	[0·0, 6·1]	
Any solicited AE	n (%) Obs	11 (35·5) 23	12 (40·0) 41	40 (67·8) 107	**0·0038**
	[95% CI]	[21·1, 53·1]	[24·6, 57·7]	[55·1, 78·3]	
Pairwise test	vs. M	0·7946	..	..	
	vs. H	**0·0040**	**0·0220**	..	
Any related solicited AE	n (%) Obs	10 (32·3) 20	10 (33·3) 34	40 (67·8) 106	**0·0007**
	[95% CI]	[18·6, 49·9]	[19·2, 51·2]	[55·1, 78·3]	
Pairwise test	vs. M	1·0000	..	..	
	vs. H	**0·0017**	**0·0031**	..	
Any severe solicited AE	n (%) Obs	1 (3·2) 1	1 (3·3) 1	5 (8·5) 6	0.6796
	[95% CI]	[0·6, 16·2]	[0·6, 16·7]	[3·7, 18·4]	
Any solicited local AE	n (%) Obs	1 (3·2) 1	2 (6·7) 3	4 (6·8) 4	0·7827
	[95% CI]	[0·6, 16·2]	[1·8, 21·3]	[2·7, 16·2]	
Any solicited systemic AE	n (%) Obs	11 (35·5) 22	12 (40·0) 38	40 (67·8) 103	**0·0038**
	[95% CI]	[21·1, 53·1]	[24·6, 57·7]	[55·1, 78·3]	
Pairwise test	vs. M	0·7946	..	..	
	vs. H	**0·0040**	**0·0220**	..	
Any severe solicited systemic AE	n (%) Obs	1 (3·2) 1	1 (3·3) 1	5 (8·5) 6	0·6796
	[95% CI]	[0·6, 16·2]	[0·6, 16·7]	[3·7, 18·4]	
Any unsolicited AE	n (%) Obs	17 (54·8) 34	15 (50·0) 28	36 (61·0) 102	0·5849
	[95% CI]	[37·8, 70·8]	[33·2, 66·8]	[48·3, 72·4]	
Any related unsolicited AE	n (%) Obs	13 (41·9) 23	8 (26·7) 15	29 (49·2) 61	0·1310
	[95% CI]	[26·4, 59·2]	[14·2, 44·4]	[36·8, 61·6]	
Any severe unsolicited AE	n (%) Obs	3 (9·7) 3	2 (6·7) 2	2 (3·4) 2	0·4588
	[95% CI]	[3·3, 24·9]	[1·8, 21·3]	[0·9, 11·5]	
Any related severe unsolicited AE	n (%) Obs	3 (9·7) 3	1 (3·3) 1	2 (3·4) 2	0·5452
	[95% CI]	[3·3, 24·9]	[0·6, 16·7]	[0·9, 11·5]	
Any AESI	n (%) Obs	0 (0·0) 0	0 (0·0) 0	0 (0·0) 0	NC
	[95% CI]	[0·0, 11·0]	[0·0, 11·4]	[0·0, 6·1]	

n... number of participants with AE, percentages are based on N, Obs...number of events

p-value (Overall): Fisher-Freeman-Halton test between Groups L, M, H

NC... not calculable

Table 10. Summary of Adverse Events after Challenge at M6 (H2) or M12 (L, M, H1)

	Statistic	Group L (N=24)	Group M (N=23)	Group H1 (N=21)	Group H2 (N=26)

Any AE	n (%) Obs	3 (12.5) 4	0 (0.0) 0	4 (19.0) 6	5 (19·2) 13
	[95% CI]	[4.3, 31.0]	[0.0, 14.3]	[7.7, 40.0]	[8·5, 37·9]
Any related AE	n (%) Obs	1 (4.2) 1	0 (0.0) 0	3 (14.3) 4	2 (7·7) 3
	[95% CI]	[0.7, 20.2]	[0.0, 14.3]	[5.0, 34.6]	[2·1, 24·1]
Any severe AE	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	2 (7·7) 2
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[2·1, 24·1]
Any related severe AE	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	2 (7·7) 2
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[2·1, 24·1]
Any SAE	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	0 (0·0) 0
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[0·0, 12·9]
Any related SAE	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	0 (0·0) 0
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[0·0, 12·9]
Any medically attended AE	n (%) Obs	0 (0.0) 0	0 (0.0) 0	1 (4.8) 1	2 (7·7) 3
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.8, 22.7]	[2·1, 24·1]
Any related medically attended AE	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	1 (3·8) 1
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[0·7, 18·9]
Any solicited AE	n (%) Obs	1 (4.2) 1	0 (0.0) 0	3 (14.3) 4	2 (7·7) 3
	[95% CI]	[0.7, 20.2]	[0.0, 14.3]	[5.0, 34.6]	[2·1, 24·1]
Any related solicited AE	n (%) Obs	1 (4.2) 1	0 (0.0) 0	3 (14.3) 4	2 (7·7) 3
	[95% CI]	[0.7, 20.2]	[0.0, 14.3]	[5.0, 34.6]	[2·1, 24·1]
Any severe solicited AE	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	2 (7·7) 2
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[2·1, 24·1]
Any solicited local AE	n (%) Obs	1 (4.2) 1	0 (0.0) 0	2 (9.5) 2	0 (0·0) 0
	[95% CI]	[0.7, 20.2]	[0.0, 14.3]	[2.7, 28.9]	[0·0, 12·9]
Any solicited systemic AE	n (%) Obs	0 (0.0) 0	0 (0.0) 0	1 (4.8) 2	2 (7·7) 3
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.8, 22.7]	[2·1, 24·1]
Any severe solicited systemic AE	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	2 (7·7) 2
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[2·1, 24·1]
Any unsolicited AE	n (%) Obs	2 (8.3) 3	0 (0.0) 0	2 (9.5) 2	4 (15·4) 10
	[95% CI]	[2.3, 25.8]	[0.0, 14.3]	[2.7, 28.9]	[6·2, 33·5]
Any related unsolicited AE	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	0 (0·0) 0
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[0·0, 12·9]
Any severe unsolicited AE	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	0 (0·0) 0
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[0·0, 12·9]
Any AESI	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	0 (0·0) 0
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[0·0, 12.9]

n... number of participants with AE, percentages are based on N, Obs...number of events

The local tolerability profile within 14 days after the single vaccination was considered excellent at all dose levels, with less than 7% of vaccinees (4/59 in Group H) reporting any local AE (p-value overall 0.7827). Tenderness was the most common injection site reaction after the single vaccination, affecting more than 5% of subjects (3/59 in Group H) (**Fig. 16A-C**). No injection site reactions were

observed after challenge at Month 6 **(Fig. 16D)**. One mild case each of pain, tenderness and swelling was reported after challenge at Month 12 **(Fig. 16 E-G)**. Notable systemic adverse events included short-term fever, headache, fatigue and muscle pain. Rates of related systemic AEs were significantly lower in the Low and Medium dosage groups compared to the High dosage group (p-value overall 0.0007; pairwise test L vs. H 0.0017; M vs. H 0.0031). Nine individuals experienced ten related severe solicited systemic AEs **(Tables 3 and 4)**, predominantly fever, occurring within two to four days after the single vaccination: one fever case each in the Low and Medium dosage groups and five cases in the High dosage group; and one severe case of headache (H) **(Table 11)**. Following challenge at Month 6 or 12, none of the vaccinees experienced fever; two individuals in dose group H2 reported severe nausea after 6 months challenge, one case each of moderate headache and mild joint pain were reported in dose group H1 after 12 months challenge **(Table 12)**.

Table 11. Related Solicited Systemic AEs by Symptom within 14 Days after Single Vaccination

	Statistic	Group L (N=31)	Group M (N=30)	Group H (N=59)	p-value (Overall)
Fever					
severe (Grade 3)	n (%) Obs	1 (3·2) 1	1 (3·3) 1	5 (8·5) 5	0·6796
	[95% CI]	[0·6, 16·2]	[0·6, 16·7]	[3·7, 18·4]	
moderate (Grade 2)	n (%) Obs	2 (6·5) 2	3 (10·0) 4	14 (23·7) 14	0·0894
	[95% CI]	[1·8, 20·7]	[3·5, 25·6]	[14·7, 36·0]	
mild (Grade 1)	n (%) Obs	0 (0·0) 0	2 (6·7) 3	3 (5·1) 3	0·5157
	[95% CI]	[0·6, 16·2]	[1·8, 21·3]	[1·7, 13·9]	
Nausea					
severe (Grade 3)	n (%) Obs	0 (0·0) 0	0 (0·0) 0	0 (0·0) 0	NC
	[95% CI]	[0·0, 11·0]	[0·0, 11·4]	[0·0, 6·1]	
moderate (Grade 2)	n (%) Obs	0 (0·0) 0	1 (3·3) 1	2 (3·4) 2	0·6158
	[95% CI]	[0·0, 11·0]	[0·6, 16·7]	[0·9, 11·5]	
mild (Grade 1)	n (%) Obs	1 (3·2) 1	2 (6·7) 2	6 (10·2) 6	0·5509
	[95% CI]	[0·6, 16·2]	[1·8, 21·3]	[4·7, 20·5]	
Vomiting					
severe (Grade 3)	n (%) Obs	0 (0·0) 0	0 (0·0) 0	0 (0 ·0) 0	NC
	[95% CI]	[0·0, 11·0]	[0·0, 11·4]	[0·0, 6·1]	
moderate (Grade 2)	n (%) Obs	0 (0·0) 0	1 (3·3) 1	1 (1·7) 1	0·7438
	[95% CI]	[0·0, 11·0]	[0·6, 16·7]	[0·3, 9·0]	
mild (Grade 1)	n (%) Obs	1 (3·2) 1	0 (0·0) 0	1 (1·7) 1	1·0000
	[95% CI]	[0·6, 16·2]	[0·0, 11·4]	[0·3, 9·0]	
Headache					
severe (Grade 3)	n (%) Obs	0 (0·0) 0	0 (0·0) 0	1 (1·7) 1	1·0000
	[95% CI]	[0·0, 11·0]	[0·0, 11·4]	[0·3, 9·0]	
moderate (Grade 2)	n (%) Obs	2 (6·5) 2	1 (3·3) 1	7 (11·9) 7	0·4793
	[95% CI]	[1·8, 20·7]	[0·6, 16·7]	[5·9, 22·5]	
mild (Grade 1)	n (%) Obs	5 (16·1) 5	6 (20·0) 6	14 (23·7) 15	0·7364
	[95% CI]	[7·1, 32·6]	[9·5, 37·3]	[14·7, 36·0]	
Fatigue					
severe (Grade 3)	n (%) Obs	0 (0·0) 0	0 (0·0) 0	0 (0·0) 0	NC
	[95% CI]	[0·0, 11·0]	[0·0, 11·4]	[0·0, 6·1]	

moderate (Grade 2)	n (%) Obs	1 (3·2) 1	1 (3·3) 1	10 (16·9) 10	0·0725
	[95% CI]	[0·6, 16·2]	[0·6, 16·7]	[9·5, 28·5]	
mild (Grade 1)	n (%) Obs	4 (12·9) 4	4 (13·3) 4	8 (13·6) 8	1·0000
	[95% CI]	[5·1, 28·9]	[5·3, 29·7]	[7·0, 24·5]	
Muscle Pain					
severe (Grade 3)	n (%) Obs	0 (0·0) 0	0 (0·0) 0	0 (0·0) 0	NC
	[95% CI]	[0·0, 11·0]	[0·0, 11·4]	[0·0, 6·1]	
moderate (Grade 2)	n (%) Obs	0 (0·0) 0	1 (3·3) 1	9 (15·3) 9	0·0222
	[95% CI]	[0·0, 11·0]	[0·6, 16·7]	[8·2, 26·5]	
Pairwise tests	vs. M	0·4918	..	..	
	vs. H	**0·0249**	0·1548	..	
mild (Grade 1)	n (%) Obs	1 (3·2) 1	3 (10·0) 3	9 (15·3) 9	0·2227
	[95% CI]	[0·6, 16·2]	[3·5, 25·6]	[8·2, 26·5]	
Joint Pain					
severe (Grade 3)	n (%) Obs	0 (0·0) 0	0 (0·0) 0	0 (0·0) 0	NC
	[95% CI]	[0·0, 11·0]	[0·0, 11·4]	[0·0, 6·1]	
moderate (Grade 2)	n (%) Obs	0 (0·0) 0	1 (3·3) 1	5 (8·5) 5	0·2685
	[95% CI]	[0·0, 11·0]	[0·6, 16·7]	[3·7, 18·4]	
mild (Grade 1)	n (%) Obs	1 (3·2) 1	2 (6·7) 2	5 (8·5) 5	0·8073
	[95% CI]	[0·6, 16·2]	[1·8, 21·3]	[3·7, 18·4]	
Rash					
severe (Grade 3)	n (%) Obs	0 (0·0) 0	0 (0·0) 0	0 (0·0) 0	NC
	[95% CI]	[0·0, 11·0]	[0·0, 11·4]	[0·0, 6·1]	
moderate (Grade 2)	n (%) Obs	0 (0·0) 0	0 (0·0) 0	0 (0·0) 0	NC
	[95% CI]	[0·0, 11·0]	[0·0, 11·4]	[0·0, 6·1]	
mild (Grade 1)	n (%) Obs	0 (0·0) 0	0 (0·0) 0	1 (1·7) 1	1·0000
	[95% CI]	[0·0, 11·0]	[0·0, 11·4]	[0·3, 9·0]	

n... number of participants with AE, percentages are based on N, Obs...number of events

p-value (Overall): Fisher's exact test for overall differences between groups

NC... not calculable

Table 12. Related Solicited Systemic AEs by Symptom within 14 Days after Challenge at M6 (H2) or M12 (L, M, H1)

	Statistic	Group L (N=24)	Group M (N=23)	Group H1 (N=21)	Group H2 (N=26)	p-value (L vs. M vs. H1)
Fever						
severe (Grade 3)	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	0 (0·0) 0	NC
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[0·0, 12·9]	
moderate (Grade 2)	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	0 (0·0) 0	NC
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[0·0, 12·9]	
mild (Grade 1)	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	0 (0·0) 0	NC
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[0·0, 12·9]	
Nausea						
severe (Grade 3)	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	2 (7·7) 2	NC
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[2·1, 24·1]	
moderate (Grade 2)	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	0 (0·0) 0	NC
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[0·0, 12·9]	
mild (Grade 1)	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	0 (0·0) 0	NC
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[0·0, 12·9]	

Vomiting						
severe (Grade 3)	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	0 (0·0) 0	NC
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[0·0, 12·9]	
moderate (Grade 2)	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	0 (0·0) 0	NC
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[0·0, 12·9]	
mild (Grade 1)	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	0 (0·0) 0	NC
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[0·0, 12·9]	
Headache						
severe (Grade 3)	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	0 (0·0) 0	NC
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[0·0, 12·9]	
moderate (Grade 2)	n (%) Obs	0 (0.0) 0	0 (0.0) 0	1 (4.8) 1	0 (0·0) 0	0.3088
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.8, 22.7]	[0·0, 12·9]	
mild (Grade 1)	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	0 (0·0) 0	NC
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[0·0, 12·9]	
Fatigue						
severe (Grade 3)	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	0 (0·0) 0	NC
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[0·0, 12·9]	
moderate (Grade 2)	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	0 (0·0) 0	NC
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[0·0, 12·9]	
mild (Grade 1)	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	1 (3·8) 1	NC
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[0·7, 18·9]	
Muscle Pain						
severe (Grade 3)	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	0 (0·0) 0	NC
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[0·0, 12·9]	
moderate (Grade 2)	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	0 (0·0) 0	NC
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[0·0, 12·9]	
mild (Grade 1)	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	0 (0·0) 0	NC
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[0·0, 12·9]	
Joint Pain						
severe (Grade 3)	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	0 (0·0) 0	NC
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[0·0, 12·9]	
moderate (Grade 2)	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	0 (0·0) 0	NC
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[0·0, 12·9]	
mild (Grade 1)	n (%) Obs	0 (0.0) 0	0 (0.0) 0	1 (4.8) 1	0 (0·0) 0	0.3088
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.8, 22.7]	[0·0, 12·9]	
Rash						
severe (Grade 3)	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	0 (0·0) 0	NC
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[0·0, 12·9]	
moderate (Grade 2)	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	0 (0·0) 0	NC
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[0·0, 12·9]	
mild (Grade 1)	n (%) Obs	0 (0.0) 0	0 (0.0) 0	0 (0.0) 0	0 (0·0) 0	NC
	[95% CI]	[0.0, 13.8]	[0.0, 14.3]	[0.0, 15.5]	[0·0, 12·9]	

n... number of participants with AE, percentages are based on N, Obs...number of events

Changes in blood cell counts were observed in one third of participants after the single vaccination; most commonly Leukopenia, Neutropenia and Lymphopenia (**Table 13**). Severe cases were observed across all groups: two cases of neutropenia in the Low and one case in the Medium dose group; two cases of lymphocytopenia in the High dose group. After challenge, no severe cases were reported and

a significant reduction in the occurrence of these values in comparison to post single vaccination (paired signed rank test difference at Day 7 after single vs after challenge, p-value < 0.0001, **Table 13)** was observed.

Table 13. Related unsolicited AE up to 28 days after vaccination (Groups L, M and H) and re-vaccination (challenge) (Groups L, M, H1 and H2)

		After Vaccination			After Re-vaccination			
						Paired Signed rank test		
	Statistics	Group L (N=31)	Group M (N=30)	Group H (N=59)	Group H2 (N=26) Group L (N= 22) Group M (N=22) Group H1 (N=18)		Day 7 after vaccination vs. Day 7 after re-vaccination	Day 28 after vaccination vs. Day 28 after re-vaccinatio
Blood and lymphatic system disorders	n (%) Obs	6 (19·4) 12	4 (13·3) 7	17 (28·8) 32	0 (0) 0			
Leukopenia	n (%) Obs	6 (19·4) 6	2 (6·7) 2	14 (23·7) 14	0 (0) 0	Group H2	<·0001	0·1751
						Group L	<·0001	0.9296
						Group M	<·0001	0.7215
						Group H1	<·0001	0.5218
Neutropenia	n (%) Obs	5 (16·1) 5	1 (3·3) 3	10 (16·9) 10	0 (0) 0	Group H2	<·0001	0·1708
						Group L	<·0001	0.9765
						Group M	0·0003	0.7656
						Group H1	<·0001	0.8801
Lymphopenia	n (%) Obs	1 (3·2) 1	2 (6·7) 2	4 (6·8) 5	0 (0) 0	Group H2	<·0001	0·8339
						Group L	0·0002	0.8831
						Group M	<·0001	0.2870
						Group H1	<·0001	0.6995

n... number of participants with AE, percentages are based on N, Obs...number of events

Plasma and urine samples were screened for viremia and viral shedding by PCR as described above. Viremia peaked at Day 3 post immunization in all groups, with the highest mean genome copy equivalent (GCE) value in Group H ($2.3x10^5$ GCE/mL). GCE values in Groups L and M were considerably lower, reaching mean titers of $7.4x10^4$ and $8.9x10^4$ GCE/mL, respectively. Seven days after a single vaccination, the numbers of subjects who showed reportable viremia results were notably decreased in all study arms, with mean values of plasma viral RNA ranging from 8814.0 GCE/mL (Group L) to 27,028.0 GCE/mL (Group H). No subject in any dose arm showed a reportable viremia result on Day 14 **(Fig. 17A)**. No viremia was detected after challenge at Day 180 or Month 12 **(Fig. 17B)**. Urinary shedding was detected in a single subject from Group L at Day 7 following vaccination

($1.1x10^4$ GCE/mL) **(Fig. 18A)** and was not detected at all after challenge at Day 180 or Month 12 **(Fig. 18B)**.

Post-hoc analyses on solicited AEs were performed in order to separate AEs arising before and after re-vaccination. In addition, a statistical comparison of rates of abnormal lymphocyte, neutrophil, and leukocyte counts between 7 and 28 days after single and any re-vaccination was performed.

Example 4. Immunogenicity studies

A. Neutralizing antibody titers and seroprotection conferred by the single-shot CHIKV-Δ5nsP3 vaccine

Secondary objectives of the clinical trial included the immune response after the single vaccination, measured by CHIKV-specific neutralizing antibodies, identification of the optimal dose level of the live-attenuated vaccine candidate, assessment of immunogenicity of CHIKV-Δ5nsP3 after challenge and assessment of antibody persistence up to Month 12 after a single vaccination. Neutralizing antibodies to the vaccine were evaluated using a microneutralization assay (μNT), which is based on a colorimetric CPE readout. Briefly, equal volumes of serial two-fold dilutions of serum samples were mixed with CHIKV-Δ5nsP3 (at a concentration resulting in 100% CPE) and incubated for 1-2h at 37°C, prior to transfer onto Vero cells plated in 96 well plates. After several days, inhibition of Vero cell infection was observed by assessing cell viability. The neutralizing titer is defined as the reciprocal serum dilution which induces 50% protection from cell death (μNT_{50}) compared with the virus control lacking neutralizing antibody. Titers below the quantification limit ($\mu NT_{50}<20$) were given the value of 10. Seroconversion was defined as reaching a CHIKV-specific neutralizing antibody titer of at least 20 for baseline seronegative subjects; i.e., $\mu NT_{50} \geq 20$.

The immunogenicity analyses were a comparison of the Geometric Mean Titers (GMTs) and Seroconversion Rates (SCRs) in the per-protocol (PP) population between the dose Groups L, M and H, at Day 28 (i.e. 28 days after vaccination) by ANOVA (factors dose group covariate study site). In addition, GMTs and Geometric Mean Fold Increases (GMFIs) were compared overall and pair-wise (Tukey's HSD test) between dose groups at all time points. All analyses were done in SAS (Version 9.3).

All three vaccine dosages were highly immunogenic after a single vaccination. At 14 days after the single vaccination, 100% of subjects in all dosage groups seroconverted. (Seroconversion was defined as subjects achieving a CHIKV-specific neutralizing antibody titer of at least 20 [$\mu NT_{50}\geq20$]). Furthermore, seroconversion rates in all dosage groups were sustained until Month 12 **(Fig. 19A)**. At least a 16-fold increase in antibody titers at Day 28 was observed in 96.3% or more subjects in all dosage groups. By Day 28, the highest CHIKV-specific neutralizing GMTs ranged from 592.6 to

686.9, representing a more than 60-fold increase in titers over baseline (**Fig. 19B**). Peak measured titers of individuals reached up to 10,240 (Groups M and H).

A lack of an anamnestic response, i.e., a booster effect of the challenge dose, in 100%, 100%, 94.4% and 96.2% of subjects, in Groups L, M, H1 and H2, respectively, was observed following challenge, indicating sterilizing immunity as characterized by a less than or equal to a four-fold rise in antibody titers as compared to pre-challenge titers (**Fig. 20A** and **Table 14**). Prior to challenge, the Group H2 GMT persisted at 452.5 (range 40-2560) and remained unchanged 28 days post challenge at 490.2 (range 80-2560) (**Fig. 20B**). Similarly, 28 days after challenge at Month 12 (Month 13), antibody levels in Groups L, M and H1 remained the same as prior to challenge (**Fig. 20B**).

Table 14. Rates of participants within ≤4-Fold Increase in Neutralizing Antibody Titer 28 days after Challenge at Month 6 and Month 12.

	Statistics	Group L (N=23)	Group M (N=23)	Group H1 (N=20)	Group H2 (N=26)
Reaching <=4-fold increase	n /N (%) [95% CI]	22/22 (100) [85.1, 100]	22/22 (100) [85.1, 100]	17/18 (94.4) [74.2, 99.0]	25 /26 (96·2) [81·1, 99·3]

n... number of participants

Setting the seroprotective threshold When transposing the seroprotective threshold established by Yoon et al. 2015 (*supra*) to the results of the current study, a titer of >1:10 is achieved by Day 14 in 100% of the subjects. Since the PRNT assay used by Yoon et al. and the microneutralization assay used within our study are based on the same principle, albeit in a different format and tested against different viruses, results are not directly comparable. As discussed herein, the PRNT determines virus neutralization by reduction of plaques using the attenuated CHIKV strain 181/clone 25, whereas the µNT determines neutralization of the attenuated CHIKV-Δ5nsP3 by reduction of virus-induced cytopathic effect. But even using the conservative seroprotective threshold of μNT_{50} ≥20 as applied in the current Phase 1 study, all subjects developed neutralizing antibody titers by Day 14, which were sustained throughout Month 12 following a single vaccination (**Fig. 19A**). Stressing this even further by using an unlikely seroprotection threshold titer of μNT_{50} ≥40 to ≥80, still nearly 100% of subjects across the different doses would be protected after a single vaccination with CHIKV-Δ5nsP3 by Day 14 (**Figs. 21A** and **21B**). When applying even an unreasonably high threshold titer of μNT_{50} ≥160, greater than 90% of subjects across all doses would be protected after a single vaccination with CHIKV-Δ5nsP3 at least until Month 12 (**Fig. 21C**). Based on the high and persisting geometric mean antibody titers elicited by the single-shot live-attenuated CHIKV vaccine CHIKV-Δ5nsP3, neutralizing antibody levels obtained should be well above the surrogate endpoint indicative of protection.

Microneutralization PRNT titers against Asian CHIKV strain suggest robust cross-neutralization elicited by the CHIKV-Δ5nsP3 vaccine

For assessment of cross-neutralizing activity of antibodies elicited by the CHIKV-Δ5nsP3 vaccine, a panel of sera from the clinical study from different time points were also tested in a µPRNT assay for neutralizing capacity against the attenuated heterologous CHIKV strain 181/clone 25 of the Asian genotype. A total of 111 single sera (including 37 pre-vaccination samples) and 5 human serum pools associated with VLA1553-101 study were tested. One µPRNT result was invalid due to the sample crossing the 50% neutralization threshold twice. The correlation between positive titers (n=75) measured by µNT and µPRNT was calculated using the Pearson correlation coefficient. As shown in **Fig. 22**, there was a highly statistically significant correlation of the neutralization titers of sera tested against the vaccine strain CHIKV-Δ5nsP3 (del5nsP3), based on LR2006-OPY1 of the East Central South African (ECSA) genotype and CHIKV strain 181/clone 25 of the Asian genotype. It was observed that antibodies induced by the CHIKV-Δ5nsP3 vaccine also efficiently neutralized the 181/clone 25 Asian CHIKV strain. Furthermore, neutralizing antibody titer values against both strains were highly similar in spite of being obtained using different assay formats (µNT v. µPRNT). This data strongly suggests that the CHIKV-Δ5nsP3 vaccine may confer protection against more than one strain of CHIKV.

While cross-neutralization between different CHIKV genotypes has already been shown in the literature, the results obtained during the feasibility study provided further insight into the cross-neutralizing ability of the CHIKV-Δ5nsP3-induced antibodies. Due to differences in assay systems, slight differences in the reported results were nevertheless expected. To support the results obtained from the µPRNT, anti-CHIKV total IgG antibodies were quantified by ELISA, using a CHIKV virus-like particle (E1, E2 and C1 proteins from West African strain 37997) and results were compared.

Methodology

In the course of clinical development, serum samples selected from the current study were tested using a micro-neutralization test (µNT) which measured the neutralization of CHIKV-Δ5nsP3, a micro-plaque reduction neutralization test (µPRNT) which measured neutralization of 181/clone 25 CHIKV and a Chikungunya virus-like particle (VLP)-based IgG ELISA based on the 37997 West African 37997 CHIKV strain. The purified CHIKV virus-like particles (VLPs) for ELISA comprised viral proteins C, E1 and E2 from the West African strain. Serum samples were selected based on neutralization titer obtained during clinical testing to span the titer range and dependent on sample availability. A panel of 111 CHIKV-Δ5nsP3 human serum samples were included in this comparability study. All three assays were compared in terms of correlation of results and assay characteristics.

Comparison of CHIKV µNT, µPRNT and IgG ELISA

A sub-set of the 111 clinical serum samples were analyzed in µNT, µPRNT and ELISA assays. The correlation between log-transformed titers measured with µNT, µPRNT and ELISA was calculated using the Pearson correlation coefficient (Pearson r), where a value of "1" indicates total positive linear correlation and a value of "0" indicates no linear correlation. Samples with titers below the lower limit of quantification (LLOQ) as well as positive controls were excluded from the correlation analysis. As shown in **Fig. 22A**, the value obtained for Pearson r with regard to µNT and µPRNT was 0.6724, indicating a moderately strong positive correlation. Moreover, the fact that titers obtained with µPRNT and µNT were comparable indicates a cross-protective ability of CHIKV-Δ5nsP3 against the Asian lineage of CHIKV (181/clone 25).

By contrast, the Pearson r value obtained for µPRNT and ELISA results was 0.1991, indicating only a weak positive correlation (see **Fig. 22B**). This finding is also reflected in the narrower distribution of titers measured by ELISA compared to titers measured with µPRNT. Presumably, functional differences of CHIKV antibodies, which become apparent in neutralization assays, are not detectable by ELISA. While ELISA measures only total CHIKV-specific IgG antibodies, µPRNT detects CHIKV-neutralizing antibodies of all isotypes. Nevertheless, all samples with positive ELISA titers also showed positive µPRNT titers, an observation supporting a predictive value of ELISA in anti-CHIKV immune responses. All post-vaccination samples from day 14 and later tested positive in ELISA. As CHIKV strains derived from different CHIKV lineages than the vaccine strain CHIKV-Δ5nsP3 (ECSA) were employed in both µPRNT (strain 181/clone 25 Asian) and ELISA (West African strain 37997), the ability of CHIKV-Δ5nsP3 to induce to cross-neutralizing antibodies is further supported.

Early seroconversion A total of ten Visit 1B samples (Day 7±1 day post vaccination) from all groups were included in a study to analyze test performance with samples collected during the early phase of the immune response (see **Table A1**). All of the samples tested had IgG levels below the LOD in the CHIKV IgG ELISA, indicating the absence of CHIKV-specific IgG antibodies at this early time point after vaccination. However, all except for two Visit 1B samples tested positive in µPRNT and all tested positive in µNT assays, due to the presence of CHIKV-neutralizing IgM antibodies at this early time point.

Table A1. ELISA, µPRNT and µNT results for CHIKV-Δ5nsP3 trial sera from visit 1B (day 7±1 post vaccination).

ELISA (ELU/mL) (VLP 37997 WA)	**µPRNT$_{50}$** (CHIKV 181/clone 25 Asian)	**µNT$_{50}$** (CHIKV-Δ5nsP3 ECSA)
<14.7	<10	20
<14.7	21	40
<14.7	29	20

<14.7	14	20
<14.7	40	40
<14.7	<10	20
<14.7	30	40
<14.7	45	20
<14.7	76	40
<14.7	31	20

Patient sera from CHIKV-Δ5nsP3 trial neutralized wild-type CHIKV Serum samples from the clinical trial (n=47 single sera) were analyzed to quantify wild-type chikungunya virus (Indian Ocean/ECSA lineage [La Reunion strain; wt CHIKV-LR] and West African strain; wt CHIKV-WA 3797) neutralizing antibodies. Sera collected at different visits of individual subjects were analyzed by PRNT (see **Fig. 23**). All post VLA1553-101 vaccination samples obtained at Day 14 or later demonstrated substantial neutralizing activity against both wild-type La Reunion CHIKV and a heterologous strain of the West African lineage.

Overall, the neutralization capacity of a particular serum for the attenuated CHIKV-Δ5nsP3 strain as assessed by μNT correlated well with its neutralization capacity for two wild-type CHIKV strains as assessed by PRNT. The results not only demonstrated the cross-neutralizing capacity of the CHIKV-Δ5nsP3 vaccine, but also showed the comparability of titer values obtained using the μNT assay and the PRNT assay.

B. GMT values from CHIKV-Δ5nsP3 clinical trial sera and convalescent human sera comparable

GMTs conferred by natural exposure to CHIKV Antibodies conferred by natural infection are hypothesized to provide life-long protection against CHIKV fever (Galatas, et al. and Nitatpattana, et al.; *supra*); therefore, the μNT titers observed in the present clinical samples were compared with neutralizing antibody titers in individuals convalescing from natural infection. Fourteen serum samples from individuals recovered from Chikungunya infection (kindly provided by World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) through the University of Texas Medical Branch (UTMB) or purchased from SeraCare and Biomex) were tested in the CHIKV-Δ5nsP3 μNT assay as used in the current clinical study. The neutralization titers of convalescent sera from all three sources were comparable (see **Table A2**). Furthermore, titers were similar to those observed after a single vaccination with CHIKV-Δ5nsP3, which reached GMT values up to 2560 at Day 28 in all dose groups.

Table A2. Neutralization titers from convalescent serum samples from naturally-infected patients.

Sample	**μNT_{50}**		**Sample**	**μNT_{50}**
Sera Care #2	640		UTMB #4	1,280
Sera Care #8	1,280		UTMB #5	2,560
Sera Care #10	2,560		UTMB #6	5,120

Biomex	1,280	UTMB #7	1,280
UTMB #1	1,280	UTMB #8	1,280
UTMB #2	2,560	UTMB #9	1,280
UTMB #3	2,560	UTMB #10	5,120

SEQUENCES

SEQ ID NO: 1 - Nucleotide sequence of the CHIKV-Δ5nsP3

GATGGCTGCGTGAGACACACGTAGCCTACCAGTTTCTTACTGCTCTACTCTGCAAAGCAAGAGAT
TAATAACCCATCATGGATCCTGTGTACGTGGACATAGACGCTGACAGCGCCTTTTTGAAGGCCCT
GCAACGTGCGTACCCCATGTTTGAGGTGGAACCAAGGCAGGTCACACCGAATGACCATGCTAAT
GCTAGAGCGTTCTCGCATCTAGCTATAAAACTAATAGAGCAGGAAATTGACCCCGACTCAACCAT
CCTGGATATCGGCAGTGCGCCAGCAAGGAGGATGATGTCGGACAGGAAGTACCACTGCGTCTG
CCCGATGCGCAGTGCGGAAGATCCCGAGAGACTCGCCAATTATGCGAGAAAGCTAGCATCTGCC
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SEQ ID NO: 2

Amino acid sequence of E2 protein from LR2006_OPY1 Chikungunya virus strain—amino acids 339-742 from structural polyprotein GenBank Accession: ABD95938.1 (1-1248 aa)

STKDNFNVYKATRPYLAHCPDCGEGHSCHSPVALERIRNEATDGTLKIQVSLQIGIKTDDSHDWTKLR
YMDNHMPADAERAGLFVRTSAPCTITGTMGHFILARCPKGETLTVGFTDSRKISHSCTHPFHHDPPVI
GREKFHSRPQHGKELPCSTYVQSTAATTEEIEVHMPPDTPDHTLMSQQSGNVKITVNGQTVRYKCNC
GGSNEGLTTTDKVINNCKVDQCHAAVTNHKKWQYNSPLVPRNAELGDRKGKIHIPFPLANVTCRVPK
ARNPTVTYGKNQVIMLLYPDHPTLLSYRNMGEEPNYQEEWVMHKKEVVLTVPTEGLEVTWGNNEPY
KYWPQLSTNGTAHGHPHEIILYYYELYPTMTVVVVSVATFILLSMVGMAAGMCMCARRRCITPYELTP
GATVPFLLSLICCIRTAKA

Some E2 variants identified herein

SEQ ID NO: 3

E168K variant of E2 protein from Chikungunya virus

STKDNFNVYKATRPYLAHCPDCGEGHSCHSPVALERIRNEATDGTLKIQVSLQIGIKTDDSHDWTKLR
YMDNHMPADAERAGLFVRTSAPCTITGTMGHFILARCPKGETLTVGFTDSRKISHSCTHPFHHDPPVI
GREKFHSRPQHGKELPCSTYVQSTAATTEEIKVHMPPDTPDHTLMSQQSGNVKITVNGQTVRYKCNC
GGSNEGLTTTDKVINNCKVDQCHAAVTNHKKWQYNSPLVPRNAELGDRKGKIHIPFPLANVTCRVPK
ARNPTVTYGKNQVIMLLYPDHPTLLSYRNMGEEPNYQEEWVMHKKEVVLTVPTEGLEVTWGNNEPY
KYWPQLSTNGTAHGHPHEIILYYYELYPTMTVVVVSVATFILLSMVGMAAGMCMCARRRCITPYELTP
GATVPFLLSLICCIRTAKA

SEQ ID NO: 4

G55R variant of E2 protein from Chikungunya virus

STKDNFNVYKATRPYLAHCPDCGEGHSCHSPVALERIRNEATDGTLKIQVSLQIRIKTDDSHDWTKLR
YMDNHMPADAERAGLFVRTSAPCTITGTMGHFILARCPKGETLTVGFTDSRKISHSCTHPFHHDPPVI
GREKFHSRPQHGKELPCSTYVQSTAATTEEIEVHMPPDTPDHTLMSQQSGNVKITVNGQTVRYKCNC
GGSNEGLTTTDKVINNCKVDQCHAAVTNHKKWQYNSPLVPRNAELGDRKGKIHIPFPLANVTCRVPK
ARNPTVTYGKNQVIMLLYPDHPTLLSYRNMGEEPNYQEEWVMHKKEVVLTVPTEGLEVTWGNNEPY
KYWPQLSTNGTAHGHPHEIILYYYELYPTMTVVVVSVATFILLSMVGMAAGMCMCARRRCITPYELTP
GATVPFLLSLICCIRTAKA

SEQ ID NO: 5

E247K variant of E2 protein from Chikungunya virus

STKDNFNVYKATRPYLAHCPDCGEGHSCHSPVALERIRNEATDGTLKIQVSLQIGIKTDDSHDWTKLR
YMDNHMPADAERAGLFVRTSAPCTITGTMGHFILARCPKGETLTVGFTDSRKISHSCTHPFHHDPPVI
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ARNPTVTYGKNQVIMLLYPDHPTLLSYRNMGEEPNYQEEWVMHKKEVVLTVPTEGLEVTWGNNEPY
KYWPQLSTNGTAHGHPHEIILYYYELYPTMTVVVVSVATFILLSMVGMAAGMCMCARRRCITPYELTP
GATVPFLLSLICCIRTAKA

SEQ ID NO: 6

G82R variant of E2 protein from Chikungunya virus

STKDNFNVYKATRPYLAHCPDCGEGHSCHSPVALERIRNEATDGTLKIQVSLQIGIKTDDSHDWTKLR
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KYWPQLSTNGTAHGHPHEIILYYYELYPTMTVVVVSVATFILLSMVGMAAGMCMCARRRCITPYELTP
GATVPFLLSLICCIRTAKA

SEQ ID NO: 7

H232Y variant of E2 protein from Chikungunya virus

STKDNFNVYKATRPYLAHCPDCGEGHSCHSPVALERIRNEATDGTLKIQVSLQIGIKTDDSHDWTKLR
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GREKFHSRPQHGKELPCSTYVQSTAATTEEIEVHMPPDTPDHTLMSQQSGNVKITVNGQTVRYKCNC
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GATVPFLLSLICCIRTAKA

Further preferred aspects:

1. A pharmaceutical composition comprising i) a combination of an attenuated chikungunya virus as defined by the nucleic acid sequence of SEQ ID NO: 1 and variants thereof, wherein said variants have a nucleic acid sequence that is at least 99% identical to SEQ ID NO: 1 and comprises the entire deletion mutation of nsP3 as in SEQ ID NO: 1; and ii) pharmaceutically acceptable excipients, characterized in that said pharmaceutical composition is substantially safe and is able to induce a sustained protective immune response against a chikungunya virus in a human after a single dosage, wherein said dosage is about 10^3 to $5x10^4$ $TCID_{50}$/dose, preferably about 10^3 to $2x10^4$ $TCID_{50}$/dose.

2. The pharmaceutical composition of aspect 1, wherein said dosage is about 5×10^3 $TCID_{50}$/dose.

3. The pharmaceutical composition according to aspect 1 or 2, wherein said attenuated chikungunya virus variant expresses an E2 protein with at least one mutation, particularly an E protein as defined by SEQ ID Nos: 3-7.

4. The pharmaceutical composition of any one of aspects 1 to 3, wherein said attenuated chikungunya virus variant expresses an E2 protein with an E168K mutation as defined by SEQ ID NO: 3.

5. A pharmaceutical composition which is a one-shot vaccine essentially consisting of i) a mixture of CHIKV-Δ5nsP3 which expresses an E2 protein as defined by SEQ ID NO: 2 and a CHIKV-Δ5nsP3 which expresses an E2 protein as defined by SEQ ID NO: 3; and ii) pharmaceutically acceptable excipients, wherein said mixture is delivered at a dose of about 10^3 to $5x10^4$ $TCID_{50}$/dose, preferably about 10^3 to $2x10^4$ $TCID_{50}$/dose.

6. The pharmaceutical composition of any one of aspects 1 to 5, wherein said composition is a liquid frozen composition.

7. The pharmaceutical composition of any one of aspects 1 to 5, wherein said composition is a lyophilized composition.

8. The pharmaceutical composition of any of aspects 1 to 7, wherein the pharmaceutically acceptable excipients essentially consist of sucrose, potassium phosphate and sodium citrate and, optionally, magnesium chloride, D-sorbitol, L-methionine and recombinant human serum albumin rHSA.

9. The pharmaceutical composition according to any one of aspects 1 to 8, wherein said pharmaceutically acceptable excipients essentially consist of about 5% (w/v) sucrose, about 10 mM potassium phosphate, about 25 mM sodium citrate and about 0.01% (w/v) recombinant human serum albumin (rHSA).

10. The pharmaceutical composition according to any one of aspects 1 to 8, wherein said pharmaceutically acceptable excipients essentially consist of about 5% (w/v) sucrose; about 5 mM potassium phosphate; about 25 mM sodium citrate; about 5 mM MgCl2; about 0.5 % (w/v) D-sorbitol; about 10 mM L-methionine; and about 0.01% (w/v) recombinant human serum albumin(rHSA).

11. The pharmaceutical composition of any one of aspects 1 to 10, wherein said composition is able to increase serum antibody titers to said virus in a human by at least 1 log relative to a control within about 14 days from primary immunization.

12. The pharmaceutical composition of any one of aspects 1 to 10, wherein said composition is able to increase serum antibody titers to said virus in a human by at least 1 log relative to a control within about 7 days from primary immunization.

13. The pharmaceutical composition of any one of aspects 1 to 12, wherein said composition is able to stimulate seroconversion in at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, up to 100% of vaccinated subjects within 7 days of vaccination, wherein seroconversion is defined as reaching a CHIKV-specific antibody titer of at least 10.

14. The pharmaceutical composition of any one of aspects 1 to 12, wherein said composition is able to stimulate seroconversion in at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, up to 100% of vaccinated subjects within 14 days of vaccination, wherein seroconversion is defined as reaching a CHIKV-specific antibody titer of at least 10.

15. The pharmaceutical composition of any one of aspects 1 to 14, wherein the protective immune response lasts for at least 6 months.

16. The pharmaceutical composition of any one of aspects 1 to 14, wherein the protective immune response lasts for at least 12 months.

17. The pharmaceutical composition of any one of aspects 1 to 14, wherein the protective immune response lasts for at least 24 months.

18. The pharmaceutical composition of any one of aspects 1 to 17, for use in a method of treating or preventing a Chikungunya virus infection.

19. A method of treating or preventing a Chikungunya virus infection in a subject in need thereof, comprising administering an effective amount of the pharmaceutical composition according to any one of aspects 1 to 17.

1A. A liquid frozen or lyophilized life chikungunya vaccine formulation comprising: a) an effective amount of at least one strain of life chikungunya virus; b) about 1 to 50% (w/v) sugar; c) about 1 mM to about 20 mM phosphate; d) about 1 mM to about 50 mM of at least one carboxylate; e) optionally about 1 mM to about 10 mM $MgCl_2$; f) optionally about 0.1 % to about 5 % (w/v) D-sorbitol; g) optionally about 1 mM to 20 mM L-methionine; and h) optionally about 0.001% to about 0.1 % (w/v) human serum albumin.

2A. The formulation of aspect 1A, wherein the human serum albumin is a recombinant human serum albumin.

3A. The formulation according to any preceding aspects wherein said at least one carboxylate is selected from the group consisting of succinate, citrate, fumarate, tartarate, maleate and lactate.

4A. The formulation according to any preceding aspects wherein said sugar is selected from the group consisting of sucrose, mannitol, lactose, sorbitol, dextrose, fucose and trehalose.

5A. The formulation according to any preceding aspects wherein the concentration of sugar is between about 1 to about 10%; the concentration of phosphate is between about 1 to about 10 mM; and said at least one carboxylic acid is citrate or succinate at a concentration between about 10 to about 30 mM.

6A. The formulation according to any preceding aspects further comprising: k) at least one diluent selected from the group consisting of tissue culture medium, saline and water to volume.

7A. The formulation according to any preceding aspects wherein the pH is between about pH 5.0 to about pH 8.0, preferably between pH 7.0 and pH 7.5, most preferred pH 7.3.

8A. The formulation according to any preceding aspects wherein said phosphate is selected from the group consisting of monophosphates, polyphosphates and phosphorylated compounds.

9A. The formulation according to aspect 8A wherein said monophosphate is potassium phosphate.

10A. The formulation according to any preceding aspects wherein formulation comprises an effective amount of at least one strain of chikungunya virus; b) sucrose at a concentration of about 5% (w/v); c) potassium phosphate at a concentration of about 5 mM to about 10 mM; d) sodium

citrate at a concentration of about 25 mM; e) MgCl2 at a concentration of about 10 mM; f) D-sorbitol at a concentration of about 0.5 % (w/v), g) L-methionine at a concentration of about 10 mM; and h) recombinant human serum albumin at a concentration of about 0.01% (w/v).

11A. The formulation according to any preceding aspects wherein formulation comprises an effective amount of at least one strain of chikungunya virus; b) about 5% (w/v) sucrose; c) about 5 mM potassium phosphate; d) about 25 mM sodium citrate; e) about 10 mM MgCl2; f) about 0.5 % (w/v) D-sorbitol, g) about 10 mM L-methionine; and h) about 0.01% (w/v) recombinant human serum albumin.

12A. The formulation according to any preceding aspects wherein said chikungunya virus is selected from an attenuated chikungunya virus of SEQ ID NO: 1; variants with 99% sequence identity to SEQ ID NO: 1 of which have the 60aa deletion as in SEQ ID NO: 1; and/or combinations thereof.

13A. The formulation according to any preceding aspects wherein said chikungunya virus comprises essentially an attenuated chikungunya virus of SEQ ID NO: 1 and a variant with 99% sequence identity to SEQ ID NO: 1 and having the 60aa deletion as in SEQ ID NO: 1.

14A. The formulation according to any preceding aspects wherein said at least one strain of chikungunya virus is selected from an attenuated chikungunya virus population that comprises substantially 2 variants, said variants expressing the E1 wild type amino acid sequence as encoded in the relevant part of nucleic acid sequence SEQ ID NO: 1 and wherein one variant expressing the wild type E2 structural protein as defined in SEQ ID NO:2 and wherein the other variant expressing the E168K mutation in the E2 structural protein as defined in SEQ ID NO:3.

15A. The lyophilized chikungunya vaccine formulation according to any preceding aspects wherein said chikungunya virus is an attenuated chikungunya virus population that comprises substantially 2 variants, said variants expressing E2 structural proteins as defined by the amino acid sequences of SEQ ID NO: 2 and SED ID NO: 3 (with E168K) and wherein said 2 variants have a combined dose between about 103 and 2 x 10^4 $TCID_{50}$/dose and a target potency of about 5 x 10^3 $TCID_{50}$/dose.

16A. The lyophilized chikungunya vaccine formulation according to any preceding aspects wherein said chikungunya virus is an attenuated chikungunya virus population that comprises one or more variants and wherein the variant has one or more mutations in E2 which mutations are shown in the group of variants encoding for an E2 amino acid sequence with E168K (SEQ ID NO: 3), G55R (SEQ ID NO: 4), E247K (SEQ ID NO: 5), G82R (SEQ ID NO: 6) and/or H232Y (SEQ ID NO: 7).

17A. A method of preparing chikungunya virus vaccine formulations, comprising:

(a) cultivating a chikungunya virus and mixing the chikungunya virus with a concentrated stabilizing solution to form a virus bulk; and, optionally,

(b) dialyzing the virus bulk to form a chikungunya virus vaccine solution; wherein the vaccine solution comprises a) about 1 to 50% (w/v) sugar; b) about 1 mM to about 20 mM phosphate; c) about 1 mM to about 50 mM of at least one carboxylate; d) about 1 mM to about 10 mM MgCl2; e) about 0.1 % to about 5 % (w/v) D-sorbitol; g) about 1 mM to 20 mM L-methionine; and f) about 0.001% to about 0.1 % (w/v) human serum albumin.

18A. The method of aspect 17A, wherein the stabilizing solution comprises a) about 5% (w/v) sugar; b) about 20 mM phosphate; c) about 25 mM citrate; d) about 10 mM MgCl2; e) about 0.5 % (w/v) D-sorbitol; f) about 10 mM L-methionine; and g) about 0.01% (w/v) human serum albumin.

19A. The method of aspect 17A or 18A, wherein the dialyzing is done to form a formulation of any of aspects 1A to 16A.

20A. The method of aspects 17A-19A further comprising the step of lyophilizing the vaccine solution.

1B. A pharmaceutical unit dosage composition comprising i) an attenuated chikungunya virus; and ii) one or more pharmaceutically acceptable excipients, characterized in that said pharmaceutical composition is able to induce a sustained protective immune response against a chikungunya virus in a human after a single dosage, wherein said unit dosage composition comprises about 10^3 to 5 x 10^4 $TCID_{50}$/dose, preferably about 10^3 to 2 x 10^4 $TCID_{50}$/dose.

2B. The pharmaceutical composition according to aspect 1B, wherein said attenuated chikungunya virus comprises an RNA genome corresponding to the DNA sequence as defined by SEQ ID NO: 1 (CHIKV-Δ5nsP3) and/or one or more variants thereof, wherein said variant has a nucleic acid sequence that is at least 99% identical to SEQ ID NO: 1 and comprises the entire 60 amino acid deletion in nsP3 as in SEQ ID NO: 1.

3B. The pharmaceutical composition of aspect 1B or 2B, wherein said dosage is about 5 x 10^3 $TCID_{50}$/dose.

4B. The pharmaceutical composition according to aspect 2B or 3B, wherein said attenuated chikungunya virus variant expresses an E2 protein with at least one mutation compared with the wild-type E2 protein as defined by SEQ ID NO: 2, particularly an E2 protein as defined by any of SEQ ID Nos: 3-7.

5B. The pharmaceutical composition of any one of aspects 2B to 4B, wherein said attenuated chikungunya virus variant expresses an E2 protein with an E168K mutation as defined by SEQ ID NO: 3.

6B. A pharmaceutical composition for use as a one-shot vaccine for the prevention or treatment of chikungunya virus infection, wherein the composition comprises or consists of i) CHIKV-Δ5nsP3 which expresses an E2 protein as defined by SEQ ID NO: 2, a CHIKV-Δ5nsP3 which expresses an E2 protein as defined by SEQ ID NO: 3 or a mixture thereof; and ii) one or more pharmaceutically acceptable excipients, wherein said composition is administered to a subject at a dose of about 10^3 to 5 x 10^4 $TCID_{50}$/dose, preferably about 10^3 to 2 x 10^4 $TCID_{50}$/dose.

7B. The pharmaceutical composition of any one of aspects 1B to 6B, wherein said composition is a liquid frozen composition.

8B. The pharmaceutical composition of any one of aspects 1B to 6B, wherein said composition is a lyophilized composition.

9B. The pharmaceutical composition of any one of aspects 1B to 8B, wherein the pharmaceutically acceptable excipients essentially consist of sucrose, potassium phosphate and sodium citrate and, optionally, magnesium chloride, D-sorbitol, L-methionine and recombinant human serum albumin rHSA.

10B. The pharmaceutical composition according to any one of aspects 1B to 9B, wherein said pharmaceutically acceptable excipients essentially consist of about 5% (w/v) sucrose, about 10 mM potassium phosphate, about 25 mM sodium citrate and about 0.01% (w/v) recombinant human serum albumin (rHSA).

11B. The pharmaceutical composition according to any one of aspects 1B to 9B, wherein said pharmaceutically acceptable excipients essentially consist of about 5% (w/v) sucrose; about 5 mM potassium phosphate; about 25 mM sodium citrate; about 5 mM $MgCl_2$; about 0.5 % (w/v) D-sorbitol; about 10 mM L-methionine; and about 0.01% (w/v) recombinant human serum albumin(rHSA).

12B. The pharmaceutical composition of any one of aspects 1B to 11B, wherein said composition is able to increase serum antibody titers to said virus in a human by at least 1 log relative to a control within about 14 days from primary immunization.

13B. The pharmaceutical composition of any one of aspects 1B to 11B, wherein said composition is able to increase serum antibody titers to said virus in a human by at least 1 log relative to a control within about 7 days from primary immunization.

14B. The pharmaceutical composition of any one of aspects 1B to 13B, wherein said composition is able to stimulate seroconversion in at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, up to 100% of vaccinated subjects within 7 days of vaccination, wherein seroconversion is defined as reaching a neutralizing CHIKV antibody titer of at least 10, preferably at least 20.

15B. The pharmaceutical composition of any one of aspects 1B to 13B, wherein said composition is able to stimulate seroconversion in at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, up to 100% of vaccinated subjects within 14 days of vaccination, wherein seroconversion is defined as reaching a neutralizing CHIKV antibody titer of at least 10, preferably at least 20.

16B. The pharmaceutical composition of any one of aspects 1B to 15B, wherein the protective immune response lasts for at least 6 months.

17B. The pharmaceutical composition of any one of aspects 1B to 15B, wherein the protective immune response lasts for at least 12 months.

18B. The pharmaceutical composition of any one of aspects 1B to 15B, wherein the protective immune response lasts for at least 24 months.

19B. The pharmaceutical composition of any one of aspects 1B to 15B, wherein the protective immune response confers life-long protection against CHIK virus disease.

20B. The pharmaceutical composition of any one of aspects 1B to 19B, for use in a method of treating or preventing a Chikungunya virus infection.

21B. A method of treating or preventing a Chikungunya virus infection in a subject in need thereof, comprising administering an effective amount of the pharmaceutical composition according to any one of aspects 1B to 17B.

CLAIMS

What is claimed is:

1. A liquid frozen or lyophilized live chikungunya vaccine formulation comprising: a) an effective amount of at least one strain of live chikungunya virus; b) about 1 to 50% (w/v) sugar; c) about 1 mM to about 20 mM phosphate; d) about 1 mM to about 50 mM of at least one carboxylate buffering agent; e) optionally about 1 mM to about 10 mM $MgCl_2$; f) optionally about 0.1 % to about 5 % (w/v) D-sorbitol; g) optionally about 1 mM to 20 mM L-methionine; and h) optionally about 0.001% to about 0.1 % (w/v) human serum albumin.
2. The formulation of claim 1, wherein the human serum albumin is a recombinant human serum albumin (rHSA).
3. The formulation according to any preceding claims wherein said at least one carboxylate buffering agent is selected from the group consisting of succinate, citrate, fumarate, tartarate, maleate and lactate.
4. The formulation according to any preceding claims wherein said sugar is selected from the group consisting of sucrose, mannitol, lactose, sorbitol, dextrose, fucose and trehalose, preferably sucrose or trehalose, most preferably sucrose.
5. The formulation according to any preceding claims wherein the concentration of sugar is between about 1 to about 10%; the concentration of phosphate is between about 1 to about 10 mM; and said at least one carboxylic acid is citrate or succinate at a concentration between about 10 to about 30 mM.
6. The formulation according to any preceding claims further comprising: k) at least one diluent selected from the group consisting of tissue culture medium, saline and water to volume.
7. The formulation according to any preceding claims wherein the pH is between about pH 5.0 to about pH 8.0, preferably between pH 7.0 and pH 7.5, most preferably pH 7.3.
8. The formulation according to any preceding claims wherein said phosphate is selected from the group consisting of monophosphates, polyphosphates and phosphorylated compounds.
9. The formulation according to claim 8 wherein said monophosphate is potassium phosphate.
10. The formulation according to any preceding claims wherein formulation comprises an effective amount of at least one strain of chikungunya virus; b) sucrose at a concentration of about 5% (w/v); c) potassium phosphate at a concentration of about 5 mM to about 10 mM; d) sodium citrate at a concentration of about 25 mM; e) $MgCl_2$ at a concentration of about 10 mM; f) D-

sorbitol at a concentration of about 0.5 % (w/v), g) L-methionine at a concentration of about 10 mM; and h) recombinant human serum albumin at a concentration of about 0.01% (w/v).

11. The formulation according to any preceding claims wherein formulation comprises an effective amount of at least one strain of chikungunya virus; b) about 5% (w/v) sucrose; c) about 5 mM potassium phosphate; d) about 25 mM sodium citrate; e) about 10 mM $MgCl_2$; f) about 0.5 % (w/v) D-sorbitol, g) about 10 mM L-methionine; and h) about 0.01% (w/v) recombinant human serum albumin.

12. The formulation according to any preceding claims wherein said chikungunya virus is selected from an attenuated chikungunya virus of SEQ ID NO: 1; variants with 99% sequence identity to SEQ ID NO: 1 of which have the 60aa deletion as in SEQ ID NO: 1; and/or combinations thereof.

13. The formulation according to any preceding claims wherein said chikungunya virus comprises essentially an attenuated chikungunya virus of SEQ ID NO: 1 and a variant with 99% sequence identity to SEQ ID NO: 1, wherein the variant also has the 60aa deletion in the nsP3 protein as in SEQ ID NO: 1.

14. The formulation according to any preceding claims wherein said at least one strain of chikungunya virus is selected from an attenuated chikungunya virus population that comprises substantially two variants, said two variants both expressing the E1 wild type amino acid sequence as encoded in the relevant part of nucleic acid sequence SEQ ID NO: 1 and wherein one variant expresses the wild type E2 structural protein as defined in SEQ ID NO:2 and wherein the other variant expresses the E168K mutation in the E2 structural protein as defined in SEQ ID NO:3.

15. The lyophilized chikungunya vaccine formulation according to any preceding claims wherein said chikungunya virus is an attenuated chikungunya virus population that comprises substantially two variants, said two variants expressing E2 structural proteins as defined by the amino acid sequences of SEQ ID NO: 2 and SED ID NO: 3 (with E168K), respectively, and wherein said two variants have a combined dose of between about 10^3 and 2 x 10^4 $TCID_{50}$/dose and a target potency of about 5 x 10^3 $TCID_{50}$/dose.

16. The lyophilized chikungunya vaccine formulation according to any preceding claims wherein said chikungunya virus is an attenuated chikungunya virus population that comprises one or more variants and wherein the variant has one or more mutations in E2 which mutations are shown in the group of variants encoding for an E2 amino acid sequence with E168K (SEQ ID NO: 3), G55R (SEQ ID NO: 4), E247K (SEQ ID NO: 5), G82R (SEQ ID NO: 6) and/or H232Y (SEQ ID NO: 7).

17. A method of preparing chikungunya virus vaccine formulations, comprising:

(a) cultivating a chikungunya virus and mixing the chikungunya virus with a concentrated stabilizing solution to form a virus bulk; and, optionally,

(b) dialyzing the virus bulk to form a chikungunya virus vaccine solution; wherein the vaccine solution comprises a) about 1 to 50% (w/v) sugar; b) about 1 mM to about 20 mM phosphate; c) about 1 mM to about 50 mM of at least one carboxylate; d) about 1 mM to about 10 mM $MgCl_2$; e) about 0.1 % to about 5 % (w/v) D-sorbitol; g) about 1 mM to 20 mM L-methionine; and f) about 0.001% to about 0.1 % (w/v) human serum albumin.

18. The method of claim 17, wherein the stabilizing solution comprises a) about 5% (w/v) sugar; b) about 20 mM phosphate; c) about 25 mM citrate; d) about 10 mM $MgCl_2$; e) about 0.5 % (w/v) D-sorbitol; f) about 10 mM L-methionine; and g) about 0.01% (w/v) human serum albumin.

19. The method of claim 17 or 18, wherein said dialyzing step results in a formulation of any of claims 1 to 16.

20. The method of any one of claims 17 to 19 further comprising the step of lyophilizing the vaccine solution.

21. The vaccine formulation of any one of claims 1 to 16 for use in a method of vaccinating a human subject to stimulate a protective immune response against Chikungunya virus in said subject.

22. A method of treatment or prevention of a Chikungunya virus infection comprising administering an effective amount of the vaccine formulation as defined by any one of claims 1 to 16 to a subject in need thereof.