RESEARCH ARTICLE

Development and evaluation of an inactivated Chikungunya virus vaccine

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ABSTRACT

Background: Chikungunya (CHIK) is a debilitating viral disease that can result in high levels of morbidity and, sometimes, even mortality. It is caused by the chikungunya virus (CHIKV), which belongs to the *Togaviridae* family. Chikungunya disease is characterized by a triad of symptoms: fever, rash, and joint pain. It is generally treated with painkillers and non-steroidal anti-inflammatory drugs (NSAIDs). Chikungunya is a vaccine-preventable disease and various vaccine platforms are being used, including recombinant protein subunit, live-attenuated, inactivated, viral vector, virus-like particles (VLPs), and nucleic acids (DNA and RNA). *Objective:* The inactivated (killed) virus platform is one of the safest, as there is no chance of reversion to the wild-type virus. Since this uses a traditional vaccine platform, its efficacy and safety are time-tested. Therefore, the study was aimed at developing an inactivated vaccine against CHIKV. *Methods:* Chikungunya virus was isolated from 53 sera samples. These were then purified, cloned, and sequenced. The 53 isolates were narrowed down to five CHIKV isolates cultured in African green monkey kidney (Vero) cells. The fastest growing isolate was used as the candidate vaccine. It was inactivated using three chemicals – β-propiolactone (BPL), formaldehyde, and binary ethyleneimine (BEI). These were formulated with and without *Mycobacterium w* (Mw) and used for immunization of Balb/c mice to evaluate the immunogenicity of these formulations. The neutralizing antibody titers were estimated using the plaque reduction neutralization test with a 50% end-point ($PRNT_{50}$). *Results:* Purification, cloning and sequencing of the samples confirmed that these were indeed CHIKV isolates. Cell culture of the isolates revealed that five isolates grew better than the rest. The isolate AHD4 grew the fastest and was chosen as the candidate vaccine. It was formulated with BPL, formaldehyde, and BEI, with and without Mw. The six formulations were tested for immunogenicity in eight Balb/c mice in each group. The BEI-inactivated CHIKV formulated with Mw elicited the highest neutralizing antibody response. *Conclusion:* The BEI-inactivated CHIKV formulated with Mw is the best candidate vaccine that could be taken forward for further evaluation in rhesus monkeys.

Keywords: Chikungunya, CHIKV, Vaccine, Virus, Inactivation, Immunization.

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INTRODUCTION

Chikungunya (CHIK) is a mosquito-borne viral disease that is of high public health importance in India. It is spread primarily by the bite of infected *Aedes aegypti* mosquitoes and to a lesser extent by *Aedes albopictus* mosquitoes. The term *"chikungunya"* is derived from the word *"kungunyala"*, which in the African Kimakonde language means *"that which bends up"*. This alludes to the contorted and stooped posture exhibited by patients afflicted by the disease caused by the chikungunya virus (CHIKV).[1]

CHIKV is an alphavirus belonging to the *Togaviridae* family. Its genome consists of a positive sense, single stranded RNA molecule that is approximately 12 kb in length. The viral genome consists of two large open reading frames (ORFs), which encode four non-structural (nsP1, nsP2, nsP3, and nsP4) and five structural (capsid, E1, E2, E3, and 6K) proteins and is flanked by 5′ and 3′ NTR sequences.[2] CHIKV isolates across the globe are classified into three genotypes. This is based on phylogenetic analyses of cDNA sequences encoding the E1 protein. These three genotypes are East/Central/South African (ECSA), West African, and Asian.[3-5]

CHIKV infection causes an acute febrile illness that is marked by a triad of symptoms, namely, fever, rashes, and joint pain. Epidemiological studies have clearly shown that CHIKV originated from Africa about 200-300 years ago.[6,7] CHIK was first detected in 1952-1953 in the Makonde plateau in Tanganyika, which is modern-day Tanzania.

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Although all age groups are affected by CHIK disease, children bear the brunt of the burden, as they suffer much more than adults. The incubation period of CHIKV ranges between 4-7 days from the time of the mosquito bite. In the acute phase of the disease, the major symptoms include joint and muscle pain, high fever, extreme weakness, headache, vomiting, and rashes. Various types of neurological syndromes and non-neurological manifestations characterize the chronic phase. Notably, it has been reported that the excruciating joint pain that the patients experience can persist for months and even up to two years.

Over the past couple of decades, there have been large outbreaks of CHIK disease in South-East Asia.[8] The first Indian outbreak of CHIK was reported from Kolkata in 1963, which was followed by several other major outbreaks in

various parts of the country. For example, in 1964 there were outbreaks in Chennai, Puducherry, and Vellore. The following year, there were outbreaks in Vishakhapatnam, Rajahmundry, Kakinada, and Nagpur. After a gap of seven years, Barsi (Solapur, Maharashtra) experienced an outbreak in 1973.[9] After these outbreaks, CHIKV seemed to vanish from the country. It again re-emerged after a gap of 32 years in 2005 and caused an explosive outbreak that affected 13 states.[10] The states to be affected first included Andhra Pradesh, Gujarat, Karnataka, Kerala, Madhya Pradesh, Maharashtra, and Tamil Nadu. All age groups and both genders were affected by the virus. Investigations revealed that the CHIKV isolates were that of the African genotype, meaning that they were vastly different from those that circulated in 1963-1973, which were of the Asian genotype.[11] A major cause for concern in recent years has been the occurrence of deaths arising from CHIKV infections, which was not reported earlier. Therefore, it has been strongly felt by the scientific community that it is imperative to develop a CHIKV vaccine to reduce mortality and save precious lives. The present study focuses on developing an inactivated (killed) vaccine against CHIKV and testing its immunogenicity in mice.

MATERIALS AND METHODS

Virus Isolation and Purification

Fifty-three blood samples were collected from patients who exhibited symptoms that were typical of CHIK disease. After sera separation, the serum samples were tested in mice pups and cell culture to establish whether they produced any cytopathic effects (CPE). The serum samples were separated into two groups – Group 1: Mice testing and Group 2: Cell culture testing.

In Group 1, 3-day-old Balb/c mice pups were injected intra-cerebrally (IC) with the sera samples from the patients after dilution (1:10) in 1X PBS (pH 7.2). In Group 2, the sera samples were added to a 70% confluent monolayer of Vero cells, derived from African green monkey kidneys and incubated for 72 hours at 37°C at 5% CO_2 concentration and observed for development of CPE. The mice pups showed symptoms of CHIK disease within the next 3 days and the Vero cells also exhibited CPE within 3 days post-infection.

The brains of infected mice pups that exhibited retarded movements were harvested and the clarified brain homogenate was used to infect Vero cell monolayers, as described above. The culture supernatants derived from both groups (Group 1 and 2) were removed and plated on monolayers of Vero cells for plaque formation. Plaques developed within 3 days and yielded viruses that were further subjected to two rounds of plaque purification. The purified virus isolates were cultured to scale up, increase their volume, and prepare virus stocks for use in the next stage.

Molecular Cloning and Sequencing

The genomic RNA was extracted from the plaque-purified virus isolates. The isolated RNA was reverse-transcribed by RT-PCR using SuperScript™ III One-Step RT-PCR System (Invitrogen, Waltham, MA, USA) and Pfu DNA polymerase (Promega, Madison, WI, USA) according to manufacturer's instructions. PCR primers were designed to amplify the CHIKV non-structural protein 4 (NS4). PCR then amplified this region of the genome. The DNA amplicon encoding the NS4 protein was then cloned into pCR™ 2.1-TOPO™ TA vector (Invitrogen, Waltham, MA, USA) according to the manufacturer's protocol and then sequenced using the BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA) using cDNA-specific forward and reverse primers. Sequence analysis confirmed that the isolated viruses were indeed CHIKV. Full genome sequencing of one of the CHIKV isolates (IND-06-Guj) was also carried out. The genome of this CHIKV isolate was found to be a linear RNA strand of 11,829 bp in length. The whole genome sequence (Accession: JF274082.1; GI: 326902023) was uploaded on the GenBank® database (National Institutes of Health, Bethesda, MD, USA).

CHIKV Culture

Beginning with 53 CHIKV isolates, the number was narrowed down to five, based on their growth characteristics in Vero cell culture. These five CHIKV isolates were named CHIKV AHD1, CHIKV AHD2, CHIKV AHD3, CHIKV AHD4, and CHIKV AHD5. Subsequently, the fastest-growing isolate that reached the highest titer was further propagated in Vero cells that had been adapted to grow in fetal calf serum (FCS)-free media. Subsequently, the culture supernatant containing the virus was concentrated roughly 25-fold in a Tangential Flow Filtration System (Millipore, Burlington, MA, USA). The concentrated virus was loaded over a sucrose gradient (20-60%) and ultra-centrifuged. Fractions enriched with the virus were pooled and aliquoted for later use.

CHIKV Inactivation

CHIKV was inactivated using three different chemicals to see which one was the best. These included β-propiolactone (BPL), formaldehyde, and binary ethyleneimine (BEI). CHIKV was efficiently inactivated by all three chemicals. Since Vero cells are highly susceptible to CHIKV infection, inactivation of the virus was tested by passaging the preparation in these cells. This involved four blind passages in Vero cells, followed by plaque titration. After four passages, no virus could be detected, thereby indicating that CHIKV had been fully inactivated and the formulation was ready for immunization of mice.

Mice Immunization

Groups of 8-week-old Balb/c mice were used for the immunization studies. The mice were divided into six groups with eight mice in each group (n=8). These groups included the following immunogens:

Group 1: BPL-inactivated CHIKV
Group 2: BPL-inactivated CHIKV + *Mycobacterium w* (Mw)
Group 3: Formaldehyde-inactivated CHIKV
Group 4: Formaldehyde-inactivated CHIKV + Mw

Group 5: BEI-inactivated CHIKV
Group 6: BEI-inactivated CHIKV + Mw
Three micrograms of each of the above immunogens were formulated with the adjuvant alhydrogel. Inactivated Mw (also known as *Mycobacterium indicus pranii*) was used in half of the vaccine formulations as it has been established that it enhances the innate immune response and is approved for use in humans. Mice were immunized intramuscularly (IM). Following the primary immunization, the mice were administered two booster doses at 2- and 5 weeks post-immunization. Mice were bled from the retro-orbital plexus one week after the second booster dose and the sera were tested for neutralizing antibodies by the plaque reduction neutralization test with a 50% end-point ($PRNT_{50}$).

Plaque Reduction Neutralization Test ($PRNT_{50}$)

Serum samples from individual mice obtained one week after the second booster dose were assayed for CHIKV neutralizing activity by $PRNT_{50}$, as described elsewhere. [12] Briefly, two-fold serial dilutions of mice sera were heat-inactivated at 56°C for 30 minutes. These were then incubated with ~100 plaque-forming units (pfu) of CHIKV at 37°C for one hour before being added to a 6-well tissue culture plate containing ~70% confluent monolayer of Vero cells. The inoculum was removed after one hour and the cell monolayer overlayed with tissue culture medium containing 1% agarose. The plate was incubated for 72 hours at 37°C/5% CO_2 for plaque development. The percent neutralization was calculated from the difference in the number of plaques obtained in the absence and in the presence of immune sera. The reciprocal of the highest serum dilution giving at least 50% neutralization, was regarded as the CHIKV neutralization titer.

RESULTS

Culture of CHIKV Isolates

CHIKV isolates were cultured in Vero cells in medium containing FCS. The Vero cells were infected with the CHIKV isolates at a multiplicity of infection (MOI) of 0.1 for 1 hour at 37°C. Afterwards, the monolayer of Vero cells was washed and incubated in fresh culture medium. Viruses released into the medium after infection were collected at specific time points (6, 12, 18, 24, 30, and 36 hours post-infection) and their titers were determined. The viral growth kinetics curve clearly shows that of the five CHIKV isolates, CHIKV AHD4 isolate grew much quicker and to the highest titer than the other isolates. Hence, this isolate was chosen for further studies (Figure 1).

Culture of CHIKV AHD4 Isolate

Monolayers of Vero cells cultured in FCS-free medium were infected with CHIKV isolate AHD4 at MOI of 0.1 for 1 hour at 37°C. The cell monolayer was washed and incubated in fresh culture medium. Viruses released into the medium after infection were collected at specific time-points (6, 12, 18, 24, 30, and 36 hours post-infection) and their titers were

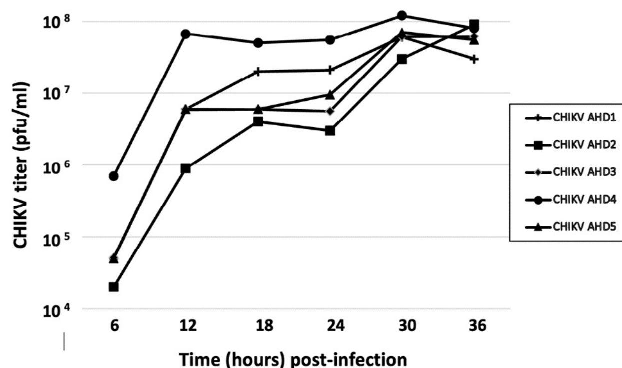


Figure 1: Growth kinetics of CHIKV isolates in Vero cells

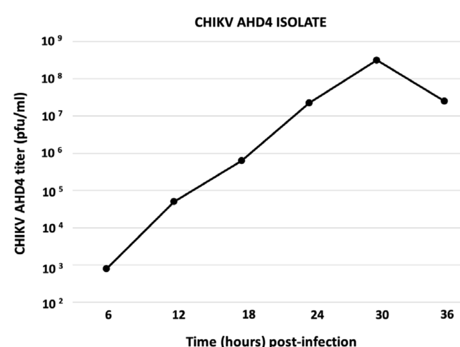
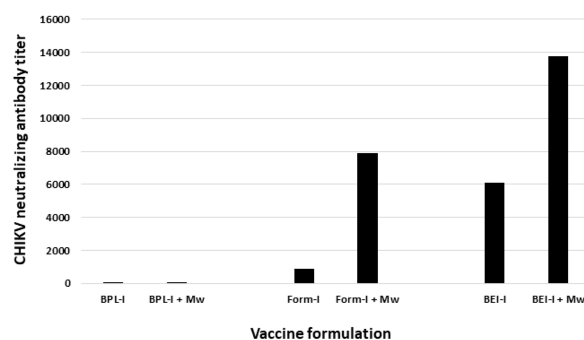


Figure 2: Growth of CHIKV AHD4 in Vero cells cultured in FCS-free medium

determined. The AHD4 isolate grew to high titers in Vero cells adapted to FCS-free medium and reached peak titers around 30 hours post-infection (Figure 2).

Mice Immunization

Groups of Balb/c mice (n=8) were immunized with the following formulations: (i) BPL-Inactivated CHIKV (BPL-I), (ii) BPL-Inactivated CHIKV combined with Mw (BPL-I + Mw), (iii) Formaldehyde-Inactivated CHIKV (Form-I), (iv) Formaldehyde-Inactivated CHIKV combined with Mw (Form-I + Mw), (v) BEI-Inactivated CHIKV (BEI-I), and (vi)



BPL-I: BPL-inactivated CHIKV; BPL-I + Mw: BPL-inactivated CHIKV + *Mycobacterium w*; Form-I: Formaldehyde-inactivated CHIKV; Form-I + Mw: Formaldehyde-inactivated CHIKV + *Mycobacterium w*; BEI-I: BEI-inactivated CHIKV; BEI-I + Mw: BEI-inactivated CHIKV + *Mycobacterium w*

Figure 3: Geometric mean neutralizing antibody titer in mice immunized with different formulations of inactivated CHIKV vaccine

BEI-Inactivated CHIKV combined with Mw (BEI-I + Mw). Following the primary immunization, two booster doses were administered at 2- and 5 weeks post-immunization. Mice were bled one week after the second booster dose and the sera were tested for CHIKV neutralizing antibodies by the $PRNT_{50}$ assay. It was clearly evident that the formulation containing BEI was the most immunogenic. Notably, the geometric mean neutralizing antibody titer was significantly enhanced when BEI was formulated with Mw (Figure 3).

DISCUSSION

Various vaccine platforms have been used over the years to develop CHIKV vaccines. These include protein subunits, virus-like particles (VLPs), viral vectors, and nucleic acid platforms.[13] Recombinant protein subunit vaccines use a small part (subunit) of the virus instead of the whole virus. The immunogenic protein is most commonly expressed in *E. coli*, though other expression systems, such as yeast, are also available nowadays. Some subunit vaccines incorporating E1 and E2 envelope proteins have shown good efficacy in animal models, such as mice.[14, 15] VLPs, as the name suggests, are like viruses, but not totally, as they don't contain any genetic material, thereby making them non-infectious and extremely safe to use as candidate vaccines. Viral structural proteins (C-E3-E2-6K-E1) have been successfully expressed in several cell lines, which yielded proteins that self-assembled into VLPs. These were subsequently purified by buoyant density gradient sedimentation and used for immunization of rhesus monkeys [16] and humans.[17] Viral vector-based platforms are another useful strategy to deliver antigens into cells. These use a viral vector that carries the transgene and delivers it to the target cells; therefore, they can be considered analogous to the famous Trojan horse described in Greek history. Viral vector-based vaccines have used many types of viruses, including vesicular stomatitis virus (VSV),[18] modified vaccinia virus Ankara (MVA), [19] measles virus (MV), [20] and adenovirus (Ad). It is quite plausible that pre-existing antibodies to human Ad could be present, as almost all humans are exposed to this virus. Therefore, Oxford University developed a chimpanzee adenovirus vector (ChAdOx1) to evade these pre-existing antibodies, thereby averting any cross-reaction. Notably, ChAdOx1 is extremely safe to use in humans. [21] In recent decades, nucleic acid-based vaccine candidates have been studied intensely across the globe. A codon-optimized DNA vaccine encoding the CHIKV envelope (E) proteins (E1, E2, E3) generated robust antigen-specific humoral and cellular immune responses, including neutralizing antibodies in mice and rhesus monkeys and also protected immunized mice against virus challenge. [22] An mRNA vaccine expressing CHIKV E2-E1 antigens, encapsulated in lipid nanoparticles (mRNA-LNP) induced potent humoral and cellular responses in C57BL/6 mice. [23]

Inactivated (killed) vaccines, together with live-attenuated vaccines, constitute the category of traditional vaccine platforms, which have also been employed for the development of CHIKV vaccines. These are so called because they have been in use for well over a century, and there is a high level of confidence regarding their safety and efficacy. In the case of inactivated vaccines, the virus is killed and is, therefore, very safe as there is no chance of reversion of the virus to its wild type. Inactivation is carried out either by treatment with formaldehyde [24] or UV irradiation.[25] Both these approaches elicited neutralizing antibodies in non-human primates. In case of the former, the Asian strain of CHIKV was formalin-inactivated and administered 28 days apart in 16 soldiers. Neutralizing antibodies were generated in all the soldiers two-weeks after the second dose. No local or systemic side effects were noted. Longitudinal studies were not conducted to study how long the antibodies persisted. This CHIKV candidate vaccine was not developed any further.[24] In the present study, besides formaldehyde, BPL and BEI were also used and the latter yielded high titers of CHIKV-specific antibodies. Another inactivating agent has been reported in the literature. This is the use of 1,5 iodonapthyl azide (INA) for inactivating CHIKV. However, the neutralizing capacity of the neutralizing antibodies were lower than that in the present study. [26] Another chikungunya candidate vaccine, developed by the US National Institutes of Health (NIH) under its Small Business Innovation Research (SBIR) Program has used hydrogen peroxide as an inactivating agent. However, the efficacy of this CHIKV candidate vaccine is not known. [27] This chemical inactivation approach, especially using formaldehyde, has been used for inactivating CHIKV and other viruses, including Japanese encephalitis virus (JEV) [28] and dengue virus (DENV). [29]

To the best of the author's knowledge, this is the first instance where multiple chemicals have been used simultaneously to inactivate CHIKV and make a head-to-head comparison with reference to immunogenicity in mice. Notably, the generation of high titers of neutralizing antibodies following immunization could be used as a surrogate marker for vaccine efficacy. Importantly, the immunogenicity studies yielded promising results, indicating that this CHIKV candidate vaccine could be further evaluated in rhesus monkeys.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

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PEER-REVIEWED CERTIFICATION

During the review of this manuscript, a double-blind peer-review policy has been followed. The author(s) of this manuscript received review comments from a minimum of two peer-reviewers. Author(s) submitted revised manuscript as per the comments of the assigned reviewers. On the basis of revision(s) done by the author(s) and compliance to the Reviewers' comments on the manuscript, Editor(s) has approved the revised manuscript for final publication.