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Vaccine

Development of an inactivated candidate vaccine against Chandipura virus (*Rhabdoviridae*: *Vesiculovirus*)

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ABSTRACT

A Vero cell based vaccine candidate against Chandipura (CHP) virus (*Rhabdoviridae*: *Vesiculovirus*), was developed and evaluated for immunogenicity in mice. Virus was purified by ultracentrifugation on 30% glycerol cushion followed by differential centrifugation on 10–60% sucrose gradient and inactivated with β-propio lactone at a concentration of 1:3500. The inactivated product was blended with aluminium phosphate (3%) and immunized 4-week-old Swiss albino mice. Neutralizing antibodies in the range of 1:10 to 160 and 1:80 to 1:320 was detected with 85% and 100% sero-conversion after 2nd and 3rd dose, respectively. All the immunized mice with antibody titer above 1:20 survived live virus challenge. The vaccine candidate has potential to be an efficient vaccine against CHP virus.

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1. Introduction

Chandipura virus (CHPV), a member of the family *Rhabdoviridae*, *genus Vesiculovirus* was implicated as the etiological agent for an encephalitis-like highly fatal outbreak in several districts of Andhra Pradesh (AP) and Maharashtra during 2003 [1]. CHPV, a novel virus causing febrile illness in man was discovered accidentally during an outbreak investigation of dengue/chikungunya-like illness in Nagpur district, Maharashtra, India. The virus was isolated on a few subsequent occasions from *Phlebotomus* sand flies and humans; however, due to low or no pathogenicity to humans, not much attention was given to the virus until its resurgence in a virulent form in 2003, killing 183 children in AP [1]. The case fatality rate (CFR) was >56% and most deaths occurred within 48 h of onset of symptoms. The outbreak was comparatively severe in Gujarat as 18 children died out of 23 admitted and the CFR was 78.3% [2]. Most of the deaths occurred within 24 h. Sporadic cases are reported every year from the endemic areas of AP and Maharashtra [3,4]. Patients are treated symptomatically as no effective vaccines or therapeutics is available. Development and evaluation of a subunit vaccine was reported recently which produced good immunogenicity in laboratory animals. An attempt was therefore made to develop a tissue culture based inactivated vaccine candidate against the virus.

2. Materials and methods

2.1. Propagation and purification of CHPV in Vero E6 cell line

Virus stock was prepared in Vero E6 cell line using a plaque purified strain of CHPV (Strain no. 034627). The cell line and the virus were EM screened for 10 consecutive passages to rule out contamination. The cells were grown in 150 cm^2 tissue culture flasks (Nunc, Denmark) in DMEM supplemented with 10% FBS (Invitrogen, USA). Virus infection, harvest, virus quantitation and storage were carried out as described earlier [5]. In brief, the cells when formed a confluent monolayer, were infected with 1 MOI of virus and incubated for 1 h at 37 °C with intermittent rocking at every 15 min. After incubation, the inoculum was discarded; cells were washed twice with PBS and fed with maintenance medium (DMEM containing 2% FBS). Cells were incubated at 37 °C, observed under an inverted microscope for the appearance of cytopathic effects (CPE) and harvested when more than 75% cells exhibited CPE. The cultures were freeze–thawed thrice, centrifuged the cell lysate at 5,000 rpm in a refrigerated centrifuge for 30 min, and collected the supernatant. It was transferred onto tubes containing 5 ml 30% glycerol in NTE buffer and ultra centrifuged at 32,000 rpm for 3 h at 4 °C. The virus pellet was suspended in 50 μl NTE buffer, pooled, loaded over a 10–60% sucrose gradient and centrifuged at 32,000 rpm for 2 h. The translucent zone between 60% sucrose and 10% sucrose was collected carefully as it contained the maximum virions and ultra centrifuged at 32,000 rpm for 1 h as above. The pellet was collected, suspended in NTE buffer and stored at −70 °C until use.

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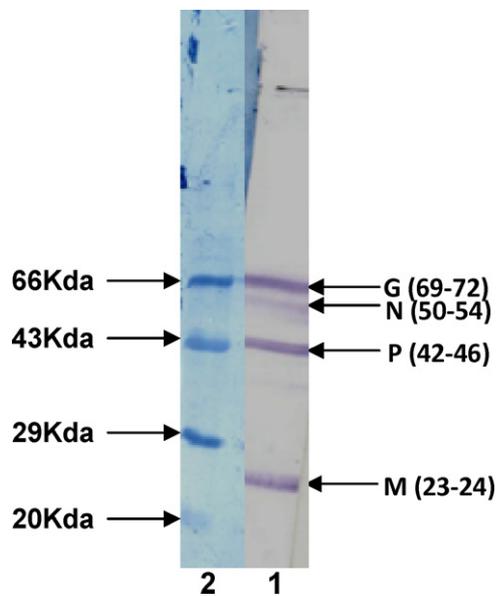
Fig. 1. Western Blot analysis of gradient purified CHPV. (Lane 1) CHPV protein profile. Gel of purified virus on NC paper probed with anti CHPV antibody (raised in mice) followed by anti mouse antibody tagged with AP enzyme. (Lane 2) Molecular weight marker stained with amido black after transferring the gel to NC paper.

2.2. SDS-PAGE and immunoblotting

The amount of protein in purified live virus and inactivated virus was estimated using Lowry's method [6]. The known amount of protein was separated on 12% resolving and 4% stacking SDS-PAGE in a mini electrophoresis unit (Bio-Rad, USA) at 100 V for 2 h. The proteins were transferred at 100 V for 45 min onto a nitrocellulose membrane in wet condition using a trans-blot apparatus (Bio-Rad, USA). The unoccupied binding sites were blocked with 2% (w/v) bovine serum albumin (BSA) in PBS (pH 7.4) for 1 h at room temperature. Prior to immunoassay, the membranes were washed thrice for 5 min each in wash buffer comprising 0.1% (W/V) BSA in PBS with 0.05% (v/v) Tween 20 (Sigma, USA). It was incubated with primary antibody (hyper immune serum raised in mice against CHPV at a dilution of 1:100) for 1 h at 37 °C. The membrane was washed with wash buffer and incubated with anti-mice AP conjugate (Sigma, USA) at a dilution of 1:25,000. The membrane was washed twice with PBST and developed with BCIP/NBT pre-mixed substrate solution till the bands appeared. Washing the membranes in distilled water terminated the reaction.

2.3. β-Propio lactone (BPL) inactivation kinetics

BPL (Sigma, St. Louis, MO, USA) was mixed with the virus stock to get a concentration of 1:3500 and stirred continuously on a magnetic stirrer at 4 °C. Aliquots of 100 μl was collected at every 15 min, BPL activity was neutralized by adding 100 μl of 2% sodium thiosulphate and stored at −70 °C until the completion of experiment. Samples were collected from '0' to 150 min. The sequential samples were titrated in RD cells and the inactivation kinetics was determined (Fig. 3). The sample, which did not induce CPE in the cell line was determined as completely inactivated product and the time point was taken as time of complete inactivation (t). For mice immunization studies, CHPV was treated with BPL for double the time required for complete inactivation ($2 \times t$).

2.4. Aluminium phosphate ($AlPO_4$) adsorption

The stock solution (10 mg/ml) of $AlPO_4$ (Sigma Chemical Co., USA) was diluted in inactivated CHPV suspension to get final concentrations of 1, 2 and 3 mg/ml and mixed thoroughly on magnetic stirrer for 1 h at 4 °C and tested for maximum binding. The samples were centrifuged at 14,000 rpm for 5 min at 4 °C, pellet and supernatant were collected and virus titer was determined as described earlier [4]. For immunization, the inactivated virus was precipitated with $AlPO_4$ (3 mg/ml) as it gave maximum binding. Both positive control and negative controls were also tested simultaneously. The former constituted of purified CHPV with $AlPO_4$ while the negative control constituted normal Vero tissue culture fluid mixed with same concentration of $AlPO_4$.

2.5. Immunization of mice and antibody determination

Three- to 4-week-old Swiss albino mice were pre-bled and tested for N-antibody to CHPV. Mice immunization was done sub-cutaneously in the abdominal area. Test mice were immunized with the $AlPO_4$ adsorbed inactivated CHPV (0.1 ml/mice). Positive controls were mice immunized with purified live virus adsorbed with $AlPO_4$ and negative controls were mice inoculated with normal TCF adsorbed with $AlPO_4$ at the same concentration as for test samples. Bleeding was done 21 days after each dose through the retro-orbital route after light anesthesia. Sera was separated and screened for CHPV antibodies by neutralization test (NT).

2.6. Challenge of immunized mice with live virus

The immunized mice were challenged 21 days after the third dose with 20 μl of live virus (100 pfu) by intra cranial (i/c) route and observed for 14 days. An additional set of positive and negative controls were also used for challenge experiment to verify the technique and virus infectivity. Normal (unimmunized) mice of the same age were used for the study. The positive control received live virus while the negative control received only DMEM. All the mice were kept under ideal conditions and observed for sickness and death.

2.7. Potency test

To determine the potency of the vaccine candidate, 10 mice were immunized with different dilutions (1:5, 1:10 and 1:50) of the vaccine. Mice were bled and the antibody titers were determined. The immunized mice were also challenged with live virus as above and observed. Positive and negative controls were also used in the study.

2.8. Statistical analysis

Log GMT values for different doses were compared by paired t test using SPSS 11 software. The survival analysis was carried out in PASW Statistics 18. The replicas of each experiment were combined together in order to increase the sample size (30 mice) for each experiment. The Kaplan–Meier plot was used to show the % survival with respect to time. Since the mice were followed up to 14 days, the mice remaining after 14th day were treated as Censored (survived). Three different tests (Log Rank test, Breslow's test and Tarone–Ware test) were used for comparing the mean survival times of different groups.
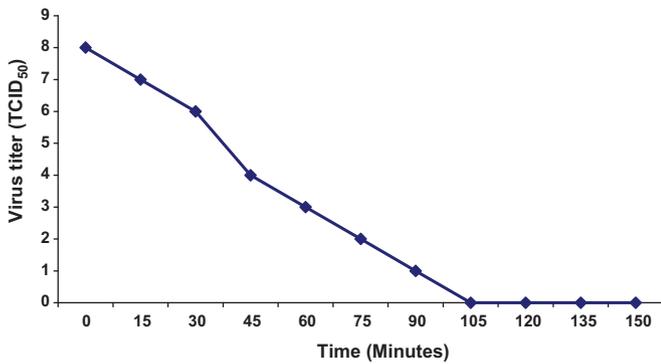
Fig. 2. Inactivation kinetics of CHPV with BPL (1:3500).

3. Results

3.1. CHPV propagation, purification and inactivation

Though CHPV replicated in several systems, Vero E6 cell line was used for large scale propagation due to the ease in downstream processing [4]. Chicken embryos, though produced highest titer of virus, was found difficult to purify. Among the various methods employed to purify CHPV from Vero E6 tissue culture fluid (TCF), ultra centrifugation with 30% glycerol cushion followed by discontinuous sucrose gradient (10–60%) gave maximum virus yield with lowest level of contaminating proteins. The protein profiles of the purified products were analyzed by SDS-PAGE and Western blot. The SDS-PAGE profile clearly demonstrated the presence of specific CHPV protein bands. Western blot analysis clearly demonstrated the G, N, P and M proteins at the equivalent molecular weights between the ranges of 23 and 72 kDa as observed in CHPV prior to inactivation (Fig. 1).

3.2. Inactivation of CHPV by BPL

Complete inactivation of CHPV with a titer of Log_{10} 8 $TCID_{50}$/ml with BPL at a concentration of 1:3500 was achieved at 105 min at 4 °C. Inactivation was gradual and steady as the drop in titer was approximately 1 log per 15 min (Fig. 2). The complete inactivation of CHPV was demonstrated in *in vitro*, *in ovo* and *in vivo* for three consecutive passages.

3.3. Immunogenicity in mice

NT studies in tissue culture demonstrated the production of antibodies was dose dependant. The antibody titer (NT) increased with every dose reaching the maximum after the 3rd dose (Fig. 3). Comparable increase in antibody titer was also observed when different dilutions of the vaccine were used for immunization though at a very low level. After two dose, the NT titer ranged between 1:10 and 1:160. However, after the third dose, NT antibody titer increased and ranged between 1:40 and 1:320. The antibody titer obtained after immunization with live virus is given in Fig. 4. Sero conversion rate after the second and third dose was 85 and 100%, respectively. Mice with NT titer above 1:20 survived the challenge test.

3.4. Statistical analysis

All the three tests showed that the mean survival time for 1:50 virus was significantly more than that of each of 1:5 vaccine, 1:10 vaccine and 1:50 vaccine ($p < 0.01$ for each comparison). The mean survival times of the vaccine groups 1:5, 1:10 and 1:50 do not differ significantly ($p > 0.05$ for each pair) (Fig. 5).

4. Discussion

CHPV has emerged as an important pediatric encephalitis-causing pathogen with very high case fatality rate in central India [7]. The disease is predominantly rural and majority of the patients belong to the low socio-economic class of the society. The virus is transmitted through sand flies, which breed in cracks and crevices around human habitats. The high case fatality rate and rapid progression of the disease warranted the need for an effective vaccine for immunization of children in the endemic areas. A recombinant candidate vaccine using CHPV G-protein was reported to have developed recently with 90% protection in mice [7,8]. The gene (G-gene) was expressed in baculovirus expression system, HPLC purified and used for mice immunization. Three doses were given at an interval of 4 weeks at a concentration of one microgram. Anti-CHPV IgG antibodies were observed as early as 2nd week of immunization. Live virus challenge through IC route demonstrated 90% protection. They also observed neutralization of homologous and heterologous viruses with the antisera. The investigators also demonstrated the induction of both arms of immune response. The vaccine was evaluated in combination with commercially available DPT vaccine to determine the efficacy of the combination in a view to cover the local endemic population. The combination yielded identical results as observed for CHPV vaccine [9].

However, no clinical trials have been reported with the above vaccine. We, therefore, made an attempt to develop a Vero E6 cell based, BPL inactivated vaccine against CHPV. Vero E6 cell line has been selected for propagation of the virus due to the ease in management, scale up and downstream processing. It has also
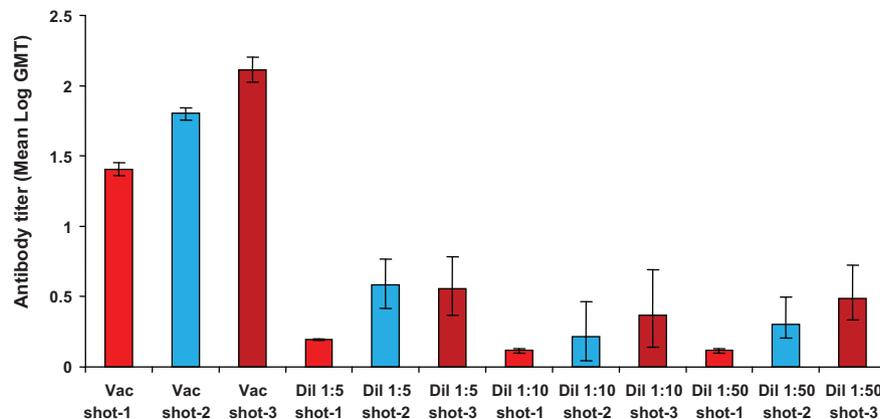
Fig. 3. Comparison of antibody titer at different dose and dilution of vaccine (neat vaccine protein concentration was 10 μg/ml).

Comparison of antibody titer at different dose of purified virus
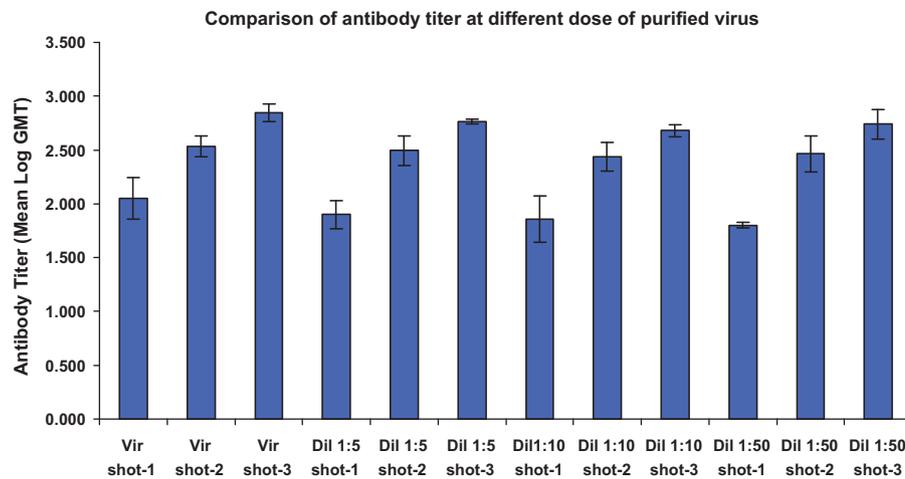
Fig. 4. Comparison of antibody titer at different dose of purified virus.
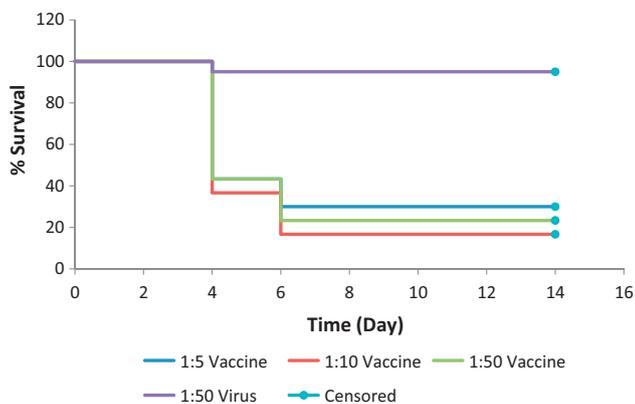
Fig. 5. Survival curve for challenge experiment (survival plot; Kaplan–Meier plot).

been approved by regulatory authorities for vaccine development against several human pathogens [10–12].

In the study, several techniques were used to standardize CHPV purification without significant loss in biological activity. The use of cellophane sulphate (Millipore India Ltd, Bioscience division) for purification did not yield CHPV of biological activity though it was used successfully to purify rabies virus as claimed by the manufacturer. Gel filtration Chromatography using Sephacryl S-200 (Sigma Chemical Co., USA) was also used as per the manufacturer's instructions to purify the virus. However, the yield of purified CHPV with biological activity was very low. CHPV purification using column chromatography was also not successful due to the low yield of virus with biological activity. The use of ultracentrifugation on 30% glycerol cushion followed by centrifugation of discontinuous sucrose gradient (10–60%) was found effective to achieve the highest amount of purified virus as demonstrated by SDS-PAGE and Western blot (Fig. 1).

BPL has emerged as an important inactivating agent and is being extensively used in vaccine production as it retains the antigenicity and immunogenicity of the viral proteins [10,12,13]. In addition, several BPL inactivated candidate vaccines (poliomyelitis, severe acute respiratory syndrome, and human immunodeficiency virus) are currently being evaluated in animal models and human clinical trials [14]. BPL has several advantages over other chemicals as it completely hydrolyses when incubated at 37 °C to an isomer of lactate and β-propionic acid which are non-toxic degradation product found normally in human body as a by-product of fat metabolism. In addition to virus inactivation BPL also serve as an internal bac-

tericide and fungicide as it reacts with the nucleic acid of these microbial contaminants and degrade the organism [15].

As an adjuvant, alum (aluminium phosphate) at a concentration of 3 mg/ml was used in the present study as it has been demonstrated to enhance the immunogenicity of viral vaccines. Adjuvants are being used to improve the immunogenicity of weak antigens especially to enhance the speed and duration of the immune response as well as to increase the efficiency of a vaccine or toxoid by increasing the availability of the antigen in the lymphatic system [16–19]. Complete binding of CHPV was demonstrated in the present study.

The immunogenic potential of the BPL inactivated CHPV vaccine was assessed by determining the neutralizing antibodies generated after three successive doses of vaccine. Neutralization also can be used as marker to determine protective efficacy. The study therefore demonstrates the efficacy of the vaccine in protecting immunized mice. However, the vaccine should be evaluated in non-human primates before it can be considered for use in humans.

5. Conclusion

The study demonstrates development of an efficient vaccine candidate against CHPV. Even the two dose vaccine was found to be efficient to give 100% protection in 85% immunized mice. Antibody titer after the third dose ranged between 1:80 and 1:320. Mice which demonstrated neutralizing antibody titer above 1:20 survived live virus challenge through intra cranial route. The study also demonstrated BPL as an efficient inactivating agent to inactivate CHPV retaining the immunogenic and antigenic properties of the virus.

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