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RESEARCH ARTICLE

# IDENTIFICATION OF IMMUNODOMINANT EPITOPES OF E1 AND NUCLEOCAPSID PROTEINS OF CHIKUNGUNYA VIRUS WITH NEUTRALIZING CAPACITY

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**ABSTRACT**

Chikungunya (CHIK) disease caused by mosquito transmitted *Alphavirus*. Chikungunya showed worldwide epidemics including some fatal cases. Thus there is need for a safe and effective therapy for Chikungunya. In this study we delineated linear dominant B and T cell epitopes of envelope E1 and nucleocapsid proteins with neutralizing potential *in vitro*. Immunogenicity of synthesized peptides, encapsulated in PLGA micro particles was done intramuscularly with CpG ODN as adjuvant in outbred and inbred H-2$^d$ mice. Few peptides showed high antibody peak titer with robust memory response. Peptides of E1 and Capsid proteins showed antibody peak titer in ranging 1,18,000 to 2,40,000. Peptides E1P1, E1P4, E1P10 of E1 protein and CP3, CP5, CP9 of capsid protein were found major B cell epitopes. E1P4, E1P9 and CP6 peptides were identified as T cell epitopes, confirmed by T cell lymhoproliferation assay and cytokines profile. Peptides E1P4, E1P9 and CP6 induced Th1 and Th17 type of immune response. During antibody isotyping E1P1, E1P4, E1P10, CP3, CP9 peptides showed equal distribution of IgG1, IgG2a and IgG2b subclass. More importantly E1P1, E1P4, E1P10, CP3 & CP9 peptide specific antibodies showed *in vitro* neutralization (>90%) to CHIKV in plaque reduction neutralization test (PRNT$_{90}$). Overall identified epitopes can be assembled in multiple antigenic peptides (MAPs) for developing effective subunit vaccine for CHIKV.

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## INTRODUCTION

Chikungunya virus (CHIKV) is an arbovirus belonging to genus *alphavirus* (Suhrbier *et al*., 2012). CHIKV infection generally characterized by high fever, headache, skin rash, myalgia, and polyarthraligia (Simon *et al*., 2011). Disease symptoms usually resolve within 2 weeks, but in some cases joint swelling, joint rigidity, arthralgia can persist for months to years (Borgherini *et al*., 2007; Hoarau *et al*.,2010; Schilte *et al*.,2013; Sissoko *et al*., 2009; Staikowsky *et al*., 2008). CHIKV infection has been reported in many countries (Powers *et al*., 2007, Salje *et al*., 2016). Widespread distribution of CHIKV was due to better survival in a novel transmission vector, *Aedes albopictus* (Parola *et al*., 2006; Tsetsarkin *et al*., 2007). Single nucleotide change in E1 glycoprotein (Schuffenecker *et al*., 2006) had shown to promote its infection to midgut cells of *Ae. albopictus* moreover better transmitted by *Ae. aegypti* (Tsetsarkin *et al*., 2007; Vazeille *et al*., 2007). CHIKV have positive-sense, single-stranded RNA genome with two open reading frames (ORFs), encodes four nonstructural proteins (nsP1, nsP2, nsP3 and nsP4) and five structural proteins viz. Capsid, E1, E2, E3 and 6K (Jose *et al*., 2009). E2 glycoprotein interacts with a cellular receptor while E1 glycoprotein mediates virus fusion to host cell under low pH conditions (Fields *et al*., 2013). Outbreaks in 2004-2007 badly affect Indian Ocean region and other geographical area with complexity of disease (Rezza *et al*., 2007; Simon *et al*., 2006; Sissoko *et al*., 2009) . Recently Chikungunya affected places like Caribbean and the Americas region (Weaver *et al*., 2015). Many laboratories are working for diagnosis and vaccine design for CHIKV throughout the world. In the present study, Immunodominant epitopes of E1 and Capsid protein were identified by studying humoral and cell mediated responses of peptides in murine model. Eleven sequences of E1 protein and nine sequences of capsid protein were synthesized as predicted by DNASTAR and TEPITOPE software. E1P1, E1P4 and E1P10 peptides of E1 and CP3, CP5 and CP9 peptides of capsid protein showed B cell response through competitive immunoassay, direct binding assay and high antibody titer with memory response. E1P4 and E1P9 peptides of E1 and CP6 peptide of Capsid demonstrated T cell response as proved by lymphoproliferative response &

cytokine profile. Interestingly E1P4 peptide represents overlapping B and T cell response. $PRNT_{90}$ assay proved E1P1, E1P4, E1P10, CP3 and CP9 epitopes have neutralizing specificity to Chikungunya virus.

## MATERIALS AND METHODS

### *Selection and synthesis of peptides*

On the basis of hydrophilicity, hydrophobicity, secondary structure, antigenicity index, amphipathicity from DNASTAR software and binding time (*T*1/2) of peptides to MHC molecules (TEPITOPE software), 11 peptide sequences of E1 protein and 9 peptide sequences of capsid protein (African S27 strain) spanning entire proteins were identified and synthesized using Fmoc chemistry. Peptides were cleaved from solid support and crude peptides were purified by Gel filtration and later by HPLC using C18 column.

| Peptides | Sequences | Sequence No. |
|---|---|---|
| (E1 protein) | | |
| E1P1 | KCCGTAECKDKNLPDYS | 3344-3360 |
| E1P2 | DAENTQLSEAHVEKSESCKT | 3380-3399 |
| E1P3 | FGDIQSRTPESKDVYA | 3483-3498 |
| E1P4 | AASKKGKCAVHSMTN | 3604-3618 |
| E1P5 | DAPSLTDMSCEVPACTHSSDF | 3575-3595 |
| E1P6 | ASAYRAHTASASAK | 3402-3415 |
| E1P7 | AYANGDHAVTVKDAKF | 3429-3444 |
| E1P8 | NAVTIREAEIEVEGNSQ | 3618-3634 |
| E1P9 | VCSTQVHCAAECHPPKDH | 3652-3669 |
| E1P10 | GPMSSAWTPFDNKIVVY | 3447-3463 |
| E1P11 | YKGDVYNMDYPPFGAGRPGQ | 3463-3482 |
| Peptides Sequences | (Capsid protein) | Sequence No. |
| CP-1 | FYNRRYQPRPWAPR | 2483-2496 |
| CP-2 | PRPRPQRQAG | 2504-2513 |
| CP-3 | VPQQKPNNNRKNKKQRQKKQ | 2530-2549 |
| CP-4 | APQNEPKQKKQPPQ | 2550-2563 |
| CP-5 | KKPAQKKKKPGRRERMC | 2564-2580 |
| CP-6 | LVGDKVMKPAHV | 2603-2614 |
| CP-7 | SKFTHEKPEGYYNW | 2650-2663 |
| CP-8 | GKPGDSGRPIFD | 2682-2693 |
| CP-9 | WNKDIVTKITPDGADDW | 2719-2735 |

### *Experimental Mice*

Outbred and Inbred H-$2^d$ mice were used throughout the study. Six to eight weeks old outbred mice were obtained from Experimental Animal Facility, AIIMS, New Delhi, India. BALB/c (H-$2^d$) mice were procured from National Institute of Nutrition, Hyderabad. Six to eight mice were used in each group. All the animals were kept in pathogen free condition. Experiments were conducted in accordance with the guidelines of AIIMS ethics as well as CPCSEA, Ministry of Social Justice, Government of India.

### *PLGA Micro particle preparation, particle size and morphology*

PLGA microspheres with entrapped peptides were prepared by double solvent evaporation process (water-in-oil-in-water) using poly (DL-lactide-*co*-glycolide; 50:50) (Sigma) (Ali *et al*., 2013).The entrapment percentage for different peptides within microspheres were in the range of 50-55% as quantified by Bicinchoninic acid (BCA) method (Sigma, IL, USA). Microsphere's size was determined by laser diffraction (Malvern Instrument, UK) and the size was between 2-6µm. Morphology of microspheres was observed by scanning electron microscopy (Phillips, CM 10) and found to be spherical in nature.

### *Immunization and sera collection*

Each group of mice was immunized intramuscularly on day 0 using PLGA microspheres (used as delivery vehicle) containing 40µg of peptides equivalent was suspended in 40 µl of PBS with 5µg of CpG-ODN 1826 (TCCATGACGTTCCTGACGTT) (Coley Pharmaceuticals, USA). Booster dose contained 20µg of peptide and 5µg of CpG was given on 23 and 42 day. To raised the sera against E1 protein or virus lysate, a primary dose of 30µg in Freund's complete adjuvant (FCA) was used on day 0 followed by booster dose of 15µg in Incomplete Freund's adjuvant (IFA) on 23 and 42 day. Bleeds were collected from retro-orbital plexus on the different day 0, 28, 42, 60, 90, 120. Antisera was separated and stored at −20 $^0$C till use.

### *Competitive immunoassay by ELISA*

Antibodies were raised in outbred mice with native E1 protein and virus lysate (E1 and virus lysates were provided by Dr. M.M.Parida, DRDE, Gwalior, INDIA). Varying amounts of peptides (0.125-256µg) of E1 protein were incubated with optimal dilution of E1 antisera (which gave absorbance 1.0 at 1:8000 dilutions) for 2 h at 37 $^0$C. Similarly Peptides of Capsid protein were incubated with virus lysate antisera (1:12000 dilutions with absorbance 1.0). We used virus lysate or its antisera for study of capsid' peptide only not for peptides of E1 protein. The Ag-Ab complex was transferred onto the ELISA plates previously coated with E1 protein/virus lysate (VL) (100ng/well), after blocking(5% milk powder) and proper washings with PBS-T, goat anti mouse IgG-HRPO (1:1000 dilution) was added and color was developed with orthophenylenediamine as chromogen. Finally absorbance was taken at 492 nm. The amount of peptide required for 50% inhibition with E1/virus lysate was calculated. The results were expressed as the % antibody binding in presence of competitor as compared to the control value (without competitor).

### *Direct binding assay*

To check the immunoreactivity of E1 peptides with E1 antisera and Capsid's peptides with virus lysate antisera or *vice versa*, direct binding assay was performed using ELISA protocol. The ELISA plates were coated with different peptides (200ng/well) of E1 or Capsid protein. After blocking and washings E1 and VL antisera raise in mice was added at a dilution of 1:200 onto the ELISA plates and incubated at 37$^0$C for 2h. Color was developed as usual. To check the reactivity of peptides antisera with E1/virus lysate, plates were coated with E1 and Virus lysate (100ng/well) and peptide antisera was added at 1:200 dilutions. Color was developed as described earlier.

### *Peptide Specific peak antibody titer*

Peak antibody titer for each peptide was estimated by ELISA. Microtiter plate (96 well, Immulon IIHB, Dynatech) was coated with 200 ng/well of each peptide (l00 µl/well) and kept overnight at 4$^0$C. Plates were blocked with 5% milk powder. After washing, two-fold serial dilution of respective peptide antisera, was added and color was developed as described above. The titers were expressed as the highest sera dilution giving an absorbance higher than that of pre-immune sera.

### *IgG isotyping*

IgG subclass (IgG1, IgG2a, IgG2b and IgG3) estimation was done as per manufacturer's instructions using isotyping kit ISO-2 (Sigma, USA).

### *Memory recall response*

B cell memory response was evaluated after immunization with E1/VL (capsid peptide). As soon as there was a fall in peak titer, mice were immunized with 20µg of E1 or virus lysate containing 5µg CpG ODN on day 120 and bleed was collected on day 135. Serum was separated and peak antibody titers were measured as described above.

### *In vitro T cell proliferation assay*

To identifying T cell epitopes outbred and inbred H-$2^d$ mice were immunized with 40µg equivalent peptide in PLGA microspheres on day 0 with 5µg CpG ODN. Booster dose of 20µg was given on day 10. Another group of mice were immunized with 30µg of E1 or virus lysate on day 0 followed by booster dose of 15µg on day 10. Spleen was isolated on day 21. Splenocytes (devoid of B cells by panning with anti mouse immunoglobulin) were cultured ($2\times10^5$ cells/well) in triplicate wells of 96 well plate in RPMI 1640 medium (300µl/well) with 10% fetal calf serum (FCS), gentamycin and streptomycin (50 µg/ml). The cells were stimulated *in vitro* using following variables.

1. Mice were immunized with E1 protein and splenocytes *in vitro* stimulated with E1 protein (5, 10 and 25 µg/well) and peptides (10, 25, 50µg/well).

Mice were immunized with virus lysate and splenocytes *in vitro* stimulated with virus lysate (5, 10 and 25 µg/well) and peptides of capsid protein (10, 25, 50µg/well).

2. Mice were immunized with individual peptides of E1/capsid and splenocytes *in vitro* stimulated with E1/virus lysate and cognate peptide.

Phytohaemagglutinin (PHA) (2µg/well) was used as a positive control. 200µl of culture supernatant was collected from each well after 72h. The cells were pulsed with [$^3$H] thymidene and after 18h harvested onto glass fiber discs. Thymidene incorporation was measured by liquid scintillation counter. Stimulation index (SI) was calculated by counts per minute (CPM) in the presence of an antigen divided by CPM in the absence of an antigen. The data were presented as mean SI ±SD of triplicate wells.

### *Estimation of Cytokines*

IL2, IL-4, IL-10, IL-17, IFN- , TNF- cytokines level were estimated in the culture supernatant by sandwich ELISA as per manufacturer's instructions (e-Biosciences, USA). Cytokine concentration was calculated from the standard curve and data were presented as mean±SD

### *Neutralization assays*

Plaque reduction neutralization test ($PRNT_{90}$) was used for measuring neutralizing capacity of antipeptide antisera. Two fold serial dilutions of peptide specific antisera was incubated with 200 plaque-forming units (PFU) of CHIKV (DRDE-06) for 90 min at 37°C. Antibody and virus complex (200µl/well) was transferred onto 24-well cell culture plates (Corning NY) containing a confluent monolayer of vero cells. The virus cell mixture was incubated for 1 hr at $37^0$C with the plate rocked every 20 min. Each well was then overlaid with 0.4% genepure LE agarose/ DMEM medium layer (ISC BioExpress Kaysville,UT) and plates were incubated for 48 hr at 37 $^0$C. The agarose layer was removed and the wells were covered with a fixative/staining solution (40% methanol and 0.25 % crystal violet) for five minutes. Then plates were rinsed with DDW to remove the fixative/staining solution. The neutralizing antibody titers were expressed as a reciprocal of dilution of serum, which gave a 90% reduction of plaque formation compared to the plaque number in the virus control.

### *Statistical analysis*

The data on peptide specific IgG, IgG subclass, T cell proliferation and cytokine levels were compared by non-parametric Kruskal-Wallis one-way analysis of variance by ranks. The level of significance (*p* value) were compared between different interval bleeds & mean antibody titer in sera was compared with student's *t*- test.

## RESULTS

### *Competitive immunoassay of E1 antisera with their respective peptides and virus lysate antisera with peptides of capsid protein*

Increasing amounts of individual peptides (0.125 to 256µg) were incubated with E1 or virus lysate antisera. Among the individual Peptides E1P1, E1P4, E1P10/CP3, CP5, CP9 competed with anti-E1/anti-virus lysate sera in a dose dependent manner (Fig. 1). Varying amounts of peptides viz. E1P1, E1P4, E1P10, CP3, CP5 and CP9: 8µg, 16µg, 32µg, 28µg, 8µg and 14µg respectively, required for binding to 50% of the antibodies. Rest peptides (E1P2, E1P3, E1P5-9, E1P11, CP1-2, CP4 and CP6-8) did not compete even at higher concentration of competitor (Fig.1A and B). However, under similar experimental conditions, 3.5 µg of E1 and 3.9 of virus lysate showed 50% inhibition with its own antisera. The study indicates that E1P1, E1P4, E1P10, CP3, CP5 and CP9 are potential B cell epitopes of E1 and capsid protein. E1P1, E1P4, CP5 and CP9 can be considered as dominant competitors because they require low concentration of peptide to get 50% binding with its respective sera.

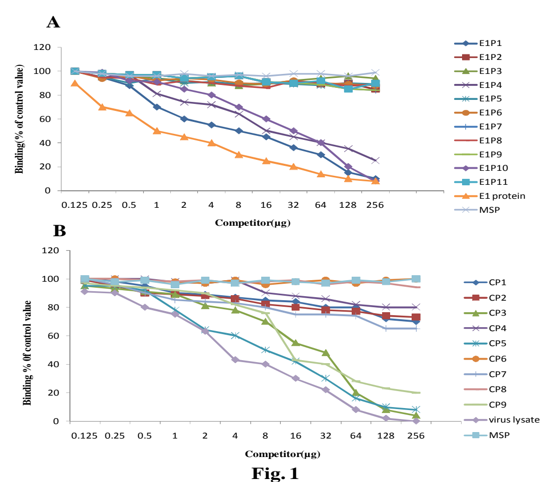



**Fig. 1** **(A)** Competitive immunoassay of different peptides of E1 protein with E1 antisera.**(B)** Competitive immunoassay of peptides of capsid protein with virus lysate antisera. Increasing amount of peptides (0.125-256µg) were incubated with optimal dilution of E1 or virus lysate antisera for 2 h at 37 $^0$C. The Ag-Ab complex was transferred onto the ELISA plates previously coated with E1 protein/virus lysate (100ng/well). Results are expressed as the percentage of the antibody binding in presence of competitor as compared to the control value (without competitor). Peptide of MSP (merozoite surface protein) was used an unrelated peptide control.

### *Immunoreactivity of E1 antisera with its peptides and virus lysate* antisera with capsid's peptides

Immunoreactivity of peptides with anti-E1 and anti-virus lysate sera raised in outbred and inbred mice was tested by direct binding assay. Peptides E1P1, E1P4, E1P10/CP3, CP5, CP9 showed maximal immunoreactivity with their respective E1/virus lysate antisera. Among different bleeds the sera on day 60 showed the maximal immunoreactivity. Peptides E1P2, E1P3, E1P5-9, E1P11, CP1-2, CP4 and CP6-8 did not show significant immunoreactivity with the antisera (Fig2. A). In conclusion E1 and virus lysate induced antibodies to multiple epitopes and peptides that showed competitive immunoassay as well as direct binding assay appears the same.

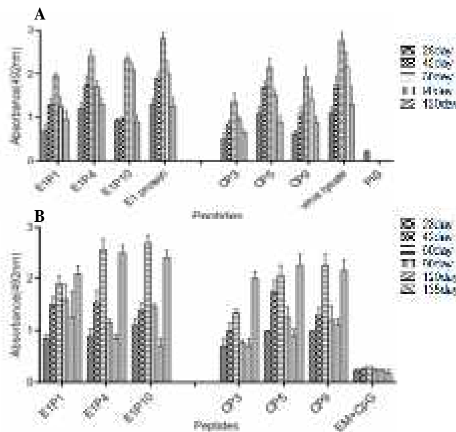


Fig. 2

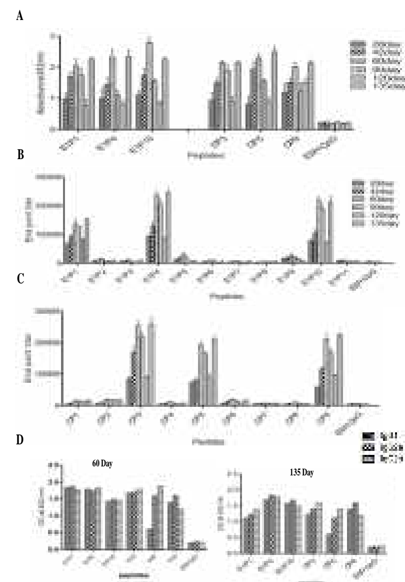
**Fig. 2 (A)** Direct binding assay of peptides of E1/Capsid with E1/virus lysate antisera was done by ELISA, 200ng/100µl peptides were coated onto ELISA plates and E1 protein or virus lysate antisera were added at 1:200 dilutions. Preimmune sera (PIS) were taken as negative control **(B)** Direct binding assay of E1/virus lysate with peptides antisera of E1/capsid raised in outbred mice, 100ng/100µl of E1/virus lysate were coated on ELISA plates and individual peptides antisera were added at 1:200 dilutions. Empty microspheres (EM) + CpG ODN antisera were taken as negative control. Data of two independent experiments expressed as Mean ± SD.

### *Humoral response of peptides in terms of antibody peak titer*

Antisera raised with individual peptides of E1 or Capsid protein was analyzed for peak antibody titer by ELISA. E1P1, E1P4 and E1P10 peptides of E1 and CP3, CP5 and CP9 peptides of capsid showed high antibody titer in all the interval bleeds. E1P1, E1P4, E1P10/CP3, CP5, CP9 showed significant ($p < 0.001$) antibodies titer ranging from 1,18,000 to 2,10,000/180000 to 240000 in outbred mice on day 60 (Fig. 3A and B). Antibody peak titers increased continuously from 28 day to 60 day and later declined on day 120. In case of H-$2^d$ mice antibody titers were slightly higher compared to outbred mice (Fig. S1 B and C). However rest of the peptides E1P2-3, 6-9, 11 and CP1, 2, 4, 6-8 showed very low antibodies titer as compared to other peptides. The peptides E1P1, E1P4, E1P10, CP3, CP5 and CP9 that showed peak Abs titer also showed direct binding assay and competitive immunoassay. Results indicate that E1P1, E1P4, E1P10 peptides of E1 protein and CP3, CP5, CP9 peptides of capsid protein induced robust IgG response in both H-$2^d$ and outbred strains.

### *Immunoreactivity of E1 peptides antisera with E1 protein and capsid peptides antisera with virus lysate.*

The antisera of different interval of the peptides E1P1, E1P4 and E1P10 had shown immunoreactivity with E1 protein and CP3, CP5 and CP9 antisera with virus lysate in outbred (Fig. 2B) and H-$2^d$ (Fig. S1A) mice. E1P1, E1P4, E1P10, CP3, CP5 and CP9 showed maximal immunoreactivity as compared to rest of the peptides. Overall E1P1, E1P4, E1P10/CP3, CP5, CP9 antisera recognized epitopes on E1 protein/virus lysate during antigen-antibody interaction assay.



FigS1

**Fig. S1 (A)** Direct binding assay of peptides antisera of E1/capsid protein with E1protein/virus lysate raised in H-$2^d$ mice. 100ng/100µl of E1/virus lysate were coated on ELISA plates and individual peptides antisera were added at 1:200 dilutions. Empty microspheres (EM) + CpG ODN antisera were taken as negative control. Data of two independent experiments expressed as Mean ± SD.**(B)** Antibody peak titer of peptides of E1 protein in H-$2^d$ mice **(C)** Antibody peak titer of peptides of capsid protein in H-$2^d$ mice. 200ng of peptides were coated on ELISA plate and peptide antisera were added with twofold serial dilutions following goat-antimouse-IgG -HRPO was added and color was measured at 492 nm. EM + CpG ODN were used as negative control. The titers were expressed as the highest serum dilution giving an absorbance higher than that of negative control. Data of two independent experiments expressed as Mean ± SD. **(D)** Estimation of Peptide specific IgG isotype antibodies (IgG1, IgG2a and IgG2b) in H-$2^d$ mice on days 60 and 135. Experiments were done twice and data are represented as mean absorbance of pooled sera.

### *Recall memory response*

E1P1, E1P4, E1P10, CP3, CP5 and CP9 showed secondary or memory response through generation of high antibody levels on 135 day after giving a booster dose on day 120. (Fig.3A and B; Fig.S1B and C).

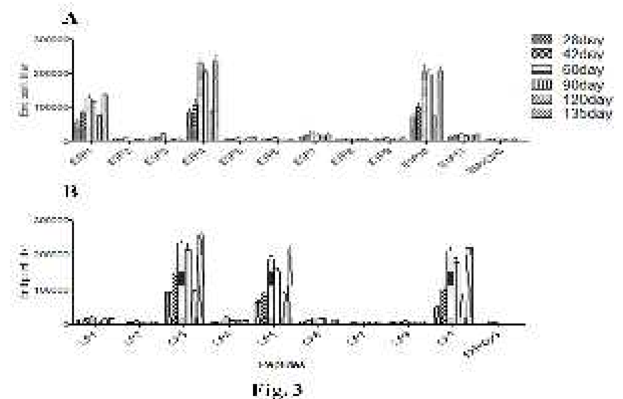


Fig. 3

**Fig.3** Peptides specific IgG peak titer of sera of different intervals in outbred mice **(A)** Antibody peak titer of peptides of E1 protein. **(B)** Antibody peak titer of peptides of capsid protein. 200ng of peptides were coated on ELISA plate and peptide antisera were added with twofold serial dilutions following goat-antimouse-IgG -HRPO (1:1000) dilution was added and finally color was measured at 492 nm. EM + CpG ODN were used as negative control. The titers were expressed as the highest serum dilution giving an absorbance higher than that of negative control. Data of two independent experiments expressed as Mean ± SD.

Memory response was slightly higher or similar to 60 day sera, it indicates that peptides E1P1, E1P4, E1P10, CP3, CP5 and CP9 had the ability to generate good memory response which showed effectors function on re-exposure to native E1 protein and virus lysate.

### *IgG subclass estimation*

IgG subclass were estimated in pooled antisera of day 60 and 135. In case of outbred mice antisera E1P1, E1P4, E1P10, CP3 and CP9 showed high levels of IgG1 and IgG2a/IgG2b subtypes represent Th1 & Th2 biased immune response. IgG2a/IgG2b subtypes was dominant (P<0.05) for CP5 peptide (Fig. 5B).

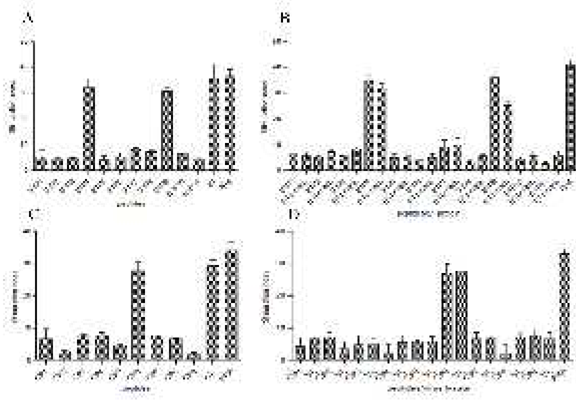



**Fig. 4** T cell epitope mapping using $H^3$-thymidine incorporation assay showing stimulation index (SI). **(A)** Outbred mice were immunized with E1 protein and splenocytes in vitro stimulated with E1 protein and individual peptides. **(B)** Outbred mice were immunized with individual peptides of E1 protein and splenocytes stimulated with E1 protein and cognate peptides. **(C)** Outbred mice were immunized with virus lysate and splenocytes in vitro stimulated with virus lysate and peptides of capsid protein. **(D)** Outbred mice were immunized with individual peptides of capsid protein and splenocytes stimulated with virus lysate and cognate peptides. Splenocytes were cultured ($2 \times 10^5$ cells/well) in 96 well plates in RPMI 1640 medium with 10% fetal calf serum, gentamycin and streptomycin. Phytohaemagglutinin (PHA) was used as a positive control. Stimulation index (SI) was calculated by counts per minute (CPM) in the presence of an antigen divided by CPM in the absence of an antigen. Results of three independent experiments was expressed as Mean SI value ± SD.

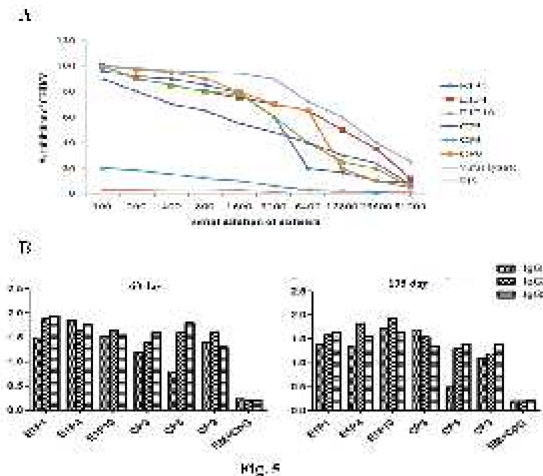



**Fig. 5 (A)** *In vitro* neutralization of CHIKV by peptide specific antibodies using vero cells. Serial dilutions of Abs were incubated with 200 PFU of CHIKV ( DRDE 06). Plaques were counted and titers were expressed as the reciprocal of antibody dilution. EM + CpG ODN antisera were taken as negative control. **(B)** Estimation of peptide specific IgG isotypes antibodies (IgG1, IgG2a and IgG2b) in pooled sera from outbred mice on days 60 and 135. ELISA plates were coated with peptides and antisera were added at fixed 1:200 dilutions. HRP- conjugated goat antimouse IgG1, IgG2a and IgG2b were used. Experiments were done twice and data was represented as mean absorbance of pooled antisera.

It indicate that IgG subtypes depend on peptide immune nature. Isotypes levels were highest on 60 day compared to other day antisera. Similar results of subclass distribution were obtained in H-$2^d$ strain too (Fig. S1 D) indicating that both strains induced similar isotypes profile. However IgG3 level was insignificant in both the strain (data not shown).

### *T cell proliferation assay*

E1 primed splenocytes from outbred mice when *in vitro* stimulated with individual peptides or E1 protein, E1P4 and E1P9 showed significant ($p$>0.001) stimulation index(SI) as compared to other peptides. Peptides E1P4 and E1P9 showed SI 32±1.60 and 30±4.98 at 25µg/well concentration of peptides. E1 protein showed SI 35±3.05 at 5µg/well conc. of E1 protein (Fig.4A). Peptide primed splenocytes of outbred mice stimulated with cognate peptides or E1protein again E1P4, E1P9 showed the best SI value on stimulation with same peptide and E1 protein (Fig.4B). In case of capsid protein stimulation of virus lysate primed splenocytes with capsid's peptide or virus lysate, CP6 showed SI 27±6.11 at 25µg/well and virus lysate showed SI 28±4.62 at 5µg/well. Stimulation of capsid's peptide primed splenocytes with same peptide or virus lysate only CP6 showed significant SI value (Fig. 4C & D). Again E1P4, E1P9 and CP6 peptides consistently showed high ($p$ < 0.001) SI value as compared to other peptides in H-$2^d$ mice also (Fig S2A, B, C and D). In conclusion peptides E1P4, E1P9 and CP6 showed high lymhoproliferation in outbred and inbred H-$2^d$ mice and can be considered as T cell epitopes on E1 and Capsid protein.

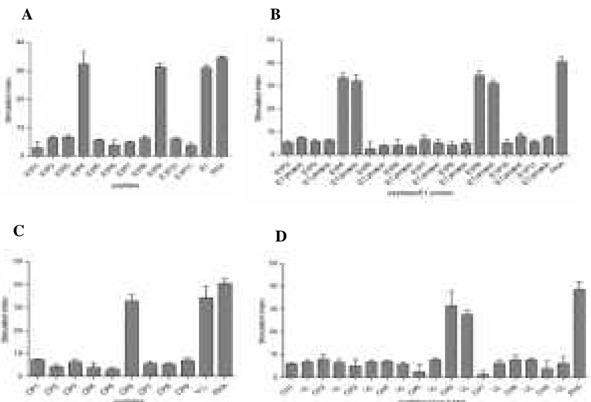



**Fig.S2** T cell epitope mapping using $H^3$-thymidine incorporation assay showing stimulation index (SI). **(A)** H-$2^d$ mice were immunized with E1 protein and splenocytes in vitro stimulated with E1 protein and individual peptides. **(B)** H-$2^d$ mice mice were immunized with peptides of E1 protein and splenocytes stimulated with E1 protein and cognate peptides. **(C)** H-$2^d$ mice were immunized with virus lysate and splenocytes in vitro stimulated with virus lysate and peptides of capsid protein. **(D)** H-$2^d$ mice mice were immunized with peptides of capsid protein and splenocytes stimulated with virus lysate and cognate peptides. Splenocytes were cultured ($2 \times 10^5$ cells/well) in 96 well plates in RPMI 1640 medium with 10% fetal calf serum, gentamycin and streptomycin. Phytohaemagglutinin (PHA) was used as a positive control. Stimulation index (SI) was calculated by counts per minute (CPM) in the presence of an antigen divided by CPM in the absence of an antigen. Results of three independent experiments were expressed as Mean SI value ± SD.

### *Evaluation of Th1, Th2 and Th17 type response*

After *in vitro* stimulation of E1 protein primed splenocytes with E1 protein and peptides of E1 protein and virus lysate primed splenocytes with capsid protein and peptides of capsid; E1P4, E1P9 peptides of E1 protein and CP6 peptide of capsid protein showed IL-17A level significantly higher as compared to other peptides and control value. The level of IL-17A were found in the range 226-369 pg/ml, P<0.005 (Fig. 6A). E1P4, E1P9 and CP6 peptides induced significantly high IFN- level in the range 719-1018 pg/ml, $p$ <0.001 (Fig 6A). IL-2 level was significantly higher (310-437pg/ml, P<0.001) with peptide

E1P4, E1P9 and CP6 as compared to others peptides 32-78pg/ml (Fig 6 A). E1 protein induced significant level of IL-4 and IL-10 and moderate level of IL-2 cytokine. Virus lysate showed significant level of IL-2, IL-17, TNF- and IFN- and moderate level of IL-4 cytokine. Significantly high (P<0.001) levels of TNF- was observed for E1P4 followed by E1P9 and CP6 (P<0.005) peptides. Peptides primed splenocytes when stimulated with cognate peptides again same peptides showed significant level of IL-17 (317-448pg/ml, P<0.001). Rest cytokines showed similar level as in above formulation (Fig 6B). IL-4 level was insignificant for all peptides in all type of stimulation.

Fig-6

**Fig. 6** Cytokine levels in culture supernatant of splenocytes of outbred mice. **(A)** E1 protein primed splenocytes were stimulated with E1 protein or its peptides and virus lysate primed splenocytes were stimulated with virus lysate or capsid's peptides. **(B)** Peptides primed splenocytes *in vitro* stimulated with cognate peptides. Data are representative of three independent experiments and shown as Mean ± SD. Empty microspheres stimulated cells were taken as control. VL- Virus Lysate, EM – Empty microsphere

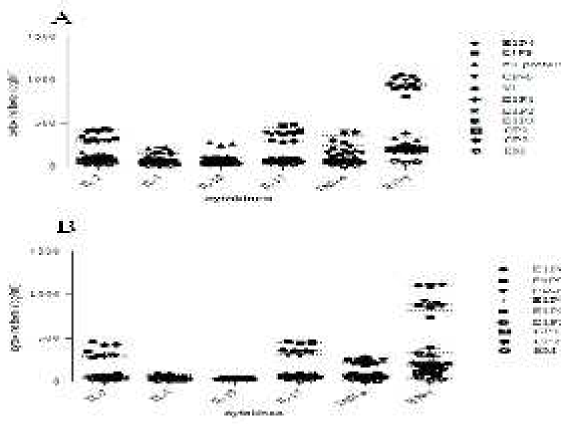


Fig S3

**Fig.S3** Cytokine levels in culture supernatant of splenocytes of H-$2^d$ mice. **(A)** E1 protein primed splenocytes were stimulated with E1 protein or its peptides and virus lysate primed splenocytes were stimulated with virus lysate or capsid peptides. **(B)** Peptides primed splenocytes *in vitro* stimulated with cognate peptides. Data are representative of three independent experiments and shown as Mean ± SD. Empty microspheres stimulated cells were taken as control. VL- Virus Lysate, EM – Empty microsphere.

Cytokine profile in culture supernatant of splenocytes of H-$2^d$ mice was same as in outbred mice, shown in Fig S3. Finally results conclude that peptides E1P4, E1P9 and CP6 showed significantly high level of IL-2, IL-17, TNF- , IFN- cytokines in culture supernatant of splenocytes of outbred and inbred H-$2^d$ mice.

### *In vitro neutralization assay*

Plaque reduction neutralization test ($PRNT_{90}$) assay was performed to validate the neutralizing capacity of peptides specific antisera against CHIKV. Peptides antisera of 60 day were used for in vitro neutralization assay. We selected antisera of E1P1, E1P4, E1P10, CP3, CP5 and CP9 for *in vitro* neutralization assay. Antisera of E1P1 at 1:400; E1P4, E1P9 at 1:200; CP3 at 1:100; CP9 at 1:800 dilutions showed 90% inhibition of CHIK virus through antibody neutralization (Fig-5A). However CP5 did not show significant neutralization.

## DISCUSSIONS

Many studies are currently investigating the development of safe and effective vaccines to prevent CHIKV diseases. Present study demonstrates the domnant B and T cell epitopes of E1 and capsid protein. These peptides generate higher humoral and cell mediated responses in outbred and inbred H-$2^d$ mice. Peptide based study showed therapeutic results for many disease like cancer, HIV, influenza etc. (Asahara *et al.*, 2013; Lin *et al.*, 2013; Mohan *et al.*, 2014**).** For development of a peptide based vaccine the primary request is to identify dominant antigen(s) and its epitopes that could be capable to elicited protective immune response. In case of CHIKV investigators focused on non structural and structural protein for vaccine purpose through generation of neutralizing antibodies that showed protection against CHIKV infection (Bao *et al.*, 2013; Fric *et al.*, 2012; Roy *et al.*, 2014; Tretyakova *et al.*, 2014; Weber C *et al.*,2015). Earlier study described monoclonal antibodies against envelope E1 protein showed protection against alphavirus infection (Wust *et al.*, 1989). A recent study showed that live attenuated vaccine protect mice and nonhuman primates after CHIKV challenge (Plante KS., 2015).Thus epitopes based subunit vaccine can be a option for CHIKV. Based on algorithms prediction, epitopes were synthesized and their humoral and cell mediated responses were studied in outbred and H-$2^d$ mice. Peptides entrapped in PLGA microspheres (used as a delivery vehicle) were immunized by intramuscular route with CpG ODN 1826 as an adjuvant. PLGA microspheres and CpG ODN was used to enhance immunogenicity of the peptides. E1P1, E1P4, E1P10, CP3, CP5 and CP9 peptides showed high peak antibody titer and sustained over longer duration with strong memory response after boosting with native protein. E1P1, E1P4, E1P10/ CP3, CP5 and CP9 showed competitive immunoassay and immunoreactivity with E1/virus lysate antisera, indicate the presence of monoclonal antibody specific to these epitopes. However all the peptides antisera were tested with unrelated peptides to avoid the unspecific reactivity. Mouse monoclonal antibody against the E1 protein had shown to inhibit the virus assembly during *in vitro* study (Masrinoul *et al.*, 2014). Epitope specific antibody of E1 protein can be helpful in protection against the CHIKV. IgG isotype play a major role in protection against infectious disease. IgG2a/IgG2b isotype were dominant for CP5 peptide while rest of the peptides E1P1, E1P4, E1P10, CP3 and CP9 showed equal distribution of IgG1 and IgG2a/IgG2b subclass. However in case of CHIKV IgG1 and IgG2b subtypes helps in protection against CHIKV (Fric *et al.*, 2012; Masrinoul *et al.*, 2014). When E1 and virus lysate primed splenocytes were *in vitro* stimulated with respective peptides, E1P1, E1P9 and CP6 showed maximum lymphocyte proliferation as compared to rest of the peptides and *vice versa* in outbred as well as H-$2^d$ mice. Thus these epitopes can be helpful to activate T cell immune response against the CHIKV. CpG ODN is well known to activate B-cell and plasmacytoid dendrite cells via TLR9 (Allacher *et al.*, 2010). In the present study, we used CpG ODN as an adjuvant to induces humoral and cell

mediated response. CpG ODN enhanced peptide immune response effectively. Finally we evaluate the cytokine profile of T cell proliferating peptides because cytokine profile shape the function of immune system and are necessary to activate the both native and adaptive immune response. Tumor Necrosis Factor (TNF- ) cytokine produced by macrophage/ monocytes during acute inflammation play an important role in activation of innate and adaptive response (Barksby *et al*., 2007; Vilcek and Lee, 1991). Interestingly lymphoproliferative peptides showed significant level of TNF- but E1 protein did not show that much level of this cytokine. The levels of IL-4 cytokine were significantly higher for E1 protein compared to rest of the peptides. Only E1 protein produced higher level of IL-10, indicate the presence of immunosuppressive sequence in E1. IL-17 is a major inflammatory cytokine and promotes the up regulation of other inflammatory cytokines and chemokines. In our study IL-17 levels were significantly higher for T cell proliferating peptides confirmed these peptides have a capacity to induced inflammatory response during CHIKV infection. Virus infection to a new cell can be prevent by neutralizing antibodies because epitope specific antibody can block binding sites of the virus and help in the prevention of virus infection. Earlier study has shown CHIKV clearance is easy to the host in the presence of the neutralizing antibodies (Fric *et al*., 2012; Goh *et al*., 2013). Antisera of E1P1, E1P4, E1P10, CP3 and CP9 showed *in vitro* neutralization for CHIKV. Thus peptides which have neutralizing capacity are very crucial for vaccine design for CHIKV. In conclusion peptides E1P1, E1P4, E1P10, CP3, CP5 and CP9 showed B cell response, while peptides E1P4, E1P9 and CP6 showed T cell response. Peptide E1P4 showed both B and T cell type responses. E1P1, E1P4, E1P10, CP3 and CP9 showed *in vitro* neutralization for CHIKV. However this study is limited to murine model but MAP (Multiple Antigen Peptide) of neutralizing epitopes E1P1, E1P4, E1P10, CP3 and CP9 can be used for further study in primates model to develop a subunit vaccine against Chikungunya virus.

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**Conflict of interest**

The authors declare no conflicting financial interests.

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