Abstract
Phage display peptide libraries are widely used in various protein-protein interaction studies to determine the immunological binding of proteins and epitope mapping of different targets. In the present study, the peptide library is used to identify the Fab binding epitope site of Tetanus Toxoid (TT). The peptides were screened against Fab by biopanning, using a random peptide 12-mer phage display library. Clones were selected based on the binding activity by phage ELISA and one of the peptide sequence (DTMSYTPNIHLL), which showed highest binding activity towards the Fab was cloned into pGEX-4T1 vector for expression and purified using glutathione agarose affinity chromatography. The binding activity of the purified peptide was demonstrated by ELISA and Immunoblot analysis. Further, analysis of the peptide sequence revealed homology of four amino acids (YTPN) to heavy chain sequence of TT. F13 Fab significantly binds to the YTPN epitope of the TT antigen as indicated by ELISA and may have potential in diagnostics.

Keywords: Tetanus toxoid, Fab, Phage display, Peptide, ELISA.

Introduction
Tetanus is potentially a life threatening disease, caused by a potent neurotoxin viz. tetanus toxin produced by anaerobic bacterium Clostridium tetani. Tetanus toxin is a single polypeptide (150 kDa), which consists of a heavy chain harboring a cellular receptor binding domain and a light chain with zinc metalloprotease activity (1-4). Though tetanus is preventable by vaccination and also post-exposure prophylaxis, lack of awareness and timely intervention lead to approximately one million cases of tetanus annually, which results in 0.3-0.5 million deaths globally (5).

Present treatment for tetanus involves usage of animal derived polyclonal antibodies but side effects like serum sickness induced by the host immune response to foreign protein limits its usage. Other limitations of the animal derived immune serum include prolonged immunization procedure, risk of the recipient to certain zoonosis and batch-to-batch variation in the therapeutic efficacy of the antiserum. Advent and usage of recombinant antibodies and peptides to some extent able to overcome the limitations of conventional antibodies. Further, advances in phage display technology where displaying of biologically active materials like peptides or proteins on the surface of bacteriophage particles has facilitated the isolation of specific peptides with affinity towards various targets and potential to serve as prophylactic, therapeutic candidates or diagnostic reagents (6-15).
In the present study, we have utilized the latter approach to identify a peptide, which showed significant binding to F13 Fab using a peptide phage library. The selected peptide was further characterized as a GST tagged protein. The peptide sequence revealed the presence of a four amino acid stretch, also present in sequence of the TT heavy chain and is shown to be responsible for the immune response.

Material and Methods

Antibodies, bacterial strains, vectors and chemicals: The F13 Fab used for epitope mapping was selected using human naïve library. The peptide library kit was purchased from New England Biolabs (NEB) (USA) for selection of peptides. The bacterial strain *E. coli* BL21 (DE3) used for over expression of peptide and vector pGEX-4T1 was purchased from Invitrogen (USA) and GE Healthcare (UK) respectively. The plasmid isolation mini prep kits, DNA-gel extraction kits and PCR purification kits were purchased from Qiagen (Germany). The bacterial strain *E. coli* ER2738 used for the propagation of the recombinant phages was purchased from NEB (USA). Nuclease free water and T4 DNA Ligase were purchased from GeNei (India). Glutathione agarose used for purification of GST tagged proteins and all other fine chemicals used were purchased from Sigma Chemical Company (USA).

Epitope mapping of anti-Tetanus F13 Fab fragment: The F13 Fab antibody epitope was mapped using a linear phage display peptide library of random peptide 12mers fused to a minor coat protein of M13 phage. Briefly, Maxisorp immunotube (Nunc, Denmark) was coated with 100µg/ml of F13 Fab in 0.1M NaHCO3 (pH 8.6) buffer and incubated overnight at 4°C with gentle agitation. The tube was then washed with tris buffered saline (TBS) at room temperature (RT) for 1h under gentle agitation. The unbound phages were washed out with TBST and the bound phages were eluted with 1ml of 0.2M Glycine-HCl (pH 2.2) and pH neutralized with 150 µl of 1M Tris-HCl buffer (pH 9.1). The eluted phages were infected by inoculating 20 ml of *E. coli* ER2738 culture and then incubated at 37°C with vigorous shaking for 4h. The culture was centrifuged at 10,000 rpm for 10min and the supernatant was transferred to a sterile 50 ml tube. To the supernatant, PEG/NaCl (1/6 volume of supernatant) was added and allowed the phages to precipitate on ice for 1h. The PEG/NaCl precipitate was centrifuged at 10,000 rpm for 15min. The supernatant was discarded and the pellet was dissolved in 1ml of sterile PBS. The suspension was centrifuged at 10,000 rpm for 5min to pellet the residual cells. The supernatant containing phages were re-purified by adding 1/6 volume of PEG/NaCl and incubated on ice for 1h followed by centrifugation at 10,000 rpm for 10min. The supernatant was discarded and the pellet was dissolved in 200 µl of sterile PBS. The amplified phages were titrated on LB/IPTG/Xgal plates and used for further rounds of biopanning. The clones obtained after third round of biopanning were checked for binding activity by phage ELISA.

Phage ELISA: Analysis of phage peptides binding to F13 Fab was performed by ELISA using bacterial supernatants according to Phage display peptide library kit instructions. Thirty individual colonies selected after third round of biopanning were inoculated into 3ml cultures of ER2738 cells at mid log phase. The cultures were harvested after incubation at 37°C for 4h and 30 min and centrifuged at 10,000 rpm for 10 min. The pellets obtained were stored at 4°C for phagemid isolation and the supernatants containing phages were collected for analysing in phage ELISA. For phage ELISA, Maxisorp microtiter plates were coated with F13 Fab (300ng/well) and incubated overnight at 4°C. The wells were blocked with blocking buffer for 1h at RT. The plate was washed thrice with TBST and

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phages (1x10⁷) were added and incubated for 2h at RT. The plate was again washed with TBST and incubated with anti-M13 mouse antibody conjugated with HRP (1:6000) for 1h at RT. The bound phages were detected with 3, 3, 5, 5-tetramethylbenzidine peroxidase substrate (Thermo Scientific, USA). The absorbance was read at 450nm using a microplate reader (BIO-TEK, US). The experiment was performed in triplicate and the data shown are the representative of mean and standard deviation.

**Phagemid DNA extraction and sequencing:** The Phagemid DNA was isolated from the bacterial cells using plasmid purification kit according to the manufacturer’s instructions. The DNA was eluted in 50 µl of TE buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA). The phagemid DNA was sequenced using 96 GIII primer.

**Cloning of F13 Fab binding peptide into pGEX-4T vector:** The peptide, which showed highest binding activity in phage ELISA was selected for cloning and expression in bacterial system. The total DNA stretch of specific peptide (68bp) has been synthesized as two complimentary primers. An equal concentration of complimentary primers of the peptide were taken in 1.5ml tube and denatured at 95°C for 5min. The DNA strands were allowed to renature by auto-annealing. The annealed DNA product and pGEX-4T, vector were double digested with EcoRI and NcoI restriction endonucleases according to the manufacturer’s instructions (NEB, USA) and purified using gel purification kit. The digested DNA fragments were ligated into pGEX-4T, vector using T4 DNA ligase. The ligated product was transformed into chemically competent TOP 10 cells of E. coli strain and plated onto LB agar plates (supplemented with 100µg/ml of ampicillin). The plates were incubated overnight at 37°C and individual colonies were picked from each plate, inoculated into LB medium containing ampicillin (100µg / ml) and cultured overnight at 37°C in an orbital shaker. The overnight grown culture was used for isolation of plasmids by plasmid mini prep kit. The positive clones were confirmed by restriction enzyme digestion with EcoRI and PstI. The restriction digestion of pGEX-4T, vector alone served as a negative control.

**Expression and purification of peptide:** The positive pGEX-4T, TTE1 clone was transformed into BL21 (DE3) cells. Individual colonies were picked and grown in LB media containing ampicillin (100µg/ml). The culture was induced with 1mM IPTG once the O.D of the culture reached ~0.6 AU at 600nm. Post induction the culture was grown for 4h at 30°C followed by centrifugation at 5000 x g for 20 min at 4°C to collect the bacterial pellet. The pellet was resuspended in lysis buffer and sonicated. The lysed sample was centrifuged at 10,000 rpm for 30 min and the supernatant was purified by glutathione agarose column chromatography. The bound protein was eluted using reduced glutathione and dialysed against PBS. The dialysed protein was stored at -20°C for further characterization.

**Binding activity of peptide by ELISA:** ELISA was performed by coating a 96 well microtiter plate (Nunc, Denmark) with 300ng/well of F13 Fab in 50mM carbonate buffer (pH 9.6) and incubated overnight at 4°C. The plate was washed thrice with TBST and blocked with 1% bovine gelatin, followed by washing with TBST. The peptide was added to the wells in a two fold serial dilution with the highest concentration of 1 µg /100 µl and incubated at 37°C for 1h. The plate was then washed five times with TBST and binding of the peptide with F13 Fab was detected by addition of anti-GST HRPO conjugate followed by TMB substrate. The reaction was quenched by 1.25M H₂SO₄. Absorbance was measured at 450nm using a microplate reader (BIO-TEK, US). The experiment was repeated thrice to evaluate the concentration dependent binding activity of peptide towards F13 Fab and the data shown are the representative of mean and standard deviation.
Immunoblot analysis of purified peptide: The purified peptide was electrophoresed by 12% SDS-PAGE and immunoblotted onto a PVDF membrane (Hybond-C, GE Health care, USA) using a transblot apparatus (Bio-Rad, USA) following the manufacturer’s instructions. The blot was probed with anti-GST peroxidase conjugate (Pierce, USA) and developed using 0.05% 3,3-diaminobenzidine tetra hydrochloride (DAB) (Sigma, USA) and 0.3% hydrogen peroxide in PBS.

Determination of specificity of peptide by Immunoblot: The purified peptide was immunoblotted as mentioned above. The blots were incubated with F13 Fab and E. coli lysate respectively and washed thrice with PBST followed by probing with anti-human IgG (Fab specific) HRPO conjugate (Sigma, USA). The blot was developed as mentioned above.

Indirect ELISA for epitope specificity: A 96 well microtiter plate was coated with 200 ng/well of peptide in 50mM carbonate buffer (pH 9.6) and incubated overnight at 4°C. The plate was washed thrice with TBST and blocked with 1% bovine gelatin by incubating for 1h at 37°C followed by washing thrice with TBST. The F13 Fab was added in a two fold serial dilution with highest concentration of 2 µg /100 µl and incubated at 37°C for 1h. The unrelated Fab fragments (2µg/100µl of anti-JEV Fab, anti-Rabies Fab and anti-ChikV Fab) were used as a control. The plate was incubated at 37°C for 1h and was washed five times with TBST and dried. The binding activity of the F13 Fab with peptide was detected by addition of His-probe and a chromogenic substrate TMB. The reaction was stopped by the addition of 1.25M H₂SO₄ and the absorbance was measured at 450nm using a microplate reader (BIO-TEK, US). The experiment was performed in triplicate and the data shown are the representative of mean and standard deviation.

Results
Biopanning against F13 Fab: The F13 Fab was screened using human naïve phage library against TT as described previously (16). The Phage display peptide library was used to identify the epitope region of F13 Fab with high binding affinity to TT. Three rounds of biopanning was performed with the library against F13 Fab and the eluted phages exhibited an increase in phage output from round 1 to round 3 of the selections (Table 1) indicating enrichment of F13 Fab specific phage peptides.

Phage ELISA: Phage ELISA shows that there is an incremental enrichment of the specific phages on subsequent rounds of biopanning using the amplified phages (Fig. 1a), whereas no increase in absorbance values was observed for the control (Bovine serum albumin). The eluted peptide phages after three rounds of biopanning were used to infect the E. coli ER2738 cells and plated on LB/IPTG/Xgal (Fig. 1b). A total of 30 individual clones were tested for binding by phage ELISA against F13 Fab and amongst them seven clones showed positive binding to F13 Fab (Fig. 1c).

Analysis of peptide sequence: Ten clones from each round of biopanning were selected for sequence analysis. The Phagemid DNA was purified according to the manufacturer’s protocol and sequenced. The sequence primer 96 GIII was used for peptide DNA analysis. The DNA sequence was translated and the analyzed peptide sequences are shown in Fig. 2. Amongst them one of the clone, yielded a sequence DTMSYTPNIHLL, which had a possible epitope, viz. TTE1, which was further selected for characterization.

Cloning of TTE1 into pGEX-4T, vector: The DNA fragment of TTE1 was cloned into pGEX-4T, vector between EcoRI and NolI restriction enzyme sites. The plasmid was transformed into BL21 (DE3) cells and the transformed colonies were screened for positive clones by restriction digestion with EcoRI and PstI enzymes. A clear mobility shift was observed in the insert as compared to the control on 1% agarose gel. The positive clones yielded a 1047 bp fragment (Fig.
Identification and Characterization of Novel Binding

Fig. 1a. Enrichment of F13 Fab specific peptide phages after each round of biopanning. The output phages were incubated with F13 Fab detected by horse radish peroxidase (HRP) conjugated anti-M13 antibody. The BSA served as negative control. The bars indicates the standard deviation of OD values. 1b. Blue colour plaques are recombinant peptide phages after 3rd round of biopanning infected with E. coli ER2738 cells plated on LB/IPTG/Xgal plate. 1c. Phage ELISA showing the binding activity of different individual peptide phage clones against F13 Fab and BSA used as negative control. The bars indicates the standard deviation of OD values.

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>1st ROUND</th>
<th>2nd ROUND</th>
<th>3rd ROUND</th>
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<td>DTMSTYTPNHLL</td>
<td>DTMSTYTPNHLL</td>
<td>DTMSTYTPNHLL</td>
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<tr>
<td>KLFLAKYLLVPF</td>
<td>MHHRYTPQSSIN</td>
<td>DTMSTYTPNHLL</td>
<td>DTMSTYTPNHLL</td>
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<tr>
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<td>HLWRRQHNVPYAI</td>
<td>LDSNIFSRRGMV</td>
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<tr>
<td>DNAAVHLRLHTG</td>
<td>QIPYTSTPSAT</td>
<td>TLAVLDTNPHT</td>
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Fig. 2. Sequencing analysis of different clones after each round of biopanning. The enriched peptide sequence after 3rd round is highlighted.
3, Lane 1-7) whereas, the pGEX-4T1 vector alone yielded a 979 bp fragment (Fig. 3, Lane 8) as expected. Positive clones were also confirmed by DNA sequencing and termed as pGEX-4T1-TTE1 (Data not shown).

**Expression and purification of TTE1 peptide:**
The TTE1-GST fusion protein was expressed in *E. coli* and the soluble fraction obtained post cell lysis was purified using a standard glutathione agarose column. SDS-PAGE analysis of the purified protein revealed a band at ~27 kDa (Fig. 4a), which was also detected by anti-GST and F13 Fab in western blot analysis (Fig. 4b, Fig. 4c respectively).

**Indirect ELISA for epitope specificity:** An indirect ELISA was performed to determine the specificity of F13 Fab towards TTE1-GST

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**Figure 3.** Agarose gel electrophoresis analysis of TTE1 peptide cloned into pGEX-4T1. Lanes 1-7: Positive clones, Lane 8: Vector control, Lane M: DNA standard marker.

**Figure 4a.** Detection of purified TTE1-GST protein on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Lane M: Protein molecular size standard. Lane 1: Purified protein.

**Figure 4b.** Western blot analysis of purified TTE1-GST protein probed with anti-GST antibody. Lane M: Pre-stained protein molecular size standard. Lane 1: Purified TTE1 protein.

**Figure 4c.** Reactivity of TTE1 peptide with F13 Fab in immunoblot analysis. Lane M: Pre-stained protein molecular size standard. Lane 1: Purified TTE1-GST protein. Lane 2: *E. coli* lysate as a negative control.

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The titration of F13 Fab against TTE1-GST peptide revealed a concentration dependent reduction in the binding signal, whereas unrelated Fabs like anti-JEV Fab, anti-Rabies Fab and anti-ChikV Fab did not show any binding activity to TTE1-GST peptide (Fig. 5a, 5b).

Discussion

There is a vast body of literature where phage display technology has shown to play a vital role in the development of antibody fragments and antibody engineering. A major application of phage display technology is in epitope mapping, which provides information for developing peptide vaccines and diagnostic tools (17-24). Indeed, screening of high affinity peptides from a large diversified phage displayed peptide library against desired target requires less effort, time and resources (25). The choice of biopanning method and strategy is crucial in the selection of binding sequences that are specific for a particular domain. Tetanus toxin comprises of two polypeptide chains, viz. heavy and light chains connected by disulfide linkage. The heavy chain contains a toxin binding domain and a pH dependent translocation domain, which allows the toxin to bind to gangliosides and enters into cytosol respectively. Whereas, the light chain acts as an endopeptidase which once released in cytosol cleaves synaptobrevin 2, blocking release of GABA and glycine to motor neurons causing prolonged muscle transactions. Tetanus toxin is highly immunogenic and most of the individuals exhibit cellular immune responses to a few identical TT epitopes (26-30). Previous studies identified that tetanus toxin contains multiple epitopes, including some universal epitopes. However, the study based on tetramer guided
epitope mapping revealed the presence of 36 distinct epitopes on the tetanus toxin. Most of the required epitopes, which are presented across the tetanus toxin has been reported to have high affinity towards T cells (31).

The F13 Fab used in this study was successfully selected in our laboratory, using human naive library against TT. The Fab was expressed as a periplasmic fraction in *E. coli* and purified by affinity chromatography. We used phage display peptide library to identify the epitope of F13 Fab on TT. To maintain the sequence diversity among the binding phage and enrich the specifically bound phages, three rounds of biopanning were carried out. To eliminate unbound phages from immunotube, washing steps were increased progressively 10 to 30 times from first to third round of biopanning. Finally ten clones were selected based to phage ELISA showing highest binding. On peptide sequence analysis, three clones revealed identical sequences whereas, another two had a common YTPN motif. The selected 12-mer peptide sequence was cloned and expressed as a GST-fusion protein in *E. coli*. The ~27 kDa protein was purified to >90% homogeneity using glutathione agarose column chromatography, under non-denaturing conditions. The protein was found to exhibit binding to F13 Fab as indicated by immunoblotting and ELISA, whereas it did not bind to other unrelated Fabs. Indeed, an earlier study by our group F13 Fab was reported to bind to the tetanus toxoid (16).

The analysis of TTE1 peptide revealed the sequence as YTPN from the peptide library and showed sequence similarity with the corresponding amino acid position at 1180-1183 on heavy chain of TT. Further analysis revealed that YTPN was found to be present in one of the predominant CD4 T cell epitope of TT and also shown in sequence YNGLKFIIKRYTPNNEIDSF (TT1170-1189).

**Conclusion**

The study concludes that a specific epitope in TT has been identified using the F13 Fab by phage display peptide library. The binding activity of the purified peptide was demonstrated by ELISA and Immunoblot analysis. Further, analysis of the peptide sequence revealed homology of four amino acids (YTPN) to heavy chain sequence of TT. F13 Fab significantly binds to the YTPN epitope of the TT antigen as indicated by ELISA. The potential of this peptide as an immunizing agent or diagnostic reagent would be broaden in future.

**References**

8. Hallahan, D., Geng, L., Qu, S., Scarfone, C., Giorgio, T., Donnelly, E., Gao, X. and


