Abstract

Diphtheria is caused by *Corynbacteria diphtheria* and it is one of the contagious diseases with a mortality rate of 5% to 10% worldwide. Though the mass immunization of diphtheria vaccine reduces the mortality, but the immediate effective treatment for this disease involves the administration of anti-diphtheria polyclonal antibodies or diphtheria antitoxin (DAT) produced from Equines. However the availability of anti-diphtheria polyclonal antibody is very limited due to the less number of manufacturers. Hence the development of monoclonal antibodies (mAbs) with neutralizing capability may act as a potent alternate candidate to DAT or as an effective therapeutic agent. In the present study, we have developed and characterized five mouse monoclonal antibodies against diphtheria toxoid. The specificity of the mAbs was established by its non-reactivity towards other toxins by indirect ELISA (Enzyme linked Immunosorbent assay) and competitive ELISA with the commercial mAb. The non-competing mAbs were used to develop immunocapture ELISA for the quantification of toxoid content in in-process samples during manufacture of the vaccine. The $r^2$ value obtained by the regression analysis was 0.996. This ELISA can be adapted to measure the toxoid content and blending of the vaccine can be performed based on the estimated toxoid. The neutralizing activity of the mAbs against diphtheria toxin was performed by *in vitro* cell based neutralization assay using Vero cells. The cytotoxicity assay demonstrated that mAb neutralized the toxin in a concentration dependent manner. We have further shown that the mAb binds to the receptor binding domain of diphtheria toxin and it blocks the toxin from binding to the heparin-binding epidermal growth factor like growth factor by ELISA. These monoclonal antibodies may have a potential in development of therapeutics and diagnostics.

Key words: Monoclonal antibodies, Diphtheria toxin, ELISA, *In vitro* neutralization

Introduction:

*Corynebacterium diphtheria* is a gram positive bacterium that causes the infectious disease, diphtheria in the upper respiratory tract of humans (1). The potent toxin produced by *Corynebacterium* plays a major role in inhibition of protein synthesis in eukaryotic cells resulting in cell death (2). The classical form of diphtheria results when the bacterium is infected with a bacteriophage carrying the structural gene for biosynthesis of the toxin responsible for clinical disease. The clinical presentation includes a fibrous, adherent pharyngeal membrane and causes severe systemic toxicity, myocarditis and
Peripheral neuritis. Diphtheria toxin is produced as a single polypeptide chain with the disulfide bond linking two fragments which is fragment A and fragment B. Fragment A is known as catalytic domain, a potent enzyme that acts intra cellular to block protein synthesis. Fragment B domain contains a translocation and receptor region. It is responsible for the recognition of receptors on mammalian cells and translocation of fragment A into cells (3,4).

Until recently, diphtheria was known to be a rare disease in industrialized countries with well-established routine childhood vaccination programs (5). After primary vaccination, anti-diphtheria antibodies wane in absence of boosting. As waning of antibodies in adults has been documented in various studies in Australia, New Zealand, Germany and Poland (6). The importance of maintaining adequate population immunity against diphtheria has drawn attention when diphtheria epidemic re-emerged in several eastern European countries in 1990s, with a high proportion of adult cases (7). Diphtheria is preventable by vaccination, however the disease seems to continue due to regional variations in compliance to vaccination, insufficient booster regimens and deterioration of the immune system. According to WHO, 4,500-5,500 cases were reported annually, with the majority occurring in India and Indonesia (8).

The protective role of antisera raised in horses against the toxin to treat human diphtheria as an alternative were reported earlier in the late nineteenth century (3, 9). However, the usage of equine polyclonal antiserum has limitations causing serum sickness, allergic reactions to the recipient and high regulatory requirements for the safe manufacture of blood derived products (10,11). Hence, development of high binding and neutralizing monoclonal antibodies would be safe and better alternative to equine DAT and many resource poor countries would benefit greatly (12).

According to World Health Organization (WHO), circulating diphtheria antitoxin level of 0.01 IU/ml, as determined by the neutralization test, provides basic clinical immunity against the disease, and a higher titer would be required to provide full protection (13). The discovery of potent neutralizing antibodies against the diphtheria toxin holds great promise as potential therapeutics (14). Murine monoclonal antibodies against diphtheria can be used as an alternative to equine serum (15). These monoclonal antibodies (mAbs) can also be used for diagnosis and in maintaining of quality in vaccine manufacture (16). The evaluation of potency in diphtheria toxoid vaccine for lot release is being performed in guinea pigs through intradermal challenge test or by performing serological assays in mice (17). To demonstrate that the vaccine batches induce a protective immune response, a quite large number of animals are required and this in vivo tests accuracy and precision was found to remain contentious. Vaccine manufacturers are determining the potency by in vitro ELISA based assays which are correlating well with the in vivo tests. Monoclonal antibody assays are being used in diphtheria vaccines to demonstrate the safety (18).

In the present study, we describe the identification of an anti-diphtheria antibodies isolated from antibody secreting cells generated from immunized mice. The selected monoclonal antibodies were characterized for their binding activity and specificity towards diphtheria toxin by ELISA based methods. These antibodies shown neutralization of toxin in a cell based assays and also prevented the toxin from binding to the receptor HB-EGF. Selected the two non-competing antibodies and developed an immunocapture ELISA for the quantification of diphtheria toxoid content in vaccine in-process samples.
**Materials and methods:**

**Reagents and chemicals:** Diphtheria toxoid standard (1100 Lf/ml) was purchased from NIBSC (UK). Myeloma partner SP2/mIL-6, Human MAb against diphtheria toxoid 16M3F10.1C (HB-8363) and Vero cells were obtained from ATCC (USA). Hypoxanthine-aminopterin-thymidine (HAT), Hypoxanthine-thymidine (HT) and Goat anti-mouse IgG Fc specific HRPO conjugate was obtained from Sigma (St. Louis, Missouri, USA). Isotype kit was purchased from Roche Diagnostics (Mannheim, Germany).

**Immunization of mice:** All the animal experiments were approved by Institutional Animal Ethics Committee (IAEC) and performed accordingly. The animals were purchased from National Institute of Nutrition (NIN), Hyderabad. Four to six weeks old female BALB/c mice were hyper-immunized with diphtheria toxoid. Briefly, mice were immunized intraperitoneal with diphtheria toxoid (50 μg/dose/animal) emulsified with equal volume of Freund’s complete adjuvant. After an interval of two weeks, booster doses were administered intraperitoneal with diphtheria toxoid (25 μg/dose/animal) mixed with equal volume of Freund’s incomplete adjuvant. Subsequently, after one week interval, blood was collected from the retro-orbital sinus of the mice and incubated at room temperature for 4 hours. Serum was separated from blood by centrifugation at 10,000 rpm for 5 minutes and tested for end-point titer by indirect ELISA. After last booster, mice were kept under observation for one month. Four days prior to fusion, diphtheria toxoid was administered to mice by intravenous route (10 μg/animal) for three consecutive days and were kept under observation for a day before performing spleenectomy.

**Generation of monoclonal antibodies:** The development of hybridomas was performed by fusing myeloma partner, Sp2/m IL-6 and hyper immunized mouse spleenocytes as per standard procedure (19). Briefly, 10 x 10⁶ cells of mouse myeloma partner (Sp2/m IL-6) were suspended in DMEM medium. High titer mouse spleenocytes, 100 x 10⁶ cells were isolated and fused with Sp2/m IL-6 cells at a ratio of 10:1 in the presence of polyethylene glycol (PEG 1500). After fusion, hypoxanthine-aminopterin-thymidine (HAT) medium was used as selective media for the growth of hybrid cells. Hybridomas were seeded into microtiter tissue culture plates at 1 x 10⁵ cells per well and incubated at 37°C in 7% CO₂. Medium was changed every four days and replenished with fresh HAT growth medium. Hybridomas were grown for a total of 14 days in HAT medium and gradually adapted to hypoxanthine-thymidine (HT) medium and further passaged for 7 days. Hybridomas were monitored daily for growth or contamination under an inverted microscope (Olympus CK2). The confluent hybridomas were primarily screened against diphtheria toxoid by indirect ELISA. Three rounds of limiting dilution were performed to establish the monoclonality of the progeny hybridomas.

**Binding of hybridomas by Indirect ELISA:** Diphtheria toxoid, diphtheria toxin and non-toxic mutant of diphtheria toxin (CRM-197) were dissolved in carbonate bicarbonate buffer (0.1 M), pH 9.6, at a final concentration of 5 μg/ml respectively and subsequently, 100 μl of each antigen was coated in respective wells in Maxisorp 96-well micro titer plates. These plates were incubated overnight at 4°C. The wells were washed thrice with phosphate buffered saline (PBS) containing 0.05% (v/v) Tween-20 (PBST) to remove any unbound antigen and the remaining active surfaces in the wells were blocked with 2% (w/v) skimmed milk powder in PBST for 1 hour at 37°C. The plates were again washed with PBST and 100 μl of hybridoma culture supernatants, negative controls (PBST, unimmunized mouse serum, myeloma culture fluid) and the positive control (immunized mouse serum) were added to respective wells and incubated at 37°C for 1 hour. After completion of the incubation step, the plates were washed thoroughly with PBST and goat anti-mouse IgG Fc specific HRPO conjugate (1:25,000) was added to each well (100 μl/well) and incubated for 1 hour at 37°C. The unbound secondary antibody was removed by washing with washing with PBST and 3, 3′, 5, 5′-Tetramethylbenzidine (TMB) with H₂O₂ (0.03% v/v) was added to the wells as a
chromogenic substrate. The reaction was stopped after 10 minutes with 1.25M sulfuric acid. Absorbance was recorded at 450 nm using ELISA plate reader (Molecular Devices).

**Purification of mAbs by Affinity chromatography:** The mAbs were affinity purified employing Protein G Sepharose™ 4 Fast Flow as per manufacturer’s recommendations. All the buffers required for the purification were prepared and filtered using 0.45 μm bottle top filter units and stored at 2-8°C until use. Briefly, the resin was packed into a suitable column (XK 16/20) and equilibrated with 5-10 column volumes of 10mM Tris, pH 7.5 (Buffer A) at a flow rate of 2 ml/minute. The filtered TCF was prediluted with the equilibration buffer, maintained on ice bath and passed through the resin at a flow rate of 1ml/minute to ensure higher residence time and a maximum binding of the IgG to the resin. The unbound proteins were washed with 5-10 column volumes of Buffer A at a flow rate of 2 ml/minute. The bound mAbs were eluted in small fraction volumes of 10 ml using 5-10 column volumes of 0.1M Glycine, pH 2.5-3.0 (Buffer B) at a flow rate of 1ml/minute. The eluted fractions were neutralized with 1M Tris, pH 9.0 by adding 1/10th volume of the fraction. The eluted fractions were analyzed by SDS-PAGE and immunoblotting. Elution fractions containing the MAb were pooled and dialyzed against PBS, and were concentrated using PEG-4000. The protein concentration was estimated by bicinchoninic acid (BCA) method. The sample was stored at 20 °C till further analysis.

**Immunoblot analysis of purified mAbs with diphtheria toxin:** SDS-PAGE was performed as previously described (20). Diphtheria toxin(10μg) were loaded onto a 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis under denaturing conditions. The proteins were electrophoretically transferred onto polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore) using standard techniques. Western blot analysis of diphtheria toxin using purified monoclonal antibodies was performed as described (21). The unbound active surfaces of the PVDF membrane were blocked with 5% (w/v) skimmed milk powder in PBST at 37 °C for 2 hours. The membrane was washed and incubated with the purified mAbs at 37 °C for 2 hours. After incubation, membrane was washed and incubated with anti-mouse IgG HRPO conjugate (1:10,000) (Sigma-Aldrich) at 37 °C for 1 hour. The reaction was visualized with diaminobenzidine tetra hydrochloride (DAB) (Sigma-Aldrich) chromogen.

**Binding specificity of mAbs toward unrelated antigens by Indirect ELISA:** *Clostridium septicum*, *Clostridium perfringens* type B, *Clostridium perfringens* type D and *Clostridium sordelli* toxin were dissolved in carbonate bicarbonate buffer (0.1 M), pH 9.6, at a final concentration of 5 μg/ml respectively and subsequently, 100 μl of each antigen was coated in respective wells in Maxisorp 96-well micro titer plates. These plates were incubated overnight at 4°C. The wells were washed thrice with PBST to remove any unbound antigen and the remaining active surfaces in the wells were blocked with 2% (w/v) skimmed milk powder in PBST for 1 hour at 37°C. The plates were again washed with PBST and respective purified antibodies, negative controls (PBST, unimmunized mouse serum, myeloma culture fluid) and the positive control (immunized mouse serum) were added to respective wells and incubated at 37°C for 1 hour. After completion of the incubation step, the plates were washed thoroughly with PBST and goat anti-mouse IgG Fc specific HRPO conjugate (1:25,000) was added to each well (100 μl/well) and incubated for 1 hour at 37°C. The unbound secondary antibody was removed by washing with PBST and TMB substrate was added to the wells. The reaction was stopped after 10 minutes with 1.25M sulfuric acid and absorbance was recorded at 450 nm using an ELISA plate reader (Molecular Devices).

**Isotyping of mAbs:** Isotyping analysis of the mAbs was performed to identify the monoclonality of the clones using isostrip method as per the manufacturer’s instructions (Roche, Germany). Briefly, 150 μl of MAb culture supernatant was added into individual tubes with colored latex beads and agitated. The isostrips were positioned...
in each tube for 5 minutes and then observed for the blue bands at the places indicated for light chain type and different sub-classes of heavy chain.

**Biotinylation of mAbs:** Antibodies were biotin labeled as previously described (22). Briefly, mAbs were dialyzed against 0.1 M Sodium bicarbonate buffer and incubated overnight at 4°C. Biotin solution (EZ-Link™ Sulfo-NHS-SS-Biotin) was added to each antibody as per manufacturer instructions. The mixtures were incubated at room temperature for 1 hour and dialyzed against PBS to remove free biotin. Biotinylated mAbs were aliquoted and stored at -20°C for further studies.

**Competitive ELISA:** Competitive ELISA was performed to identify the antibodies binding to the same epitope by an indirect ELISA. Diphtheria toxoid was dissolved in carbonate bicarbonate buffer (0.1 M), pH 9.6, at a concentration of 5 μg/ml and coated at 100 μl/well in Maxisorp 96 well microtiter plates. After overnight incubation at 4°C and subsequent blocking with 2% (w/v) skimmed milk powder in PBST for 1 hour at 37°C, equal volumes of purified antibodies (native and biotin conjugated) were added to respective wells and incubated for 1 hour at 37°C. After completion of the incubation step, these plates were washed thoroughly with PBST and streptavidin HRPO conjugate (1:25,000) was added to each well and incubated for 1 hour at 37°C. The unbound avidin was removed by washing with PBST and TMB substrate was added to the wells. The reaction was stopped after 10 minutes with 1.25M sulfuric acid and absorbance was recorded at 450 nm using ELISA plate reader (Molecular Devices).

**Determination of cytotoxic dose of diphtheria toxin:** To determine the cytotoxic dose on Vero cells the assay was performed according to the method described (24). Briefly, diphtheria toxin (1 Lf/ml) was added to 96 well tissue culture plate and serially diluted in Dulbecco’s modified Eagles medium (DMEM). A suspension of Vero cells were prepared in complete medium with approximately 2 x 10^5 cells/ml, 2.5 x 10^5 cells/ml and 3 x 10^5 cells/ml and 50 μl of each cell suspension was added to the wells of three micro titer plates respectively. The plates were incubated at 37°C in the presence of 5% CO₂ for 96 hours. The cytotoxic effect was observed under inverted microscope. Cell debris was removed by washing the wells with PBS for three times. Crystal violet
dye (0.03%) was added to each well and incubated the plate at room temperature for 40 minutes. Subsequently, post washing the stained cells were eluted from the wells by adding 70% ethanol (200 μl/well) and incubated at room temperature for 20 minutes. The absorbance of the eluted cells was measured at 595 nm using an ELISA plate reader (Molecular Devices).

**In vitro neutralization assay:** Neutralizing ability of selected mAbs was evaluated by cytotoxicity assay. Briefly, 1 μg of purified MAb was serially diluted (50 μl/well) then diphtheria toxin 0.062 Lf/ml (cytotoxic dose) was added in 1:1 ratio. Plates were incubated at 37°C for 1 hour. Vero cells were added to each well at a density of 2.5 × 10⁵ cells/ml and incubated at 37°C in 5% CO₂ for 96 hours. The cytotoxic effect was observed under inverted microscope. The cell debris was removed by washing the cells with PBS for three times. The crystal violet dye (0.03%) was added to each well and incubated the plate at room temperature for 40 minutes. Subsequently, post washing the stained cells were eluted from the wells by adding 70% ethanol (200 μl/well) and incubated at room temperature for 20 minutes. The absorbance of the eluted cells was measured at 595 nm.

**Receptor-blocking ELISA:** ELISA plates were coated with 1 μg/ml (100 μl/well) of recombinant human Heparin binding epidermal growth factor (rhHB-EGF, R&D Systems), and incubated overnight at 4°C. 1 Lf/ml of diphtheria toxoid and different concentrations of mouse monoclonal antibodies were added in a 96-well round-bottom plate and incubated for 1 hour at 37°C. Antigen-antibody mixture was transferred to the rhHB-EGF coated ELISA plate and incubated for 1 hour at 37°C. The plates were washed thoroughly with PBST and binding activity was detected by addition of Diphtheria anti-toxin (Equine polyclonal serum, NIBSC Standard 0.01 IU/ml). Plates were incubated for 1 hour at 37°C and washed thoroughly with PBST followed by incubation with Anti-Equine IgG HRPO conjugate (1:10,000) for 1 hour at 37°C. Finally the reaction was developed using TMB substrate. The reaction was stopped after 10 minutes with 1.25M sulfuric acid. Absorbance was recorded at 450 nm using an ELISA plate reader (Molecular Devices) (25).

**Results:**

**Development of mAbs against diphtheria toxoid:** The mAbs were generated and developed from mouse hybridomas according to standard techniques, as detailed in Materials and Methods. The hybridomas were developed by fusion of spleenocytes from mouse showing good seroconversion to diphtheria toxoid by indirect ELISA, with mouse myeloma partner Sp2/mIL-6. A total of 960 clones were selected on HAT medium and screened against diphtheria toxoid.

![Fig. 1. Determination of cross reactive mAbs with diphtheria toxin, CRM-197 and diphtheria toxoid by indirect ELISA.](image-url)
by indirect ELISA. Selected five clones (2C4.1C, 3B5.1C, 7G3.1C, 8D7.1C and 9F4.1C) that shown high binding activity with diphtheria toxoid by indirect ELISA. These were cloned for three rounds of limiting dilution for the establishment of monoclonality.

**Binding of mAbs to diphtheria toxoid, toxin and CRM-197 by indirect ELISA:** The selected mAbs were tested for cross reactivity towards diphtheria toxin, toxoid and CRM-197. Out of five selected mAbs, four mAbs (2C4.1C, 3B5.1C, 7G3.1C, and 9F4.1C) were found to cross react with diphtheria toxin and CRM-197, while one MAb 8D7.1C showed specific binding towards diphtheria toxoid as shown in Fig. 1.

**Purification of monoclonal antibodies:** The matured culture supernatants of the selected clones were affinity purified by Protein-G Sepharose column (26) as discussed in Materials and methods section. The purified mAbs was analysed under 12% reduced SDS-PAGE gel which showed bands at ~50 and ~25 kDa (Fig.2). These purified mAbs were used for further studies.

**Binding of mAbs with diphtheria toxoid:** Immunoblotting analysis of the five selected purified mAbs against diphtheria toxoid revealed that 2C4.1C, 3B5.1C, 7G3.1C and 9F4.1C showed binding to diphtheria toxoid whereas 8D7.1C showed no binding (Fig. 3).

**Binding of mAbs with diphtheria toxoid sub fragments:** Diphtheria toxoid was trypsinized as mentioned in methods section and tested purified mAbs against fragments A and B of diphtheria toxoid. MAbs viz. 2C4.1C, 7G3.1C and 9F4.1C recognized the subunit fragment B, whereas, 3B5.1C reacted with subunit fragment A diphtheria toxoid and 8D7.1C showed no binding towards fragment A or B (Table 1).
Table 1. Analysis of binding of mAbs against diphtheria toxin fragments A and B.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Clone ID</th>
<th>Fragment B (37 kDa)</th>
<th>Fragment A (21 kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2C4.1C</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>3B5.1C</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>7G3.1C</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>9F4.1C</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>8D7.1C</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Isotype analysis of mAbs: The isotyping of the monoclones were analyzed to identify the subclass of immunoglobulin of the antibodies. All the tested monoclones were identified as sub-class of IgG. The purified monoclonal antibodies 2C4.1C, 3B5.1C, 7G3.1C, 8D7.1C and 9F4.1C on isotyping showed that all of them were IgG, heavy chain with kappa light chain.

Specificity of mAbs: MAbs specificity were tested against toxin produced by different clostridial species (*Clostridium septicum*, *Clostridium perfringens* Type C) by indirect ELISA. Varying concentrations of the purified monoclonal antibodies (2C4.1C, 3B5.1C, 7G3.1C, 8D7.1C and 9F4.1C) were evaluated for binding against clostridial toxins. The mAbs did not show any cross reactivity with clostridium toxins as shown in Fig.4.

Fig. 4. Specificity of mAbs (2C4.1C, 3B5.1C, 7G3.1C, 9F4.1C and 8D7.1C) were tested against diphtheria toxin, *Clostridium septicum* and *Clostridium perfringens* Type C by Indirect ELISA.

Fig. 5. Competition assay between Biotin labelled 3B5.1C and developed mAbs against diphtheria toxoid.

Praveen et al
Competitive ELISA: The purified mAbs were tested for competition against diphtheria toxoid by a competitive ELISA (27). Purified monoclonal antibodies 2C4.1C, 3B5.1C, 7G3.1C, 8D7.1C and 9F4.1C were biotinylated as described in methods. No competition was observed between the mAbs 2C4.1C and 3B5.1C and also with the biotinylated mAb 3B5.1C. The results indicates that mAbs are binding to different epitopes on diphtheria toxoid (Fig.5). Similarly, no competition was observed between Human mAb 16M3F10.1C (ATCC) and the in house developed mAbs (Fig. 6).

Immunocapture ELISA: Immunocapture ELISA was developed to quantify the antigen in bulk diphtheria toxoid vaccine samples. Two non-competing mAbs were selected, one mAb (mAb 2C4.1C) as capture and mAb 3B5.1C which was biotinylated as an antigen detection. The optimal dilutions of the monoclonal antibodies to be used for developing a sandwich ELISA were selected based on the checker board titration calibrated with the standard antigen. The mAbs were able to quantify the diphtheria toxoid of different lots and showed a linear regression ($r^2=0.99$) as shown in Fig.7. The repeatability of the assay was established using six different standard concentrations, and repeated four times as per the standard qualification guidelines. The assay linearity at six different concentrations showed good curve fit and % of CV was less than 20% at each standard concentration. The recovery rate of the developed sandwich ELISA method with coefficient of variation of 5.88–16.43% indicates that the method possesses good sensitivity and provides a reliable quantitative rate (Table 2).

Cytotoxic effect of diphtheria toxin: Vero cells were seeded at the rate of $2.5 \times 10^5$ cells/ml were found suitable for monolayer cell sheath formation.

![Fig. 6. Competition assay between Human mAb (16MF10, ATCC) and in house developed mAbs against diphtheria toxoid.](image)

![Fig. 7. Validation of sensitivity of diphtheria toxoid (Lf/ml) on Immunocapture ELISA.](image)
Cytotoxic effect of diphtheria toxin was tested on Vero cells at varying concentrations of the toxin (Lf/ml). Results indicated that minimum cytotoxicity effect of diphtheria toxin on Vero cells was found to be 0.0625 Lf/ml as shown in Fig. 8.

In vitro neutralization assay: The purified monoclonal antibodies (2C4.1C, 3B5.1C, 7G3.1C, 8D7.1C and 9F4.1C) were tested for neutralizing activity against diphtheria toxin by cytotoxicity assay. Results revealed that mAbs 2C4.1C, 7G3.1C and 9F4.1C were able to neutralize the cytotoxic effect of diphtheria toxin in a concentration dependent manner, whereas, mAbs 3B5.1C and 8D7.1C did not neutralize the toxin (Fig. 9).

Receptor-blocking ELISA: Receptor blocking ELISA was performed to identify whether mAbs were recognizing receptor domain of diphtheria toxin and inhibit its binding to the receptor (29). The mAbs at varying concentrations were pre-incubated with 1 Lf/ml of diphtheria toxin and then the solution was allowed to bind HB-EGF.

Table 2. Repeatability of the sandwich ELISA was assessed by inter-test variances of four independent tests with five experimental blends.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Description</th>
<th>Test-1</th>
<th>Test-2</th>
<th>Test-3</th>
<th>Test-4</th>
<th>Mean Lf/ml</th>
<th>Standard deviation</th>
<th>% Coefficient of variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Standard</td>
<td>3446</td>
<td>3143</td>
<td>3130</td>
<td>3596</td>
<td>3328.75</td>
<td>233.47</td>
<td>6.9</td>
</tr>
<tr>
<td>2</td>
<td>Sample-A</td>
<td>4010</td>
<td>4241</td>
<td>3312</td>
<td>2760</td>
<td>3580.75</td>
<td>585.5</td>
<td>16.43</td>
</tr>
<tr>
<td>3</td>
<td>Sample-B</td>
<td>3724</td>
<td>4175</td>
<td>3572</td>
<td>3907</td>
<td>3844.5</td>
<td>225.5</td>
<td>5.88</td>
</tr>
<tr>
<td>4</td>
<td>Sample-C</td>
<td>3359</td>
<td>4030</td>
<td>3022</td>
<td>3110</td>
<td>3380.25</td>
<td>430.02</td>
<td>13.01</td>
</tr>
<tr>
<td>5</td>
<td>Sample-D</td>
<td>2644</td>
<td>3684</td>
<td>3368</td>
<td>2612</td>
<td>3077</td>
<td>518.57</td>
<td>16.3</td>
</tr>
<tr>
<td>6</td>
<td>Sample-E</td>
<td>3300</td>
<td>3860</td>
<td>3657</td>
<td>3025</td>
<td>3460.5</td>
<td>322.71</td>
<td>9.36</td>
</tr>
</tbody>
</table>

Fig. 8. Validation of sensitivity of diphtheria toxin (Lf/ml) on Vero cells assay.

Fig. 9. Neutralizing activity of mAbs by In vitro Vero cell cytotoxicity assay.
receptor. ELISA results indicated that mAbs 2C4.1C, 7G3.1C and 9F4.1C shown inhibition of binding of diphtheria toxin to the receptor in a concentration dependent manner, further no blocking was observed for mAbs 3B5.1C and 8D7.1C (Fig.10).

Discussion:
Diphtheria is known to be a localized infection of mucous membranes or skin caused by toxigenic strains of Corynebacterium diphtheriae and it is characterized by the presence of a pseudomembrane at the site of infection. A massive release of toxin into the body will cause lethal necrosis of the heart and liver. Vaccination essentially remains effective in elimination of infection, however the individuals are known to infected by bacteria and becoming asymptomatic carriers and possibility of transmitting to others. Diphtheria anti-toxin (DAT) has been the focus for the treatment of diphtheria infection for several years. The global incidence of disease has decreased constantly though it remains endemic in many parts of the globe and resulting in outbreaks. Due to limitations in availability and shortage in supply globally, an alternative to diphtheria anti-toxin which is safe and more readily available would be desirable to meet the need of DAT. Monoclonal antibodies have the potential to replace the anti-toxin and it can circumvent the adverse effects like hypersensitivity reactions, risk of contamination due to blood derived products etc. (30). As the discovery of hybridoma technology by Kohler and Milstein (1975) brought a new dimension in the diagnosis and therapeautic usage of monoclonal antibodies for several diseases which includes in inhibition of angiogenesis, cancer treatment and as an immune suppressor after organ transplantations etc.(27, 31, 32, 33). The monoclonal antibodies have many potential applications and do not depend upon the polyclonal serum. Monoclonal antibodies are more specific and sensitive when compared to the polyclonal serum. Hence monoclonal antibodies are being used in therapeutics and in development of diagnostics (34).

In the present paper, we describe the monoclonal antibody generation, expression, purification and immunological characterization of an anti-diphtheria antibodies and their application for the quantification of antigen in in-process samples during vaccine production by ELISA. Monoclonal antibodies for diphtheria toxoid have been screened for their activity by ELISA. Based

![Fig. 10. Receptor binding inhibition assay of mAbs by ELISA. 2C4.1C, 7G3.1C and 9F4.1C mAbs are shown to block diphtheria receptor (HB-EGF) binding with diphtheria toxin.](image-url)
on the reactivity, five mAbs were selected for further studies. The lack of cross reactivity with other bacterial antigens confirmed the specificity of the mAbs for the defined antigen. Further, the mAbs did not compete with each other and with the commercial mAb as well. This indicates that both in house developed mAbs and commercial mAb bound to different epitopes on diphtheria toxoid as shown by competitive ELISA. Immunoblot analysis revealed that mAbs 2C4.1C, 7G3.1C and 9F4.1C bound to the fragment B whereas 3B5.1C bound to the fragment A (35).

Among the five mAbs developed, four mAbs were shown cross reactive to diphtheria toxin, diphtheria toxoid and CRM-197. Our results are in complete agreement with the results reported (36), where the mAbs developed against diphtheria toxoid have shown cross reactivity with CRM-197. However, one mAb 8D7.1C showed high specificity towards diphtheria toxoid and did not show cross reactivity with diphtheria toxoid and CRM-197. These mAbs can be used for assessing the complete inactivation of diphtheria toxin (37). The combination of mAbs helps in determination of the vaccine quality i.e., one mAb that binds to diphtheria toxoid but not toxoid (8D7.1C) while other detect both toxin and toxoid which can be used as a positive control (2C4.1C, 3B5.1C 7G3.1C and 9F4.1C) during inactivation process. Isotyping analysis demonstrated that, the developed clone belongs to IgG1 heavy chain and Kappa light chain isotype.

Diphtheria toxoid vaccination has been included as a part of the regular national vaccination program (38). The production of diphtheria toxoid is relatively easier but to ensure consistency of batches and to maintain appropriate quality is found to be critical. Diphtheria toxoid is not a well-defined biological agent and routine testing requires both in vitro and in vivo assays (39). In vitro testing would be more beneficial as it helps in reduction and replacement of animal usage for batch release criteria. However this assay needs to be validated and correlated well with in vivo assays (40). During the process of vaccine development, it is essential to establish vaccine characteristics as good quality and protective levels of antigen in the batches (41).

Quantification of antigen is essential for blending of antigen in vaccine batches, and these quantifications are being performed through the ELISA based methods (42). The mAbs developed were used in the development of an in-house ELISA for the quantification of antigen in in-process samples during manufacture of vaccine. The ELISA showed a perfect linear fit with R^2 value of >0.98. Quantitation of diphtheria toxoid antigen in vaccine preparations using mAbs offers a cheap, simple and convenient way of estimation of antigen by immunocapture ELISA without the loss of antigen (43). Similar antigen assays utilizing antibodies to capture and quantify tetanus toxoid have been described (44,45) and their value for quality control testing continues to be explored (46).

Diphtheria pathogenicity is caused by binding to the R domain of diphtheria toxin B-sub unit to the cell receptor (47, 48). Blocking or interfering with R region plays a significant role in preventing diphtheria toxin to bind to the cells. Three of the mAbs in our study recognized fragment B and thus further studies to evaluate their neutralization potential in an in vitro neutralization assay were studied as described earlier (15). Indeed, the neutralization test using cell culture as an alternative to in vivo method were also recommended by World Health organization (WHO) and the European Pharmacopoeia to reduce the animal usage (49). These in vitro assays are found to be specific and sensitive when compared to the conventional methods like flocculation test and neutralization test performed in rabbits and guinea pigs. Though the animal neutralization test considered as the golden standard it involves cumbersome and limitations for large scale screening and ethical reasons (50). The mAbs were tested for neutralizing capability using Vero cell based assay (51). MAbs 2C4.1C, 7G3.1C and 9F4.1C were found to exhibit neutralization potential and competed with almost same degree of DAT blocking diphtheria toxin to the cells (52). This
was further substantiated when preincubation of mAbs with Vero cells also inhibited the toxicity of diphtheria toxin suggesting the blocking of diphtheria toxin binding to the receptor by mAbs (53).

Furthermore the receptor blocking ELISA reaffirmed the binding of mAbs (2C4.1C, 7G3.1C and 9F4.1C) to HB-EGF receptor. On the contrary, 3B5.1C (specific to fragment A of toxin) and 8D7.1C (specific to diphtheria toxoid) were unable to inhibit the diphtheria toxin activity as expected.

**Conclusion:**

Diphtheria was an infectious disease and spread worldwide even in the vaccination era. There are several reports where the disease was more susceptible to children and adults. Disease can be treated with antibiotics along with the antibiotics and diphtheria polyclonal produced in Equine. Due to the low production of diphtheria anti-toxin, monoclonal antibodies can act as an alternate for treatment of disease. Developed monoclonal antibodies are capable for neutralizing and in quantification of diphtheria toxoid. The epitope mapping of the mAbs to be further established. These antibodies needs to be further characterized for a possible application in the development of therapeutics and diagnostics.

**Abbreviations:**

mAbs, monoclonal antibodies; CRM-197, Cross reative material, a non-toxic mutant of diphtheria toxin; IC-ELISA, immune capture Enzyme linked immunosorbent assay; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; WHO, World Health Organization.

**References:**


prepared from outdated human blood. Vox Sang, 16: 491–495.


A solution for the increasing lack of equine DAT for therapeutic use. Virulence, 7:613-615.


routine, experimental and real-time aged diphtheria toxoids by in vitro analytical techniques. Vaccine. 25, 6863-6871.


