CHAPTER 3
ANALYTICAL METHODS

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Analytical Methods

This chapter mainly discusses the detailed methodologies, which were followed for analytical purposes. Tetanus toxoid and Diphtheria toxoid were analyzed for their quality using a set of various physicochemical and immunological characterization techniques as described in the following sections. Apart from these, specific methods, which were used for specific studies are also described in this chapter.

3.1. Materials and Reagents:

3.1.1 Materials:

Tetanus Toxoid, Diphtheria Toxoid, Equine Polyclonal IgG (whole molecule) against TT (500IU/ml), Equine Polyclonal IgG (whole molecule) against DT (1200IU/ml) were generous gift samples of Serum Institute of India Ltd, Pune.

Bovine Serum Albumin, Tris (hydroxymethyl-amino-methane), Sodium Dodecyl Sulphate (SDS), Acrylamide, Bisacrylamide, Bromophenol blue, Glycine, Coomassie brilliant blue R-250, Ammonium persulphate, N,N,N,N'-Tetra-methyl ethylene-diamine (TEMED), Gel-filtration Molecular Weight Marker (Cat. No.151-1901 for HPLC molecular weight calibration), IEF standard pI range 4.45-9.6 (Bio-Rad, Cat. no. 161-0310) were purchased from Bio-Rad, USA.

Molecular weight marker Cat. No. HMW-H for SDS-PAGE were purchased from Banglore Genei, India

Sodium Chloride, HCl, Glycerol, Methanol, Glacial acetic acid, Potassium dihydrogen phosphate, Sodium Hydroxoide, Sodium carbonate, Sodium Bicarbonate, Potassium Chloride, Disodium hydrogen Phosphate were purchased from Qualigens, India.

Sodium azide; Secondary Antibodies- IgG, Antihorse IgG (whole molecule), Alkaline Phosphatase conjugate antibody developed in rabbit; pNPP Sigma Fast ™ tab; Tween20 were purchased from Sigma, USA.

2-Mercaptoethanol was purchased from Ubichem, India.

3.1.2 Reagents:

3.1.2.1. Reagent-A: Alkaline copper tartarate solution, Bio-Rad, USA

3.1.2.2. Reagent-B: Dilute Folin Reagent, Bio-Rad, USA

3.1.2.3. Resolving gel buffer: Tris (hydroxymethyl-amino-methane) (18.10 gm) and 0.4 gm SDS were dissolved in 50 ml Milli-Q and pH was adjusted to 8.9 with concentrate HCl. Final volume was made up to 100 ml with Milli-Q water.

3.1.2.4. Spacer gel buffer: Tris (hydroxymethyl-amino-methane) (5.9 gm) and 0.4 gm SDS were dissolved in 50 ml Milli-Q water, pH was adjusted to 6.7 with conc HCl and final volume was made upto100 ml with Milli-Q water.

3.1.2.5. 30% Acrylamide: Acrylamide (29.25 gm) and 0.75 gm Bisacrylamide were dissolved in 50 ml Milli-Q water and volume was made up to 100 ml with Milli-Q water. Solution was filtered through 0.22 μ, 47 mm membrane (Pall) and stored at 4°C in an amber colored bottle.

3.1.2.6. Sample buffer for SDS-PAGE: Sample buffer was prepared by performing following addition and mixing:

Spacer gel buffer	1.25 ml
10% SDS in Milli-Q	2.00 ml
2-Mercaptoethanol	0.50 ml
Glycerol	2.50 ml
0.5% Bromophenol blue	0.20 ml

3.1.2.7. Electrode buffer: Electrode buffer was prepared by dissolving 6.32 gms of Tris, 4 gm of Glycine and 1 gm of SDS in about 700 ml of Milli-Q water and volume was made up to 1000ml.

3.1.2.8. Staining solution: 0.8 gm of Coomassie brilliant blue R-250 was dissolved in 200 ml of Methanol and 28 ml of Glacial Acetic acid. Finally, 200 ml of Milli-Q water was added.

3.1.2.9. Destaining solution: Destaining solution was prepared by addition of 50 ml Methanol and 70 ml of Glacial Acetic acid to 500 ml of Milli-Q water and final volume was made up to 1000ml.

3.1.2.10. 10% Ammonium persulphate (APS): Freshly prepared in Milli-Q water.

3.1.2.11. Mobile Phase : Potassium dihydrogen phosphate solution was prepared(0.1 M) by dissolving 13.6 g of KH_2PO_4 in 700 ml of Milli-Q water, pH was adjusted to 7.2 using

0.1N NaOH, volume was made up to 1000ml. Solution was filtered using 0.22 µ, 47mm nylon membrane (Pall) under vacuum.

3.1.2.12. Sodium azide (0.05%): 0.5 gm of sodium azide was dissolved in 900 ml of Milli-Q water and volume was made up to 1L. Solution was filtered using 0.22 µ, 47mm membrane filter under vacuum.

3.1.2.13. Ready anode 7 mM phosphoric acid buffer, Bio-Rad, Cat. no. 161-0761

3.1.2.14. Ready cathode 20 mM lysine and 20 mM arginine buffer, Bio-Rad, Cat. no. 161-9762.

3.1.2.15. Sample buffer (1x) for IEF: 50% glycerol

3.1.2.16. Gel staining solution for IEF: Bio-Rad, Cat. no. 161-0434.

3.1.2.17. Coating Buffer (pH 9.6): Sodium carbonate (1.59 gm), Sodium bicarbonate (2.93 gm), and Sodium azide (0.2 gm) were dissolved in Milli-Q water and volume made up to 1000 ml with Mill-Q water

3.1.2.18. Dilution and Washing Buffer (pH 7.4): Sodium Chloride(8 gm),Potassium Chloride (0.2 gm), Disodium hydrogen Phosphate(1.15 gm), Potassium dihydrogen phosphate (0.2gm) were dissolved in Milli-Q water and filtered through 0.22µ membrane filter to this Tween 20 (0.5 ml) was added and volume was made up to 1000ml using MilliQ water.

3.1.2.19 Blocking Buffer (pH 7.4): Disodium hydrogen phosphate (1.78 gm), Sodium dihydrogen phosphate (1.56 gm), Bovine Serum Albumin (0.25 gm) was dissolved in Milli-Q water and filtered through 0.22µ membrane filter and volume was made up to 1000ml using MilliQ water.

3.2. Analytical Methods

3.2.1 Determination of Protein Content by DC Protein Assay:

Protein content of the samples was determined using DC protein assay (modified Lowry method) for accurate protein loading for SDS-PAGE, HPLC analysis and pI determination of toxoids.

The Bio-Rad DC Protein Assay is the colorimetric assay for the determination of protein concentration in presence of reducing agents as well as detergents. The reaction is similar to the well-documented Lowry assay, but with the following improvement: The

reaction reaches 90% of the maximum color development within 15 minutes thereby saving valuable time and the color changes not more then 5% in 1 hour and 10% in two hour after the addition of reagents.

The assay is based upon the reaction of protein with an alkaline copper tartrate and Folin reagent. As with the Lowry assay, there are two steps, which lead to color development: the reaction between protein and copper in an alkaline medium, and subsequent reduction of Folin reagent by the copper-treated protein. Color development is primarily due to the amino acids tyrosine and tryptophan, and to a lesser extent, cystine, cytosine, and histidine. The proteins affect the reduction of the Folin reagent by loss of 1,2 or 3 oxygen atoms, thereby producing one or more of several possible reduced species which have characteristic blue color with maximum absorbance at 750 nm and the minimum absorbance at 405 nm. ***(Lowry, O.H., 1951; Peterson, G.I., 1979)***

3.2.1.2 Requirements:

A. Instruments:

a. UV-Visible Spectrophotometer, Shimadzu UV-1601

b. Vortex mixer- Remi

B. Chemicals and Reagents:

a. Bovine Serum Albumin

b. Reagent-A: Alkaline copper tartarate solution.

c. Reagent-B: Dilute Folin Reagent.

3.2.1.3 Procedure:

100 µl of sample +500 µl reagent-A were mixed in a test tube and vortexed. 4 ml of reagent-B was added to the above mixed solution and immediately vortexed after 15 minutes, absorbance was measured at 750 nm.

3.2.2 Determination of Limes Flocculation Units (Lf):

Antigenicity measurements are generally applied to characterize and quantify antigens. By definition, measurement of antigenicity requires the use of antibodies. Their specificity makes them excellent reagents, even in very complex matrices. Flocculation is an antigenicity determining test. ***(Leenaars P.P.A.M. 2001).***

In flocculation test, an antigen (toxoid) reacts with specific antibody (antitoxin) in

presence of electrolytes. At optimum concentration of both the reactants at 50°C, antigen-antibody complexes form floccules which appear in tube over a period of time *(WHO 77.1 Rev 1; WHO 77.2 Rev 1)*.

3.2.2.1 Sample Preparation:

Tetanus Toxoid or Diphtheria Toxoid was diluted to give an expected concentration of about 40 - 90 Lf units per ml. No dilution was required when Lf-units of the test sample was less than 90.

3.2.2.2 Requirements:

A. Chemicals and Reagents:

a. Saline solution (0.85% NaCl)

b. Standard Tetanus antitoxin diluted at 100 IU/ml or Standard Diphtheria antitoxin diluted at 100 IU/ml (Serum Institute standard)

3.2.2.3 Procedure:

The procedure followed was as mentioned in WHO manual for the production of tetanus toxoid vaccine *(WHO manual, 1977)*. In brief, the method followed was as mentioned below:

Water bath was maintained to 50°C. Increasing amounts of specific reference antitoxin (100 IU/ml) in a suitable range between 0.30 to 1 ml with an increment of 0.05 ml (broad range) per tube was dispensed in flocculation tubes. Volume was made up to 1.0 ml in each tube with 0.85% normal saline. Test toxoid sample (1.0 ml) was added in each tube and thoroughly mixed by inverting them thrice and again incubated till floccules were observed. Lf/ml of the test sample was re-confirmed by repeating the test in narrow range at a difference of 0.01 ml or 0.02 ml of anti toxin.

Antibody concentration in the tube showing flocculation first was multiplied by the dilution factor of the toxoid to get the Lf/ml of toxoid.

The first tube to flocculate is defined as the one, which contains the amount of antitoxin closest in equivalence to the amount of antigen to the sample.

3.2.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE):

Electrophoretic techniques can reveal similar physical properties of protein antigen as liquid chromatography. In addition, it provides more information about protein-protein

interactions when performed under denaturing and reduced conditions *(Metz B., 2003).* It provides more realistic picture of purity and molecular weight.

SDS-PAGE has been routinely used for several decades for the analyses of protein based vaccine components. This technique is used by many researchers for the characterization of tetanus toxoid and proved to be capable of differentiating between the toxoids of different characteristics *(Leenaars P.P.A.M., 2001).*

3.2.3.1 Requirements:

A. Instruments:

a. Mini Protein (P3) Electrophoresis System (Bio-Rad)

b. Power Pac-300 (Bio-Rad)

c. Gel-Documentation System (Bio-Rad)

B. Reagents:

a. Resolving gel buffer:

b. Spacer gel buffer:

c. 30% Acrylamide:

d. Sample buffer:

e. Electrode buffer:

f. Staining solution :

g. Destaining solution:

h. Ammonium persulphate (10%) :

3.2.3.2 Procedure:

The SDS-PAGE was carried out according to the procedure of ***Laemmeli* (*1970*)** and modified by ***Fling* (*1986*)** using Mini-Protein III slab cell (Bio-Rad, USA). Running gel was casted with 7% resolving gel followed by 1 cm 4% stacking gel on top of it (**Table 1a**). Total size of the gel was 7.5 x 10 cm, thickness 0.75 mm.

About 10-20 µg of protein mixed with sample buffer was loaded in separate wells and electrophoresed using tris-glysine-SDS buffer (tank buffer) pH 8.3 at room temperature. Initially, voltage was kept 50 V constant for 15 min (until samples reached close to resolving gel) which was then increased to 80 V for approx. 90 min till the dye reached

about 0.5 cm before the end of resolving gel. A standard molecular weight marker was also loaded in each gel. After completion of electrophoresis the gel was removed and stained with 0.2% Coomasie Brilliant Blue-R250 for 45 min and then destained using destaining reagent.

Gel documentation system (Bio-Rad) was used to photograph the gel followed by molecular weight determination and densitometric scanning was done using Quantity - One software (Bio-Rad).

Addition of reagents to prepare 7% gel was done as follows:

7% mini-gel (for two gels)		
	Resolving gel	**Spacer gel**
Milli-Q	6.2 ml	3.5 ml
30% acrylamide	2.8 ml	1.0 ml
Gel buffer	3.0 ml	1.5 ml
APS	75 µl	50 µl
TEMED	5.0 µl	2.5 µl

3.2.4 High Performance Liquid Chromatography (HPLC):

HPLC is a highly sensitive and accurate method for the determination of purity as well as molecular weight of a protein. It has been a method of choice for the quality control testing of many of the biotechnology oriented products *(Federici M.M., 1994)*. Many of the researchers have used this technique for the characterization of tetanus toxoid using size exclusion as a separation principle *(Lyng J., 1990; Gupta R. K., 1994; Doshi J. B., 2003)*. Even this technique has been suggested to be an useful tool for the rapid purification of tetanus toxoid *(Silveira I. A.,1994)*. HPLC was used for qualitative and quantitative analysis of TT and DT.

3.2.4.1 Requirements:

A. Instruments:

a. Shimadzu Class LC-10AT VP binary gradient HPLC with a UV-Visible SPD-10A VP detector

b. Protein-Pak 300SW (Waters) column having fractionation range, Native Globular proteins: 10-400 kD; Random coil: 2-150 kD

B. Reagents:

a.Mobile Phase:

b. 0.05% Sodium-azide:

c. Sample Preparation: The toxoid samples were diluted to the final required concentration (in Lf/ml) to prepare standard curve. Standard and unknown samples were filtered through 0.22 μ acrodisc (Pall). In case if any precipitates were observed the toxoid samples were centrifuged at 10,000 rpm for 15 min before filtration.

d. Gel-filtration Molecular Weight Marker:

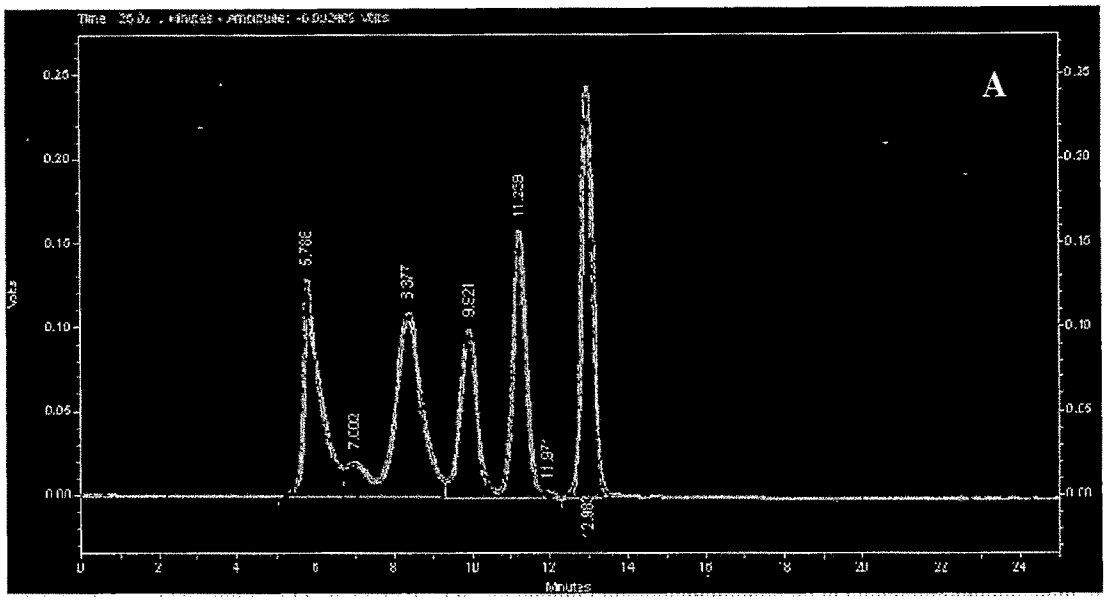
3.2.4.2 Procedure:

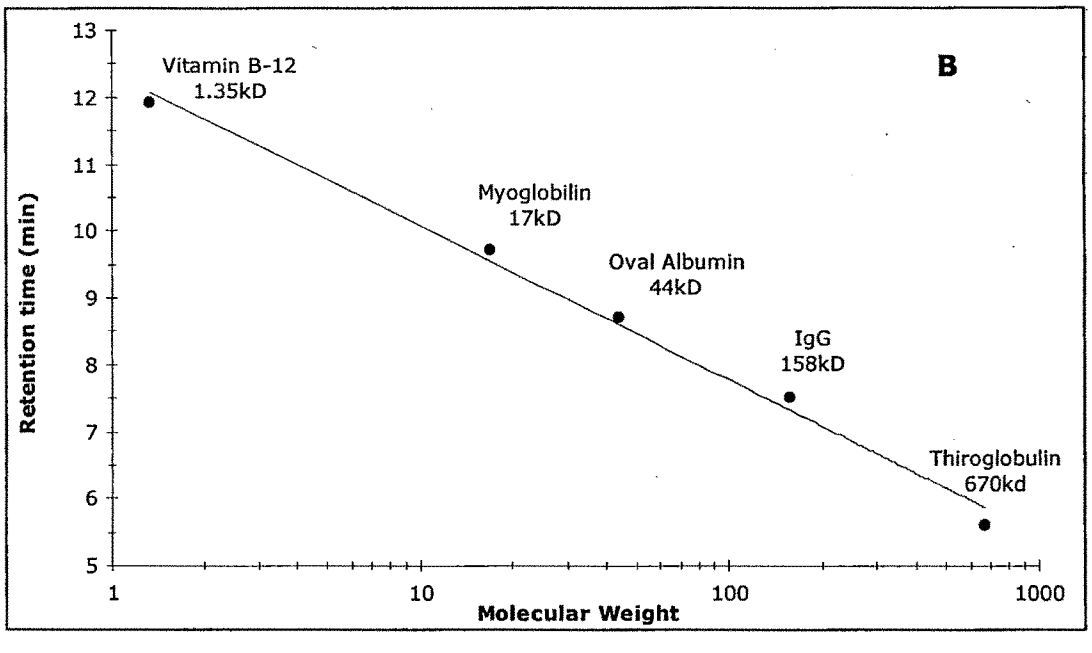
Required parameters were programmed using Class VP software (Schimadzu, Japan). Program parameters were: 1) Flow rate -1 ml/min 2) Detection wavelength- 280 nm 3) Run time -25 min.

The column was equilibrated by passing at least 100-200 ml of mobile phase. 20 μL of sample was loaded using Hamilton syringe. System was calibrated for determination of molecular weights of the standard and unknown samples of TT using Gel Filtration Molecular weight markers. A standard calibration curve was plotted with retention time of each marker protein versus corresponding molecular weight on log scale as shown in **Figure a20**.

After completion of the analysis, the mobile phase was changed to Milli-Q water and column was flushed with about 100 ml of Milli-Q. Then about 100 ml of storage solution (sodium azide) was passed through column. The flow rate was reduced to zero and then system was switched off.

Figure a20: Standard curve for molecular weight marker (A) HPLC profile (B) Calibration curve





A log linear relationship was obtained in the molecular weight range of the 1.35kD to 670kD protein, given by the regression **Equation 1.**

$$y = -1.0013\text{Ln}(x) + 12.382,\ R^2 = 0.9922 \ldots \textbf{Equation 1}$$

3.2.5. Determination of Isoeelctric point of TT and DT by Isoelectric Focussing:

The pI of a protein can be simply defined as a pH at which net charge on protein is zero and protein has no electrophoretic mobility in an isoelectric focusing run. pI value of a protein can be affected by changes in primary structure, post transnational modifications and denaturation. Hence, it is used to monitoring such alterations *(Garfin D. E., 2001; Branchu S., 1999).*

3.2.5.1 Requirements:

A. Instrument:

a. Mini-protein III electrophoresis unit

b. HPLC column (Gel-filtration) Protein pak-300SW (Waters, USA)

B. Reagents

a. Ready anode buffer

b. Ready cathode buffer

c. Pre-cast isoelectric focussing gel 4%, pH range 3-10 (Bio-Rad)

d. IEF standard pI range 4.45-9.6

e. Sample buffer (1x) :

f. Gel staining solution for IEF

g. Destaining solution:

3.2.5.2 Procedure:

The precast IEF gel was assembled on electrophoresis apparatus. The anode and cathode buffers were poured to their respective locations. Samples were diluted using appropriate amount of sample buffer and loaded about 20-25 µg per well. The IEF marker was loaded in the first well of gel. Constant current of 250 V was applied for one hour followed by 500 V for two hours. After completion of electrophoresis, gels were removed and stained for 45 min followed by destaining for 2-3 hours.

Gel documentation system (Bio-Rad) was used to photograph the gel and further analysis for pI determination was done using Quantity One software (Bio-Rad).

3.2.6 Estimation of TT using ELISA:

TT from various samples was estimated by an adapted direct enzyme-linked immunosorbent assay (ELISA) method after modification. ***(Gupta, R.K., 1996)***

3.2.6.1 Requirements:

A. Instrument:

a. Nunc-Immuno™ Plate with Maxisorb™ surface

b. Bio-Rad Microplate washer

c. Bio-Rad Microplate Incubator,

d. BioRad Microplate Reader 550 with Microplate Manager™ software

B. Reagents:

a. Toxoid Standard- Tetanus toxoid Pool 184 (3840 Lf/ml).

b. Primary antibody- Equine Polyclonal IgG (whole molecule) against TT (500IU/ml) [2µl of stock solution, volume made up to 10 ml with dilution buffer] (1:5000dilution], Serum Institute of India Ltd, Pune.

c. Secondary Antibodies- IgG, Antihorse IgG (whole molecule), Alkaline Phosphatase conjugate antibody developed in rabbit. [3 µl stock, volume made up to 3ml with dilution buffer (1: 1000 dilution), Sigma.

d. para-Nitro Phenyl Phosphate substrate: pNPP Sigma tab- 1 tablet of substrate and 1 tablet of buffer was dissolved in 5 ml MilliQ water to get working solution.

e. Coating Buffer (pH 9.6):

f. Dilution Buffer (pH 7.4):

g. Washing Buffer (pH 7.4):

h. Blocking Buffer (pH 7.4):

3.2.6.2 Procedure:

TT measured by an adapted direct enzyme-linked immunosorbent assay (ELISA) method. TT standard curve was prepared by adsorbing flat bottomed 96 well ELISA plates (Nunc-Immuno™ Plate) with 100µl of two fold dilutions of TT in 50 mM Bicarbonate Coating buffer of pH 9.6 and incubated at 37.5^{0}C for 1 hr. Plates were washed 4 times with 300µl of PBS-T-0.05% Washing buffer using Bio-Rad Microplate

washer and blocked using 0.5% BSA in 10mM PB i.e. Blocking buffer, 100µl per well and incubated for 1 hr at 37.5°C for 1 hr followed by washing step. To this 1:5000 dilution of Primary antibodies, 100µl was added and incubated for 1 hr at 37.50C followed by washing cycle. Secondary antibodies, 100µl per well was added and incubated followed by washing cycle. To this 100µl of p-Nitrophenyl phosphate substrate was added and incubated at room temperature for 30 minutes in dark. Plate was read at 450nm on BioRad Microplate Reader 550 and data was analyzed using Microplate Manager™ software. Optical density at 450nm was plotted against concentration of TT (Lf/ml). Unknown samples were suitably diluted to obtain optical density in standard concentration curve range.

3.2.7 Estimation of DT using ELISA:

DT from various samples was estimated by an adapted direct enzyme-linked immunosorbent assay (ELISA) method after modification.***(Gupta, R.K., 1996).*** Similar method as described for TT was used except instead of TT specific primary antibodies DT specific Primary Polyclonal IgG antibodies (whole molecule) against DT (1200IU/ml) (1:5000dilution] were used. Optical density at 450nm was plotted against concentration of DT (Lf/ml). Unknown samples were suitably diluted to obtain optical density in standard concentration curve range.

3.2.8 Estimation of TT specific IgG from hyper immunized mice sera using ELISA:

TT specific IgG from hyperimmunized mice sera was estimated by an adapted direct enzyme-linked immunosorbent assay (ELISA) method after modification. ***(Xing, D.K.L.,1996; van der Luben, I.,M.,2003)***

3.2.8.1 Requirements:

A. Instrument:

a. Nunc-Immuno™ Plate with Maxisorb™ surface

b. Bio-Rad Microplate washer

c. Bio-Rad Microplate Incubator,

d. BioRad Microplate Reader 550 with Microplate Manager™ software

B. Reagents:

a. Toxoid Standard- Tetanus toxoid Pool 184 (3840 Lf/ml).

b. Primary antibody- Equine Polyclonal IgG (whole molecule) against TT (500IU/ml)[2µl of stock solution, volume made up to 10 ml with dilution buffer] (1:5000dilution], Serum Institute of India Ltd, Pune.

c. Secondary Antibodies- IgG, Anti-horse IgG (whole molecule), Alkaline Phosphatase conjugate antibody developed in rabbit. [3 µl stock, volume made up to 3ml with dilution buffer. (1: 1000 dilution), Sigma, USA.

d. para-Nitro Phenyl Phosphate substrate:

e. Coating Buffer (pH 9.6)

f. Washing Buffer (pH 7.4)

h. Blocking Buffer (pH 7.4)

3.2.8.2 Procedure:

ELISA was performed by coating Flat bottomed 96 well ELISA plates with 100µl of 1Lf/ml TT in 50 mM bicarbonate coating buffer of pH 9.6. Plate was allowed to incubate at 37.5^{0}C for 1 hr and washed 4 times with 300µl of PBS-T-0.05% of Washing buffer using BioRad Microplate washer. Blocking buffer of 0.5% BSA in 10mM phosphate buffer, 100µl per well was added and incubated for 1 hr at 37.5^{0}C for 1 hr followed by washing step. To this two fold dilutions of appropriate concentration of the primary antibodies, 100µl, was added and incubated for 1 hr at 37.5^{0}C followed by washing of the plate. Secondary antibodies, 100µl per well was added and incubated followed by washing. To this 100µl of p-Nitrophenyl phosphate substrate was added and incubated at RT for 30 minutes in dark. Plate was read at 450nm on BioRad Microplate Reader 550 and data was analyzed using Microplate Manager™ software. Optical density at 450nm was plotted against the concentration of primary TT specific IgG (IU/ml). This standard curve was utilized to determine IgG levels in hyperimmunized mice, which was used for the preparation of standard curve, by using it as primary antibody, for the estimation of IgG in experimental mice.

3.2.9 Estimation of TT specific IgG experimental mice sera using ELISA:

TT specific IgG in experimental mice was performed using the same method described for estimation of TT specific IgG in hyperimmunized mice except the Standard curve is prepared using Mouse TT specific IgG obtained from hyperimmunized mouse sera instead of Equine TT specific IgG, as primary antibodies.

3.2.10 Estimation of DT specific IgG from hyperimmunized mice sera using ELISA

DT specific IgG in hyperimmunized mice performed using the same method described for estimation of TT specific IgG in hyperimmunized mice except the Diphtheria Toxoid is used instead of TT and DT specific Primary Equine Polyclonal IgG antibody (whole molecule) against DT (1200IU/ml)[1:5000dilution], instead of TT specific primary equine antibodies.

3.2.11 Estimation of DT specific IgG from experimental mice sera using ELISA

DT specific IgG in experimental mice was performed using the same method described for estimation of DT specific IgG in hyperimmunized mice except the Standard curve is prepared using Mouse DT specific IgG obtained from hyperimmunized mouse sera instead of Equine DT specific IgG, as primary antibodies.

3.2.12 Estimation of TT specific IgA from Fecal matter extracts, Intestinal Lavage solution and Intestinal washings of mice using ELISA

TT specific IgA from different samples of mice was estimated by an adapted direct enzyme-linked immunosorbent assay (ELISA) method after modification. (***van der Luben, I.,M.,2003)***

3.2.12.1 Requirements:

A. Instrument:

a. Nunc-Immuno™ Plate with Maxisorb™ surface

b. Bio-Rad Microplate washer

c. Bio-Rad Microplate Incubator,

d. BioRad Microplate Reader 550 with Microplate Manager™ software

B. Reagents:

a. Toxoid Standard- Tetanus toxoid Pool 184 (3840 Lf/ml).

b. Secondary Antibodies- Anti-mouse IgA (Whole molecule), Alkaline Phosphatase conjugate antibody. [3 µl stock, volume made up to 3ml with dilution buffer. (1: 1000 dilution), Sigma, USA.

c. para-Nitro Phenyl Phosphate substrate: pNPP Sigma tab- 1 tablet of substrate and 1 tablet of buffer was dissolved in 5 ml MilliQ water to get working solution.

d. Coating Buffer (pH 9.6)

e. Washing Buffer (pH 7.4)

f. Blocking Buffer (pH 7.4)

3.2.12.2 Procedure:

ELISA was performed by coating Flat-bottomed 96 well ELISA plates with 100µl of 1Lf/ml TT in 50 mM bicarbonate coating buffer of pH 9.6. Plate was allowed to incubate at 37.5^{0}C for 1 hr and washed 4 times with 300µl of PBS-T-0.05% of Washing buffer using BioRad Microplate washer. Blocking buffer of 0.5% BSA in 10mM phosphate buffer, 100µl per well was added and incubated for 1 hr at 37.5^{0}C for 1 hr followed by washing step. To this different dilution of unknown samples, 100µl, was added and incubated for 1 hr at 37.5^{0}C followed by washing of the plate. Secondary antibodies, 100µl per well was added and incubated followed by washing. To this 100µl of p-Nitrophenyl phosphate substrate was added and incubated at RT for 30 minutes in dark. Plate was read at 450nm on BioRad Microplate Reader 550 and data was analyzed using Microplate Manager™ software. Optical density at 450nm was used to compare the IgA levels at different points.

3.2.13. Estimation of DT specific IgA from Fecal matter extracts, Intestinal Lavage solution and Intestinal washings of mice using ELISA

DT specific IgA from different samples of mice was estimated using same method as that of TT specific IgA form different samples except that DT 1Lf/ml was used instead of TT for coating the plates.

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