CHAPTER 5
FORMULATION DEVELOPMENT

Chapter 5

Formulation Development

5.1 Experimental:

In the preformulation studies, Chitosan, Sodium Tripolyphosphate, Glycerol and Propylene glycol were found to be compatible with tetanus toxoid. Hence, the same excipients were used for the preparation of chitosan microparticles. Chitosan was selected for formation of microparticles as it is biodegradable, soft tissue compatible and mucoadhesive polymer. Chitosan is a polycationic polymer, therefore reactions with negatively charged components, either ions or molecules, can lead to the formation of a network through ionic bridges between polymeric chains. As ionic crosslinking is a simple and mild procedure, it was used to entrap **Tetanus** and **Diphtheria toxoids**, which usually denature due to thermal or chemical crosslinking procedures.

5.1.1. Materials and Methods:

Chitosan (CS) was obtained as a gift sample from Central Institute of Fisheries Technology, Cochin and was used without any modification and purification. (Molecular weight--6,00,000 daltons, Degree of deacetylation >85%)

Tetanus toxoid (TT, 3840 Lf/ml), Diphtheria toxoid (DT, 4500 Lf/ml) and Primary antibodies [IgG, horse polyclonal against TT, 500 IU/ml and against DT, 1200 IU/ml] were a generous gift from Serum Institute of India Ltd., Pune.

Ethylene diaminetetraacetic acid (EDTA), Propylene glycol, Glacial acetic acid, Sodium chloride, Sodium sulphate, Potassium chloride, Sodium carbonate, Sodium bicarbonate, Potassium chloride, Disodium hydrogen phosphate, Sodium dihydrogen phosphate, Potassium dihydrogen phosphate, Methanol were purchased from SD fine chemicals, Mumbai.

Tris (hydroxymethyl-amino-methane), Sodium Dodecyl Sulphate (SDS), Acrylamide, Bisacrylamide, 2-Mercaptoethanol, Glycerol, Bromophenol blue, Glycine, Coomassie brilliant blue R-50, Ammonium persulphate, N,N,N,N'-Tetra-methyl ethylene-diamine (TEMED), Bovine Serum Albumin, Alkaline phosphatase conjugated anti-mouse IgG, Alkaline phosphates conjugated anti-horse IgG, Alkaline phosphates conjugated anti-mouse IgA; Tween 20, p-Nitrophenyl phosphate (pNPP), Sodium azide, PEG 3350, Fetal Calf Serum were purchased from Sigma, United States of America.

Sodium tripolyphosphate (STPP) was purchased from National Chemicals, Baroda.

Soybean trypsin protease inhibitor was purchased from Sisco Research Lab, Mumbai and Phenylmethane suphonyl fluoride (PMSF) was purchased from HiMedia Labs, Mumbai. All other reagents were of analytical grade.

Flat bottomed 96 well ELISA plates (Nunc-Immuno™ with Maxisorb™ surface) were purchased from Nunc, Denmark. Bio-Rad Microplate washer, BioRad Microplate Reader 550 was used to read ELISA plates and data was analyzed using Microplate Manager™ software.

5.1.2. Solutions and Reagents:

5.1.2.1 Chitosan solution:-

Chitosan (2gm) was added to sterile 100ml Milli Q water containing Glacial acetic acid (1ml) under continuous stirring using magnetic stirrer. After overnight stirring chitosan solution was obtained which was filtered through sterile Dacron cloth to get 20 mg/ml solution of chitosan.

5.1.2.2 Sodium Tripolyphoshphate solution:-

Sodium Tripolyphosphate (0.560 gm) was dissolved in 20ml Milli Q water and passed through sterile 0.2μ filter to get 28mg/ml solution of STPP.

5.1.2.3 Sodium Carboxymethylcellulose (SCMC) solution:-

SCMC, 2gm, was added to 1000 ml Milli Q water under continuous stirring using magnetic stirrer. The solution was filtered through sterile Dacron cloth filter.

5.1.2.4 Phosphate buffer saline (pH 7.4):-

Phosphate buffer saline (PBS, 7.4) was prepared by dissolving Sodium chloride (8 gm), Potassium chloride (0.2 gm), Disodium hydrogen phosphate (1.15 gm), Potassium dihydrogen phosphate (0.2 gm) in 800 ml of Milli Q water. The pH was adjusted to 7.4 and volume was made up to 1000 ml using Milli Q water and passed through 0.22μ membrane filter.

5.1.2.5 Lavage Solution:-

Lavage solution was prepared by dissolving Sodium chloride (25mM), Sodium sulphate (40mM), Potassium chloride (10mM), Sodium bicarbonate (20mM), and Polyethylene

Glycol (PEG, average MW 3350, 48.5mM) in 400 ml of Milli Q water. Volume was made up to 500ml using Milli Q water. The solution was filtered through sterile dacron cloth.

5.1.2.6 Protease Inhibitor cocktail / Homogenization buffer:-

Protease inhibitor cocktail buffer was prepared by dissolving EDTA (50mM), BSA (1%), Soybean trypsin inhibitor (0.1 mg/ml) and 2 mM PMSF in 1000ml of Phosphate buffer saline, pH 7.4.

5.1.3. Characterization:

Characterization of Chitosan (CS), Sodium Tripolyphosphate (STPP), Tetanus Toxoid (TT) and Diphtheria Toxoid (DT) was carried out for various properties.

5.1.3.1 Chitosan (CS):

5.1.3.1.1 Solution pH:- pH of 2mg/ml solution in 1% acetic acid was determined using Global digital pH meter, DPH-500.

5.1.3.1.2 Solution Viscosity:- Viscosity of 1% solution in 1% acetic acid was determined using Brookefield Rheometer, Brookefield Engineering Labs, USA with small volume adapter.

5.1.3.1.3 Fourier transform Infra-Red Spectroscopy (FTIR):-

FTIR of the Chitosan powder was taken using Potassium bromide technique on Shimadzu FTIR.

5.1.3.2 Sodium Tripolyphosphate (STPP):

5.1.3.2.1 Solution pH:- pH of 1mg/ml solution in Milli Q was determined using Global digital pH meter, DPH-500.

5.1.3.3 Tetanus Toxoid (TT) and Diphtheria Toxoid (DT):-

Concentration in terms of Limes flocculation (Lf) units of TT and DT samples were measured using Flocculation test. Protein content was determined using Detergent Compatible (DC) Protein Assay method. Molecular weight of TT and DT was determined using Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) and HPLC. Isoelectric point (pI) was determined using Isoelectric Focusing (IEF) technique.

5.1.3.3.1 Determination of Limes Flocculation (Lf) Units:-

Limes flocculation (Lf) units of TT and DT were determined using Flocculation Test as described in Analytical Methods section. Appropriate dilutions of the TT and DT were allowed to react with equine anti-tetanus IgG and anti-diphtheria IgG respectively at 50^{0}C in constant temperature water bath. Lf was calculated by observing the tube, which gave the flocculation reaction at earliest.

5.1.3.3.2 Determination of Protein Content by DC Protein Assay:-

As protein content of the toxoids is important for optimum protein loading in case of SDS-PAGE, HPLC analysis and pI determination, Protein content of TT and DT samples was determined using DC protein assay (modified Lowry method) as described in Analytical Method section.

Calibration Plot was prepared using BSA as a standard protein in the concentration range of 20 µgm/ml to 100µgm/ml.

5.1.3.3.3 Determination of Molecular Weight of TT and DT:

5.1.3.3.3.1 Using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE):- Molecular weight of TT and DT was determined using SDS-PAGE analysis method described in Analytical methods section. SDS-PAGE gel of TT and DT was photographed using Gel Doc system and analyzed using Qunatity One™ software.

5.1.3.3.3.2 Using High Performance Liquid Chromatography (HPLC):-

HPLC was performed as per the method described in Analytical Method section. Retention time of the main peak of the standard TT and DT was compared with retention time of molecular weight markers. Molecular weight of TT and DT was calculated using the linear relationship between the Retention time in minutes (Rt) and Molecular weight of markers.

5.1.3.3.4 Determination of Isoelectric point:-

Isoelectric point (*p*I) of TT and DT was determined using Isoelectric Focusing method as described in Analytical Methods section.

5.1.3.3.5 Quantitative estimation of TT and DT using HPLC:

5.1.3.3.5.1 Quantitative estimation of TT:- From molecular weight determination by HPLC, TT was found to have retention time of 7.24 min. Hence, calibration of TT was done by HPLC at Rt value of 7.24 min. Calibration plot was prepared by using serial two

fold dilution of the stock solution in the range of 6.25 to 100 Lf/ml of TT and Area Under the Curve (AUC) at Rt 7.24 was measured. AUC was plotted against the respective concentration to obtain the calibration plot, which was used for the estimation of unknown TT.

5.1.3.3.5.2. Quantitative estimation of DT:- From molecular weight determination by HPLC, DT was found to have retention time of 8.11. Hence, Calibration of DT was done by HPLC at Rt value of 8.11min. Calibration plot was prepared by using serial two fold dilution in the range of 6.25 to 100 Lf/ml of DT and AUC at Rt 8.11min was measured. AUC was plotted against the respective concentration to obtain the calibration plot, which was used for the estimation of unknown DT.

5.1.3.3.6 Quantitative estimation of TT and DT using ELISA:

5.1.3.3.6.1 Quantitative estimation of TT:

ELISA was performed using method as described in Analytical Methods Section. Standard curve of TT was prepared by making two fold serial dilutions in the range of 0.0390 Lf/ml to 0.001207 Lf/ml. Calibration plot was used for the determination of the unknown TT concentration.

5.1.3.3.6.2 Quantitative estimation of DT

ELISA was performed using method as described in Analytical Methods Section. Standard curve of DT was prepared by making two fold serial dilutions in the range of 0.0390 Lf/ml to 0.001207 Lf/ml. Calibration plot was used for the determination of the unknown DT concentration.

5.1.4. Preparation of Plain Chitosan Microparticles:

Chitosan solution, 2ml, was diluted upto 40ml with Milli Q water and to this 1mg /ml solution of STPP was added drop-wise till there was formation of turbidity which marked crosslinking and microparticle formation. This was verified by observing under Olympus microscope. Then the exact quantity of STPP required to crosslink CS was optimized.

5.1.4.1 Optimization of amount of STPP to cross-link CS:-

CS amount was fixed at 40mg and optimum amount of STPP required to cross-link CS was determined by measuring change in percent transmittance (%T) at 600nm using

Shimadzu-1601 UV-Visible Spctrophotometer. pH was monitored during the reaction using Global digital pH meter, DPH-500. Chitosan (40mg) was taken from stock solution of chitosan (2% in 1% acetic acid Milli Q water) and volume was made up to 40ml to get 1 mg/ml solution using MilliQ water. To this, 1ml of STPP 2mg/ml solution was added and allowed to react for 10 min on magnetic stirrer. After 10min, %T was measured. The step was repeated till %T became constant. %T was plotted against the amount of STPP added.

5.1.4.2 Confirmation of CS-STPP complexation using FTIR:-

FTIR was taken to confirm the formation of complex between CS and STPP. After completion of complexation of CS with STPP, the dispersion was kept for freezing at – 70^{0}C in Remi Deep Freezer for 24 hrs and then lyophilized using Heto Drywinner Lyophilizer, Denmark. FTIR of the lyophilized powder and Chitosan powder was taken using Shimadzu FTIR spectrophotometer. FTIR of Chitosan powder was overlapped with Chitosan-STPP (CS-STPP) complex and shift or absence of peaks was examined.

5.1.5. Preparation of TT Loaded Microparticles:

TT loaded microparticles were prepared by adding 1000Lf TT in chitosan solution and to this 1ml STPP was added at every 10 minutes. Different variables which thought were found to affect the entrapment of TT in to CS microparticles like viscosity of reaction medium, pH of reaction medium and rate of addition of STPP were optimized.

5.1.5.1 Optimization of experimental conditions for entrapment of TT:–

After optimizing ratio of Chitosan to STPP [1.16:1 (w:w)], other variable formulation conditions were optimized so as to entrap 1000Lf of TT per batch. Phase A was prepared by taking 2 ml of CS from stock solution. To this, TT (1000Lf) solution was added and volume was made up to 40ml. To Phase A, 1ml of 2.8 mg/ml solution of STPP was added after every 10 min. Entrapment efficiency was calculated by measuring the unentrapped TT from supernatant obtained after centrifugation using ELISA method as described in Analytical method section. %EE was calculated by using **Equation 1.**

$$\%EE = \left[\frac{Total\ amount\ of\ TT\ added - Unentrapped\ TT}{Total\ amount\ of\ TT\ added}\right] X\,100 \quad \text{.....Equation 1}$$

5.1.5.1.1 Effect of viscosity:-

Effect of viscosity of the reaction medium on entrapment was studied using following solvent compositions in which CS was added: Water, Propylene Glycol (PG), Glycerol, Water : Glycerol (50:50), Water : PG (50:50). Final volume was 50 ml (40ml CS + 10 ml STPP).

5.1.5.1.2 Volume of water:-

Volume of water was optimized by determining encapsulation efficiency in different volumes i.e. 25ml (15ml CS+10ml STPP), 50 ml (40ml CS+ 10ml STPP) and 60ml (50ml CS +10ml STPP) of the final reaction medium volume.

5.1.5.1.3 Effect of pH:-

Effect of pH on the entrapment of TT was checked at pH 4.5, 6 and 7. The pH of the reaction medium was adjusted to pH 4.5, 6 and 7, using 1% sodium carbonate solution.

5.1.5.1.4 Effect of rate of STPP addition:

Effect of rate STPP addition was checked at four levels: 1ml per min, 5ml per min, 1ml per 5min and 1ml per 10 min after mixing chitosan and TT.

5.1.5.2 Preparation of DT loaded CS microparticles:–

DT loaded CS microparticles were prepared using the optimized experimental conditions that were used for encapsulation of TT. Entrapment efficiency was calculated by measuring the unentrapped DT from supernatant obtained after centrifugation using ELISA method as described in Analytical method section. %EE was calculated using **Equation 2.**

$$\%EE = \left[\frac{Total\ amount\ of\ DT added - Unentrapped DT}{Total\ amount\ of\ DT\ added}\right] X\,100 \quad \text{.....Equation 2}$$

5.1.6. Optimization of Toxoid loading in CS microparticles:

After the experimental conditions for toxoids loading were optimized, optimization of final formulation capable of providing maximum toxoid loading was conducted. Following formula was used to prepare microparticles and to study entrapment:-

Phase A- Chitosan-40 mg,

MilliQ water-qs 50ml

Phase B- STPP-28mg,

MilliQ water-qs 10ml

5.1.6.1 Tetanus Toxoid Loading:-

Maximum TT Entrapment was studied by adding aliquots of TT solution in the range of 100Lf to 10,000 Lf into Phase A from stock solution and allowed to mix after making the volume upto 50 ml. To this, 1ml of Phase B was added dropwise after every 5 min interval. Unentrapped TT was measured using ELISA as per the method described earlier.

5.1.6.2 Diphtheria Toxoid Loading:-

DT loading optimization was done following the same procedure as TT but using aliquots of DT in the range of 100Lf to 10,000 Lf. Unentrapped DT was estimated using ELISA as per the method described earlier.

5.1.7. Optimization of the Particle size of Final Optimized Formulation:

Particle size was measured using Malvern Particle Size Analyzer, UK, Model 2000 SM. The initial formulation (Batch 1W) prepared in water was found to have large particles with mean diameter of 125.76μ with very broad particle size distribution. The particle size was reduced when the batch was prepared in Propylene Glycol instead of water (Batch 2PG) but the entrapment was very poor. (**Table 17**)

Table 17: Particle size optimization Batch 1W to 2PG

Batch No.	Process parameters
1W	Product prepared in water, Not processed
2PG	Product prepared in PG, Not processed

Therefore, the batch prepared in water was processed for particle size control using Emulsiflex C-5, Canada (E-C5) as given in the **Table 18.**

Table 18: Particle size optimization Batch 3WE to 6WEP

Batch No.	Process parameters
3WE	2cycles without pressure
4WEP	2cycles without pressure +1 cycle with 5000psi
5WEP	2cycles without pressure +2 cycles with 5000psi
6 WEP	2cycles without pressure + 4 cycles with 5000psi

For still finer size, formulation prepared in water was centrifuged, dispersed in 20ml PG and processed using Emulsiflex C-5 as shown in **Table 19**

Table 19: Particle size optimization Batch 7PGEP to 9PGEP

Batch No.	Process parameters
7PGEP	2 cycles without pressure + 2 cycles with 5000 psi
8PGEP	2cycles without pressure + 4 cycles with 5000 psi
9 PGEP	2cycles without pressure + 6 cycles with 5000 psi

For size control, the formulation prepared in water was centrifuged, dispersed in 20ml PG and processed using CAT 560 Silverson type Homogenizer as shown in **Table 20**

Table 20: Particle size optimization Batch 10PGH1.45 to 15PGH9.45

Batch No.	Process parameters
10PGH1.45	For 1min at 4500 rpm
11PGH2.90	For 2min at 9000 rpm
12PGH3.45	For 3min at 4500 rpm
13PGH5.45	For 5min at 4500 rpm
14PGH6.45	For 6min at 4500 rpm
15PGH9.45	For 9min at 4500 rpm

For a more efficient size reduction both Emulsiflex C-5 and CAT 560 Silverson type Homogenizer were used as shown in **Table 21**

Table 21: Particle size optimization Batch 16PGHE to 27DT

Batch No.	Process parameters
16PGHE	For 3min at 4500 rpm + 2 pressure cycles with 5000 psi pressure
17PGEH3	2 cycles without pressure + 4 cycles with 5000 psi pressure +3 min at 4500 rpm
18PGEH6	2 cycles without pressure + 4 cycles with 5000 psi pressure +6 min at 4500 rpm
19PGEH3	2 cycles without pressure + 2 cycles with 5000 psi pressure +3 min at 4500 rpm
20PGEH6	2 cycles without pressure + 2 cycles with 5000 psi pressure+6 min at 4500 rpm
21PGEH3	2 cycles without pressure + 4 cycles with 5000 psi pressure+ 3 min at 4500 rpm
22PGEH6	2 cycles without pressure + 4 cycles with 5000 psi pressure + 6 min at 4500 rpm
23PGEH8	2 cycles without pressure + 4 cycles with 5000 psi pressure +8 min at 4500 rpm
24PGEH9	2ml 2% SCMC + 2 cycles without pressure + 4 cycles with 5000psi cycles + 9 min at 4500 rpm
25PGEH12	2ml 2% SCMC+ 2 cycles without pressure + 4 cycles with 5000psi cycles + 12 min at 4500 rpm
26Plain	2ml 2% SCMC+ 2 cycles without pressure + 4 cycles with 5000psi cycles + 12 min at 4500 rpm
27 TT	1500Lf TT in CS, 2ml 2% SCMC+ 2 cycles without pressure + 4 cycles with 5000psi cycles + 12 min at 4500 rpm
27 DT	1500Lf DT in CS, 2ml 2% SCMC+ 2 cycles without pressure + 4 cycles with 5000psi cycles + 12 min at 4500 rpm

The final optimized procedure to give the required particle size distribution is as follows.

Phase A: Chitosan- 40 mg,

TT- 1500Lf,

MilliQ water qs- 100ml

Phase B: STPP- 28 mg,

MilliQ water qs- 20ml

Procedure- Phase A was allowed to mix at 1000 rpm on magnetic stirrer. To phase A, 1ml of phase B was added drop wise after every 5min. After completion of addition, the system was allowed to cure for 20 min under continuous stirring. The bottle was centrifuged in Remi K-70, industrial centrifuge, at 2000 rpm for 15 min and supernatant was removed carefully. The sediment was redispersed in 20 ml PG and to it 5 ml of 2%

Sodium carboxymethylcellulose was added and proper mixing was ensured. This suspension was passed through the Emulsiflex C-5 (2 cycles without pressure) to ensure proper mixing. After cooling the system for 10 minutes at 4°C it was again passed through Emulsiflex C-5 (4 cycles at 5000 psi pressure). The product obtained was kept at 4°C for 15 minuets and processed using CAT-560 homogenizer for 12 minutes at 4500 rpm in ice bath and stored in refrigerator till further use.

5.1.8. Transmission Electron Microscopy (TEM):

TEM was performed, after sonicating the 27TT and 28DT batch, using Philips CM200 TE microscopal resolution of 0.23nm under the 200kV of voltage.

5.1.9. Environmental-Scanning Electron Microscopy (E-SEM):

E-SEM of Batch 1W, 27TT and 28DT was taken using Quanta 200 FEI SE Microscope.

5.1.10. Effect of high shear rate on the conformational changes:

There was a possibility that the high shear rate used in the particle size reduction using Emulsiflex C-5 and CAT-560 Silverson type Homogenizer, may cause conformational changes or fractionation in the TT and DT structure adversely affecting its antigenicity. Hence, the toxoids were subjected to similar homogenization procedure as followed for particle size optimization and estimated using SDS-PAGE and HPLC. TT and DT (1500 Lf) was taken and volume was made up to 8ml using PG, to this 2ml 2% SCMC was added and processed using the procedure described in particle size optimization section. The sample processed were analyzed using SDS-PAGE and HPLC methods as described in Analytical Methods Section.

5.1.11. Preparation of TT/DT adsorbed vaccine for parenteral use:

Tetanus adsorbed vaccine (10Lf/ml) or Diphtheria adsorbed vaccine (25Lf/ml) for parenteral use was prepared using Tetanus toxoid Pool No. 187 containing 3840 Lf/ml) and Diptheria toxoid lot no. C6 containing 4500 Lf/ml respectively, using following procedure.

Soultion A was prepared by dissolving Aluminum Chloride(10 gm), Sodium Chloride(1.8 gm) and Sodium Acetate(1.36 gm) in 1000ml Water for Injection.

Soultion B was prepared by dissolving Trisodium Phosphate(15.6 gm) in 1000ml water for injection. Both the solutions were sterilized at 15psi, 121°C for 30 min.

Solution A (500ml) was taken and stirred under aseptic condition on magnetic stirrer using pre-sterilized magnetic bar. To this, 2.604 ml of Tetanus toxoid or 11.11 ml of Diphtheria toxoid was added and allowed to stir for 5 minutes followed by addition of 200ml of solution B. Aluminum phosphate was formed in situ which adsorbed the toxoid. The maximum adsorption occurred at pH 6.3, the pH was adjusted using 7% sodium carbonate solution. To this, 5 ml Thiomersal solution (2%) was added and allowed to stir for 12 hours and then was filled aseptically into 10ml vials for further use. *(WHO manual 77.1 Rev 1and 77. 2 Rev 1)*

5.1.12. Release study of TT and DT in 0.1 N HCl and Phosphate Buffered saline (pH 7.4):

From Batch 27 TT containing 1500Lf TT and 28DT containing 1500Lf DT, 1 ml was taken and added in 5ml of 0.1N HCl or PBS and placed in incubator shaker at 100rpm and 37.5^{0}C. Samples, 100µl, were withdrawn after 30, 60, 120 minutes and replaced by fresh 0.1N HCl or PBS. Withdrawn samples were added in 900µl of 50mM bicarbonate coating buffer (pH 9.6) and were analyzed using direct ELISA for estimation of TT and DT.

5.1.13. Bioadhesion Testing:

Bioadhesion testing was performed by measuring the force of detachment method. Layer of the formulation, 100µl, being tested was formed on the polycarbonate membrane and it was covered with another polycarbonate membrane. The force required to measure the detachment of second membrane was measured digitally in dynes.

5.1.14. Rheological studies:

Rheological properties of the Batch 27TT was studied using Brookefield Rheometer, model LV, spindle- SC4-16 and Rheocalc V 1.40 software. Geometric up-down program with Start RPM of 10, End RPM of 10, Step RPM of 20 and Corner RPM of 100 was used.

5.1.15. Sedimentation study:

From Batch 26 Plain, 27 TT and 28 DT, 10 ml final product was taken and kept in 10ml measuring cylinder, at 25^{0}C. The sedimentation rate was measured by noting the height of sediment till there was no change.

5.1.16. Redispersion Study: To check the redispersion property of the sediment formed it was inverted manually and number of inversion cycles were required to redisperse the

sediment were recorded and again kept for sedimentation and height of sediment was measured.

5.1.17. Stability Studies:

Stability of the Batch 27TT and 28 DT was studied at 4^0 and 25^0C for three months. Samples were withdrawn after every 15 days and analyzed for leached TT and DT using ELISA in the supernatant as described above.

5.1.18. In-Vivo Studies

Local and systemic immune responses were measured in 5-6 weeks old BALB/c mice weighing 15-20gms. The Ethical Committee for Laboratory Animals of The M.S. University of Baroda, Gujarat, India, approved all animal experiments.

5.1.18.1 Hyperimmunization of mice:-

Ten BALB/c mice were hyperimmunized using modified hyperimmunization protocol by administering through intraperitoneal route 10Lf/ml TT or 25Lf/ml of DT adsorbed on aluminium phosphate on 1st, 2nd, 3rd, 15th, 16th and 17th day. Food was withdrawn 6 hours before administration of the dose but water was available ad libitum. Blood was withdrawn from retro-orbital plexus on 22nd day and allowed to clot to collect sera, which was pooled and distributed in aliquots and stored at -20°C. ***(Lavelle, E.C., 1999)***

5.1.18.2 Estimation of TT and DT specific IgG in hyperimmunized mice using ELISA:-

Estimation of TT and DT specific IgG was performed as per the ELISA method described in Analytical Methods Section. Standard curve for estimation of TT specific IgG was prepared using two fold serial dilution of Equine anti-TT IgG in the range of 0.001950 to 0.000121 IU/ml. Standard curve for estimation of DT specific IgG was prepared using two fold serial dilution of Equine anti-DT IgG in the range of 0.0039 to 0.000244 IU/ml.

5.1.18.3 Systemic Immune response study:-

In-vivo study was carried out in BALB/c mice by administering encapsulated TT and DT formulation through oral route. Systemic immune response was determined by measuring the IgG level in serum and Local immune response was determined by measuring IgA level in Intestinal Lavage, Intestinal Washings and Fecal Matter extracts. Following procedures were used.

5.1.18.3.1 Dose Response Relationship study :-

Protocol 1 for Dose Response Relationship study.

	Animal Group (n=5)	Sample
Batch 27TT and 28 DT to be tested (AG= TT/DT)	1.	CS-AG – 20
	2.	CS-AG – 40
	3.	CS-AG – 60
	4.	AG – 20
	5.	AG – 40
	6.	AG – 60
	7.	AG-Ads 10Lf for TT and AG-Ads 25 Lf for DT (i.m.)
Vaccination	On 1st,2nd, 3rd, 15th, 16th and 17th day	
Sampling	After 5 hour fasting, 1000µl blood to be taken on 22nd Day	
Sample Treatment	Centrifuge blood obtained from each animal at 14000 rpm and store sera at - 20 ^{0} C, analyzed for level of IgG using ELISA.	

5.1.18.3.2 Assessment of Systemic Immune Response:-

Protocol 2 for Assessment of Systemic Immune Response

	Animal Group (n=10)	Sample
Batch 27 TT and 28 DT to be tested (AG= TT/DT)	1	CS-AG
	2	CS-plain
	3	AG-plain
Vaccination	On 1st,2nd, 3rd, 15th, 16th and 17th day	
Sampling	After 5hrs fasting 700 µl blood to be taken on 14th, 22nd and 29th day	
Sample Treatment	Centrifuge blood from each animal at 14000 rpm for 20 min and pool obtained sera of each group and store at -20^0 C, analyzed for level of IgG using ELISA.	

5.1.18.3.3 Estimation of TT and DT specific IgG in experimental mice using ELISA:

Estimation of TT and DT specific IgG was performed as per the ELISA method described in Analytical Methods Section. Standard curve for estimation of TT specific IgG was prepared using two fold serial dilution of mouse anti-TT IgG from hyperimmunized mice in the range of 0.0019 to 0.000118 IU/ml.

Standard curve for estimation of DT specific IgG was prepared using two fold serial dilution of mouse anti-DT IgG from hyperimmunized mice in the range of 0.0011 to 0.000069 IU/ml.

5.1.18.4 Assessment of Local Immune Response in BALB/c Mice:-

5.1.18.4.1 Procedure for Collection of intestinal lavage fluid:

Each mouse was kept in glass petri dish and four doses of 0.5ml of the lavage solution were administered orally at 15 min intervals. Thirty minutes after the last dose of lavage solution, the mice were administered 0.1 mg Pilocarpine intraperitoneally. Discharged intestinal contents, over the next 20 min, were collected in plastic tube containing 0.5 ml of protease inhibitor cocktail buffer and frozen at -70°C until tested. *(Grewal, H.M.S., 2000; Forrest, B.D., 1992)*

5.1.18.4.2 Procedure for Collection of faecal samples:

Five pieces (~100mg) of freshly voided faeces from each mouse were collected and incubated for 15 min in 4ml of homogenization buffer on ice, followed by mashing it with a blunt needle. The IgA containing supernatants, obtained by centrifuging the suspension for 25 min at 13000 rpm at 4°C, were stored at -20°C until tested for IgA levels. *(Grewal, H.M.S., 2000; Forrest, B.D., 1992).*

5.1.18.4.3 Procedure for Collection of Intestinal washings:

Intestinal wash was collected by flushing off small intestine with 4ml of a mixture of equal volume of PBS and 50mm EDTA containing 0.1mg/ml of soybean trypsin inhibitor. It was vortexed and centrifuged for 10 min at 1000 rpm. Supernatant (appx 3ml) was mixed with 50µl of 100mM PMSF in 95% ethanol and centrifuged at 2000 rpm for 20min at 4°C. The supernatant, 2ml, was mixed with a further 40µl of 100mM PMSF and 40µl of 1% NaN_3. After standing for 15 min, 200µl of Fetal Calf Serum was added and the fluid was stored at - 20°C until tested. *(Elson, C.O., 1984; Challacombe, S.J., 1997)*

5.1.18.4.4 Protocol 3 for assessment of Local Immune Response.

	Animal Group.(n=10)	Sample
Batch 27TT and Batch 28DT to be tested (AG= TT/DT)	1	CS-AG-40
	2	AG-40
Vaccination	On 1st,2nd, 3rd, 15th, 16th and 17th day	
Sampling	5 hour fasting is required before dosing and before taking any sample: 1. Faeces (5 no., in the morning) from each mouse on 4th,8th, 14th, 18th and 22nd day. 2. Intestinal Lavage Fluid from each mouse on 4th, 8th, 14th and 22nd day. 3. Intestinal washings on 22nd day.	
Sample Treatment	Use specific method for each sample	

5.1.18.5 Estimation TT and DT specific IgA in experimental animals using ELISA:

Estimation of TT/DT specific IgA in samples of fecal extracts, intestinal lavage solution and Intestinal washings was done using ELISA as described in Analytical Methods Section.

5.2 Results and Discussion

5.2.1. Characterization of Chitosan (CS):

5.2.1.1 pH of solution:-

The pH of 2mg/ml Chitosan solution in acetic acid was found to be 4.6.

5.2.1.2 Viscosity of solution:-

Viscocity of 1% solution in 1% acetic acid as determined using Brookefield Rheometer was 132cps. Chitosan is a copolymer of β-(1-4)-linked 2-aceatamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose. Recently Berth et al determined the radius of gyration of Chitosan *(Berth, G., 2000)* and established the relationship between the molecular weight and radius of gyration of Chitosan in aqueous solution, and further revealed that Chitosan behaved like a guassian coil instead of the worm like chain model found in common polyelectrolytes. At low pH, the primary amine along the backbone of Chitosan is fully protonated, which may be responsible for higher viscosity.

5.2.1.3 Fourier transform Infra-Red spectroscopy (FTIR):-

FTIR of chitosan powder showed salient peaks which were attributed to different functional groups. (**Figure 11 & Table 22**)

Table 22: FTIR peaks of chitosan powder

Peak (cm^{-1})	Functional group
3408 & 3101.3	- OH stretch
2993.3 & 2851.5	Aliphatic C-H stretching
1215 & 1031	Free amino group
1384	-C-O stretch of primary -OH group

5.2.2. Solution pH of STPP:- pH of 2mg/ml solution in Milli Q was found to be 9.7.

5.2.3. Characterization of Tetanus and Diphtheria Toxoid:

5.2.3.1 Limes flocculation units of TT and DT:-

Limes flocculation units of TT and DT were determined using Flocculation Test. The tube in which flocculation is observed first is the one in which concentration of antigen and antibody is equivalent. The concentration of antibody of this tube (IU/ml) is assigned as concentration of antigen (Lf/ml). *(Lyng, J., 1987).* Tube 4 flocculated first

followed by Tube 5 and Tube 3 in case of TT, indicating that Tube 4 contained equivalent amount of TT and anti-TT IgG. Same pattern of flocculation was observed in case of DT indicating that Tube 4 contained the equivalent amount of DT and anti-DT IgG although the dilution factor was different as shown in **Table 23 and 24.** After considering the dilution factor, the Lf was found to be 3840Lf/ml for TT and 4500 Lf/ml for DT. The observed values of Limes flocculation units of toxoids were same as that of the labeled claims, indicating accuracy of the method.

Table 23: Flocculation test for TT

Equine Anti-Tetanus IgG: 500 IU/ml

Dilution = 1:5

Tetanus toxoid dilution = 1:40

Tube No.	1	2	3	4	5	6
Normal saline (ml)	0.10	0.08	0.06	0.04	0.02	0.00
Anti-tetanus IgG (ml)	0.90	0.92	0.94	0.96	0.98	1.00
Tetanus Toxoid (ml)	1.00	1.00	1.00	1.00	1.00	1.00
Flocculation sequence			F3	F1	F2	
Toxoid Lf/ml	**3840 Lf/ml**					

Table 24: Flocculation test for DT

Equine Anti-Diphtheria IgG: 1200 IU/ml

Dilution = 1:12

Diphtheria toxoid dilution = 1:50

Tube No.	1	2	3	4	5	6
Normal saline (ml)	0.25	0.20	0.15	0.10	0.05	0.00
Anti-Diphtheria IgG (ml)	0.75	0.80	0.85	0.90	0.95	1.00
Diphtheria Toxoid (ml)	1.00	1.00	1.00	1.00	1.00	1.00
Flocculation sequence			F3	F1	F2	
Toxoid Lf/ml	**4500 Lf/ml**					

5.2.3.2 Determination of Protein Content by DC Protein Assay:-

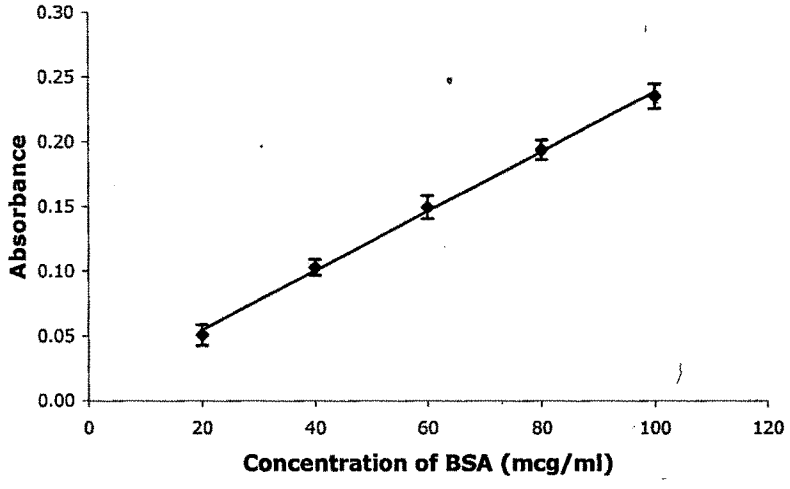
DC Protein assay is a modified Lowry Method. The assay is based upon the reaction of protein with an alkaline copper tartarate and Folin reagent, which produces characteristic blue color, which can be measured at 750 nm. ***(Lowry, O.H., 1951; Peterson, G.I., 1979).*** Calibration plot of BSA concentration against Absorbance at 750

nm was found to be linear in the range of 20 µg/ml to 100 µg/ml. **(Table 25 & Figure 26).**

Table 25: Standard curve for DC Protein assay

BSA (µg/ml)	Absorbance at 750 nm (Mean± S.D.)
20	0.0505 ± 0.008
40	0.1028 ± 0.006
60	0.1492 ± 0.008
80	0.1933 ± 0.007
100	0.2345 ± 0.009

Figure 26: Standard curve for the DC protein assay.



DC Protein assay regression equation:

$$y = 0.0023x + 0.0085; R^2 = 0.9979 \ldots\ldots \textbf{Equation 3}$$

Equation 3 was used for estimation of protein content, which was found to be 22.1 mg/ml for TT and 15.52 mg/ml for DT. Protein 'N' was calculated from total protein content which was found to be 3.536 mg/ml for TT and 2.483 mg/ml for DT.

5.2.3.3 Molecular weight determination:

5.2.3.3.1 Molecular weight determination Using SDS-PAGE:-

SDS-PAGE is a highly sensitive electrophoretic technique in which proteins are analyzed in denaturing conditions. Sodium Dodecyl Sulphate (SDS) binds and denatures proteins, giving them a negative charge. When such proteins move through the polyacrylamide gel of specific concentration under an electric field, separation occurs purely on the basis of molecular weight. *(Wilson, K., 2000, Lavelle, E.C., 1999)* The molecular weight of the toxoids obtained by comparing the Rf value of the sample and molecular weight markers using Quantity One™ software of Bio Rad Gel Doc system was found to be in between 160-170 kDa for TT and 60-65 kDa for DT. **(Figure 27 & 28)**

Figure 27: SDS-PAGE of TT (M- Molecular weight marker & 1 to 7- samples)

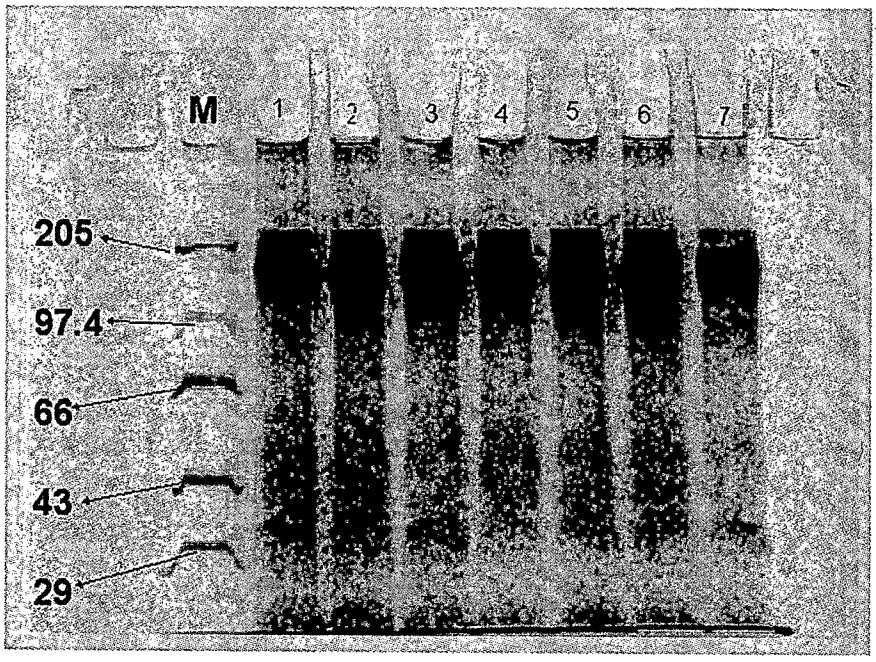
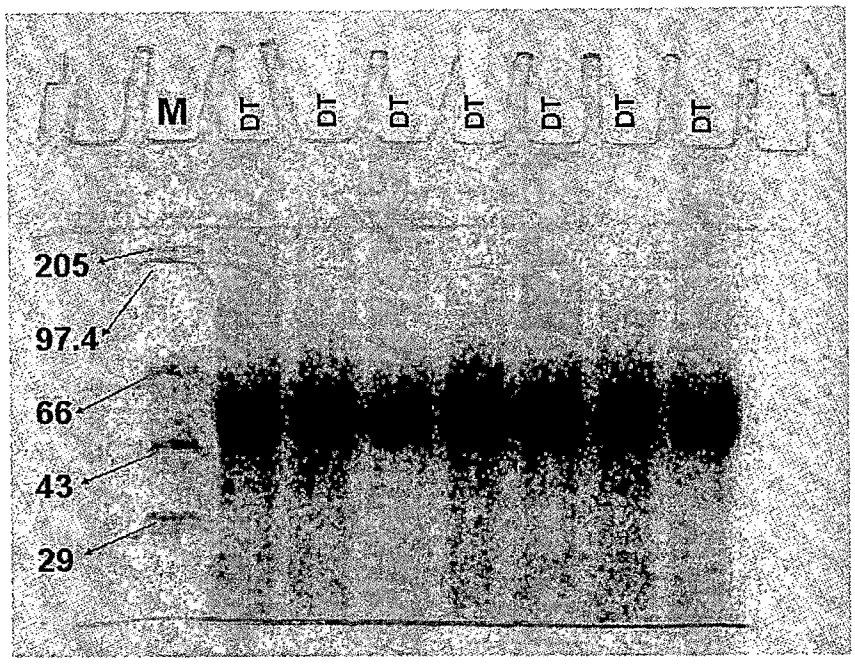


Figure 28 : SDS-PAGE of DT (M- Molecular weight marker & DT samples)



5.2.3.3.2 Molecular weight determination using High Performance Liquid Chromatography:-

HPLC is a highly sensitive and accurate method for the determination of purity as well as molecular weight of a protein. *(Federici M.M., 1994).* The molecular weight of the toxoids was found by comparing the retention time of the molecular weight markers. From the data, **(Figure 29 & 30)** TT had a molecular weight of ~160-165 kDa with retention time of 7.24 minutes and DT had molecular weight of ~60-65 kDa with retention time of 8.12 minutes.

However, Molecular weights of TT and DT were slightly higher as compared to the reported molecular weight of the toxins, which are 150 kDa for tetanus toxin and 58kDa for Diphtheria toxin. This shift may be due to toxoidation using formaldehyde, which leads to crosslinking between molecules of toxin as well as crosslinking with proteins in the culture medium. *(Rappuoli, R., 1994).*

It is worth noting that the values of molecular weight obtained by both methods, SDS-PAGE and HPLC, were almost similar, indicating that both methods complement each other.

Figure 29: HPLC chromatogram of TT

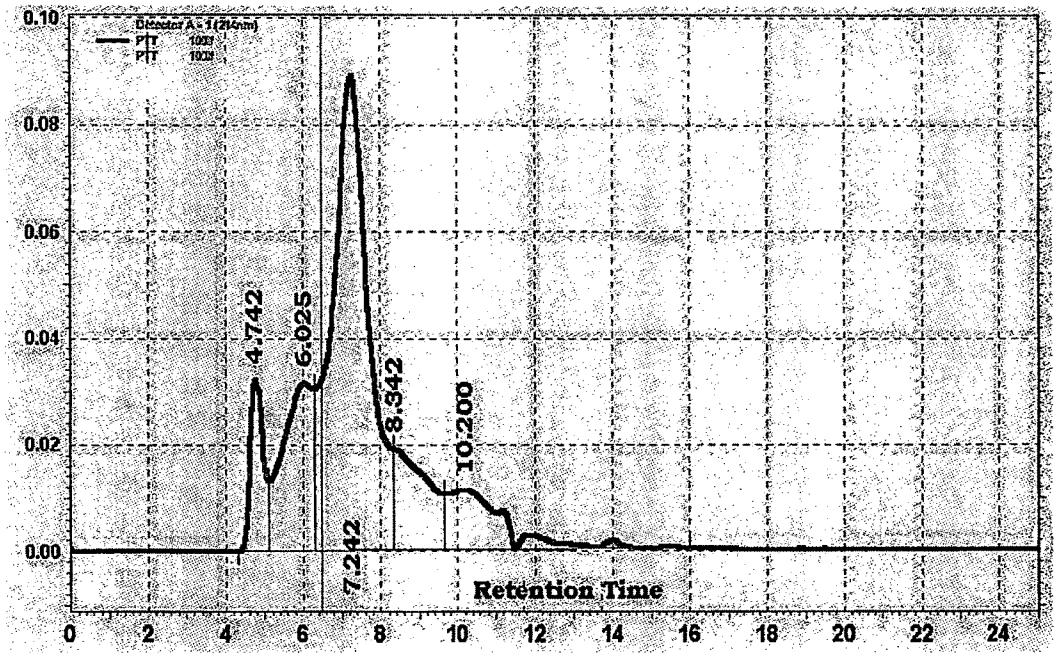
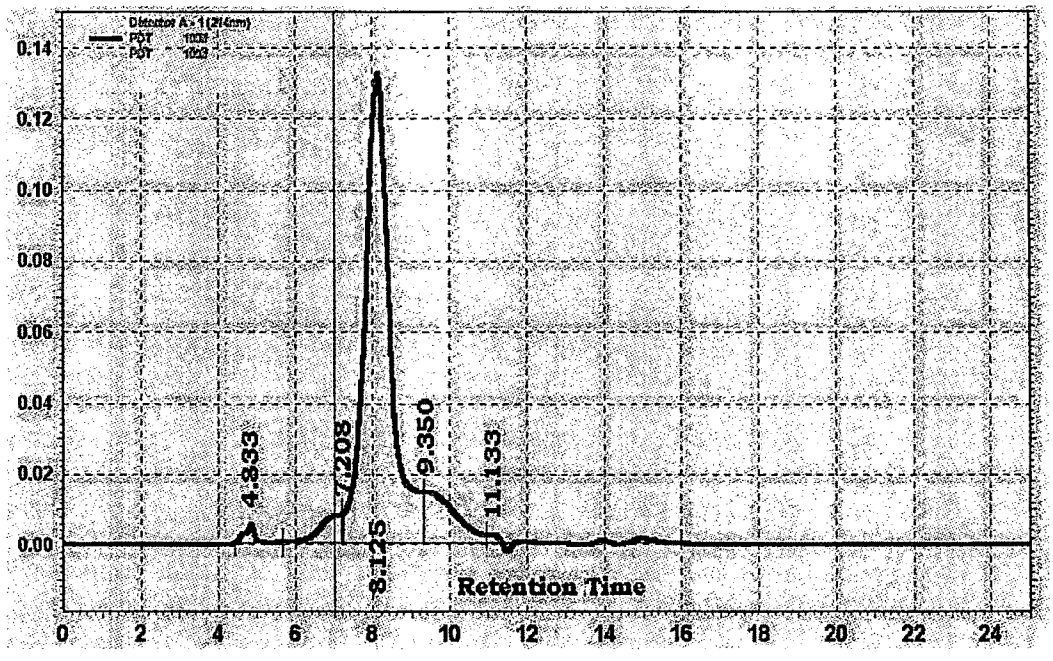


Figure 30: HPLC chromatogram of DT



5.2.3.4 Quantitative estimation of TT and DT:

5.2.3.4.1 Quantitative estimation of TT and DT using HPLC:

The relationship between the toxoid concentration and the Area Under Curve of the main peak can be used to obtain linear relationship, which was used to estimate the unknown concentration of TT and DT.

5.2.3.4.1.1 Quantitative estimation of TT:-

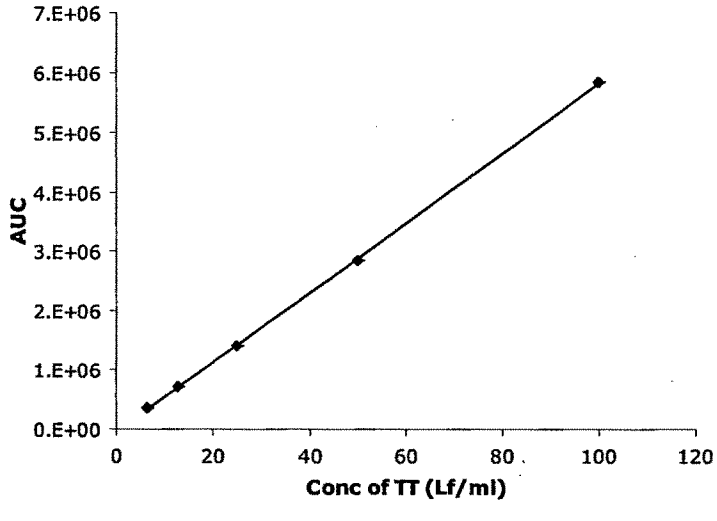
Linear relationship was obtained when Area Under the Curve (AUC) at Rt 7.24 min was plotted against the respective concentration given by Regression **Equation 4. (Table 26 and Figure 31)**

$$y = 58475x - 32101;\ R^2 = 0.9999 \ldots\ldots \textbf{Equation 4}$$

Table 26: Standard curve for estimation of TT using HPLC

Concentration (Lf/ml)	AUC ± S.D.
6.25	349874 ± 323.147
12.50	711844 ± 212.132
25.00	1416218 ± 174.655
50.00	2858291 ± 784.885
100.00	5832834 ± 516.188

Figure 31: Standard curve for estimation of TT using HPLC



5.2.3.4.1.2 Quantitative estimation of DT:-

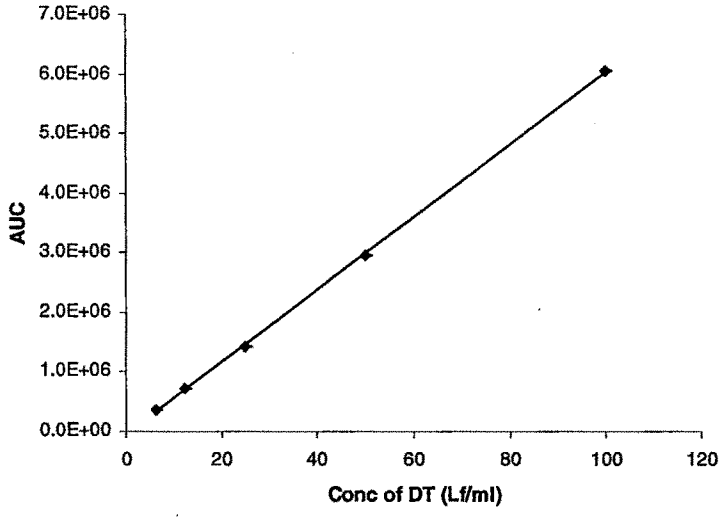
Linear relationship was obtained when Area Under the Curve (AUC) at Rt 8.11 min was plotted against the respective concentration given by Regression **Equation 5. (Table 27 and Figure 32)**

$$y = 60761x - 54514;\ R2 = 0.9998\ldots.\ \textbf{Equation 5}$$

Table 27: Standard curve for estimation of DT using HPLC

Concentration (Lf/ml)	AUC ± S.D.
6.25	357876 ± 77.781
12.50	714099 ± 71.417
25.00	1431796 ± 63.639
50.00	2955469 ± 657.609
100.00	6040595 ± 313.955

Figure 32 : Standard curve for estimation of DT using HPLC



The HPLC chromatogram of particular toxoid can also be for Qualitative purpose as shifts in the chromatogram gives information regarding aggregation of the Toxoid leading to appearance of high molecular weight peak before the main peak or degradation of the Toxoid leading to appearance of low molecular weight peaks after the main peak.

5.2.3.4.2 Quantitative estimation of TT and DT using ELISA:

Though HPLC is a highly sophisticated and sensitive technique for the quantitative estimation of TT and DT, it is not cost effective mainly due to the high cost Instrument, Running costs and cost of Protein Pack column. Moreover, lower limit of detection using HPLC is 6.5 Lf, which is also limiting factor when the unknown concentration is very low. Therefore ELISA was developed for the quantitative estimation of TT and DT, which is not only a highly specific and sensitive technique but also gives information about the antigenicity of the toxoids as it utilizes toxoid-specific antibodies.

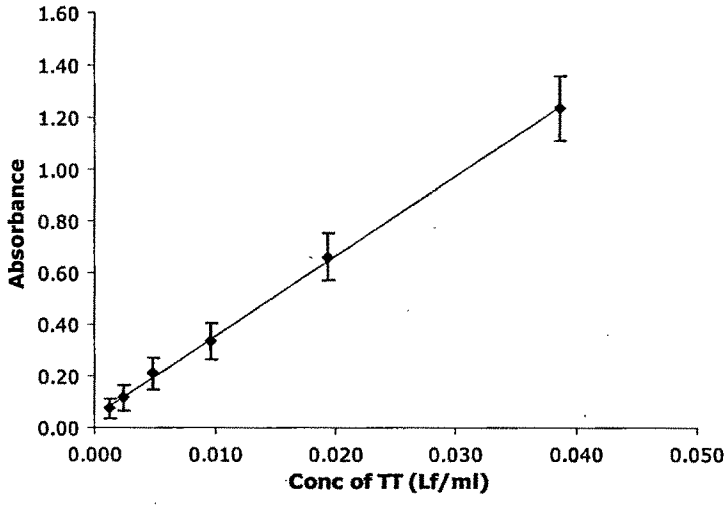
5.2.3.4.2.1 Quantitative estimation of TT:- The standard curve for TT by ELISA was obtained in the range of 0.0012 to 0.0386 Lf/ml **(Table 28 and Figure 33),** unknown concentration was determined using regression **Equation 6.**

$$y = 30.993x + 0.0462,\ R^2 = 0.9993 \text{.....} \textbf{Equation 6}$$

Table 28: Standard curve for TT using ELISA

Concentration of TT (Lf/ml)	Abs ± S.D.
0.001207	0.074 ± 0.0382
0.002414	0.116 ± 0.0502
0.004828	0.209 ± 0.0636
0.009656	0.338 ± 0.0702
0.019312	0.661 ± 0.0912
0.038624	1.236 ± 0.1230

Figure 33 : Standard curve for TT using ELISA



5.2.3.4.2.2 Quantitative estimation of DT:-

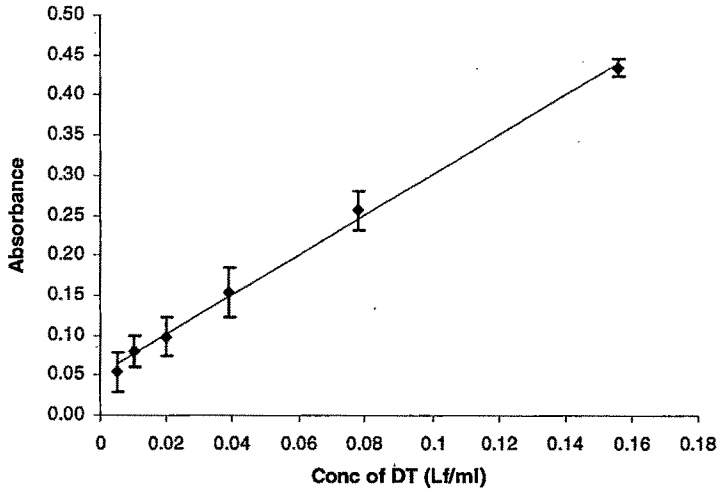
The standard curve for DT by ELISA was obtained in the range of 0.005 to 0.156 Lf/ml **(Table 29 and Figure 34)**, unknown concentration was determined using regression **Equation 7.**

$$y = 2.4908x + 0.0513, R^2 = 0.9973 \ldots \textbf{Equation 7}$$

Table 29: Standard curve for DT using ELISA

Concentration of DT (Lf/ml)	Abs ±S.D.
0.005	0.054 ± 0.024
0.010	0.080 ± 0.019
0.020	0.098 ± 0.024
0.039	0.153 ± 0.031
0.078	0.256 ± 0.024
0.156	0.434 ± 0.010

Figure 34: Standard curve for DT using ELISA



5.2.3.5 Isoelectric point (pI) of TT and DT:

Isoelectric focusing (IEF) makes use of the electrical charge properties of molecules to focus them in defined zones in the separation medium. There is specific pH for every protein at which the net charge it carries is zero. This isoelectric pH value, termed as isoelectric point, or pI, is a characteristic physicochemical property of every protein. *(Garfin, D.E., 2000; Mosher, R.A., 1992; Wilkins, M.R., 1997)* and is also important to understand the ionization properties of a protein. The pI values of TT and DT can be very instrumental in understanding the ionic interaction with chitosan during the encapsulation as well as release studies. The pI was calculated by comparing the Rf of the sample band with the Rf of pI markers using Qunatity One ™ software and found to be in between 4.8-5.0 for TT and 4.65– 4.8 for DT **(Figure 35 & 36)** *(Garfin D. E., 2001).* The pI value was found to be nearly same for both the toxoids, indicating that the reaction medium pH requirements will be same for both the systems.

Figure 35: Isoelectric Focusing of TT

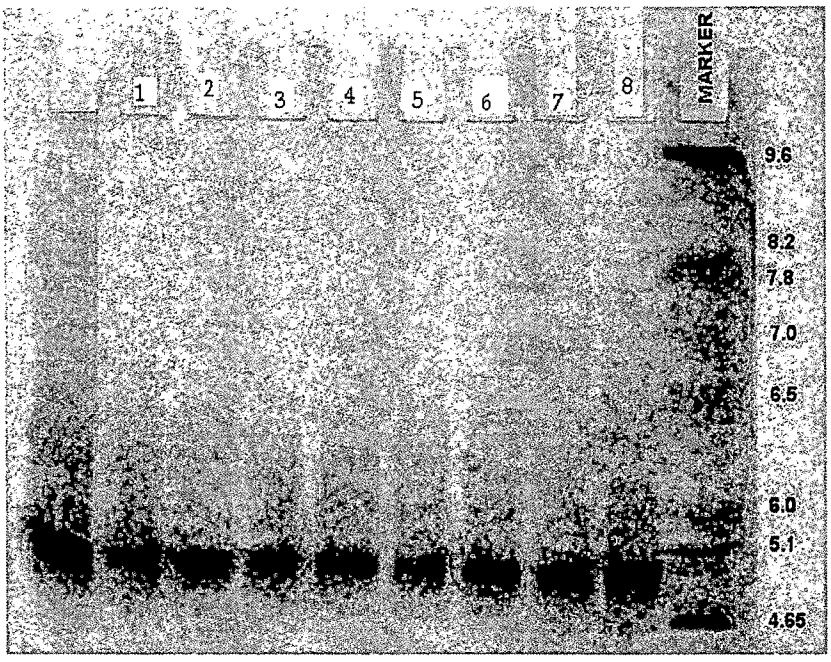
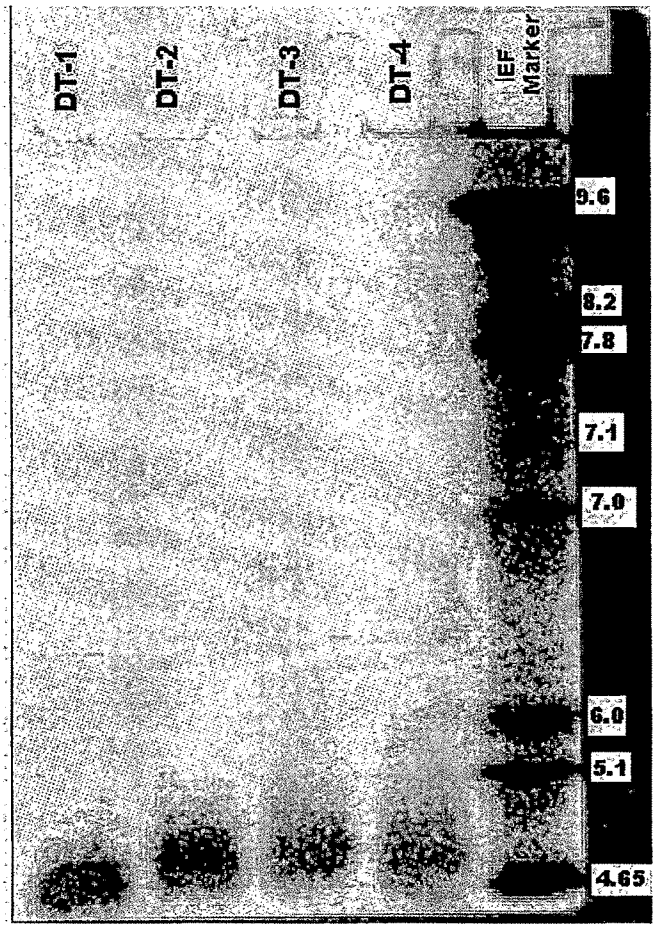


Figure 36: Isoelectric Focusing of DT



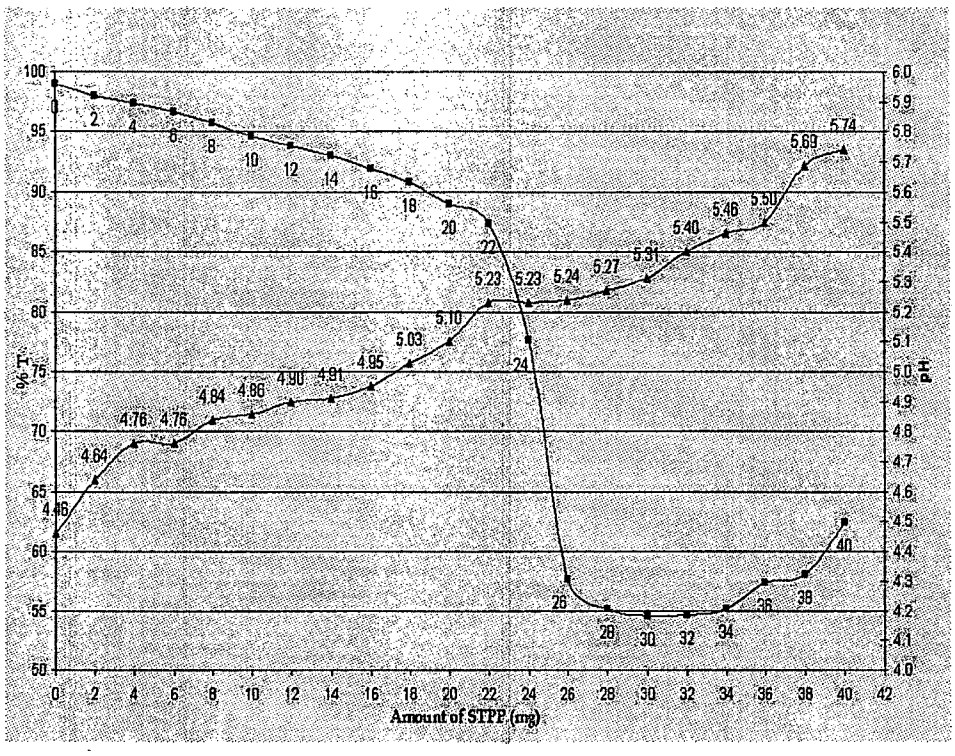
5.2.4. Preparation of microparticles:

5.2.4.1 Optimization of Amount of STPP to Crosslink CS:-

Ionic crosslinker STPP was also found to be compatible with TT in the preformulation studies, therefore it was used for crosslinking of CS. Reactions of chitosan with negatively charged components can be demonstrated by IR spectra *(Mi, F.L, 1999)*, turbidimetric titration *(Shu, X.Z.,2001)* or viscometry *(Brack, H.P.,1998)*. Chitosan microparticles can be formed by the addition of either covalent or ionic crosslinker. Ionic crosslinking is a simple and mild procedure as compared to covalent crosslinking. *(Xu, Y., 2003; Berger, J., 2004)*.

The crosslinking reaction can be monitored by pH measurement as addition of tripolyphosphate leads to the release of OH^- ions. To study the required amount of STPP for complete crosslinking of chitosan, change in %T was measured as a function of increased amount of STPP. %T was plotted against the amount of STPP added as shown in **Figure 37.**

Figure 37: Effect of addition of STPP on pH and %T of the CS.



The plot clearly shows that the %T decreased with addition of STPP. The rate of decrease of %T was slow and steady till 22mg of STPP was added. The rate of decrease in %T was

very fast after 22 mg up till 28mg of STPP was added, after which %T remained constant before increasing after 34 mg of STPP. The %T decreases as the free protonated amino groups are crosslinked by phosphate groups of STPP, decreasing solubility of chitosan. The reduction in %T with increasing concentration of STPP can be attributed to the crosslinking of the protonated amino groups of chitosan by phosphate ions of STPP. The increase in %T after complete crosslinking is may be due to the dilution of the system.

The amount of STPP required to crosslink 40 mg of chitosan was calculated from the point at which %T remained constant and was found to be 28mg.

The pH of the system during this phase was found to be 5.2 to 5.4. The pH of the system increased with the addition of STPP, remained constant in between and again increased in last phase. The pH was below the isoelectric point of chitosan (pH 6.5) indicating that the decrease in %T was only due to ionic interaction of the protonated amino groups of chitosan and phosphate groups of the STPP and not due to the coacervation-phase separation reaction.

5.2.4.2 Fourier Transform Infra Red Spectroscopy of CS and CS-STPP complex:-

FTIR spectroscopy was used to prove the formation of ionic complex between CS and STPP. ***(Mi, F.L, 1999; Brack, H.P., 1998)*** The FTIR spectra of lyophilized CS microparticles crosslinked using STPP was overlapped with that of plain CS powder as shown in **Figure 38.** A band at 3408 cm^{-1} has been attributed to –OH group stretching vibration in Chitosan matrix. In chitosan microparticles, a shift from 3408 to 3298 cm^{-1} was observed, and the peak of 3298 cm^{-1} had widened, which may be due to increased hydrogen bonding. Same observations were reported by **Yu, J.H. (1999)** for formation of chitosan films. In CS-STPP complex, instead of the shoulder peak of 1650 cm^{-1}, a new sharp peak at 1631 cm^{-1} was observed. The 1600 cm^{-1} peak of –NH2 bending vibration observed in chitosan powder shifted to 1535 cm^{-1} in CS-STPP complex, which may be attributed to the tripolyphosphate group of STPP linking with amino group of chitosan. ***Knaul*** observed similar results in the study of Chitosan film treated with phosphate (NaH_2PO_4), and attributed it to the linkage between phosphate and amino group ***(Knaul, J.Z., 1999)***. Hence, the FTIR studies indicate that the ionic interaction between phosphate groups of STPP and amino groups of CS were responsible for formation of complex between STPP and CS. These observations are in line with results of FTIR study reported by ***Xu,Y. (2003)*** for interaction between CS-STPP for formation of CS nanoparticles.

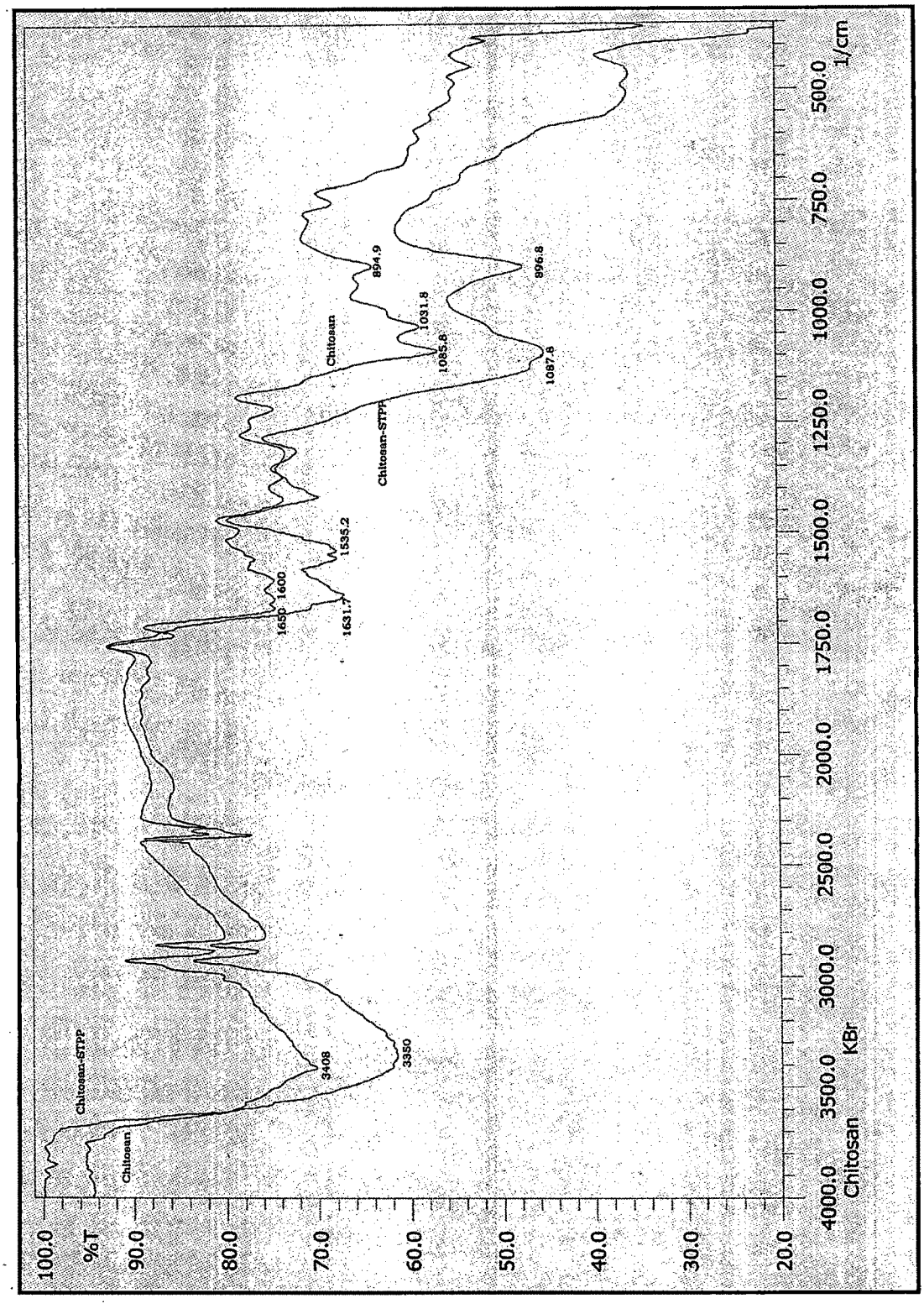


Figure 38: FTIR of Chitosan and Chitosan-STPP complex.

5.2.5. Preparation of TT loaded microparticles:

5.2.5.1 ELISA for unentrapped TT :-

Entrapment efficiency was calculated by measuring the unentrapped TT from supernatant obtained after centrifugation using ELISA method as described in Analytical method section. Different reported colorimetric methods for calculating %EE like Lowry *(Lowry, O.H., 1951; Peterson, G.I., 1979; Coppi, G., 2001)*, Bicinchoninic acid method *(Wiechelman, K.J., 1988; Wilson, K., 2000; Spiers, I.D., 2000; Alonso, M.J. 1994)*, Bradford method *(Wilson, K., 2000)* and Ninhydrin method *(Brewer, J.M., 1995)* were tried. But in all cases, there was interference of chitosan as it also showed color formation which absorbed in the same range as the vaccine. Also, absorbance of the blank was variable, therefore we could not use any of these methods. Hence, modified ELISA method was used for the estimation of TT, which is more specific, reproducible, sensitive and accurate.

5.2.5.2 Optimization of Experimental conditions

Various experimental conditions were optimized to achieve maximum TT loading and desirable product characteristics

5.2.5.2.1 Viscosity of the reaction medium:

Viscosity of the reaction medium was changed by different solvent compositions and its effect on entrapment of TT was studied.

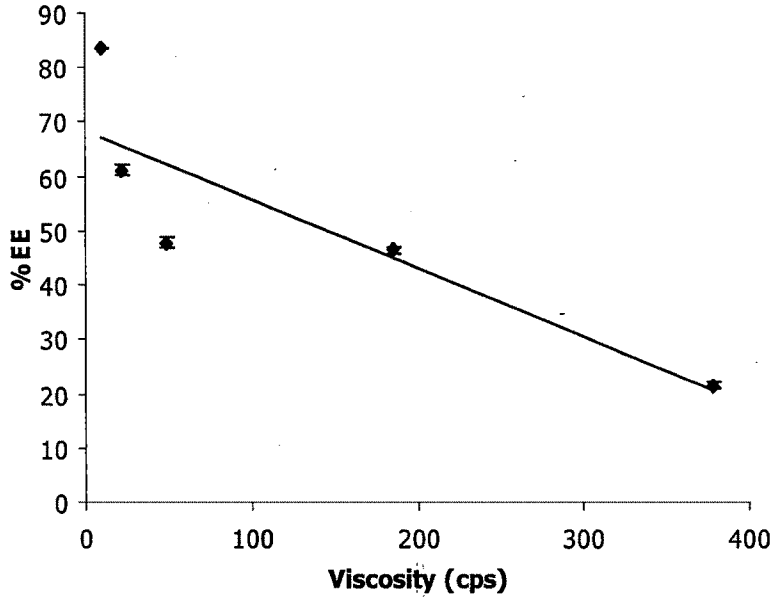
The results showed that entrapment efficiency decreased with increase in viscosity of the reaction medium **(Table 30 and Figure 39)**. The relationship was found to linear and given by the regression **Equation 7**

$$y = -0.1265x + 68.344; R^2 = 0.7573 \ldots\ldots \textbf{Equation 7.}$$

Table 30: Effect of viscosity of reaction medium on the entrapment of TT

Solvent composition	Viscosity (cps)	%EE± S.D.
Glycerol	377.9	21.54 ± 0.72
Water: Glycerol (50:50)	185.0	46.34 ± 0.61
Propylene glycol	48.00	47.81 ± 0.90
Water: PG (50:50)	22.50	61.20 ± 0.87
Water	9.600	83.50 ± 0.12

Figure 39: Effect of viscosity of reaction medium on the entrapment of TT



This may be due to Reduction in interaction between the CS and STPP due to increased viscosity, leading to low entrapment and also the extended uncoiled conformation ***(Liu, W.G, 2002)*** of chitosan may be hindered due to high viscosity reducing the interaction with TT and STPP due to steric hindrances. The entrapment was found to be maximum in water as compared to the other solvent compositions. Therefore, water was selected as the reaction medium.

5.2.5.2.2 Volume of the water:

Effect of volume of water as a reaction medium was studied from 25ml to 60ml. The entrapment was found to be nearly 100% in 60ml volume. This can be attributed to the extended conformation of chitosan due to dilution effect at higher volumes enabling maximum interaction between CS-TT and CS-TT-STPP. **(Table 31)**

5.2.5.2.3 pH of reaction medium:

Among the formulation variables that could affect the chitosan microparticles, pH appears to be the one of important parameters because pH is known to influence the amount of protein incorporated into polymer nano- and microparticles. *(Calvo, P.,1997; Elgersma, A.V.,1990; Barichello, J.M.,1999.)* It also affects chitosan conformation *(Roberts, G.A.F., 1992)* and its reaction with tripolyphosphate anions *(Mi, F.L, 1999)* required for preparation of chitosan microparticles. At the same time, pH also has significant effects on the chemical and conformational changes and stability of proteins *(Brange, J., 1992)* and would govern its interaction with STPP and chitosan during microparticle formation **(Ma,Z.,2002)**. Hence, the effect of pH on the entrapment of TT was checked. The entrapment decreased as the pH shifted towards neutral pH. **(Table 31)** This observation strengthens the importance of maintaining the reaction medium pH below pKa value of CS which is 6.5. As the pH approaches the pKa value, CS will precipitate due to the reduced solubility rather than reduced availability of the ionic amino groups due to ionic interaction with STPP. Due to this, the ionic interaction between the CS and STPP will remain incomplete, thus giving low entrapment. The pH of the reaction medium is also important from TT / DT - CS interaction point of view. The isoelectric point (pI) of TT and DT is in between 4.8-5.0 and 4.65- 4.8, respectively. Below pI value, the toxoid will be negatively charged, giving rise to maximum ionic interaction with positively charged CS. Also, proteins can adsorb very efficiently onto polymers at pH around their isoelectric point (pI) because of the minimization of electrostatic repulsion, increased conformational stability and smaller specific surface area *(Coppi, G., 2002)* during the encapsulation process. At the same time, the presence of zwitterionic toxoid molecules in the globular state can favor their hydrophobic association with chitosan along with H-bonding as shown in *in-silico* molecular modeling study. pH 4.5 was chosen as the initial pH of the reaction. As the initial pH of the CS solution is 4.5 and pH, after addition of required STPP was 5.3, which is below the pKa of CS and just above the pI of toxoids, the reaction medium was used as such without adjusting the initial pH.

5.2.5.2.4 Rate of STPP addition:

Rate of STPP addition was checked as the change in pH is directly proportional to rate of addition of STPP. It was observed that %EE increased as rate of addition decreased **(Table 31)**. Faster the rate of addition of STPP and lesser will be the interaction period

between CS-TT or CS-DT and CS-TT-STPP or CS-DT-STPP and lower will be the entrapment. Hence, a slow addition rate of 1ml per 5min was optimized as it gave 99% EE.

Table 31: Effect of Volume of water, pH of reaction medium and rate of STPP addition on %EE of TT

Variable	%EE ± S.D.
Volume of water (ml)	
25	61.70 ± 0.92
50	85.20 ± 0.63
60	99.10 ± 0.05
pH	
4.5	99.20 ± 0.98
6	83.10 ± 0.12
7	76.80 ± 1.02
Rate of addition of STPP	
5ml/min	85.07 ± 0.26
1ml/min	71.20 ± 0.13
1ml/5min	99.10 ± 0.05
1ml/10min	99.24 ± 0.12

5.2.6. Preparation of DT loaded microparticles:

Experimental conditions optimized for TT were used for the preparation of the DT loaded microparticles. Unentrapped DT was estimated using DT specific ELISA as described in Experimental Method and Quantitative Estimation of DT Section.

%EE was determined using **Equation 2.**

5.2.7. Optimization of Toxoid loading in CS microparticles:

Maximum possible entrapment of TT and DT in the CS microparticles was studied by loading the vaccines up to 10,000Lf as per the formula given in experimental section. A very high 97-99% entrapment was found for all toxoid loadings up to 10,000Lf/batch for TT and DT. **(Table 32 and 33)**

The high entrapment is probably due to very strong ionic interaction between the TT/ DT and CS at acidic pH. Capable of attracting several cationic species, the STPP could function as a crosslinker that can bind the positively charged CS-TT/CS-DT intermediate into an electrostatically stabilized CS-STPP-TT or CS-STPP-DT complex. ***(Ma, Z., 2002).*** The complex formed is an ionic and not a covalent complex. Moreover, this had not changed the antigenicity of the TT or DT as we could detect TT and DT using ELISA. These results are inline with results reported by ***van der Lubben (2003)*** who reported CS-DT microparticle formation by adsorption method and determination of %EE using ELISA. This proves the very excellent protein loading capacity of chitosan microparticles, which can be advantageous in administering large doses of toxoids / vaccines in a small volume like in case of polio drops.

However, as the amount of entrapped toxoid increased, aggregation of the microparticles also increased. This aggregation may be due to surface adsorption of toxoids rather than complete encapsulation. This aggregated system showed very high settling rate and broad particle size distribution. Therefore, batch containing 1500Lf was taken for final studies in both cases as these batches showed least aggregation and 99.52 and 98.25% entrapment for TT & DT, respectively.

Table 32: Loading of Tetanus Toxoid in CS microparticles

TT (Lf/batch)	% EE ± S.D.
500	99.15 ± 0.08
1000	98.57 ± 0.12
1500	**99.52 ± 0.17**
2500	98.78 ± 0.53
3000	99.49 ± 0.04
3500	98.87 ± 0.08
4500	98.14 ± 0.29
5500	97.09 ± 0.73
7000	98.84 ± 0.39
8500	99.04 ± 0.03
10,000	98.05 ± 0.51

Table 33: Loading of Diphtheria Toxoid in CS microparticles

DT (Lf/batch)	% EE ± S.D.
500	98.26 ± 0.59
1000	97.99 ± 0.68
1500	**98.25 ± 0.76**
2500	98.63 ± 0.75
3000	98.21 ± 0.16
3500	98.75 ± 0.34
4500	98.91 ± 0.38
5500	97.93 ± 0.41
7000	97.51 ± 0.53
8500	98.81 ± 0.65
10,000	98.91 ± 0.87

5.2.7.1 Collaboration of ELISA with HPLC:

Loading of the toxoids was also confirmed using HPLC analysis of the supernatant. HPLC chromatogram of the plain batch containing neither TT nor DT is shown in **Figure 40** Main peaks are observed at Rt of 9.19 min and 11.31min. **Figure 41** shows the overlapped HPLC chromatogram of CS plain and 40 Lf TT standard, showing that the peak of CS does not overlap the peak of the toxoid. **Figure 42** shows the chromatogram of supernatant of batch containing 8500Lf TT showing that there is no peak at 7.25 min

(Rt of TT) indicating that all the TT is entrapped within the microparticles. This results can be confirmed by ELISA results showing 99.04 ±0.0 3% EE.

Figure 40: HPLC chromatogram of Plain Chitosan batch supernatant

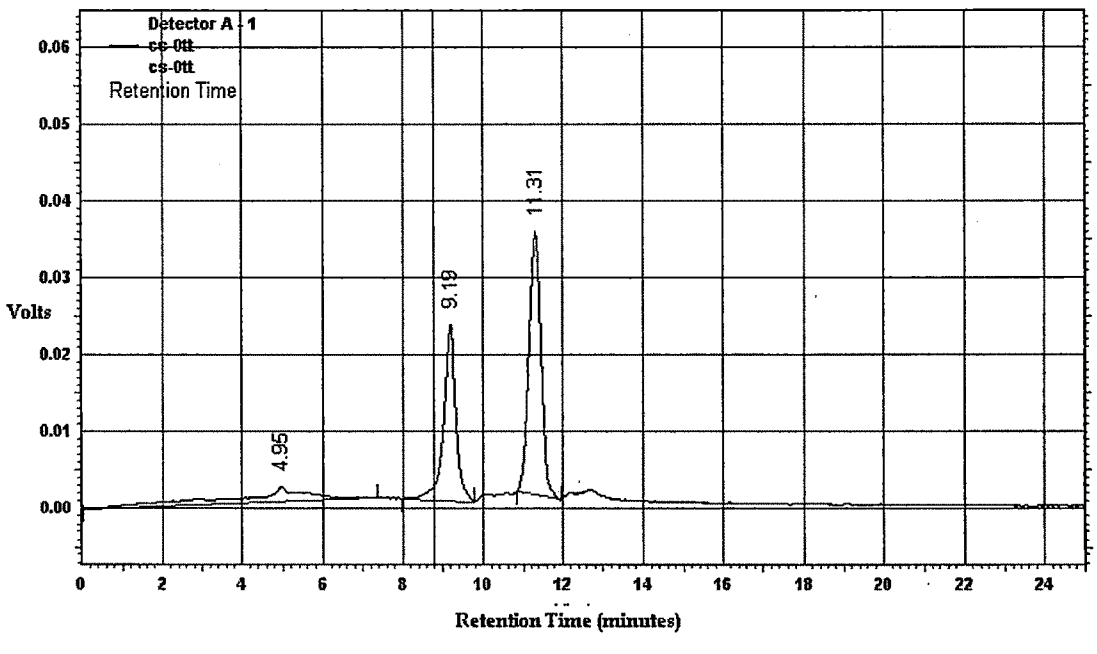


Figure 41: HPLC chromatogram of CS and plain 40 Lf TT

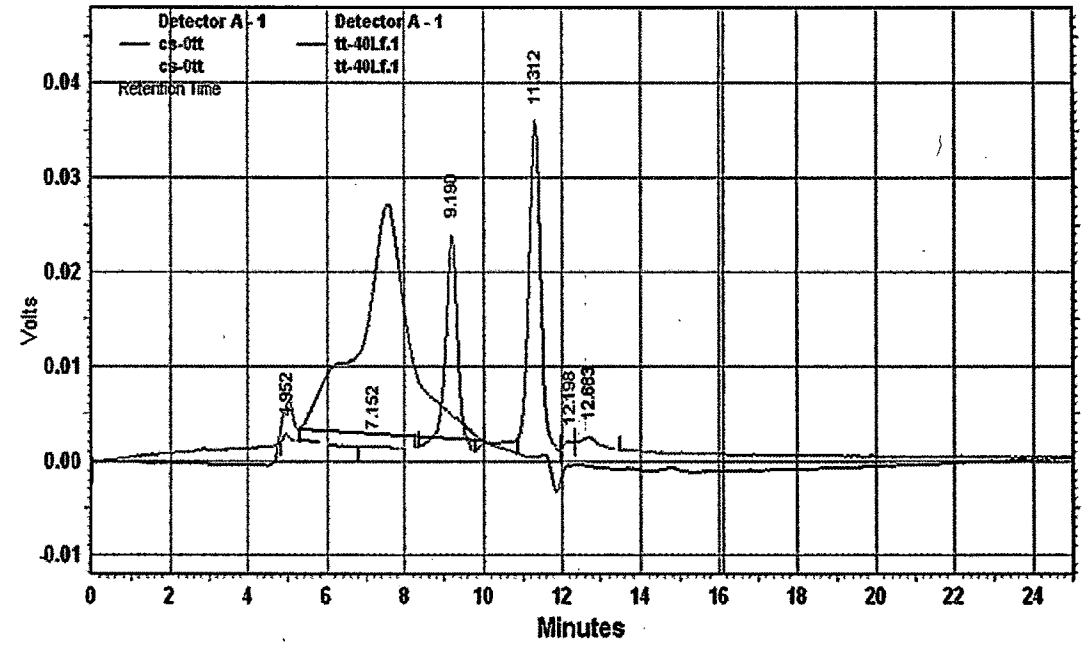


Figure 42: Chromatogram of supernatant of CS-TT batch containing 8500Lf TT

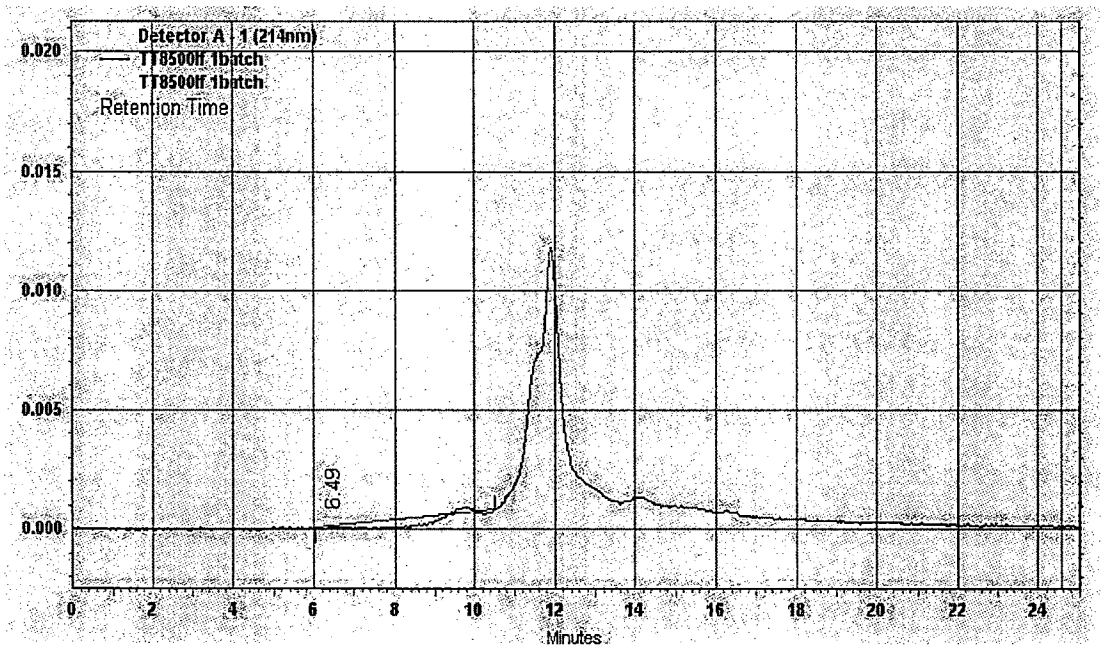


Figure 43: Chromatogram of Std DT and supernatant of batches for determination of unentrapped DT

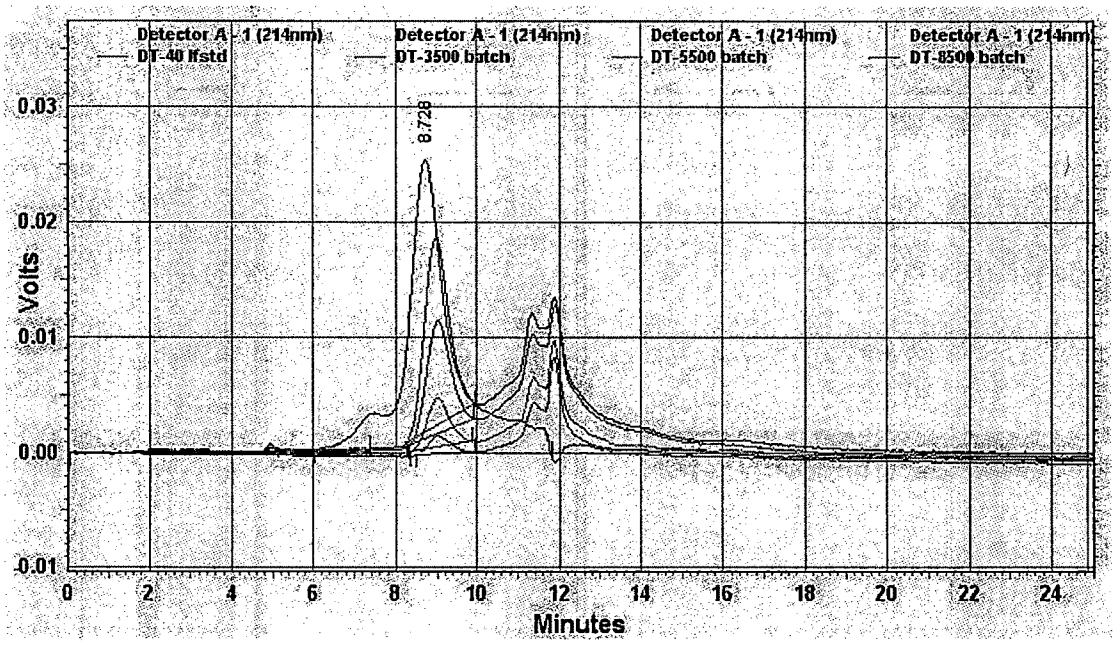


Figure 43 Shows the overlap of chromatogram of standard DT (40 Lf) and chromatogram of supernatant of CS-DT batches 3500, 5500, 8500 Lf. There was change in shape of DT peak. Supernatant of the batches show presence of some amount of DT and additional

peak in between Rt of 11 to 12 min. This peak was absent in chromatogram of Std 40 Lf DT, therefore, the additional peak may be attributed to some low molecular weight chemical used in the preparation of the microparticles. These batches showed %EE of ~98% when analyzed using ELISA. Thus, results of ELISA are confirmed by HPLC, indicating that high amount of toxoids can be incorporated into the CS microparticles.

5.2.8. Optimization of the Particle size of Final Optimized Formulation:

A number of researchers have repeatedly demonstrated the uptake of microparticles across the gut following oral administration. ***(O'Hagan, D.T., 1990)*** There are different reports about the importance of the particle size for the Peyer's Patch targeting. ***Le Fevre (1980)*** evaluated the effect of particle size on the uptake of polystyrene microparticles and showed that 5.7µm particles were taken up into PPs, but 15.8µm particles were not. ***Ebel (1990)*** showed that in mice, 2.65 µm polystyrene particles were taken up to a greater extent than particles of 9.13µm. ***Eldridge et al (1990)*** suggested that the upper limit for particle uptake into mouse PPs was about 10µm. Eldridge further suggested that smaller particles (< 5 µm) were transported systemically in the lymph while larger particles (> 5 µm) were remained lodged in PPs. There are studies where particle size range of 1 to 50µm was used for the oral vaccine delivery. ***(Whittum-Hudson, J.A.,1996).***

Hence, after optimizing the loading, it was very important to control the size of the microparticles below 10µm. Particle size was measured using Malvern Particle Size Analyzer, UK, Model 2000 SM. The initial formulation (Batch 1W) prepared in water was found to have large particles with mean diameter of 125.76µ with very broad particle size distribution. The particle size was reduced when the batch was prepared in Propylene Glycol instead of water (Batch 2PG) but the entrapment was very poor. **(Table 34)**

Table 34: Particle size optimization

	Particle Size* (µm)			
Batch No.	**D(10)**	**D(50)**	**D(90)**	**Dmean**
1W	20.79	34.74	299.43	125.76
2PG	11.15	24.36	53.28	29.47

Therefore, the batch prepared in water was processed for particle size control using Emulsiflex C-5, Canada (E-C5) as given in the **Table 35.**

Table 35: Particle size optimization (Batch 3WE - 6WEP)

	Particle Size*(µm)			
Batch No.	D10	D50	D90	Dmean
3WE	10.65	31.19	144.5	60.713
4WEP	10.62	29.01	85.10	40.26
5WEP	11.97	24.87	52.62	30.836
6 WEP	12.68	27.16	58.05	32.106

When the batch was prepared in water was processed using EC-5 (Batch 3WE- 6 WEP), there was reduction in particle size but still D90 and Dmean were above 32 µm. This may be due to aggregation of the particles when processed using E C-5. Therefore batch was centrifuged and the sediment was redispersed in 20ml PG and processed using Emulsiflex C-5 as shown in **Table 36**

Table 36: Particle size optimization (Batch 7PGEP-9PGEP)

	Particle Size* (µm)			
Batch No.	D10	D50	D90	Dmean
7PGEP	9.299	24.35	69.56	37.211
8PGEP	9.51	19.76	38.68	22.269
9 PGEP	9.687	20.65	40.2	23.138

There was reduction in the particle size as observed for batch 7 PGE, but when the batch was subjected to 6 cycles with 5000 psi pressure there was increase in particle size as observed for Batch 9 PGEP. Therefore another equipment, CAT-560 Silverson type homogenizer (CAT-560) was used. Formulation prepared water was centrifuged and dispersed in 20ml PG and processed using CAT 560 Silverson type Homogenizer as shown in **Table 37**

Table 37: Particle size optimization (Batch 10PGH1.45 - 15PGH9.45)

	Particle Size* (µm)			
Batch No.	**D10**	**D50**	**D90**	**Dmean**
10PGH1.45	9.051	21.22	44.39	25.173
11PGH2.90	10.52	24.70	49.76	27.84
12PGH3.45	8.573	17.37	34.32	19.726
13PGH5.45	8.473	17.85	35.70	20.307
14PGH6.45	6.270	13.09	28.49	15.6
15PGH9.45	7.922	16.55	33.28	18.90

Batch 10 PGH1.45 showed broader particle size distribution as compared to batch 9 PGEP, therefore RPM was increased up to 9000 (Batch 11PGH2.90), which further increased Dmean and D90. Hence, RPM of 4500 was used for different time intervals for Batch 12 PGH3.45 to 15 PGH9.45. Still, the targeted D mean of 10µm could not be achieved. Therefore formulation prepared in water was centrifuged and dispersed in 20ml PG and processed using E C-5 and CAT 560 in combination as shown in **Table 38.**

Table 38: Particle size optimization (Batch16PGHE-27DT)

	Particle Size* (µm)			
Batch No.	**D10**	**D50**	**D90**	**Dmean**
16PGHE	12.89	26.79	52.36	30.152
17PGEH3	9.277	18.29	34.90	20.565
18PGEH6	9.817	23.73	119.5	73.969
19PGEH3	4.032	17.14	83.03	32.73
20PGEH6	3.992	12.39	88.08	31.64
21PGEH3	3.648	10.38	73.93	27.325
22PGEH6	5.224	11.05	25.44	15.234
23PGEH8	5.625	14.24	69.83	26.866
24PGEH9	5.594	12.17	27.74	18.191
25PGEH12	5.254	12.31	27.15	18.172
26Plain	**5.220**	**10.058**	**25.451**	**15.234**
27 TT	**4.570**	**9.944**	**23.652**	**15.154**
27 DT	**5.219**	**12.769**	**25.458**	**14.623**

** D(10), D(50), D(90) indicates 10%,50%,90% of the particles have diameter lesser than the given value.*

For batch 16PGHE first CAT-560 and then EC-5 was used but it showed Dmean of 30.152 µm. Therefore after this all batches were treated using EC-5 first and then using CAT-560.

Batch 22PGEH6, which was processed first with E C-5 and then with CAT-560 showed Dmean of 15.234 µm and D90 of 25.44 µm, indicating better particle size distribution as compared to earlier batches but still the Dmean was above the targeted particle size of 10µm. Therefore it was processed for to 2 more minutes using CAT-560, but it led to broader particle size distribution (Batch 23PGEH8)

So it was decided to try other options of either of adding peptizing agent or increasing the viscosity barrier. Addition of 2% sodium Carboxymethylcellulose (SCMC) was found to control the particle size distribution. Adsorption of Na CMC molecules on the particles and its effect on the viscosity of the reaction medium both may be responsible for increasing the barrier to the particle-particle interaction, preventing aggregation of the particles. Batch 24PGEH9 processed after addition of 2% SCMC had Dmean of 18.191 µm and D90 of 27.74 µm. The Batch 25PGEH12, where CAT-560 process time was increased up to 12 min, showed narrow particle size distribution with Dmean of 18.172 µm and D90 of 27.15 µm. Further processing at 4500 rpm for more than 12 minutes, started to give aggregated system indicated by increase in the mean particle size and broadening of the particle size distribution. Same conditions were applied for Batch 26 Plain, 27TT and 28 DT. The particle size was having narrow particle size distribution but the mean particle size was above 15µ.

Table 39 and **Figure** 44 shows the particles size distribution of 26 Plain, 27 TT and 28 DT batches obtained using Malvern Particle Size analyzer. There was no change in particle size of the TT and DT loaded batches as compared to Plain CS batch, indicating that there was no effect of entrapment of toxoids on the particle size distribution. The size distribution for all the batches show anearly bell shaped distribution curve, indicating that the formulation and size reduction procedure was reliable.

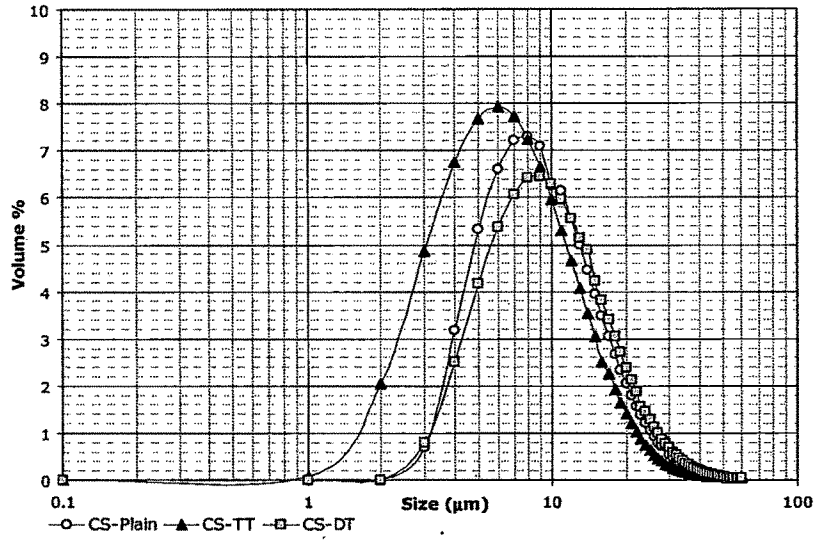
Thus, the final optimized batch had particle size from 4 µm to 25 µm with mean particle size of 15 µm. It was assumed that there will immediate absorption of 10-40% particles and remaining particles will remain lodged in PPs wherein it will release the antigen slowly, giving sustained effect. It was also expected that chitosan, which is having very strong mucoadhesive properties will enhance the residence time of the microparticles in

the intestine leading to maximum contact time for the delivery system to release the antigens.

Table 39: Particle size distribution for 26Plain, 27 TT and 28 DT batches

Size (μm)	Volume %			Size (μm)	Volume %			Size (μm)	Volume %		
	CS	CS-DT	CS-TT		CS	CS-DT	CS-TT		CS	CS-DT	CS-TT
0.1	0	0	0	20	2.05	2.39	1.4	46	0.1	0.11	0.07
1	0	0	0.08	21	1.79	2.12	1.19	47	0.09	0.1	0.07
2	0	0	2.05	22	1.56	1.87	1.02	48	0.09	0.09	0.07
3	0.7	0.79	4.85	23	1.37	1.55	0.86	49	0.08	0.08	0.07
4	3.18	2.51	6.74	24	1.19	1.46	0.74	50	0.07	0.07	0.07
5	5.32	4.16	7.67	25	1.04	1.28	0.63	51	0.07	0.07	0.07
6	6.61	5.36	7.92	26	0.92	1.13	0.54	52	0.06	0.06	0.06
7	7.21	6.07	7.72	27	0.8	0.99	0.47	53	0.06	0.06	0.06
8	7.3	6.41	7.25	28	0.71	0.88	0.4	54	0.06	0.06	0.06
9	7.09	6.45	6.64	29	0.82	0.77	0.34	55	0.05	0.06	0.06
10	6.25	6.28	5.97	30	0.54	0.68	0.3	56	0.05	0.05	0.06
11	6.13	5.96	5.3	31	0.48	0.6	0.26	57	0.05	0.05	0.06
12	5.56	5.56	4.66	32	0.42	0.53	0.23	58	0.04	0.05	0.06
13	4.99	5.13	4.06	33	0.39	0.47	0.2	59	0.04	0.04	0.06
14	4.44	4.88	3.52	34	0.33	0.41	0.18	60	0.04	0.04	0.06
15	3.94	4.23	3.04	35	0.3	0.37	0.16	61	0.04	0.04	0.05
16	3.47	3.81	2.51	36	0.27	0.32	0.14	62	0.04	0.04	0.05
17	3.05	3.41	2.24	37	0.24	0.29	0.13	63	0.04	0.03	0.05
18	2.67	3.04	1.92	38	0.21	0.26	0.12	64	0.04	0.03	0.05
19	2.34	2.7	1.64	39	0.19	0.23	0.11	65	0.03	0.03	0.05
43	0.13	0.15	0.08	40	0.18	0.2	0.1	66	0.03	0.03	0.05
44	0.12	0.13	0.08	41	0.16	0.18	0.09	67	0.03	0.03	0.05
45	0.11	0.12	0.08	42	0.14	0.16	0.09	68	0.03	0.03	0.05

Figure 44: Particle size distribution curve for 26Plain, 27 TT and 28 DT



5.2.8.1 Scanning Electron Microscopy (SEM):- Environmental SEMs taken for Batch 1W, Batch 27 TT and Batch 28 DT are shown in **Figure 45, 46, 47,** respectively

Figure 45 is E-SEM of Batch 1W, which was not processed for particle size reduction. The picture clearly shows that instead of discrete particles there are lumps probably due to aggregation of the particles, which clearly shows the role of SCMC and particle size reduction treatment. However, discrete, non-aggregated particles are clearly visible in the E-SEM of Batch 27 TT (**Figure 46**) and Batch 28 DT (**Figure 47**). As the formulation is a suspension, the background is not clear and all particles are embedded in suspension matrix.

Figure 45: E-SEM of Batch 1W

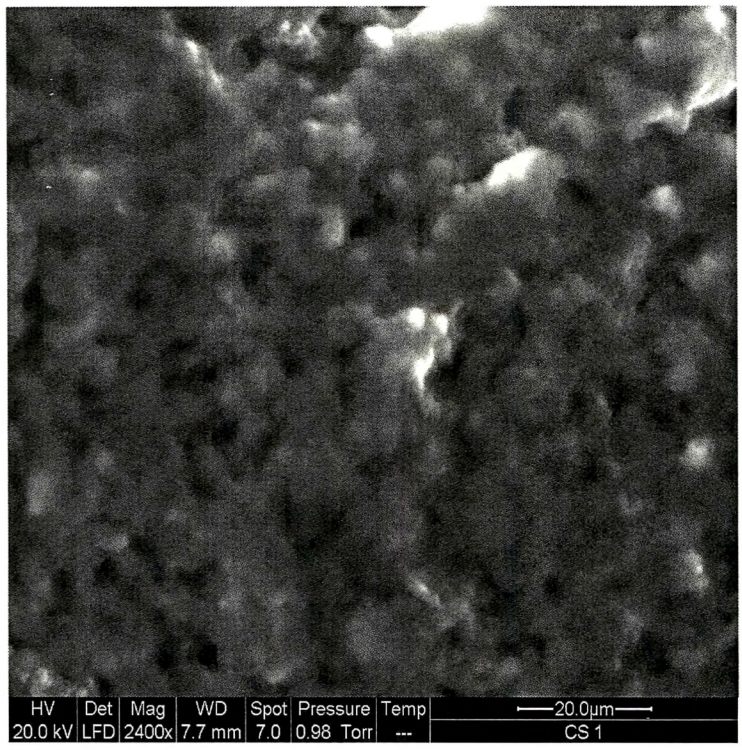


Figure 46: E-SEM of Batch 27TT

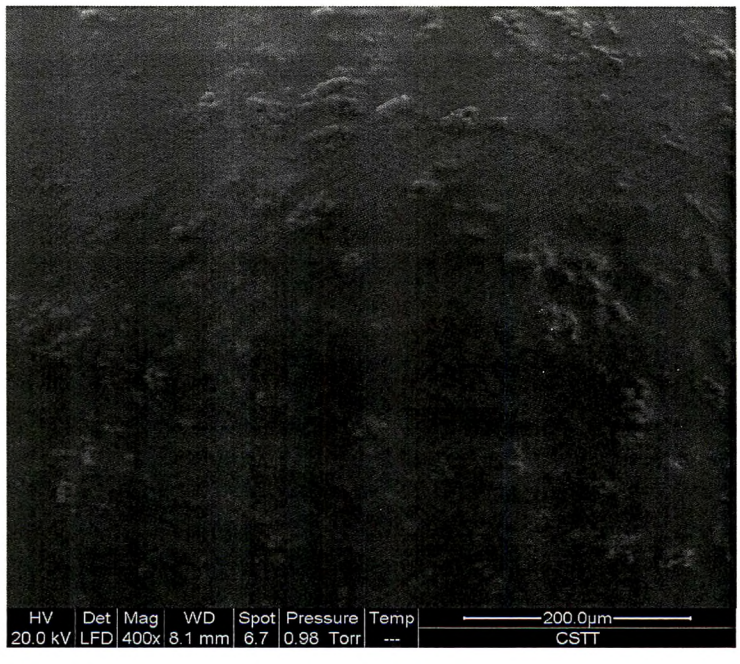
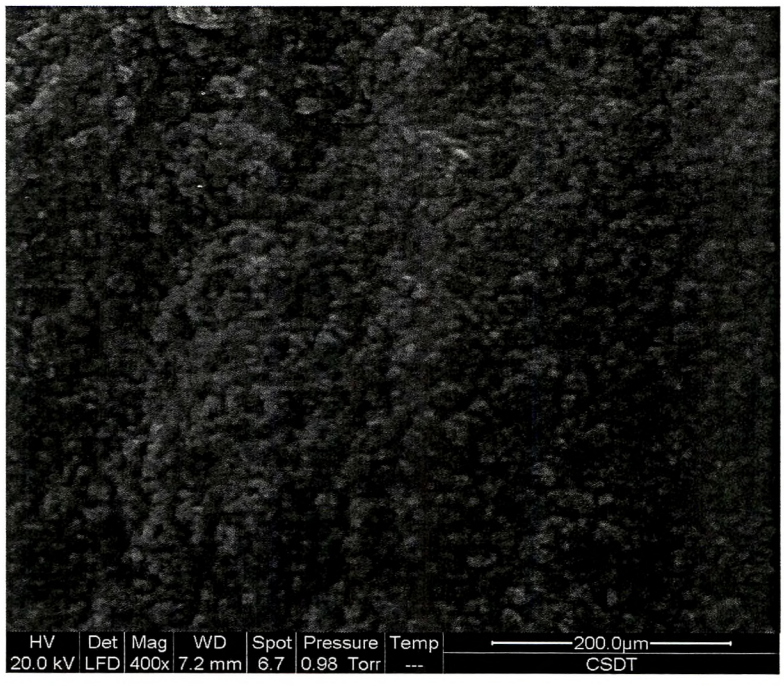


Figure 47: E-SEM of Batch 28DT



5.2.8.2 Transmission Electron Microscopy (TEM):- The TEM shows discrete non aggregated, solid and spherical particles measuring less than 1µm. **(Figure 48 & 49).** This indicates that the final formulation also contained nanoparticles along with microparticles.

Figure 48: TEM of 27 TT Final formulation.

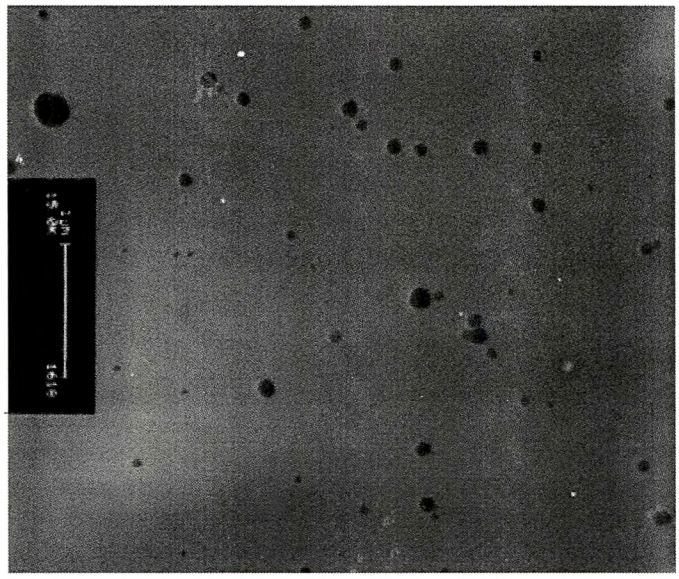
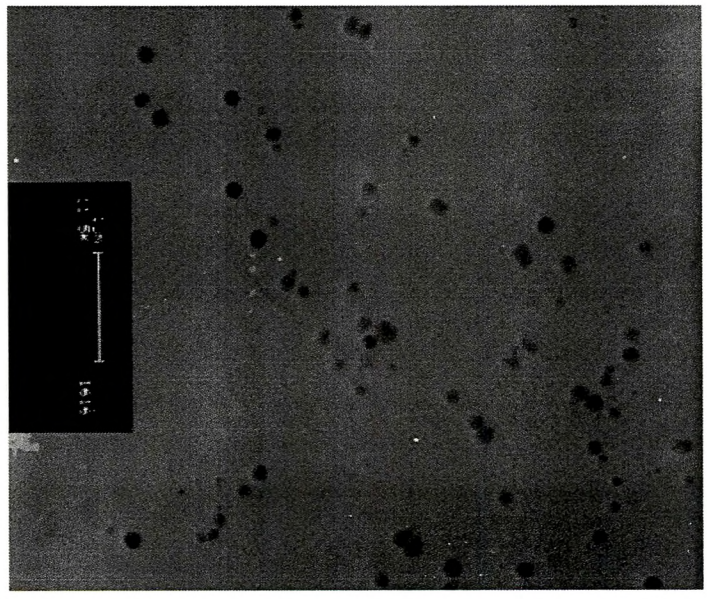


Figure 49: TEM of 28DT final formulation.

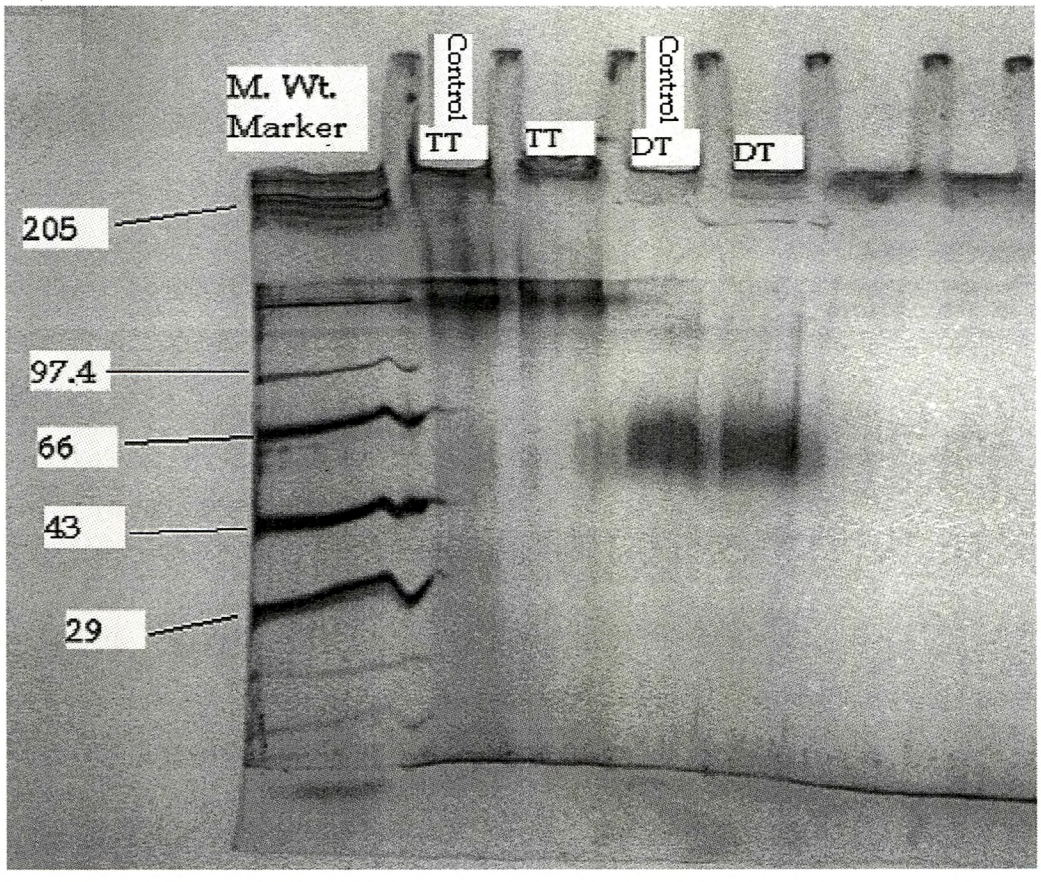


5.2.9. Effect of high shear rate on conformational changes:-

5.2.9.1 Study of change in conformations using SDS-PAGE:-

The effect of high shearing rate as well as pressure of compressed air which may affect the conformations of TT and DT was studied using SDS-PAGE as it has been used as a main tool for studying the stability of entrapped antigen and confirming protein integrity. *(Spiers, I.D., 2000; Lavelle, E.C.,1999)*. The results (**Figure 50**) of SDS-PAGE of processed TT and DT, showed no change as compared to control. This clearly shows that the process parameters of particle size reduction method were antigen friendly.

Figure 50: SDS-PAGE of processed samples of TT and DT.



5.2.9.2 Study of change in conformations using HPLC:-

HPLC chromatogram of processed sample of TT and DT as shown in **Figure 51** and **52** indicate that the respective peaks of the vaccines are retained. Both chromatograms show identical peaks at Rt of around 12 min, which may be attributed to sodium CMC, which was used as dispersing agent.

In addition, the chromatogram of DT showed an additional peak at Rt of 5.09 min which may be attributed to aggregated DT which acts as high molecular weight compound, resulting in faster elution from the column.

These observations suggest that there are no conformational changes or breakdowns of the TT and DT molecules during the particle size reduction processing.

Thus, the results of both SDS-PAGE and HPLC studies show that the entrapment and pressure homogenization procedures adopted for particle size reduction had not affected the conformation of the toxoids, indicating that antigenicity of the vaccines shall be maintained after the entire entrapment and size reduction processing.

Figure 51: HPLC chromatogram of sample of TT, processed using Emulsiflex C-5 and Silverson type homogenizer

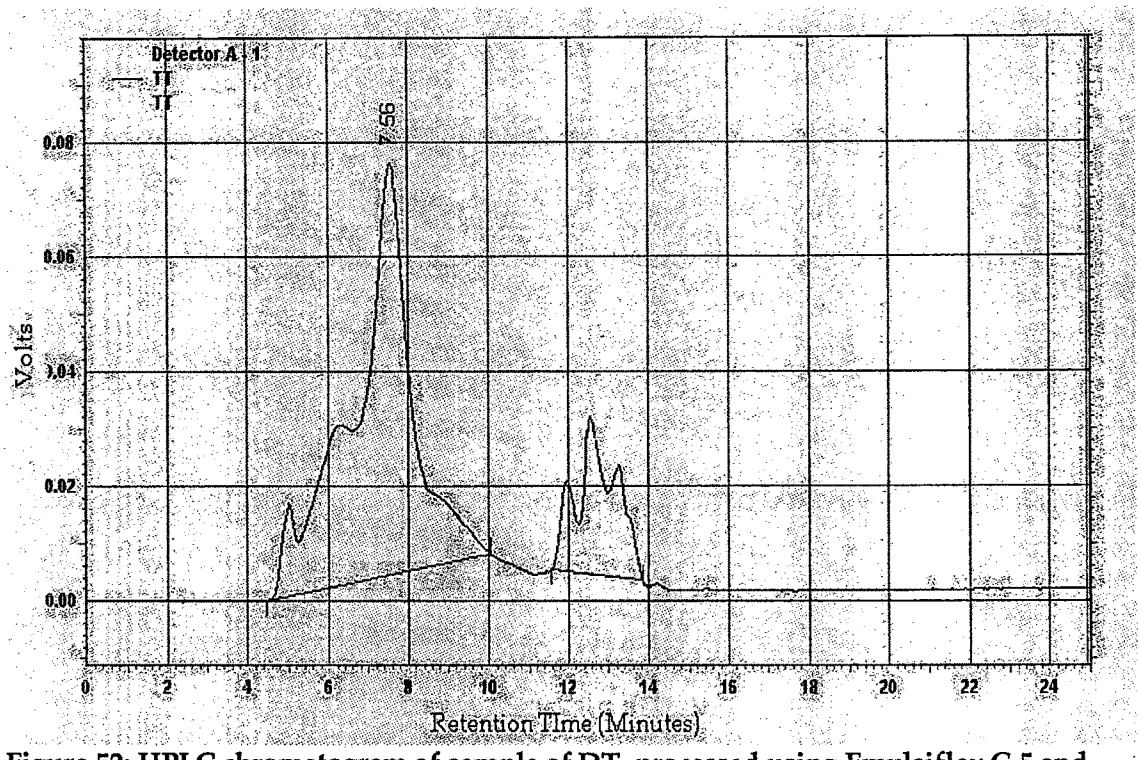
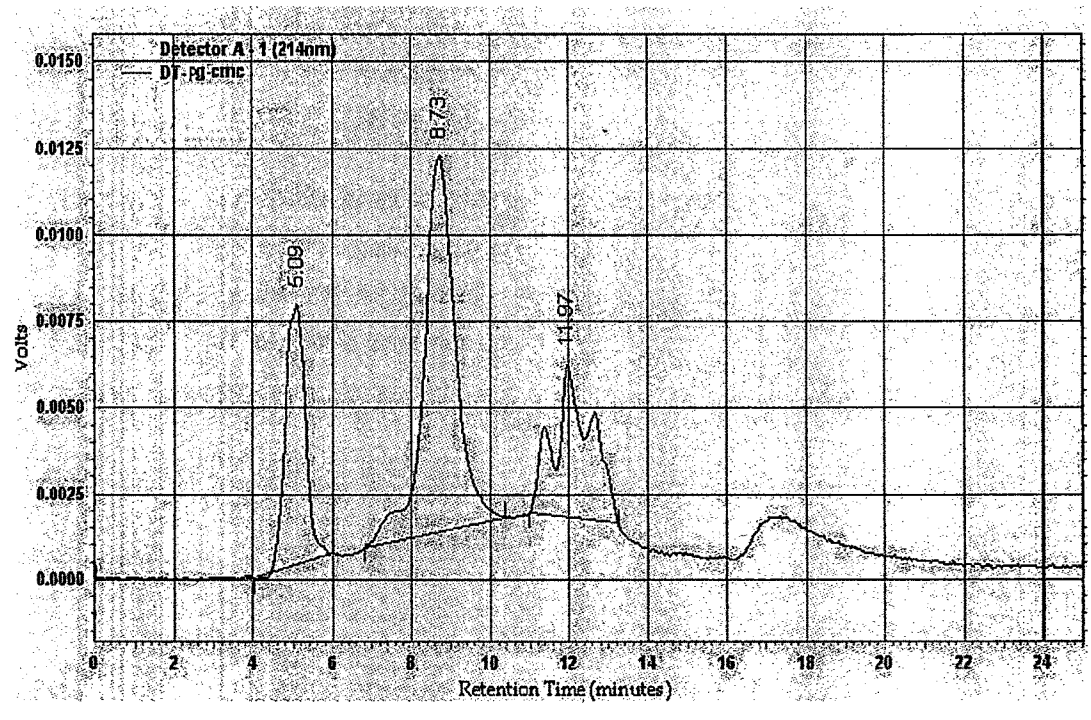


Figure 52: HPLC chromatogram of sample of DT, processed using Emulsiflex C-5 and Silverson type homogenizer.



5.2.10. Release study from 27TT and 28DT batches:

In order to ensure that encapsulated toxoids are protected from the external luminal conditions and they are delivered in the form of microparticles up to the PP's present in lower intestine, it is essential that microparticles should remain intact in gastric acidic medium as well as in intestinal alkaline medium. As the gastric emptying time is up to 2 hours, the study was carried for 2 hrs in gastric conditions simulated by use of 0.1 N HCl.

There was only 2.4% & 2.1% release of the TT and DT respectively from loaded chitosan microparticles, in 0.1N HCl **(Table 40).** This amount can be attributed to the surface bound toxoids.

Table 40: Release study of TT and DT in 0.1N HCl and PBS (7.4)

Time (min)	TT Cumulative % Release		DT Cumulative % Release	
	0.1 N HCl	PBS (7.4)	0.1 N HCl	PBS (7.4)
30	0.658	--	0.509	--
60	1.681	--	1.531	--
120	2.401	--	2.101	--

Study in PBS (pH 7.4) showed no release of TT and DT up to 2 hours. The reason may be that optimized formulation conditions had ensured there is complete complexation of chitosan and STPP giving maximum crosslinking and crosslinking density leading to maximum entrapment of toxoids. Further, the ionic interaction between the TT & CS and DT & CS might have prevented their release in PBS.

The stability of chitosan microparticles in gastric pH (0.1N HCl) and intestinal medium pH [PBS (pH 7.4)] is important from the delivery point of view of entrapped TT to PP's, as the soluble TT and DT is incapable of formation of memory cells. This observation is line with observations reported by **van der Lubben et al, 2003** who observed there was only very negligible i.e. only 2% release of the DT in 0.1N HCl and no release in PBS (pH 7.4) buffer from DT adsorbed on preformed chitosan microparticles.

5.2.11. Bioadhesion testing:

For drug delivery purpose bioadhesion implies attachment of drug carrier system to a specified biological location, which is gastrointestinal tract in oral delivery of toxoids using chitosan. *(Ahuja, A.,1997).* Chitosan is reported to have mucoadhesion properties, which is also responsible for the permeation enhancement of the associated drugs. *(Aungst, B.J., 2000).* Mucoadhesive properties of the chitosan are useful in increasing the intestinal transit time of delivery system. FITC-labeled chitosan coated ethylcellulose microparticles were retained at the middle parts of the small intestine for more than 8 hrs, which is due to mucoadhesive properties of the coated chitosan *(Takishima, J.,2002).* By increasing the intestinal retention time, the chances of absorption of the toxoids increases as it decreases the washing of the formulation along with intestinal content due to propulsive movement of the GI tract.

The force required to break the adhesive bond between membrane and the formulation was measured digitally.

Force of detachment in dynes for Batches 26 Plain, 27 TT and 28 DT were determined and are given in **Table 41.** 27TT and 28DT values are more as compared to 26 Plain values, indicating that incorporation of the toxoids resulted in increased bioadhesion property of the microparticles.

It was assumed that due to increased bioadhesion properties chitosan microparticles containing TT and DT will remain attached on the intestinal wall or lodged on the PPs for a longer time. Due to close proximity with the intestinal wall it will also reduce the contact with intestinal lumen enzymes. CS-TT or CS-DT microparticles will be processed directly by enzymes present in PPs, which will lead to degradation of microparticles and release of TT and DT in the lymphatic system, against which antibodies will be formed.

Table 41: Bioadhesion testing

Batch	Detachment force (Avg ± S.D) (dyens)
26 Plain	13.167 ± 1.457
27 TT	22.657 ± 1.066
28 DT	18.967 ± 1.934

5.2.12. Rheological Study:

As the final product is a suspension stabilized by the use of sodium carboxymethylcellulose, its rheological properties was studied for following factors: the viscosity of suspension as it affects the settling of the dispersed particles, the change in flow properties of the suspension when the container is shaken and when the product is poured from the bottle.

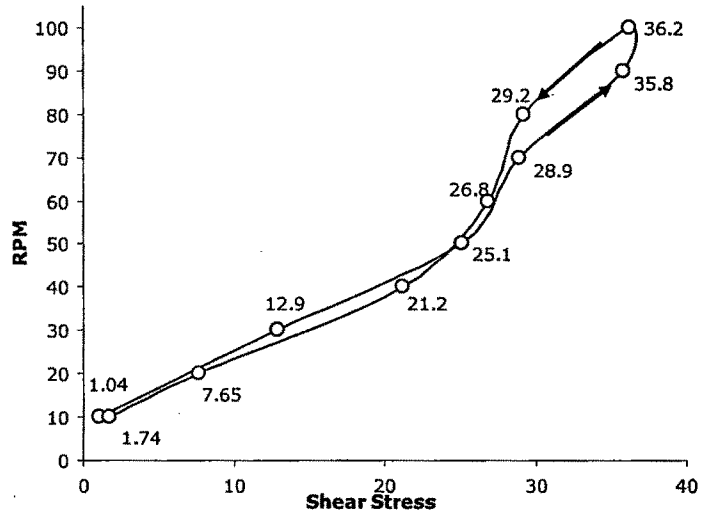
SCMC is a suspending agent having thixotropic as well as pseudoplastic properties and it proved to be useful since it forms gel on standing giving stable suspension due to high viscosity and the aggregation of the particles will be avoided due to reduced random movement of the suspended particles. It becomes fluid when disturbed or when shear is applied by shaking for removal of the product from the container so that the ease of the administration is ensured. This will also ensure compliance with content uniformity test. The hysteris loop of SCMC is also observed in the **Table 42** and **Figure 53**. Curve is showing both the pseudoplastic and thixotropic characteristics.

Table 42: Rheological characterization of CS-TT formulation

No.	RPM*	Torque(%)	Viscosity (cps)	Sh Str (D/cm^2)	Sh Rate (sec^{-1})
1	10	0.3	36.0	1.04	2.9
2	30	3.7	148.0	12.9	8.7
3	50	7.2	172.8	25.1	14.5
4	70	8.3	142.3	28.9	20.3
5	90	10.3	137.3	35.8	26.1
6	100	10.4	124.8	36.2	29
7	80	8.4	126.0	29.2	23.2
8	60	7.7	154.0	26.8	17.4
9	40	6.1	183.0	21.2	11.6
10	20	2.2	132.0	7.65	5.8
11	10	0.5	60.0	1.74	2.9

** One minute at each RPM step*

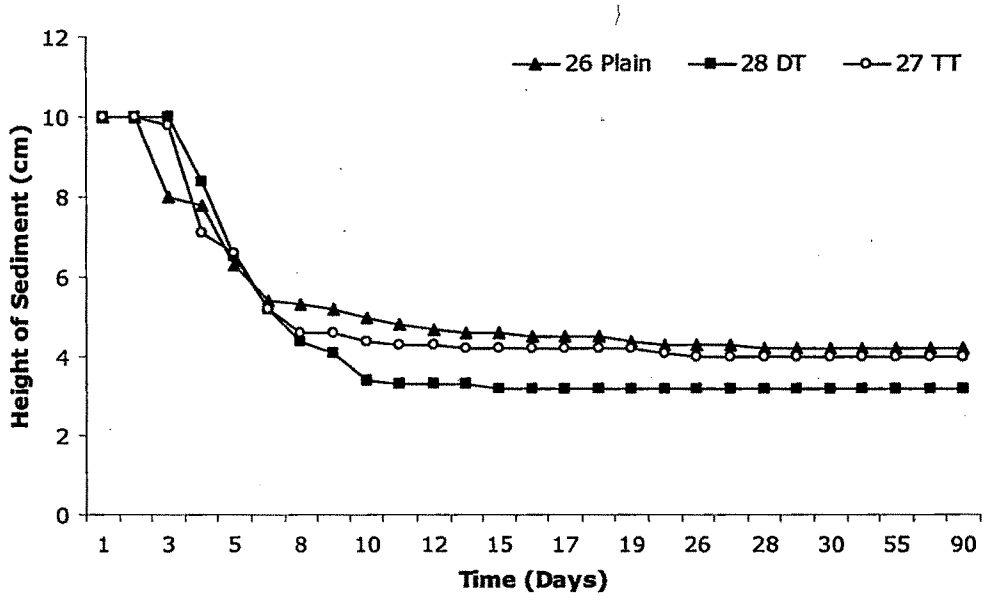
Figure 53: Rheological characterization of CS-TT final formulation



5.2.13. Sedimentation study:

From Batch 26 Plain, 27 TT and 28 DT, 10 ml final product was observed for sedimentation. The sedimentation profile of Plain and loaded chitosan microparticles is shown in the **Figure 54**. The rate of settling was fast for first 8 days after which it remained constant. There was no difference in sedimentation profile of Plain and Loaded chitosan microparticles.

Figure 54: Sedimentation profile of Batch 26 Plain, 27TT and 28 DT.



5.2.14. Redispersion study:

Ease of redispersion and Uniformity of dosing are the main requirements of suspension system. The only shear that occurs in a suspension during storage is due to settling of the suspended particles; this force is negligible and may be disregarded. But the cake formed should not be hard but should be easily redispersed when container is shaken to remove product. *(Martin, P.P.,1991)*. Therefore the product was shaken to check the redispersion of the formulation. It was completely redispersed after 3 times manual inversion of the cylinder. It was found to have good redispersion property, indicating that the sediment formed was not hard.

5.2.15. Stability Study:

Stability of the Batch 27TT and 28 DT was studied at 4⁰ and 25⁰C for three months and examined for leaching of the toxoid **(Table 43)**. There was very negligible leaching of TT and DT in both conditions.

Table 43: Stability of final batch

	TT (% leached)		DT (% leached)	
Time (Days)	4⁰C	25⁰C	4⁰C	25⁰C
30	0.752 ± 0.014	0.512±0.009	0.261±.0.018	0.398±0.008
60	1.216± 0.025	1.726± 0.083	0.851± 0.035	1.203± 0.079
90	1.568± 0.072	1.962± 0.098	1.029± 0.045	1.587± 0.082

This study shows that the product can be stored at RT also up to 3 months. This is of great significance from the transportation and administration point of view. As it is a oral delivery system it can be self administered by the patients thus eliminating the need of medical supervision and storage in refrigerator conditions is also not required as it is stable at RT. This will be of great help in increasing immunization coverage and avoiding the lapses in booster dose administration, especially in far flung rural areas where as appropriate storage facilities (refrigerator, cold chain) are not available.

5.2.16. Hyperimmunization of mice:

Hyperimmunized mice serum IgG was used as standard for the quantification of the IgG levels in the experimental animal serum. Hyperimmunization was done via intraperitoneal route, which is known to produce higher titre of antibodies.

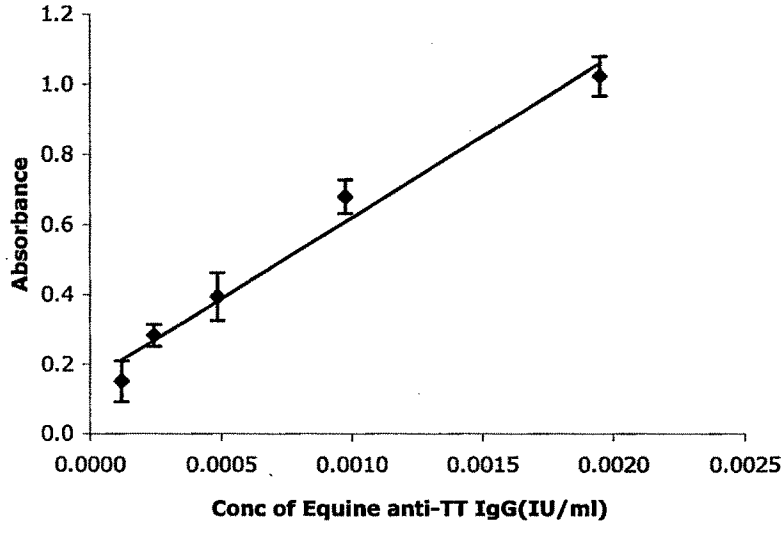
5.2.17. Estimation of TT and DT specific IgG in hyperimmunized mice using ELISA:

IgG levels were estimated using the ELISA where polyclonal equine TT-specific IgG and DT– specific IgG were used. Standard curve is shown in **Table 44** and **Figure 55** for TT and **Table 45** and **Figure 56** for DT.

Table 44: Standard curve Equine anti-TT IgG as standard.

Conc of Equine anti-TT IgG (IU/ml)	Abs± S.D.
0.000121	0.151 ± 0.059
0.000243	0.284 ± 0.031
0.000485	0.395 ± 0.068
0.000975	0.680 ± 0.048
0.001950	1.023 ± 0.056

Figure 55: Standard curve using Equine anti-TT IgG as standard.



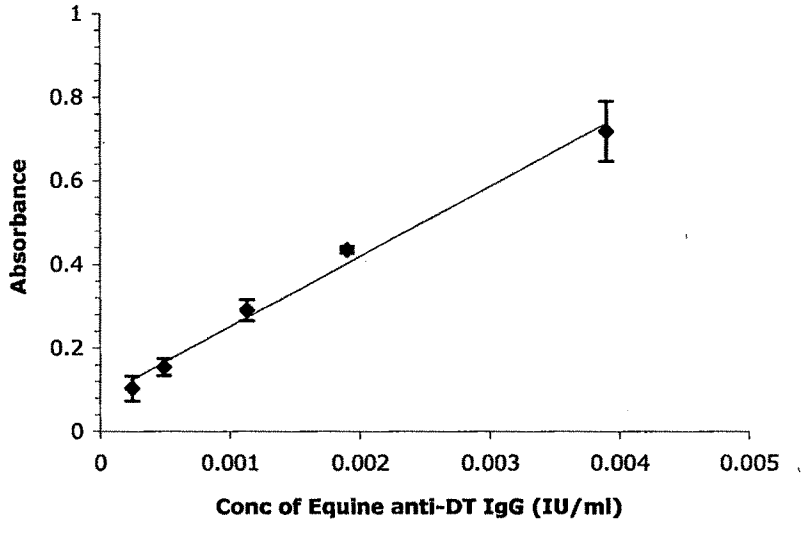
Relationship between Absorbance and conc of Equine anti-TT IgG was found to be linear and given by regression **Equation 8.** IgG levels in mice hyperimmunized with TT was calculated using Equation 8 and found to be 0.245 IU/ml

$$y = 462.96x + 0.1572, R^2 = 0.9779 \ldots\ldots \textbf{Equation 8}$$

Table 45: Standard curve using Equine anti-DT IgG as standard.

Conc of Equine anti-DT IgG (IU/ml)	Abs± S.D.
0.000244	0.103 ± 0.004
0.000488	0.155 ± 0.025
0.001130	0.291 ± 0.030
0.001900	0.435 ± 0.020
0.003900	0.719 ± 0.025

Figure 56: Standard curve using Equine anti-DT IgG as standard.



Relationship between Absorbance and conc of Equine anti-DT IgG was found to be linear and given by regression **Equation 9.** IgG levels in mice hyperimmunized with DT was calculated using **Equation 9** and found to be 2.224 IU/ml

$$y = 167.61x + 0.0838, R^2 = 0.9906 \ldots\textbf{Equation 9}$$

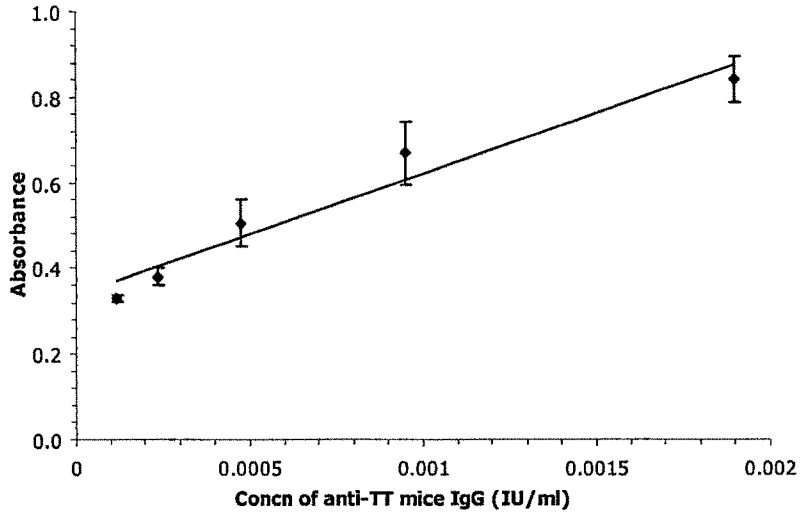
5.2.18. Estimation of TT and DT specific IgG in experimental mice using ELISA:

As hyperimmunized animal sera gave very high titre of 0.245 IU/ml TT specific IgG and 2.224 IU/ml of DT specific IgG, it was diluted to obtain standard curves which were used for the estimation of respective IgG level in experimental animals, as shown in **Table 46 & 47** and **Figure 57 & 58,** respectively for TT and DT

Table 46: Standard curve using TT specific mice IgG as standard.

Conc of anti-TT mice IgG	Abs ± S.D.
0.000118	0.330 ± 0.009
0.000235	0.379 ± 0.021
0.000475	0.505 ± 0.056
0.000950	0.670 ± 0.074
0.001900	0.840 ± 0.054

Figure 57: Standard curve using anti-TT mice IgG as standard.



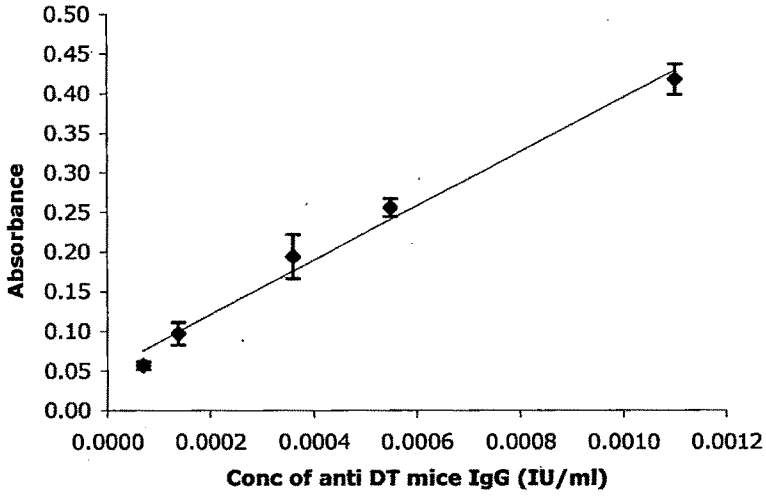
Relationship between absorbance and conc of anti-TT mice IgG was found to be linear and given by regression **Equation 10.** TT Specific IgG levels in experimental mice were calculated from **Equation 10**

$$y = 284.05x + 0.3357,\ R^2 = 0.951 \ldots\ldots \textbf{Equation 10}$$

Table 47: Standard curve using anti-DT mice IgG as standard.

Conc of anti-DT mice IgG (IU/ml)	Abs± S.D.
0.0000688	0.057± 0.01273
0.0001375	0.097± 0.00071
0.0003600	0.194± 0.00495
0.0005500	0.256± 0.01414
0.0011000	0.418± 0.02758

Figure 58: Standard curve using anti-DT mice IgG as standard.



Relationship between absorbance and conc of anti-DT mice IgG was found to be linear and given by regression **Equation 11**. TT Specific IgG levels in experimental mice were calculated from **Equation 11**

$$y = 343.43x + 0.0522, R^2 = 0.9871 \ldots\ldots \textbf{Equation 11}$$

5.2.19. Assessment of Systemic Immune Response:

5.2.19.1 Dose Response Relationship:-

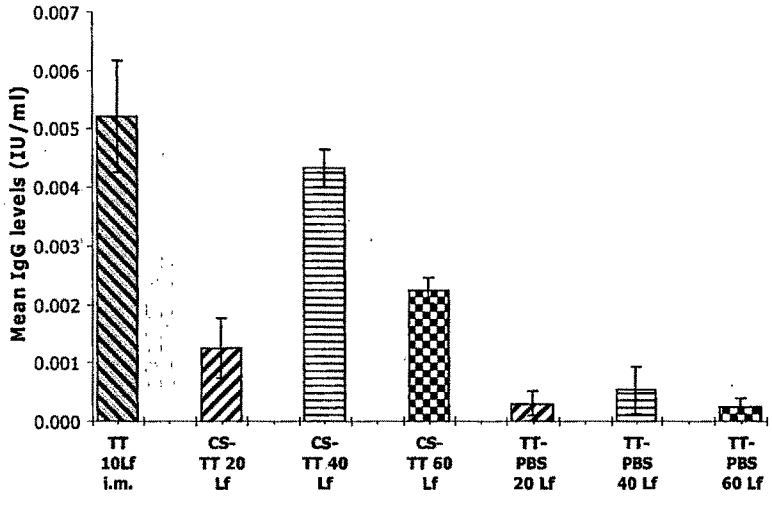
The aim of this in vivo study was to determine a dose-response relationship following oral vaccination with TT and DT.

Dosing, sampling and sample treatment was done as per Protocol 1. Required dose of encapsulated TT was calculated and corresponding volume of the Batch 27TT was administered. Intramuscular injection of 10Lf adsorbed TT vaccine (TT 10Lf i.m.), 20Lf (CS-TT 20LF), 40 Lf (CS-TT 40Lf), 60 Lf (CS-TT 60 Lf) from 27TT batch and Plain TT 20 Lf (TT-PBS 20Lf), 40Lf (TT-PBS 40Lf) and 60 Lf (TT-PBS 60Lf) was administered as per Protocol 1.

Table 48: TT specific IgG titres on 22nd day.

Formulation	Mean IgG titre (IU/ml ±S.E.M.)
TT 10Lf i.m.	0.00520 ± 0.000957
CS-TT 20 Lf	0.00125 ± 0.000521
CS-TT 40 Lf	0.00432 ± 0.000318
CS-TT 60 Lf	0.00224 ± 0.000230
TT-PBS 20 Lf	0.00030 ± 0.000206
TT-PBS 40 Lf	0.00053 ± 0.000406
TT-PBS 60 Lf	0.00025 ± 0.000132

Figure 59: TT specific IgG titres on 22nd day.

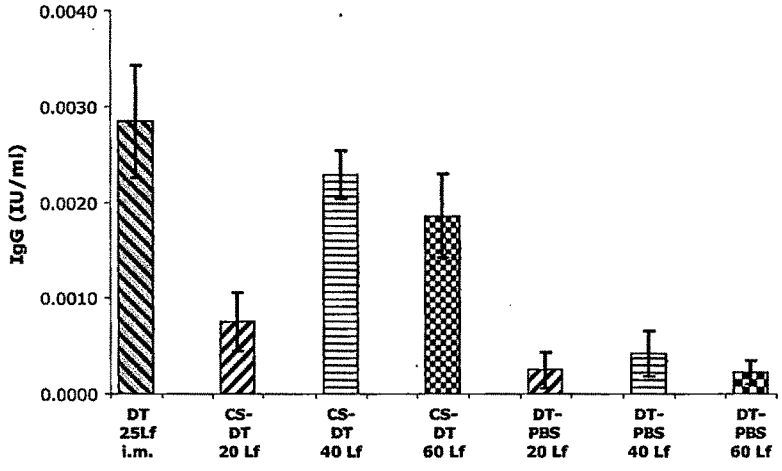


Similar study was carried out for Batch 28DT. Required dose of encapsulated DT was calculated and corresponding volume of the Batch 28DT was administered. Intramuscular injection of 25Lf adsorbed DT vaccine (DT 25Lf i.m.), 20Lf (CS-DT 20LF), 40 Lf (CS-DT 40Lf), 60 Lf (CS-DT 60 Lf) from 28DT batch and Plain DT 20 Lf (DT-PBS 20Lf), 40Lf (DT-PBS 40Lf) and 60 Lf (DT-PBS 60Lf) was administered as per Protocol 1.

Table 49: DT specific IgG titres on 22nd day.

Formulation	Mean IgG titre (IU/ml± S.E.M.)
DT 25Lf i.m.	0.002845 ± 0.000583
CS-DT 20 Lf	0.000753 ± 0.000306
CS-DT 40 Lf	0.002288 ± 0.000252
CS-DT 60 Lf	0.001855 ± 0.000437
DT-PBS 20 Lf	0.000247 ± 0.000185
DT-PBS 40 Lf	0.000415 ± 0.000234
DT-PBS 60 Lf	0.000223 ± 0.000131

Figure 60: DT specific IgG titres on 22nd day.



As evident from **Figure 59 and 60,** dose-dependent response towards the antigens could be induced at 3 weeks after oral vaccination with TT and DT loaded chitosan microparticles. All mice vaccinated with CS-TT and CS-DT showed a strong and significant enhancement in IgG titres against TT and DT, respectively whereas only minor immune responses were observed in the groups vaccinated with TT and DT in PBS. This clearly shows the generation of systemic immune response most probably through a effective uptake of the CS-TT and CS-DT microparticles by the PPs.

Mice vaccinated with 20Lf TT and DT entrapped in chitosan microparticles were found to have a significantly lower systemic immune response than the groups vaccinated with 40 or 60 LF TT and DT entrapped in chitosan microparticles. The response obtained for 60 Lf was found to be lower than 40 Lf TT and DT entrapped in chitosan. The reason may be that all PPs may become saturated with particles at a particular volume

equivalent to 40Lf dose and hence may not be capable of taking up the excess particles required to incorporate 60Lf dose.

However, the systemic immune response of the mice vaccinated with 40 Lf TT and DT entrapped in chitosan microparticles was less as compared to that of mice intramuscularly vaccinated with 10Lf TT and 25 Lf DT adsorbed on Aluminium Phosphate. This indicates that for oral vaccination, approximately 4 and 1.6 times a much higher dose would be required for TT and DT respectively to get similar immune response as that of positive control. At this dose, the immune response is approximately 144 and 5.6 times higher than the IgG titres observed after oral administration with TT in PBS and DT in PBS, respectively.

IgG levels obtained on 22nd day after administration of different TT formulations was statistically compared using Student's t-test. Difference in IgG titres after 10Lf TT administered through i.m. route were compared with orally administered 20, 40 and 60 Lf CS-TT for level of significance. Difference in IgG titres after 20, 40 and 60Lf CS-TT were also compared with IgG titres obtained after administration of 20, 40 and 60 Lf TT in PBS for level of significance. (**Table 50**)

Table 50: TT specific IgG titres on 22nd day, comparison using Student's t-test.

	Student's t-test					
Formulation	**TT i.m. Vs CS-TT 20**	**TT i.m. Vs CS-TT 40**	**TT i.m. Vs CS-TT 60**	**CS-TT20 Vs TT-PBS 20**	**CS-TT20 Vs TT-PBS 40**	**CS-TT20 Vs TT-PBS 60**
't' value	3.624	0.873	3.008	2.597	5.937	5.405
Significance Level at 'P' value	<0.02	No Significance	<0.05	<0.05	<0.002	<0.002

The IgG levels were significantly lower in case of 20Lf CS-TT ($P<0.02$) and 60Lf CS-TT ($P<0.05$) when compared with IgG levels after i.m. 10Lf TT. But the IgG levels after 40Lf CS-TT were not significantly different as compared to mean IgG levels after i.m. 10Lf TT. The mean IgG levels after 20, 40 and 60 Lf CS-TT were significantly higher than 20Lf TT-PBS ($p<0.05$), 40Lf TT-PBS ($p<0.002$) and 60 Lf TT-PBS ($p<0.002$) respectively.(**Table 50**)

The level of significance between Oral route and i.m. route and microencapsulated DT with DT in PBS was compared statistically using Student's t-test. (**Table 51**)

Table 51: DT specific IgG titres on 22nd day, comparison using Student's t-test.

	Student's t-test					
Formulation	**DT i.m.Vs CS-DT 20**	**DT i.m.Vs CS-DT 40**	**DT i.m. Vs CS-DT 60**	**CS-DT20 Vs DT-PBS 20**	**CS-DT20 Vs DT-PBS 40**	**CS-DT20 Vs DT-PBS 60**
't' value	3.174	0.875	1.357	2.395	5.432	3.569
Significance Level at 'P' value	<0.02	No Significance	No Significance	<0.05	<0.001	<0.02

The IgG levels were significantly lower in case of 20Lf CS-DT ($p<0.02$) but the IgG levels after 40Lf CS-DT and 60 Lf CS-DT were not significantly different as compared to mean IgG levels after i.m. 25Lf DT. As the 40 Lf CS-DT dose gave nearly same response as that of 60Lf CS-DT, it was taken for comparison. The mean IgG levels after 20, 40 and 60 Lf CS-DT were significantly higher than 20Lf DT-PBS ($p<0.05$), 40Lf DT-PBS ($p<0.001$) and 60 Lf DT-PBS ($p<0.02$) respectively. **(Table 51)**

From this study, it was concluded that 40Lf oral dose of TT and DT could elicit a systemic immune response. However this study was done at only 1 time point i.e. on 22nd day. To properly understand the kinetics of the immune response, Protocol 2 was used.

5.2.19.2. Assessment of Systemic Kinetic Immune Response:

Kinetics of the immune response was studied as per Protocol 2. Main drawback of the in vivo studies is the biological variation which can seldom be controlled. This can be minimized by increasing the number of animals, therefore in this study, 10 animals per group were used for the evaluation of the immune response to encapsulated 27TT and 28DT batch.

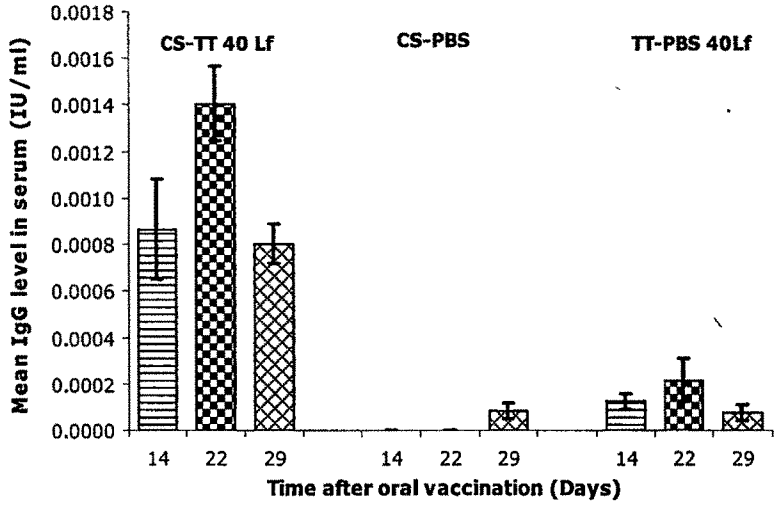
5.2.19.2.1 Assessment of Systemic Kinetic Immune Response for TT:-

Systemic immune response study was performed over a period of 29 days. Kinetics of immune response was studied by taking samples on day 14, 22 and 29 after oral administration of encapsulated TT (CS-TT), chitosan plain microparticles (CS-PBS) and TT in PBS (TT-PBS).

Table 52: TT specific IgG titres.

	Mean IgG Titre (IU/ml ± S.E.M.)		
	CS-TT 40Lf	CS-PBS	TT-PBS 40Lf
14	0.0008 ± 0.0002	0.0	0.00012 ± 0.00003
22	0.0014 ± 0.0001	0.0	0.00021 ± 0.00009
29	0.0008 ± 0.00008	0.00008 ± 0.00003	0.00007 ± 0.00003

Figure 61: TT specific IgG titres.



At all sampling days, as shown in **Table 52** and **Figure 61** for **Batch 27TT**, no measurable IgG titres could be detected in groups orally fed with plain chitosan microparticles alone but very minor response was detected on 29th day. Very minor immune response could be detected after 14 days of oral vaccination with TT in PBS, which was enhanced to some extent after booster dose on 22nd day but the level was very low on 29th day. Following oral administration of TT loaded microparticles, measurable titres were found on 14th day which increased substantially on 22nd day, after booster dose. On 29th day the IgG level was low as compared to 22nd day but it was still much higher as compared to TT in PBS on 29th day. Highly elevated IgG titres could be found in CS-TT as compared to TT-PBS on all days of sampling indicating that the antigenicty of the antigens was retained after the sntire loading and particle size reduction operation and that the it was capable of delivering the vaccines orally in a safe and effective form.

The mean IgG level after oral vaccination with **Batch 27TT**, 40 Lf CS-TT on 14th, 22nd and 29th day were 10.1, 11.08 and 31.5 times higher than the level obtained after 40Lf TT-PBS. The titres with CS-TT were significantly higher than that of CS-PBS and TT-PBS, at the significance level as shown in **Table 53** on all sampling days.

Table 53: Statistical comparison of IgG titres from different formulations of TT using Student's t-test.

	Student's t-test					
Formulation	**CS-TT Vs CS-PBS**			**CS-TT Vs TT-PBS**		
Sample day	14	22	29	14	22	29
't' value	5.061	10.336	7.573	3.499	6.298	7.746
Significance Level at 'P' value	<0.002	< 0.0001	< 0.0001	< 0.01	< 0.0001	< 0.0001

From this in vivo experiment, it was concluded that the systemic immune response against TT was strongly enhanced after entrapment of vaccine in chitosan microparticles, whereas TT in PBS induced only minor immune responses. It was also observed that immune response was measurable on 14th day and increased after booster dose, indicating the formation of memory cells after initial dosing. Thus, the positive outcome of these studies have proved that the hypothesis of the present project was based on sound reasoning and that encapsulation in CS not only protected the antigen but also retained its antigenicity and facilitated its oral transport and absorption, most probably through the Peyer's patches and elicited a positive immune response.

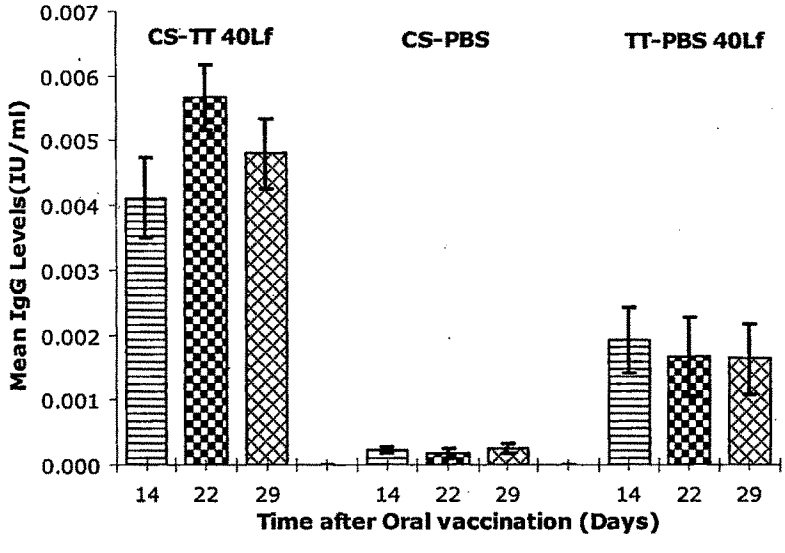
5.2.19.2.2 Assessment of Systemic Kinetic Immune Response for DT:-

Systemic immune response to orally administered encapsulated DT (CS-DT), chitosan plain microparticles (CS-PBS) and DT in PBS (DT-PBS) was studied over a period of 29 days using Protocol 2.

Table 54: DT specific IgG titres.

	Mean IgG titre (IU/ml)± S.E.M.		
	CS-DT 40Lf	**CS-PBS**	**DT-PBS 40Lf**
14	0.0041 ± 0.0006	0.00023 ± 5.067E-05	0.0019 ± 0.0005
22	0.0056 ± 0.0004	0.00018 ± 7.768E-05	0.0016 ± 0.0006
29	0.0048 ± 0.0005	0.00024 ± 7.387E-05	0.0016 ± 0.0005

Figure 62: DT specific IgG titres.



As evident from **Table 54** and **Figure 62**, at all sampling days, very minor IgG titres could be detected in groups orally fed with plain chitosan microparticles alone, which may be due to non specific IgA stimulation but could not be confirmed. Very minor immune response could be detected after 14 days of oral vaccination with DT in PBS, which was enhanced to some extent after booster dose on 22nd day but the level was very low on 29th day. Following oral administration of DT loaded microparticles, measurable titres were found on 14th day which increased substantially on 22nd day, after booster dose. On 29th day the IgG level was low as compared to 22nd day but still the level was very high as compared to DT in PBS on 29th day. Highly elevated IgG titres could be found in CS-DT as compared to DT-PBS on all days of sampling.

The mean IgG level after oral vaccination with **Batch 28DT**, 40Lf CS-DT on 14th, 22nd and 29th day were 2.1415, 3.4030, and 2.9505 times higher than the level obtained after 40Lf DT-PBS. The titres with CS-DT were significantly higher than that of CS-PBS and DT-PBS, at the significance level as shown in **Table 55** on all sampling days.

Table 55: Statistical comparison of IgG titres from different formulation of DT using Student's t-test.

	Student's t-test					
Formulation	**CS-DT Vs CS-PBS**			**CS-DT Vs DT-PBS**		
Sample day	14	22	29	14	22	29
't' value	6.187	10.857	8.227	2.733	5.118	4.117
Significance Level at 'P' value	<0.001	<0.0001	<0.0001	<0.02	<0.0001	<0.001

From this in vivo experiment, it was concluded that the systemic immune response against DT was strongly enhanced after entrapment of vaccine in chitosan microparticles, whereas DT in PBS only induced only feeble immune responses. It was also observed that immune response was measurable on 14th day and increased after booster dose, indicating the formation of memory cells after initial dosing. This shows the significance of encapsulation of toxoids in chitosan microparticles. As observed during in-vitro studies, microparticles were intact in simulated gastric and intestinal conditions with only some release in gastric media and no release in intestinal media. This proves that microparticles must be taken up as such by intestinal mucosa probably through the PPs. It also indicates that there is formation of memory cells, resulting in increased response after administration of booster doses.

5.2.20 Assessment of Local Immune Response

For the TT and DT dose of 40Lf, which resulted in similar systemic responses as the positive control, the specific anti-TT and anti-DT IgA production in faeces, intestinal lavage and intestinal washings was determined over a period of 22 days to assess generation of local immune response.

5.2.20.1 Local immune response in intestinal lavage:-

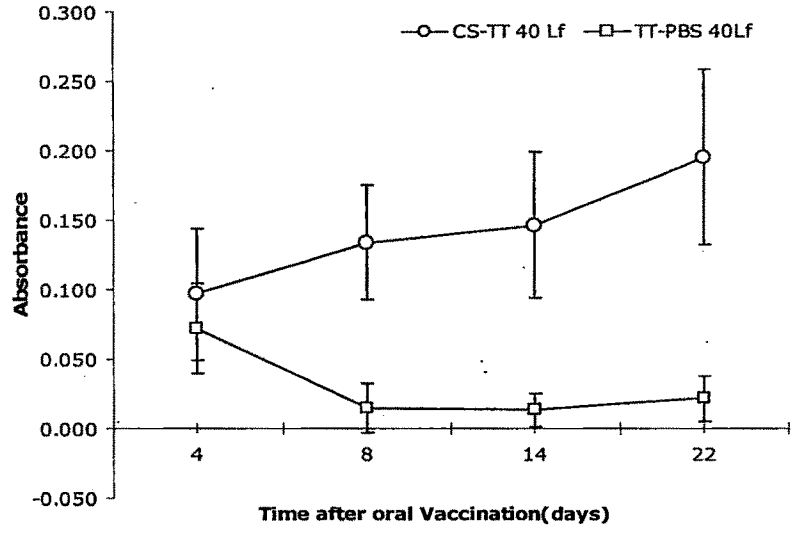
5.2.20.1.1 Local immune response in intestinal lavage for TT:-

IgA levels in intestinal lavage were studied by using Batch 27TT, Protocol 3 was used. IgA levels were determined using ELISA and shown in **Figure 58.**

Table 56: TT specific IgA levels in Intestinal Lavage

	Mean IgG Titre (IU/ml)± S.E.M.	
Sample Day	**CS-TT 40 Lf**	**TT-PBS 40Lf**
4	0.0965 ± 0.0470	0.0718 ± 0.0323
8	0.1338 ± 0.0413	0.0142 ± 0.0176
14	0.1460 ± 0.0525	0.0131 ± 0.0118
22	0.1949 ± 0.0630	0.0216 ± 0.0163

Figure 63: TT specific IgA levels in Intestinal Lavage



Local immune response in terms of IgA levels in the gastrointestinal tract started after 4 days and reached a maximum in about 22 days as shown in **Table 56** and **Figure 63 for Batch 27TT.** There was increase in IgA level after booster dose in the CS-TT but such trend was not observed for TT-PBS.

Statistical comparison of the data using Student's t-test is shown in **Table 57,** indicates that the IgA levels were not significantly different on 4th day sample but were significantly higher on 8th, 14th and 22nd day samples in CS-TT as compared to TT in PBS on respective days.

Table 57: Comparison of TT specific IgA levels in Intestinal Lavage using Student's t-test.

	Student's t-test			
Formulation	**CS-TT 40 Lf Vs TT-PBS 40 Lf**			
Sample Day	4	8	14	22
't' value	0.4317	2.6609	2.4652	2.8582
Significance Level at 'P' value	No Significance	<0.05	<0.05	<0.05

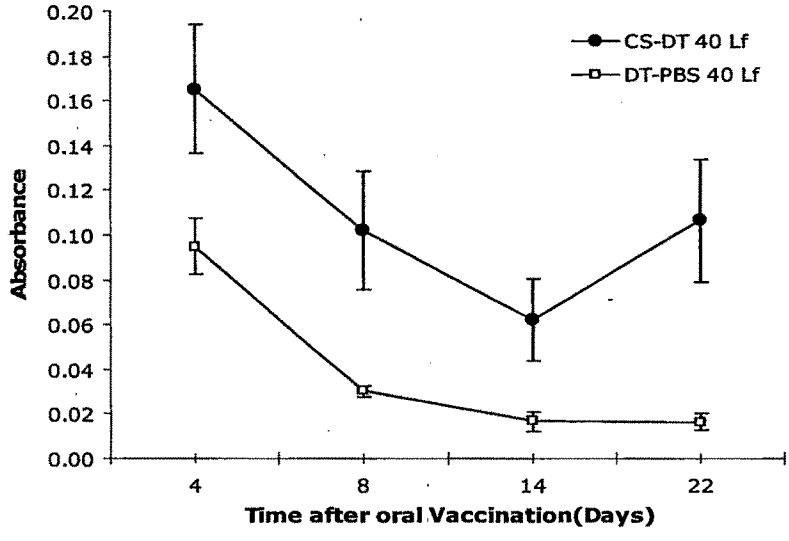
5.2.20.1.2 Local immune response in intestinal lavage for DT:

IgA levels in intestinal lavage were studied by using Batch 28DT, Protocol 3 was used followed. IgA levels were determined using ELISA and shown in **Table 58 and Figure 64.**

Table 58: DT specific IgA levels in Intestinal Lavage.

Mean IgG titre (IU/ml)± S.E.M.		
Sample Day	CS-DT 40 Lf	DT-PBS 40 LF
4	0.1651 ± 0.0287	0.0948 ± 0.0126
8	0.1020 ± 0.0264	0.0303 ± 0.0025
14	0.0621 ± 0.0184	0.0166 ± 0.0044
22	0.1065 ± 0.0273	0.0165 ± 0.0038

Figure 64: DT specific IgA levels in Intestinal Lavage.



The result show that IgA levels were high after the initial dosing i.e. on the 4th day and were significantly different than IgA levels obtained after administration of DT in PBS. There was decrease in IgA level in 8th day sample but still it was statistically significantly higher than the same day sample of DT in PBS. There was rise in local immune response in the gastrointestinal tract i.e. IgA levels, after booster dose but such trend was not observed for DT-PBS.

Statistical comparison of the data using Student's t-test is shown in **Table 59** shows that The levels were significantly higher on 4th, 8th, 14th and 22nd day sample in CS-DT compared to DT in PBS on respective days.

Table 59: Comparison of DT specific IgA levels in Intestinal Lavage using Student's t-test.

Student's t-test				
Formulation	CS-DT 40 Lf Vs DT-PBS 40 Lf			
Sample Day	4	8	14	22
't' value	2.239	2.705	2.409	3.266
Significance Level at 'P' value	p<0.05	<0.05	<0.05	<0.01

5.2.20.2 Local immune response in Faeces:

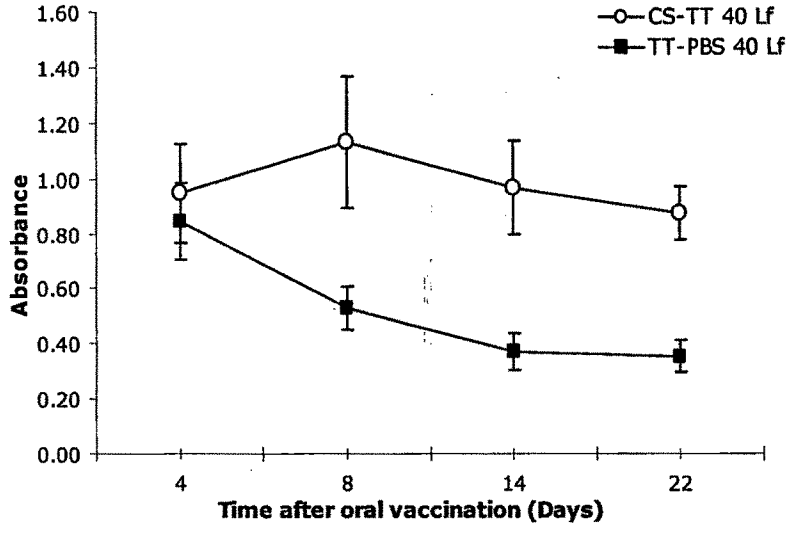
5.2.20.2.1 Local immune response in faeces for TT:

Local immune response in Faeces was studied by extracting the IgA using homogenization buffer and determining them using ELISA and shown in **Table 60** and **Figure 65.**

Table 60: TT specific IgA levels in Fecal Matter.

TT IgG titre (IU/ml) ± S.E.M.		
Sample Day	CS-TT 40Lf	TT-PBS 40Lf
4	0.9466 ± 0.1798	0.8449 ± 0.1388
8	1.1325 ± 0.2381	0.5270 ± 0.0793
14	0.9660 ± 0.1687	0.3714 ± 0.0664
22	0.8747 ± 0.0988	0.3554 ± 0.0589

Figure 65: TT specific IgA levels in Fecal Matter.



The local immune response in the gastrointestinal tract started after 4 days i.e. day after the priming was stopped. IgA level in 8th day sample was more as compared to 4th day sample indicating that there was continuous formation of IgA. There was higher IgA level on 22nd day as compared to TT in PBS. There was increase in IgA level after booster dose in the CS-TT but such trend was not observed for TT-PBS.

Statistical comparison of the data using Student's t-test is shown in **Table 61** indicates that IgA levels were not significantly different on 4th day but were significantly higher on 8th, 14th and 22nd day sample in CS-TT as compared to TT in PBS on respective days as shown in **Table 61.**

Table 61: Comparison of TT specific IgA levels in Fecal matter using Student's t-test.

Student's t-test				
Formulation	**CS-TT 40Lf Vs TT-PBS 40Lf**			
Sample Day	**4**	**8**	**14**	**22**
't' value	0.447	2.412	3.277	4.512
Significance Level at 'P' value	No significance	<0.05	<0.01	<0.001

5.2.20.2.2 Local immune response in faeces for DT:

Local immune response was studied in Faeces, by extracting the IgA using homogenization buffer. DT specific IgA levels in faeces were studied after administration of Batch 28DT, Protocol 3 was followed. IgA levels were determined using ELISA and shown in **Table 62** and **Figure 66.**

Table 62: DT specific IgA levels in Fecal Matter

DT IgG titre (IU/ml) ± S.E.M.		
Sample Day	**CS-DT 40Lf**	**DT-PBS**
4	1.0880 ± 0.2396	0.7401 ± 0.0847
8	0.6511 ± 0.0835	0.5160 ± 0.0966
14	0.4873 ± 0.0654	0.2795 ± 0.0403
22	0.6255 ± 0.1018	0.2063 ± 0.0450

Figure 66: DT specific IgA levels in Fecal Matter

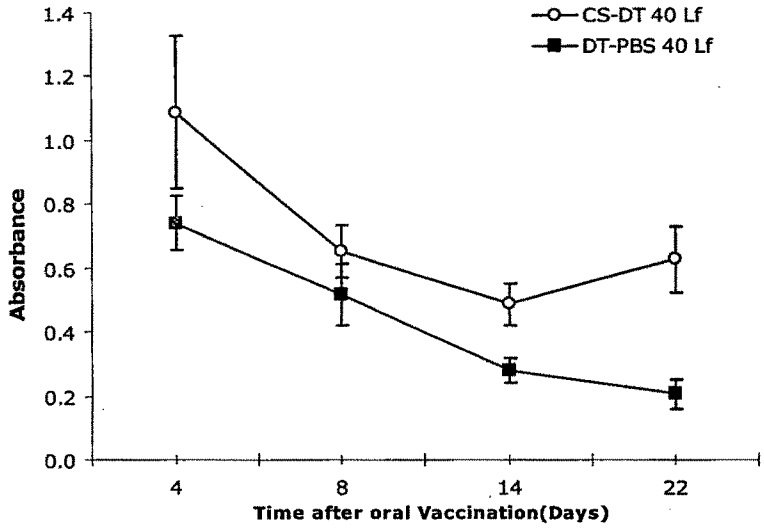


Figure 66 shows IgA levels in the faeces for **Batch 28DT and DT-PBS.** The local immune response in the gastrointestinal tract started after 4 days but there was decrease in IgA level in 8th day sample and 14th sample. But there was increased IgA level on 22nd day, which comes after administration of booster dose on 15th, 16th and 17th day. There was increase in IgA level after booster dose in the CS-DT but such trend was not observed for DT-PBS. Statistical comparison of the data using Student's t-test is shown in **Table 63.** The results show that IgA levels were not significantly different on 4th day sample. The levels were significantly higher on 8th, 14th and 22nd day in CS-DT sample as compared to DT in PBS on respective days as shown in **Table 63.**

Table 63 : Comparison of DT specific IgA levels in Fecal matter using Student's t-test.

Student's t-test				
Formulation	**CS-DT 40Lf Vs DT-PBS 40Lf**			
Sample Day	**4**	**8**	**14**	**22**
't' value	1.368	1.562	2.819	3.855
Significance Level at 'P' value	No significance	No significance	<0.02	<0.005

5.2.20.3 Local immune response in Intestinal washings:

5.2.20.3.1 Local immune response in Intestinal washings for TT:

At the end of the local immune response studies in intestinal lavage, faeces the study animals were sacrificed and intestinal washings of the animals were analyzed for the IgA levels.

The results **(Table 64)** show that the levels were significantly higher, when compared using Student's t-test, in CS-TT ($p < 0.001$) than TT-PBS on the 22nd day.

Table 64: TT specific IgA level in Intestinal washings.

	Abs ± SEM	't' value	Significance Level at 'P' value
CS-TT	0.488 ± 0.049	6.012	<0.001
TT-PBS	0.152 ± 0.025		

5.2.20.3.2 Local immune response in Intestinal washings for DT:

Immune response for **Batch 28DT** of encapuslated DT was studied in intestinal washings of the animals at the end of study by following Protocol 3.

Table 65 shows IgA levels in the intestinal wash taken after sacrificing animal on the last i.e. on 22nd day. The levels were significantly higher, compared using Student's t-test, in CS-DT ($p < 0.001$) than DT-PBS.

Table 65: DT specific IgA level in Intestinal washings

	Abs ± S.E.M.	't' value	Significance Level at 'P' value
CS-DT	0.166 ± 0.013	4.283	<0.001
DT-PBS	0.109 ± 0.006		

Though this study is a single end point study, it gives clear differentiation between levels of TT and DT specific IgA obtained after administration (priming and booster doses) of TT and DT chitosan microparticles and plain TT and DT in PBS. These results of Intestinal washing study complement the observation of the previous studies in

Intestinal Lavage and Faeces extract that there was enhanced response to the chitosan microencapsulated TT and DT as compared to TT or DT in PBS.

Thus, the results of the oral vaccination studies in mice showed that the systemic and local mucosal immune response was strongly enhanced after incorporating the vaccine into chitosan microparticles. Systemic immune response was dose dependent. Induction of immune response shows that the antigenicity of the TT and DT was maintained during the microencapsulation and size reduction process.

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