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Full Length Research Paper

Large scale recovery of tetanus toxin and toxoid from fermentation broth by microporous tangential flow filtration

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Abstract

The commercial production of purified tetanus toxoid mainly depends on the effective separation of the bacterial toxin and toxoid from large volumes of fermentation broth of *Clostridium tetani* (Harvard 49205) vaccine strain. Tangential flow or cross-flow filtration system was used as rapid drive in the processing of immunobiological assays of tetanus toxin. Tetanus toxoid was prepared by detoxifying the culture filtrates of *C. tetani* and further purified by ultrafiltration, salt fractionation and adsorption onto aluminium phosphate. Present study deals with the separation of tetanus toxins using a microporous membrane (0.22 µm) and concentration of tetanus toxoids using an ultrafiltration membrane (30 kDa, NMWL pore size) with operational variables like average trans-membrane pressure (ATP), cross flow rate, flux. Under the best conditions, >96% recovery was achieved. Additionally, potency control of 10 batches of tetanus toxoid, prepared from the filtered toxins/toxoid lots by microporous tangential flow filtration system, was evaluated by *in vitro* passive haemagglutination (PHA) assay and the results obtained in the *in vitro* PHA were compared with *in vivo* toxin neutralization (TN) test. An excellent correlation between *in vitro* test and *in vivo* TN test was observed by Spearman's correlation coefficient. It reveals that the process development in which employing available equipment and the *in vitro* PHA is a promising alternative to the toxic TN test in the potency assay of tetanus vaccine.

Key words: Tetanus toxin, Seitz filtration, tangential flow filtration, microfiltration module, flux, tetanus toxoid, immunogenicity test.

INTRODUCTION

Tetanus is an infectious bacterial disease caused by a highly toxinogenic strain of bacillus *Clostridium tetani*. Under favorable anaerobic conditions in necrotic wounds, and with dirt, this ubiquitous bacillus may produce tetanospasmin, a highly potent neurotoxin. This bacterial toxin is the second most potent toxin, known after botulinus toxins with a minimal lethal dose of less than 2.5 ng kg^{-1} of human body weight and blocks inhibitory neurotransmitters in the central nervous system and causes the muscular stiffness and spasms typical of generalized tetanus (WHO, 2006). Tetanus toxin is synthesized intracellularly by *C. tetani* as a single polypeptide chain with a molecular weight of 150 kDa during the logarithmic phage. After cell lysis, the toxin is released into the surrounding medium and cleaved by endogenous protease enzyme to give an NH_2-terminal light chain of 50 kDa (toxic moiety) and COOH-terminal heavy chain of 100 kDa (binding). The light and heavy chains are held together by disulfide bridge (Plotkin and Orenstein, 1999).

Now-a-days, basic immunization of infants against teta-

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tetanus is usually given with the combined adsorbed Diphtheria-Tetanus-Pertussis (DTP) vaccine, which results in formation of protective tetanus antitoxin in serum. Presently, many manufacturer produce DTP group of vaccines for mass immunization program using large volume capacity bioreactors. In these bioreactors, after the end of their incubation period, the concentration of toxin in supernatant usually reaches 80 to 120 floccula-tion units. The separation of bacterial cells, detoxification, concentration and sterile filtration of toxoids are some of the major unit operations in the processing of large volumes of fermentation media in the production of bacterial vaccines.

The separation of cells is achieved traditionally by centrifugation or by the dead-ended depth filtration method. Such solid-liquid separation methods are time consuming and toxin loss may exceed 25%. Furthermore, membrane filters separate components and suspensions on the basis of molecule size and it is hard to validate a depth filtration system, which is mandatory under current Goods Manufacturing Practices (cGMP). In the conventional method, the dead-end filtration, incorporating filter pads of suitable pore sizes, the separation must provide complete retention of cells and maximal passage and recovery of soluble end-product. When tetanus fermentation broth is clarified, the large bacterial mass blocks most of the dead ended filtration pads requiring still higher inlet pressures or change of pads altogether. The clarified toxin solution is then polished by passing it through pad filters to produce sterile tetanus toxin. Additionally, the pads also adsorb the active material, resulting in reduced yields. For these reasons, an alternative bio-separation technique was looked at seriously, that could be validated, and the trials were conducted. Membrane-based methods have been widely suggested for the processing of bioactive species (Reid and Adlam, 1974; Tanny et al., 1980; Brown and Kavanogh, 1987). In modern filtration techniques, microporous tangential flow filtration (TFF) system, the particles or solutes retained by membrane are continuously removed in the retentate flowing tangentially across the membrane surface. The clarified solution flows through the membrane into permeate, also called the cross flow filtration. The TFF system provides a practical and economical alternative to the dead ended filtration. The entire operation conforms to the GMP norms and can be validated as and when required (Rao et al., 1992; Levine and Castillo, 1999).

In this study, the conventional depth filtration method normally used for the clarification of tetanus culture broth has been successfully replaced with that of microporous TFF system for large scale recovering of tetanus toxin at a commercial level and further use of a similar system for concentration of crude tetanus toxoid. The study establishes operation parameters like ATP, cross flow rate, flux that optimizes overall filtration, concentration and result in improvement in the preparation of purified tetanus toxoid. The TFF system was completed and validated for GMP point of view. This paper also describes immunogenicity tests on the tetanus vaccines, prepared from the filtered toxin/toxoid lots by TFF system, the *in vivo* toxin neutraliza-tion (TN) test and *in vitro* passive haemagglutination assay (PHA), and results were compared by Spearman's corre-lation coefficient.

MATERIALS AND METHODS

Strain and culture medium for production of toxin

The Harvard strain of *C. tetani* (obtained in lyophilized state from Central Research Institute, Kasauli, India, A National Control Authority) was used for the production of tetanus toxin in fermenter vessels (500 L). Modified Mueller Miller (MMM) medium was used for growing *C. tetani* (WHO, 1977). The fermentation vessel had a working volume of 400 L. The pH of medium was adjusted to 7.4 with 40% sodium hydro-xide solution. Sterilization of the medium was carried out by steam at 115°C for 20 min and after cooling it down to 35°C. it was inoculated with one day seed and prolonged for about one week at 35°C under continuous mild agitation and aeration. At the end of the incubation period, samples were drawn for purity checks and estimation of antigen concentration.

Clarification of fermentation broth

The fermented broth showed a pure and satisfactory concentration of the toxin (Lf mL^{-1}), it was then clarified by (i) centrifugation (ii) Seitz filtration (iii) tangential flow filtration to study the toxin recovery. Tetanus cells were centrifuged (Model-Sorvall RC 3B plus, Sorvall Instruments, New Town, Germany) at a speed of 4000 rpm. The load (tetanus culture broth filled in polypropylene containers 6 x 1 l) was distributed symmetrically around the rotating assembly and centrifuged for 60 min using the timer mode. The supernatant, which contained the toxin, was collected.

Conventional method

Using the conventional method, the alternative was Seitz filtration, which is a dead-ended depth filtration method. With this method, the fermented culture fluid was then first clarified by filtration through T500 Seitz filter pads, 20 x 20 cm, 0.45 µ pore size (Seitz Werke Bad Kreuznach, West Germany) and subsequently sterilized by filtration through EKS Seitz filter pads, 20 x 20 cm, 0.22 µ pore size using a filter press assembly (Straussburger, West Germany) which hold 14 pads. This operation was carried out at 35°C under 12 *psi* pressure. After collecting 80 to 100 L of filtrate, physiological saline was pumped into the body to filter the balance toxin.

Tangential flow filtration method

This method was carried out using a Millipore's Prostak system and Pellicon system with relevant open-channel modules for rapid clarification of tetanus culture broth and concentration of crude toxoid, respectively. These open-channel modules are tangential flow stacked plate membrane devices with open feed channels and are available with both microporous membranes (Prostak MF modules) and ultrafiltration membranes (Pellicon, UF modules). The separation between ultrafiltration and microfiltration is based on the pore size of the membranes. The Prostak system (Sys No. TFF05P178; Cat.No.BM5AN8834; Millipore) includes prostak holder, Rotary Lobe pump (Jhonson Pumpen AG, Wadenswil, Switzerland) coupled with a motor and the speed of the same is controlled through a variable speed controller and a feed tank. The

insulation consists of temperature and pressure gauges, flow sensors, air flow meter and sanitary piping. A sanitary type diaphragm valve was also installed on the retentate line, which is used in controlling the trans-membrane pressure, across the membrane. The Prostak holder is designed to accommodate modules with serpentine flow path from feed channel to retentate channel.

The Prostak MF modules (Catalogue No PSGV AG201, 0.22 µm pore size) are made of void free composite microporous hydrophilic polyvinylidene diflouride (PVDF) membrane with the Millipore (Molshein, France) trade name Durapore. The material is autoclavable, a very low protein-binding characteristic and has broad chemical compatibility. The system with open-channel modules is fully on-line steam sterilizable. The filter area can be varied from 10 to 50 ft^2. In the Prostak system, five 20 ft^2 Prostak MF modules were installed in series and the system was operated at a cross flow of 2000 L h^{-1} at an operating temperature of 23°C.

Toxoid preparation

After the clarification of fermented broth in the Prostak system, the filtrate containing the toxin was detoxified by adding 0.5% (v/v) formaldehyde (35-40% AR grade). The containers were incubated at 36°C for 4 weeks to obtain the crude toxoid and then the specific toxicity was checked. Once the toxoid passed the specific toxicity test, it was then concentrated using the Pellicon system, described subsequently (WHO, 1977; Pasteur Institute of India, 1991).

Concentration of toxoid using Pellicon TFF system

Concentration is a pressure driven membrane process used to concentrate, separate or purify macromolecules. The separation is based on molecular weight of the macromolecule. The membrane is a thin semi-permeable polymeric material that will retain macromolecules and allow smaller dissolved solutes to pass through the membrane. The pore sizes of ultrafiltration membranes are in the range of 0.001 to 0.1 µm and the retentative abilities of ultrafiltration membranes are described by the nominal weight limit (NMWL) (Zeman and Zydney, 1996).

Concentration of crude tetanus toxoid was determined using a Pellicon TFF system with ultrafiltration membranes. The ultrafiltration membranes are available in a range of molecular weight cut-offs of 500 Daltons (Da) to 1000 KDa and offer filtration areas of 0.5 (5.5 ft^2) and 2.5 m^2 (27.5 ft^2). The Pellicon TFF system (Sys No. TFF05C184; Cat.No.BM5CN9473; Millipore) accommodates an acrylic filter holder and other insulations as in the case of Prostak TFF system. The basic material of construction is modified polyethersulfone with a pore size of 30 kDa (NMWL) with the trade name Biomax-30. The material has a higher flux, excellent chemical resistant, integrity testable, void-free structure for higher yield and reliability. The batch size at this stage of concentration of tetanus toxoid was in the range of 800 to 1000 L. The molecular weight of tetanus toxoid is about 150 kDa, which includes the monomer, dimers and oligomers of various sizes. The area of the device Biomax 30K NMWL cassettes was 50 ft^2. Two cassettes were installed in the acrylic holder of the Pellicon system and the system was operated at a cross flow of 1000 L h^{-1} at an operating temperature of 25°C.

Both the TFF systems were flushed with sterile distilled water as recommended by the manufacturer. The clean water flux was measured to assess the cleaning efficiency after each trial. After optimization of the operating parameters based on flow and pressure excursion experiments, the volume of permeate (clarification of broth by Prostak system) and retentate (concentration of toxoid by Pellicon system) was measured over time until the known initial volume of the broth/toxoid had been reduced 10-fold (10x level). At this point, the cell and module wash started to recover completely the balance toxin/toxoid retained.

To clean the modules between experiments, the TFF systems were flushed with sterile normal saline followed by warm distilled water (40°C). During cleaning procedure, the pump was operated at a higher velocity to flush out all material. Then the prostak and pellicon systems were sanitized with sodium hypochlorite (600 ppm) and 0.1 N sodium hydroxide solutions, respectively. A 2% formalde-hyde solution was used as storage agent for both the modules to ensure membranes still wet and to prevent microbiological growth without damage to the filter. Prior to the formaldehyde step, the clean water flux was measured to assess the cleaning effectiveness.

Validation of the TFF system

The selection of a membrane, as specified earlier, was mainly guided by the possibility of validation of the system. Validation of the system included: (i) determination of the pre-and post-operation integrity testing of the membrane device, which ensured repeated use, (ii) removal of the storage agent, i.e., formalin from the modules. Formalin was removed by flushing the system with distilled water. Samples were collected at different intervals. To 3 ml sample, 0.5 ml chromotropic acid was added and the mixture was placed in boiling water bath for 5 min. The presence of formalin was confirmed by the development of pink colour, (iii) checking absence of *C. tetani* – as the membrane used was of pore size 0.22 µ, the absence of *C. tetani* from the clarified toxin needed to be validated. This was carried out by passing a representative sample of 250 ml through the GVWP disc (Durapore PVDF 0.22µ disc), which was placed on the surface of MMM agar. On another MMM agar plate, overnight grown culture of *C. tetani* in fluid thioglycollate medium was streaked as a positive control, (iv) sanitization of the system – for sanitizing the Prostak TFF and Pellicon TFF systems, sodium hypochlorite and NaOH were used, respectively. The cleaning procedure ensured removal of the cleaning agent. Removal of sodium hypochlorite (below 10 ppm)/NaOH was confirmed by comparing serially diluted sample of sodium hypochlorite/NaOH with a universal indicator.

TT vaccine lot preparation

The crude tetanus toxoid was concentrated, purified by fractionation with ammonium sulphate and adsorbed onto aluminium phosphate gel. It is supplied as a sterile solution in physiological buffer usually with preservative. Ten licensed lots of adsorbed tetanus toxoid vaccine, containing 10 Lf mL^{-1} of tetanus toxoid adsorbed onto 3.0 mg mL^{-1} of aluminium phosphate were used for immunogenicity study.

Analytical methods

Flocculation test (Lf)

The immunological precipitation test was carried out to determine the concentration of toxin or toxoid, which is useful for calculating the antigen content. The antigen content is a good indicator of consistency of production. The tetanus antitoxin (100 Lf ml^{-1}) supplied by the quality control division of this institute was standardized against the international reference reagent of tetanus toxoid. Lf per ml of toxin/toxoid was determined by the method of Ramon as explained in the WHO manual (WHO, 1994a).

Minimum lethal dose (MLD)

The MLD test was performed on mice with a weight range of 17 to 20 g. The purpose of the test is to confirm that the strain used really produces toxin. The MLD of tetanus toxin was determined by the method described in the WHO manual (WHO, 1994b).

Protein nitrogen assay

The total protein nitrogen content of the toxin/toxoid is determined by the method described by Kjeldahl, which is the standard method included in pharmacopeias (WHO, 1994c). The ratio of toxin/toxoid concentration (Lf ml^{-1}) to protein nitrogen concentration (mg PN ml^{-1}) denoted the antigenic purity of toxin/toxoid and was expressed as Lf per mg of protein nitrogen.

Immunogenicity/potency test

The potency test was done on the tetanus vaccines to predict the effectiveness of vaccine in humans by using animal models (guinea pigs and mice). The classical *in vivo* TN test and serological *in vitro* PHA have been employed to determine the end point of tetanus antitoxin content (WHO, 1990; Galazka, 1993). The TN test was performed in mice and it measured the amount of antitoxin, which could neutralize the toxic activity of the toxin. The TN test was the reference assay for the PHA in use. It was standardized in order to correlate them with a known level of immune response seen in the *in vivo* test.

In vivo toxin neutralization test

The *in vivo* TN test on pooled sera was performed in mice at Lp/200 dose level of tetanus toxin according to the Indian Pharmacopoeia potency method (Indian Pharmacopoeia, 1985). The Lp/200 toxin dose level is defined as the minimum amount of tetanus toxin which when mixed with 0.005 (1/200) IU tetanus antitoxin causes a defined degree of tetanic paralysis in mouse of a defined weight in four days. The level of antitoxin was calculated against the standard and expressed in IU/ml. The Lp/200 dose of the toxin was 0.00008 Lf. The tetanus antitoxin levels of most serum pools were tested at end points level.

The total protein nitrogen content of the toxin/toxoid is determined using Kjeldahl Method.

In vitro passive haemagglutination assay

The *in vitro* PHA was performed by the method described in the WHO manual (WHO, 1997). According to which, the purified antigen was covalently coupled to tannin-treated turkey erythrocytes. Briefly, pooled serum samples for individual TT vaccine lots were prediluted at 1/20 in PBS, pH 7.2. Each serum samples and standard tetanus antitoxin were tested in duplicate. For 2-fold dilution, all the wells were filled with 50 µl of PBS, pH 7.2 except for wells A12-D12, which were filled with 25 µl of PBS, pH 7.2. All the wells of row 1A-10A were filled with 50 µl of 1/20 diluted serum and the well of row 11A was filed with 50 µl of PHA reference serum (1 IU/ml) and mixed well. The 50 µl of the mixture in the wells of the row 1A-11A were transferred to the next wells of the row 1B-11B and mixed well and the transferring and mixing process was continued through all the rows from B-H. To equalize the volumes, 50 µl from each of the wells of the row 1H-11H were ultimately discarded. A volume of 25 µl of the 1/20 prediluted positive control serum was added in the wells A12-D12, which served as positive control and the wells E12-H12, used as a negative control with PBS only. To all the wells was then added 50 µl of the diluted red blood cell suspension. Microtitre plates were incubated at room temperature for 20 min. The tetanus antibody titre in the serum samples was estimated by optical reading of agglutination and expressed in IU/ml by multiplying the reciprocal of the specific titre of the reference serum with the sensitivity of the test serum. The titration of the tetanus reference serum was found to be 0.0078 IU/ml. The PHA titres were converted into antitoxin units (AU or IU) by running in parallel with the test sera and a reference serum with known antitoxin content, determined and calibrated against the WHO standard serum in IU/ml.

Statistical analysis

The Spearman's correlation coefficient was used to compare the relationship between *in vitro* PHA method and *in vivo* TNT. These statistical analyses were done at KMCH College of Health Sciences, Coimbatore, India using the Statistical Package of Social Sciences – SPSS software, version 11.0 (SPSS Inc.2000-01).

RESULTS AND DISCUSSION

Clarification by traditional methods

A high biomass was produced by stationary pot culture method and fermentor cultivation (Pasteur Institute of India 1991). Applying the methods of centrifugation and Seitz filtration, a volume of 100 L culture was processed as a single batch. Two and four trails were carried out by centrifugation and Seitz filtration, respectively and the output of each batch was 80 to 100 L filtrate. The Lf titre was estimated and the total titre was calculated. An average toxin recovery of about 95% was obtained. Even though the recovery is good, there is an inherent danger of potentially hazardous aerosol formation, spillage and the process is maximum exposure and labor intensive. Table 1 shows the result of four clarification trials by Seitz filtration method. From Table 1, it is clear that the maximum toxin recovery obtainable was about 88 to 90%. This was mainly because of the high hold-up volume, which resulted in dilution. Further, there must be some toxin loss due to adsorption by the filter pads. The initial feed pressure (4-8 psi) increased to 12 psi due to the build-up of cell debris from fermentation broth and the flow through the filter was reduced considerably. Moreover, the process seriously lacked scale-up criteria. This method required 150 to 200 filter pads to filter 400 L broth. The output thus obtained was subjected to another depth filtration using EKS pads and 28 pads were required to obtain a sterile product. The procedure detailed required change of pads during the process. The process time for a 400 L batch was usually about 8 to 9 h including post operation sanitation. A slight variation in inlet pressure results in a turbid output, needing yet another clarification cycle. The problem was encountered in 14 out of 40 batches processed in a year. Cost of each filter pads is very expensive.

Clarification and concentration by tangential flow filtration method

In the recent past, the demand for tetanus toxoid has been very high. To enhance production, our organization introduced a pilot scale bioreactor to produce 400 L of culture in the batch mode. Before this study, the standard method for clarification was Seitz filtration. The tetanus culture broth was divided into 4 lots of 100 L. Considerable loss in the yield of toxin was noticed apart from the process being time consuming and labor intensive. In order to find a suitable solution to the above problems, a preliminary trial

Table 1. Tetanus toxin recovery in production batches clarified by Seitz filtration.

Trail number	Volume of culture broth (L)	Flocculation unit in culture broth (Lf/ml)	pH	MLD (in million)	Volume of toxin collected (L)	Flocculation unit in toxin (Lf/ml)	Recovery (%)[a]
SF-1	100	120	7.60	8	106	90	88.8
SF-2	100	100	7.53	4	102	80	89.1
SF-3	100	110	7.55	8	104	85	90.0
SF-4	100	120	7.58	8	107	90	89.2

MLD- Minimum lethal dose (WHO requirements); [a]Recovery is calculated based on the total Lf titre in the broth after reducing 10% of the initial volume (which is equivalent to the volume of the biomass).

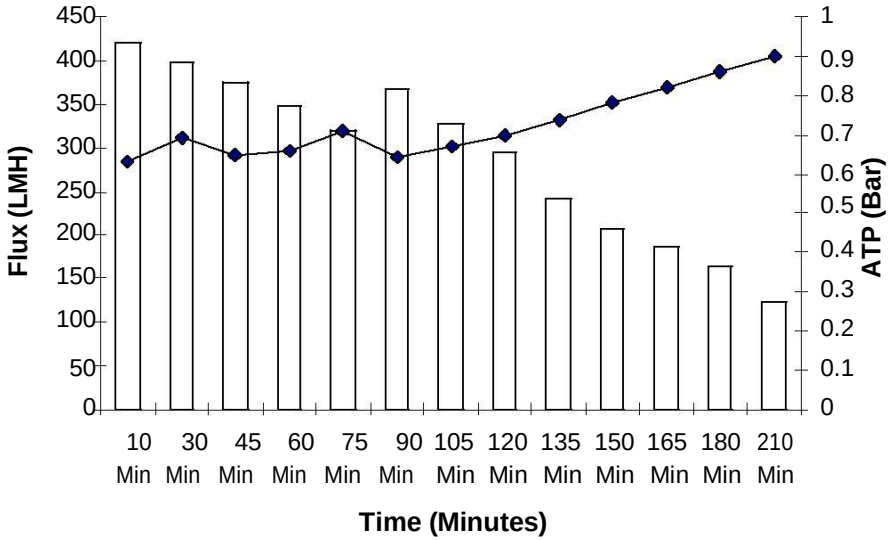

Figure 1. Product flux profile of tetanus toxin clarified through Prostak modules. Conditions: 0.22-µm pore size; 100-ft^2 filter area; process volume 400.

was conducted on a pilot unit of TFF system using two open-channel 2 ft^2 modules with hydrophilic PVDF membrane of 0.22 µm pore size (Type GVPP; Durapore) by the Millipore India team under the initiative of UNICEF. The trials showed better recovery and also effective and econo-mical clarification of the tetanus toxin fermentation due to good flux. The data were scaled up for processing a 400 L batch and also processed with the commercial-scale data on the other unit operation. According to Ravetkar et al. (2001) the tetanus toxin recovery was >95% for a batch volume of 100 L.

Unlike “dead-end” filtration, the product fluid flows tangentially across the surface of the membrane. Briefly, the product fluid present in the feed tank enters the system through the pump and flows into the feed manifold of Prostak/Pellicon holder through the strainer in order to separate coarse particles on the fluid then flows into the Prostak/Pellicon modules and tangentially across the membrane of the different plates. Materials smaller than the pore size are able to pass through the membrane as filtrate (or permeate), which can exist from the module at its outlet and flows out of the filtrate tubing and collected in a collection tank. The membranes retain large particles (mammalian and bacterial cells, particles and high molecular weight organics). The retained material is called retentate (or concentrate), which is returned to the feed tank due to the action of the pump. This sweeping action helps to keep retained material from settling and eventually restricting the flow.

The experimental trails with Prostak module at maximum pump speed (pump speed 400) and diaphragm valve (60 to 80% open) showed an initial permeate flow rate of 9 L/min at a cross-flow rate of 1800 L/h. The permeate flow rate gradually reduced to 4.5 L/min at the end. The flux is expressed in liters per square meter per hour (LMH) and the decay profile is given in Figure 1. An average trans-membrane pressure (ATP) was initially 8.7 psi. There was a gradual increase of ATP as the cell concentration increased and stabilized at 0.9 bar during the cell wash step (Table 2).

An average trans-membrane pressure (ATP) of 0.8 bar was kept by adjusting the pump speed (300 rpm) and diaphragm valve (30% open) in the retentate line in the case of the Pellicon module. The ATP was increased, as the concentration of low molecular weight species was so high and stabilized at 1.1 bar. Comparing both the modules.

Table 2. Average trans-membrane pressure (ATP) during tetanus toxin clarification using Prostak modules.

Volume in feed tank (L)	Permeate volume (L)	Concentration factor (X)	Pressure inlet (P_{IN}) bar	Pressure outlet (P_{OUT}) bar	Pressure permeate (P_{PER}) bar	ATP[a] bar (P_{in} + P_{OUT} - P_{PER})
400	0	1	0.8	0.4	0.0	0.60
200	200	2	0.8	0.4	0.0	0.60
150	250	3	0.8	0.5	0.0	0.65
125	275	4	0.9	0.5	0.0	0.70
110	290	5	1.0	0.5	0.0	0.75
100	300	6	1.0	0.6	0.0	0.80
88	312	8	1.0	0.6	0.0	0.80
80	320	10	1.2	0.6	0.0	0.90

[a]Average value in five trails; Conditions: 0.22-µm pore size; 20- ft^2 filter area.

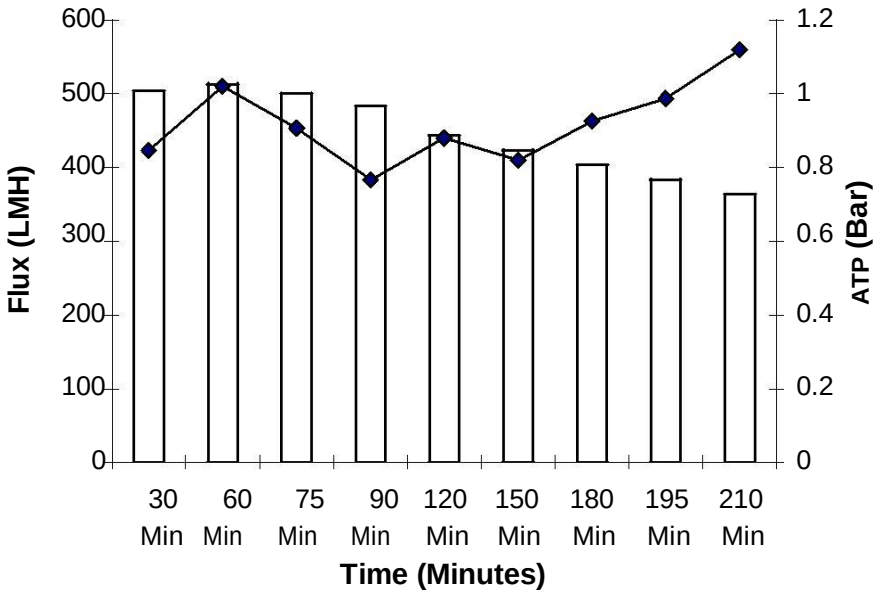

Figure 2. Flux decay of tetanus toxoid concentrated through Pellicon modules. Conditions: 30 kD pore size; 50-ft^2 filter area; process volume 950.

modules, there was no great change in ATP, denoting that the cross flow was optimal. The concentration polarization layer (gel layer) formed by the bacterial cells and low molecular weight species was acting to retard the flow of toxin and toxoid either through the polarization layer or the membrane itself as evidenced by the rapid reduction in product flux at the start (Figure 2). The product flux obtained is similar to the pattern observed in the Prostak system. The higher flux obtained in the Prostak TFF sys-tem is mainly due to the large filtration area (100 ft^2) and higher capacity pump, which gives an enhanced cross flow rate. The TFF permitted the more rapid removal of macro-molecules such as formaldehyde than the traditional dia-lysis process; it reduces the level of residual formaldehyde by about 50 fold (Vancetto et al., 1997).

The recovery rate for tetanus toxin and toxoid using the Prostak modules (5 trails) and Pellicon modules (5 trails), respectively are given in Tables 3 and 4. Consistently, >96% yield was obtained in all the trials. Additional trials were conducted in the Prostak modules to find out the effect of pH and MLD on toxin recovery. The batches from routine production were selected in such a way that the pH and MLD at the time of harvesting ranged from 7.0 to 7.6 and 1 to 8 million, respectively. The recovery was identical (>96%) in all the experiments and required 4.5 h including post operation sanitation for the filtration and concentration of 400 L batch fermentation broth and 950 L crude toxoid, respectively. The benefits of TFF system for clarification are (i) minimal exposure of the working personnel as it is a closed operation and no spillage, (ii) no recurring cost of pads (iii) Savings on autoclaving charges for sterilization of filter assemblies, (iv) savings on disposal expenses like decontamination and incineration of used pads, (v) no problem of final sterile filtration, as 0.2 $\propto$ filtered output was

Table 3. Tetanus toxin recovery by tangential flow filtration.

Trail number[a]	Volume of culture broth (L)	Flocculation unit in culture broth (Lf/ml)	Volume of saline wash (L)	Volume of toxin collected (L)	Flocculation unit in culture broth (Lf/ml)	Recovery (%)[b]
PRO -1	400	110	120	480	80	97.0
PRO-2	400	95	120	470	70	96.2
PRO -3	400	80	120	470	60	98.0
PRO-4	400	120	120	490	85	96.4
PRO-5	400	90	120	480	65	96. 3

[a]Toxin recovery using Prostak modules (PRO-1to5). [b]Recovery is calculated based on the total Lf titre in the culture broth after reducing 10% of the initial volume (which is equivalent to the volume of the biomass).

Table 4. Tetanus toxoid recovery by tangential flow filtration.

Trail no.[a]	Volume of toxoid (L)	Flocculation unit in toxoid (Lf/ml)	Volume of saline wash (L)	Volume of toxoid collected (L)	Flocculation unit in toxoid (Lf/ml)	Recovery (%)[b]
CON-1	970	70	30	29	2300	98.2
CON -2	960	60	30	35	1600	97.2
CON –3	970	90	30	33	2600	98.2
CON –4	950	85	30	31	2600	99.8
CON –5	980	65	30	30	2100	98.7

[a]Toxoid recovery using Pellicon modules (CON-1to 5); [b]Recovery is calculated based on the total Lf titre in the crude toxoid after concentration of the initial volume.

used.

The decline in flux with time in both the modules suggested a dynamic gel layer formed on the membrane surface as evidenced by the reduction in Lf titre. As the cell concentration increased, so too did the instantaneous concentration of toxin in the permeate (Figure 3). The presence of a significant concentration of toxin in permeates was also indicative of toxin retention by the above layer. The optimal cross flow and membrane wash procedure enhanced the recovery of toxin/toxoid to a great extent.

The establishment of an effective cleaning strategy was our next goal. The same Prostak / Pellicon modules were used for 5 successive clarification/concentration and cleaning cycles. After sanitation with sodium hypochlorite /0.1 N NaOH and cleaning, the normalized water permeability (NWP) was calculated and compared with the original value. The NWP of Prostak module after each cleaning cycle was over 90% of the original value (742.87 /h/m²/bar at 25°C) indicative of correct cleaning protocols. Further, the same Prostak and Pellicon modules were employed for the clarification of toxin and concentration of toxoid, respectively from regular production batches. Over a span of 6 years, about 60,000 L of culture broth and about 40,000 L of crude toxoid were clarified and concentrated, respectively before irreversible clogging set in.

The toxin clarified by Seitz filtration or centrifugation needed polishing filtration prior to sterile filtration through 0.22 µm filter pads. The quality of the toxin filtrate obtained by TFF was excellent as judged by its bright, shiny appearance and the ease with which it passed through a sterile cartridge. The toxin obtained from 5 trials in the Prostak module was detoxified to toxoid by treatment with 0.5% (v/v) formaldehyde. The toxoid obtained from 5 trials in the Pellicon module was ammonium sulphate fractionated, dialyzed and finally sterile filtered to yield a purified toxoid ready for formulation into a vaccine. The potency control of these formulated tetanus vaccine was evaluated by the *in vivo* TNT and *in vitro* PHA according to the methods described in this study (Indian Pharmacopoeia, 1985; WHO, 1997). Table 5 summarizes the antigenic purity and tetanus antitoxin levels determined by *in vivo* TNT and *in vitro* PHA in pooled sera from guinea pigs immunized with different TT vaccine. The antigenic purity ranged from 1500 to 2000 Lf per mg of protein nitrogen. According to the WHO requirements, the purity of TT for the preparation of vaccine should not be less than 1000 Lf per mg/PN (WHO, 1990). All the preparations had anti-genic purities higher than the minimum purity required by the WHO regulations.

An *in vitro* PHA method for titration of tetanus antitoxin in sera of guinea pigs immunized with tetanus vaccines is shown in Figure 4. The results obtained in the *in vitro* PHA were compared with the *in vivo* TNT used routinely to perform the potency control of tetanus component in adsorbed vaccines. The correlation coefficient was bet-ween the *in vitro* and *in vivo* tests by Spearman's corre-lation coefficient (Figure 5). According to this coefficient, a perfect correlation, positive or negative, was determined

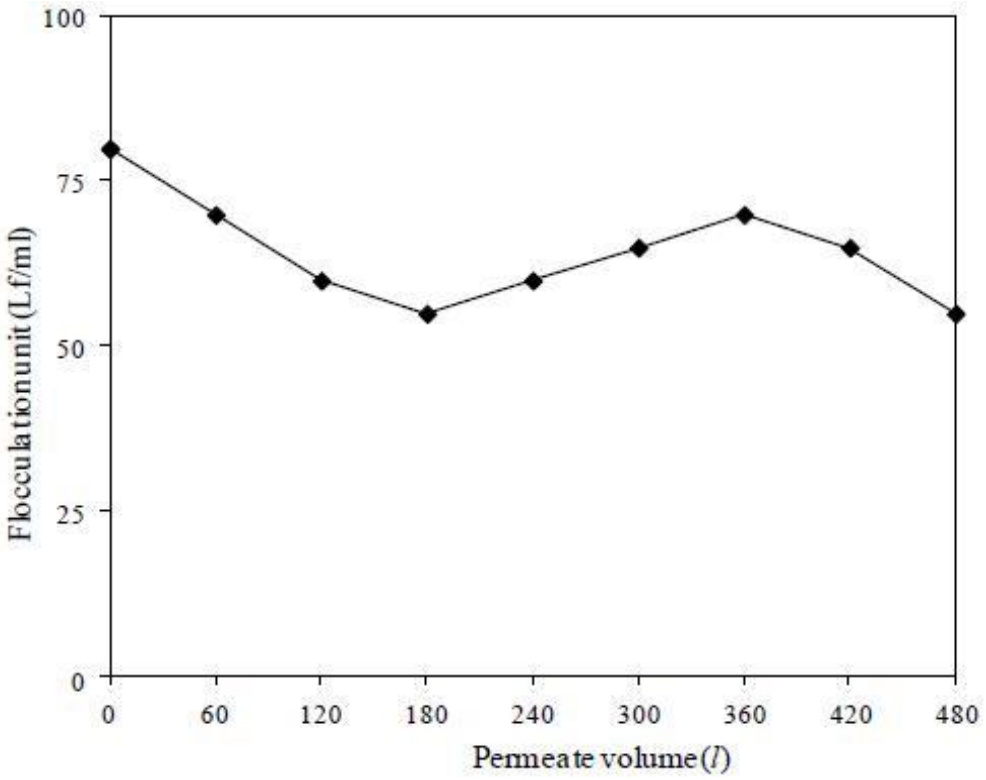

Figure 3. Lf titre of tetanus toxin clarified through Prostak modules. Conditions: 0.22-µm pore size; 100-ft^2 filter area; process volume 400.

Table 5. Potency of tetanus toxoid vaccines estimated by *in vivo* TNT and *in vitro* PHA.

Vaccine lots[a]	Antigenic purity Lf/mg PN	Tetanus antitoxin levels (IU/ml) determined by	
		***In vivo* TNT**	***In vitro* PHA**
TFF-TT-1	1970	6	5
TFF-TT-2	1984	7	10
TFF-TT-3	1388	4	2.5
TFF-TT-4	1870	5	5
TFF-TT-5	1913	5	5
TFF-TT-6	1639	4	2.5
TFF-TT-7	1373	2	2.5
TFF-TT-8	1724	4	2.5
TFF-TT-9	1785	4	5
TFF-TT-10	1477	3	5

[a]TT vaccine lots from clarified toxin (Prostak modules), detoxified, concentrated (Biomax modules), purified and coded as TFF-TT (TT vaccine).

by an approximated value of r = 1.0 or r = -1.0, respectively. In this work, correlation coefficient between *in vitro* PHA method and *in vivo* TNT was r = 0.655 and significant at the 0.05 level (2 tailed). This set of results confirms an excellent degree of correlation among the results obtained by the *in vitro* and *in vivo* tests. The TNT is expensive,timeconsuming,requireswell-trainedpersonnel, a large number of animals and a relatively large amount of serum. Introduction of the PHA procedure in potency testing of tetanus vaccines contributes in two ways to improvement of animal welfare. First, replacement of the traumatic lethal paralytic technique by serum titration results in a considerable refinement of the *in vivo* TNT. Secondly, a substantial saving in number of animals is

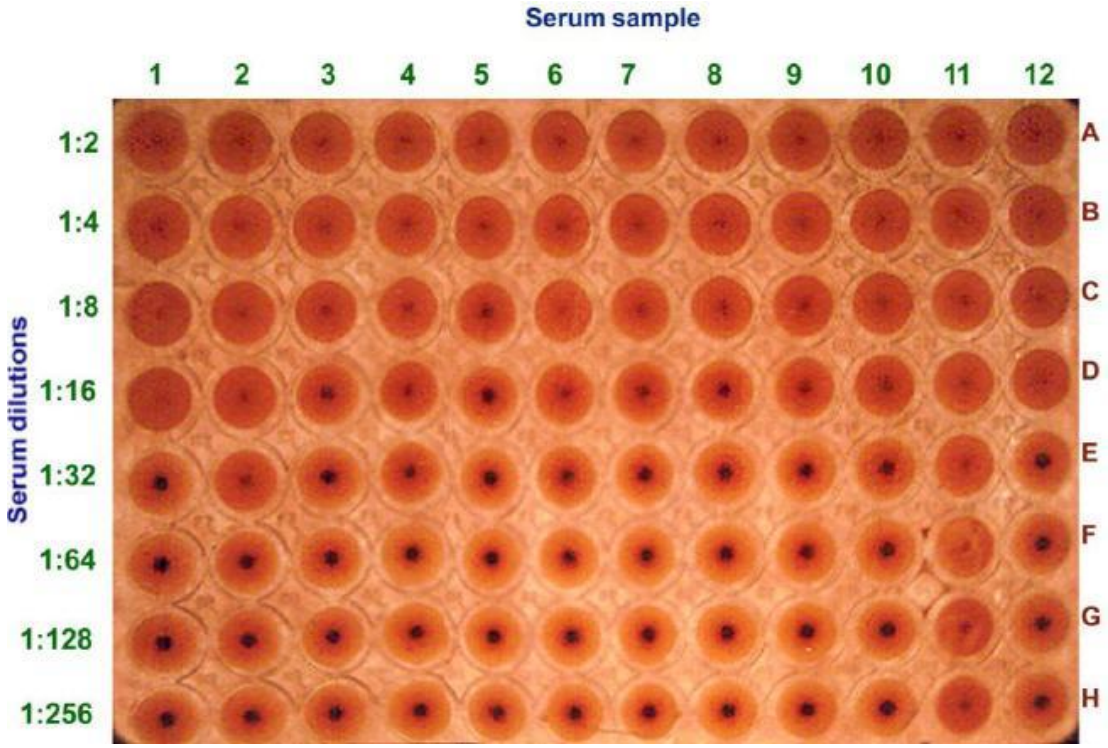

Figure 4. Passive haemagglutination test using TRBC.

feasible as quantitative data (antitoxin units, IU mL^{-1}) are generated instead of qualitative data (paralysis or letha-lity). Comparing the results, it was evident that the PHA is simple, specific, inexpensive, more sensitive, reprodu-cible and the results of PHA are determined within 20 min as observed by Relyveld et al. (1996). The PHA is therefore a useful test for monitoring antitetanus anti-bodies in epidemiological surveys and in high-risk groups and also widely used to compare the effectiveness of different vaccines and immunization schedules. The PHA standardized in our laboratory is sufficient for determi-ning the potency of the antitoxin content of the serum of the immunized guinea pigs. The minimum protective level of tetanus antitoxin in serum is 0.01 IU/ml (Galazka, 1993).

The introduction of a fermentor is a prerequisite to enhance toxin production. The fermentation broth must be clarified quickly otherwise deterioration will take place due to the presence of proteolysis enzymes (Pasteur Institute of India, 1991). Conventional methods like centrifugations or Seitz filtration are either cumbersome or time consu-ming. The other drawbacks are a lack of scale-up criteria or environmental problems like aerosol formation. Also, the depth filtration is not a closed process (due to the need to change pads during the process) and the working personnel are exposed to the toxin. Another GMP aspect observed is the inability to test integrity of pads and possible leaching of extractable matter. A modern filtration method, like what we described in this study, is the need of the hour. Operational parameters like cross flow, ATP and filtration areas are based on the pressure excursion test (unpublished data). The Millipore systems used in this study combine gentle but high-velocity cross flow, allowing a higher recovery of toxin/toxoid without the loss of biological activity associated with other techniques. An effective protocol suitable for bench-scale and pilot-scale production units is presented in this article. The higher recovery is mainly due to the low hold-up volume and weak protein binding nature of the membrane materials. The reusable modules maintain integrity over a large number of runs. The modules are completely sealed from the environ-ment; generate no aerosols and both the TFF systems can be validated as per the GMP requirement. In the present report, we showed that it is a versatile system for pro-cessing hundreds of liters in hours, rather than days and we also evaluated the suitability of the PHA as a sero-logical method for the titration of tetanus antitoxin in sera of immunized guinea pigs which gives comparable results to the *in vivo* TN method. When consistency in production and quality control is well established, the PHA method is superior to the TNT both in precision and in reducing the number of animals.

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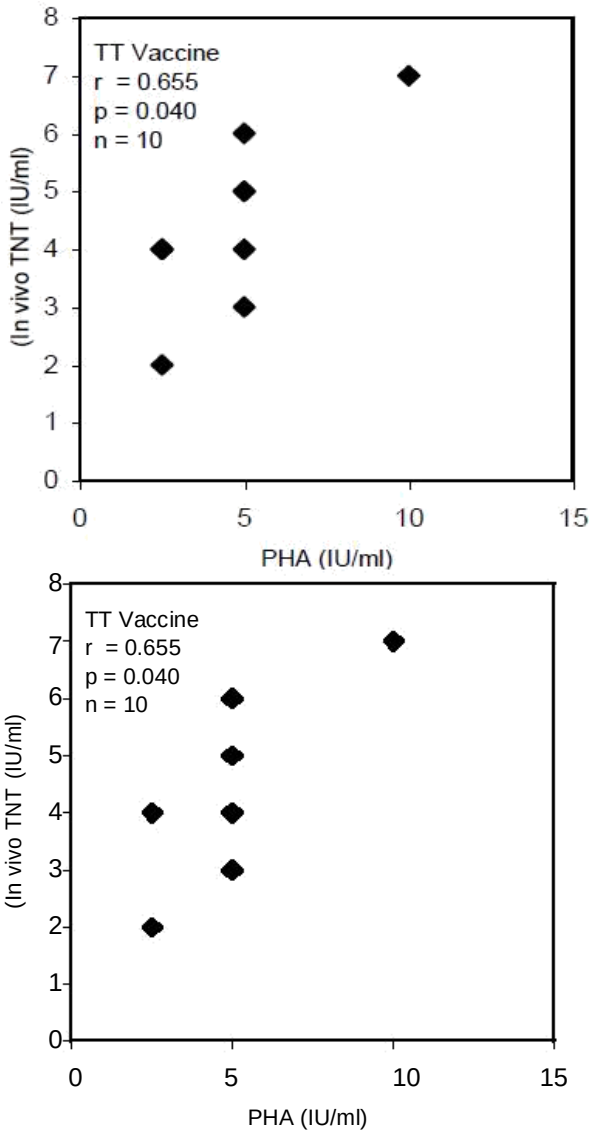

Figure 5. Spearman's correlation coefficient was applied to estimate the correlation between *in vitro* PHA and *in vivo* neutralization test in TT vaccine lots. n, lots of vaccine.

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REFERENCES

Brown DE, Kavanogh PR (1987). Cross-flow separation of cells. Process Biochem. 22:96-101.

Galazka AM (1993). The immunologic basis for immunization series: Tetanus document. Geneva, World Health Organization. WHO/EPI/GEN/93.13.

Indian Pharmacopoeia (1985). Monograph on DTP vaccine (Adsorbed), DT vaccine (Adsorbed) and Tetanus vaccine (Adsorbed) Vol. I, 3rd Edition.

Levine HL, Castillo FJ (1999). In Biotechnology, Quality assurance and validation, drug manufacturing technology Series, In: Avis KE, Wagner CM and Wu VL, editors. Inter Pharma Press Inc., Baffalo Grove: Illinoisp. p. 154.

Pasteur Institute of India (1991).Manual for the production and standardization of Diphtheria-Tetanus-Pertussis vaccine (DTP). Pasteur Institute of India, Coonoor, TamilNadu, India.

Plotkin SA, Orenstein WA (1999). Tetanus toxoid, 3rd edition, Vaccines. p441-474.

Rao YUB, Mahadevan MS, Michaels LS (1992). Evaluation of microporous tangential-flow filtration in the production of Diphtheria and Pertussis vaccines. Pharm. Technol. 16:102-110.

Ravetkar SD, Rahalkar SB, Kulkarni CG (2001). Large scale processing of tetanus toxin from fermentation broth. J. Sci. Ind. Res. 60:773-778.

Reid DE, Adlam C (1974). Large-scale harvesting and concentration of bacteria by tangential flow filtration. J. Appl. Bacteriol. 41:321-324.

Relyveld EH, Huet M, Lery L (1996). Passive hemagglutination tests using purified antigens covalently coupled to turkey erythrocytes. Dev. Biol. Standardization 86:225-241.

Tanny GB, Mireman D, Pistole T (1980). Improved filtration technique for concentration and harvesting bacteria. Appl. Environ. Microbiol. 40:269-273.

Vancetto MDC, de Oliveira JM, Prado SMA, Fratelli F, Higash HG (1997). Tetanus toxoid purification: A case study. Nature Biotechnol. 15:807 – 808.

WHO (1990). Requirements for diphtheria, tetanus, pertussis and combined vaccines. (Revised 1989) Technical Report Series 800

WHO (1977). Manual for the production and control of vaccines. Tetanus toxoid. BLG/UNDP/77.2 Rev.1.

WHO (1990). Requirements for tetanus vaccine (adsorbed). Tech. Rep. Series 800, WHO, Geneva, pp.109-126.

WHO (1994a). Production and control of tetanus vaccine. A training curriculum, World Health Organization. WHO/VSQ/GEN/94.5.

WHO (1994b). Production and control of tetanus vaccine. A training curriculum, World Health Organization. WHO/VSQ/GEN/94.7.

WHO (1994c). Production and control of tetanus vaccine. A training curriculum, World Health Organization. WHO/VSQ/GEN/94.6.

WHO (1997). Manual of laboratory methods for testing of vaccines used in the WHO Expanded Programme on Immunization, World Health Organization, Geneva. WHO/VSQ/97.04.

WHO (2006). Weekly Epidemiological Record. 20(81):197-208.

Zeman LJ, Zydney AL (1996). Microfiltration and Ultrafiltration. Dekker, New York.