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Original Article

Ultrafiltration before detoxification enhance the purity and efficacy of tetanus toxin during large-scale production of tetanus toxoid vaccine

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ABSTRACT

The most effective way to control tetanus (caused by *Clostridium tetani*) is immunization with tetanus toxoid vaccine. However, the production of tetanus vaccine is a complex process including growth of bacteria, harvesting of toxin, and then conversion of that toxin into potent toxoid vaccine. During production, purification of vaccine either at toxin stage or at toxoid stage is essential. In the present study, effect of purification using ultrafiltration at toxin stage was evaluated on four commercial batches of tetanus vaccine in terms of antigenic content (lf/ml), purity in terms of protein nitrogen, antigenic potential by minimum lethal dose and potential efficacy by maximal toxic value. The standard reference methods as per the WHO manual for the production and quality control of tetanus vaccine were followed. A significant increase in the antigenic content, purity, antigenic potential, and potential efficacy of all four batches was observed after ultrafiltration. The results indicate that ultrafiltration before detoxification is an effective method of tetanus toxin purification. It simultaneously increase the quality of the toxin along with the removal of contaminating agents, which otherwise results in adverse effects during use.

Key Words: Tetanus, WHO, Tetanus Vaccine, Ultrafiltration, Production

INTRODUCTION

Despite of significant improvements in human health care, certain bacterial infections such as tetanus always have been a great health problem. Tetanus develops with the production of a potent neurotoxin, tetanospasmin, produced by bacillus *Clostridium tetani*. Tetanus toxin is a single polypeptide chain of 150 kDa and is released into the surrounding medium as NH2- terminal light chain of 50 kDa (toxic moiety) and COOH terminal heavy chain of 100 kDa (binding), both held together by disulfide bridge [2]. The bacterial toxin is effective even at a minimal lethal dose of less than 2.5 ng kg-1 of human body weight [1]. Tetanus toxin induces death in absence of an effective immunity; therefore, active immunization is essential to prevent death caused by tetanus [3]. Tetanus immunisation has been made obligatory during childhood as a part of regular vaccination schedule [4-6].

Production of potent tetanus vaccine is a multi-step process. Tetanus vaccine is produced as tetanus toxin by cultivating *C. tetani* in suitable media, and toxin thus produced is further detoxified to obtain highly immunogenic tetanus vaccine [7]. For the purpose of commercial production, *C. tetani* is grown anaerobically in a medium containing mixture of various proteins, and other constituents. After sufficient growth, bacterial mass is separated from liquid by either centrifugation or dead ended depth filtration. Usually, such solid liquid separations are time consuming with low toxin yield. Such methods are also difficult to validate, otherwise mandatory under current good manufacturing practices. In addition, the production of more pure product is another aspect need to be looked simultaneously. The tetanus toxin at this stage is crude and contains several impurities such as proteins of media origin etc. Such impurities remain in vaccine as its component, and led to unwanted side effects during usage. Also the presence of such impurities affects the quality and immunogenicity of the vaccine. Therefore, to reduce these untoward reactions, purification of tetanus toxin at this stage is essential [8-9]. Ultrafiltration is such a technology, which

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works on the principle of separating molecules based on their molecular weight. By employing ultrafiltration, tetanus toxin can be made free from impurities, and hence can increase the quality, purity and immunogenicity of tetanus vaccine [8, 10-11]. Central Research Institute, Kasauli (CRIK) India is a major manufacturer and supplier of tetanus vaccine to Government of India for expanded programme on immunization. Recently, CRIK has started the production of tetanus vaccine in new cGMP compliant facility. In order to produce pure and more immunogenic tetanus toxin and as a part of process standardization and validation in new cGMP facility; present study was undertaken to evaluate the significance and role of ultrafiltration in purification of tetanus toxin during large-scale production. Comparisons were done based on *in vitro* and *in vivo* parameters.

MATERIALS AND METHODS

Production of Tetanus Toxin: The work was carried out in new cGMP DPT facility, Central Research Institute, Kasauli, India. Institutional ethical committee approved the study protocol. The Harvard strain of *C. tetani* was used for the production of tetanus toxin in fermenter vessel (500 L). Modified Mueller Miller (MMM) medium was used for growing *C. tetani* [12]. The pH of medium was adjusted to 7.4. Fermenter was inoculated with seed and was maintained for 7 days at 35°C under continuous mild agitation and aeration. At the end of the incubation period, toxin was harvested.

Separation of Tetanus Toxin: Using the conventional method, the Seitz filtration, which is a dead-ended depth filtration method, was used. With this method, the fermented culture fluid was first clarified by filtration through T500 Seitz filter pads (20x20 cm, 0.45µ, Seitz Werke, Germany) and was subsequently sterilized by filtration through EKS Seitz filters (20x20 cm, 0.22µ, Germany). The filtrate collected was used as crude toxin. Ultrafiltration was carried out using a Millipore's Pellicon system with ultrafiltration membranes (Pellicon, UF modules). The separation was based on the pore size of the ultrafiltration membranes. The Pellicon TFF system accommodates an acrylic filter holder for polyethersulfone 30 kDa (NMWL) ultrafiltration modules. The material has a higher flux, excellent chemical resistant, integrity testable, void-free structure for higher yield and reliability. The tetanus toxin was passed through the pellicon system and filtrate obtained was used as ultra-filtrated toxin.

***In vitro* test:**

Ramon's lime flocculation: The Ramon's Lime flocculation test was performed to test the antigenic content of tetanus toxin and was expressed as lf/ml of sample [12] using an in-house antitetanus serum calibrated against the 2nd international standard for tetanus antitoxin (NIBSC, Potters Bar, UK). The concentration of reference anti-tetanus serum gives Lf-equivalent/ml [13]. The determination of sample Lf content was based on fact that formation and subsequent precipitation of antigen–antibody complexes is the most prominent when their concentrations are equal. Briefly, equal volumes of reference antiserum in increasing concentration and toxin samples were mixed and placed in water bath preheated at 50ºC. Tubes were observed continuously, and the tube with the fastest flocculation was selected. The time required for flocculation (Kf) was also recorded.

Protein Nitrogen (PN2) Estimation: The estimation of PN2 concentration indicates the antigenic content/ purity of the tetanus toxin. The PN2 content was estimated by Kjeldhal's method. Test sample (depending upon the lf/ml content) was taken in a clean test tube and volume was made to 20ml by distilled water. To this, 50% TCA was added and was kept in water bath at 52ºC for 10 minutes followed by centrifugation at 5000rpm for 30 minutes. Sediment was taken in a Kjeldhal flask and a pinch of digestion mixture ($CuSO_4$ and KSO_4 in 1:3) and concentrated H_2SO_4 was added. Flask was kept at 50ºC for 2hrs. After heating, 50% NaOH and distilled water was added. 0.01% Methyl red indicator was added and flasks were kept for distillation. After distillation, reaction mixture was titrated against N/100 NaOH with pink to straw yellow as end point. PN2 content was calculated as = (blank -sample) X0.14 / sample volume. For purity calculation, lf/ml content was divided by PN2 content [12].

***In vivo* test:**

Maximum Toxin Value (MTV): Based upon the Lf value, equal volume of toxin was mixed with increasing amount of antitoxin and volume was made to 1ml with normal saline. 3 Swiss albino male mice of approximately 18-20grams were injected with 0.5ml of mixture per dilution group via subcutaneous route. Test was carried out in triplicate for both crude and ultra-filtrated toxin. Mice were observed for 4 days for the progressive symptoms of typical tetanus [12].

Minimum Lethal Dose (MLD): 1ml of test toxin (crude and ultra-filtrated) was mixed with 9ml of sterile peptone water and was serially diluted to a dilution of 1/8000000000. 1 ml of toxin dilution was injected into mice (Swiss albino, male, 18-20g) via subcutaneously. Mice were observed for 4 days for the progressive symptoms of typical tetanus or death. Positive control containing pure undiluted

toxin and a negative control containing peptone water were also included [12].

Statistical Analysis: All the experiments were repeated three times to confirm the reproducibility of experiments. Results were analysed statistically by Student's *t* test using SPSS 11.05.

RESULTS AND DISCUSSION

The need of tetanus vaccine will continue since immunity to tetanus is induced only by immunization because recovery from clinical tetanus does not result in protection against further episodes [14]. Due to effectiveness of vaccination, it has been made essential in WHO recommended expanded programme on immunization [15]. However, the production of potent tetanus vaccine is a complex process. In the present study, four different commercial batches of tetanus toxin (TT1-TT4) were produced. These batches were distributed in two equal parts. For the study, one part was processed conventionally and was used as crude toxin (CT), and second part was used as ultra-filtrated toxin (UT) after Ultrafilteration. Quality of both the toxins (crude and ultra-filtrated) was evaluated based on their toxicity content in terms of Lf/ml content, purity in terms of PN2 content and its antigenicity and potential efficacy in terms of MLD and MTV. The result of the present study indicates that quality of tetanus toxin, as assessed by Lf/ml content, increased significantly after ultrafiltration in all four tetanus toxin batches ($p \leq 0.01$, Table 1). The measurement of toxin content (defined in Lf/ml) is a good indicator for the consistency of commercial production. In the present study, Lf/ml of the toxin was measured by the Ramon's version of the flocculation test method. It is WHO recommended method, and is generally used by vaccine manufacturers as in-process control test. This reaction between the antigen and the antibody provides additional information on antigenic quality due to a correlation between time factor and quality of the antigen. The time required for the formation of this complex is known as the Kf value, and depends on the ratio of antigen and specific antitoxin. The Kf value reflects the quality of the antigen [16]. In the present study, Kf value found to be decreased significantly after ultrafiltration in all four samples ($p \leq 0.01$, Table 1) indicating that quality of toxins improved after ultrafiltration. The results indicate ultrafiltration as an effective method for the purification of tetanus toxin. It has been assumed that purification of toxin before detoxification result in a purer product, and hence is expected to remove components that are likely to cause adverse reactions in humans. However, the method of purification should be such that no substances are incorporated into the final products that are likely to cause adverse reactions in humans [16]. The ultrafiltration cassettes used in the present study were of inert nature and hence did not cause any kind of product contamination [8].

As per the WHO recommendation, each toxin should be tested for antigenic strength and purity by determining the antigen concentration in Lf and the concentration of protein (non-dialyzable) nitrogen. The purity of tetanus toxin for the preparation of vaccine should not be less than 1000 Lf per mg/PN [16]. In the present study, all four sample showed significant increase in their PN2 concentration, which was otherwise less than that of WHO recommendations, and hence were not suitable for the further processing as commercial product. All the preparations had antigenic purities higher than the minimum purity recommended by WHO. The result indicates that purification of tetanus toxin by ultrafiltration before detoxification increase the antigenic quality of the toxin, and hence can yield purer form of tetanus toxoid vaccine. Crude toxins and ultra-filtrated toxins were further evaluated in mice for their antigenic content by MLD and MTV test. Since good antigenic content is primary requirement for a good quality toxoid vaccine, therefore, their antigenic content was evaluated *in vivo* for their ability to cause symptomatic tetanus and potential efficacy in mice. MLD test indicate the minimum amount of toxin required to kill/ develop typical tetanus symptoms while MTV gives information about the maximum amount of toxin present, which can be neutralised by specific antitoxin [12, 16-18]. Both these test bridge a link between the antigenic content of toxin, and hence the potency or potential efficacy of the toxoid produced from that toxin. In the present study, MLD value remained unchanged in all toxin samples indicating that both (CT and UT) were equally potent after ultrafiltration. This indicates that ultrafiltration does not affect the antigenic quality of the toxin. On the other hand, significant increase in the MTV value of all four samples was observed indicating the significant increase in the toxicity of the toxin, which correlates with the simultaneous increase in the potential efficacy of toxoid made from that toxin. The result of both these test further add up to increase in quality and purity of tetanus toxin after ultrafiltration.

The approach adopted by most manufacturers is to obtain the best possible quantity and quality of toxin during commercial production, and to convert it into toxoid without any contaminating proteins. The purity and yield of toxin is also a good indicator to monitor product consistency [16-18]. The fundamental requirements of tetanus toxoid

vaccines are demonstrated safety and potency. It recalls for the production of purified toxoid to minimize adverse reactions on use [8]. The results of present study suggest ultrafiltration as an effective method for the purification of tetanus toxin before detoxification in order to remove contaminating impurities while simultaneously increasing the quality & purity of toxin in order to achieve more pure form of tetanus toxoid vaccine.

CONFLICT OF INTEREST: None to be declared.

Table 1: Table showing the results of Lf/Ml, protein nitrogen content, maximal toxic value and minimum lethal dose in the tetanus toxin before (crude toxin, CT) and after ultrafiltration (ultra-filtrated toxin, UT).

Sr. No.	Parameters	Toxin 1		Toxin 2		Toxin 3		Toxin 4	
		CT	UT	CT	UT	CT	UT	CT	UT
1.	Lf/ml	50±1.2	260±4.5 ($p \leq 0.001$)	45±0.9	220±3.3 ($p \leq 0.001$)	55±1.0	260±6.6 ($p \leq 0.001$)	50±1.7	240±4.2 ($p \leq 0.001$)
2.	Kf (in minutes)	4±0.22	3±0.15	3±0.11	2±0.15	3±0.25	2±0.11	3±0.14	2±0.15
3.	PN2 purity	1078.8±15.5	1265.8±14.4 ($p \leq 0.01$)	865.4±16.8	1013.8±12.8 ($p \leq 0.001$)	1037.7±11.5	1198±14.6 ($p \leq 0.01$)	649.3±13.5	1017±15.5 ($p \leq 0.001$)
4.	MLD (in millions)	4±0.07	4±0.17	2±0.17	2±0.09	4±0.13	4±0.12	4±0.16	4±0.11
5.	MTV	40±2.5	50±3.1 ($p \leq 0.01$)	35±1.7	45±1.5 ($p \leq 0.01$)	40±2.2	45±2.0 ($p \leq 0.01$)	40±2.3	50±2.6 ($p \leq 0.01$)

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