International Journal of Biology Research
ISSN: 2455-6548, Impact Factor: RJIF 5.22
www.biotechjournals.com
Volume 1; Issue 2; May 2016; Page No. 32-35

Developmental stages of tetanus vaccines and its effect on experimental animals

[1] Ayesha Zaman, [2] Karishma Noor, [3] Abdul Rehman M, [4] Salma Afridi, [5] Muhammad Waqas, [6] Aneeqa Iqbal,

[7] Dr. Mujaddad Ur Rehman, [8] Madia Ayaz, [9] Dr. Muhammad Ayub Jadoon

[1, 2, 4, 5, 6, 7, 9] Department of Microbiology, Abbottabad University of Sciences and Technology Pakistan.
[3] Department of Microbiology, university of central Punjab Lahore Pakistan.
[8] Ayub medical complex Abbottabad, Pakistan.

Abstract
Clostridium tetani is a gram positive spore forming flagellated bacteria. These bacteria produce the toxin which is responsible for causing tetanus. These toxins mainly affect the neurons of infected persons causing paralysis, lockjaw and other complications. Once the toxins reached to neurons then they are not preventable. Tetanus is vaccine preventable disease i.e. the disease is controlled by vaccinating the individuals. Tetanus have high mortality rate in newborn babies therefore it is necessary to vaccinate the pregnant women by tetanus toxoid to protect the mother as well as ne born baby. Tetanus toxoids are used as vaccines for controlling tetanus the toxoids are prepared from *C. tetani* toxins b inactivating them by use of formalin. The toxins were extracted from *C. tetani* by culturing them in broth and then incubated in fermentation medium to produce the toxins. The toxins were then filtered by use of filter paper and in this study the effect of toxins were observed on mice's by injecting to them intramuscularly. The toxin shows no affect when injected in low dose such as 0.1 micro liters but when the doses increased the symptoms starts to appear. The symptoms of toxin on mice's were muscle spasm, paralysis and eventually death for leaving them untreatable. The toxoids prepared from these toxins were also tested on rabbits for checking its potency the vaccinated animal sera was then taken and checked the immune response i.e. antitoxins in the sera of vaccinated animals. As doses of toxoids increased the immune response appeared was high.

Keywords: C. tetani, tetanus vaccines, effect on experimental animals

1. Introduction
Tetanus was first described before more than 3000 years in Egypt which was spread throughout the world. Tetanus was a main and fatal health problem before the discovery of passive immunization in 1893 and discovery of active vaccination in 1923, while it is still the major health problem in developing countries. The mortality rate of tetanus in each year is about 1 million deaths cases, in which the most common cases is neonatal tetanus which is about 400 000 death cases in new born babies (Dietz *et al.*, 1996) [9]. Tetanus is acute and fatal disease which is caused due to toxin produced by Clostridium tetani which mainly affect the neurons. The genera of Clostridium tetani is clostridia which is a large group of anaerobic gram positive bacteria these bacteria are rod shaped and spore forming. They are mostly present everywhere in environment, and are also a part of the intestinal flora of domestic animals such as horses, chickens, and even also found in humans intestines. These bacteria produce endospores which are in rod shaped but wider than rod shaped bacteria, due to which their shape appear like drumstick. The most common and severe diseases caused due to the toxins produced by this group of bacteria are tetanus of which the causative agent is *C. tetani*, and botulism which is caused due to the toxins of *C. botulinum*. *C*lostridium *tetani is* obligate anaerobic rod shaped flagellated bacteria which lose their flagella after the production of spores (Cato *et al.*, 1986) [5]. Spores of *C. tetani* become kill after boiling for at least 15 minutes but it can't ensures that all spore of tetani will kill on boiling, some spores even after boiling survive. Ensure and safe technique for killing all spores and acquiring sterility is autoclaving at 120°C, for 15 minutes. *C. tetani* causes localized infections. Spores of *C. tetani* are responsible for causing infections not only spores cause the infection but also they are transmitted to body via contamination of wounds and also sometimes transmitted from getting injuries by contaminated objects (Savage *et al.*, 2007) [16]. Once the toxin of tetani reached and invade the neurons of a person then the anti-toxin cannot be effective to neutralize the action of these toxins. The gene of these toxins are encoded and present on a 75 kilo base plasmid which produce these toxin as a single polypeptide chain having molecular weight of 150 000 (Finn *et al.*, 1984; Eisel *et al.*, 1986) [12, 10].

When *C. tetani* or its spores transmitted to body its starts the production of toxins inside the body and these toxins adheres in the central nervous system of infected persons, where it affect i.e. inhibit the function of neurotransmitters, such as glycine and gammaaminobutyric acid, due to which the nerve impulses become affected and causing paralysis and lockjaw etc (Borrow *et al.*, 2006) [3]. The neonatal tetanus cases occurs due to unhygienic practices during birth of baby, mostly the spores transmitted at a time of cutting of umbilical cord or dressing after delivery if the instruments used for such purposes are contaminated by spores of tetani. It is a fatal disease for newborn babies, its fatality rate in new born babies are up to 70 to 100% (WHO, 2005) [18]. The tetanus disease in newborn babies is the main cause of deaths of babies in most of the countries having the mortality rate in hospitalized patients are up to 55 percent to 86 percent (Ali *et al.*, 2002) [1].

The neonatal tetanus cases are only preventable by maternal immunization with tetanus toxoid. By maternal immunization

about 725,000 cases of neonatal tetanus prevented each year but still about 270,000 newborns death case and 30,000 women death cases occurs due to tetanus worldwide (Butt *et al.*, 2005, Junejo *et al.*, 2010) [4, 14]. Initial studies on immunization against tetanus shows that use of both intrathecal anti-tetanus serum (ATS) or tetanus immunoglobulin (TIG) are effective (Geeta *et al.*, 2007) [13] but the recent studies shows that intrathecal tetanus immunoglobulin treatment is more effective for both mild and moderate tetanus cases.

Maternal and Neonatal Tetanus (MNT) prevention and control is to decrease the neonatal cases up to 1 or less than 1 out of 1000 live births each year worldwide. The main 3 strategies for controlling MNT cases as recommended by WHO/UNICEF/UNFPA are: vaccinate all pregnant women in high risk area by at least 2 doses of tetanus toxoid (TT2) while vaccinate all childbearing age women by giving them of at least 3 doses of tetanus toxoid (TT3) and promoting the hygienic practices during the delivery of all pregnant women.

The tetanus cases in the United States was dramatically decreased by the use of tetanus toxoid and improving the wound management from 560 cases (1947) to 48 cases in 1987 which was a record low cases (Pan American Health Organization, 2002; CDC, 2000) [15, 7]. *C tetani* spores are ubiquitous so for protection of all age groups from tetanus it is necessary to maintain subsequent antitoxin levels by means of taking appropriate timed boosters. It is thought that the immunization from tetanus toxoid remain persists for at least ten years (CDC, 1991) [6]. Protection from tetanus disease is acquired by development of neutralizing antibodies against tetanus toxin. The minimum protective level from tetanus is having at least 0.01 IU/mL of tetanus antitoxin in serum (Wassilak *et al.*, 1999) [17].

2. Types of tetanus vaccines

The tetanus vaccines are in multiple combination of tetanus toxoid with diphtheria toxoid and pertussis antigen in a specific concentration to prevent diphtheria, tetanus, and pertussis diseases globally. The vaccines used for prevention of tetanus are in different types some are single used only for tetanus TT while some are combined with diphtheria toxin DT used for prevention of both tetanus and diphtheria. One dose of DT vaccines contains 6.7 to 25Lf diphtheria toxoid and 5 to 7.5Lf tetanus toxoid which are mainly used in children for primary immunization and boosting. While Td vaccine is used in adults for boosting and primary immunization which contains low dose of diphtheria i.e. less than 2Lf/dose but having a similar dose of tetanus toxoid as mentioned above.

Some tetanus vaccines are combination of tetanus toxoid, diphtheria toxoid and pertussis antigen. DTP vaccines are used for control of three diseases which are diphtheria, pertussis and tetanus. They are available in various forms and are given in 0.5 mL doses. The most common forms of DTP vaccines are five which are DTwP, DTaP, Tdap, DT, and Td. Three forms of these vaccines which are DTwP, DTaP and DT are used for the children which are younger than 7 years, while the other two forms which are Tdap and Td are used for individuals which are 7 years or older than 7 years. The lower case mentioned in above vaccines names such as "d" and "p" shows that the concentration of diphtheria and pertussis toxoids in these vaccines are reduced as compared to other which is in combination. These reduced concentrations of one from other are due to prevent adverse effects in adults. While the alphabets such as "a" in "ap" in names of above vaccines indicates that the pertussis toxoids are acellular.

3. Methodology

For diphtheria and tetanus vaccine the potency and amount of toxoid were recorded in International Units (IU) and in Limits of Flocculation (Lf). The potency test for all of these toxoids was carried out in experimental animals which were mice's, rabbits and guinea pigs.

4. Preparation of tetanus toxoid

The first step for production of tetanus toxoid vaccine is to prepare the tetanus toxins and then inactivate them so that we can get the tetanus toxoid. For preparation of tetanus toxins the *C. tetani* was cultured in NIH Islamabad Pakistan b using BHI broth. This BHI broth also contains 0.5 percent east extract+0.5 percent NaCl+2 percent glucose at a ph of 7.4. The *C. tetani* was inoculated in such broth and then incubated at 37 degree centigrade for overnight. The next day after incubation the cultured *C. tetani* was inoculated in fermentation medium so that we get the toxins of *C. tetani*. When we inoculate the cultured bacterial strain in fermentation medium they are placed again in incubator for at least 5 to 7 days. After incubation the toxins present in this media was purified and filtered for further use by testing them on experimental animals (Demain *et al*., 2005) [8].

The purified toxin was then inactivated for preparation of toxoid and which then we may use it for immunization against tetanus. The toxin was inactivated by addition of Formalin and incubating at 30 to 45 degree centigrade (EL-Helw, 2007) [11]. We may add the formalin to fermentation medium having tetanus toxin before filtering toxin from fermentation medium and then filtered them b use of filter paper so we get the toxoid but by using such step the toxin if you want to test will not extracted because b use of formalin the toxin become inactivated in that medium.

After use of formalin the toxin become inactivated and we may use it for immunization purposes against tetanus. The filtered toxoid was added b glycerin and aluminium phosphate or hydroxide. The glycerin was used for the purpose of preservation while aluminium phosphate or hydroxide was used as adjuvant (Barile *et al*., 1970) [2].

The prepared toxoid now was able to use for immunization purposes they were tested first on experimental animals for checking its potency.

5. Experimental animals

The animals used for this study were mice, pigs and rabbits at NIH Islamabad Pakistan. The toxin which we filtered at earl stage was used for its pathogenicity on our experimental animals. The toxin was injected to mice's at very low concentration and after some time the action of toxin by injecting it into mice was noted. These mice's become parlaysed due to such toxin and leaving them for some more time the mice's become dead due to these toxins. Due to appearance of paralysis symptoms it proves that these toxins are neurotoxins i.e. affecting the neurons of mice's and they become paralysed.

The prepared toxoid was also injected intramuscularly to mice's, rabbits and pigs for checking its potency and clarifying that whether we can use these prepared toxoid as

vaccine for immunization or not. After injecting the doses of these toxoid to experimental animals the serum were then taken from these animals used for our experiment after some das and tested for presence of immune response that heather the immune response activated against them or not.

6. Results

The toxins were injected in low dose and in high dose from 0.1 to 0.7 micro liters to mice's having 21 gram weights for observing its effect on them. After injecting the toxin intramuscularly to mice's in low dose, the mice's shows no symptoms and are normal for about 24 hours. After 24 hours the mice's shows muscle spasm, weight loss and paralysis. The mice's which were injected by low dose of toxin were leaved for some more time and after week about 15 to 20 percent of mice's become dead due to low dose of toxins. Some mice's were injected by high dose of toxin due to such high dose the mice's become dead within 24 to 48 hours as enlisted in Table 1.

Table 1: Symptoms appeared in mice's due to different doses of tetani toxin

DOSE	SMYPTOMS	
0.1 micro liter	Normal	No death
0.2 micro liter	Weight loss	15-20 percent died
0.5 micro liter	Muscle spasm	Died in Week
0.7 micro liter	Paralyzed	All died in 48 hours

After injecting toxin to experimental animals and the symptoms appeared due to these toxins. Then the prepared toxoid was injected intramuscularly or subcutaneously to them and leaving them for 24 hours and then observing the effect of toxoid against these toxins. The toxoid become neutralizes the symptoms appeared due to toxins when injected earlier. But if injected after some time of symptoms the toxoids can't able to neutralize them.

The toxoids were also injected to normal rabbits having weight of 2 to 3 kg. After injecting the different doses at different interval of time of prepared toxoids to these animals the sera of these animals were taken and these vaccinated sera were tittered for measuring the immune response of body against this toxoid and preventing the tetanus shown in table 2.

Table 2: Titer of antitoxins measured in serum of vaccinated animal

Animal	Doses	Antitoxin titer in serum IU/ml
Rabbits	1 ml	5
	3 ml	10
	5 ml	15
	7 ml	20

7. Discussion

Tetanus is an acute and fatal disease which is caused due to the toxins of *C. tetani*. Different types of vaccines are present for acquiring immunization against tetanus disease. These types of vaccines are based on combination of tetanus toxoid with diphtheria toxoid and pertussis antigen and also based on the concentration of these combinations. For the preparation of tetanus toxoid the toxin of *C. tetani* required so for this purpose the *C. tetani* was cultured in liquid medium and incubated for overnight then the cultured bacteria were again incubated in fermentation medium so that the toxin from these cultured bacteria extracted for the preparation of toxoid. For such purpose the formalin was added to fermented medium so that the extracted toxins become neutralized and filtered for further usage. The glycerin and aluminium phosphate or hydroxide was added to filtered toxoid for preservation and as adjuvant for use of immunizing the body against *C. tetani*.

The extracted toxins were tested on mice's having weight of about 21gm and observe its effect on the body. The effect of these toxins were mainly on neurons and the symptoms which were appeared in low dose of toxin was paralysis while leaving them for some more time without treating or in high dose these mice's become dead.

The prepared toxoid were also tested on rabbits and guinea pigs after injecting them in different doses for different interval of time the vaccinated sera were observed for immune response appeared due to these toxoids. The stud shows the positive results of these toxoids when prepared and injected against the affected animals from toxin of *C. tetani* b removing the paralysis and weakness within 48 hours.

8. References

1. Ali M, Mazhar A, Irshad HM. Tetanus neonatorum. *Pak Paed J.* 2002; 26:187-190.
2. Barile MF, Hardegree MC, Pittman M. Immunization against neonatal tetanus in New Guinea. 3 The toxin-neutralization test and the response of guinea pigs to the toxoids as used in the immunization schedules in New Guinea. *Bull. WHO* 1970; 43:453-459.
3. Borrow R, Balmer P, Roper HM. Tetanus Update. The immunological basis for immunisation series Module 3, *Tetanus update. WHO*, 2006.
4. Butt TK, Shazia M, AliL, Hamid H. Neonatal seizures - etiology, treatment and outcome. *Pak Paed J.* 2005; 29:151-156.
5. Cato EP, George WL, Finegold, SM, Smeath PHA, Mair, NS, Sharpe ME, *et al.* Genus Clostridium praemozski 1880, *Bergey's manual of systemic bacteriology*, Baltimore: Williams and Wilkins, 1986; 2:1141-200.
6. Centers for Disease Control and Prevention (CDC). Recommendations of the Advisory Committee on Immunization Practices (ACIP). Diphtheria, tetanus, and pertussis: recommendations for vaccine use and other preventive measures. MMWR 1991; 40(RR-10):1-28.
7. Centers for disease control and prevention, Guidelines for control of Tetanus, Atlanta, GA-CDC, (2000).CDC. Tetanus -- United States, 1987 and 1988. MMWR 1990; 39:37-41.
8. Demain AL, Gerson DF, Fang A. Effective levels of tetanus toxin can be made in a production medium totally lacking both animal (e.g. brain heart infusion) and dairy proteins or digests (e.g. casein hydrolysates). *Vaccine* 2005; 23(46-47):5420-5423.
9. Dietz V, Milstien JB, van LF, *et al.* Performance and potency of tetanus toxoid: implications for eliminating neonatal tetanus. *Bull World Health Organ* 1996; 74:619-28.
10. Eisel U, Jarausch W, Goretzki K, *et al*. Tetanus toxin: primary structure, expression in E coli, and homology with botulinum toxins. *EMBO J*. 1986; 5:2495-2502.
11. EL-Helw HA. A New medium for production of the tetanus toxin. *J. Egypt. Vet. Med. Assoc*. 2007; 67(1):125-131.

12. Finn CJ, Silver RP, Habig WH, *et al*. The structural gene for tetanus neurotoxin is on a plasmid. *Science* 1984; 224:881-884.
13. Geeta MG, Krishna KP, Mathews L. Intrathecal tetanus immunoglobulin in the management of tetanus. *Indian J Pediatr*. 2007; 74:43-45.
14. Junejo AA, Abbasi KA, Bouk GR. Profile of tetanus in children at Children Hospital Chandka Medical College, Larkana. *Med Channel* 2010; 16:211.
15. Pan American Health Organization. Newsletter 2002; 24(3):6.
16. Savage EJ, Nash S, McGuiness A, Crowcroft NS. Audit of tetanus prevention knowledge and practices in accident emergency departments in England. *Emerg Med J*. 2007, 417-421
17. Wassilak SGF, *et al*. Tetanus Toxoid. In: Plotkin SA and Orenstein WA, eds. Vaccines. 3rd ed. Philadelphia, PA: WB Saunders Company, 1999, 441-474.
18. WHO. Maternal and Neonatal Tetanus Elimination by 2005. WHO, 2000. (Available at www.who.int/vaccines_documents last accessed on 1.10.2011)