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Original article

Comparative analysis of antigenic strength and *in vivo* serum antibodies concentration of tetanus toxoid vaccine adsorbed in Pakistan

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ABSTRACT

Clostridium tetani produce tetanospasmin, a potent exotoxin; that causes tetanus or lockjaw disease. Scientists developed an anti-tetanus toxoid to protect the body from the spasm's neurotoxic effect. In Pakistan recently, 478 cases of neonatal tetanus were reported. The study was carried out at The National Control Laboratory for Biologicals Islamabad, aiming to decipher the effectiveness of the most frequently used tetanus toxoid vaccine adsorbed in Pakistan in comparison to standard reference vaccine having earlier known consistent values. The vaccines included domestic public sector, domestic private sector, imported private sector I, and imported private sector II. The triplicate experiments on purebred Swiss albino mice were performed by immunizing with Tetanus toxoid and then tested parallel with standard reference vaccine. Various analytical tests were performed on the test organism that included flocculation test/identity test, antibody quantification using enzyme-linked immunosorbent assay (ELISA), potency test, abnormal toxicity test, osmolality, pH test, liquid sub-visible particle test, and sterility test. Results of all the vaccines were compared in comparison with the standard reference vaccine. Absorbances of test vaccines were recorded at the lowest dilution by ELISA. The domestic private sector, imported private sector I, imported private sector II and standard reference vaccine were flocculated at mean dilution (Mean: 0.24, 95% CI: 0.1903–0.2897), and the domestic public sector was flocculated at mean dilution (Mean: 0.23, 95% CI: 0.2052–0.2548). All the products were found within the normal ranges where it was concluded that the maximum average titer of 2.81 was observed at dilution $10^{-1.6}$, indicating that these vaccines were adequate/suitable for the prevention of tetanus.

1. Introduction

Clostridium tetani is an environmental, spore-forming, obligatory anaerobic rod persisting in soils, manures, and the gastrointestinal tract of animals (Adeel et al., 2016). This organism produces two types of toxins during the growth phase, i.e., tetanospasmin and tetanolysin. Tetanospasmin causes tetanus that is encoded by plasmid present in all toxigenic strains. Tetanolysin has an uncertain role in the pathogenesis of tetanus (Alouf, 2006). Tetanus is a neuromuscular dysfunction caused by an exotoxin (tetanospasmin) of *C. tetani*. Lockjaw is still a potentially life-threatening disease and is usually associated with an infected puncture wound (Ajmal et al., 2019). According to the WHO reports, in 2017, 12,509 cases of tetanus were reported worldwide, and out of accounted cases, 2,266 cases were neonatal tetanus. In Pakistan, 478 cases of tetanus were reported, and all are neonatal tetanus (Brüggemann et al., 2003).

The incubation period of tetanus is 3–21 days; however, the incubation period of neonatal tetanus is 4–14 days. Clinically tetanus is classified into three different forms, i.e., localized, general-

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ized, and cephalic. (Khakheli et al., 2019). WHO generalized tetanus being most prevalent with 80% pervasive cases that lead to trismus spasm, neck contraction, and abdominal muscle rigidity. Localized tetanus occurs in patients with compromised immunity, while cephalic tetanus is rare and associated with head and face wounds (Bugno et al., 2018). For the first time, Emile Roux and Alexandre Yersin described the bacterial toxins concept in 1889 and proved the idea through animal model testing of diphtherial disease (Hawgood, 1999). The toxins are multidomain proteins having distinct modes of action and multiple cellular targets. The study of bacterial toxins leads to the development of a vaccine by deactivating toxins (toxoids). It is one of the significant medical achievements in preventing devastating infectious diseases in the twentieth century (Kumar et al., 2022).

The toxins of *C. diphtheriae*, *C.tetani,* and *C. botulinum* were discovered in 1889, 1890, and 1896 respectively (Larsson et al., 1999). Tetanus neurotoxin consists of three functional domains: receptor attachment, membrane translocation, and cleavage of a host protein required for synaptic vesicle exocytosis. Tetanus neurotoxin is unequally transported in motor neurons (Montes et al., 2019). The seven different serotypes of tetanus neurotoxins named A to G belong to the clostridial neurotoxin family. This family consists of heavy and light chains bound by a disulfide bond. Light chain act as zinc-endopeptidases is specific for neurotransmitter release apparatus. Synaptobrevin and SNAP-25 protein are essential for the attachment of synaptic vesicles at the nerve terminal. Soluble N-ethylmale-imide-sensitive factor-attachment protein receptors (SNAREs) play an indispensable role in membrane attachment events in eukaryotic cells (Yoo et al., 2021). Neurotransmitter released is blocked by clostridia neurotoxin *in vivo* by selective proteolysis of synaptic SNAREs (Nesil and Erbas, 2021; Stalpers et al., 2021). Tetanus toxoid is a Food and Drug Administration (FDA), USA approved vaccine that protects the body from the effects of clostridium neurotoxins, which block the inhibitory neurotransmitter and lead to spasms (O'Neil et al., 2021).

The last few decades have witnessed a sharp decline in tetanus prevalence in developed countries due to a proper tetanus immunization program. However, in developing countries like Pakistan, the prevalence of neonatal tetanus is still high. It was essential to determine whether the mother is not adequately vaccinated during pregnancy or if the vaccine is not potent against the disease. In this study, one possible reason related to vaccine efficacy was studied. It was crucial to predict the antigen content and protective level (in terms of Antibodies' response) of such tetanus toxoid vaccine adsorbed (domestic formulated and imported). Ramon titration method identifies the product's antigen content, and the reaction is called flocculation. The antigen content is expressed either in Lf/ml or Lf/dose (Pirazzini et al., 2022). The toxin neutralization test is a reliable and widely used technique for the measurement of tetanus toxoid.

In Pakistan, different domestically formulated and imported tetanus toxoids are being used for the prevention of tetanus. Domestic formulated includes private and public sector organizations located in Islamabad whereas two other types of tetanus toxoid vaccine adsorbed imported from private sector organizations approved by World Health Organization (WHO) are used in Pakistan. Previously, no such comparative study of tetanus toxoid vaccine adsorbed had been conducted in Pakistan. The present study was conducted to test the efficacies comparison of domestic formulated tetanus vaccine, imported tetanus vaccine, and standard reference vaccine adopted by the WHO expert committee on biological standardization.

2. Materials and method

A total of four different domestic and imported brands of tetanus toxoid vaccine adsorbed were tested, i.e., domestic public and private sector formulated vaccine and imported private sector vaccine. Standard reference vaccine was taken from National Control Laboratory for biological (NCLB), Islamabad, Pakistan. The *in vivo* study was conducted in the animal house of NCLB Islamabad. The research organisms, i.e., purebred *Swiss albino* mice (*BALB/c* Strain), weighing 14–16 g for testing, were purchased from the National Institute of Health, Islamabad. Various experiments were performed to evaluate the efficiencies and immunogenicity level of the tetanus toxoid vaccine adsorbed of four different brands.

2.1. Ethical permission

Animal testing was performed after the approval of research and ethical permission from the Committee on Research Ethics and Animal Research of the Department of Life Sciences, Abasyn University, Islamabad campus, Islamabad, Pakistan.

2.2. Identity Test/Lf test

The identity/Lf test was performed using the flocculation technique. The test's principle was to incubate a fixed amount of toxoid with varying amounts of known antitoxin in the presence of electrolytes and record the dilution which one first flocculates. Kf-Flocculation rate (time for the first tube to flocculate). One hour of flocculation time is convenient for tetanus toxoid vaccine adsorbed. The vaccine samples were prepared in the conical tube by adding 1 g of sodium citrate into 10 ml of well-mixed vaccine followed by incubation at 37 °C for 24–48 h (Varacallo et al., 2021). The clear supernatant was obtained after centrifugation and transferred into another conical tube. The testing was performed on a pre-heated water bath (Memmert Water bath, WNE 14-Memmert, GmbH®) at 50 °C. The test tubes were arranged, and equine origin anti-tetanus serum was added, followed by the addition of saline water until the tube volume reached 1 ml. 1 ml of sample supernatant was added into each test tube; 1/3rd of the tube was immersed in the water bath and mixed through gentle shaking. The flocculation time was noted after 15 min of incubation at 50 °C and the results were noted as indicated in Table 2.1 for all test samples and standard reference vaccine. The following formula was used for Lf/ml determination.

Total Lf/ml = Volume of ATS used in 1st Flocculation Tube × Lf of Standard ATS.

2.3. Mice immunization by tetanus toxoid vaccine adsorbed

Healthy and active mice were divided into six groups i.e. (1) Domestic public sector (2) Domestic private sector (3) Imported private sector I (4) Imported private sector II (5) Standard reference vaccine (6) Control group. Each sample test group, standard reference vaccine group, and mice control group had 16 mice which is further divided into four subgroups according to the dilution and each subgroup contained 4 mice as indicated in Table 2.3. The control group of mice was not injected at all.

Blood samples from the tail of individual mice were taken through a 1 ml sterile disposable syringe following the standard operative procedure; the separated serum was stored at −20 °C. Feed and water were added to each cage of mice and the mice were left for further two days. The test vaccine and standard reference vaccine were diluted with normal saline up to the dilution of $10^{-2.5}$ as illustrated in Table 2.2 and in supplementary Fig. 2.2. After 48 h of observation, the mice were checked for being healthy and active as all the mice gained weight, proving them to be more beneficial. Sixteen disposable syringes from 4 groups of test vaccines and sixteen from the standard reference vaccine were filled up

Table 2.1
Flocculation Test Dilution.

Particulars	Tube No.									
	1	2	3	4	5	6	7	8	9	10
Anti-tetanus serum (ml)	0.10	0.12	0.14	0.16	0.18	0.20	0.22	0.24	0.26	0.28
Normal Saline (ml)	0.9	0.88	0.86	0.84	0.82	0.80	0.78	0.76	0.74	0.72
Tetanus toxin /toxoid (ml)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Lf /ml	10	12	14	16	18	20	22	24	26	28
Flocculation observed				3rd	1st	2nd				

Table 2.2
Dilution of vaccines for potency test.

S. No.	Amount of Tetanus Toxoid	Normal Saline	Dilutions	Log of Dilution
01	01 ml from vial	09 ml	100	10^{-1}
02	05 ml from 1st Dilution	05 ml	50	$10^{-1.3}$
03	05 ml from 2nd Dilution	05 ml	25	$10^{-1.6}$
04	05 ml from 3rd Dilution	05 ml	12.5	$10^{-1.9}$
05	05 ml from 4th Dilution	05 ml	6.25	$10^{-2.2}$
06	05 ml from 5th Dilution	05 ml	3.125	$10^{-2.5}$

Table 2.3
Mice arrangement in cages for diluted vaccine administration.

Sample type	No. of Mice	Withdraw blood before vaccination	Dilution				Withdraw blood on day 28 after vaccination
			10^{-16}	10^{-19}	10^{-22}	10^{-25}	
Domestic Public Sector (Group-01)	16	16	4	4	4	4	16
Domestic Private Sector (Group-02)	16	16	4	4	4	4	16
Imported Private Sector I (Group-03)	16	16	4	4	4	4	16
Imported Private Sector II (Group-04)	16	16	4	4	4	4	16
Standard Reference Vaccine (Group-05)	16	16	4	4	4	4	16
Mice Control (Group-06)	16	–					16

to the mark of 0.5 ml. After disinfection of the mice's tail with 70% isopropyl alcohol, each mouse from the concerned group was subcutaneously administered 0.5 ml of all the diluted test vaccines near the spine as given in (Fig. 2.1). However, the control mice were not injected at all. All the mice, their feed, water, and temperature were observed for 28 days regularly.

On the 28th day after administering the vaccine, mice were weighed, and upon being healthy, the blood samples were taken from the tail vein of the mice from all the groups of mice, test vaccinated, standard reference vaccine group, and control untreated group of mice. The whole blood was transferred into sterile disposable sterile test tubes that were already labeled according to the dilution and group and transferred to the lab for further investigations. The serum from mice blood was separated through the high-speed centrifugation and the supernatant serum was stored at −20 °C. The mice were transferred from cages to a glass jar and an adequate amount of chloroform was provided into the jars. All mice expired within a few minutes and were packed in a biosafety red plastic bag and transferred into the incineration department of the National Institute of Health (NIH), Islamabad, Pakistan for proper disposal through incineration (Fig. 2).

2.4. Antibodies quantification by ELISA

For checking the titer of tetanus IgG antibodies, the sample was processed (incubated and washed) according to the standard methodology suggested by Biotech Lifesciences ELISA classic tetanus IgG™ kit USA (Cat # MBS9907879). The reaction procedure was followed step by step according to the manufacturer of the kit, and the test reading was noted using an ELISA plate reader (Platos-R 496, AMP Diagnostics, Austria) as shown in (Fig. 2.3).

2.5. Abnormal toxicity test/general safety test

For investigating the adverse effects of all four test vaccines and standard reference vaccines, an abnormal toxicity test (ATT) as a non-specific vaccine safety test was performed following the WHO protocols [21]. 0.5 ml human dose of test vaccines and the standard reference vaccine was injected intraperitoneally into two mice of each group as illustrated in Table 2.4.

2.6. Sterility test

Sterility Testing was performed to confirm whether the product was free from any viable microorganisms. For this measurement, the Fluid Thioglycolate Medium (FTM) and Tryptic Soy Broth (TSB) were used. The log sheet for the test was maintained, and each test tube was checked for any irregularities, such as microbial growth presence or leakage. One test-tube each from FTM and TSB was marked as syringe control (S/C) and medium control (M/C). 1 ml of domestic public sector vaccine was added both in FTM and TSB tubes. The same procedure was adopted for the other three vaccines and also for the standard reference vaccine. For syringe control, took 1 ml of the medium from FTM and 1 ml from TSB with separate sterile syringes and re-inoculated it in the same tube. 1 ml of tap water was added to FTM and TSB tubes as a positive control. FTM Tubes, S/C, M/C, and P/C were incubated at 30–35 °C and TSB Tubes S/C, M/C, and P/C were placed in an incubator at 20–25 °C for 14 days [22].

2.7. pH test

To specify the acidic or essential nature of the vaccine, two-point calibration was performed. The electrodes' tips were soaked

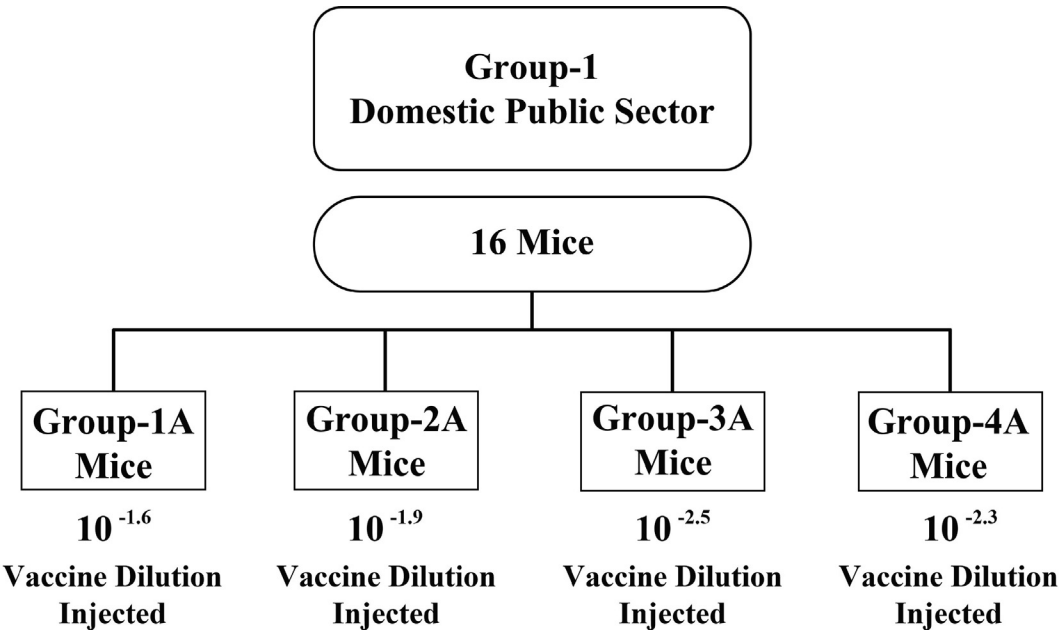

Fig. 2.1. Group categorization of mice.

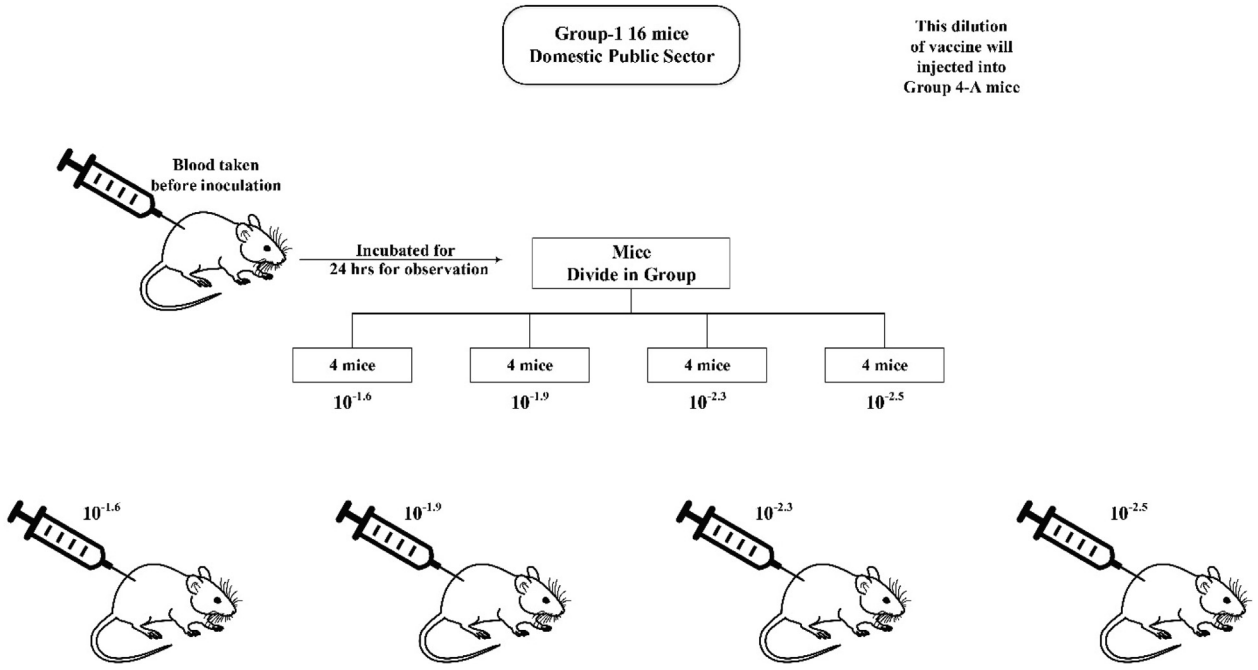

Fig. 2. Group-vise division of mice in 4 different dilutions.

Table 2.4
Mice arrangement in the cages for abnormal toxicity test.

Sample type	Number of mice	Route of administration
Domestic Public Sector (Group-01)	2	Intraperitoneal
Domestic Private Sector (Group-02)	2	Intraperitoneal
Imported Private Sector I (Group-03)	2	Intraperitoneal
Imported Private Sector II (Group-04)	2	Intraperitoneal
Standard Reference Vaccine (Group-05)	2	Intraperitoneal
Mice Control (Group-06)	2	No vaccine injected

in 25 mm of the domestic public sector test sample, swirled, and left for a while. The pH meter was an auto endpoint that displayed the pH of the test sample. The electrode was rinsed with distilled water and blotted the electrode tip with a lint-free tissue, and the results were recorded. The test was performed in triplicate to the obtained mean of results. The same procedures were adopted for the other three samples and the standard reference vaccine.

2.8. Liquid subvisible particle count test

The particle counting was performed by irradiating the sample with a laser diode for measuring the size and distribution of parti-

cles in the vaccine, and detection was performed using the scattered light methodology using the particle counter (Liquid Particle Counter for USP 788: APSS-2000, Particle Measuring Systems, USA). One gram of sodium citrate hydrated through digital balance was poured into 10 ml of well-mixed vaccine. The samples were incubated at 37 °C for 24–48 h (Rhyner, 2011). The clear supernatant obtained after centrifugation was transferred into another falcon tube. 500 ml of filtered distilled water was placed in a suction port. The machine was calibrated with a standard calibrating particle of varying sizes (5, 10, 15, 20, and 25). 400 ml of filtered distilled water was placed in a beaker in port, and Zero counts were adjusted in the background. All vaccine samples and standard reference vaccines were allowed to sip into the machine, and the readings were noted. The test was performed in triplicate.

2.9. Osmolality test

Osmolality testing was performed to test the number of solute particles in the sample. The sampler tip was placed onto the sample that acquired 20 µl of the sample (vaccine). The excess sample was wiped off and pushed into the cradle using an osmometer 3320. The same procedure was used for the rest of the samples and standard reference vaccine.

3. Results

3.1. Identity Test/Lf testing

The vaccine samples were tested parallel with the standard reference vaccine. After 28 min of testing, the first tube flocculated, having a dilution of 0.22 of the domestic public sectors. The 2nd and 3rd test tubes flocculated in the dilution series of the domestic public sector after 32 and 34 min were 0.24 and 0.23 respectively. The first floccules appeared in the domestic private sector sample after 23 min at dilution 0.26. Other floccules in domestic private sector dilution series were observed at dilution 0.28 and 0.24 at 25 and 28 min, respectively. Imported private sector I sample showed the first flocculation after 25 min at dilution 0.24. Flocculation in the other two test tubes showed a dilution of 0.26 and 0.22 at 26 and 28 min, respectively. In detail, the first flocculation in the imported private sector II samples was observed at dilution 0.26 after 26 min. Flocculation in imported private sector II dilution series test tubes appears after 29 and 30 min at dilutions 0.24 and 0.22 systematically. Flocculation in standard reference vaccines was also noted at a different time interval. The first flocculation was observed in 0.26 dilutions in 25 min. In the 2nd test tube, flocculation appeared in dilution 0.22 after 26 min, and in the 3rd test tube, flocculation was observed in 0.24 dilutions after 28 min as shown in Table 3.1.

The domestic public sector sample and domestic private sector results were calculated as 22 Lf/ml and 26 Lf/ml. The imported private sector I and imported private sector II results were 24 Lf/ml and 26 Lf/ml. However, the results of the standard reference vaccine were found as 26 Lf/ml as given in Table 3.2. A non-significance ($p = 0.12$) difference was found between the flocculation of domestic and imported vaccines.

3.2. Mice immunization by tetanus toxoid vaccine adsorbed

On day-28, after immunization with four different tetanus toxoid vaccines adsorbed and a standard reference vaccine, all mice's blood sample were tested through an ELISA kit (Biotech Lifesiences ELISA classic tetanus IgG™ Kit, USA). By variable dilutions, as illustrated in Fig. 3.1; A significant difference ($p = 0.00$) in the absorbance of all the test and standard reference vaccines was noted. The absorbance of the domestic public sector sample at the lowest dilutions $10^{-1.6}$ was recorded as 2.58. The other three dilutions, $10^{-1.9}$, $10^{-2.3,}$ and $10^{-2.5,}$ were recorded as 2.31, 1.92, and 1.45, respectively. Negative and positive controls were noted as 0.03 and 3.48, respectively. The lowest dilution $10^{-1.6}$ reading was 2.93 for domestic private sector vaccines. The three other dilutions $10^{-1.9}$, $10^{-2.3,}$ and $10^{-2.5}$ reading were 2.47, 2.09, and 1.72. Imported private sector I sample reading was noted as 2.72 at the lowest dilution $10^{-1.6}$ Dilutions$10^{-1.9}$, $10^{-2.3,}$ and $10^{-2.5}$ reading were noted as 2.35, 1.98, and 1.64, respectively. The absorbance of imported private sector II samples at the lowest dilution of $10^{-1.6}$ was recorded as 2.98. Dilutions $10^{-1.9}$, $10^{-2.3,}$ and $10^{-2.5}$ absorbances were noted as 2.43, 2.15, and 1.79, respectively. Standard reference vaccine absorbance was calculated as 3.01 at the lowest dilution of $10^{-1.6}$. The absorbance of the other three dilutions $10^{-1.9}$, $10^{-2.3,}$ and $10^{-2.5}$ was recorded as 2.73, 2.26, and 1.93, respectively. A non-significant ($p = 0998$) difference was observed between tested vaccines were observed, where the maximum mean optical density (OD) value (Mean: 2.24, 95% CI: (0.9675–3.5125) was found with the standard reference vaccine and the lowest mean OD value (Mean: 1.97, 95% CI: (0.7602–3.1932) was obtained with domestic public sector vaccine as shown in Table 3.3.

3.3. Abnormal toxicity testing

No symptoms of illness were observed after 7 days. Also, no mice died within 7 days of observation. All mice remained healthy throughout the test and gained weight.

3.4. Sterility test

No visible growth and turbidity was observed in any tested samples present in FTM and TSB after day 7th and 14th observations. All samples and standard reference vaccines were found sterile and free of microbes.

3.5. pH test

The domestic public sector and domestic private sector sample results were recorded as 7.2 and 7.3, respectively. Results of an imported sample of the private sector were noted as 7.2 and 7.1 systematically. The test was performed in triplicate. The normal pH range of the adsorbed tetanus vaccine was 6.6–7.5. the pH of all the samples was found in the normal range (Table 3.4). A non-significant difference was observed in pH of both domestic and imported vaccine.

3.6. Liquid sub visible particle count test

Liquid sub visible particle counts of the domestic vaccine, imported vaccine, and standard reference vaccine were found within the normal range as mentioned in Table 3.5.

3.7. Osmolality test

The numbers of solute particles in all test and standard reference vaccines were found in their normal range as mentioned in Table 3.6. Osmolality of both domestic and imported vaccine showed no significance difference and were found in normal range.

3.8. Statistical Analysis:

All data were collected in an excel sheet for preliminary descriptive analysis. Analysis of Variance (ANOVA) in Statistical Package for the Social Sciences version 23.0 was used to determine the difference between five categories of vaccines (Local Public

Table 3.1
Comparative Analysis of identity test of toxoid vaccine.

Vaccine	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	P value
Domestic Public Sector	3	0.2300	0.01000	0.00577	(0.2052-0.2548)	0.945
Domestic Private Sector	3	0.2400	0.02000	0.01155	(0.1903–0.2897)	
Imported Private Sector I	3	0.2400	0.02000	0.01155	(0.1903–0.2897)	
Imported Private Sector II	3	0.2400	0.02000	0.01155	(0.1903–0.2897)	
Standard Reference Vaccine	3	0.2400	0.02000	0.01155	(0.1903–0.2897)	

Table 3.2
Comparison of identify test (LF/mL) between domestic and imported toxoid vaccine.

Vaccine	N	Mean Flocculation LF/mL	Variance	F-Test	p value
Domestic Public Sector	3	22	2	2.4	0.12
Domestic Private Sector	3	26	2		
Imported Private Sector 1	3	24	2		
Imported Private Sector 2	3	26	2		
Reference Standard	3	26	2		

F-Test: Fisher Exact Test.
p value greater than 0.05 was considered non-significant.

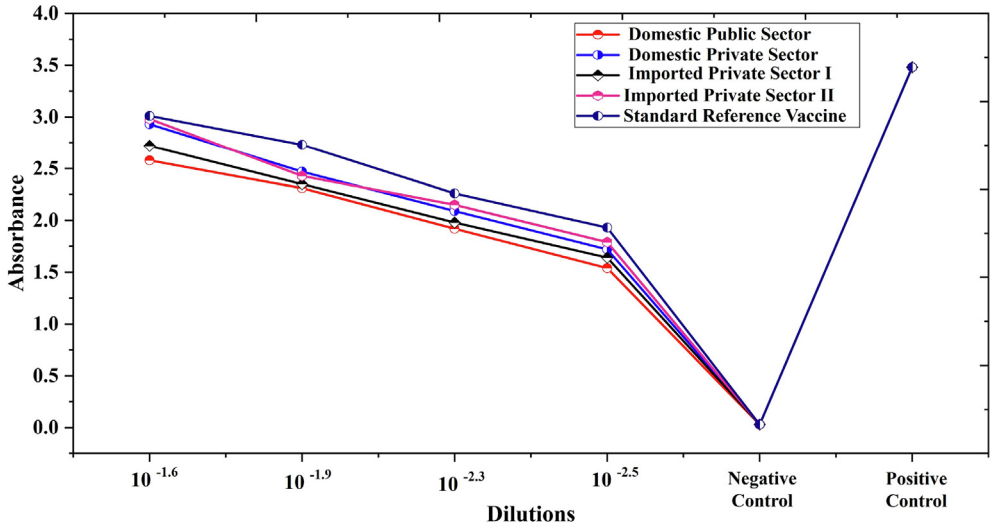

Fig. 3.1. Absorbance spectrum of Toxoid Vaccine through ELISA.

Table 3.3
Comparative Analysis of Toxoid Vaccine through ELISA.

Vaccine	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	P value
Domestic Public Sector	6	1.9767	1.15918	0.47323	(0.7602–3.1932)	0.996
Domestic Private Sector	6	2.1200	1.19643	0.48844	(0.8644–3.3756)	
Imported Private Sector I	6	2.0333	1.16923	0.47734	(0.8063–3.2604)	
Imported Private Sector II	6	2.1433	1.19666	0.48853	(0.8875–3.3991)	
Standard Reference Vaccine	6	2.2400	1.21253	0.49502	(0.9675–3.5125)	

Table 3.4
Comparison of pH between domestic and imported tetanus toxoid vaccine adsorbed.

Vaccine	N	Mean pH	Variance	F-Test	p-value
Domestic Public Sector	3	6.6	0.01	2.4	0.12
Domestic Private Sector	3	6.5	0.01		
Imported Private Sector I	3	6.5	0.01		
Imported Private Sector II	3	6.7	0.01		
Standard Reference Vaccine	3	6.5	0.01		

F-Test: Fisher Exact Test.
p-value greater than 0.05 was considered non-significant.

Table 3.5
Comparison of particle count between domestic and imported toxoid vaccine.

Vaccine	Particle size	N	Mean of Particles	Variance	F-Test	p-value
Domestic Public Sector	10 µm	3	1253	25	173251.41	7.40E-41
Domestic Private Sector		3	1017	9		
Imported Private Sector I		3	1488	100		
Imported Private Sector II		3	1190	144		
Standard Reference Vaccine		3	1248	144		
Domestic Public Sector	25 µm	3	235	25		
Domestic Private Sector		3	197	9		
Imported Private Sector I		3	190	16		
Imported Private Sector II		4	169	4		
Standard Reference Vaccine		3	140	4		

F-Test: Fisher Exact Test.
p-value less than 0.05 was considered as significant.

Table 3.6
Comparison of Osmolality between domestic and imported tetanus toxoid vaccine adsorbed.

Vaccine	N	Mean Osmolality	Variance	F-Test	p value
Domestic Public Sector	3	298	144	0.75	0.58
Domestic Private Sector	3	290	25		
Imported Private Sector I	3	294	25		
Imported Private Sector II	3	285	225		
Standard Reference Vaccine	3	291	49		

F-Test: Fisher Exact Test.
p-value greater than 0.05 was considered non-significant.

Sector sample, Local Private Sector sample, Imported Private Sector I, Imported Private Sector-II, and Reference Standard). The data with a p-value less than 0.05 was considered significant.

4. Discussion

Tetanus vaccines are commonly used vaccines worldwide for the prevention of lockjaw disease. The incidence of tetanus has decreased in the developing and developed world due to its widespread use in immunization programs (Humeau et al., 2000). The incidence of neonatal tetanus increases with the use of substandard tetanus toxoid vaccine adsorbed (Hassan et al., 2016). The vaccine may lose efficacy/potency due to the breakdown of the cold chain and the excipient used for the preservation and enhancement of immunogenicity of the vaccine (Jódar et al., 2004).

The present study was designed to investigate the reason for the high prevalence of neonatal tetanus in Pakistan despite the extensive use of the tetanus toxoid vaccine adsorbed. It was essential to determine whether the mother is not properly vaccinated against tetanus during pregnancy or if the vaccine is not potent against the disease. It also explored the efficacies and effectiveness of the domestic formulated vaccine and imported vaccines. It also enabled us to know the efficacy of public sector formulated vaccines and private sector formulated vaccines. The study resulted in several novel tests. Another possible reason for the high prevalence of neonatal tetanus is the cultural diversity of unhygienic childbirth procedures and cord care is also a contributor to the occurrence of newborn tetanus (Bennett et al., 1996; Quddus et al., 2002). Unskilled midwives, parents' lack of education, lack of maternity care, geographical position and climatic distribution of Clostridium tetani spores, and remote settled population are some of the factors which contribute to this lethal disease (Hassan et al., 2016).

Measurement of tetanus toxoid antigen through flocculation test (LF/ml) gives us results within specified limits set by WHO of all tests and standard reference vaccine. The comparison showed that domestic formulated vaccine contains an adequate amount of antigen contents compared to imported vaccines. Domestic public sector formulated vaccines found a low amount of antigen contents as compared to private sector vaccines but the results of all tests vaccines came under the specified limits set by WHO. The same type of study was performed by (Shi et al., 2019) for the quantitative estimation of diphtheria and tetanus toxoid vaccine adsorbed. The flocculation was observed between 30 and 100 Lf/ml in a study conducted by (Duit et al. (2019)), whereas in this study, the floccules were observed between 10 and 50 Lf/ml.

The selection of mice species is the most important step in animal studies. Potency levels differ significantly with animal species and a lot of tetanus toxin use. Vaccines that have adequate potency produce less titer of antibodies due to animal species variability (Hardegree et al., 1972). Keeping the problem in mind model organisms' Swiss Albino Mice (BALB/c Strain) which is well documented were used for immunization of tetanus toxoid vaccine adsorbed.

Immunogenicity of tetanus toxoid vaccine adsorbed is assessed for many years through toxin neutralization test which is costly and difficult. Comparatively to the toxin neutralization test a new approach ELISA was used by (Gupta and Siber, 1994) for the evaluation of tetanus antibodies which is cost-effective, more stable at 2–8 °C and its protocol is simple. Good correlation was found by comparative analysis of titer in serum of immunized mice determines by toxin neutralization test and by ELISA. Given the above study for the quantitative estimation of antibodies concentration in mice serum, an ELISA test was performed. At various dilutions the absorbance of test samples and standard reference vaccine were noted, Negative and positive control was also run parallel with the test. The standard reference vaccine showed the highest absorbance on the lowest and highest dilutions. Domestic private sector and imported private sector II samples showed great similarity in absorbance from lowest to highest dilutions. The absorbance of Imported private sector I and domestic public sector was found less in each dilution compared to the other two vaccines. It was concluded that imported private sector II vaccines produced the highest concentration of antibodies and domestic public sector vaccines produce the lowest antibodies concentration. Negative control absorbance was noted at zero and positive

control was found at peak. These results indicate the accuracy of the test. Furthermore, it also confirmed that the BALB/c strain of mice is a choice of animal for the potency testing of tetanus toxoid vaccine adsorbed. It is also confirmed from the testing of all these vaccines and standard reference vaccines that all the tested vaccines are adsorbed adequately on aluminum phosphate by producing an excellent immunogenic response.

Every injectable product used for cure and prevention must be sterile and free from microbial contamination as described in United States Pharmacopeia (USP) chapter 71 and European pharmacopeia (Ph. Eur.) chapter 2.6.1. It requires two different types of media and two different ranges of temperature for 14 days of incubation period due to extensive personnel requirements for test and quality assurance. All the test samples and standard reference vaccines were tested against positive control, negative control, and syringe control. The results of all test samples, standard reference vaccine, and negative control were found satisfactory, and no microbial contamination was observed. The appearance of turbidity and microbial contamination in positive control was the evidence that the test was performed in standard operative procedures that fulfill all the requirements of USP.

The pH is an important indicator in the stability study for the acidic and alkaline nature of a product. Every component present in a product/vaccine must be stable in a specified limit of acidic and alkaline environments set by the manufacturer or by the respective pharmacopeia. Determination of pH is necessary for the assurance of stability of the components present in vaccines. All the test samples and standard reference vaccines were tested for pH. These vaccines were found within a range of specified limits set by manufacturer/pharmacopeia.

Every parenteral product used for the treatment and prevention of disease in humans must be clear from visible particles and must be within the specified limit case of subvisible particles. The number of subvisible particles is based on the size of particles present in products. Large size microparticles must be less in number as compared to small size microparticles as indicated in the pharmacopeia. All the test samples and standard reference vaccines were tested for liquid sub-visible particle count and came under the limits specified by WHO or the respective pharmacopeia.

5. Conclusion

It was concluded from this study that the reason for the high prevalence of neonatal tetanus in Pakistan was not the use of substandard vaccines. The vaccines use against tetanus in Pakistan, whether it is formulated in Pakistan or imported from other countries are suitable, efficient, and quite potent. The other possible reason for neonatal tetanus could be the lack of parent education, ignorance of maternity care including low or no tetanus vaccination during pregnancy, unskilled midwives, the unhygienic childbirth procedure, and cord care are also contributors to the occurrence of neonatal tetanus, geographical position & climatic distribution of *Clostridium tetani* spores, and remote settled population away from health care facilities are some of the possible factors which contribute to this lethal disease (tetanus).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2022.103337.

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