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Quality Assessment of Some Brands of Tetanus Toxoids Marketed in Open Markets in South-Eastern Nigeria

V. U. Chigozie[1], P. M. Eze[1*], C. R. Chukwunwejim[1], C. C. Abba[2], C. S. Nworu[3] and C. O. Esimone[1]

[1]Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, NnamdiAzikiwe University, Awka, Nigeria.
[2]Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences, NnamdiAzikiwe University, Awka, Nigeria.
[3]Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Authors CSN and COE designed and supervised the study. Authors VUC and PME managed the analyses of the study and the literature searches. Authors CRC and CCA proofread and edited the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: This study was designed to verify the immunogenicity (potency) and the safety of tetanus toxoid vaccines marketed in three large open drug markets in South-Eastern Nigeria.
Methodology: Tests for Sterility, formaldehyde concentrations, specific toxicity, endotoxin, and immunogenicity (potency) were conducted on three different brands of tetanus toxoids (Brand α - from Ariaria Drug Market, Aba-Abia State; Brand β - from Ogbete Drug Market, Enugu State; and Brand γ - from Bridge-Head Drug Market, Onitsha-Anambra State).
Results: All vaccine brands studied passed the sterility testing, but did not comply with the 2011 BP specifications on free formaldehyde concentration, which stipulates that the free formaldehyde concentration should not exceed 0.02%. The three vaccine brands did

**Corresponding author: Email: ezep2004@hotmail.com;*

not show specific, abnormal, or general toxicity, but contained different amounts of endotoxins. The result of the potency testing showed that the three brands were immunogenic and elicited specific antibodies against tetanus toxin; but brand γ was the most immunogenic since it elicited the highest titers of total IgG, IgG1, and IgG2a followed by brand α, and then brand β.

Conclusion: Generally, the quality control tests carried out on these three commercial brands of tetanus toxoids marketed in Nigeria showed that they do not comply with all the pharmacopeial standards on quality and safety required for vaccines of this nature. Therefore, we conclude that some of the tetanus toxoids marketed in open drug markets in Nigeria are substandard and may be responsible for the failures of these vaccines used for immunization in the country.

Keywords: Tetanus toxoid; vaccine; quality control; sterility test; endotoxin; immunization; potency; safety.

1. INTRODUCTION

Tetanus is a life-threatening condition that is characterized by the symptom of prolonged contraction of skeletal muscle fibers and caused by tetanospasmin, a neurotoxin produced by the Gram-positive, rod-shaped, obligate anaerobic bacterium *Clostridium tetani*. Infections leading to possible tetanus generally occur through wound contamination and often involve a cut or deep puncture wound. As the infection progresses, muscle spasms develop in the jaw and elsewhere in the body [1-2]. Tetanus is an international health problem, as *C. tetani*spores are ubiquitous, but is more common in hot, damp climates with soil rich in organic matters. The disease occurs almost exclusively in persons unvaccinated or inadequately immunized [3].

Tetanus (the neonatal form in particular) remains a significant public health problem in developing countries [4]. An estimated 400,000 deaths occurred annually from neonatal tetanus (NT) prior to 1989 when the World Health Organization (WHO) adopted the goal of eliminating NT as a public health problem worldwide [5,6]. To achieve this, and to control non-neonatal tetanus (non-NT) as well, the WHO recommends that newborns be passively protected at birth by the antepartum administration of at least two doses of tetanus toxoid (TT) to their mothers and that all children subsequently receive at least three doses of diphtheria-tetanus-pertussis (DTP) vaccine [7,8].

In Nigeria the mortality rate of tetanus has been reported to be in the range of 26%-60% [9] and also, tetanus accounts for up to 20% of neonatal deaths [10]. Even with widespread adoption of the WHO prenatal prime-boost TT vaccination strategy, death rates due to NT and non-NT are still high in the Nigeria, despite being among the 12 WHO Expanded Programme on Immunization (WHO/EPI) priority countries [4].

Tetanus infection can be prevented by proper immunization and by post-exposure prophylaxis [7]. For the various TT vaccination strategies to be effective, the TT vaccine in circulation and use must be sufficiently immunogenic. Therefore, the potential factors affecting the immunogenicity of TT need to be evaluated if NT is to be eliminated and if non-NT is to be controlled.

The quality of TT imported and marketed in open markets in Nigeria is a growing concern as the country wages the war against sub-standard drugs. Developing countries, such as Nigeria, which do not yet have manufacturing capacity for immunological products like the TT vaccines, rely heavily on the importation of these products. For this reason and because of the many cases of NT and non-NT in patients that had received adequate prime-boost TT immunization, it is very important to regularly assess the quality of TT marketed in the open drug markets in the country. This study was therefore designed to assess the quality of TT vaccines marketed in large open drug markets in Nigeria.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Test vaccines

Three different commercial brands of TT vaccines were purchased from the three major open drug markets in South-Eastern Nigeria.The samples were obtained and the study conducted between the months of April and May, 2011. Brands α, β, and γ were purchased from Ariaria Drug Market, Aba-Abia State; Ogbete Drug Market, Enugu State; and Bridge-Head Drug Market, Onitsha-Anambra State respectively. The test TT vaccine brands were either sealed in ampoules or in multi-dose vials. The identity and profile of the various vaccine brands are presented in Table 1. All the TT vaccine brands analyzed were imported from India. The samples were stored appropriately at 2-8ºC during analysis, and they were all analyzed before their specified expiry dates.

2.1.2 Reagents, chemicals and culture media

The following reagents were used in this study: Methanol (Sigma Aldrich, Germany), Chloroform (Sigma Aldrich, Germany), Phenyl-hydrazine (Qualikems, USA), ABTS Peroxidase substrate (solution A&B) (KPL, USA), Horse Radish Peroxidase-labeled anti-mouse IgG, IgG1, and IgG2a (KPL, USA), Bovine Serum Albumin (BSA) Solution (KPL, USA), ABTS stop solution (KPL, USA), Imidazole buffered saline (KPL,USA), Coating Buffer, Tween-20 (KPL, USA), Glycerol solution (KPL, USA), Concentrated Hydrochloric acid (BDH, England,) and Isopropyl alcohol (IPA) (BDH, England), Lyophilized endotoxin (generated from *E. coli*: 055:B55) (Lonza, USA), LAL lysate (0.125 sensitivity) (Lonza, USA), Normal Saline (Fluka, England) and Phosphate Buffered Saline (PBS) pH7.2 and Mice Serum (primary antibodies). Culture media used include Thioglycollate Broth (Lab M, UK), Nutrient Broth (ANTEC, UK), and Sabouraud Dextrose Broth (Micro-Master, India). All reagents and culture media used in the analyses were of analytical grade.

2.1.3 Animals and test microorganisms

Guinea pigs of both sexes (weighing between 250-350g) and Swiss albino mice of both sexes (weighing between 14-20g) obtained from the animal facility of the Faculty of Pharmaceutical Sciences, NnamdiAzikiwe University, Anambra State-Nigeria were used in this study. Clinical Isolates of *Escherichia coli* and *Aspergillus niger,* obtained from the Microbiology Laboratory of Bishop Shanahan Hospitals, Nsukka, Enugu State Nigeria, and *Clostridiumsp.,* isolated from soil sample near the Faculty of Pharmaceutical Sciences, NnamdiAzikiwe University, Anambra State-Nigeria, were also used in this study.

Table 1. Identity and profile of the three sample brands of tetanus toxoid (TT) vaccines studied

S/N	Brand code	Manufacturer	NAFDAC registration number	Batch number	Date of manufacture	Expiry date	Price
1	Brand α	DANO Vaccines, India.	Unregistered	1025 A	Nov., 2009	Nov., 2012	1000 Naira (7 USD)
2	Brand β	SII Vaccines, India.	Unregistered	018L0003C	Not indicated	Mar., 2013	1500 Naira (10 USD)
3	Brand γ	Biological E Vaccines, India	04-6587	AE001A	Nov., 2009	Oct., 2012	1500 Naira (10 USD)

2.2 Methods

2.2.1 Sterility testing

Sterility test was carried out on the various brands of TT vaccines in accordance to the methods described in the 2011 British Pharmacopoeia (BP) [11]. Five (5) mL of sterile double strength Nutrient Broth, Sabouraud Dextrose Broth (containing 0.04% chloramphenicol) and Thioglycollate Broth were added respectively to sterile test test-tubes (for the vaccines samples). The test vaccine ampoules were disinfected with 70% alcohol and air dried; then crack-opened with sterile forceps. Five (5) mL of the vaccines samples were transferred into tubes containing the different culture media and the samples were further diluted in a 10-fold serial dilution process with the respective culture media to dilutions of 10^{-1}, 10^{-2}, 10^{-3} 10^{-4} and 10^{-5}. This is to sufficiently neutralize any possible antimicrobial agents contained in the samples. Positive control test tubes containing Nutrient broth, Sabouraud Dextrose and Thioglycollate Broth were respectively inoculated with 1 mL of a suspension of *E. coli, A. niger, and Clostridium sp.* respectively, while uninoculated test tubes containing the respective culture media were used as the negative controls. All test tubes, including the tubes containing dilutions of the various test samples and controls were plugged with sterile cotton wool, and then incubated aerobically at 35-37ºC (for bacterial cultivation) and 20-25ºC (for fungal cultivation) for 14 days. Tubes containing the Thioglycollate medium for the cultivation of anaerobic organisms were placed in an anaerobic candle jar suitable for the growth of anaerobic organisms. All tubes were observed during the incubation period for presence or absence of growth. Samples which show no evidence of microbial growth complies with the test for sterility.

2.2.2 Testing for free formaldehyde concentrations in vaccines samples

The residual formaldehyde concentrations in the brands of TT vaccines were determined by a previously described method [12] which was also adopted by the Centre for Disease, Control, and Prevention (CDC). One (1) mL of each of the vaccine samples and 3 mL of distilled water were added into a separating funnel; followed by the addition of Phenyl-hydrazine, concentrated hydrochloric acid, methanol, and chloroform. The reaction mixture was shaken for about 30 seconds for proper reaction of formaldehyde with phenyl-hydrazine to form formaldehyde- phenylhydrazone, which was extracted from the reaction mixture by the chloroform. The compound formaldehyde- phenylhydrazone is trapped in the chloroform layer; this chloroform layer was collected gently from the funnel via the tap of the separating funnel. The absorbance was measured using a UV-Vis Spectrophotometer (Jenway® Model 6505, UK) at 529nm. The calibration curve was prepared using the OD values of

formaldehyde- phenylhydrazone formed by a similar reaction of serial dilutions of standard formaldehyde solution and measured also at 529 nm.

2.2.3 Specific toxicity testing

Specific toxicity testing of the various brands of TT vaccines was determined according to the procedure outlined in the 2011 British Pharmacopoeia (BP). Fifteen healthy naïve guinea pigs (weighing between 240 - 350g) were randomized into three groups of five animals for per sample vaccine. The guinea pigs were injected subcutaneously with 5-times single human dose (SHD) as stated on the label of each of the tetanus vaccine. The animals were then observed for 21 days for signs and symptoms of tetanus infection.

2.2.4 Endotoxin concentration testing

The level of endotoxin in the TT vaccines test brands was determined by the limulus amoebocyte lysate (LAL) gel clot endpoint assay. This test was carried out in a laminar flow cabinet and all glassware used was de-pyrogenated in a hot air oven (Thermolab, UK) at a temperature of 250ºC for 2 hours. In the determination of the endotoxin levels of the various TT vaccine brands, several dilutions of each of the vaccines (1/8, 1/16, 1/32, 1/64, 1/128, and 1/256) were prepared using LAL reagent water. Endotoxin sample from *E. coli* (055:B5) was reconstituted with 5mL of LAL reagent water as labelled and vortexed for 15 min to give a 20EU/mL standard endotoxin solution. The standard endotoxin solution was further diluted to give a 1EU/mL solution. Two-fold serial dilutions of the standard 1EU/mL endotoxin solution were made in LAL water and mixed properly by 1 min vortexing. The lysate was reconstituted with 5.2mL of LAL reagent water and swirled gently to avoid destruction of the protein. Taking care to avoid any endotoxin contamination, 0.1mL of each dilution of the various TT vaccines brands were carefully transferred into a pyrogen-free reaction tube followed by the addition of 0.1mL of the reconstituted lysate.

Immediately following the addition of the lysate to each tube, the content was mixed thoroughly and the tube placed in an incubating block for 1 h at 37ºC±1ºC. This was repeated for each dilution of the control standard endotoxin. After incubation, the result was taken by carefully removing the assay tubes and inverting to 180ºC. Duplicates were included for all the TT vaccine dilutions and endotoxin dilutions. This assay can be carried out as a qualitative or quantitative test. The quantity of endotoxin present in the TT vaccines was obtained by taking the product of denominator of the endpoint dilution fraction and the Lysate sensitivity.

2.2.5 Vaccine potency and immunogenicity testing

The potency of the tests vaccine brands was determined using the Antibody Induction Method (AIM) in accordance with the provision of the 2011 British Pharmacopoeia.

2.2.5.1 Immunization of mice

Fifteen (15) mice of similar weight were randomized into 3 groups (n=5) and each mice were injected (subcutaneously) with 250µL of a 1:20 dilutions of the TT vaccines in normal saline. The animals were fed with standard livestock pellets and allowed clean drinking water *ad libitum* and then bled after 18 days through the retro orbital plexus using a heparinized capillary tube. Sera samples were recovered after centrifugation of the blood samples at 4000rpm for 30 min. and stored in -20ºC freezer until analyzed.

2.2.5.2 Antibody measurement by ELISA

TT-specific antibody levels in the collected sera samples were determined by indirect ELISA technique. A Titretek® micro-well ELISA plate was coated with 100µL of a 1:10 dilution of TT in 1x coating buffer and incubated overnight at 4ºC. The coated plate was washed 3 times with a 1x wash solution containing 0.05% Tween 20 in imidazole buffered solution. The nonspecific binding sites were blocked with 300µL 1% solution bovine serum albumin (BSA) in PBS and incubated for 1 hour at room temperature. After the incubation, the micro-wells were washed 3 times again with 1x wash solution and then 100µL of 1/20 dilutions of the mice sera in 0.1% BSA were added into the wells in duplicates and incubated at room temperature for 1hour. After the incubation, each of the micro-well was washed 3 times with 1x wash solution and 100µL of the horse radish peroxidase (HRP)-conjugated secondary antibodies (IgG, IgG1, and IgG2a) diluted in 0.1% BSA solution was added into each well and incubated for another 1 hour at room temperature. After the incubation of the secondary antibodies, the wells were washed 3 times and 100µL of ABTS peroxidase substrate was added into each well for 15 min. The optical density of the colored enzyme-substrate (HRP-ABTS) reaction product in the plate was read at 405 nm using Thermomax® ELISA plate reader.

3. RESULTS AND DISCUSSION

3.1 Results

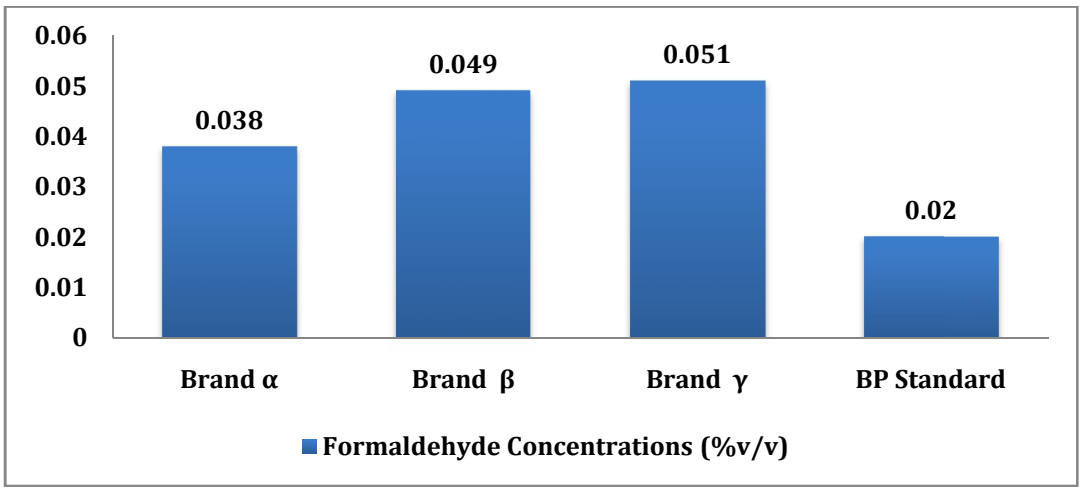

Fig. 1. Free formaldehyde Concentrations in the Various Tetanus Toxoid Brands

3.2 Discussion

All test vaccine brands studied passed the sterility testing since none of the test vaccine brands produced any visible growth in the growth media after fourteen (14) days of incubation, which is in line with the provisions of the British Pharmacopoeia (BP) (see Table 2).

The result of the formaldehyde concentration test revealed that the three test TT vaccine brands did not comply with the BP specification on free formaldehyde concentration which

stipulates that the free formaldehyde concentration should not exceed 0.02% [11]. Free formaldehyde is a measure of residual formaldehyde concentration left and acceptable in the final product (TT vaccines) after the toxoiding process [13]. The result showed that the free formaldehyde concentrations in the three toxoid vaccine brands were way beyond the acceptable concentration in TT vaccine as stipulated by the BP [11]. The free formaldehyde concentrations calculated from the standard plot were 0.0380% for Brand α, 0.049% for Brand β, and 0.051% for Brand γ. Brand α (Dano vaccines) contained 90% more than the required formaldehyde concentration; Brands β and γ contained 145 and 115% more than the required formaldehyde concentration respectively (see Fig. 1). The result also revealed that Brand γhas the highest free formaldehyde concentration; followed by Brand β and then Brand α, which has the least free formaldehyde concentration. Formaldehyde, which is the detoxifying (toxoiding) agent in the TT vaccines, is a tissue irritant and some patients are particularly very sensitive to high formaldehyde concentration in TT vaccine. The three brands of vaccines tested may cause local reactions and tissue damage at the site of injection. This shortcoming alone is enough to disqualify these products from being marketed to consumers in Nigeria, but they are marketed and used freely.

The results of the specific toxicity tests showed that the three vaccine brands had no specific, abnormal, or general toxicity (Table 3). This implies that the vaccines were not contaminated with traces residual tetanus toxin in the vaccines. The guinea pigs used for this study survived a five times single human dose (2.5mL) injection of the test TT brands (administered subcutaneously). Hence, the test vaccines were considered to have negligible or no specific toxicity as specified in the 2011 British Pharmacopoeia. Specific toxicity signifies the presence of the Clostridial toxins in the formol toxoid, or the possibility of toxoid reversal; which can induce tetanus symptoms. These symptoms include fever, severe muscular discomfort, spasm followed by possible bone fracture, then death [11]. What dictated the choice of animal (guinea pigs) is the exquisite sensitivity of guinea pigs to tetanus toxins (tetanospasmin) in a very minute concentration [13].

The result of the endotoxin test carried out on the various toxoid vaccines is presented in Table 4. Vaccines are supposed to be endotoxin free; but the result of this test showed that all the three test vaccine brands contained different amounts of endotoxins. Endotoxin is very difficult to get rid-off in bacterial vaccines like TT, but concentrations present in the product should be such that does not generate fever when administered to a patient. It is well known that even a small amount (1ng = 10EU) of endotoxin can cause a change in the physiology of humans [14]. In a preclinical research sponsored by Novartis vaccines and Diagnostics on the acceptable level of endotoxin concentration in vaccine formulation, toxoid vaccines (such as TT) were reported to have the highest endotoxin values due to the complexity of these vaccines and the fact that they are all derived from bacteria with minimal purification [15]. The implication of endotoxin, a Toll-Like Receptors (TLR)-4 agonist, contamination of vaccine products is the distortion of the physiology and other consequent adverse effect such as fatal generalized Schwartzman reaction, depending on the endotoxin concentration [16-18]. Endotoxin triggers macrophages to release interleukin-1 which is carried to the hypothalamus of the brain where it causes a resetting the body's thermostat to a higher temperature thereby causing pyrexia [19]. The effects of endotoxin on the central nervous system, cardiovascular system, kidney, liver, hypothalamus, and lymphatic systems have been documented in experimental animal studies [20,21].

Table 2. Sterility test results of the various brands of tetanus toxoid

Growth conditions	Test organisms	Growth medium	Vaccine brands			Controls	
			Brand α	Brand β	Brand γ	Positive	Negative
Aerobic	*Escherichia coli*	Nutrient Broth	-	-	-	+	-
	Aspergillus niger	Sabouraud Dextrose Broth	-	-	-	+	-
Anaerobic	*Clostridium tetani*	Thioglycollate Medium	-	-	-	+	-

- : growth absent; + : growth present

Table 3. Specific toxicity test of the different vaccine brands

Vaccine Brands	Brand α	Brand β	Brand γ
Animals Used	Guinea Pigs	Guinea Pigs	Guinea Pigs
Weight Range	250-350 g	250-350 g	250-350 g
Injected Volume	2.5mL (5 x SHD)	2.5 mL (5 x SHD)	2.5 mL (5 x SHD)
Observation	No symptoms of tetanus or death	No symptoms of tetanus or death	No symptoms of tetanus or death

SHD= Single human dose

Table 4. Endotoxin levels in the tetanus toxoid vaccines determined by the limulus amebocyte lysate assay

Vaccine dilutions	Tetanus toxoid brands		
	Brand α	Brand β	Brand γ
Undiluted	+	+	+
1/8	+	+	+
1/16	+	+	+
1/32	+	+	+
1/64	+	-	+
1/128	+	-	-
1/256	-	-	-
Endotoxin unit estimation	16 EU/mL	4 EU/mL	8 EU/mL

+ = Firm gel; - = No gel; EU = Endotoxin Unit

TT vaccines serve to protect the recipient by eliciting antibodies in the serum that prevent infection, interfere with microbial spread, or sequester and nullify pathogenic toxins. The potency of the various TT brands was assessed by immunization experiment in which the antibody responses of mice were determined by ELISA techniques after administering the same dose (4 IU) of the vaccines brands to groups of mice subcutaneously. The potency of the various TT brands is directly related to their immunogenicity. In the potency study, the TT specific total IgG, IgG1 and IgG2a were measured in sera samples of mice immunized with the three test TT vaccine brands. As seen in Fig. 2, the results of the immunological study on commercial TT vaccine brands show that immunization with all the TT brands elicited specific antibodies against tetanus toxin; but Brand γ was the most immunogenic since it elicited the highest titers of total IgG, IgG1, and IgG2a followed by Brand α, and then Brand β. The purpose of TT vaccination is to evoke specific neutralizing immunoglobulins against tetanus toxin. Therefore, these vaccines are capable of inducing immunoglobulin G (IgG) subtype which is involved in neutralization of tetanus toxin in event of tetanus.

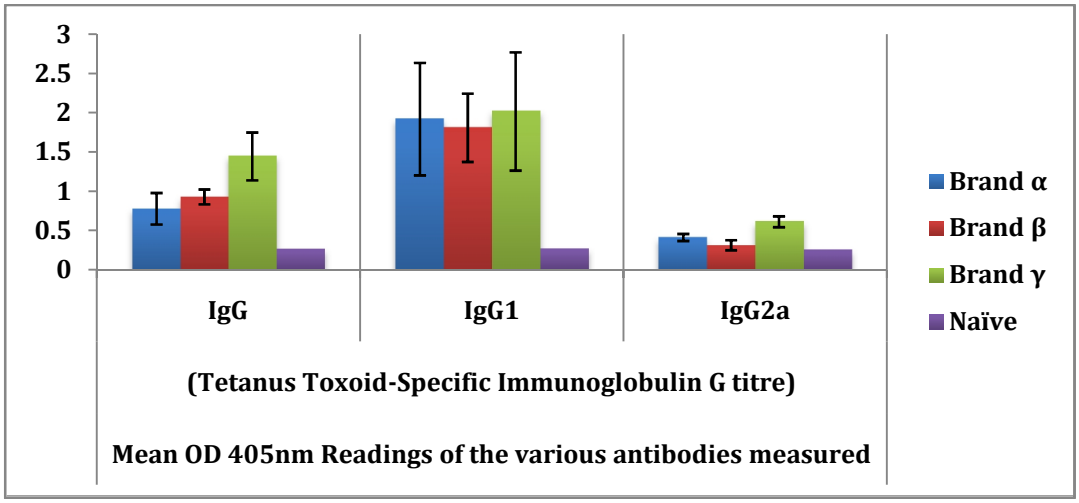

Fig. 2. Total Immunoglobulin G Responses of Mice to the Brands of Tetanus Toxoid under Study

In Nigeria, apart from the open markets, it can be quite difficult to purchase these vaccines except by buying directly from the manufacturers. The costs of the vaccines are shown in Table 1 and these vaccines cannot be said to be too expensive. Because these vaccines are mainly sourced not by individuals, but by hospitals or other health institutions that carryout vaccination programmes, the availability of safe and effective vaccines is possible regardless of the costs. Therefore vaccines of good quality should be of utmost importance regardless of the costs in safeguarding the lives of the people.

4. CONCLUSION

The results of the quality control carried out on three commercial brands of TT marketed in open markets in South-Eastern Nigeria showed that the vaccines did not comply with all the pharmacopeia standards required for vaccines of this nature. The outcome of this research underscores the need for more stringent quality control and regulation of vaccines marketed in open drug market where proper storage is not guaranteed. Marketing and use of vaccines of doubtful sources should be prohibited outrightly.

CONSENT

Not applicable.

ETHICAL APPROVAL

All authors hereby declare that "the principles of laboratory animal care" (NIH publication No. 85-23, revised 1985), were adopted, and experiments were examined and approved by the appropriate ethics committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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