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Evaluation of the Efficacy and Safety of Hepatitis B Vaccines Sold in Open Markets in South Eastern Nigeria

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Abstract

This study was carried out to determine the compliance of different brands of Hepatitis B vaccines marketed in open markets across South-Eastern Nigeria with Pharmacopoeial standards for safety and immunogenicity. Four different commercial brands of hepatitis B vaccines (Hepavax, Euvax, Sii and Engerix brands) were purchased from open markets across the five states of South-Eastern Nigeria. The toxicity and immunogenicity of the different vaccine brands were evaluated using animal model. Using 70 mice, the immunogenicity of the vaccines was evaluated by quantifying Hepatitis B-specific antibody levels after immunization. Similarly, safety of these vaccines was evaluated by investigating the sterility, endotoxin levels, and changes in the weight, liver enzymes (ALT) and white blood cell count (WBC) The various brands of hepatitis B vaccines appeared sterile and conformed to Pharmacopeial requirements for parenteral products. All the vaccine samples were all endotoxin-free and non-toxic. The total white blood cell (WBC) count showed no significant increase at 18 and 72 hours in the test animals after administration of the vaccines. Except in animals immunized with Euvax 1 brand, there was no significant change ($P < 0.05$) in the weights of the test animals at Day 8 after immunization compared to the control. There was no statistically significant difference ($P > 0.05$) between the ALT levels produced by various vaccine brands. All the vaccines induced the production of Hepatitis B specific antibodies. All the tested brands appeared immunogenic. However, while three brands conformed to the Pharmacopeial safety profile, more studies are needed to further evaluate the safety of Euvax because on unsatisfactory weight changes observed in this study.

Keywords: Hepatis B Vaccines; Nigeria; Commercially Available; Efficacy; Immunogenicity; Safety;

Introduction

Hepatitis B is a blood-borne and sexually transmitted infection. In countries where the disease is highly endemic, perinatal and horizontal transmission is evident [1,2,3]. Hepatitis B virus (HBV) infection is a serious global public health problem and is endemic in Africa, including Nigeria [4]. It is the commonest cause of Chronic Liver Disease (CLD) in Nigeria [5]. HBV infection affects about one-third of the world's population [1]. The outcome of HBV infection varies greatly from person to person. In most of the cases the infection is cleared spontaneously, however, about 5% of adults will develop chronic infection [6]. By contrast, 40 – 90 % of children who are born to HBV-infected mothers will progress to develop a persistent liver disease [7]. The infection can be asymptomatic or symptomatic, may take the form of acute or chronic liver disease but it is potentially fatal. Adults that acquire acute infection usually recover or can be managed by supportive therapy and relief of symptoms via provision of adequate nutrition and fluid replacement, while the chronic type is ultimately fatal [8,9,10]. HBV has been reported to be a leading cause of liver cancer, and an important contributor to cirrhosis as well as end-stage liver disease requiring liver transplantation. In appreciation of the above-outlined issues WHO have recommended that hepatitis B vaccines should be included in national immunization programmes [10]. The most effective way of reducing global incidence is vaccination as well as effective enforceable public health policies [11]. Since the introduction of preventive vaccination programs against hepatitis B in over 170 countries, the number of new infections is continuously decreasing [2,12]. The use of vaccination to prevent diseases is the greatest public health success of the last century [11]. Complete vaccination induces protective antibodies in more than 95% of vaccinated subjects [13]. Efforts have been intensified by many researchers/scientists, companies and Governments all over the world to improve and manage available vaccines as well as develop new ones [14].

Hepatitis B recombinant Vaccine (HBV) is a preparation

of purified hepatitis B surface antigen (HBsAg) that has been produced by recombinant DNA techniques. The preparation should satisfy all the production and quality control requirements [14]. General requirements, such as tests for potency, purity, toxicity, pyrogenicity, and sterility will apply as much to hepatitis B vaccines made by recombinant DNA methods [15]. Generally, there are side effects associated with HBV. Reactions, such as local pain, myalgia and transient fever occur mostly within 24 hours. There are also documented cases of fever, rash, soreness or swelling where the shot was given, or temporary pain and stiffness in the joints [16,17].

The quality of hepatitis B vaccines marketed in open markets in Nigeria is a growing concern as the country wages war against sub-standard drugs especially as the country does not manufacture such immunological product, but rely heavily on the importation of these products. The number of pharmaceuticals imported has more than doubled in the past decades [14,18]. The globalization of pharmaceutical industries supply chain appears to be increasing, and as such, increasing the risk of pharmaceutical consumers being exposed to drug products or immunologic products that have been contaminated, counterfeited or mislabelled [14]. In addition, economically motivated adulteration of pharmaceutical raw materials is on the rise [15,19]. Hence, the chances of the purchase and usage of these products that may be fake, substandard, adulterated, contaminated, counterfeited or mislabeled are high. The overall effect of these is that a patient may not obtain the desired effect at the required time [16,20]. This study was therefore designed to determine the compliance of different brands of Hepatitis B vaccines marketed in open markets across South-Eastern Nigeria with Pharmacopoeial standards for safety and immunogenicity. It is our expectation that the results of this study will provide safety and immunologic information on Hepatitis B vaccines commercially available in South Eastern Nigerian markets.

Material and Methods

Study area/ Vaccines collection

Different commercial brands of hepatitis B vaccines were purchased from open markets across five states of South-Eastern Nigeria (Anambra, Imo, Abia, Enugu and Ebonyi States). Vaccines were transported in an insulated vaccines carrier and stored in the storage facility of Pharmaceutical Microbiology and Biotechnology, Nnamdi Azikiwe University within 2-3 h of collection. Temperature at collection was 4 ± 1°C and before storage 5 ± 1°C. Study was conducted within 1 month of vaccine collection and the temperature of the storage facility was charted daily. The study was approved by the Ethics Committee of Chukwuemeka Odumegwu Ojukwu University Teaching Hospital, Awka **(COOUTH/AA/VOOL.1.003).**

Animal Studies

Albino rats of both sexes, weighing 45-70 g each, were used. They were housed in the animal house in the Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University Awka under the standard condition of temperature (25 ± 5°C) and relative humidity (60 ± 5 %) and fed with standard pellets diet and water. The animals were kept to acclimatize for about a week before they were randomly divided into the different experimental groups. The use of animals in this research was in accordance with the guidelines approved by the Animal Ethical committee, Nnamdi Azikiwe University Awka Nigeria.

Sterility Testing

Sterility testing was carried out on the different vaccine brands by the Direct Inoculation Method as described by the European Pharmacopoeia and a modification of Haruka, et al. [17,21,22].

Toxicity testing

Effect of Vaccine on Liver (Alanine aminotransferase Test)

The animals were divided into 7 groups of 10 animals per group and each brand of hepatitis B vaccine administered to each group respectively. One group of the test animals received normal saline (0.9%) as the control. Alanine aminotransferase (ALT) in the serum of the test animals was determined using Randox diagnostic kit (Randox Laboratories Ltd., England) with 2,4-dinitrophenylhydrazine as substrate as described by Reitman and Frankel [23].

Briefly, 0.1ml of test animal serum and 0.5ml of 2,4-dinitrophenylhydrazine were mixed and followed by incubation at 37°C for 30 min. The colour reagent (0.5ml) was added and the mixture was mixed properly and allowed to stand at room temperature for another 20 min. After which 5 ml of 0.4 M NaOH solution was added to terminate the reaction. The absorbance was read at 546 nm against the blank after 5 min.

The activity of the enzyme was extrapolated from the standard calibration curve obtained from an absorbance-enzyme activity table of values provided by the manufacturer. Enzyme activity was expressed in IU/L protein. One unit of alanine aminotransferase activity was defined as the amount of protein that liberated one micromole (1μm) of pyruvate per ml / min under experimental condition.

Effect of Vaccines on Total White Blood Cell Count

Total white blood cell count (Total leukocyte count) was determined by Haemocytometer method as described by Schalm, et al. [24]. The animals were divided into 8 groups of 10 animals per group and each brand of hepatitis B vaccine administered to each group respectively. Two groups of test animals respectively

received cyclophosphamide (400 mg/Kg) and normal saline (0.9%) as the controls. The WBC count of the animals was first determined before administration of the vaccines, and also determined 18 and 72 hrs after vaccination. A total of 0.02 ml of blood was collected with pipette into a small test tube containing 0.38 ml of 2% acetic acid tinged with gentian violet to make a 1:20 dilution of the blood sample. The diluted sample was loaded on to the Neubauer counting chamber, and all cells on the four corner squares were counted using a light microscope at x10 objective. The number of cells counted for each blood sample was multiplied by 50 to obtain the total white blood cell count per microlitre of blood.

Effect of Vaccines on Animal Body Weight (Animal Body Weight Change Test)

The animals were divided into 8 groups of 10 animals per group and each brand of hepatitis B vaccine administered to each group respectively. Two groups of test animals respectively received cyclophosphamide (400 mg/Kg) and normal saline (0.9%) as the controls Weights of the animals in each group were recorded before vaccination and then recorded daily for 7 days. The change in weight over the 7 day period was calculated.

Endotoxin testing

The level of endotoxin in the hepatitis B vaccines test brands was determined by the limulus amebocyte lysate (LAL) gel clot endpoint assay using the method described by Chigozie, et al. [25]. Briefly, 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 5 mL of LAL reagent water as labeled and vortexed for 15 min to give a 20 EU/mL standard endotoxin solution. The standard endotoxin solution was further diluted to give a 1 EU/mL solution. Two-fold serial dilutions of the standard 1 EU/mL endotoxin solution were made in LAL water and mixed properly by 1 min vortexing. The lysate was reconstituted with 5.2 mL of LAL reagent water and swirled gently to avoid destruction of the protein. Taking care to avoid any endotoxin contamination, 0.1 mL of each dilution of the various hepatitis B vaccine brands were carefully transferred into a pyrogen-free reaction tube followed by the addition of 0.1 mL 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 37°C ± 1°C. 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 180°C. Duplicates were included for all the hepatitis B vaccine dilutions and endotoxin dilutions. The quantity of endotoxin present in the hepatitis B vaccines was obtained by taking the product of denominator of the endpoint dilution fraction and the Lysate sensitivity.

Vaccine Immunogenicity Testing

Vaccination of animals and antibody measurement was carried out using the method described by Mahboubi, et al. [26].

Vaccination of mice

Ten (10) mice of similar weight were randomized into 8 groups (Groups A to H). Groups A to F were respectively injected intraperitoneallly with 0.5mL of 4ug/mL of the different brands of hepatitis B vaccines. Group G was injected with 0.5 mL of 0.9 % normal saline and Group H was administered with 400 mg/Kg of cyclophosphamide orally. The animals were fed with standard livestock pellets and allowed clean drinking water *ad libitum* and then bled after 28 days through the retro orbital plexus using a heparinized capillary tube. Sera samples were recovered after centrifugation of the blood samples at 4000 rpm for 30 min and stored in -20°C freezer until analyzed.

Antibody measurement by ELISA

Hepatitis B-specific antibody levels in the collected sera samples were determined qualitatively by indirect ELISA technique using a CUSABIO® mouse HBsAb ELISA Kit (Wuhan Hi-tech Medical Devices Park, China). Briefly the microtiter well plate was correctly labeled to accommodate all six (6) vaccine test samples and the controls. Blank wells into which no solution will be added were also labeled. A 50µl of negative control and positive control were added to their respective wells and a 50µl of the samples were added to their respective wells. Thereafter a 50µl of HRP-conjugate was added to each well (not to blank well). The microtiter plate was covered with an adhesive strip and incubated for 30 minutes at 37°C. Each well was aspirated and washed, and the process repeated four times for a total of five washes. Washing was done by filling each well with Wash Buffer (200µl) using a multi-channel pipette and allowing the plate to stand for 20 seconds. After the last wash, any remaining Wash Buffer was removed by aspirating or decanting. The plate was then inverted and blot dried against clean paper towels. Following which a 50µl of chromogen Substrate A and 50µl chromogen Substrate B were added to each well. The plate was then incubated for 15 minutes at 37°C while protected from light. Thereafter 50µl of Stop Solution was added to each well and the plate gently tapped to ensure thorough mixing. The optical density (OD) of each well was determined within 10 minutes, using a Thermomax® ELISA plate reader set to 450 nm and the OD of the blank was subtracted from those of the samples and controls to get their actual ODs.

Statistical Analysis

Data were analyzed using Anova and Student's T-test. A p-value of ≤ 0.05 was considered statistically significant.

Results

As observed in table 1, all the vaccine brands used in the study passed the test for sterility, since they showed no visible growth in the double strength media employed after 14 days of incubation in line with the provisions of the European Pharmacopoeia (2005). Similarly, all the brands of Hepatitis B vaccines were endotoxin free (Table 2). The results of the toxicity testing on the vaccine brands (Figure 1, Tables 2 & 3) showed that all brands evaluated complied to pharmacopoeial requirements to be nontoxic. From the results of the total white blood cell (WBC) count (Table 2) it can be observed that there was no significant increase ($P > 0.05$) in the WBC at 18 and 72 hours in the test animals after administration of the vaccines. The results of weight change tests show that except the animal groups treated with Euvax 1 brand and cyclophosphamide which recorded reduction in weight on the eight day after administration, other groups of test animals administered with the other vaccine brands recorded weight gain on the eight day after vaccine administration (Table 4). There was no statistically significant difference ($P > 0.05$) between the ALT levels produced by various vaccine brands and the control (Figure 1). The results of the total WBC count, body weight test and ALT test therefore shows that the various vaccines brands studied were safe and nontoxic to the test animals (Table 5). From the table 4, it could be observed that all vaccine samples tested were immunogenic as they were able to induce the production of antibodies specific for the viral antigen contained in the vaccines

Table 1: Result of Sterility Test carried out on the Brands of Hepatitis B Vaccines.

Growth Conditions	Test Organisms	Growth Medium	Brands of vaccines						Controls	
			Hepavax 1	Hepavax 2	Sii	Euvax 1	Euvax 2	Engerix	Positive	Negative
Aerobic	E. coli	Nutrient Broth	-	-	-	-	-	-	+	-
	A. niger	Sabouraud Dextrose Broth	-	-	-	-	-	-	+	-
Anaerobic	Clostridium sp	Thioglycollate Medium	-	-	-	-	-	-	+	-

Key: + = microbial growth, - = No microbial growth

Table 2: Mean WBC count of test animals administered with test vaccines, cyclophosphamide and normal saline

		A Hepavax	B Sii	C Euvax 1	D Euvax 2	E Hepavax 2	F Engerix	G Cyclophosphamide	H Normal saline
WBC Before Immunization	Mean	56.7	64.5	69.6	55.2	63.9	65.7	65.6	64.7
	SD	2.86	7.05	5.87	4.24	6.06	4.08	4.55	7.41
After Immunization (18 hrs)	Mean	67.7	72.1	73.3	67.6	71.2	58.4	37.5	71.3
	SD	12.57	8.45	3.59	4.45	9.62	7.97	4.27	8.01
After Immunization (72 hrs)	Mean	74	86.3	72.6	64.6	72.1	82.8	34.3	57.5
	SD	4.16	11.34	6.06	7.82	12.21	5.49	5.52	6.84
P-value		0.81	0.26	0.15	0.73	0.39	0.15	0.62	NA

SD: standard deviation **NA:** not applicable

Table 3: Mean body weights (g) of test animals administered with test vaccines, cyclophosphamide and normal saline.

Brands		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Hepava1	Mean	22.22	22.19	21.17	22.03	22.2	23.46	23.49	24.31
	SD	2.6	2.61	2.58	2.71	2.78	2.91	3.02	2.83
Sii	Mean	24.75	25.99	25.73	27.15	25.22	26.44	26.56	27.2
	SD	2.49	2.6	2.49	2.5	2.6	2.7	2.55	2.53
Euvax 1	Mean	22.37	21.97	21.72	23.05	22.36	21.57	21.42	21.84
	SD	2.68	2.94	2.89	3.11	3.07	2.96	2.9	2.96
Euvax 2	Mean	23.05	22.6	22.76	24.04	24.41	25.15	23.48	24.5
	SD	7.04	6.57	6.51	6.6	6.45	6.42	5.55	5.57
Hepavax 2	Mean	25.91	25.67	26.22	28.31	27.61	29.1	28.8	29.34
	SD	2.81	3.43	3.34	3.36	3.2	3.29	3.43	3.49
Engerix	Mean	24.96	23.6	25.18	24.44	24.67	24.67	24.83	25.22
	SD	3.67	3.12	3.32	3.18	3.051	3.05	3.05	3.08
Cyclophosphamide	Mean	23.32	23.84	22.45	23.05	21.94	21.38	20.12	20.73
	SD	3	2.94	3.49	3.06	3.15	3.16	3.03	2.93
Normal saline	Mean	23.49	24.37	23.2	23.52	23.08	24.5	23.14	24.13
	SD	2.15	3.16	3.34	3.21	3.33	3.47	3.47	3.33

SD: Standard Deviation **NA:** Not applicable

Table 4: Results of the immunogenicity study carried out on the different test vaccine brands

Brands	Mean Optical density (OD)	Mean OD of Samples - Mean OD of Blank	Mean OD of sample /Mean OD of negative control	Immunogenicity
Hepavax	0.3824	0.3379	4.118	Positive
Sii	0.7067	0.6622	8.0695	Positive
Euvax 1	0.5747	0.5303	6.4611	Positive
Euvax 2	0.7844	0.7399	9.0161	Positive
Hepavax 2	0.6752	0.6308	7.6856	Positive
Engerix	0.5402	0.4958	6.0407	Positive
Cyclophosphamide	0.1751	0.1307	1.5921	Negative
Normal saline	0.1583	0.1139	1.3875	Negative
Blank	0.044429	0	NA	NA
Positive control	1.216	1.1716	NA	NA
Negative control	0.1265	0.0821	NA	NA

OD (sample)/OD (negative control) ≥2.1: Positive

OD (sample/OD (negative control) ＜2.1: Negative **NA:** Not applicable

Table 5: Summary of results of safety analyses carried out to evaluate the various vaccine brands

Brands	Sterility Test	Endotoxin test	ALT test	TWBC count	Body weight change test	Potency/ Immunogenicity testing
Hepavax 1	Pass	Pass	Satisfactory	Satisfactory	Satisfactory	Immunogenic
Hepavax 2	Pass	Pass	Satisfactory	Satisfactory	Satisfactory	Immunogenic
Sii	Pass	Pass	Satisfactory	Satisfactory	Satisfactory	Immunogenic
Euvax 1	Pass	Pass	Satisfactory	Satisfactory	Unsatisfactory	Immunogenic
Euvax 2	Pass	Pass	Satisfactory	Satisfactory	Satisfactory	Immunogenic
Engerix	Pass	Pass	Satisfactory	Satisfactory	Satisfactory	Immunogenic

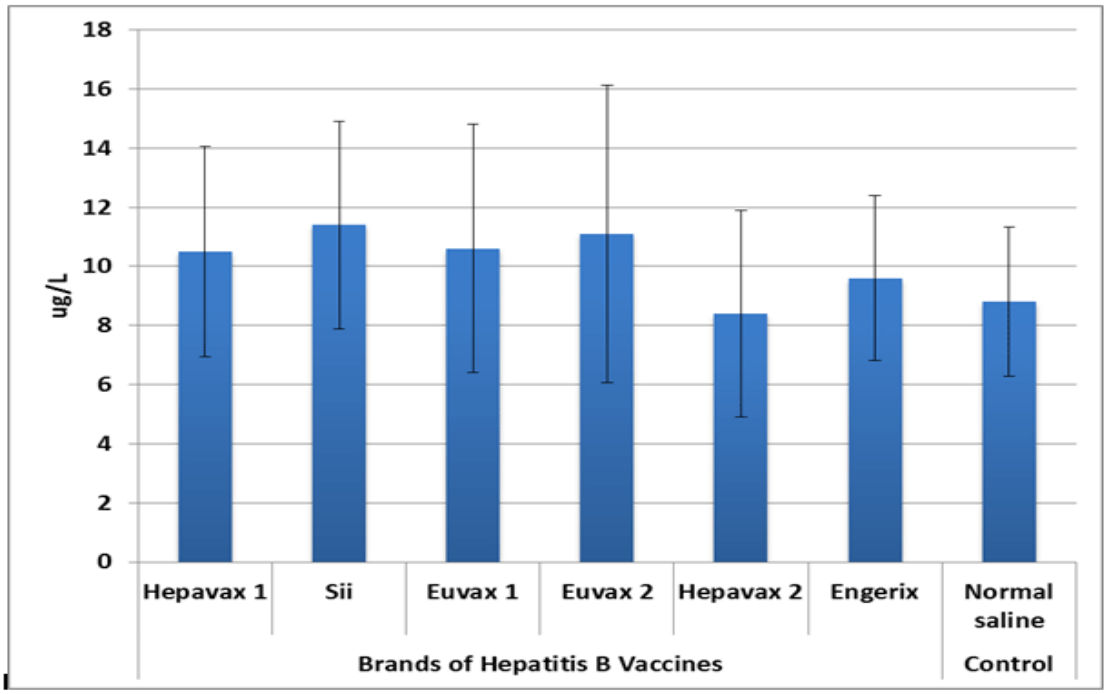

Figure 1: Result of the ALT test carried out on the test vaccine samples.

Discussion

In many countries, immunization programmes for HBV are implemented. Despite this, HBV prevalence is not decreasing [27]. This may be due to incomplete vaccination or inefficacy of the immunization programme [28]. The WHO (2010) stated that because of the diversity in the reactivity of vaccines containing HBsAg produced by different manufacturing processes and to which different adjuvants or immunostimulants have been added, recombinant hepatitis B vaccines produced by different manufacturers must be considered as different products. Thus the brands of hepatitis B vaccines (all imported and used in Nigeria) were evaluated for their quality, safety and performance. The purpose of this study was to determine the compliance of these vaccines marketed in open markets in South-Eastern Nigeria with Pharmacopoeial standards for safety and immunogenicity [17].

WHO has developed recommendations for ensuring quality, safety and efficacy of recombinant hepatitis B vaccines [10,16,17]. The Limulus amoebocyte lysate (LAL) test is currently employed to detect and quantify endotoxins in various biological products for parenteral administration and this test is widely used as a convenient method due to its high sensitivity, its reliability and its simple handling [29]. All the brands of Hepatitis B vaccines were endotoxin free (Table 5). Yihui, et al. stated that endotoxins should be completely removed in the final products for parenteral administration because of the high toxicity; hence, the determination for amounts of remaining endotoxin in these products is of great importance [29]. The monitoring and reporting of endotoxins and other contaminants in vaccines might be useful in understanding some of the adverse effects observed in vaccine recipients. Among many quality control tests, conventional animal safety tests are performed to detect vaccine toxicity because residual vaccine toxicity has the potential to cause adverse reactions [30]. The safety of vaccines has been assessed using several animal tests, including the body weight change test and white blood cell counts [31,32]. European Union recommends that vaccines be considered safe only after testing according to laid down procedures. They recommended that the assessment of safety in the target species should be based on clinical reaction and laboratory animals' weight gain compared with unvaccinated controls [32,33]. In our findings, except the animal groups treated with Euvax 1 brand and cyclophosphamide which recorded reduction in weight on the eight day after administration, other groups of test animals administered with the other vaccine brands recorded weight gain on the eight day after vaccine administration (Table 3). The loss in the weight recorded by the animals that received the Euvax 1 is a concern, since all the animals were given same food and kept in the same environment. It could be that the vaccine was safe at the time of collection and/or may have been affected by any slight breech in the cold-chain since vaccines are biological products and failures are bound to occur when vaccines are mishandled / not refrigerated [34]. The body weight change test has been used to infer the toxicity of substances and a good correlation of the

body weight loss with a vaccine's toxicity has been shown [35]. Good vaccines should not cause a decrease in the weight of the mice 3 days post-vaccination, produce a less than 60% mean weight gain per mice compared with the control at 7 days post-vaccination or greater than 5% animal death during the 7 days observation period nor any sign of illness on the animals [36,37]. Similarly, the Alanine aminotransferase (ALT) test was used to evaluate the liver toxicity of the vaccine brands. ALT is an enzyme present in hepatocytes and is a marker of liver cell inflammation [38,39]. ALT is typically elevated in individuals with acute viral hepatitis and it rises dramatically in acute liver damage [39]. Elevations are often measured in multiples of the upper limit of normal (9 to 60µg/L) [38]. In this study there was no statistically significant difference ($P > 0.05$) between the ALT levels produced by various vaccine brands and the control (Figure 1). Sustained and intermittent elevations in ALT beyond the upper limit of normal are indicative of hepatic inflammation and correlate with an increased risk of progressive liver disease [40]. Therefore all the vaccine brands tested are safe to the liver. The safety of the vaccines may be attributed to good storage conditions, the absence of residual toxin and chemicals in the vaccines and lack of degradation products in the vaccines.

Vaccines serve to protect the recipients by eliciting antibodies in the serum that prevent infection, microbial spread and/or nullify pathogenic toxins [25]. The potency of the various HBV brands was assessed by immunization experiment in which the antibody responses of mice were determined by ELISA techniques after administering the same dose (0.5mL of 4ug/mL) of the vaccines brands to groups of mice intraperitoneallly. The potency of the various HBV brands is directly related to their immunogenicity. All the vaccines tested were immunogenic as their ratio of Optical density (sample) to that of negative control is ≥ 2.1.

Conclusions

All the tested brands appeared immunogenic. However, while three brands conformed to the Pharmacopeial safety profile, more studies are needed to further evaluate the safety of Euvax because on unsatisfactory weight changes observed in this study. The need for continued monitoring and quality control of these products in the country to prevent the introduction and proliferation of fake, substandard, adulterated, or contaminated brands is worthwhile. Marketers of vaccines should strive to store these products under the recommended temperature conditions and the government on their own should improve power supply so as to encourage this storage habits.

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