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STABILITY AND STERILITY STUDIES ON THE ROUTINE IMMUNIZATION VACCINES IN USE IN SOUTH-EAST NIGERIA

Angus Nnamdi Oli[1]*, Remigius Uche Agu[2] and Charles Okechukwu Esimone[1]

[1]Department of Pharmaceutical Microbiology and Biotechnology, Nnamdi Azikiwe University, Faculty of Pharmaceutical Sciences, Agulu Campus, Anambra State, Nigeria

[2]Biopharmaceutics and Drug Delivery Laboratory, College of Pharmacy, 5968 College Street, PO Box 15000, Dalhousie University, B3H 4R2, Halifax, NS, Canada

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***Correspondence for Author:**
Angus Nnamdi Oli
Department of Pharmaceutical Microbiology and Biotechnology, Nnamdi Azikiwe University, Faculty of Pharmaceutical Sciences, Agulu Campus, Anambra State Nigeria.
oli_an@yahoo.com

ABSTRACT

Objectives: The study sets to evaluate the stability and sterility of the vaccines used in the routine immunization programme in the five states of the south-east geo-political zone of Nigeria (Abia, Anambra, Ebonyi, Enugu and Imo States). **Methods:** The stability studies carried out include: thermal stability test by colony count for the Bacillus Calmette-Guérin (BCG) Vaccines, physical examinations on all the vaccines and shake test on the Diphtheria-Tetanus-Pertussis (DPT), Hepatitis B and Pentavalent vaccines. Test for sterility was carried out using tests for bacteria and fungi contamination. The bacteria endotoxin quantification test (Limulus Amebocyte Lysate test) was also done to test for the presence or absence of pyrogens/endotoxins. **Key findings:** The BCG vaccines have mean percentage potency losses of 13.67±4.45 > 12.99±5.15 > 12.44±4.36 > 12.05±4.98 > 10.71±6.62 for from Anambra, Abia, Enugu, Imo and Ebonyi respectively. The shake test showed that the adsorbed vaccines (DPT, Hepatitis B and Pentavalent) had no granules or floccules. The vaccines' mean settling times were 25.33 ±2.52, 20.60 ±2.07 and 25.50 ±0.71 minutes for DPT, Hepatitis B and Pentavalent vaccines used as control respectively and 72.00±17.32, 63.20±12.28, 63.50±2.12 minutes for the vaccine samples respectively. On physical examination, none of the vaccines had any visible change in appearance. The Limulus Amebocyte Lysate (LAL) test did not detect any bacteria endotoxin in the viral vaccine except in the bacteria vaccines (BCG and DPT). However, the

endotoxin concentration was within the Pharmacopeias limit for vaccines and biological products. The tests for bacteria and fungi contamination did not reveal any contaminant. **Conclusions:** The vaccines showed good and acceptable sterility and stability profiles.

Keywords: Routine-Immunization, Vaccine, Stability, Sterility, South-east Nigeria.

1. INTRODUCTION

While vaccines and other biological medicines play important role in improving world health and will continue to contribute to public health even in the foreseeable future [1], their effectiveness depends on whether they are sterile and have been stored at appropriate temperatures (which affects their stability). Stability of vaccines relate to the ability of vaccines to retain the chemical, physical, microbiological and biological properties within specified limits throughout the shelf-life [2] while sterility means freedom from the presence of viable contaminant microorganisms. Vaccine stability has a major impact on the success of any immunization programme because they are sensitive biological substances that progressively lose their potency on storage over time [3]. Vaccines lose their potency much faster when stored outside their recommended temperature. Stability studies are needed to ensure that vaccines within their shelf lives, storage period or period of use, still have the required characteristics that support quality, safety and efficacy [4]. Once a vaccine loses its potency, it becomes dead because returning it to correct storage condition cannot revive it. Storage of vaccines at the correct recommended temperature conditions is, therefore, of prime importance in order that full vaccine potency is retained up to the time the vaccine will be administered to the end user [5]. Although all vaccines are heat-sensitive, some are far more sensitive than others. The Expanded Programme on Immunization vaccines can be arranged in their order of decreasing heat stability in the following order; Hepatitis B > BCG > DPT = Yellow fever > Measles > Oral Polio Vaccine (OPV) and light sensitivity in the following order: Yellow fever = Measles = BCG > OPV > Hepatitis B > DPT [6]. All losses of potency are cumulative and so any new exposure, however small, will increase the damage to the vaccine [7]. Due to a cumulative damage, a vaccine may be completely destroyed and all its potency lost even though it is still within its shelf life.

All biological products, including vaccines, administered by the parenteral route are required to be sterile [8, 9]. Sterility testing determines whether or not the vaccines have microbial contaminations and/or pyrogens because vaccines are supposed to be sterile products. Vaccine potency loses had been reported in Nigeria [10] and other parts of the world (11, 12).

A few studies on the effect of temperature on the stability and hence potency of Oral Polio Vaccine (OPV) exist [13, 14] but no studies in Nigeria has been done to investigate the stability of any other vaccine used in routine immunization.

The aim of this study is to evaluate the stability and sterility of the vaccines used in the routine immunization programme in south-east geo-political zone of Nigeria and to what extent has poor maintenance of cold-chain affected them.

2. MATERIALS AND METHODS

2.1. VACCINE COLLECTION AND STORAGE

The vaccines used for the studies were collected from the cold-chain facilities in the various states after due authorization by their ministries of health. They were transported in vaccine carrier and stored (within 4 hours of collection) in a storage facility in the immunization unit of Nnamdi Azikiwe University Teaching Hospital, Nnewi. The vaccines were all within their shelve lives.

2.2. THERMAL STABILITY STUDY

The principle: BCG vaccines were first stored at 37°C for 30 days. Their potencies were then determined through viability test. These were compared with a control stored at 4^0C. The loss in culturable particles after the incubation period of 30 days should be not more than 20% [15].

The test: A vial of BCG vaccine from each state was dissolved with the provided diluents. Then, 25 µl of the vaccine solution was diluted in 1000ml 0.9 % sodium chloride (normal saline) to give 1: 40,000 dilutions. Fifty micro liter volume of the dilution was spread on to previously prepared soybean-casein digest agar solid medium and incubated at 37°C for 30 days. The number of culturable particles (CPs) was determined by colony counts using a microscope (XSZ-107BN; #: 000678). Ten replicate plates were used and result reported as mean ±SD. A control vaccine which potency had been previously compared with the labeled potency of the vaccines was also plated in ten replicate plates and used to compare the potency of the vaccines stored at 37°C.

2.3. PHYSICAL EXAMINATION

The vaccines tested were measles vaccine, hepatitis B vaccine, OPV, DPT vaccine, pentavalent vaccine, yellow fever vaccine and BCG vaccine. The vaccine samples were

examined for any visible change in appearance as soon as they were collected and observations recorded daily for two weeks**.**

2.4. SHAKE (SETTLING TIME) TEST

The vaccine containers were shaken vigorously after overnight freezing at -20˚C. The presence of granules or floccules when shaking was effected, or the formation of sediment at the bottom of the container within 30 minutes after shaking, with clear liquid above, was checked and the result noted. The time required for the sediments to settle was also recorded. The vaccines that have floccules or granules or that have sediments settling before 30 minutes are considered sub-standard because these suggest that the vaccine had been frozen [16]. The size of the granules increases on repeated freezing and thawing cycle [17].

2.5. TESTS FOR BACTERIAL AND FUNGAL CONTAMINATION

The vaccines tested were measles vaccine, hepatitis B vaccine, OPV, DPT vaccine, pentavalent vaccine, yellow fever vaccine and BCG vaccine. The vaccine samples were examined for bacterial and fungal contamination. Five vessels (Group A) were filled with 120ml of Soybean Casein Digest medium (SCD), (also known as Trypticase Soy Broth (TSB)) from HiMedia Laboratories to detect aerobic bacteria contaminants. Another five vessels (Group B) were also filled with 120ml of BD™ Fluid Thioglycollate Medium (FTM) to detect anaerobic bacteria contaminants. Then, another 10 vessels (Group C) were filled with 40ml of soybean casein digest medium to detect fungal contaminants. All the vessels were first incubated at 30 - 35˚C for 14 days to confirm that they are sterile and later inoculated with 0.2ml of test vaccines (except for BCG which was 0.05ml). Groups A and B vessels were again incubated at 30 - 35˚C for another 14 days and observed for bacterial growth. Positive controls containing *Escherichia coli* and *Clostridia spp* for the TSB and FTM respectively were also set up. Group C vessels are again incubated at 20 - 25˚C for another 14 days and observed for fungal growth. Positive control containing *Candida albicans* was also set up.

2.6. LIMULUS AMEBOCYTE LYSATE TEST FOR PYROGENS

This was carried out to using Genscript ToxinSensorTM Chromogenic LAL endotoxin assay kit (cat. No. L00350). The specimen and reagent preparations were done according to manufacturer’s specification.

TEST PROCEDURE

This was done as recommended by the manufacturer. A volume of 100µl of four endotoxin standards of concentrations (0.1, 0.05, 0.025 and 0.01 EU/ml) and test samples were dispensed into endotoxin-free vials in quadruplicates (except for HBV, OPV and DPT which were in triplicates) and mixed manually by gentle swirling for 30 seconds, care being taken to avoid foaming/bubbles. A blank sample vial containing 100µl of LAL reagent water was also included. A volume of 100µl of reconstituted LAL reagent was added to each vial. The vials were capped and incubated for 45 minutes at 37°C±1°C after mixing the content thoroughly by gentle swirling. Reconstituted Chromogenic substrate solution, 100µl, was added to each vial and then capped, followed by gentle swirling to mix well. The vials were incubated at 37°C±1°C for 6 minutes. This was followed by consecutive addition of 500µl each of reconstituted color-stabilizer #1 (stop solution), color-stabilizer #2 and color-stabilizer #3 to each vial with gentle swirling (after each addition) to mix well. The absorbance of each reaction vial was read at 545 nm using distilled water as blank to adjust the spectrophotometer (APEL: PD-303 spectrophotometer, made in Japan) to zero absorbance.

3. RESULTS

The study showed a good working condition of the cold-chain facilities in all the five states that make up Eastern Nigeria as at the time of vaccine collection. The vaccines showed good thermal stability as well as good sterility profile**.**

3.1. THERMAL STABILITY STUDY OF BCG VACCINE

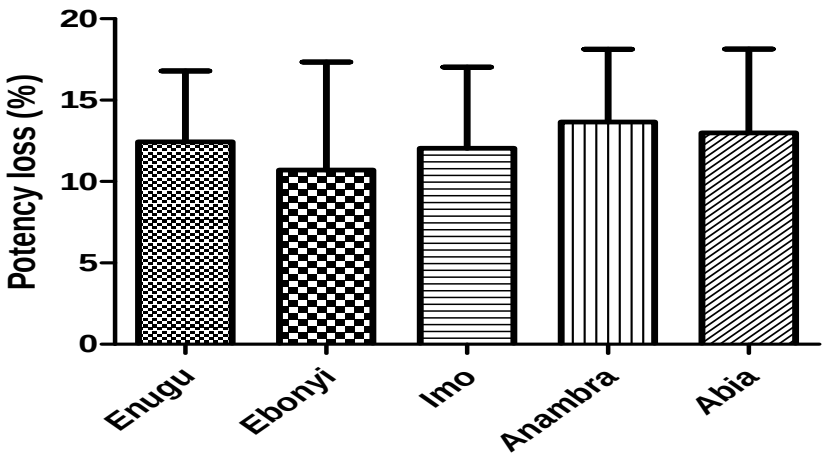

Figure 1: Thermal Stability study of BCG Vaccine

The figure 1 above gives a graphic picture of the thermal stability study on the BCG vaccines from the five states that make the South-east geopolitical zone of Nigeria. The sample from Anambra state showed a greater loses of potency compared to the samples from the other states. However, it must be appreciated that none of the vaccines loss up to 20 % of their labeled potency showing that they were all within the acceptable official potency limits. One-way analysis of variance shows there is no statistical difference (at $\alpha < 0.05\%$) in the means of the potency losses observed for the vaccines (P value = 0.7653). The vaccines have comparable thermal stability. Also, Bartlett's test for equal variances showed no significant difference in the variances at $P < 0.05$ (P value = 0.7216). None had an unacceptable stability as none had a potency loss of more than 20 % of the control.

3.2. Physical Examination of the Vaccines

Every vial of vaccine collected was immediately examined physically for possible discolorations but none showed any visible change in colour as shown in Table 2. They were transported within 4 to 5 hours to the storage facility in Nnamdi Azikiwe University Teaching Hospital, Nnewi.

Table 1: Physical Examination

Vaccine	OPV	DPT/HBs Ag/Hib	DPT	BCG	Yellow Fever	Measles	Hepatitis B
Physical Exami-nation	No observable change	No observable change	No observable change	No observable change	No observable change	No observable change	No observable change

3.3. Shake Test for the DPT, Hepatitis B and Pentavalent Vaccine

The result of the Settling Time (Shake) Test carried out on the DPT vaccines, Pentavalent Vaccines and the Hepatitis B Vaccines is as shown in Table 3. The vaccines' mean settling times were 25.33 ±2.52, 20.60 ±2.07 and 25.50 ±0.71 minutes for DPT, Hepatitis B and Pentavalent vaccines used as control respectively and 72.00±17.32, 63.20±12.28, 63.50±2.12 minutes for the vaccine samples respectively. In all the vaccines sampled, there were no floccules, no granules and no precipitate/sediment seen 30 minutes after over-night freezing, thawing and shaking. This implies that it is not likely that they had been previously stored at freezing temperature except the vaccines used as control (all the control vaccines had sediments settling within 30 minutes).

Table 2: Shake Test

Vaccines	Settling Time (Minutes)														Appearance	
	V		W		X		Y		Z		Control (C)		Samples (S)		Control (C)	Samples (S)
	C	S	C	S	C	S	C	S	C	S	Mean	SD	Mean	SD		
DPT	NC	NC	NC	NC	28	82	25	52	23	82	25.33	±2.52	72.00	±17.32	P	NP
Hepatitis B	21	62	23	52	22	52	18	81	19	69	20.60	±2.07	63.20	±12.28	P	NP
Pentavalent	26	62	25	65	NC	NC	NC	NC	NC	NC	25.50	±0.71	63.50	±2.28	P	NP

NOTE: NC means "Not Collected"

Pentavalent = DPT/HBsAg/Hib, V = Anambra, W = Abia, X = Imo, Y = Enugu, Z = Ebonyi

NP = Floccules and/or granules not present, P = Floccules and/or granules present

3.4. TESTS FOR BACTERIAL AND FUNGAL CONTAMINATION

Tables 3 shows the result of Test for bacterial contamination carried out on the sampled vaccines. Vessels 11 and 12 are the positive controls for SCD (testing for aerobes) and FTM (testing for anaerobes) respectively. The result revealed that the vaccines are all free of bacteria contaminants as supported by the result of the physical examination on the vaccines. The vaccines contain no extraneous colour suggestive of bacterial contamination.

Table 4 shows the result of the Test for Fungal contamination of the vaccines sampled. This showed that the vaccines were also free of fungal contaminants.

Table 3: Test for bacterial Contamination

Vaccine	Replicate Vessels containing Vaccines and controls											
	1	2	3	4	5	6	7	8	9	10	11	12
Measles	-	-	-	-	-	-	-	-	-	-	+	+
OPV	-	-	-	-	-	-	-	-	-	-	+	+
DPT	-	-	-	-	-	-	-	-	-	-	+	+
YF	-	-	-	-	-	-	-	-	-	-	+	+
HBV	-	-	-	-	-	-	-	-	-	-	+	+
BCG	-	-	-	-	-	-	-	-	-	-	+	+
Pentavalent	-	-	-	-	-	-	-	-	-	-	+	+

Notes: - means No Growth while + mean Growth, Vessels 11 and 12 contains *Escherichia coli* and *Clostridia spp* respectively as positive controls for aerobic and anaerobic organisms

Table 4: Test for Fungal Contamination

Vaccine	Replicate Vessels containing Vaccines and controls										
	1	2	3	4	5	6	7	8	9	10	11
Measles	-	-	-	-	-	-	-	-	-	-	+
OPV	-	-	-	-	-	-	-	-	-	-	+
DPT	-	-	-	-	-	-	-	-	-	-	+
YF	-	-	-	-	-	-	-	-	-	-	+
HBV	-	-	-	-	-	-	-	-	-	-	+
BCG	-	-	-	-	-	-	-	-	-	-	+
Pentavalent	-	-	-	-	-	-	-	-	-	-	+

Note: (-) means No Growth and (+) means Growth. Vessel 11 contained *Candida albicans* as the positive control.

3.5. Limulus Amebocyte Lysate (LAL) Test

Figure 2 below gives a graphic view of bacteria endotoxin concentrations in Endotoxin Units per milliliter (EU/mL) in the vaccines. The concentrations were calculated from the graph shown in figure 3 below. It is understandable that the bacterial vaccines especially the DPT and the pentavalent vaccines, contain some levels of endotoxin but this level is neither clinically nor statistically significant (P value = 0.2592). Also, there is no linear trend significance (at $\alpha < 0.05$) in the endotoxin concentrations between the vaccines (P value = 0.1072). The negative values for the viral vaccines means there was no detectable bacterial endotoxins in the vaccines. Pentavalent vaccine, which is a combination of viral and bacterial vaccine, also contained minimally detectable endotoxin.

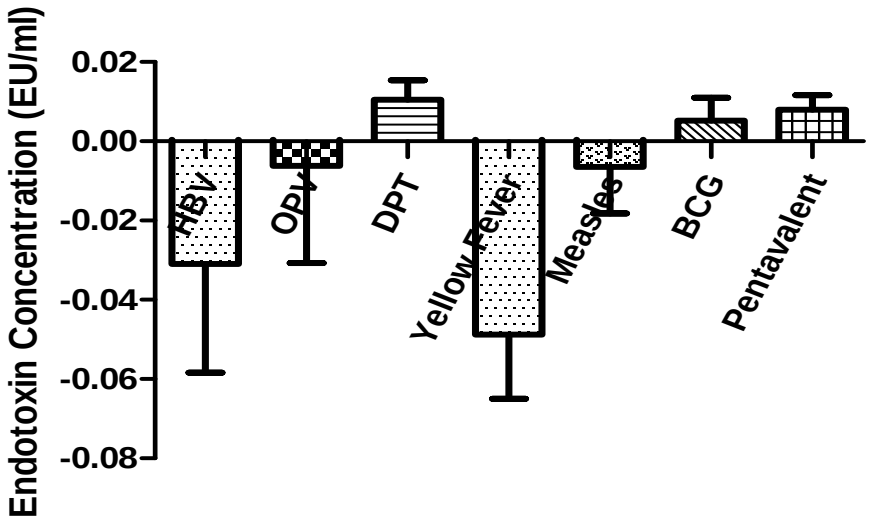

Figure 2: Endotoxin Concentration (EU/mL) in the Vaccines

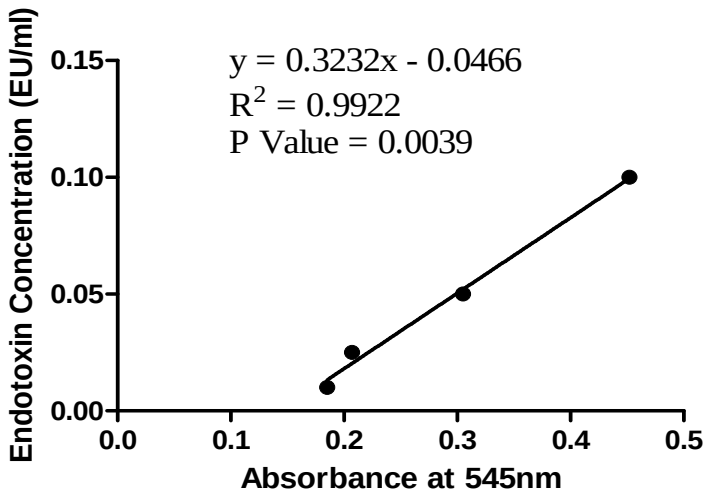

Figure 3: Standard Curve for Quantifying Endotoxin in Chromogenic Assay

4. DISCUSSION

Figure 1 above shows the thermal stability test for the BCG vaccines sourced from the five states that make up the South-east, Nigeria. The labeled potency of the BCG vaccine is 1 – 33 x 10^5 CFU per dose of 50µl which is (2.5 – 82.5 CFU) x 40,000 per dose of 50µl. EUROPEAN PHARMACOPOEIA, 2007, recommended that the number of viable units after a thermal stability test should not be less than 80 per cent of the unheated vaccine (the control stored at 4 °C). The mean percentage potency losses for the five vials tested were 13.67±4.45 > 12.99±5.15 > 12.44±4.36 > 12.05±4.98 > 10.71±6.62 for vaccine from Anambra, Abia, Enugu, Imo and Ebonyi respectively which are all less than the cut-off point of 20%. This suggests that the vaccines batches tested are still stable and may still be used for immunization. Storage outside the recommended temperature cannot guaranty the vaccine's full immunogenicity [18].

From Table 1 above we see the result of the physical examination carried out on the vaccines. It was observed during samples collections that the cold-chain stores were all equipped with stand-by generators. This may have contributed positively to the maintenance of the cold chain required for the vaccines storage. Aluminum-containing adjuvants have been used to enhance the immune response to vaccines made from killed, inactivated and subunit antigens by adsorbing the antigen [19, 20]. Long exposure to high temperature may result in some

changes in the physical characteristics of the aluminum compound used as an adjuvant in vaccine preparation. When vaccines containing aluminum-salt adjuvants are frozen or lyophilized the vaccine preparations frequently demonstrate a significant decrease in potency due to the aggregation of adjuvant particles during freezing or lyophilization [21, 22].

Table 2 shows the result of the shake test. All the vaccines tested passed the test and so are believed not to have been damaged by freezing. Freezing destroys completely and permanently the potency of an adsorbed vaccine [23, 24, 25] by breaking down the lattice structure formed by bonds between the adsorbent and the antigen in a vaccine [17]. Freeze-damaged vaccines had been shown to have lower immunogenicity and may cause sterile abscesses and/or other kinds of local reactions [16, 26].

Tables 3 and 4 demonstrate that all the vaccines tested are truly free from bacteria and fungi contamination. Current Good Manufacturing Practice must have been observed during their manufacture. Vaccines and other biological products and indeed all parenteral products are required to be free from fungal and bacterial contaminations.

Figure 2 shows the endotoxin concentrations in the vaccines. Zero and negative values of endotoxin concentration reveals that the product contains no endotoxin and so is safe for use. It was observed that BCG, DPT and Pentavalent vaccines contain 0.005174±0.005772, 0.010417333±0.004944 and 0.007965±0.003654 EU/ml respectively of endotoxins. These are within the acceptable endotoxin limits for biological products such as vaccine [8, 27].

4.1. CONCLUSIONS

The vaccines tested were all in good conditions suggesting that cold-chain facilities were working as at the period the vaccines were sampled. It also suggests that cold-chain system is maintained in the states. It was observed at the time of collection that the cold-chain facilities were all supported with stand-by generators.

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This study is part of the doctorial research work being undertaken by Oli AN.

COMPETING INTERESTS

The vaccines were donated by Abia, Anambra, Ebonyi, Enugu and Imo States' Ministries of Health.

The funders and the vaccines donors had no role, whatsoever, in the design of the study, the collection and analysis of data thereof, decision to publish, or preparation of the manuscript.

Ethical Issues

The work described in this article was approved by the Ethics Committee of Nnamdi Azikiwe University Teaching Hospital, Nnewi (Approval #: NAUTH/CS/66/Vol.4/220).

Authors' Contribution

Oli AN wrote the first draft of the manuscript, designed and implemented the study as well as did data analysis and interpretations, Agu RU revised the draft critically and cross-checked for important intellectual content while Esimone CO conceptualized the study and also revised the manuscript critically.

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