

Full Length Research Paper

Potency and immunogenicity of bacillus calmette guerin (BCG) vaccines used in routine immunization programme in South-East, Nigeria

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The efficacy of every vaccine depends in part on appropriate vaccine handling from the manufacturer to its utilization. There is need for continuous monitoring of vaccine to ensure that cold-chain system is maintained throughout the product life-span. This study aims to validate the BCG vaccines used in immunization in South-East, Nigeria. The potency of the vaccines was determined by viable count on Soybean-Casein-Digest Agar after incubation of 50 μ l of 1: 40,000 dilutions of the vaccines at 37°C for 30 days. Ten replicate plates were used and result reported as mean \pm SD. The viable counts in the vaccines were compared with the labeled potency on the vials. The immunogenicity test was done by Antibody Induction Method. This involves measuring the neutralizing antibodies in a control group (given physiological saline) and immunized group after 30 days using the enzyme-linked immunosorbent assay (ELISA). Anti-tuberculosis antibodies' concentration was determined by absorbance measurement at 450nm wavelength. Viable counts in the BCG vaccine samples were 54.6 \pm 11.79 > 51.91 \pm 11.35 > 48.18 \pm 15.33 > 44.91 \pm 16.29 > 44.55 \pm 15.69 CFU/50 μ l (dilution factor was 40,000). The immunogenicity test shows that the IgG titers for the BCG vaccines from control, Enugu/Ebonyi, Imo, Anambra and Abia were 0.645, 1.567, 1.507, 1.451 and 1.286 respectively while the IgM titres were 0.689, 0.736, 0.805, 0.792 and 0.715 respectively. One way analysis of variance shows that there is statistical difference in the IgG antibody titer produced by the control compared to the vaccines (P value < 0.0001). The IgG antibody was enough to confer protection. Neither the control nor the vaccines from the states produced enough protective IgM. There is no statistical difference in the IgM antibody titer produced by the control compared to the vaccines (P value = 0.1058). The vaccines were all within their labeled potency and have good immunogenicity profile.

Key words: Immunogenicity, potency, BCG vaccine, routine immunization programme, South-East, Nigeria.

INTRODUCTION

Bacillus Calmette-Guerin (BCG) vaccine is a live bacterial vaccine given for protection against severe forms of

childhood tuberculosis. The bacillus strain was isolated from bovine tuberculosis and attenuated by French

scientists Calmette and Guerin (Nitin, 2013). Vaccination with BCG vaccine still remains the standard for TB prevention in most countries because of its efficacy in preventing life-threatening forms of TB in infants and young children. It is not expensive and usually requires only one administration in either newborns or adolescents (World Health Organization, 2006a; Fine et al., 1999). Currently there is no suitable alternative to BCG vaccine, so it will remain in use for the foreseeable future and may continue to be used as a prime vaccine in a prime-boost immunization schedule in conjunction with new TB vaccines. (Ho et al., 2005). Tuberculosis was declared a global emergency by World Health organization in 1993, and *Mycobacterium tuberculosis* is now considered to be responsible for more adult deaths than any other pathogen. (World Health organization, 2013). Nigeria is classified as a high TB, high multiple drug resistance (MDR-TB) burden country. (World Health organization, 2012). Although TB infection and disease in children are believed to be sentinel events that indicate level of transmission within a community and effectiveness of TB control programs, (Gie et al., 2009) there are few accurate data worldwide on the true incidence of childhood TB. (Orogade et al., 2013). BCG vaccine is reported to be of varying efficacy in preventing tuberculosis. Some studies in Nigeria showed 69.8% Mantoux Tuberculin Skin Test (TST) sensitivity 4 weeks post BCG vaccination (Babaniyi, 1990; Odujinrin and Ogunmekan, 1992) while meta-analysis of BCG efficacy of published studies using 12 case control and 8 prospective studies revealed a decreasing efficacy of about 50% (OR 0.49, CI 0.38-0.7). (Colditz et al., 1994).

More so, it has been identified that failure to adhere to required protocols for storage and handling can reduce vaccine potency, (Lala and Lala, 2003; Indian Academy of Paediatrics, 1990 and 2001; World Health organization, 2006a; b) resulting in inadequate immune responses in patients, as well as inadequate protection against disease. The efficacy of every vaccine depends in part on appropriate vaccine handling from the manufacturer to its utilization; therefore, there is need for continuous monitoring of vaccine to ensure that cold-chain system is maintained from the manufacturer to the end user. This study aims to validate the BCG and measles vaccines used in immunization in South-East, Nigeria.

METHODOLOGY

Collection of the vaccine samples

The BCG vaccines were donated freely by the Ministries of Health of Ebonyi, Enugu, Imo, Abia and Anambra States. They were collected from the cold-chain stores in the respective states,

transported in vaccines carrier and stored in the storage facility in Nnamdi Azikiwe University Teaching Hospital, Nnewi within three and half hours of collection. The temperature of the storage facility was charted daily throughout the period of vaccine storage. The studies were conducted within 1 month of vaccine collection.

Potency evaluation

A vial of BCG vaccine was taken from each state and their potencies were determined through viability test. The viability test involved determining the number of culturable particles (CPs) by colony counts on Soybean-Casein Digest Agar solid medium after incubation of 50µl of 1: 40,000 dilutions (25 µl diluted in 1000ml normal saline previously maintained at 0 to 4°C) of the vaccines at 37°C for 30 days. Ten replicate plates were used and result reported as mean ±SD. The number of culturable particles in the vaccines is compared with the labeled potency on the vaccine vials.

Immunogenicity evaluation

Animals

Albino mice (weight, sex and age matched) accommodated under standard conditions (temperature: 26 ± 2°C, relative humidity: 45 ± 2%) and provided with standard pellet diet and water were used. The weight range of the mice was 20 to 30 g. The study was done in Pharmacology and Toxicology Laboratory of Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Agulu Campus and in Chemical Pathology laboratory of Nnamdi Azikiwe University Teaching Hospital, Nnewi. Ethical approval was gotten from The Ethics Committee of Nnamdi Azikiwe University Teaching Hospital, Nnewi (Approval #: NAUTH/CS/66/Vol.4/220).

Antibody development in the immunized animals, Bleeding and Serum extraction

Modified Antibody Induction Method (British Pharmacopeia, 2011; Maheshwari et al, 1998), with ELISA (Lagranderie et al., 1996) was used. Briefly, 45 mice of similar weight and sex were distributed into 5 groups of 9 mice. The first 4 groups were immunized with one human dose of the test BCG vaccines, by intra-peritoneal injection. The last group (control) was immunized with physiological saline. The animals were fed daily for 30 days and their cages cleaned daily. The animals were checked daily for any abnormality. On the 31st day, heparinized capillary tube was inserted via the eye (just below the eye ball) of each mouse to allow the blood flow into separate sterile Eppendorff tubes. The clotted blood was centrifuged at 2,683.2 g for 10 min and the sera were carefully pipetted out, transferred into another sterile Eppendorff tubes and preserved by freezing at -20°C until ready to use.

Antibody measurement

The level of BCG neutralizing antibodies in the serum samples were determined by ELISA method.

BCGIgG and IgM concentration for the BCG vaccines

Reagents preparation: All specimens and reagents were brought

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to room temperature before use and prepared according to the ELISA kits' manufacturer's (Ratio Diagnostics – Germany) protocol. The wash solution was prepared by diluting 100 ml of the wash concentrate with 900 ml of distilled water making sure that a homogenous solution was formed.

Assay procedure: Ten microlitres of the serum samples were diluted with 1 ml of sample diluents (provided with the kit) and 100µl of the diluted samples and the three ready-to-use controls/standards were pipetted in duplicate into respective wells of a 96-well plate pre-coated with purified and inactivated tuberculosis antigen. Sorbent M, 30µl volume, was pipetted into the wells containing diluted samples. The well A1 contained the substrate blank. The plates were incubated for 45 min at 37°C. The wells were aspirated and washed four times for 30 seconds with wash solution (300 µl/well) using automatic microplate washer (Stat Fax – 2600, model #: H009775). The anti-mouse IgG or IgM monoclonal antibodies labeled with horseradish peroxidase conjugate of volume 100 µL was pipetted into each well except the blank well for the BCG IgG and IgM ELISA plates respectively. The plate was incubated for 45 min at 37°C. The wells were aspirated and washed automatically 4 times for 30 seconds and then blotted and dried by inverting on to absorbent tissue paper. Tetramethylbenzidine (TMB) Chromogenic solution (volume 100 µl) was pipetted into each well (including the blank well) and the plate incubated at room temperature for 15 min, avoiding direct sunlight. A volume of 100 µl of stopping solution was pipetted into each well. The degree of enzymatic turnover of the substrate (that is, the absorbance) is measured within 30 min of adding the stopping solution at 450nm wavelength using Stat Fax - 2100 microplate reader (manufacturer – Awareness Technology, USA) and this is proportional to the quantity of the anti-tuberculosis IgG or IgM antibodies present. The results are recorded as a ratio of the absorbance of the serum samples and that of the cut-off (the reagent control standard). Report is shown as mean ± standard error in the mean.

RESULTS

Table 1 shows the result of viability test carried out on the BCG vaccines from each state. The dilution factor = 40,000. The test showed the number of culturable particles (in CFU) available after an incubation period of 30 days. The values obtained give the potency of BCG vaccines. The sample from Anambra has the highest number of culturable particles (in CPU) compared with samples from other states. Nevertheless, at < 0.5 level of significance, a One-way analysis of variance (P Value = 0.4357) showed that the mean potencies of the samples are not significantly different. Bartlett's test for equal variances (P Value = 0.7495) showed that there is no significant difference. This means that the samples are equally potent. Their CFU per dose of 50µL falls within the labeled potency. The potency of the vaccine is usually given as a range: 1 – 33 x 10⁵ CFU = (2.5 – 82.5 CFU) x 40,000 per dose of 50µL. Tables 1 and 2 showed the IgG and IgM antibodies titers in the albino mice 30 days post-immunization.

The mean IgG titers were 1.568, 1.506, 1.451, 1.287 and 0.645±0.028 for Enugu/Ebonyi State, Imo State, Anambra State, Abia State respectively while the mean IgM titers were 0.736, 0.805, 0.792, 0.715 and 0.689. The

interpretation of the manufacturers of the ELISA kits used:

Protective Antibody – Results > 1.1

Doubtful Protection – Results = ± 10 % of the Cut-off (i.e. 1±0.1)

No Protection (Too low or No Antibody) – Results < 0.9

The vaccines, but not the physiological saline, produced enough protective IgG antibodies. Figure 1 is a graphic presentation of the antibodies titer to tuberculosis evoked by the BCG vaccine. One-way analysis of variance at $\alpha < 0.05$ shows that the mean IgG antibody evoked by the vaccines and the control differ significantly (P Value < 0.001) and the Bartlett's test for equal variances shows that the variances also differ significantly (P Value = 0.0226). Dunnett's multiple comparison test show that the means differ significantly from that of the control. The general interpretation is that the vaccines produced enough IgG to confer protection except the control. There is therefore statistical difference in the protection (antibody titer) produced by the control compared to the vaccines from the states. However, the case is difference as regards to the IgM antibody. One-way analysis of variance and Bartlett's test for equal variances all showed no significant different at $\alpha < 0.05$. Also, Dunnett's Multiple Comparison Test shows that the mean IgM antibody titers produced by the vaccines do not differ significantly (statistically) with that of the control. It is interpreted that the State samples and the control had too low an antibody levels or doubtful protection. This could be because of the time of collection of the blood sample which was one month post immunization. Immunoglobulin M (IgM) is the first antibody produced after immunization and in BCG vaccination. They do not exist at this time instead BCG-induced T cells which produce cytokines that contribute to macrophage activation and control of *M. tuberculosis*. (Lagranderie et al., 1996).

DISCUSSION

The values (Table 1) when compared with the labeled potency on the vaccine vials showed that the vaccines still retained their potency. The labeled potency was 1 – 33 x 10⁵ = (2.5 – 82.5) x 40,000 CFU per 50 µl while the vaccines from Ebonyi, Enugu, Imo, Anambra and Abia had observed potency of 48.18 ± 15.33, 44.55± 15.69, 44.91 ±16.29, 54.64± 11.79 and 51.91± 11.35 (x 40,000) CFU per 50 µl respectively. A rapid alternative method, the ATP assay, was modified and developed by Staten Serum Institute (Jensen et al., 2008). The method was evaluated by collaborative study for its suitability for viability testing of BCG vaccine and for use in the temperature stability test and found to be good (Ho et al.,

Table 1. The potency of the BCG vaccines (Value × 40,000 CFU/dose of 50µl).

State	N	Potency (Mean±SEM)	P-value
Vaccines from Ebonyi at 4°C	10	47.6±4.85	0.436
Vaccines from Enugu at 4°C	10	44.5±4.96	
Vaccines from Imo at 4°C	10	44.9±5.15	
Vaccines from Anambra at 4°C	10	54.6±3.73	
Vaccines from Abia at 4°C	10	51.9±3.59	

Table 2. BCG IgG titer in the immunized mice.

State	N	IgG Titer (Mean±SEM)	P-value
Enugu /Ebonyi	9	1.568±0.0402*	< 0.001
Imo	9	1.506±0.0427*	
Anambra State	9	1.451±0.0498*	
Abia State	9	1.287±0.0139* ^a	
Control	9	0.645±0.028	

= Significant when compared with Control (P < 0.001) a = Significant when compared with Enugu/Ebonyi State, Imo State and Anambra State (P<0.01) Note: Control was physiological saline.

Table 3. BCG IgM titer in the immunized mice.

State	N	IgM Titer (Mean±SEM)	P-value
Enugu/Ebonyi	9	0.736±0.0316	0.106
Imo	9	0.805±0.0305	
Anambra	9	0.792±0.0410	
Abia	9	0.715±0.0302	
Control	9	0.689±0.0390	

2008). Also, Kairo et al. (1999) developed Tetrazolium salt assay method for rapid determination of viability of BCG vaccines. Nevertheless, the culturable viable count method which estimates colony forming units (CFU) per container is still considered as the 'gold standard' for testing viability of BCG vaccine and as a surrogate marker for potency for this live vaccine (European Pharmacopoeia, 2007; World Health Organisation 1987). It is also the 'gold standard' the thermal stability test (European Pharmacopoeia, 2007; Ho et al., 2011). It is generally considered that antibodies play a minor role in the immune protection against tuberculosis (Tables 2 and 3). However, antibodies facilitate the elimination of mycobacteria by increasing opsonization of the microorganisms (Hanekom, 2005; Kaufmann, 1993). A strong antibody response to BCG vaccination results in high proliferation rates of CD4 T lymphocytes (Petricevich

et al., 2001) and splenocytes as well as long-lasting and strongest Th1 type cellular immune responses (Lu et al., 2013; Lu et al., 2014). CD4 molecules are primarily located on T helper cells (Th cells) (Miceli & Parnes, 1993). Parra et al., (2009) proposed a new assay method for correlates of protection. The assay involves biomarker panels and growth inhibition bioassay of mycobacteria.

Conclusion

The immunogenicity of the BCG vaccines from the five states is significantly different from that of the control and their observed potencies were all within the labeled potencies on the vials. The vaccines are therefore still within the acceptable potency and immunogenicity margin. These margin. These suggest adequate cold-chain maintenance

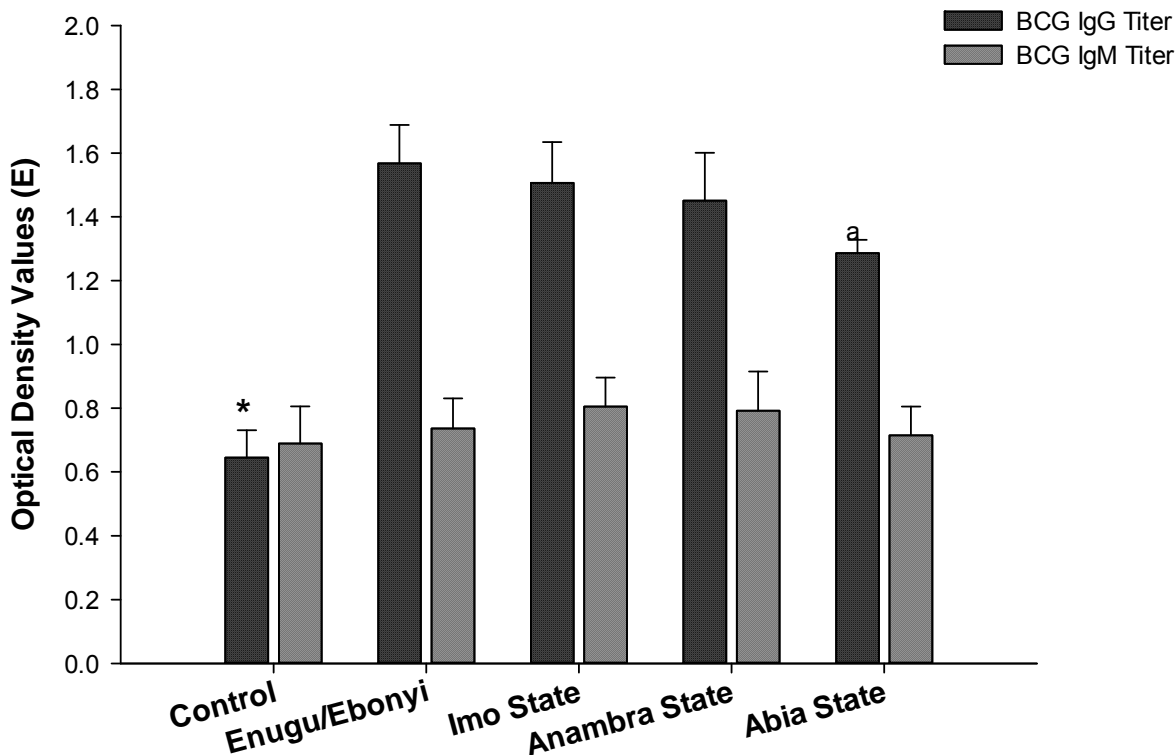


Figure 1. BCG vaccines antibodies titers for IgG and IgM in the immunized mice. The immunoglobulin G (IgG) and IgM responses were analyzed in albino mice immunized intraperitoneally with vaccines from the States and with physiological saline at 30-day post-vaccination. Each point represents the mean \pm SD of triplicate readings of the sera diluted 1/100. * = significant when compared with all the States ($p < 0.001$) a = Significant when compared with Enugu/Ebonyi State, Imo State and Anambra State ($P < 0.01$). Note: Control was physiological saline.

system in South-eastern Nigerian central stores as of the time the vaccines were collected. Continuous monitoring of efficiency of the cold-chain maintenance is thereby recommended.

CONFLICTS OF INTEREST

There are no conflicts of interest and neither the funders nor the vaccine donors had any influence whatsoever in the study design, data collection, manuscript writing nor decision to publish the work.

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