Congo Red Binding Test for the National Program on Immunization Vaccines in use in Anambra, Ebonyi and Enugu states of Nigeria

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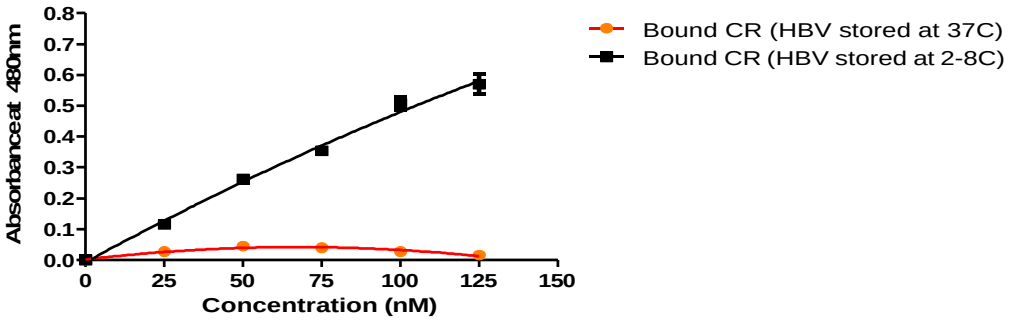
The present study was to give an over-view of the potency of the vaccines used for National Immunization programme using Congo red binding test and to provide a quick method of estimating the stability/ potency of the vaccines when a more rigorous method is not feasible for some reasons. This is an experimental study carried out in the department of Chemical Pathology Laboratory of Nnamdi Azikiwe University Teaching Hospital, Nnewi between December 2011 and September 2012. The solutions that contained the vaccine samples and the controls were incubated at 37^0C for 48 hours, centrifuged and the absorbance of the supernatant fluid measured. The absorbance at 480nm of unbound Congo red dye in Phosphate buffered saline solution in the absence and presence of the vaccines were measured and recorded. The amount of Congo red bound was calculated by subtracting the absorbance of Congo red in the supernatant (vaccine present) from the absorbance of Congo red in PBS solution (vaccine absent). The absorbance of the different concentrations of Congo red dye in PBS alone (i.e. unbound dye) was determined before each set of experiment. The use of Congo red binding test may provide a quick method for the estimation of the potency and stability of immunization vaccines. By this Congo red binding assay, the vaccines tested passed as there was a continuous increase in the amount of dye bound by the vaccine samples. The vaccines tested are potent enough to be used for immunization. It was observed at the time of vaccine collection that the storage facilities in all the three states' vaccine stores had adequate power supply - the National Electricity supply being supplemented with standby Generators.

Keywords: Congo red dye, National Program on Immunization, Vaccine, Binding Test, South-Easthern Nigeria.

INTRODUCTION

Vaccination has proved to be the most cost effective way of preventing disease and avoiding treatment cost (Brass 1993; Creech *et al.,* 2009; Duclos 2004; Gendon 2002). It has remained an important intervention for reducing morbidity and mortality world over especially in children in developing countries (Feller, 2012). In some cases, disease prevention can be for life (Breiman *et al.,* 2012; Yomayuza *et al.,* 2012). It has been variously advocated to replace *in vivo* vaccine potency testing on laboratory animals with convenient *in vitro* testing (Aggerbeck and Heron 1996; Hendriksen 2009; Hong and Hendriks 1999; Poirier *et al.,* 2010; Reeve *et al.,* 2011). This is more so



Data Set: ±SD, n = 4

Figure 1. Congo red Adsorption Test for Hepatitis B Vaccine (Batch. # - WVA11002)

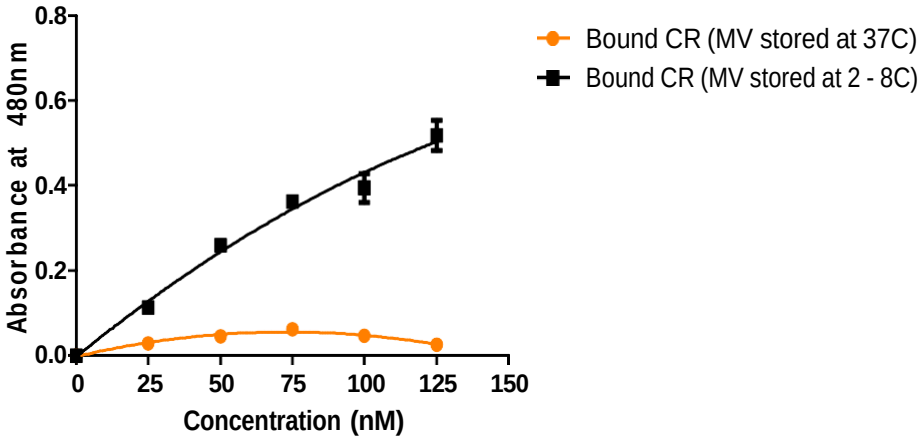
advocated in developing countries because good quality laboratory animals and proper animal facilities are not always available to carry out the needed quality control tests (Hong and Hendriks 1999). The current neutralization test (NT) technique for potency/ immunogenicity studies has been flawed because of its inherent disadvantages such as the use of tissue culture and/or lots of laboratory animals, the use of live pathogens (wild types) or their toxins which possess health risks to laboratory workers, time consuming, stress and labour intensive and so cannot be used for large scale population or epidemiological studies (Ivanov and Dragunsky, 2005). “The stability of vaccines has a major impact on the success of immunization programmes worldwide” (Knezevic, 2009). A major responsibility of every country is to ensure that vaccines used for immunization remain stable and potent on its way from the manufacturer, through the distribution channels, to the end users or vaccine recipients (Knezevic 2009, WHO 2006, Galazka *et al.,* 1998). WHO emphasizes the role of National Regulatory Authorities (NRAs) and National Control Laboratories (NCLs) in overall vaccine evaluation, including stability assessment (Knezevic, 2009). In developing countries, a quick method of estimating the stability/potency of the vaccines used in national immunization is needed when logistics problems has been encountered and in this way avoid substantial distress and suffering of laboratory animals imposed by the neutralization test. In our study, we tried to apply Congo red binding test to validate or invalidate the vaccines used for the national immunization. The theory behind the Congo red binding test is that a continuous decline in the vaccines ability to absorb or bind Congo red dye compared to a control stored at normal storage temperature means a decrease in vaccine potency and stability.

METHODOLOGY

The vaccines used were generously donated by the ministries of health and a teaching hospital in the states sampled. Three vials of each vaccine sample (one vaccine from a batch except for the yellow fever vaccine which came from two different batches) were used for the tests. Phosphate buffered saline (PBS) was prepared extemporaneously and the pH adjusted to 7.4 before sterilization by autoclave. Modified Smalley et al 1995 technique was employed to determine the amount (plotted as Absorbance) of Congo red dye that is bound by each vaccine sample. Briefly, four replicate solutions of different concentrations of Congo red in PBS (pH 7.4) were prepared and 250µl of the vaccines were added (except for BCG which was 100 µl). Samples were incubated at 37^0C for 48 hours and then centrifuged at 4,000 revolutions per minute for 10 minutes. The absorbance at 480nm (APEL: PD-303 Spectrophotometer, made in Japan) of the unbound dye in the supernatant fluid was measured. The amount of Congo red bound was calculated by subtracting the absorbance of Congo red in the supernatant from the absorbance of Congo red in PBS solution. The absorbance of the different concentrations of Congo red dye in PBS alone (i.e. unbound dye) was determined before each set of experiment. A negative control was set up by using vaccines, of same batch with the sample, which had been stored at 37^0C for 10 months. All chemicals used were purchased by the investigator.

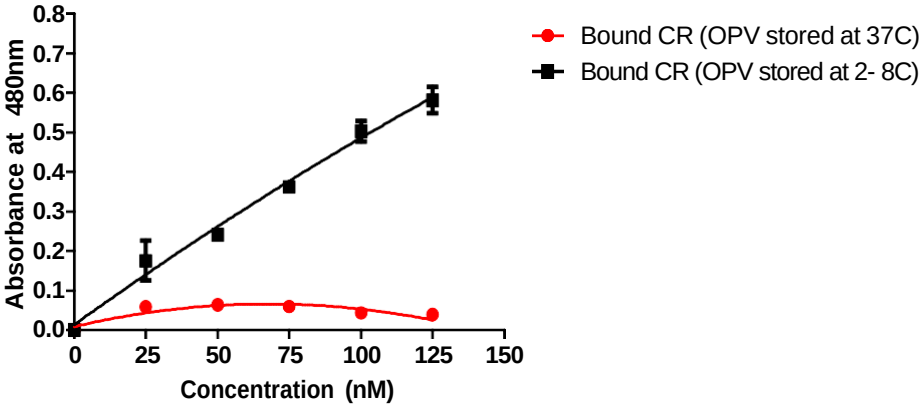
RESULT AND DISCUSSION

The Hepatitis B Vaccine sample studied had a steady increase in the amount of Congo red dye adsorbed while the negative control vaccine adsorbed the dye sluggishly up to 75nm and thereafter there was a steady decline in the amount of the dye adsorbed. The potency of vaccines and indeed all biological products is affected, among other things, by temperature (Milstien et al., 2002). The negative control vaccine sample (stored at 37^0C for 10 months) failed while the test the vaccine samples were



Data Set: ±SE, n = 4

Figure 2. Congo red Adsorption Test for Measles Vaccine (Batch # - 004N1007)



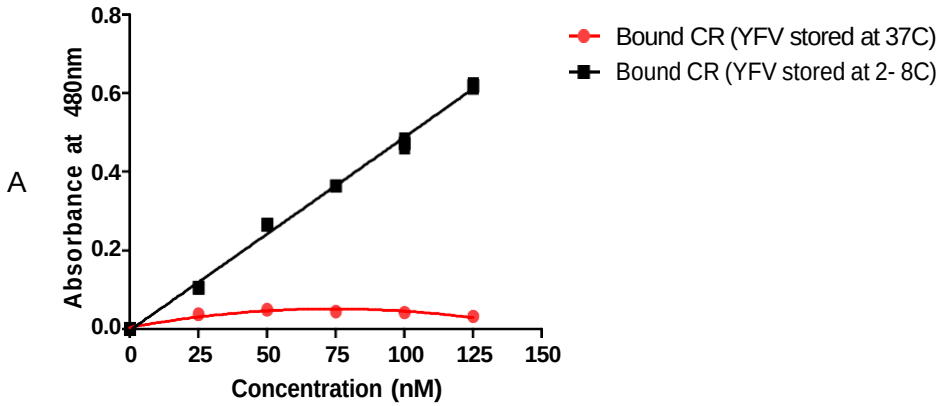
Data Set: ±SD, n = 4

Figure 3. Congo red adsorption Test for Oral Polio Vaccine (Batch # - 113920).

successful. (Figure 1)

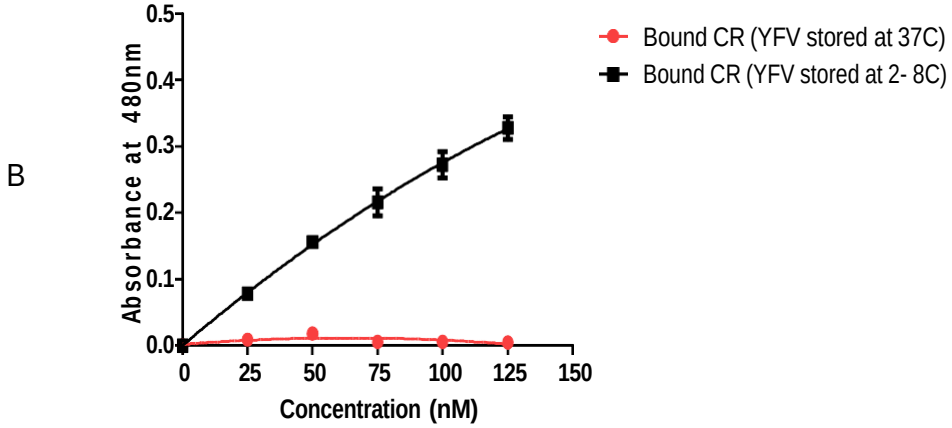
The Measles vaccines are live vaccines (contain live attenuated/weekend viruses). Live vaccines are generally known to stimulate a range of immune responses that occur naturally. Figure 2 above shows that there was a progressive increase in the quantity of the dye adsorbed by the measles vaccine sample. In the negative control vaccine, there was a marked difference in the quantity of dye adsorbed. After the 75nm concentration, the amount began to decrease progressively. The overall quantity of the Congo red in the negative control vaccine (stored at 37^0C for 10 months) could not reach up to 0.1 value of absorbance showing that the potency has been seriously compromised. Related work showed that Congo red binding test is useful for selection of virulent culture for vaccine production (Friedman et al., 2001). This is in line with our finding that the live measles used in this formulation is still active unlike the negative control vaccine. Some other researchers have used other methods to assay the potency/immunogenicity of measles vaccine (Griffin et al 2001, Cohen et al., 2007).

The Oral polio vaccine (OPV) used in eastern Nigeria is a trivalent oral poliovirus vaccine formulated from live polio virus. Figure 3 above shows that the OPV samples tested adsorbed continuously an increasing amount of the dye – almost parallel to the quantity of the dye in the solute PBS. The negative control vaccine did not adsorb up to 0.1 absorbance value and the value declined progressively after 50nm concentration. This shows a progressive loss of potency of the negative control vaccine as opposed to the test vaccines which have good potency/stability. Similar works done in the northern part of the country confirmed the potency of OPV used in the national immunization programme (Muhammad *et al.,* 2010a; Muhammad *et al.,* 2010b). Other researches had evaluated the potency of OPV used in their areas (Muhammad *et al.,* 2010a; Muhammad *et al.,* 2010b; Eswaran *et al.,* 2003) using various methods and found



Data Set: ±SD, n = 4

Figure 4a. Congo red adsorption Test for Yellow Fever Vaccine (Batch # - G5410).



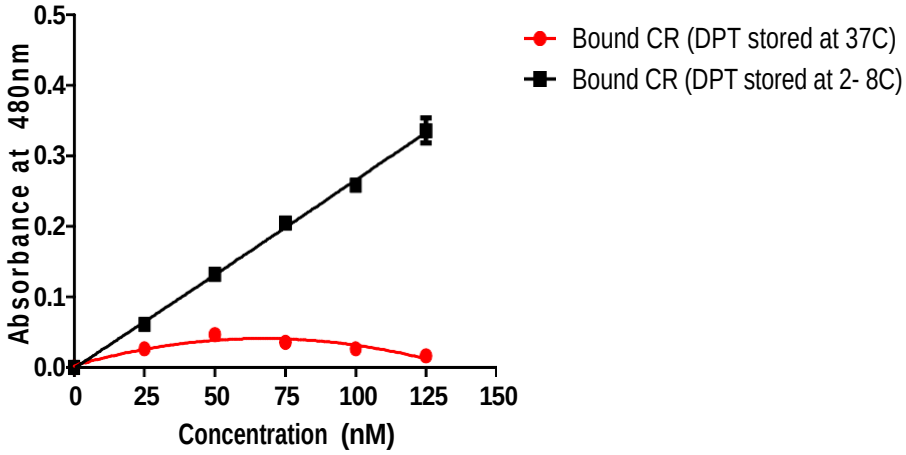
Data Set: ±SD, n = 4

Figure 4b. Congo red adsorption Test for Yellow Fever Vaccine (Batch # - 126).

them to be of the right potency. As a way of replacing the *in vivo* Neutralization method of potency and immunogenicity testing, some researchers have suggested the ELISA technique in line with WHO recommendation that a surrogate test should be reproducible and give accurate result as the *in vivo* test (Ivanov and Dragunsky 2005; Knezevic, 2009).

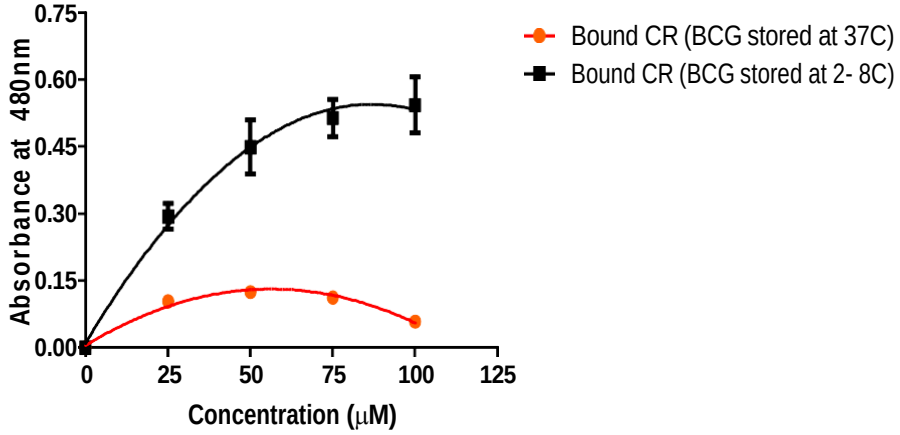
As shown in Figures 4a and 4b the two batches of the Yellow fever vaccine (YFV) tested showed similar result except that the negative control batch number 126 almost did not adsorbed the dye signifying that it was more affected by the storage condition than the batch number G5410. The vaccine samples progressively adsorbed the dyes as the dye concentration increases. The samples were therefore adjudged to be good and may be used for immunization programme. IgM antibodies capture ELISA (MAC-ELISA) and ELISA inhibition methods for the detection of antibodies against dengue virus had been modified and suggested for diagnosis, surveillance and yellow fever vaccine evaluation (Va´zquez *et al.,* 2003).

The Diphtheria-Pertussis-Tetanus (DPT) vaccine samples (Figure 5) showed a positive Congo red binding test. There was an increasing amount of the dye bound by the vaccine but in the negative control, the amount of the dye bound decreased progressively after the 50nm concentration and the peak dye adsorption could not reach 0.1 absorption value showing deterioration in the vaccine stability. As the DPT vaccine samples tested showed a continuous increase in the quantity of dye bound, they may be said to be of the right stability/potency and may be used for immunization. The intra-cerebral challenge test (Kendrick test) for the determination of the potency of whole cell vaccines has been considered unfit because of animal welfare and technical reasons but more precise methods based on determining the ribosyltransferase activity in arrays with receptor-binding



Data Set: ±SD, n = 4

Figure 5. Congo red adsorption Test for DPT Vaccine (Batch # - 0000111).



Data Set: ±SD, n = 4

Figure 6. Congo red adsorption Test for BCG Vaccine (Batch # - 037G1043).

assays has been suggested (Corbel and Xing, 2004).

Bacillus Calmette-Guérin (BCG) vaccine sample – lyophilized – adsorbed the dye progressively up to a plateau at 75µm concentration giving the concentration of maximum adsorption. The vaccine was saturated at this point. The control sample adsorbed the dye up to 50µm concentration after which there was a progressive decline, showing loss in the vaccine potency. It is important to note the high concentration used in the test which proved that the vaccine can be saturated – above which it can adsorb no more even though the vaccine may be potent. (Figure 6)

STATISTICAL ANALYSIS

The determination of CR binding was carried out in quad-ruplicate using Graphpad Prism 5 software. The results are expressed as the mean ± standard deviation.

CONCLUSION AND RECOMMENDATION

The use of Congo red binding test may provide a quick overview of the potency and stability of immunization vaccines.The vaccines tested passed the test as there was a continuous increase in the amount of dye bound by the vaccine samples. The vaccines tested are potent enough to be used for immunization. Solar refrigerators are also advised (Muhammad *et al.,* 2010a; Muhammad *et al.,* 2010b) to support the standby generators and the National Electricity - Nigeria being in the tropic. The use of Congo red binding test is in line with the 3Rs-concept: Replacement, Reduction and Refinement of animal tests (Hendriksen, 2009; Metz *et al.,* 2002).

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Competing Interests

None exists

Authors' Contridution

Oli AN' wrote the first draft of the manuscript, 'Oli AH' performed the statistical analysis, 'Uzodinma SU' revised the draft critically for important intellectual content, Nnadozie OJ performed part of the experiment and did data collection, 'Ele GN' managed the literature searches Okeke IJ revised the final draft and cross-checked for important intellectual content, 'Esimone CO' conceptualized the study.

Ethical Approval

This was gotten from Nnamdi Azikiwe University Teaching Hospital, Nnewi (Approval #:NAUTH/CS/66/Vol. 4/220) although it is not needed for the study because neither human nor animal was used.

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