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## EVALUATION OF LEVAMISOLE AS AN ADJUVANT FOR TYPHOID FEVER VACCINE FORMULATION

<sup>1\*</sup>Odimegwu, Damian C.; <sup>1</sup>Onah Agatha I.; <sup>1</sup>Ifudu France; <sup>2</sup>Oli Angus N.

<sup>1</sup>University of Nigeria, [Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences], Nsukka, Enugu State, Nigeria

<sup>2</sup>Nnamdi Azikiwe University, [Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences], Awka, Anambra State, Nigeria

[damian.odimegwu@unn.edu.ng](mailto:damian.odimegwu@unn.edu.ng); [nonsodimegwu@yahoo.co.uk](mailto:nonsodimegwu@yahoo.co.uk)

### ABSTRACT

There is a need for robust adjuvant for typhoid fever vaccine, as available licensed vaccines are saddled with challenges which include the need for revaccination after few years, which a suitable adjuvant can overcome. Efficacious typhoid fever vaccines are of importance because typhoid fever is a communicable disease that is endemic in developing countries characterized by poor water supply, sanitary conditions and increasing population. Vaccination with an effective vaccine is an effective control measure. Levamisole an antihelmintic drug with immunomodulatory activity was evaluated for possible adjuvant effect to *Salmonella typhi* vaccine. The blood culture results showed its utility as an adjuvant. Formulated typhoid vaccine and levamisole combination group had the lowest microbial load ( $17.0 \pm 6.8$ ) after challenge with live and viable *Salmonella typhi*., followed by formulated vaccine alone ( $27.6 \pm 5.2$ ) thus emphasizing the usefulness of levamisole as an adjuvant. The mice body weight assessment results also emphasizes this, as the mice in these treatment groups had an interrupted body weight decrease. Our result therefore shows levamisole may function as an adjuvant in *Salmonella typhi* vaccine formulation.

**Keywords:** *Salmonella typhi*, typhoid fever, levamisole, adjuvant.

## INTRODUCTION

The main aim of vaccination is to generate a strong immune response to an administered antigen, one that is able to provide a long term protection against an infection. However, many of the new vaccine candidates which are based on protective antigens are inherently less immunogenic than the whole cell inactivated or live attenuated vaccines or multi-component conjugate vaccines that were developed in the past, thus the increasing need for addition of immunological adjuvants in novel vaccines developed in recent times [1-3]. Available and licensed typhoid fever vaccines are not exception to this, the injectable Vi capsular polysaccharide vaccine, (Typhim Vi) which is administered as a single injection, given at least 2 weeks before potential exposure are not very immunogenic thus requires a repeat dose every 2 years after the primary dose if continued or renewed exposure is expected [4]. This vaccine like other polysaccharide vaccines, does not elicit an adequate immune response in very young children thus is recommended only for adults and children with age  $\geq 2$  years. The oral live attenuated vaccine (Vivotif, manufactured from the Ty21a strain of *Salmonella* serotype typhi) approved for adults and children aged  $\geq 6$  years, needs to be administered in 4 doses on alternating days over 1 week before potential exposure. This oral Ty21a vaccine although immunogenic, also requires repeat doses with the entire 4-dose series every 5 years if continued or renewed exposure to *Salmonella* serotype typhi is expected [5]. An effective adjuvant can eliminate the need for revaccination or at least ensure revaccination at a longer time interval. Also the stringent cold-chain requirements and the need for precise dosing intervals for the multiple dose of Ty21a vaccine add logistic and compliance challenges to the widespread use of this vaccine in resource-poor countries. Adjuvant can allow immunization with fewer doses of

this oral vaccine hereby enhancing compliance and overcoming logistic hurdles [6-8], it can also enable reductions in the quantity of antigen contained in an individual vaccine dose [6-8] as each orally administered Ty21a vaccine capsule contains  $2-10 \times 10^9$  viable *Salmonella enterica* serotype Typhi Ty21a and  $5-50 \times 10^9$  nonviable *Salmonella enterica* serotype Typhi Ty21a [5] which made it unsafe for children less than 6 years of age. Adjuvants can also be used to increase the response to these vaccines in children and in the general population by increasing mean antibody titre and / or fraction of the subject that become protectively immunized [6-8]. Very immunogenic, safe and efficacious typhoid fever vaccines are important because, it is one of the surest ways of averting the menace of typhoid fever as typhoid fever caused by *Salmonella enterica* serotype Typhi (*S. typhi*) is an important public health problem globally. It is a major cause of death in the developing countries [9-12]. In 2000, it was estimated that over 2.16 million episodes of typhoid occurred worldwide, resulting in 216 000 deaths [10-12]. Although improved water quality and sanitation constitute ultimate solutions to this problem as the infection is commonly acquired from water or food contaminated by the faeces of an infected person. Vaccination in high-risk areas is a potential control strategy recommended by WHO for the short-to-intermediate term [12]. This importance of vaccination is heightened by increasing resistance of the aetiological agent to antimicrobial agents, including fluoroquinolones, in many parts of the world [13]. In 2003, the World Health Organization recommended strategies to reduce typhoid fever which are routine immunization of school-aged children, immunization of children beginning at 2 years of age in sites where typhoid fever occurs in younger children [12] and immunization of travelers going to endemic areas.

Levamisole which was evaluated in this study as an adjuvant to typhoid fever vaccine is an imidazole-thiazole group derivate, which has been reported to be an effective and safe broad spectrum anthelmintic commonly used in veterinary and human medicine [14]. The anti parasitic action appears to be tied to its agonistic activity towards nicotinic receptors in nematode muscles which results in spastic paralysis with the net effect of paralyzing the worm, which is then excreted live [15-17]. Levamisole equally has immunomodulatory properties. Renoux and Renoux (1971) in [18] were the first to report the immunostimulatory properties of this drug followed by its use in various disease cases such as HIV [19], advanced malignancy [20], leprosy [21-22], colon cancer [23] etc. Studies have also shown that levamisole is able to enhance both humoral and cellular immune responses in normal poultry birds [24], in diseased and stressed poultry birds [25-26]. These effects of levamisole on the immune system are complex. Levamisole can stimulate formation of antibodies to various antigens, enhance T-cell responses by stimulating T-cell activation and proliferation, potentiate monocyte and macrophage functions including phagocytosis and chemotaxis, and increase neutrophil mobility, adherence, and chemotaxis [16-17; 23]. Symoens and Rosenthal [27] summarized levamisole as a drug that enhances the immune response by restoring phagocyte and T-lymphocyte functions in immunodeficient hosts, but does not increase immune response above normal level in the immunologically competent host. They also described it as a drug found to increase the protective effects of some vaccines and its potential advantage in various chronic and recurrent infections, immunodeficient conditions and neoplastic diseases in man and animals [14]. Adverse affects of levamisole are mild and infrequent

and include rash, nausea, abdominal cramps, taste alteration, alopecia, arthralgia, and a flu-like syndrome [17; 24]. We choose to evaluate levamisole as an adjuvant to this typhoid fever vaccine because there has been report of its adjuvant activity to killed viral vaccines [28-29].

## METHODS

### Animals

Twenty-five (25) young female Swiss albino mice (8 - 9 weeks old) purchased from faculty of veterinary medicine, University of Nigeria Nsukka and kept under standard pathogen-free conditions in an animal facility of the department of pharmacology and toxicology of the University of Nigeria, Nsukka were used. These animals were fed with standard feed and water *ad libitum* throughout the study period.

### Drug material

Pure sample of the drug, Levamisole hydrochloride (the hydrochloride salt of levamisole) from Jopan Pharmaceuticals, Lagos State, Nigeria, was used in the study.

### Typhoid fever vaccine

The vaccine used was prepared locally by heat denaturation method following the steps described by [3].

### Collection and isolation of *Salmonella typhi*

*Salmonella typhi* was obtained from the University of Nigeria Teaching Hospital, Ituku-Ozalla Enugu. Pure culture of the isolate was prepared by subculturing into freshly prepared *Salmonella-shigella* agar by streak method and then incubated at 37°C for 24 hours. After incubation, distinct colonies were again transferred/subcultured into freshly prepared and sterilized nutrient broth and incubated for another 24 hours.

### Determination of bio-load

Ten-fold serial dilution of the broth culture of microorganism was carried out. Exactly 1ml of the broth culture was collected and transferred aseptically into a test tube containing 9ml of sterile water and this was tagged  $10^{-1}$ ; from this test tube ( $10^{-1}$ ) another 1ml was transferred into another test tube containing 9ml of sterile water (tagged  $10^{-2}$ ) this was continued to the ninth test tube ( $10^{-9}$ ) and the last 1ml (i.e. from this test tube) transferred into a beaker to be discarded appropriately. Nine well-labeled nutrient agar plates corresponding to the nine test tubes ( $10^{-1}$  to  $10^{-9}$ ) were each divided into 8 sections and from each test tube/dilution (e.g.  $10^{-2}$ ), one drop each on the 8 sections were made (a total of 8 drops of that same dilution) on the corresponding nutrient agar plate. The plates were then incubated at  $37^{\circ}\text{C}$  for 24 hours, after which the viable cell count (using an appropriate dilution, i.e. one that is clear enough to be counted) was done to determine the bioload or concentration of the microorganism.

### Determination of death time of the microorganism

A loopful was transferred from the selected dilution test tube at time 0, into a recovery test tube containing nutrient broth. The test tube with its content was then heated (at a temperature of about  $55\text{-}60^{\circ}\text{C}$ ) and at various time intervals (i.e. 10, 20, 30, 40, 50, 60, 70, 80, 90 minutes), subculturing into the respective recovery test tubes (labeled according to the time). The recovery test tubes were then incubated for 48 hours at  $37^{\circ}\text{C}$ , after which the test tubes were examined for microbial growth (indicated by turbidity) so as to determine the death time.

### Formulation of *Salmonella typhi* vaccine

The selected broth culture was centrifuged at 3000 revolutions per minute for 5 minutes. The supernatant was then removed with the aid of the micropipette and the cells washed twice with normal saline by centrifuging and removing the supernatant in each case. The cells were then re-suspended in a specified volume of normal saline (5ml) and heated (at the same temperature) for a period of time equivalent to the predetermined death time. The vaccine, thus formulated, was then aseptically transferred into bijoux bottles and made up with normal saline, labeled appropriately and stored in the refrigerator.

### Vaccination

The experimental animals were divided into five groups named A, B, C, D & E of five mice each and vaccinated intraperitoneally as follows; Group A received 0.05ml of distilled water only (solvent used in the levamisole solution), Group B received 5mg of levamisole/kg body weight, only, Group C received 0.4ml of normal saline only (solvent used in vaccine constitution), Group D received 0.4ml of the vaccine only (which contains  $10^8$  cells). Group E received 0.4ml of the vaccine and 5mg of levamisole/kg body weight of the mice. The vaccination was repeated once after two weeks. The above quantity of levamisole administered was contained in 0.05ml of the 2.7mg/ml solution of levamisole prepared with distilled water. The calculation done was based on their average weight.

### Weight monitoring of the experimental animals

From day 1 post second vaccination, each of the experimental animals was weighed on a daily basis using a digital sensitive weighing balance and the weight recorded accordingly. This continued till the animals were sacrificed.

### Challenge of animals with live *Salmonella typhi*

After the 1 week post second vaccination blood sample collection, the experimental animals were challenged with  $10^7$  live *S. typhi* organisms contained in 0.04ml preparation through the intraperitoneal route.

### Blood culture

One-week post challenge, blood samples were collected from each of the mice by intraocular eye puncture using the method described by [2] and 2 fold serial dilutions of the blood samples were done by diluting 25 $\mu$ L of the blood sample with 25 $\mu$ L of normal saline. 10 $\mu$ L of each of the diluted blood samples was then aseptically placed on each of the 8 respective portions of the properly labeled nutrient agar plates. This was carried out for all the mice. The plates with its contents were incubated for 24 hours at 37°C and then examined for growth of microorganisms via colony count.

### Statistical analysis

The data obtained was expressed as mean  $\pm$  standard error of mean (Mean  $\pm$  SEM). One-way analysis of variance (ANOVA) followed by Dunnet's post hoc test were used to test for significance.  $P < 0.05$  was considered significant. Graph pad prism (version 6.0) was used for the analysis.

## RESULTS

### Mice body weight

From the periodic mice body weight monitoring, the mice were observed to grow progressively in all the groups from the first day of monitoring up to the 7<sup>th</sup> day. After challenge on this 7<sup>th</sup> day a progressive decrease was noticed in all the groups (Figure 1).

### Blood culture

From the blood culture, distinct colony forming unit were seen which were counted and recorded as shown in Table 1 and graphically represented in Figure 2.

## DISCUSSION

In the course of the study, vaccination was carried out twice (at a time interval of two weeks) for enhanced immune response (the second vaccination served as a 'booster dose') before microbial challenge. Blood cultures after microbial challenge are specific assessment of microorganism presence in the challenged system [3] and it revealed a very high microbial load in the control groups A (131.5 $\pm$ 39.6), B (120.5 $\pm$ 79.5) and C (140.8 $\pm$ 37.9) with the microbial load in the control groups A and C being higher (Table 1 and Figure 2). This is attributed to the fact that distilled water (Group A) and normal saline (Group C) have no antimicrobial, prophylactic or therapeutic potentials. The levamisole treated group B has a less microbial load compared to distilled water and normal saline treated group possibly because of its reported antihelmintic effect, however the microbial load is still high. Statistical analysis of the microbial count seen in the various groups showed that the difference between the groups are significant ( $P < 0.05$ ) (Figure 2). The high microbial load seen in these groups A, B and C are as expected since they were the control groups and still went further to show that the microorganism used during the challenge are viable microbes. The typhoid vaccine treated group D and the typhoid vaccine and levamisole combination group E had a very low microbial count showing that our formulated vaccine is effective with that of the group E being lower. The low microbial count seen in these groups D (27.6 $\pm$ 5.2) and E (17.0 $\pm$ 6.8) is because of the prophylactic effect of the formulated vaccine. And the much lower microbial count seen in the group

E can be attributed to the addition of levamisole to the vaccine in this group which acted as an adjuvant potentiating the effect of the vaccine. How levamisole potentiated the effect of our formulated vaccine is unknown, however it is possible that it did so by increasing the level of serum IgG in the mice and by inducing a robust T cell proliferation as this was the mechanism reported by [23; 28] to be responsible for its adjuvant effect to viral vaccine. It is also possible that the observed adjuvant effect seen here is because levamisole potentiated monocyte and macrophage functions or increased neutrophil mobility, adherence, and chemotaxis [16-17; 23; 30-31].

The animal body weight assessment showed decrease in weight after challenge for all the groups (Figure 1). This decrease was more pronounced in the control groups A, B and C as the decrease in weight seen here was rapid (Figure 1). This is as expected since they are controls that lack antimicrobial effect and the organism used during the challenge are live and viable *Salmonella typhi*. The treated groups D and E equally had a decrease in weight after the microbial challenge but the decrease seen here was not rapid as there seems to be a struggle to overcome the microbial infestation on days 8 to 10 and 11 to 12 for the typhoid vaccine treated group D and on days 8 to 10 and 11 to 13 for the typhoid vaccine and levamisole combination group E. The struggle against weight loss seen on these days 11 to 13 as against that seen on day 11 to 12 for the typhoid vaccine alone treated group with a decrease on day 13 can still be attributed to the presence of levamisole in the combination group acting as an adjuvant. This periodic mice body weight monitoring was carried out because experimental animal body weight monitoring is important in research studies, as it provides information as regards the health status of the experimental animal especially if infection or a disease state is

involved [30] and typhoid fever infection is known to be associated with significant weight loss [10-12] hence the need for periodic weight assessment of the mice after challenge with live *Salmonella typhi*.

The results of this work shows the combined immune boosting effect of *Salmonella typhi* vaccine and levamisole against the typhoid fever infection in the experimental animals used thus suggesting the possibility of levamisole used as an adjuvant to typhoid fever vaccine formulation as it demonstrated the efficacy of combining both agents.

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### Competing Interests

Authors have declared that no competing interests exist.

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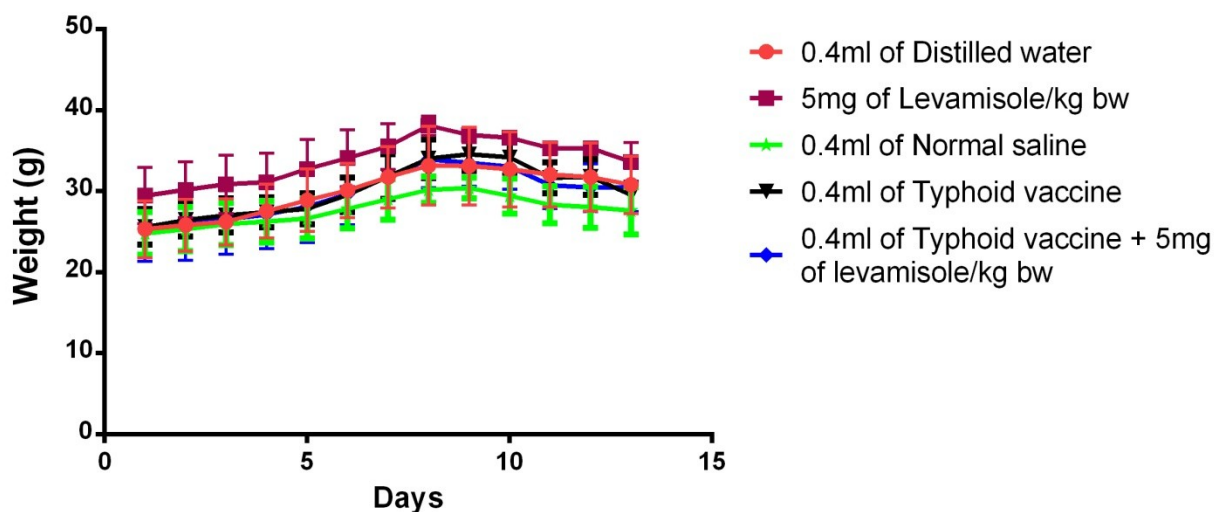
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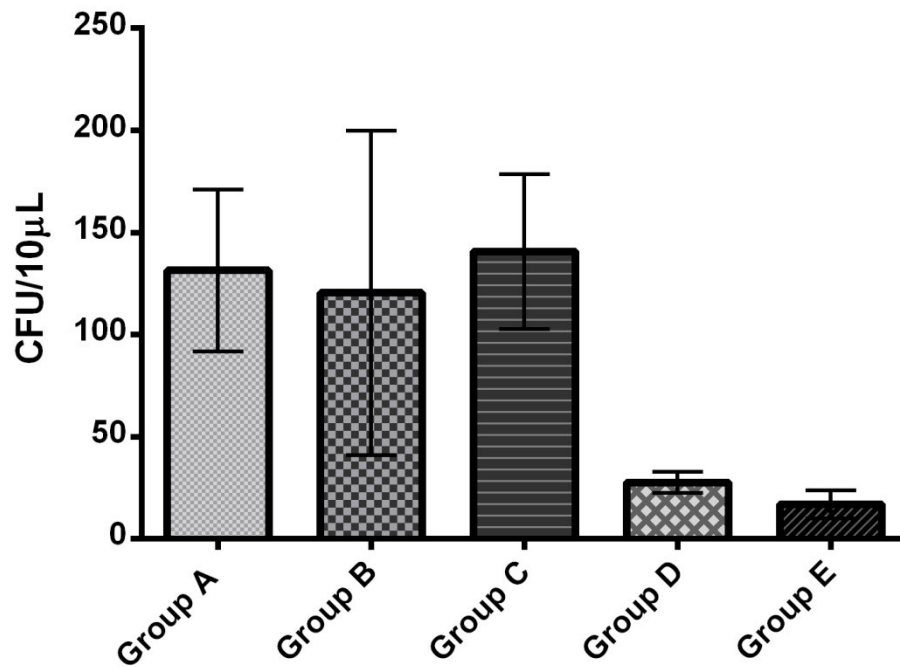


**Figure 1:** Mice body weight curve

Mice vaccinated with distilled water, Levamisole, Normal saline, typhoid vaccines and typhoid vaccine plus levamisole were monitored for changes in their weight during the course of vaccination. The periodic weight measurement was done to ascertain their health status

**Table 1:** A table of value showing number of colonies from blood culture

Mouse	Group A	Group B	Group C	Group D	Group E
1	200	-	200	36	35
2	-	200	200	21	8
3	59	-	200	43	20
4	200	41	89	15	5
5	67	-	17	23	-



**Figure 2:** A graphical representation of microbial count following blood culture. Mice vaccinated with distilled water, Levamisole, Normal saline, typhoid vaccine and typhoid vaccine plus levamisole were assessed for microbial growth in a blood culture assay. The blood culture test was done at the end of vaccination to evaluate vaccine efficacy.