



## INVESTIGATION OF THE THERMOSTABILITY AND IMMUNE RESPONSE OF AN ORAL LIVE 9R VACCINE ENCAPSULATED IN ALGINATE-COATED CHITOSAN MICROPARTICLES

EBELE BENEDETTE ONUIGBO<sup>1\*</sup>, CHRISTOPHER OSITA EZE<sup>1</sup>, ANTHONY AMAECHI ATTAMA<sup>2</sup>, EMMANUEL CHINEDUM IBEZIM<sup>2</sup>, CHIAMAKA UCHEAKONUME<sup>2</sup>

1. Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, 410001, Enugu State, Nigeria.
  2. Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, 410001, Enugu State, Nigeria
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### ABSTRACT

This study was designed to investigate the thermostability and immune response of live 9R vaccine encapsulated in alginate-coated chitosan microparticles. The microparticles were prepared by ionotropic gelation method and physically characterized for shape, particle size, encapsulation efficiency, mucoadhesion, pH and biochemical studies. Agglutination test, ELISA, toxicity test and physical examination of the birds were carried out. The particles were spherical and ranged from  $2.0\pm 0.0$  -  $3.85\pm 1.76$   $\mu\text{m}$  for the chitosan-coated live 9R vaccine and  $3.44\pm 0.66$  -  $5.57\pm 2.27$   $\mu\text{m}$  for alginate/chitosan-coated live 9R vaccine. Encapsulation efficiency was above 70 %. The mucoadhesive strength was above 11.05 dynes/cm in all the formulations. pH of formulations was stable after 5 weeks of storage with only a slight variation. There was no physical detection of toxicity in the birds. There was no significant difference in immune response between the subcutaneous live 9R vaccine group and the oral alginate/chitosan-coated live 9R vaccine group after vaccination ( $p < 0.05$ ) using plate agglutination test and ELISA. The live 9R encapsulated in alginate-coated chitosan microparticles was viable after only one week of storage at room temperature. The formulated alginate/chitosan-coated live 9R vaccine administered orally had comparable immune response with the subcutaneous live 9R vaccine.

**KEYWORDS:** Chitosan, Alginate, Live 9R vaccine, Immune, Thermostable, Subcutaneous, Oral.

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### INTRODUCTION

*Salmonella enterica* serovar Gallinarum (SG) is the etiologic agent of fowl typhoid (FT), a severe systemic disease of chickens and other galliform birds [1-3]. SG is a Gram-negative rod that is different from other *Salmonellae* because of its non-motility except *Salmonella enterica* serovar Pullorum. It causes mortality as high as 80 % in susceptible chicken especially in the young birds [4,5]. Preventive measures which are effective are flock vaccination and stringent sanitary regulations. The strain 9R of SG is routinely administered to chickens in countries with endemic fowl typhoid [1]. The rough strain provides sufficient immunity in

chicken, without showing pathogenicity for day old chicks [6]. Fowl typhoid vaccine is given as a subcutaneous injection [7]. Injections are cumbersome, uneconomical and hazardous because of needle stick injuries. For this purpose, oral vaccination of fowl typhoid vaccine is investigated in this preliminary study by encapsulating the fowl typhoid vaccine in alginate/chitosan microparticles. Preparation of a thermostable live 9R vaccine is quite innovative and it is expedient in a developing country where constant supply of electricity is a huge challenge. The formulation was prepared by a mild ionotropic gelation method. The size and encapsulation

\*Corresponding author: [ebele.onuigbo@unn.edu.ng](mailto:ebele.onuigbo@unn.edu.ng); +234-806-299-5712

efficiency were optimized using design of experiment (DE version 9.0.4) by employing the Box-Behnken technique.

Mucosal vaccines have several advantages over traditional systemic vaccines. It is more widely accepted by the public, as well as making the vaccine simpler to administer and distribute [8-10]. In addition, there is less risk of needle-stick injury or cross-contamination [11]. But there are several challenges in achieving oral delivery such as varying pH (highly acidic stomach), the presence of enzymes, first-pass effect in the liver and the intestinal barrier to drug absorption. The above reduces drug entrance into the system thereby reducing its bioavailability and the efficacy of the drug. Many polymeric microparticles have been employed in the formulation of oral vaccines [12]. They have a matrix-like structure in which a therapeutic agent is embedded within their polymeric matrix or adsorbed or conjugated onto the surface. They also protect the antigens from the adverse gastric environment [13-15]. The physicochemical properties of the microparticles such as thermostability, safety and immune responses of the birds to the oral administration compared to the injection were investigated in this preliminary study.

## MATERIALS AND METHODS

### Materials

The following materials were used as procured without further purification: sodium alginate, low molecular weight chitosan, calcium chloride, acetic acid, Tween® 80 and sodium tripolyphosphate (TPP) (all from Sigma Aldrich Co., Germany), distilled water, deionized water (National Commission for Energy Research and Development, University of Nigeria, Nsukka), Fowl typhoid vaccine (National Veterinary Research Institute (NVRI), Vom, Nigeria, Batch 05/2015), Pullorum antigen stained antigen polyvalent, Salmonella polyvalent Groups B and D SPA, negative SPA serum (Charles River, Wilmington, MA, USA). The chicks were raised from 1 day old until termination of the experiment. All bird handling and experiments were conducted following the guidelines stipulated by University of Nigeria Research Ethics Committee on animal handling and use.

### Microparticle preparation

A 5 ml volume of 0.25 % sodium alginate solution was added to a beaker and stirred at 50 rpm using an automatic magnetic stirrer for 5 min. Then 1 ml of calcium chloride was added dropwise to above solution while stirring. The prepared

Calcium/Alginate (Ca/Alg) pregel was stirred for a further 30 min, then 10 ml of 0.25 % chitosan cross-linked with 2 ml of TPP solution at pH 5.6 loaded with the live 9R vaccine was added dropwise to the Ca/Alg pregel, while stirring for 30 min to form the complex particles. This was done in seventeen runs following Box-Behnken experimental design as seen in Table 1.

### Particle size determination

The particle sizes and morphology of the microparticles were determined by digital light microscopy using Moticam 1000 1.3 mpixel (MP) live resolution, Macintosh OSX. Briefly, a drop of sample (about 0.05 ml) was smeared on slide to form a thin film which was then viewed with x40 objective lens. The mean sizes were determined and measurements were performed in triplicate.

### Drug encapsulation efficiency (%)

For quantitative determination of the fowl typhoid vaccine loading, samples were centrifuged in a cold centrifuge (Sorvall RT 6000 D) at 7500 rpm for 5 min and then the absorbance of the supernatant in the tubes was measured at 267.5 nm using a UV/VIS spectrophotometer (Spectrumlab 752S). The amount of live 9R vaccine associated encapsulated in the microparticles was calculated indirectly by the difference between the initial amount of live 9R vaccine added to the chitosan and the amount measured in the supernatant. The following equation was used to determine the EE.

$$\text{Encapsulation efficiency (EE\%)} = \frac{FT_{\text{total}} - FT_{\text{supernatant}}}{FT_{\text{total}}} \times 100 \dots 1$$

### Mucoadhesion

The bioadhesive strength of the coated microparticles was studied by evaluating the force required to detach the hydrated polymeric discs from the surface of cow intestine tissues using a Lecomte Du Nuoy tensiometer (Model Nr 3124, A Kruss Hamburg, Germany) adapted for the purpose. The total force in dynes required to completely detach the disc from the tissue was recorded. The experiment was repeated three times on different tissue surfaces for each batch of the discs. The experiment was done using SGF and SIF media. The bioadhesive force (Fb) was calculated per unit area of the polymeric disc.

$$F_b = \frac{F}{A} \dots \dots \dots 2$$

Where Fb is the mucoadhesive force, F is the force applied; A is the cross-sectional area of the tissue.

### pH stability studies

The coated microparticles were subjected to time-dependent pH analysis to determine their stability. The pH of the microparticles for each optimized run was determined using a pH meter and it was monitored for five weeks at room temperature.

### Microbiological studies

The alginate/chitosan microparticles encapsulating the live 9R vaccine, chitosan microparticles encapsulating the live 9R vaccine and the marketed live 9R vaccine were stored at room temperature for 3 weeks. Every week, loopfuls of the stored vaccines were aseptically inoculated into nutrient broth and incubated at 37 °C overnight, after which loopfuls were streaked on Mac Conkey agar to confirm viability and presence of Salmonella. Biochemical tests were done to confirm the presence of *Salmonella gallinarum*.

### Oral acute toxicity test

After vaccination, each group was treated with empty alginate/chitosan microparticles at doses 8000, 10000, 30000, 45000 and 60000 mg/kg body weight respectively. The birds were observed for loss of weight for 7 days and behavioural disorders/mortality for 14 days.

### Plate agglutination test

At 7 weeks of age, the subcutaneous live 9R vaccine group was given 0.2 ml ( $5 \times 10^7$  viable 9R vaccine strain/bird) of the vaccine, alginate/chitosan-coated live 9R group was given orally 0.2 ml ( $5 \times 10^7$  viable 9R vaccine strain/bird) of the vaccine, chitosan-coated live 9R vaccine group was given orally 0.2 ml ( $5 \times 10^7$  viable 9R vaccine strain/bird) of the vaccine while the oral live 9R vaccine group was given 0.2 ml ( $5 \times 10^7$  viable 9R vaccine strain/bird) vaccine. The vaccination was carried out once. At 9 weeks of age, about 5 ml of blood was collected from the jugular vein of each bird using sterile syringe and emptied into marked individual centrifuge tubes. Sera were prepared from blood samples to determine the circulating antibody titre by plate agglutination test. Clean microscope slides were used. Serum samples from all the birds were tested. One drop of crystal violet Pullorum antigen stained polyvalent antigen was placed on the slide. A drop of the serum samples of the immunized birds from the different groups, one group at a time, was placed next to the drop of antigen. The drops of antigen and serum

were mixed using a glass rod which was wiped clean between samples. Then, using a gentle rocking motion the tiles were rotated for 2 min and signs of agglutination observed. The results were compared with the Salmonella positive polyvalent Groups B & D SPA and the negative SPA serum. At 16 weeks of age the birds were challenged with the *S. Gallinarum* strain 9 containing virulent 85-kb plasmid.

### ELISA

Three groups were used for the experiment. The subcutaneous live 9R vaccine group, alginate/chitosan microparticles encapsulating the live 9R vaccine and negative unvaccinated vaccine. Blood was collected from the jugular vein and serum separated into tubes. The sera were stored at -26 °C until ready for ELISA. To assess the antibody response, blood was collected from the jugular vein six days after each vaccination and serum separated. A commercial ELISA kit (Biochek, Netherlands) was used according to the manufacturer's instructions to obtain optical density (OD) values. The OD values were used to calculate the adjusted E values. This was used to quantify IgG (IgY) after first vaccination, second vaccination and challenge.

OD test sample- OD standard negative control

OD standard positive control – OD standard negative control ..... (3)

### Data and statistical analysis

The results of the ELISA and plate agglutination test were expressed as mean  $\pm$  standard deviation. The group comparisons were done using one-way ANOVA and Student's T-test respectively. The significant differences in the mean values were evaluated using Sigma<sup>®</sup> plot and Microsoft Excel software package and were considered significant at  $p < 0.05$ .

## RESULTS

### Particle Size

The particle sizes ranged from  $2.0 \pm 0.0$  -  $3.85 \pm 1.76$   $\mu\text{m}$  for the chitosan-coated live 9R vaccine and  $3.44 \pm 0.66$  -  $5.57 \pm 2.27$   $\mu\text{m}$  for alginate/chitosan-coated live 9R vaccine (Table 2). The ranges were narrow with low variables. The alginate/chitosan-coated live 9R vaccine had larger particles than the chitosan-coated live 9R vaccine. There was no correlation between the particle size and increase in the ratio of the chitosan or alginate. Three sizes of the alginate-coated chitosan microparticles were

**Table 1: Variables used in the Box Behnken experimental design**

Independent variables	Symbol	Levels		
		-1	0	1
Chitosan (%)	A	0.25	0.5	0.75
Sodium alginate (%)	B	0.2	0.25	0.5
Calcium chloride (mM)	C	0.55	1.0	1.8

Dependent variables	Units	Constraints
R1 = particle size	$\mu\text{m}$	Minimize ( $<4\mu\text{m}$ )
R2 = encapsulation efficiency	%	Maximize ( $> 95\%$ )

**Table 2: Concentrations of independent variables and the responses of dependent variables**

No	Chitosan (mg/ml)	Alginate (mg/ml)	Calcium chloride (mM)	Mean particle size of chitosan-coated microparticles ( $\mu\text{m}$ )	Mean particle size of alginate-chitosan coated microparticles ( $\mu\text{m}$ )	Encapsulation efficiency (%) of alginate-coated microparticles
1	5.0*	5.0	1.0	2.33 $\pm$ 0.75	4.18 $\pm$ 0.70	98.83
2	5.0	2.5	1.8	2.66 $\pm$ 0.94	3.69 $\pm$ 0.43	99.08
3	7.5	5.0	0.55	4.22 $\pm$ 0.95	5.57 $\pm$ 2.27	96.73
4	7.5*	7.5	1.0	2.0 $\pm$ 0.0	3.84 $\pm$ 0.31	99.32
5	2.5	7.5	1.0	3.85 $\pm$ 1.76	4.38 $\pm$ 0.50	99.40
6	2.5	5.0	1.8	2.47 $\pm$ 0.75	3.50 $\pm$ 0.40	99.32
7	7.5	5.0	1.8	3.15 $\pm$ 0.84	4.79 $\pm$ 1.38	99.69
8	5.0	2.5	0.55	2.80 $\pm$ 0.89	4.50 $\pm$ 0.45	96.73
9	5.0	7.5	1.8	2.67 $\pm$ 0.94	3.44 $\pm$ 0.66	99.08
10	2.5	2.5	1.0	3.52 $\pm$ 1.89	3.54 $\pm$ 0.43	97.58
11	7.5*	2.5	1.0	2.0 $\pm$ 0.0	3.90 $\pm$ 0.14	98.51
12	2.5	5.0	0.55	3.52 $\pm$ 1.89	3.46 $\pm$ 0.75	98.76
13	5.0	7.5	0.55	3.32 $\pm$ 1.49	3.45 $\pm$ 0.02	99.20
14	7.5	7.5	1.8	3.42 $\pm$ 1.02	3.83 $\pm$ 0.27	99.19
15	2.5	2.5	0.55	3.35 $\pm$ 0.88	3.91 $\pm$ 0.30	99.19
16	5.0	5.0	0.55	2.88 $\pm$ 1.02	4.27 $\pm$ 0.34	99.36
17	5.0	5.0	1.8	3.22 $\pm$ 1.0	4.42 $\pm$ 0.74	99.55

\*Optimized batches of the microparticles based on Box-Behnken technique.

chosen as optimal from the Box-Behnken experimental design for further characterization.

### Morphology

The technique used for the preparation of the microparticles was the ionotropic gelation method and the particles appeared as near spherical and discrete particles under the microscope (Figure 1a-d). The technique produced stable and pourable microparticles.

### **Percentage drug encapsulation efficiency (PDEE)**

Seventeen microparticulate formulations were evaluated for drug encapsulation efficiency and the results of the PDEE is shown in Table 2. The encapsulation efficiency in the seventeen runs were high but did not vary greatly with a narrow range between 97.58-99.69 %.

### **Mucoadhesion**

The formulations showed sufficiently high bioadhesive strength above 11 dynescm<sup>-2</sup> (Table 3). The mucoadhesivity was highest in Run 1 due to the swelling degree of the optimal ratio of alginate:chitosan matrix (Table 3).

### **pH stability**

pH measurements were done to evaluate the stability of the microparticles after five weeks of storage at room temperature. At increasing days of storage, the pH increased but minimally, it fluctuated between 5.3 and 6.0 for all the optimized groups.

### **Microbiological tests**

The culture and sensitivity test showed a yellow growth of *Salmonella* on MacConkey agar (non-lactose fermenter) after only one week of storage at room temperature. The biochemical test was positive for *Salmonella gallinarum* as shown in Table 5 below.

### **Agglutination test**

The agglutination test yielded no appreciable agglutination in the sera of the unvaccinated, chitosan-coated live 9R vaccine group and oral live 9R vaccine group while the alginate/chitosan-coated live 9R vaccine group and the parenteral live 9R vaccine group showed strong agglutination. There was no statistical difference at ( $p < 0.05$ ) between the alginate/chitosan-coated live 9R vaccine group given orally and the parenteral live 9R vaccine group.

### **ELISA**

The E-values show that there was no significant difference in immune responses in the parenteral live

9R vaccine group, Alginate/chitosan-coated live 9R vaccine group or the unvaccinated group after the first vaccination. After the second vaccination ( $p < 0.05$ ), there was a significant rise in the titre of the Alginate/chitosan-coated live 9R vaccine group above the parenteral live 9R vaccine group and the unvaccinated group. The unvaccinated group had no increment in the titre. After the challenge, the E-value of the unvaccinated was the reverse with a high E-value above 0.8. The parenteral group had a slight rise in E-value after the challenge with the 85-kb serovar *Gallinarum* plasmid. The E-value of the alginate/chitosan group went slightly lower. There was a significant difference ( $p < 0.05$ ) post challenged between the unvaccinated group and the vaccinated groups.

### **Toxicity studies**

The birds were subjected to sub-chronic toxicity studies. The birds survived till the end of the experiment and did not show any treatment-related adverse effects. There was no loss of weight or mortality when increasing concentrations of the microparticles were administered orally. The weight gain/loss is as shown in Figure 3

### **DISCUSSION**

The particle size range obtained in this study is suitable for oral administration of vaccine. Particle size is an important consideration while formulating microparticulate systems because it plays a critical role in determining the route of administration and it affects the physical, chemical and pharmacological properties of the drug formulation [16]. Particle size equally affects release pattern and absorption of vaccine at the target site. Chitosan, alginate a calcium chloride ratios had an effect on the sizes of the particles. Particles of few micron size are more easily internalized and show better immune responses than particles of larger size which could be destroyed by the phagocytes thereby leading to poor IgG and T cells stimulation.

Shape of the particles play a vital role in internalization at the rich M cells of the Peyer's patches. They are internalized following the mechanism of uptake of bacterial pathogens that are rods or cocci by the Peyer's patches and this leads to their preferential internalization. Irregular-shaped particles are more difficult to internalize which may result in poor immune response of the cells [12].

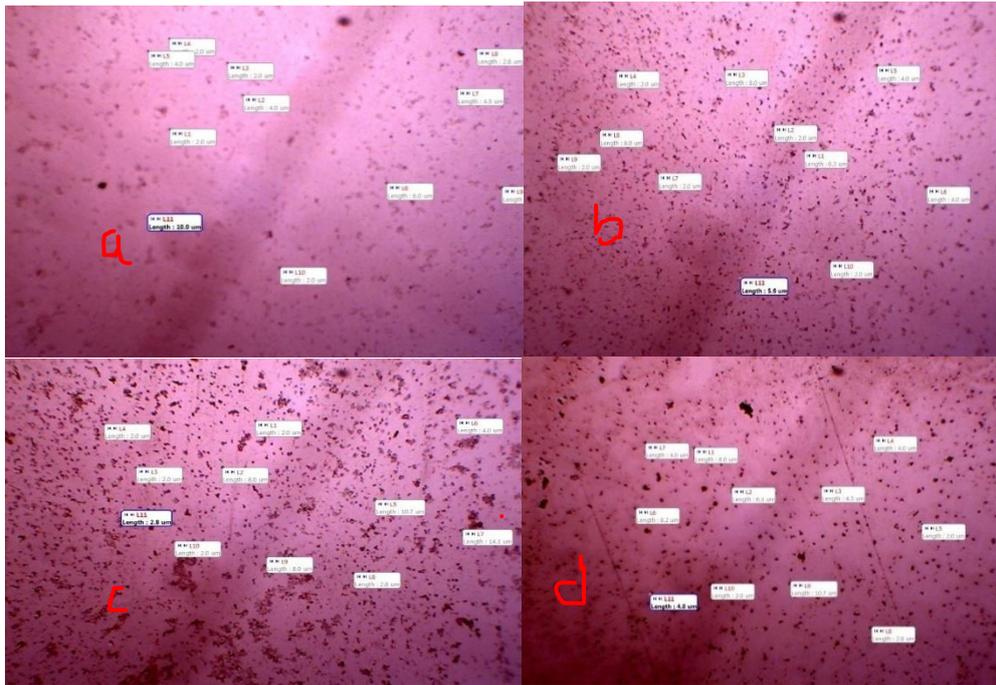


Figure 1a-d: Particle sizes of some alginate/chitosan-coated live 9R vaccine microparticles.

Table 3: Mucoadhesive strength of optimized microparticles in simulated gastric fluid and simulated intestinal fluid.

Batches of Formulation	Mucoadhesive force (dynescm <sup>-2</sup> ) ± SD in SGF.	Mucoadhesive force (dynescm <sup>-2</sup> ) ± SD in SIF
Run 1	20.49 ± 0.01	30.20 ± 0.20
Run 4	20.24 ± 0.02	20.15 ± 0.04
Run 11	20.52 ± 0.12	11.05 ± 0.06

Table 4: Stability studies using the pH of the microparticles stored for five weeks.

Optimized Batch	Day 0	Day 7	Day 14	Day 35
1	5.3	5.3	5.3	6.0
4	5.4	5.7	5.8	6.2
11	5.4	5.5	5.6	5.9

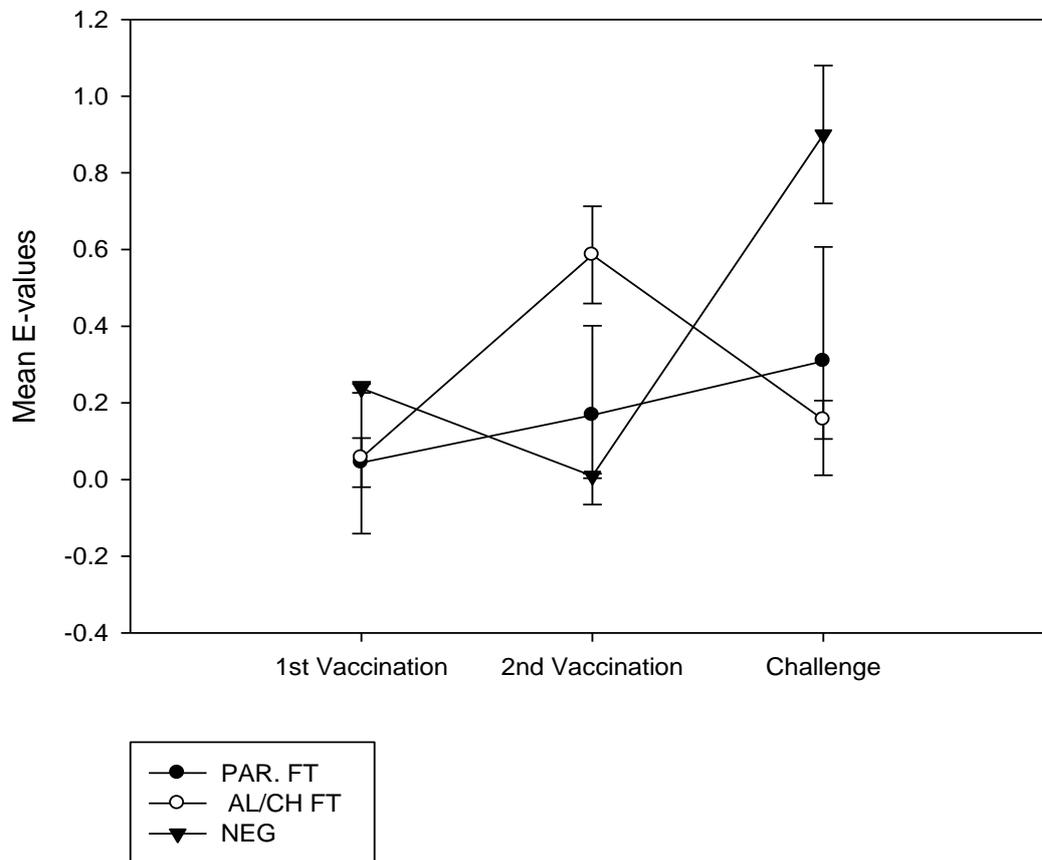
Table 5: Biochemical confirmatory tests of the presence of *Salmonella enterica* serovar Gallinarum.

Biochemical tests	Results
Glucose	Negative
Sucrose	Negative
Mannitol	Negative
Simmons Citrate	Negative
Hydrogen Sulphide	Positive
Indole	Negative
Motility	Negative

**Table 6: shows the statistical differences between the alginate/chitosan-coated live 9R vaccine and the respective vaccine formulations.**

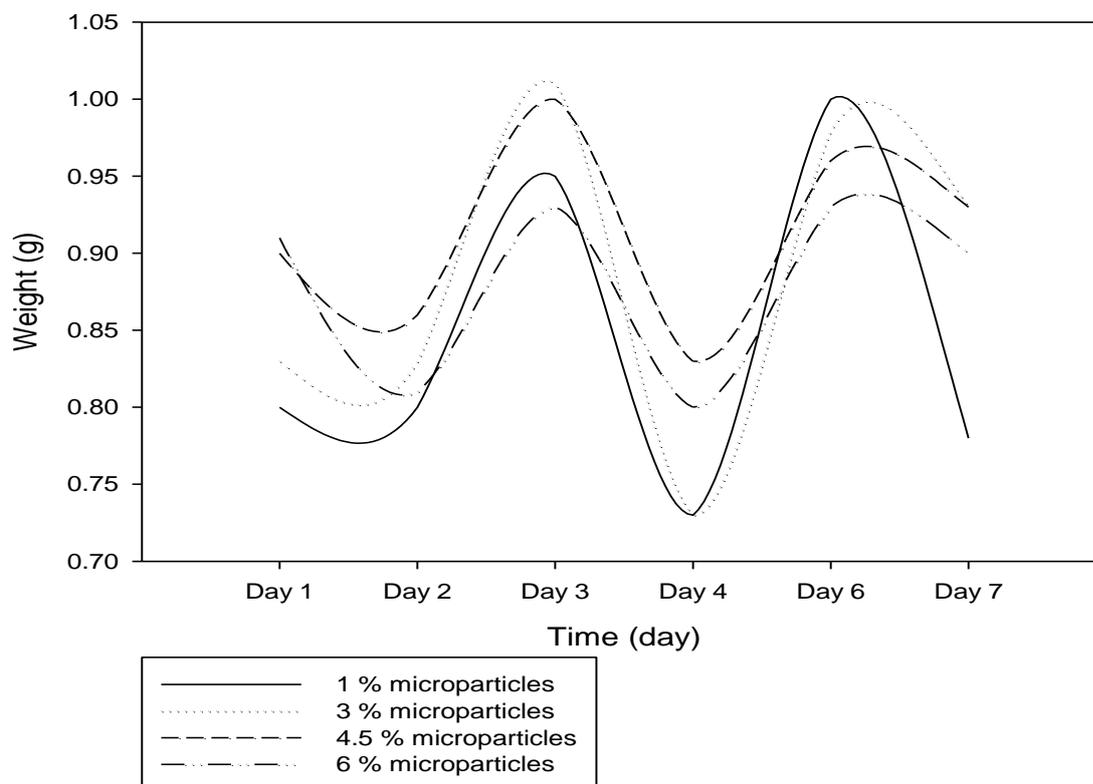
	Mean $\pm$ SD	P-value	
Alginate/chitosan- coated live 9R vaccine group	2.63 $\pm$ 1.21		
Chitosan-coated live 9R vaccine group	0.80 $\pm$ 1.23	0.001	Significant
Oral live 9R vaccine group	0.70 $\pm$ 1.20	0.000	Significant
Parenteral live 9R vaccine group	2.68 $\pm$ 0.10	0.894	Not significant
Unvaccinated negative group	0.10 $\pm$ 0.32	0.000	Significant

\*P <0.05 is significant.



**Figure 2: Graph of IgG after first vaccination, second vaccination and challenge with a 85-kb virulent *S. gallinarum* strain 9. Data represents the Mean  $\pm$ SD. There was no significant difference between the vaccinated groups after first vaccination, second vaccination and post-challenge (P < 0.05)**

## Sub chronic Toxicity studies



**Figure 3: Sub-chronic toxicity studies with increasing concentrations of the alginate/chitosan microparticles to the birds.**

Chitosan and sodium alginate microparticles are biodegradable and upon uptake by the M-cells readily release the encapsulated antigen [17]. Selection of appropriate sodium alginate concentration and coating process also plays a role in forming a stable formulation.

The encapsulation efficiency of the microparticles was high (96.73 -99.5 %) and this shows the ability of chitosan/alginate microparticles to effectively encapsulate the live 9R vaccine. Small particle size has been reported to provide a more effective surface area for vaccine to bind to the particle, thereby improving the loading efficiency [18]. Alginate is a hydrogel and would allow water-soluble biomolecules to be highly encapsulated leading to longer circulation times and better delivery. The absorption of the vaccine within the coated particles depends significantly on the ionic interaction between the charges of the chitosan, vaccine and the sodium alginate. Modifications in the pH also play a significant role in the internalization of the vaccine. It has been observed from previous works that pH

5.5 seems most favourable for interaction of chitosan and sodium alginate [15].

The mucoadhesivity was highest in Run 1 due to the swelling degree of the optimal ratio of alginate:chitosan matrix (Table 3). The swelling of the matrices increased the contact area between the two surfaces [19-21] leading to the release of adhesive sites necessary for mucoadhesive interaction. The interaction between the mucin lining the stomach and the polymers give such advantages like longer gastric residence time and consequently improved drug absorption and bioavailability of this formulation. The gel-like property formed due to the interaction between the divalent calcium ions and the anionic alginate will improve its adherence at the target site delivering the vaccine optimally and increase their biological half-life *in vivo*.

The high increase in immune response in the alginate-coated chitosan microparticles encapsulating the live 9R could be attributed to the alginate coating which is acid-stable and prevented the vaccine from being destroyed by gastric enzymes [22]. Alginate is also an adjuvant causing

antigen uptake by mucosal lymphoid tissues especially at the Peyer's patches [17]. This also shows that the microparticles did not affect the viability of the vaccine in eliciting detectable antibody titres. It also confirms that the microparticles did not yield toxic compounds that interfered with the immunogenicity of the vaccine.

The values obtained from the ELISA corroborated the findings in the plate agglutination test. There was no significant difference between the parenteral live 9R vaccine and the alginate-coated chitosan microparticles encapsulating the live 9R. There were significant differences at ( $p < 0.05$ ) between the unvaccinated and the vaccinated birds after challenge with the 85-kb plasmid *Salmonella gallinarum* as seen in the graph.

The motility test (biochemical test) showed that the sample contained *Salmonella* sp which was non-motile which confirmed the presence of *Salmonella gallinarum*. The storage conditions were not favourable for the lyophilized live 9R encapsulated in alginate-coated chitosan microparticles which did not grow on MacConkey agar after the first week of storage.

## CONCLUSIONS

This study has demonstrated that the encapsulation of live 9R vaccine in alginate-coated chitosan microparticles given to birds orally showed comparable immune responses with the marketed parenteral live 9R vaccine. It can be suggested that this antigen delivery system would be efficient for oral delivery of 9R vaccine. This would encourage farmers to adopt vaccination of their birds against fowl typhoid and reduce antibiotic abuse which is already a world health problem due to an increasing number of resistant *Salmonella* sp. The thermostability of the lyophilized live 9R vaccine in alginate-coated chitosan was poor necessitating further studies.

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