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A DNA Vaccine versus Attenuated Vaccine to Protect against *Salmonella* Infection in Chickens

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AJV and HA coordinated the study. Authors SC, AJV and HA designed the study. Authors SC and AJV did the laboratory work and managed the literature searches. Author SC took care of the chickens at the animal house. Authors SC and AJV wrote the protocol and the first draft of the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

The aim of this study was to vaccinate layer hen chickens against *Salmonella* infection. Two vaccines were assessed for efficacy and safety: a DNA vaccine containing *Salmonella* genomic DNA encapsulated in a liposome as a vector and a live attenuated *Salmonella* vaccine comprising 5 attenuated *Salmonella* serovars that were attenuated using indigenous plant extracts such as garlic and onion. The results showed that both vaccines had a high protection capacity, preventing *Salmonella* infection after challenge with a wild type of *SalmonellaTyphimurium*. Hyper-immune eggs inhibited the growth of *Salmonella* spp in vitro in immunized chickens. ELISA demonstrated the specific antibody production to LPS of *S. Typhimurium.* Post-mortem studies confirmed the presence of salmonellosis in the control group but not in immunized chickens with either vaccine. This study shows that Poultry salmonellosis can be prevented by the use of prophylactic DNA or live-attenuated vaccines (LAV).

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1. INTRODUCTION

The Poultry Industry is vulnerable to a wide range of food borne illnesses such as salmonellosis which is responsible for thousands of death globally and billions of loss in revenue to the poultry industry. Salmonellae are gram-negative, non-spore-forming facultative anaerobic bacilli [1]. *Salmonella enterica* serovar Typhimurium is a member of the family Enterobacteriaceae and is a common cause of bacterial food poisoning in humans. The fimbrial appendages are found on the surface of many enteric bacteria and enable the bacteria to bind to eukaryotic cells. *S. Typhimurium* type 1 fimbriae are characterized by mannose-sensitive hemagglutination and are assembled via the chaperone/usher pathway. *S. Typhimurium* type 1 fimbrial proteins are encoded by the fim gene cluster (fimAICDHFZYW), with fimAICDHF expressed as a single transcriptional unit [2].

Salmonella is the second most common intestinal infection in the United States. More than 7,000 cases of *Salmonella* were confirmed in 2009; however the majority of cases go unreported. The Centers for Disease Control and Prevention estimates that over 1 million people in the U.S. contract *Salmonella* each year, and that an average of 20,000 hospitalizations and almost 400 deaths occur from *Salmonella* poisoning, according to a 2011 report [3].

The most common manifestation of nontyphoid salmonellosis is mild to moderate gastroenteritis, consisting of diarrhea, abdominal cramps, vomiting, and fever. Typically, symptoms of gastroenteritis develop within 6 to 72 h after ingestion of the bacteria [4]. The very young, the elderly and immuno-compromised individuals could develop complications from salmonellosis which could result in death. The Poultry Industry and its products are of particular importance to public health bodies internationally due to the size of the industry and the large volume of consumption of poultry products by the public [5].

Salmonella is one of the most important microorganisms involved in human food borne diseases throughout the world. Food borne disease non-typhoidal salmonellosis in humans is usually a self-limiting illness confined to the gastrointestinal tract, but when infection spreads beyond the intestine, or when immunocompromised persons are affected, effective antimicrobial treatment is essential [6]. However, probably as a consequence of the extensive use of antibiotics, especially in livestock production, surveillance networks have indicated that the incidence of human salmonellosis caused by antimicrobial resistant *Salmonella* is increasing in many countries [7], from that comes out the rational of producing a prophylactic vaccine to protect against salmonellosis in chickens.

Increasing antimicrobial resistance in *Salmonella* can limit therapeutic options available for clinical cases that require antimicrobial treatment. Furthermore, illness caused by resistant *Salmonella* can be more severe and difficult to control. As an example, infections caused by *S. Typhimurium* DT104 resistance to ampicillin, tetracycline, streptomycin, cloramphenicol, and sulphonamides presented higher rates of admission to hospital and increased mortality [2].

This project seeks to contribute to safe poultry products in a cost effective way and to address public health concerns by possible helping to reduce resistant *Salmonella* strains.

Curtello et al wants to demonstrate that poultry products including eggs are protected from salmonellosis by the production of a vaccine.

In this research 6-month old Layer chickens were raised for a period of four months where they were immunized and assessed for tolerance to virulent *Salmonella* strain and degree of immunization. Over six hundred egg shells were tested in addition to chicken feaces, cloacal swabbing and anatomical organs for the presence of Salmonellae.

Antimicrobial medication did not offer 100% protection against salmonellosis [8]. Hence the need to explore new possibilities such as the development of vaccines for possible immunization of chickens against salmonellosis.

2. MATERIALS AND METHODS

2.1 *Salmonella* and Life-attenuated Vaccine Preparation

Salmonella (mixture of five *Salmonella* strains: *S. Montevideo, S. Yeerongpilly, S. Augustenborg, S. Kentucky, S Typhimurium*) was incubated at 370C in peptone broth with combinations of natural extracts. The two aqueous extracts that produced the desired effect were garlic and onion. They were prepared as follow: The fresh garlic cloves and onion were washed, and sun dried for a week. After drying, both were ground separately using electric blender and diluted 1L distilled water. Each extract was used individually and in combined concentration. Five ml of the diluted form of the extract (15 mg/ml) was incorporated in differential and selective *Salmonella* plating media. A bacteriogram was carried out to check for bacteria attenuation. The immunogen contains the 5 serovars of *Salmonella* (108 CFU of vaccine strain per chicken in 1 ml of incomplete Freund's adjuvant and was given intramuscularly at chicken breasts). The same immunogen (the 5 serovars) without incomplete Freund's adjuvant was given per-orally (108 CFU per chicken).

2.2 Preparation of DNA Vaccine and Detection of Isolated *Salmonella Typhimurium* Fragments

One colony of bacteria was picked from overnight culture. The bacteria was suspended in 30 µl of polymerase chain reaction (PCR) water and boiled for approximately three and a half minutes then chilled immediately in ice. Twenty five (25) µl of boiled cells, DNA Template solution, was added to PCR eppendorph reaction tube. Twenty five (25) µl of Deoxynucleotide triphosphate (DNTP) mixture consisting of: 2'-Deoxyadenosine-5'-Triphosphate (dATP, 20 µl), 2'-Deoxycytidine-5'-Triphosphate (dCTP,20µl), Deoxyguanosine -5'-Triphosphate (dGTP, 20µl) and 2'-Deoxythymidine-5'-Triphosphate, (dTTP, 20µl).

It made up to 1,000 µl by adding 900 µl of PCR water. 0.2 µl of Primers (they were Fim 1A and Fim 2A that encodes to fimbriae of Salmonella), in addition to 14 µl of PCR water, 0.5 µl Taq DNA Polymerase, 2.5 µl of Magnesium Chloride ($MgCl_2$) and 2.5 µl DNA polymerase buffer.

Amplification was carried out with temperature settings consisting of initial denaturation (3 min at 94°C), 30 amplification cycles (1 min at 94°C, 80 sec at 52°C and 1 min at 72°C) and the final extension (10 min at 72°C). The amplification products were visualized by staining with ethidium bromide, after electrophoresis at 0.8% agarose gel, as previously reported [9].

To prepare the immunogen genomic DNA 5 ml (25 µg/µl) plus 5 ml of the purified bacterial DNA by PCR (14 µg/µl) were diluted in PCR water (5 ml) and then mixed with 1 ml cationic liposomes (Coatsome EL series, NOF Corporation) for 60 seconds and incubated for 1 hr at room temperature (RT) according to the manufacturer's instructions [10].

Primers: The 20-mer forward primer (Fim 1A), 59-CCT TTC TCC ATC GTC CTG AA-39, has a calculated temperature of 60oC and is located between bp 586 and 605 on the fim A gene of S.Typhimurium. The 20-merreverse primer (Fim 2A), 59-GGTGTT ATC TG CCTGAC-CA-39, has a calculated annealing temperature of 60oC and is located between bp 651 and 670 on the fim A gene of *S. Typhimurium* [11].

The explanation why Fim 1A and Fim 2A were amplified was because fimbriae are used by bacteria to adhere to animal cells. In addition fimbriae carry adhesins which attach them to the substratum so that the bacteria can withstand shear forces and obtain nutrients. Our hypothesis was that the production of anti-fimbriae antibodies would interfere with the pathogenesis of salmonellosis in chickens. The empty liposomes were also filled with genomic bacterial DNA that after expression would express a large number of *Salmonella* antigens including those in cell wall, LPS among others that would cause bacteria neutralization. That was our goal and that was demonstrated in the experiment of inhibition of the bacterial growth in vitro by hyper-immune egg or purified IgY.

2.3 Immunization of Layer Chicks and Laboratory Investigation

Twenty-one 6 months-old Layer hens were purchased from a poultry farm Layer nursery. The chickens were tested in the laboratory for the presence of *Salmonella* and were negative. They were divided randomly in three groups of 7 chickens each and placed in a control biosecurity chicken house with separated compartments approximately 50 meters from the laboratory.

All chicken received oral vaccine or placebo via crop gavage. Group one received 0.5 ml (90 mg/ml) of *Salmonella* DNA vaccine (whole genomic DNA and PCR products from genomic DNA administered with a commercially available cationic liposome as a vector to make a solution to be administered orally, and intramuscular. Group two received 0.25 ml of a purified live attenuated *Salmonella* mixture, McPherson bacterial concentration approximately108 colony forming unit (CFU) orally, and intramuscularly in 1 ml of incomplete Freund's adjuvant, and group three received 0.25 ml of empty liposomes in 1 ml of sterile normal saline solution (0.9%) orally and intramuscular. Immunization dose was administered day 0, day 15, day 30 and day 60. To determine efficacy chickens were orally given 1.25 ml of wild-type *Salmonella* Typhimurium strain (3x109 CFU/bird) in 3 portions crop gavage.

The chickens were sampled and observed for 4 months and no dewormer, vitamins or medication of any sort was administrated to layer hens throughout the test period. Eggs were collected, labelled and stored in the refrigerator at 4oC until further use. The animal house was washed out with cleaning reagents twice daily and fecal content removed in keeping with a sound biosecurity practice. Thirty day post immunization, eggs from the groups was analyzed in the laboratory under aseptic conditions for the presence of Salmonella. Eggs taken from the chicken house were washed with tepid warm water (approx. 350C) before laboratory analysis was performed. An optimized *Salmonella* detection method was used to isolate Salmonella. Anti-*Salmonella* antibodies from test groups were isolated from the egg yolk by the method of Polson [12]. Egg yolks were streaked on differential and selective *Salmonella* media. Where there was no growth of

Salmonella; inhibition of *Salmonella* growth in eggs was performed and the egg yolks were inoculated with a *Salmonella* mixture of 5 *Salmonella* strains isolated from around the island, including *Salmonella* Montevideo, *Salmonella Typhimurium*, *Salmonella* Yeerongpilly, *Salmonella* Augustenborg, and *Salmonella* Kentucky. They were sensitive to a large spectrum of antibiotics including ampicillin, tetracycline, streptomycin, cloramphenicol, and sulphonamides. Using a 3 mm sterile inoculating loop the inoculated egg yolk was incubated at 370C for 24hrs then streaked onto differential and selective *Salmonella* media such as triple sugar iron urea, MacConkey agar, *Salmonella* Shigella agar, bismuth sulfite agar and brilliant green agar [8]. When no typical *Salmonella* growth was observed the test was repeated to confirm consistency of *Salmonella* hyper immune egg: presence of Immunoglobulin Y (IgY) in the egg yolk. Where *Salmonella* growth was observed on the media test were similarly repeated to confirm non-*Salmonella* hyper-immune eggs that were labeled, and stored at 40C until further testing.

Egg shells were individually swabbed prior to washing and after washing the applicator sticks were placed in 1% pre-enrichment Peptone broth and incubated for 24 hour at 37^0C. After the incubatory period a 3mm loop was used to transfer broth to differential and selective media.

2.4 Isolation of *Salmonella* from Specimens

The isolation of *Salmonella* was carried out using previously described procedures. The exterior of the cloaca of the birds was first cleaned with sterilized moistened cotton balls prior to application of the moistened cotton tips of each swab applicator. The swabs and also of caeca and crops were immediately placed in sterile screw cap test tube containing 9 ml of pre-enrichment broth (buffered peptone water 1%).

At least 2.5 g of each type of specimen was dissolved in 250 ml pre-enrichment broth (buffered peptone water 1%). The inoculated pre-enrichment broth was incubated at 37^0C for 24 hours following this incubation the pre-enrichment broth was thoroughly mixed using a vortex mixer. A 1ml aliquot of buffered peptone water 1% was added to 9 ml of enrichment broth (Selenite broth, Selenite cystein broth, and Tetrathionate broth) and further incubated at 37^0C for 24 hours. After vortexing 0.15 ml and a 3 mm loopful of inoculum was used to inoculate differential plating media such as MacConkey agar, *Salmonella* Shigella agar selective media, Bismuth Sulphite and Brilliant green agar and incubated at 37^0C for 24-48 hour.

Following incubation the cultures were examined and non-lactose fermenting colonies were selected and used to inoculate Kleiger iron agar and urea agar slants. After a further 24 hours incubation period at 37ºC colonies which gave the typical *Salmonella* Shigella reaction were inoculated to the routine line of sugars and again incubated. Confirmation was followed by slide agglutination with somatic “O” and flagella “H” antigens of Salmonella. Serological typing was performed to determine the *Salmonella* serovar.

2.5 Identification by Slide Agglutination

Presumption *Salmonella* isolates were stored on tryptose agar a room temperature until confirmation as previously described (Kauffman-White Schema, Difco, Laboratory, Detroit, and Michigan U.S.A) [3]. For each isolate each of 2 loopfuls of the growth on tryptose agar

was emulsified in one drop of normal saline solution (0.9%) on a clean microscope slide. The preparation was examined for autoagglutination.

If the organism was not self-agglutinating one drop of either “H” anti-serum or “O” anti-serum was added to each spot. After mixing the slide was agitated by gently rocking back and forth for 2 to 3 minutes. The slide was examined for agglutination. (Kauffman-White Schema, Difco, Laboratory, Detroit, and Michigan U.S.A). Identification of *Salmonella* Typhimurium serovar was performed in the *Salmonella* reference laboratory, Department of Microbiology, Faculty of Medical Sciences, The University of the West Indies.

2.6 Antibiotic Susceptibility Test

All *Salmonella* isolates tested were investigated for their antibiotic resistance with the disc diffusion test using the following discs (Difco): gentamicin (10 µg), kanamycin (30 µg), ampicillin (10 µg), amikacin (30 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), chloramphenicol (30 µg),cefazolin (30 µg), cephalothin (30 µg), cefepime (30 µg), cefotaxime (30 µg), streptomycin (10 µg), ceftazidime (30 µg), cefoxitin (30 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), norfloxacin (10 µg), tetracycline (30 µg) and imipenem (10 µg).

2.7 Purification of *Salmonella*

A 1:9 *Salmonella* suspension was made in buffered peptone water and incubated overnight at 370C. One ml of pre-enrichment broth was transferred to a 1.5 ml micro-centrifuge tube and centrifuged for ten minutes at 14,000 x g, the supernatant was carefully discarded. The pellets were re-suspended in 300 µl sterile PCR grade water by vortexing. The tube was again, centrifuged at 14,000 x g for five minutes the supernatant was discarded with care. The pellets were again re-suspended in 300 µl PCR grade water by vortexing. The micro-centrifuged tube was incubated at 1000C for 15 minutes and immediately chilled on ice. The tube was centrifuged at 14,000 x g at 40C. The supernatant was transferred to a new tube and incubated at 10 min at 1000C then chilled immediately on ice. The supernatant was stored at -200C.

2.8 Immunoglobulin-Y isolation

The chicken immunoglobulin Y (IgY) fraction was isolated by the chloroform-polyethylene glycol (PEG) method [11]. The eggs were washed with warm water and the egg yolk was separated from the egg white. The membrane was broken and the egg yolk collected and diluted 1:3 in phosphate buffered saline (PBS), pH 7.4. To 1/3 of the egg yolk mixture an equal volume of chloroform was added, the mixture was then shaken and centrifuged for 30 min (1000 x g, RT). The supernatant was decanted and mixed with PEG 6000 (12%, w/v), stirred and incubated for 30 min at room temperature. The mixture was then centrifuged as previously described. The precipitate containing IgY was dissolved in PBS (pH 7.4) at a volume equivalent to 1/6 of the original volume of the egg yolk and dialyzed against 1L of PBS (pH: 7.4 for 24 h at 4ºC). The IgY was removed from the dialysis tubing. IgY samples were stored at –20ºC.

2.9 Enzyme-linked immunosorbent assay (ELISA) for studying the presence of anti-*Salmonella* antibody in layer hens

The ELISA procedure described by Smith and collaborators was used to detect anti-*Salmonella* antibodies in the different avian avian species [12]. Ninety-six well polystyrene microplates (U-shaped bottom, Sigma-Aldrich co, St. Louis USA) were incubated with (2 µg/well) of the LPS (Sigma –Aldrich Co) from S. *Typhimurium* in coating buffer (overnight at 4oC.) The microplates were washed 4 times, with (PBS-Tween-20) and blocked with 3% non-fat milk in PBS, (25 µl/well) and incubated 1 hour at (R.T). The microplates were washed 4 times, then A 50 µl aliquot of the previously isolated egg yolk (Ig)Y solutions in concentration of 1.25 mg/ml was added (triplicates). After incubating for one hour at RT the microplates were washed 4 times and 50 µl anti-IgY-HRP conjugate diluted to 1:30000 with PBS-Tween-20 was added. After a further incubation and washing step 50 µl tetrametylbenzidine (TMB) was added. The microplates were further incubated for 15 minutes in the dark and 50ul 3M HCl was added to stop the reaction. In the absence of a microplate reader the analysis was visually done: colour development was recorded as positive and no colour development was recorded as negative. Positive and negative controls were included in the test.

2.10 Challenge with a Wild-type *Salmonella Typhimurium*

After the completion of the immunization programme. On day 65 was carried out the challenge. Chickens were given a single dose (1.25 ml) of a wild -type multiresistant strain of *Salmonella* Typhimurium (resistant to some antibiotics including cloramphenicol) by oral route (3x109 cfu/bird) equivalent to 1,000 50% lethal doses (LD50s). Via crop gavage the chicken were given the 1.25 ml in 3 portions.

2.11 Statistical Analysis

Statistical analyses were conducted using the statistical package for social sciences (SPSS) software (version 18). Differences between cases and controls were tested by the student t-test. A p value<0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Preparation of DNA Vaccine

PCR to purify *Salmonella* strains DNA (before incorporating it in the vector as a part of the immunogen formulation) was carried out; this was mixed with whole genomic DNA, thought to increase the immunogenecity of the vaccine candidate. Primers were Fim 1A and Fim 2A similar to the the Fim A gene sequence (encodes the major fimbrial unit) developed by Cohen et al, 1996 [13] gave a higher specificity for detection and purification of *Salmonella* strains.

3.2 Attenuation of *Salmonella* Strain

There was a change in all the respective media appearance. Fig. 1 shows that *Salmonella* colonies were much smaller in size in some cases (the *Salmonella* colonies became smaller than tiny needle head) and showed changes in the usually colour appearance of traditional

selective and differential media. The colour changes ranges from baby pink, neon green to psychedelic yellow. The control with no extract showed typical *Salmonella* reaction on differential and selective media. Other extracts that were used did not show a 100% growth inhibition nor did they affect hydrogen sulfite production. The plate with increased garlic concentration from 15 g to 25 g in a liter of sterile diluents showed greatest results of *Salmonella* growth inhibitionas shown in Table 1.

Table 1. Size of *Salmonella* colony. (A) Normal colony size of 4 mm, (B) Plated with garlic extract, size of 1 mm, (C) Plated with onion extract, size of 1.5 mm and (D) Plated with combination 1:1 garlic-onion extract showed no colony. The colour changes ranges from baby pink, neon green to psychedelic yellow

Variables	Mean colony diameter (mm)/SD
Salmonella only	4.1 ± 0.5
Salmonella-garlic	1 ± 0.06
Salmonella-onnion	1.5 ± 0.08
Salmonella-garlic-onnion	0 ± 0

3.3 Hyperimmune Egg Inhibits the *Salmonella* Growth in Media

A total of 637 eggs from chicken vaccinated with the live attenuated vaccine (LAV) were collected and 150 samples of individual egg yolks were inoculated with a combination of *Salmonella* strains including *S. Typhimurium* at 370 C and streaked on MacConkey agar, then *Salmonella* Shigella agar, bismuth sulphide and brilliant green agar for 24 h. The result was inhibition of the *Salmonella* growth in all samples (100%) from both live-attenuated and DNA vaccinated chickens. On the other hand the control group (150 specimens) revealed inhibition of the growth of *Salmonella* colonies in 10% (15 samples) as noted in Table 2. This was statistically significant ($p<0.001$).

Table 2. Percentage inhibition of the *Salmonella* growth by anti-*Salmonella* hyperimmune egg. Both vaccines produced a hyperimmune egg able to kill or inhibit *Salmonella* growth in media in 150 out of 150 cases and only in 15 out of 150 control specimens. This was a statistically significant inhibition ($p<0.001$).

Vaccines and Control	Inhibition percentage (%)
Live-attenuated	100
DNA	100
Control	10

3.4 Anti-*Salmonella* IgY Cause Inhibition of the Bacterial Growth

When serial dilutions of the IgY (1mg/ml) from hyperimmune egg yolks of live attenuated vaccinated chickens was incubated with a loop full (3mm) inoculum of *Salmonella* strains in a microtube containing 1mg/ml of IgY in peptone water and incubated overnight at 370C after that streaked on MacConkey agar for 24 h, and then in *Salmonella* Shigella agar, bismuth sulphide and brilliant green agar for 24 h. The result was inhibition of the *Salmonella* growth by IgY in all samples (100%). Similar results were gotten with chickens vaccinated with the DNA vaccine. In the control group there was growth inhibition of *Salmonella* colonies in 8% of the samples tested as observed in Table 3.

Table 3. Percentage inhibition of the *Salmonella* growth by purified anti-*Salmonella* IgY (Polson method). All samples of IgY tested (100%) inhibited the growth of *Salmonella* in vitro. The control group inhibited the growth of *Salmonella* but only in 8%. This was a statistically significant difference between the vaccinated group and controls (p<0.001).

Vaccines and Control	Inhibition percentage (%)
Live-attenuated	100
DNA	100
Control	8

4.5 Effect of the Challenge with the Wild-type strain of *Salmonella* on Egg Contamination

After the challenge with the wild-type strain of Salmonella; eggs where tested for the presence of Salmonella. In Table 4 is shown that eggs 12 out of 100 eggs from vaccinated chickens with the live-attenuated vaccine resulted infected; 9 out of 100 eggs were infected protecting with the DNA vaccine versus 87 out of 100 eggs from the non-vaccinated group (control) resulted contaminated with the bacterium. This indicated that vaccinated hens significantly produced eggs lesser contaminated and therefore it is possible to produce a prophylactic vaccine to protect against egg salmonellosis.

Table 4. Number of egg contaminated with *Salmonella* after challenge with a wild typed *S. Typhimurium.* There was a statistically significant difference amount the groups: live-attenuated vaccine (p<0.01) and DNA vaccine (p<0.01).

Vaccines and Control	Egg contamination percentage (%)
Live-attenuated	12
DNA	9
Control	87

4.6 Post-mortem Study for Identification of *Salmonella* in Several Organs

The presence of Salmonellae was analysed post-mortem in chickens. In the control group was detected in 3 out of 7 caeca (42.8%), in 4 out of 7 stomachs (57.1%) in the stomach and in 2 out of 7 livers (33.3%). In the vaccinated group the presence of *Salmonella* was insignificantly detected in caeca, stomach and liver as shown in Table 5.

Table 5. *Salmonella* contamination percentage in organs after challenge with the wild typed *S. Typhimurium.* Both vaccinated group had a non-significant infection with Salmonella, but the control group was statistically significant infected with the organism (p<0.001; p<0.05 and p<0.01 for caeca, stomach and liver respectively).

Vaccine and Controls	Concentration of *Salmonella* in caeca	Concentration of *Salmonella* in stomachs	Concentration of *Salmonella* in livers
Live-attenuated	10^2 CFU/g	10^0 CFU/g	10^1 CFU/g
DNA	10^2 CFU/g	10^1 CFU/g	10^1 CFU/g
Control	$2x10^7$ CFU/g	10^4 CFU/g	$2x10^5$ CFU/g

The ELISA demonstrated that 3/4 duplicated samples of IgY from vaccinated chickens showed a strong immune response (antibodies) against LPS of *S. Typhimurium* and 1/4 showed a moderate immune response. On the contrary the immune response of non-vaccinated chickens was poor or weakly as recorded in Table 6.

Fig. 1 shows ELISA with a cut-off point =0.35. Each dilution represented the mean of triplicate IgY samples (each sample from different egg). Both live attenuated vaccine (LAV-1*) and DNA-liposome vaccine (DNAV-2*) had optimal OD values up to dilutions of 62.5 µl/ml of IgY. That means that with this small dosage it is able to produce in the bird an immune response above the cut-off point. These demonstrated an effective immune response to LPS of *SalmonellaTyphimurium*. The LAV-1* had effectively the highest OD values, therefore it was the most potent vaccine of the two being evaluated.

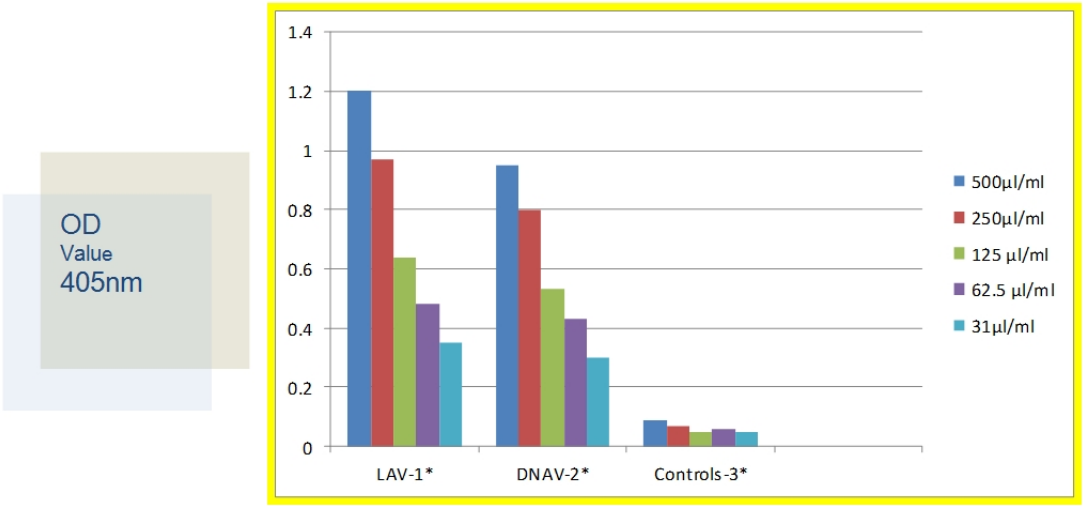

Fig. 1. Enzyme-linked immunosorbent assay (ELISA) for detection of anti-Salmonella antibodies after immnunization of groups with live-attenuated vaccine, DNA-liposome vaccine and empty liposome.

Fig. 1 shows ELISA with a cut-off point =0.35. Each dilution represented the mean of triplicate IgY samples (each sample from different egg). Both live attenuated vaccine (LAV-1*) and DNA-liposome vaccine (DNAV-2*) had optimal OD values up to dilutions of 62.5 µl/ml of IgY.

Table 6. Strength of the immune response to *S. Typhimurium* in duplicate samples of IgY from hyper-immune and non-hyperimmune egg specimens by ELISA

Strength	Vaccinated birds N=60	Non-vaccinated birds N=30
Negative or weakly	0%	100%
Moderate	12%	0%
Strong	48%	0%

6. DISCUSSION

This research showed that the onion and garlic extracts are natural antibiotics; they had the capacity to inhibit the bacterial growth in vitro. These extracts were successful used in the attenuation of 4 wild-type of *Salmonella* strain. Both garlic and onion changed all the respective media appearances, modified biochemically the mechanism of nutrition of the *Salmonella* spp. Their colonies became smaller than tiny needle head, it occurred in all selective and differential media. Hydrogen sulfite production was very much decreased in the samples. In the control group (without garlic and/or onion) the plate with increased garlic concentration from 15 grams to 25 grams in a liter of sterile diluents showed greatest results of *Salmonella* growth inhibition. We are not aware that the literature in Microbiology records the use of these indigenous plants for attenuation of pathogens to be used in the preparation of vaccines.

Anti-bacterial potentials of the extracts of two crude garlic cloves were tested against five gram negative and two gram positive multi-drug resistant bacteria isolates. All the bacterial isolates were susceptible to crude extracts of garlic. Except Enterobacter sp. and Klebsiella sp., all other isolates were susceptible when subjected to ethanolic extracts of garlic. Allicin, the active ingredient of garlic, acts by partially inhibiting DNA and protein synthesis and also totally inhibiting RNA synthesis as a primary target. Organosulfur compounds and phenolic compounds have been reported to be involved in the garlic antimicrobial activity. The antimicrobial potency of plants is believed to be due to tannins, saponins, phenolic compounds, essential oils and flavonoids [14].

Gul et al, 2012 reported that three types of extracts of garlic including aqueous extract, methanol extract and ethanol extract had been assayed separately against drug resistant Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus, Klebsiella pneumoniae, Shigella sonnei, Staphylococcus epidermidis and *Salmonella* typhi. The antibacterial activity was determined by disc diffusion method. All tested bacterial strains were most susceptible to the garlic aqueous extract [15].

The results of the inhibition of the bacterial growth by hyperimmune egg have been confirmed. A previous study by Vaillant AJ et al. [15] demonstrated that hyperimmune eggs contained large amount of specific immunoglobulins for a particular pathogen or antigen after the immunization protocol. The results confirmed previous results of the presence of anti-idiotypic antibody in the hyper-immune eggs that protect against the original organism [16].

In this study evidences that anti-*Salmonella* antibodies (IgY) are present in the hyper-immune egg of chickens immunized with live attenuated or DNA vaccine. The efficiency of liposome as a vector in vaccinology has been reported in the literature. It was recorded by Chen et al, 2009 showed that liposome-encapsulated the dense granule protein 4 (GRA4) pGRA4 enhanced the protective effect against infection of T. gondii [17].

In this current study there was a clear observation that when immunizing with the DNA encapsulated liposome vaccine, genes expression for important surface antigens were manifested or expressed and the immune system recognized them, provoking both a humoral and cellular response. This current study demonstrated humoral response to *Salmonella* only and a limitation of this research was that it could't demonstrate the cellular

response to *Salmonella* antigens but this has been reported that DNA vaccines produce both cellular and humoral immunity [18]. The ELISA was efficient in demonstrating anti-*Salmonella* immune response in vaccinated birds. It showed that in the non-vaccinated group there was a percentage of birds (17%) that were previously immunized with *S.Typhimurium*, it may be by natural infection.

Both vaccines were safe in the groups of animal immunized. There is a report of a chicken vaccinated with the DNA vaccine that got sick for 2 days but then the animal improved and showed no more problems. The post-mortem analysis was more than a clear evidence of the production of specific antibody against Salmonella. Chickens that were vaccinated with either vaccine showed higher protective level than the control group. From chicken immunized with DNA or live attenuated vaccine there was no isolation of *Salmonella* among the group. No isolate was detected from chicken immunized with the live attenuated vaccine either.

The control group naturally had more isolate and all chickens were infected with the wild type of Salmonella *Typhimuriun* because they had no protection against the microorganism. That was also shown in the experiment of the inhibition of Salmonella by hyper-immune egg or IgY, where non-immunized chicken eggs were much chicken's egg yolk samples that were contaminated with Salmonella.

Before the challenge with the wild-type strain of Salmonella, eggs where tested for the presence of Salmonella. There was statistically significant lesser egg contamination in both vaccinated groups as compared with controls ($p<0.01$). This indicates that vaccinated hens produced egg with lesser Salmonella and it is possible to produce a vaccine with the aim of protecting eggs, since eggs are of economically value and part of the nutrition of millions of people in the world. A vaccine for protection of eggs against salmonellosis was one of our aims and was fulfilled in this study.

Felgner et al. [19] found that cationic liposomes, when mixed with DNA form cationic liposome-DNA complexes with 100% entrapment of the DNA, with an overall positive charge, resulting in transfection efficiencies significantly higher than other chemical-delivery systems including anionic liposomes with encapsulated DNA, calcium-phosphate precipitation, and polycationic reagents such as DEAE-dextran or polylysine [20-21].

An observation was that chickens immunized with the DNA vaccine had egg production of 36.9% (235 eggs in 4 months) in comparison with those vaccinated with the live-attenuated vaccine that produced 637 eggs during the said period and resulted in 100% egg production. On the other hand 90% of the egg yolks among chickens from the control group lasted 6 weeks preserved at 18oC as compared to 13 and 26 weeks preservation among egg yolks from chicken vaccinated with live attenuated and DNA vaccine respectively. We did not find explanation for this finding, although we think that vaccination with either vaccine could estimulate the production of growth factors and cytokines that may act hormone-like stimulation of the laying process.

The literature of live-attenuated vaccine recently showed Escherichia coli heat-labile enterotoxin B subunit (LTB) protein used as potent mucosal adjuvant. In a study, where the effect of an attenuated *Salmonella* secreting LTB protein as an adjuvant strain (JOL1228) for a live *Salmonella* Enteritidis (SE) vaccine candidate (JOL919) was evaluated. In a single immunization experiment, chickens immunized with a mixture of JOL919 (5 parts) and JOL1228 (1 part) showed enhanced mucosal and cellular immune responses and efficient

protection against salmonellosis as compared to those unimmunized control chickens [22]. In our study we used incomplete Freund's adjuvant and it was efficacious in boosting the immune system of vaccinated animals against *Salmonella Thyphimurium* given intramuscularly.

A novel research to evaluate the usefulness of pH-sensitive fusogenic polymer (succinylated poly (glycidol) (SucPG) and 3-methylglutarylated poly (glycerol) (MGluPG)-modified liposomes as mucosal vaccine, in the induction of protective immune responses was carried out. Mice were nasally immunized with OVA-containing SucPG-modified liposomes. After immunization, significant Ag-specific Abs was detected in the serum and intestine [23]. In our study we used empty liposomes that were filled with genomic DNA and PCR-purified DNA at once and showed that immunized animals were protected against a challenge with a wild, multi-resistant strain of *Salmonella Typhimurium* proving that the delivery system (cationic liposome) was effective.

7. LIMITATION OF THE STUDY

A limitation of this research is the low number of animal used but it is a preliminary report and a larger study can be done in future. We could not find a commercially-available ELISA to measure antibodies to the products of Fim 1A and Fim 2A that encode for fimbriae neither the purified antigen (fimbria) to develop our own ELISA nor Western blotting. We accept as a limitation no using a commercially-available *Salmonella* vaccine, but we created our own, which will be evaluated in clinical trials for future licensing.

8. CONCLUSION

The preliminary results suggested that both live attenuated and DNA vaccines proved to be effective, providing protection to immunized animals. The vaccines were safe and well tolerated by the chickens, in addition these biologically products protected against egg contamination and in the challenge with the wild-type *Salmonella* Typhimurium vaccinated animal were protected. The garlic and onion can be considered as natural bacteriostatic agents that inhibited the growth of Salmonellae in vitro. In the hyperimmune egg, IgY was responsible for the *Salmonella* growth inhibition in vitro and the ELISA demonstrated moderate to strong anti-*Salmonella* immune response in vaccinated chickens, demonstrating that both live-attenuated and DNA vaccine were functional. The post-mortem studies confirmed the efficacy of both vaccines that had a high protection capacity against *Salmonella* spp. The percentage of contaminated eggs with Salmonellae was much higher in the non-vaccinated group of birds, suggesting that prophylactic vaccination of chickens against salmonellosis also protects against egg contamination.

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CONSENT

No applicable.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the ethics committee of The University of the West Indies. Mona campus. Jamaica.

COMPETING INTERESTS

The authors declared that no competing interests exist.

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