

The Use of Indigenous Plants in the Attenuation of a Live-Attenuated *Salmonella* Vaccine to Protect Against Poultry

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Abstract

The aim of this study was to develop a natural cost effective method of bacterial attenuation using indigenous plants to produce a safe live attenuated vaccine for poultry salmonellosis. Several indigenous plants were used but garlic and onion gave the expected result of *Salmonella* attenuation. Chicken were immunized with a attenuated *Salmonella* cocktail (mixture of five *Salmonella* strains: *S. Montevideo*, *S. Yeerongpilly*, *S. Augustenborg*, *S. Kentucky*, and *S. Typhimurium*). ELISA for *Salmonella* antibodies showed high titer suggesting that the indigenous plant did not affect the immunogenic capability of the vaccine. Garlic and onion can be considered as natural bacteriostatic agents that inhibited the growth of *Salmonella in vitro* contributing to a safe and effective vaccine against poultry salmonellosis.

Keywords: Garlic; Onion; Vaccine; *Salmonella*; Poultry

Introduction

Salmonella are motile, flagellated rod shaped Zoonotic pathogens which may survive with or without oxygen and do not absorb crystal violet stain. They are decolorized by alcohol due to their outer lipopolysaccharide membrane and thin peptidoglycan layer. They belong to the Enterobacteriaceae group being implicated with typhoid fever and food poisoning. This pathogen is associated with intestinal disease which may become fatal and has negatively impact on the health of individuals and various economies globally. The poultry industry is most impacted and vulnerable to the onslaught of this pernicious microbe. The Lipopolysaccharide Somatic O antigen, flagellar H and virulent V antigenic structure determines the serotype designate of *Salmonella* species [1].

The poultry industry and its products present a challenge of particular importance to public bodies internationally due to the size of the industry and the large volume of consumption of poultry products by the public [2]. The 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 lost revenue to the industry. One of the challenges the industry faces is the difficulty in eliminating infectious strains from contaminated farms.

This study incorporated *Salmonella* serotypes (*Augustenborg*, *Kentucky*, *Montevideo*, *Typhimurium* and *Yeerongpilly*) isolated from the island of Jamaica in the production of a Prophylactic Vaccine. Our aim was to develop a natural cost effective method of bacterial attenuation using indigenous plants to produce a safe live attenuated vaccine for poultry salmonellosis.

Materials and Methods

Salmonella live attenuated vaccine preparation

Salmonella cocktail (mixture of five *Salmonella* strains: *S. Montevideo*, *S. Yeerongpilly*, *S. Augustenborg*, *S. Kentucky* and *S. Typhimurium*) was incubated at 37°C in peptone broth with combinations of natural extracts. Preparation includes clean, sun dried onion and garlic cloves. After a week of drying, onion and the cloves of garlic was separately grounded using electric blender and diluted with 1 L distilled water. Each extract was used individually and in combined concentration. Five ml of the diluted 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 contained 5 serovars of *Salmonella* (10⁸ CFU of vaccine strain per chicken in 1 ml of incomplete Freund's adjuvant and given intramuscularly at chicken breast).

The immunogen (5 serovars) without incomplete Freund's adjuvant (IFA) was given orally (10⁸ CFU per chicken).

Antibiotic susceptibility test

All *Salmonella* isolates 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), ceftiofuran (30 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), norfloxacin (10 µg), tetracycline (30 µg) and imipenem (10 µg).

Separation of Immunoglobulin Y

The main immunoglobulin fraction of chicken is IgY. Eggs are important source of specific antibodies. The main isolation challenge

encountered is the high concentration of lipid interference which has to be excluded. There are a few methods to separate egg yolk plasma proteins from lipids.

Chicken immunoglobulin fraction (IgY) was isolated using chloroform-polyethylene glycol (PEG) procedure [3]. Warm tepid water was used to surface clean eggs. There was separation of the yolk from the egg white. The vitelline membrane of the egg yolk was punctured and the content collected aseptically and diluted to 1:3 ratio with phosphate buffer saline solution (PBS) at a pH of 7.4. Chloroform of equal volume was included in the mixture. The composit was shaken and centrifuged at room temperature with a gravitaional force of $1000 \times g$ for duration of 30 min. The liquid portion above the residue is poured off and thoroughly mixed with polyethylene glycol and incubated at room temperature for 30 min. The centrifugation process was repeated. IgY precipitate was dissolved in phosphate buffer saline at pH 7.4 using a volume which is equal to one sixth 1/6 of the initial volume of egg yolk. Dialysis ensued against one litre (1 L) of phosphate buffer (PBS) at 4°C for twenty four hours (24 h) at pH 7.4. The resultant IgY product was removed from the dialysed tube for storage at -20°C.

ELISA for studying the presence of anti-*Salmonella* antibodies in layer hens

ELISA was used to detect anti *Salmonella* antibodies in a variety of avian species [4]. 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 *Salmonella Typhimurium* using coating buffer ideally at 4°C plate covered with thin layer of plastic remained overnight. Removal of unbound material is achieved by washing the microplates four times with phosphate buffer saline and tween 20 detergent (PBS and Tween 20) and blot dry to remove all the liquid.

Unbound site on the surface of wells attached to plates are blocked with 25 µl per well of 3% nonfat milk with PBS buffer followed by an hour of incubation at room temperature.

Microplates were washed four times and an aliquot of 50 µl of previously isolated egg yolk IgY solution in serial dilutions of 125 µg/ml to 500 µg/ml was added in triplicates and incubated for one hour at room temperature. Washing steps were repeated, microplates washed four times with PBS buffer and tween 20 reagent plates gently tapped and blot dry by inverting on absorbent paper. 50 µl anti IgY-HRP Horseradish peroxidase conjugate was diluted to 1:30000 with PBS-Tween-20 and added for enhanced detection. Repeated incubatory and washing steps followed the addition of 50 µl tetramethylbenzidine (TMB) a chromogenic substrate. There was a repeat of the washing procedure. Microplate was incubated for an additional 15 min in the dark and 50 µl of 3M HCl added to stop the reaction. Plates were visually read for colour development as positive and no colour development as negative in addition to detection of optical density of each well with a microplate reader at an absorbance value of 450 nm. Positive and negative controls were included in the test.

Statistical analysis

Statistical analysis conducted utilised the “Software Package for Social Sciences (SPSS)”. Variation between cases and control were tested using student t-test. Probability value less than 0.05 is considered significant statistically.

Results and Discussion

There was a change in all respective media appearance. Table 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 usual colour appearance of traditional selective and differential media. The colour changes ranged from baby pink, neon green to psychedelic yellow. The control with no extract showed typical *Salmonella* reaction on differential and selective plating media. For example Dasheen extracts did not show a *Salmonella* growth inhibition. It neither affected the hydrogen sulfide production. The plates with increase garliconion concentration from 15 to 25 g in a liter of sterile diluents showed greatest *Salmonella* growth inhibition as shown in Table 1.

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

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.

Concentration (µl/ml)	Mean OD ± SD
125	0.64 ± 0.008
250	0.96 ± 0.04
500	1.20 ± 0.017

Table 2: ELISA for detection of anti-*Salmonella* antibodies;*Optical Density (OD) Readings of ELISA absorbance values at different concentrations; Cutoff point=0.35.

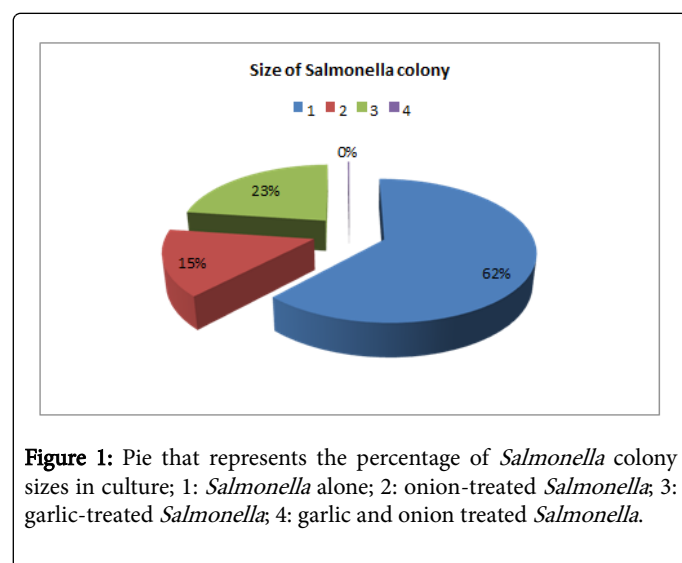


Figure 1: Pie that represents the percentage of *Salmonella* colony sizes in culture; 1: *Salmonella* alone; 2: onion-treated *Salmonella*; 3: garlic-treated *Salmonella*; 4: garlic and onion treated *Salmonella*.

Figure 1 represents the percentages of *Salmonella* colony sizes in culture, for *Salmonella* alone it was 62%, for onion-treated *Salmonella*

it was 23%, for garlic-treated *Salmonella* it was 15% and for garlic-onion treated *Salmonella* it was zero and that represents closed to 100% inhibition of the *Salmonella* growth as shown in Figure 1. So to speak there was a statistical significance ($p < 0.05$) when *Salmonella* was plated out in cultures containing indigenous plants, suggesting a mechanism of bacterial attenuation, which is useful in vaccinology.

This research showed that onion and garlic extracts are natural antibiotics with the capacity to inhibit bacterial growth *in vitro*. These extracts were successfully used in the attenuation of 4 wild-type *Salmonella* strains. Both garlic and onion changed all the respective media appearance and biochemically modify the mechanism of nutrition of the *Salmonella* spp. Hydrogen sulfide production was drastically decreased in the samples treated with indigenous plants. We are not aware that literature in Microbiology records the use of garlic and/or onions for attenuation of pathogens to be used in preparation of vaccines.

In the literature is reported that the antibacterial capabilities of extracts of two crude garlic cloves were tested against five gram negative isolates. The active ingredient of garlic: allicin, partially inhibits the synthesis, of DNA, protein and RNA as a primary target. Reports have highlighted the involvement of phenolic and organophosphate compounds in the antimicrobial activity of garlic. Plants antimicrobial efficacy is perceived to be influenced by essential oils, flavonoids, tannins, saponins and phenolic compounds [5] reported garlic extracts inclusive of aqueous, methanol and ethanol had been successfully tested against a series of resistant pathogens with the aid of disc diffusion application. One of our isolate of *S. Typhimurium* was resistant to chloramphenicol and trimethoprim/sulfamethoxazole, and the other one to ampicillin. After treatment with onion and garlic all bacterial isolate that were previously resistant to selective antibiotics became sensitive to the entire antibiotic susceptibility test. ELISA demonstrated that samples of IgY from

vaccinated chickens developed a strong immune response (antibodies) against LPS of *S. Typhimurium* as shown in Table 2. Each dilution represented the mean of triplicate IgY samples, specific for *Salmonella* spp and successfully purified by the chloroformpolyethylene glycol method (each sample from different eggs). The live attenuated vaccine had optimal OD values in all dilutions tested.

Conclusion

Garlic and onion can be considered as natural bacteriostatic agents that inhibited the growth of *Salmonella in vitro*. ELISA for anti-*Salmonella* antibodies was effective in immunodetection demonstrating that the indigenous plants did not affect the immunogenic capability of the produced live-attenuated vaccine.

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