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A Trial to Prevent Colonization of Internal Organs of Chicks by Salmonella Enterica Serovar Enteritidis Using Autogenous Bacterin

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Abstract
This study was carried out to investigate the efficacy of the locally prepared autogenous *Salmonella enterica* serovar Enteritidis bacterin in the prevention of *Salmonella enterica* serovar Enteritidis infection in broiler chicks. A total of forty (40) chicken feed samples were randomly collected and screened for the presence of *Salmonella enterica* serovar Enteritidis using pour plate technique. The isolate obtained was characterized and identified using the colonial descriptions, morphological and biochemical characteristics. The pathogenic potential of the isolate on chicks was investigated by challenging the chicks orally using 0.5 ml of the inoculum (10^8cells/ml). All chicks were kept under complete observation for 2 weeks for pathological signs and symptoms, mortalities and gross lesions of the internal organs of the chicks. The protective effect of locally prepared autogenous bacterin was investigated using *in vivo* method. The titer of antibodies produced by the vaccinated chicks was determined using micro agglutination test. Twenty-three (57.5%) samples out of 40 chicken feed samples were positive for *Salmonella enterica* serovar Enteritidis. There were significant ($P<0.05$) obvious pathological signs and gross lesions in the internal organs of the infected chicks when compared to uninfected chicks (control). The serological investigation revealed an improvement in the titer of antibodies after vaccination. The autogenous bacterin significantly ($P<0.05$) reduced the pathological features when compared to infected non protected chicks. The significant viable mean plate counts were obtained from the internal organs of the infected non-protected chicks which significantly ($P<0.05$) decreased when protected with the autogenous bacterin. The study has shown that the tested autogenous bacterin proved to be safe and effective against the isolate.

INTRODUCTION

Infections with *Salmonella enterica* occur worldwide; however, certain diseases are more prevalent in different regions. Non-typhoid salmonellosis is more common in industrialized countries whereas *enterica* fever is mostly found in developing countries (with the most cases of Asia) (Connor and Schwartz, 2005). *Salmonella* serovar Enteritidis causes salmonellosis. A person infected with *Salmonella* serovar Enteritidis bacterium usually has fever, abdominal cramps, and diarrhea beginning 12 to 72 hours after consuming a contaminated food especially eggs and undercooked chicken (Santos *et al.*, 2001). The illness usually lasts 4 to 7 days, most persons recover without antibiotic treatment. There are about 1.3 billion cases of non-typhoid salmonellosis worldwide each year and the WHO estimates that there are 17 million cases and over 500,000 deaths each year caused by typhoid fever (Chimalizeni *et al.*, 2010).

Antibiotics play a vital role in controlling the infection, examples of some good drugs include fluoroquinolones, trimethoprim-sulfamethoxazole (TMZ-SMZ), ampicillin, or third generation cephalosporin or quinolone is reasonable if susceptibilities are unknown (Burkhardt *et al.*,1997). Some side effects of antibiotics causes harm to patients, in the case of chloramphenicol and gatifloxacin they cause aplastic anemia and dysglycemia respectively. Due to the increasing incidence of antibiotic resistant and more virulent serovars, the discovery of antimicrobial agent with specific anti-*Salmonella* activities is a prevailing interest (Claesson *et al.*, 2009).

In Nigeria, the importance of controlling moulds and mycotoxins in feeds is widely known and practiced, but the control of bacteria is less well understood and frequently overlooked (Malcolm, 2004). Also indiscriminate use of antibodies and addition of growth promoters in chicken feeds contributed to the emergence of resistance among the strains of *Salmonella* species. Several studies have been carried out in order to evaluate the efficacy of antibodies, probiotics and other natural antimicrobial substances on *Salmonella* species (Malcom, 2004; Wafaa *et al.*, 2012), but *Salmonella* infections remain the primary causes of reported food poisoning worldwide and recent years (Malcolm, 2004). This study was designed to assess the efficacy of autogenous bacterin in preventing *Salmonella* serovar Enteritidis infection in broiler chicks.

MATERIALS AND METHODS

Sample Collection: A total of 40 samples of different types of poultry feeds were collected from different shops and open markets within Ihiala major market, using sterile polyethene bags, and kept in priorly disinfected cooler. The samples were brought to the laboratory in a cooler maintaining low temperature (≤4°C) using ice blocks. The collected samples were processed within six hours of its collection. Sampling was performed normally from different bags such that the product was collected from different parts of the bags. The sample was pooled and mixed properly and formed one cup of the feed sample, then 10g of the mixture was taken for analysis.

Isolation and Identification of *Salmonella enterica* serovar Enteritidis: Ten folds serial dilution was carried out on each different samples and 1.0 ml was aseptically taken from the third test tube and pour plated into the *Salmonella Shigella* Agar and incubated at 37°C for 48 h. After 48 h incubation the grown colonies were sub-cultured, characterized and identified using their colony descriptions, microscopic and biochemical characteristics.

Procurement of Chicks: A total of eighteen (18) day old chicks that are a day old of mixed sex obtained from Mrs. Eze poultry farm at Ihiala, Anambra State were used for this study. The chicks were kept in separate, thoroughly cleaned and disinfected cages and provided with feeds and water frequently.

Inoculation into the chicks: This was carried out using the method of Wafaa *et al*. (2012). Broth culture of the isolate was centrifuged at 3000 r.p.m for 10 minutes. The sediment was diluted with sterile phosphate buffer saline (PBS) and adjusted to the 10^8CFu/ml using McFarland matching Standard which is (0.6ml of 1% $BaCl_2.2H_20$ + 99.4ml of 1% concentration of H_2SO_4). Then the chicks were orally infected using 0.5 ml of the prepared inoculum.

Examination of infected chicks: The infected chicks were carefully observed for the obvious pathological signs of the challenged organism for a period of fourteen (14) days. The number of deaths was also observed. After fourteen (14) days, the infected chicks were sacrificed and gross examination of their internal organs morphologies was carried out.

Re-isolation of the organism from the infected organs: The internal organs of the infected chicks were harvested and portions were aseptically macerated in peptone water and serial diluted using ten-fold serial dilution. Samples were inoculated into *Salmonella Shigella* Agar (S.S.A) and incubated at 37°C for 24 h (Wafaa *et al.*, 2012).

Humoral activity of autogenous bacterin: A total of eighteen (18) day old chicks were used for this study. In addition, autogenous bacterin prepared from the pure culture of *Salmonella* serovar Enteritidis were also used for this study.

Preparation of autogenous bacterin: This was carried out by the modified method of Wafaa *et al.* (2012). The isolate was grown on nutrient broth at 37°C for 24 h. The culture was centrifuged at 3000 r.p.m for ten (10) minutes and the supernatant was decanted. The sediment was washed with normal saline and suspended into 1% formal saline at room temperature for 24 h. The sterile autogenous bacterin was obtained by adding equal volume of incomplete Freund's adjuvant to adjusted washed concentrate of inactivated bacterium and kept at refrigerator until when used. The autogenous bacterin was giving to the experimental chicks at first day in dose of 0.2ml/chick and boostered at a second dose at 7days in dose of 0.5ml/chick. The autogenous bacterin in the two shots was giving subcutaneously through the thigh.

Quality control tests on the prepared autogenous bacterin: The prepared autogenous bacterin was tested for purity, complete inactivation and sterility.

- Purity: this test was done before inactivation of the isolate. It was done to confirm that the broth culture of the isolate was not contaminated by other bacteria before inactivation. This was done by sub culturing the broth culture into Salmonella Shigella Agar and incubated at 37°C for 24 h. The colony was Gram stained, examined and finally confirmed using unique biochemical reactions.
- Complete inactivation test: This was carried out to ensure that the isolate was completely inactivated. Autogenous bacterin was inoculated into a *Salmonella Shigella* Agar and incubated at 37°C for 48 h. No visible growth of the isolate was seen.
- Sterility test: the prepared autogenous bacterin was confirmed to be free from any fungal contaminants by inoculating it into Sabouraud Dextrose Agar (SDA) plate and incubated at room temperature for 7 days.

Experimental design: This was carried out using the modified method of Wafaa *et al.* (2012). The chicks were grouped into two (3) groups which include group A, B and C. Each group contained six chicks each. The treatments to the group were as follows: Group A were intramuscularly administered autogenous bacterin; the 0.2 ml/chick for the first dose and boostered on the 7th day with 0.5ml/chick then challenged with 0.5ml of test organism after 14 days. Group B were infected with 0.5ml of test organism without protection. Group C were given only distilled water. The experimental chicks were carefully monitored for a period of 14 days for any obvious pathological signs.

Detection of the humoral immune response: Just before the first dose of the autogenous bacterin (zero hour),

the chicks were randomly selected and their blood were collected. Also just before the second booster dose, another blood sample was also collected on 14th day. The blood samples were allowed to separate. The separated sera were used against the isolate for agglutination reaction using micro agglutination titre techniques. The serum collected from the chicks was serial diluted using two-fold serial dilution. Then 0.1 µL of the diluted serum ($^1/_{20}$, $^1/_{40}$, $^1/_{80}$, $^1/_{160}$, $^1/_{320}$, $^1/_{640}$) was deposited on the wells of the micro titer and aseptically mixed within 1.0µL of the test isolate. This was incubated at 37℃ for 90 minutes. The agglutination result and titre value was recorded. This was repeated after 7 days (Before booster dose) and 14 days (Before challenge) (Wafaa *et al.*, 2012).

Examination of protected chicks: The protected chicks were carefully observed for the clinical manifestation of the inoculated organism for period of 2 weeks, the protection rates of the inhibitory substances were determined, and the chicks were sacrificed and gross examination of the morphologies of internal organs and intestine were carried out. Also the internal organs were harvested and some portions of these organs were cultured on *Salmonella Shigella* Agar, and incubated at 37°C for 48 h. The counts were taken and the colonies were identified morphologically and biochemically.

Statistical analysis: The data generated from this study were represented as mean ±Standard deviation and then charts. The test for significance at 95% confidence interval was carried out using student't' test (Iheukwumere and Umedum, 2013).

RESULTS

The presence of the isolate in the chicken feed samples is shown in Table 1. Out of 40(100%) chicken feed samples collected from the different retailers at Ihiala major market in Ihiala Local Government Area of Anambra State, 23(57.5%) samples were positive to *Salmonella enterica* serovar Enteritidis. *Salmonella enterica* serovar Enteritidis was characterized and identified using morphology, colony description and biochemical reaction (Table 2).

The micro agglutination antibody titres generated from the sera of broiler chicks after vaccination with locally prepared autogenous bacterin is shown in Table 3. On the first day (before first vaccination dose), the antibody titre values (ATVs) of sera samples collected from the test and control chicks was zero. On the 7th day (before booster vaccination dose), four-sixth (4/6) of the chicks vaccinated with the autogenous bacterin had maximum ATVs 1/160 whereas 1/6 and 2/6 of the remaining vaccinated chicks recorded 1/80 and 1/320titre values respectively. On the 14th day (before challenge), two-sixth (2/6) of the vaccinated chicks had maximum ATV 1/640 whereas 2/3 and 2/6 of the remaining vaccinated chicks recorded 1/160 and 1/320 respectively. There was no ATV recorded from non-vaccinated chicks after 14 days.

The obvious pathological signs of challenged isolate in broiler chicks administered autogenousbacterin are shown in Table 4 and 5. The chicks infected with the test organism without protection recorded series of obvious pathological signs of the test organism, which was significantly ($P \leq 0.05$) reduced in those chicks administered autogenous bacterin. No obvious pathological sign was recorded among the control (non-infected). The total mean viable plate counts of challenged isolate from the internal organs of chicks administered autogenous bacterin is shown in Table 6. The count was most in the lungs and least in the heart. The counts significantly ($P<0.05$) reduced among the protected chicks.

Table 1: **Presence of the isolate in chicken feed samples**

Type of feed	Positive (%)	Negative (%)	Total (%)
A	8(80)	2(20)	10(25)
B	6(60)	4(40)	10(25)
C	4(40)	6(60)	10(25)
D	5(50)	5(50)	10(25)
Total	23(57.5)	17(42.5)	40(100)

Table 2: **Characteristic and Identify of *Salmonella* serovarEnteritidis**

Parameter	*S.* serovarEnteritidis
Appearance on the media plate	Colourless with black centers
Elevation	Slightly raised
Edge	Smooth
Gram reaction	–
Morphology	Straight rods
Motility	Motile
Catalase test	+
H_2S production test	–
Indole test	–
Methyl red test	+
V.p test	–
Citrate test	+
Oxidase test	+
Galactose	+
Lactose	+
Xylitol	+/–
Mannitol	+
Inositol	+/–
Sorbitol	+
Maltose test	+
Dulcitol test	–

H_2S - Hydrogen Sulphide
V.p - Vogesproskaeur

Table 3: Micro-agglutination antibody titres in the sera of the broiler chicks protected with autogenous bacterin.

Isolate	Day	Interval	Total	Antibody titres of the chicks serum at different dilutions						
				0	20	40	80	160	320	640
S.E	0	BFVD	6	6	0	0	0	0	0	0
	7	BBVD	6	0	0	0	1	1	4	0
	14	BC	6	0	0	0	0	2	2	2
Control	0	BFVD	6	6	0	0	0	0	0	0
	7	BBVD	6	6	0	0	0	0	0	0
	14	BC	6	6	0	0	0	0	0	0

BFVD – Before First Vaccination Dose
BBVD – Before Booster Vaccination Dose
BC – Before Challenge
S.G– *Salmonella enterica* serovar Enteritidis

Table 4: Obvious pathological signs of challenge isolate in broiler chicks administered autogenous bacterin

	N= 6		
Pathological sign	**V**	**C_1**	**C_2**
Diarrhoea	1	5	0
Respiratory distress	1	6	0
Weakness	1	6	0
Anorexia	0	5	0
Dysentery	0	4	0
Alopecia	0	2	0
Death	0	4	0

N - Total number of chicks
V - Bacterin vaccination
C_1 - Infected chicks without protection
C_2 - Normal chicks

Table 5: Morphological characteristics of the visceral organs of protected chicks infected with *Salmonella enterica* serovar Enteritidis

Morphological characteristic	N= 6 V	C_1	C_2
Perihepatitis	1	5	0
Pericarditis	0	4	0
Air sacculitis	0	6	0
Haemorrhage	0	4	0
Congestion	2	6	0
Splenomegaly	0	3	0
Enterocolitis	0	6	0

N - Total number of chicks
V - Bacterin vaccinated chicks
C_1 - Infected chicks without protection
C_2 - Normal chicks

Table 6: **Total mean viable plate counts of challenge isolate from the internal organs of chicks administered autogenous bacterin**

Protection	Liver (Cfu/g)	Spleen (Cfu/g)
V	4.00 ± 1.00	7.00 ± 1.22
C1	43.00 ± 2.00	51.00 ± 2.24
C2	0.00 ± 0.00	0.00 ± 0.00

V – Bacterin Vaccinated Chicks
C1 – Infected Chicks without Protection
C2 – Normal Chicks

Table 7: **Protection rates of autogenousbacterin against *Salmonella* serovar Enteritidis**

Protection	N	D	M (%)	S	P (%)
V	6	0	0	6	100
C1	6	4	66.67	2	0^d
C2	6	0	0	6	100^a

V – Bacterin Vaccinated Chicks, C1 – Infected Chicks without Protection
C2 – Normal Chicks, N – Total Number of Chicks, D – Number of Deaths
M – Mortality Rate, S – Number of Chicks that Survived, P – Protection Rate
100^a – No Protection, 0d – Control Positive

DISCUSSION

The presence of *Salmonella enterica* serovar Enteritidis in the chicken feed samples could be traced from the feed ingredients, transportation of feeds, poor handling of the feed samples. Similar findings were reported by many researchers (Zang-Barber *et al*., 1999; Mouahid, 2001; Malcolm, 2004).

Reasonable antibody titre values recorded after the 14^{th} day corroborated with the reports of other researchers (Bubu *et al*., 2004; Wafaa *et al*., 2012) that there is enhancement of immune response against *Salmonella enterica* serovar Enteritidis infected chicks through vaccination using locally prepared autogenous bacterin.

The significant reduction in the obvious pathological signs and symptoms among the protected chicks, and absence of growth observed in the internal organs administered autogenous bacterin supports the findings of Wafaa *et al.* (2012). Several researchers have documented that the frequency of enteric bacteria re-isolation from the internal organs was significantly reduced in protected chickens (Timms *et al*., 1990; Gast *et al*., 1993; Pakpinya *et al*., 2008). Penha *et al.* (2009) found that vaccination of chickens with bacterin induced significant reduction in colonization of internal organs of chicks after re-infection. The maximum protection achieved by vaccinating the chicks could be due to the activated and boosted humoral and cellular components of immune response (Wafaa *et al.,* 2012).

CONCLUSION

From this study, it can be concluded that locally prepared autogenous *Salmonella enterica* serovar Enteritidis bacterin are effective and also safe for prevention of *Salmonella enterica* serovar Enteritidis infection in chicken

farms.

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