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Humoral Activity of Autogenous Bacterin Against Colonization of Internal Organs of Broiler Chicks by Salmonella enterica serovar Gallinarum

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Abstract
This study was carried out to investigate the humoral activity of locally prepared autogenous bacterin against *Salmonella enterica* serovar Gallinarum isolated from chicken feeds in broiler chicks. A total of forty (40) representative chicken feed samples were randomly collected and screened for the presence of *Salmonella enterica* serovar Gallinarum using pour plate technique. The isolate obtained was characterized and identified using the colonial descriptions, morphological and biochemical characteristics. The pathogenic potentials of the isolate on the broiler chicks were investigated by challenging the chicks orally using 0.5ml of the inoculum (10^8cells/ml). The protective effect of locally prepared autogenous bacterin from *Salmonella enterica* serovar Gallinarum was investigated using *in vivo* method. The titer of antibodies produced by the vaccinated chicks was determined using micro-agglutination test. Out of 40 representative chicken feed samples, 21 (52.5%) were positive for *Salmonella enterica* serovar Gallinarum. There were significant obvious pathological signs and lesions in the internal organs of the infected non-protected chicks, which decreased significantly ($p<0.05$) when protected with autogenous bacterin. 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 autogenous bacterin. The serological investigation revealed an improvement in the titer of antibodies after vaccination treatment. The *in vivo* activity showed that the locally prepared autogenous bacterin was effective in reducing the pathological changes observed from infected non-protected chicks. Thus this study showed that a dose of locally prepared autogenous bacterin is effective and safe method of preventing *Salmonella enteric* serovar Gallinarum infection in broiler chicks.

INTRODUCTION

The species are *Salmonella enterica* found in warm blooded animals and *Salmonella bongori* that is restricted to cold blooded animals particularly reptiles (Truscott and Friars, 2002). *Salmonella enteric* serovar Gallinarum causes fowl typhoid. They can cause mortality in birds of any age. Broiler parents and brown-shell egg layers are especially susceptible. Chickens are most commonly affected but it also infects turkeys, game birds, guinea fowls, sparrows, parrots, canaries and bullfinches. Infections still occur worldwide in non-commercial poultry but are rare in most commercial systems now (Pang *et al.,* 2011). Morbidity is 10-100%; mortality is increased in stressed or immunocompromised flocks and may be up to 100%. The route of infection is oral or via the naval/yolk. Transmission may be transovarian or horizontal by faecal-oral contamination, egg eating even in adults (Thompson *et al.* 2008).

The fact that *Salmonella* species are found in all areas of animal production means that total elimination is almost impossible and the main objective should be minimization. Many researchers have studied how to control the infection of *Salmonella enteric* serovar Gallinarum but there is still paucity of information on how to handle this menacing situation. Hence this work was designed to asertain the humoral activity of autogenous bacterin against *Salmonella enterica* serovar Gallinarum that has been isolated from chicken feed.

MATERIALS AND METHODS

Sample Collection: A total of 40 representative 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 ($\leq 4^0$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 to form one cup of the feed sample, then 10 g of the mixture was taken for analysis.

Isolation and Identification of *Salmonella enterica* serovar Gallinarum: Ten folds serial dilution was carried out on each different samples and 1.0ml 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 of mixed sex obtained from Mr. 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 0.5 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 organism challenged 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 using the methods of Wafaa *et al.* (2012).

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^{o}C for 24 h using the methods of 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 enterica* serovar Gallinarum were also used for this study.

Preparation of autogenous bacterin: This was carried out by the method of Wafaa *et al.*(2012). The isolate was grown on nutrient broth at 37^{oC} 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 Ferund'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 wasnot contaminated by other bacteria before inactivation. This was done by subculturing the broth culture into Salmonella Shigella Agar and incubated at 37^{o}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^{o}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 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 Awere intramuscularly administered autogenous bacterin; 0.2 ml/chick for the first dose and boostered on the 7th day with 0.5ml/chick and then challenged with 0.5ml of test organism after 14 days. Group B were Infected with 0.5 ml of test organism without protection. Group C were water giving only distilled water. The experimental chicks were carefully monitored for a period of 2 weeks 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 0.1µL of the diluted serum ($^1/_{20}$, $1/_{40}$, 1/80, $^1/_{160}$, $^1/_{320}$ and $^1/_{640}$) was deposited on the wells of the micro filter and aseptically mixed with 0.1µL of the test isolate. This was incubated at 37^{0}C for 90 minutes. The agglutination results and titer value was recorded. This was repeated after 7 days (before booster dose) and 14 days (before challenge) and this is in accordance with the methods of Wafaa *et al.* (2012).

Examination of Protected Chicks: The protected chicks were carefully observed for the obvious pathological signs of the administered test 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^{o}C for 48 h. The counts were taken and the colonies were identified morphologically and biochemically (Wafaa *et al.,* 2012).

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 farm retailers at Ihiala major market in Ihiala local Government Area of Anambra State, 21(52.5%) samples were positive to *Salmonella enterica* serovar Gallinarum. *Salmonella enterica* serovar Gallinarum was characterized and identified using its morphology, colony description and biochemical reactions (Table2). 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 seventh day (before booster dose), one-sixth ($^{1}/_{6}$) of the chicks vaccinated with the autogenous bacterin had maximum ATVs $^{1}/_{320}$ whereas $^{3}/_{6}$ and $^{2}/_{6}$ of the remaining vaccinated chicks recorded $^{1}/_{80}$ and $^{1}/_{160}$titre values respectively. On the 14th day (before challenge), two-sixth ($^{2}/_{6}$) of the vaccinated chicks had maximum ATV $^{1}/_{640}$ whereas $^{1}/_{3}$ and $^{3}/_{6}$ 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 autogenous bacterin 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 ($P<0.05$) reduced in those chicks administered autogenous bacterin. No obvious pathological sign was recorded among the control (non-infected chicks). The total mean viable plate counts of challenge 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 count significantly ($P<0.05$) reduced among the protected chicks.

Table 1: Presence of the isolate in chicken feed samples

Types of feed	Positive (%)	Negative (%)	Total (%)
A	4 (40)	6 (60)	10 (25)
B	3 (30)	7 (70)	10 (25)
C	6 (60)	4 (40)	10 (25)
D	8 (80)	2 (20)	10 (25)
Total	21 (52.5)	19 (47.5)	40 (100)

Table 2: Characteristics and identity of *Salmonella enterica* serovar Gallinarum

Parameter	*Salmonella enterica* serovar Gallinarum
Appearance on the media plate	Colourless with black center
Elevation	Slightly raised
Edge	Smooth
Gram reaction	Gram -
Morphology	Straight rods
Motility test	Non 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	+
Dulcitol	─/+
Maltose	+

H_2S ─ Hydrogen sulphide, VP ─ Voges proskauer

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.G	0	BFVD	6	6	0	0	0	0	0	0
	7	BBVD	6	0	0	0	3	2	1	0
	14	BC	6	0	0	0	0	1	3	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 Gallinarum

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	4	0
Respiratory distress	0	0	0
Weakness	1	4	0
Anorexia	0	5	0
Dysentery	0	3	0
Alopecia	0	0	0
Death	0	2	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 Gallinarum

	N= 6		
Morphological characteristic	**V**	**C_1**	**C_2**
perihepatitis	0	4	0
Pericarditis	0	0	0
Air sacculitis	0	0	0
Haemorrhage	0	4	0
Congestion	1	4	0
Splenomegaly	0	3	0
Enterocolitis	0	2	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	**Spleen (cfu/g)**	**Liver (cfu/g)**
V	9.00 ± 1.00	5. 00 ± 1.10
C_1	47.00 ± 2.2.24	39.00 ± 2.00
C2	0.00 ± 0.00	0.00 ± 0.00

V - Bacterin vaccinated chicks, C_1 - Infected chicks without protection
C_2 - Normal chicks

Table 7: Protection rates of autogenous bacterin against *Salmonella enterica*serovar Gallinarum

Protection	N	D	M (%)	S	P (%)
V	6	0	0	6	100
C_1	6	2	33.33	4	O^d
C_2	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 Death,
M - Mortality rate, S - Number that survived, P - Protection Rate, 100a - No Protection
O^d - Control Positive

DISCUSSION

The presence of *Salmonella enterica* serovar Gallinarum in the chicken feed samples could be linked to the dust, transportation of the feeds, poor handling and sanitary conditions attributed to the feed samples. Researchers like Jones and Richardson, (2004), Mitchell and McChesney, (2001), Franco, (2005), Maciorowski *et al.* (2007) and Humphrey, (2004) reported similar findings.

Reasonable antibody titre values recorded after the 14th day corroborated with the reports of other researchers (Garland, 2004; Carrique-Mas *et al.* 2007; Davies and Hinton, 2000) that there is enhancement of immune response against *Salmonella enterica* serovar Gallinarum infected chicks through vaccination using locally prepared autogenous bacterin.

The 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 (Khan *et al.,* 2003; Okamura *et al.,* 2005; Radwan *et al.,* 2007). Penha *et al.* (2009) and Priyantha (2009) found that vaccination of chickens with bacterin induced significant reduction of organ colonization after re-infection of the chickens.

The maximum protection achieved by vaccinating the chickens could be attributed to the ability of the bacterin activated and boosted the humoral and cellular components of immune response (Wafaa *et al.,* 2012). Christensen *et al.* (2002) suggested that some of these effects were mediated by cytokines secreted by immune cells stimulated with vaccination. On the other hand, vaccination has beneficial effects for chicks, particularly during the first days of life.

CONCLUSION

This study has shown the presence of *Salmonellaenterica*serovar Gallinarum in the chicken feed samples collected from different shops in Ihiala commercial market in Ihiala Local Government area of Anambra State. The locally prepared autogenous bacterin proved to be very effective in reducing *Salmonella enterica* serovar Gallinarum in broiler chicks.

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