Journal of Biology, Agriculture and Healthcare
ISSN 2224-3208 (Paper) ISSN 2225-093X (Online)
Vol. 7, No. 14, 2017

Humoral Activities of Autogenous Bacterin against Colonization of Internal Organs of Broiler Chicks by Salmonella Enterica Serovar Pullorum

Chinwe C. Ejike [*] Ikechukwu, Harmony. Iheukwumere.[2] Regina E. Amadi[1.3]
1. Department of Medical Microbiology, Chukwuemeka Odumegwu Ojukwu University
2. Department of Microbiology, Faculty of Natural Sciences, Chukwuemeka Odumegwu Ojukwu University, Anambra State, Nigeria
3. Federal Polytechnic, Nekede, Owerri, Imo State, Nigeria

Abstract
This study was carried out to evaluate the protective effects of autogenous bacterin against *Salmonella enterica* serovar Pullorum isolated from different poultry feeds in Ihiala local Government Area in Anambra state. Different types of feeds were cultured and screened for the presence of *Salmonella enterica* serovar Pullorum using pour plate method. The colonies generated from the primary isolation were sub-cultured, characterized and identified using their colony description, morphological and biochemical characteristics. The pathogenic potentials of the organism on broiler chicks were investigated by challenging the chick orally using 0.5ml of the inoculum (10^8cells/ml). The infected chicks were kept under observation 14 days for pathological signs, mortalities, and gross lesions. The protective effect of autogenous bacterin was investigated using *in vivo* method. The study revealed that *Salmonella enterica* serovar Pullorum was significantly ($P \geq 0.05$) seen more in feed type A 4(40%) compared to feed B 1(10%), feed C 2(20%) and feed D 3(30%). There was pathological lesion like perihepatitis, pericarditis, haemorrhage, congestion, spleenomegaly and entercolitis. 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 enterica* serovar Pullorum infection in broiler chicks.
Keywords: *Salmonella*, Pullorum, autogenous, bacterin

INTRODUCTION

Salmonella is a Gram negative, facultative anaerobe, non-spore forming bacteria from Enterobacteriaeae family that causes one of the most common enteric (intestinal) infections: salmonellosis (Jones, 2011). The two species of Salmonella are *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is further divided into six subspecies which are *arizone, diarizone, enterica, houtenae, indica, salamae* and each of them include over 2500 serovars (Su and Chiu, 2007) . In the 19th centuary, the causative e agent of typhoid was identified, which eventually became known as salmonella (Downes *et al*. 2001). Salmon and smith first isolated *bacillus cholerasuis*, now called *salmonella enterica* (*S. enterica*) subspecies *enteric* serovar Cholerasuis, from swine diagnosed with hog cholera. While smith was the first to actually identify the organism, Salmon was credited the discovery which came to bear his name. In any case, today the number of known strains of the bacteria totals over two thousand (Behravesh, 2008).

Salmonella enterica subspecies are found worldwide in all warm-blooded animals and in the environment. *Salmonella bongori* is restricted to cold blooded animals particularly reptiles (Behravesh, 2008). *Salmonella* grows best at a temperature of 6 – 46 ^{0}C(43 – 115 ^{0}F) ,optimum temperature of 37 ^{0} C, optimum pH of 6.5 – 7.5.Most subspecies of salmonella produce hydrogen sulfide which can readily be detected by growing them on media containing ferrous sulfate (Isrealsen et al.,2000). *Salmonella* serovar Pullorum *is* a rod shaped, gram negative, non-motile bacterium which causes infections in warm blooded animals. Although chickens are natural hosts of *Salmonella* serovar Pullorum, other birds can also become infected. Pullorum disease can be introduced into a flock by wild birds, mammals, and flies. The route of infection is oral or via the naval/yoke. Within a flock, infection is spread by bird- to-bird contact, as well as through cannibalism of infected carcasses, wound contamination, and fecal contamination of feed, water, and latter (Goldrick, 2003). Affected chicks and poultry huddle near the heat source and are weak, with poor appetites and stunted growth. Infected chicks have chalky, white droppings and affected chicks frequently have characteristic white "pasted" vents. Signs of the disease may not appear for the first five to ten days. Most deaths occur in the second or third week of life. It is best to depopulate a flock that tests positive for *Salmonella* serovar Pullorum *(*Barrow and Freita,2011) Antibiotics plays a very important roles in controlling and treatment of *salmonella* infections, In situation in which antibiotics are needed, trimethoprim/sulfamethaxazole, ampicillin or amoxicillin are the best choices (Arora and Arora 2008). Ceftriaxone, cefotaxime, or flouroquinolones are effective option for antimicrobial resistant strains.

Cephalosporin is recommended for animals at increased risk of invasive disease. But misuse and frequent use of antibiotics have lead to *Salmonella* drug resistant. Autogenous bacterin is also useful in protecting the animals from the infections, it is a killed bacterial vaccine created from the disease causing organism. This vaccine has served as a means of protecting the immune system of the animals against specific infections. It reduces the rate of diseases and death in animals. In areas where there is lack of antibiotics or vaccines, may result in high rate of diseases and mass deaths. Many reasearchers have studied different ways of humoral activities of autogenous *Salmonella* bacterin against internal colonization of bird such as Barrow (2007) stated that "Therefore the main form of controlling the presence of *Salmonella sp*. in poultry production is related to biosecurity measures and vaccination, associated with the right use of antibiotics, prebiotics and probiotics". Van-Immerseel *et al.* (2005) stated "because the level of protection offered by live vaccine strains depend on the administration route". 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 (Maciorowskiet *et al*.2007). Though hygiene program with the use of a long acting chemical treatment on the poultry feed is the only way to minimize spread of the infections. This work has been designed to check the humoral activities of bacterin against *Salmonella* serovar Pullorum that has been isolated from the chicken feed.

MATERIALS AND METHODS

Study Area: The study was conducted in Ihiala major market, Ihiala in Ihiala L.G.A., Anambra State which is located at Latitude 5.85°N and Longitude 6.86°E in Southeast geopolitical zone of Nigeria. Ihiala Community shares common borders with Uli town in Anambra state, Egbu at Imo State, and Oguisha in Imo State. Within the location of the market, the major anthropological activities are trading and domestic works.

Sample Collection: The containers used for sample collection were washed with detergent and water, and was thoroughly rinsed with water and sterilized with 70% ethanol. The containers were inverted on a swabbed bench, allowing the tiny droplets in the containers to dry up, and were aseptically closed. A total of 40 representative samples of different types of poultry feeds were collected from different shops and open market within Ihiala major market using sterile container and kept in priory 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 collected 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 Pullorum: 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 oldchicks 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).

Protection of Chick: This was carried out using the modified method of wafaa *et al*. (2013). Autogenous bacterin was used for this study.

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 Pullorum were also used for this study.

Preparation of autogenousbacterin

This was carried out by the method of Iheukwumere*et al.*(2017). The isolate was grown on nutrient broth at 37^{o}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 wasnot contaminated by other bacteria before inactivation. This was done by sub-culturing 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 A were 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 Iheukwumere *et al.* (2017).

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 Iheukwumere *et al.* (2017).

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 *et al.,* 2017).

RESULTS

The presence of the isolate in the chicken feed samples is shown in Table 1.Out of 40(100%) chicken feeds sample collected from the different farm retailers at Ihiala major market in Ihiala Local Government Area of Anambra State, 10(25)% samples were positive to *Salmonella enterica* serover Pullorum. *Salmonella enterica* serovar Pullorum was characterized and identified using its morphology, colony description and biochemical reactions (Table 2) and tested positive to catalase test, oxidase test, mannitol test, lactose test, sorbitol test and maltose test, it tested negative to hydrogen sulphide, indole test, citrate test, oxidase test, voges proskauer test, it also tested positive negative to inositol and xylitol. 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 the 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 ($p < 0.05$) reduced in those chicks administered autogenous bacterin. No obvious pathological signs were 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 shown in Table 6. The counts were more in the spleen to and less in the liver. The counts 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	1 (10)	9 (70)	10 (25)
C	2 (20)	8 (80)	10 (25)
D	3 (30)	7 (70)	10 (25)
Total	10 (25)	40 (75)	40 (100)

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

Parameter	*Salmonella enterica* serovar Pullorum
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 ─ Vogesproskauer

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 Pullorum.

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

Pathological sign	N= 6 V	C_1	C_2
Diarrhoea	1	3	0
Respiratory distress	0	6	0
Weakness	0	4	0
Anorexia	0	0	0
Dysentery	0	3	0
Alopecia	0	0	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 Pullorum

Morphological characteristic	N= 6 V	C_1	C_2
Perihepatitis	0	5	0
Pericarditis	0	4	0
Air sacculitis	0	0	0
Haemorrhage	0	3	0
Congestion	1	6	0
Splenomegaly	0	4	0
Enterocolitis	0	3	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	7.00 ± 1.22	9. 00 ± 1.10
C_1	43.00 ± 1.14	51.00 ± 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 Pullorum

Protection	N	D	M (%)	S	P (%)
V	6	0	0	6	100
C_1	6	4	66.67	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

This present study revealed that poultry feed samples collected from different farm houses located within the Ihiala major market in Ihiala local Government Area of Anambra State were positive to *Salmonella enterica* serovar Pullorum. Poultry feed samples can potentially become contaminated with *Salmonella enterica* serovar Pullorum either during harvesting, processing at the feed mill or during storage, environmental contamination as well as activities of insects, microbes and rodents. Many researchers (Maciorowski *et al*., 2007; Behravesh,2008; Iheukwumere *et al*., 2017) made the same recovery.Foods like improperly prepared egg, meat or milk infected

animals, egg or foods contaminated by the feces of infected animals are sources of salmonella and this causes infection in humans and animals. *Salmonella enterica* serovar Pullorum are consumed by animals and lives in the intestinal tracts of humans and animals and causes infection. However, outside humans and animals *Salmonella* is widely found in nature. The main reason is that Salmonella is able to survive, but not multiply, for long periods on materials that contain very low level of moisture. Consequently, Salmonella may enter anywhere in the animal production chain. Therefore, good biosecutity measures are crucial in a Salmonella control program and this is supported by findings made by many researchers (Jones, 2011; Li *et al*.,2011). The obvious pathological features such as diarrhea, respiratory distress, weakness and gross leision such as perihepatitis, congestion, hemorrhage, pericarditis, spleromegaly, enterocolitis and death seen in the infected chicks collaborated with the findings of many researchers (Maciorowski et al., 2007; Wafaa *et al*., 2012; Iheukwumere et al., 2017). The maximum protection conferred to the protected mice by the autogenous bacterin could be attributed to the ability of the autogenous bacterin to activate and boost the humoral and cellular components of the immune system. Similar conclusion was drawn by different researchers (Clinfton-Hadley et al., 2002; Wafaa *et al.,* 2012; Iheukwumere et al., 2017). The absence of growth observed in the internal organs administered autogenous bacterin supports the findings of Iheukwumere *et al.* (2017).

CONCLUSION

This study has shown the presence of *Salmonella enterica* serovar Pullorum in the chicken feed samples collected from different shops in Ihiala commercial market in Ihiala local government area of Anambra state. The *in vivo* study has shown that autogenous bacterin showed prolonged activity against *Salmonella enterica* serovar Pullorum. Therefore, the use of bacterin alongside with bio-security measures and good management practices to eradicate *Salmonella enterica* serovar Pullorum in chicken feeds should be taken into consideration both in small and large scale firms.

REFERENCEES

Arora, D.andArora, B. (2008). Text book of Microbiology, 4th edition, CBS Publishers and Distributors Private Limited, New Dehli, India.p 40–49.

Barrow,P.A. and Freitas, N.O.C.(2011) Pullorum Diseases And Fowl Thyphoid.New Thoughts On Old Diseases: *A Review Avian Pathology* 40(1):1-3.

Barrow,P.A.(2007).*Salmonella* infections: Immune and non- immune protection with vaccines. *Avian pathology* 36(1):1-13.

Behravesh,C.B.(2008).”Salmonellosis”,in control of communicable diseases manual,19th Ed. American public health association,p.535-540.

Clifton-hadley,F.A.,Breslin,M.,Venables,L.A.,Springing,K.A., Cooles,S.W.,Hougton, S. and Woodward, M.J.(2002). A laboratory study of an inactivated bivalent iron restricted *Salmonellaenteric* serovars Enteritidis and Typhimurum challenges in chickens. V*etinary Microbiology* 89:167-179.

Downes, F.P., Ito, K., Andrews, W.H, Flowers, R.S.,Siliiker, J.,Bailey,J.S.(2001). Compendium of methods for the biological examination of foods. Fourth Edition.Washinton,D.C: American Public Health Association.P.3567-3580.

Goldrick, B. (2003). Foodborne Diseases: More efforts needed to meet the Healthy People 2010 objectives. *The American Journal of Nursing*103 (3): 105–106.

Iheukwumere, I.H., Ejike, C.E. and Okeke, C.E. (2017). A trial to prevent sorbitol negative *Escherichiacoli* infections in chicks using autogenous bacitracin and probiotics. *JournalofNaturalSciencesResearch* 7(4):56–63

Isrealsen,M.,Hansen,I.D. and Jacobsen, E.(2000). Don’t grow *Salmonella* in the pellet cooler. *Feed international*17:34-38.

Jones,F.T.(2011). A review of practical salmonella control measures in animal feed.*Journal Applied Poultry Resources* 20:102–113.

Li,X.,Bethume,L.A.,Jia,Y.,Lovell,R.A.,Proescholdt,T.A.,Benz,S.A.,Kaplan,G. and Mcchesney, D.G.(2011).Surveillance of *Salmonella* prevalence in animal feeds and characterization of the salmonella isolates by serotyping and antimicrobial susceptibility: *Foodborne Pathology And Disease* 9:692-698.

Maciorowski, K., Jones, F., Pillai, S. and Ricke, S.(2007).Incidence, sources, and control of foodborne *Salmonellaspp*. in poultry feeds. *World’s Poultry of Science Journal* 60:446–457.

VanImeersal,F.,Methner,U.,Rychlick,I.,Nagy,B.,Velge,P.,Martin,G.,Foster,N.,Ducatelle,R.andBarrow,P.A(2005) .Vaccination and early protection against non -host specific salmonella serotypes in poultry;exploitation of innate immunity and microbial activity epidemiology and infection, 133:959-970.

Wafaa A. Abd El-Ghany., Soumaya, S.A., El-Shafii, M.E.,Hatem and Rehab, E. Dawood.(2012). A trial to prevent *Salmonella* Enteritidis infection in broiler chickens using autogenousbacterin compared with probiotic preparation.*Journal of Agriculture Science* 4:91 – 108.