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A Trial to Prevent Sorbitol Negative Escherichia coli Infections in Chicks using Autogenous Bacterin and Probiotics

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Abstract
This study was carried out to investigate the efficacy of locally prepared autogenous bacterin from sorbitol negative (sor$^-$) *Escherichia coli* isolated from chicken feeds as well as commercially prepared probiotic in the prevention of sor$^-$*E. coli* infection in broiler chicks. A total of forty (40) representative chicken feed samples were randomly collected and screened for the presence of sor$^-$*E. coli* using pour plate technique. The isolate obtained was characterized and identified using its morphological and biochemical characterization. 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 effects of locally prepared autogenous bacterin (B), commercially prepared probiotic (P) and autogenous bacterin plus commercially prepared probiotic (BP) were investigated using *in vivo* method. Out of 40 representative chicken feed samples, 23(57.5%) were positive for sor$^-$ *E. coli.* 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 P, B and PB. The higher 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 P, B and PB. Moreover, the serological investigation revealed an improvement in the titer of antibodies after vaccination and probiotic treatment. The tested antimicrobial agents have proved to be safe and effective against the isolate, of which BP showed the most pronounced activity.

INTRODUCTION

The serotype of *E. coli* responsible for the numerous reports of chicken infections as well as contaminated foods and beverages are those that produce shiga toxin, so called because the toxin is virtually identical to that produced by *Shigella dysenteriae* type 1(Griffin, 2012). The best known and also most notorious *E. coli* bacteria that produce shiga toxin are *E. coli* O157:H7 that causes approximately 100,000 illness, 3000 hospitalizations and 90 deaths annually in the United States. What makes this *E.coli* remarkably dangerous is its very low infectious dose and how relatively difficult it is to kill these bacteria. *E. coli* O157:H7 evolved from enteropathogenic *E. coli* serotype O55:H7 a cause of non-bloody diarrhoea, through the sequential acquisition of phage-encoded Stx2, a large virulence plasmid and additional chromosomal mutations. *E. coli* O157:H7 is a relentlessly evolving organism, constantly mutating and acquiring new characteristics, including virulence factors that make the emergence of more dangerous variants a constant threat.*E. coli* especially the O157:H7 serotype is a major threat to chicken house as well as chicks. Large numbers of *E. coli* are maintained in the chicken house environment through fecal contamination. Initial exposure to *E. coli* O157:H7 may occur in the hatchery from infected or contaminated eggs. Although most *E. coli* isolated from colibacillosis are well equipped with virulence factors that distinguish them from fecal commensal strains, systemic infections often involves predisposing environmental factors or infectious causes.

Colibacillosis is a widespread disease of chickens and turkeys, caused by a small number of pathogenic strains of *Escherichia coli* and resulting in large economic losses (Gross, 2009). It is apparently necessary to introduce interventive measures to address the root problem of *E .coli* endemics in chicken farm. The disease is treated with expensive antibiotics or chemotherapeutic agents, often resulting in the subsequent development of resistant strains that prevent continued use of a formerly effective treatment. In addition, the treatment can cause undesirable side-effects in the flock. These considerations suggest that control of colibacillosis by vaccination could be of great value. Deb and Harry (2006) found that injecting three-week-old chicks with inactivated *E. coli* 078:K80 in water or oil emulsion protected the chicken against challenged homologous pathogenic bacteria at 6 weeks of age. Panigrahy *et al.* (2009) used an oil-emulsified, inactivated *E. coli* vaccine to protect chicks against challenge with the homologous bacteria.

Probiotics is live cultures of microorganisms administered orally, acted beneficially on host health through inhibiting pathogens, enhancing intestinal immunity, and having a protective effect on the gut micro flora and improve the performance and the immunity of broiler chickens (Heavener and Huisint-Veid, 2006).

Researchers from various science related fields have studied different ways of controlling *E. coli* infections. Centre for Disease Control (2003) reported that antimicrobial therapy has been shown to reduce the

duration and severity of the symptoms of colibacillosis. Also, Huff et al. (2002) also carried out series of experiment to control as well as treat *E. coli* infections using bacteriophage aerosol spray, and from the result of the research, the extent of treatment conferred on the patient is directly proportional to the clinical side effect done to the host. There are number of publications that suggest a link to the use of antibiotics in animal production to an increase in human infections with antibiotic-resistant bacteria (Bogaard *et al.,* 2001). It appears that regardless of the actual contribution that antibiotic use in animal production has on the incidence of antibiotic-resistant infections in humans, serious consideration is being given to establishing regulations that would severely restrict the use of antibiotics in animal production. Colibacillosis continues to be a serious problem in chicken production, causing mortality and condemnations (Piercy *et al*., 2009). If antibiotic use is restricted in chicken production, it would be anticipated that colibacillosis would become an even greater problem. Therefore, there is a real need to find alternatives to antibiotic use for the prevention and treatment of colibacillosis in poultry production. Therefore, this study has been designed to explore an alternative for the prevention of colibacillosis in chicks using autogenous bacterin and probiotics.

MATERIALS AND METHODS

Sample Collection: Samples of different types of chicken feeds such as starters, growers, layers and finishers were randomly collected from both retailers and consumers of these chicken feeds.A totalof 40 samples were aseptically collected and immediately transferred into a sterile bag and transported to the laboratory in the Department of Microbiology, Faculty of Natural Science, COOU. At the laboratory, the samples were placed in a cooler containing ice to maintain the temperature and preserve the microorganisms present in the samples. These samples were subjected to various bacteriological and biochemical examinations in the laboratory.

Isolation of *Escherichia coli*: This was carried out by aseptically inoculating 1.0 ml of the sample on MacConkey agar (MAC), using pour plate method and incubated at 37°C for18-24hrs. After 24hr incubation, the grown colonies were sub-cultured, characterized and identified using their colony descriptions, morphological and biochemical characteristics (Arora and Arora, 2008).

Characterization and Identification of the Isolates: The isolates were sub-cultured on nutrient agar (Biotech), incubated invertedly at 37˚C for 24 h. The isolates were characterized and identified using their colonial and morphological descriptions Arotupin *et al.,* 2007), and biochemical reactions (Arotupin *et al.,* 2007; Uwaezuoke and Ogbulie, 2008) The colonial description was carried out to determine the appearances of the isolates on agar media plates, their sizes, edges, consistencies and optical properties of the isolates.

Preparation of test organism: The isolate was first cultured on Nutrient Agar (NA) and incubated at 37ºC for 24h.The organism was sub cultured in a Nutrient Broth (NB) and incubated at 37ºC for 24h. After incubation, the culture was centrifuged (X) at 3000 rpm for 5 minutes and the supernant was decanted. The sediment was washed twice with the aid of a phosphate buffer saline and resuspended in normal saline (0.85% Nacl). The turbidity of the suspended cells was adjusted to match the turbidity standard of 0.5 McFarland preparation which was prepared by mixing 0.6ml of 1% Bacl2.2H20 and 99,4ml of 1% concentrated H_2SO_4.The turbidity was standardized using spectrophotometer at 660nm, which was equivalent to approximately10^8 cells/ml.

Preparation of autogenous bacterin: This was carried out by the modified method of Wafaa *et al.*,(2012). The isolate was grown on nutrient agar at 37^{oC} for 24 h. Growth was harvested in normal saline and inactivated with 1% formal saline at room temperature for 24 h. Using Macfarland matching tube, washed concentrate of inactivated bacterium was suspended in normal saline to contain 10^8 Cfu/ml. 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/mouse and boostered at a second dose at 7days in dose of 0.4ml/mouse. The autogenous bacterin in the two shots was giving intramuscularly (IM) in the thigh muscles.

Quality control tests on the prepared autogenous bacterin: The prepared bacterin was tested for purity, complete inactivation, sterility and safety according to the Standard International Protocols as described by the British Veterinary Codes (Wafaa *et al*., 2012).

- 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 MacConkeyagar and incubated at 37^oc for 24 h. The colony was Gramstained, examined and finally confirmed using unique biochemical reactions.
- Complete inactivation test: This was carried out to ensure that the isolate was completely inactivated. The MacConkey agar was inoculated with the autogenous bacterin, incubated at 37^oC for 24 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.
- Safety test: Two chicks were aseptically inoculated subcutaneously with a large dose of the prepared autogenous bacterin (ten-fold of the normal dose). The chicks were observed daily for seven (7) successive

days for any signs of local reactions, clinical signs or deaths.

Probiotics used for the study: The probiotics used in this work was commercially prepared containing *Lactobacillus acidophilus* and *Streptococcus thermophilus* plus potassium, vitamin A, E and K, riboflavin and thiamine. The product was manufactured by Bomac Vets Company, U.S.A. The probiotics was giving in the drinking water in a dose of 5g/L of the drinking water for period of 14 days.

Procurement of Chicks: The poultry chicks used in this research work or study were bred in the Animal farm of the Department of Physiology, Chukwuemeka Odumegwu Ojukwu University, Uli, Anambra state. The chicks were housed in disinfected metal cages and adequately fed prior to infection.

Experimental design: The Chicks were grouped into three (3) groups which include group A, B and C Each group contained total of six chick. The treatments to the group were as follows: Group A: Blank Control (only distilled water) was given. Group B: Autogenous bacterin, 0.2 ml/mouse for the first dose and boostered on the 7^{th} day with 0.5ml/chick. Group C: Infected without treatment, 0.5 ml/chick. The experimental chicks were then exposed to the isolate via oral route after 7 days. The chicks were carefully monitored for a period of 2 weeks.

Detection of the humoral immune response: Just before the administration of the first dose of the autogenous bacterin (zero hour), the chicks were randomly selected and their blood were collected. Also just before the administration of the booster dose, another blood sample was also collected on 14^{th} day. The blood samples were allowed to separate. The separated sera were used against the isolate for agglutination reaction and the antibody titer against the isolate was determined and recorded (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 MacConkey 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). The remaining portions of the organs were subjected to histopathological examination.

Statistical Analysis: The data generated from this study were represented as mean ±Standard deviation and then charts. The statistical analysis of data generated from protective study was carried out using chi-square at 95% confidence limit (Wafaa *et al.,* 2012). The statistical analysis of other valuable data generated from this study was examined using SPSS package program version 20.0. Data were analysed by one-way Analysis of Variance (ANOVA) to determine the significant difference of the mean values at 95% confidence limit using (Wafaa *et al.,* 2012).

RESULTS

Out of 40(100%) chicken feed samples collected from the different farm houses located at Ihiala commercial market in Ihiala local government area of Anambra state, 23(57.5) samples were positive to sorbitol negative (sor^-) *Escherichia coli* (Table1). *Escherichia coli* was characterized and identified using its morphology, colony description and biochemical reactions (Table2).

The values of micro agglutination antibody titres in the sera of broiler chicks after vaccination with locally prepared autogenous bacterin is shown in Table 3. On the zero day (before first vaccination dose), the antibody titer values (ATVs) of sera samples collected from the test and control was zero. On the 7^{th} day (before booster vaccination dose), one-sixth (1/6), four-sixth (4/6), one-sixth (1/6) of the chicks vaccinated against Sor^- *E. coli* had their maximum ATVs as $^1/_{20,}\ ^1/_{40,\ 1/80}$ respectively. There was no ATV recorded for non-vaccinated chicks on the 7^{th} day. On the 14^{th} day (before challenge), two-sixth (2/6), three-sixth (3/6), one-sixth (1/6) of the chicks vaccinated against Sor^- *E. coli* had their maximum ATVs as $^1/_{80,}\ ^1/_{160}$, and $^1/_{320}$ respectively. There was no ATV recorded from non-vaccinated chicks on the 14^{th} day. Also the ATVs against Sor^- *E. coli* in the presence of probiotics were slightly higher than ATVs obtained in the presence of bacterin alone.

The obvious pathological signs of the challenge Sor^- *E. coli* isolated on the infected, protected and non-infected nor protected chickens are shown in Table 4. Respiratory distress, weakness, diarrhoea was common among the various chickens infected by Sor-*E. coli*whichsignificantly ($P<0.05$) decreased among the chickens protected with bacterin and probiotics. There were cases of death among the infected non-protected chicks which also decreased significantly ($P<0.05$) in the chicks protected with the antimicrobial substances but no record of any pathological signs and symptoms on the control chicks were made. The gross lesion in the internal organ of the infected chickens are shown in Table 5. Air sacculitis, pericarditis and lung haemorrhage were most seen in those chickens infected by Sor-*E. coli*, and they were significantly ($P<0.05$) decreased in chicks protected with the various antimicrobial agents. The non-infected non- protected chicks had no visible damage on their visceral organs.

The total mean viable plate counts of challenge isolate from the internal organs of chickens administered different antimicrobial substances are shown in Table 6. The study revealed that there was little or no visible growth observed in the internal organs of those chickens administered bacterin (B), Probiotics (P),

Bacterin vaccination plus commercially prepared probiotic (BP). The study also revealed that the total mean viable plate counts (TMPCs) from the internal organs administered (BP) were significantly ($P<0.05$) lower than the TMPCs from the internal organs of those chicks administered B, and P. There was no visible growth observed in the organs from non-infected (normal) chicks. The inhibitory substances showed more protection to the internal organs of the chicks against Sorbitol negative *E. coli*. The protection rate of bacterin (B) probiotics (P), bacterin and probiotic (BP) against the isolate are shown in Table 7. The protection rate conferred by (BP) was highest protection compared to B and P.

Table 1: Presence of sorbitol negative (sor$^-$) *Escherichia coli* in chicken feed samples

Chicken feed sample	Position sample (%)	Negative sample (%)	Total sample (%)
X	8(80)	2(20)	10(25)
Y	5(50)	5(50)	10(25)
Z	4(40)	6(60)	10(25)
M	6(60)	4(40)	10(25)
Total	23(575)	17(42.25)	40(100)

Table 2: Characteristic and identity of sorbitol negative (sor-) *Escherichia coli* (0157:H7)

Parameter	*Escherichia coli*
Appearance on MacConkey agar	Pink colonies
Elevation	Raised
Edge (mm)	Complete
Gram Reaction	–
Morphology	Rod shaped (Oblong)
Motility	+
Catalase	+
H_2s test	–
Indole	+
Citrate	+
VP test	–
MR test	+
Oxidase	–
Lactose	+
Galactose	+
Inositol	+
Xylitol	–
Mannitol	+
Dulcitol	+
Sorbitol	–
Maltose	+

VP – Voges proskaur, MR – Methyl red
H_2s – Hydrogen sulphide

Table 3: Micro agglutination antibody titres in the sera of the broiler chicks after vaccination with locally prepared autogenous bacterin against Sor$^-$*Escherichia coli*

Isolate	Day	Interval	Total	Antibody titres of the chick's serum at different dilutions						
				0	20	40	80	160	320	640
	0	BFVD	6	6	0	0	0	0	0	0
EC	7	BBVD	6	0	1	4	1	0	0	0
	14	BC	6	0	0	0	2	3	1	0
EC+P	0	BFVD	6	0	0	0	0	0	0	0
	7	BBVD	6	0	0	2	4	0	0	0
	14	BC	6	0	0	0	0	2	3	1
C	0	BFVD	6	0	0	0	0	0	0	0
	7	BBVD	6	0	0	0	0	0	0	0
	14	BC	6	0	0	0	0	0	0	0

BFVD - Before first vaccination Dose, BBVD- Before Booster vaccination Dose
BC - Before challenge, EC - *Escherichia coli*, P - Probiotic

Table 4: Obvious pathological signs and symptoms of challenge isolate on broiler chicks administered autogenous bacterin and probiotics

	N= 6				
Pathological sign	**P**	**V**	**P + V**	**C_1**	**C_2**
Diarrhoea	2	2	0	3	0
Respiratory distress	3	1	1	6	0
Weakness	2	1	1	6	0
Anorexia	1	0	0	3	0
Dysentery	2	0	0	3	0
Alopecia	0	0	0	2	0
Death	2	1	0	4	0

N - Total number of chicks, P – Probiotics, V - Vaccination
C_1 - Infected chicks without protection, C_2 - Normal chicks

Table 5: Morphological characteristics of the visceral organs of protected chicks infected with sor ⁻ *Escherichia coli*

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

N - Total number of chicks, P – Probiotics, V - Vaccination
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 with bacterin and probiotics

Protection	**Lungs (cfu/g)**	**Heart (cfu/g)**	**Liver (cfu/g)**
P	17.00± 1.63	15.00 ± 1.29	21.00 ± 1.41
V	7.00 ± 1.29	4. 00 ± 1.00	7. 00 ± 1.22
P+V	2.00 ± 1.00	0.00 ±0.00	3. 00 ± 1.00
C_1	53.00 ± 2.00	64.00 ± 2.00	61.00 ± 2.24
C2	0.00 ± 0.00	0.00 ±0.00	0.00 ± 0.00

P – Probiotics, V - Vaccination , C_1 - Infected chicks without protection
C_2 - Normal chicks

Table 7: Protection rates of bacterin and probiotics against the isolate

Protection	**N**	**D**	**M(%)**	**S**	**P (%)**
P	6	2	33.33	4	50
V	6	1	16.67	5	75
P + V	6	0	0	6	100
C_1	6	4	66.67	2	O^d
C_2	6	0	0	6	100^a

P – Probiotics, V – Vaccination, C1 - Infected chick without protection, C2 - Normal chick
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 sor- *E. coli* in the chicken feed samples could be linked to the management practices of the farm houses, dust, feed ingredients, as well as transportation of the feeds, poor handling and sanitary conditions attributed to the feed samples. Similar discoveries were reported by many researchers (Immersed *et al.,* 2002; Jones and Richardson, 2004; Alshawabkeh, 2006; Maciorowski *et al.,* 2007).

The maximum titre value attained by sor$^-$ *E. coli* bacterin was $^1/_{320}$ respectively while there was no antibody titre value recorded from non-vaccinated chicks after the 14th day. Similar reports was given by other researchers that there is enhancement of immune response against sor- *E. coli* infected chicks through vaccination using locally prepared autogenous bacterin (Davies and Breslin, 2004; Okamura *et al.,* 2004).

The *in vivo* study was carried out to determine the protection rate of locally prepared autogenous bacterin (B), commercially prepared Probiotics (P) and locally prepared autogenous bacterin plus commercially prepared probiotics (BP). The absence of growth observed in the internal organs administered BP supports the findings of Wafaa *et al.* (2012). Several other researchers have documented that the frequency of sor- *E. coli* re-isolated from the internal organs was significantly reduced in protected chickens (Khan *et al.,* 2003; Radwan, 2007). The significant decrease in TMPCs showed by the internal organs from those chickens administered BP corrorated to the findings of other researchers (Barbour *et al.,* 2003; Wafaa *et al.,* 2012). The competitive exclusion mechanism exhibited by probiotics against the pathogen sor$^-$ *E. coli* was comprehensively studied by several researchers. From their results, it was reported that probiotics maintained or increased the normal intestinal flora which are normally found in the intestinal tract of hatched chicken and these flora can exclude *E. coli* colonization (Mead, 2000; Wafaa *et al.,* 2012). The absence of visible growth of *E. coli* observed in non-infected (normal) day-old chicks supports the finding of Magdelena *et al.* (2011), who reported that during the first 3 days of life, chicken was protected from incoming antigens by increased expression of β-defensins (gallinacins 1,2,4 and 6), which made the chicks germ-free.

The maximum protection achieved by vaccinating those chickens fed with diet supplemented with commercially prepared probiotics could be attributed to the synergistic effects of the two substances. The bacterin activated and boosted the humoral and cellular components of immune response (Wafaa *et al.,* 2012) whereas the probiotics produced lactic acid that created unfavourable P^H for the growth of the *E. coli* pathogens (Alkoms *et al.,* 2000; Johansen *et al.,* 2004). The probiotics also compete with the pathogens (Wafaa *et al.,* 2012) and produced bacteriocin that was toxic to the enteric bacteria (Pascual *et al.,* 2009). The positive effect of feeding diet containing probiotic on the immune response indicates the enhancement of the formulating bacteria on an acquired immune response exerted by T and B lymphocytes. The direct effect might be related to the stimulation of lymphatic tissue, whereas the indirect effect may occur via changing the microbial population of the lumen of gastrointestinal tract or through the reduction of *E. coli* pathogen colonization. Shoeib *et al.* (2007) reported that the bursa of probiotic treated chickens showed an increase in the number of follicles with high plasma cell reaction in the medulla. Christensen *et al.* (2002) suggested that some of these effects were mediated by cytokines secreted by immune cells stimulated with vaccination and probiotic bacteria. On the other hand, vaccinating chickens fed with diet supplemented with probiotics has beneficial effects for chicks, particularly during the first days of life.

CONCLUSION

This study has shown the presence of sorbitol negative (sor-) *Escherichia coli* (*E. coli*) in the chicken feed samples collected from different farm houses in Amorka within the Ihiala commercial market in Ihiala Local Government Area of Anambra State. The *in vivo* study of the susceptibility pattern of this organism to the used antimicrobial agents, showed safe and pronounced activities with locally prepared autogenous bacterin (B) and commercially prepared probiotics (P), but locally prepared autogenous bacterin plus commercially prepared probiotics (BP) proved to be more effective.

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