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Molecular Characterization of Enteroxigenicity Profiles of Enteric Bacteria Isolated from Chicken Feeds

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Abstract
This study focused on molecular characterization of enterotoxigenicity profiles of enteric bacteria isolated from different brands of commercially produced chicken feeds sold in Anambra State. A total of 1,536 different chicken feed samples (starter, growers, finisher and layers) were collected from the consumers, retailers and wholesalers and screened for the presence of enteric bacteria using pour plate technique. The isolates were characterized and identified using their colony descriptions, biochemical and molecular characteristics. Presence of enterotoxins among the isolates was detected using Polymerase Chain Reaction (PCR) amplification of Ltx1 and Ltx2 genes. The result of this study revealed that *Escherichia coli* O157:H7 SS52 (EC), *Salmonella* serovar Typhimurium U288 (ST), *Escherichia coli* SEC470 (ES), *Salmonella* serovar Enteritidis YU39 (SY) and *Salmonella* serovar Enteritidis FM366 (SE) were isolated from the feed samples. The PCR results showed significant ($P<0.05$) amplification of heat labile-1 (Ltx1) and heat labile-2(Ltx2) enterotoxin genes from EC, SE and ST of which EC showed most pronounced results. Thus, this study has shown that EC, ST, ES, EY and SE were detected from the studied chicken feed samples. The PCR results have revealed regions that contained heat labile enterotoxins, of which this region was most significant in EC.
Keywords: Enterotoxigenicity, Enteric bacteria, Chicken Feeds, Polymerase Chain Reaction.

INTRODUCTION

Chicken diseases have contributed significantly to increase in mortality rate and economic losses in the chicken industry (Oguttu *et al.,* 2008). Globally, food borne infection and intoxications have been estimated that one billion cases of acute diarrhea occurs annually in children under the age of 5years in African, Asia and Latin America, approximately 5 million of these cases were proved fatal (Cummings *et al*., 2010). Poultry has been widely acknowledged to be a reservoir for enteric bacteria. Egg contents may be contaminated with enteric bacteria by trans-ovarian (vertical transmission) which occur when enteric bacteria are introduced from infected reproductive tissues to eggs prior to shell formation. Ready to eat chicken feed samples also serve as reservoir for enteric bacteria. They are contaminated through horizontal transmission or cross contamination by unhygienic handling, storage and preparation of this chicken feed samples thus when ingested they cause food intoxication.

Several incidence of enteric bacteria majorly enterotoxin producing strains has been reported which were traced back to contaminated chicken feeds (Brenner *et al*., 2010). Several studies focused on the study of the pathogenicity of enteric bacteria isolated from other sources (Todd, 2010; Asiton *et al*., 2012; Prince and Berry, 2012) but there is still paucity of information on the enterotoxigenicity of different strains of enteric bacteria which remains among the primary causes of reported gastroenteritis and food borne intoxication in the developing countries majorly in Ihiala Local Government Area, Anambra State, Nigeria. Thus, this study focused on the molecular characterization of enterotoxigenicity profiles of enteric bacteria isolated from chicken feeds.

MATERIALS AND METHODS

Study Area: Anambra State is a State in South-eastern Nigeria that has interstate boundaries with Delta State to the West, Imo State and Rivers State to the South, Enugu State to the East and Kogi State to the North. The State covers an area of 4,816.2 square kilometers and lies at Latitudes 6˚20' and 45.68'' North; and Longitudes 7˚04' and 19.16''east. It has a population of 4,177,828 (2006 census figure) with a population density of 860 per square kilometer. The temperature of the State ranges from 29˚C to 36.˚C with temperature range of 33.˚C. There are many human industrial activities within the State. The samples were collected randomly from Anam, Omor, Ogbunike, Onitsha, Ochanja, Ogidi, Nkpor, Ozubulu, Atani, Ihiala, Umudim, Azigbo, Igbukwu, Ufuma, Aguluzoigbo, Amikwo, Ndiokpalaeze, Nimo, Abagana, Mbaukwu and Otuocha.

Collection of Samples: A total of 1536 commercially produced chicken feed samples (starter, growesr, finisher and layers) were aseptically collected from the wholesalers, retailers and consumers. The feed types which included X (756 samples), Y (756 samples) and Z (756 samples) were aseptically collected from twenty-one (21) major towns located within Anambra State. One cup of the feed sample was aseptically collected from each feed

type by randomly collecting one Table spoon of the feed sample from each bag containing the feed type. The feed samples were mixed and homogenized to generate a representative sample for each feed type. The feed samples were collected from Broiler starter (128 samples), Grower mash (128 samples), Broiler finisher (128 samples) and Layer mash (128 samples) for each feed type (X, Y and Z) using aluminum foil. The samples were carefully labeled and classified based on the sources of collection. The feed samples were transported in cooler containing ice block for laboratory for analysis.

Culture and Isolation of Enteric Bacteria: This was carried out using the modified method of Cheesbrough (2000). One gram (1.0g) of each sample was dissolved in 5.0 ml of sterile distilled water, then make up the volume to 10.0 ml prior to serial dilution. One milliliter aliquot was aseptically transferred into a sterile test tube containing 9.0 ml of the diluent (distilled water) and from this; ten-fold serial dilutions were made up to 10^{-3}. One milliliter of the sample was plated on *Salmonella-Shigella* agar (SSA/Biotech) for *Salmonella* and *Shigella* species and MacConkey agar (MA/Biotech) for *E. coli*. All the plates in triplicates were incubated inverted at 44.5˚C for 24 h for *E. coli* and 37˚C for 24 h for other enteric bacteria.

Characterization and Identification of the Isolates: The isolates were subcultured on nutrient agar (Biotech), incubated invertedly at 37˚C for 24 h. The isolates were characterized and identified using their colonial and morphological descriptions (Cheesbrough, 2000).), biochemical reactions (Cheesbrough, 2000) and molecular characterization (Habtamu *et al.,* 2011; Gabriela *et al.,* 2014).

Extraction and purification of DNA: All strains were plated on Nutrient Agar (Biotech) and incubated at 37°C for 24 h. Bacterial genomic DNA was then extracted and purified using Zymo Research (ZR) DNA miniprepTM kit (Category No. D6005; Irvine, California, USA) (Lee *et al.,* 2009)

Determination of the quality of extracted DNA: This was carried out using mass spectrophotometer (Nanodrop). One micro litre (1µL) was aseptically dropped into a clean aperture in the chamber and the chamber was gently closed. The system was then connected to a computer system which displaced the window that revealed the quality of the sample at 260/280nm (Mohammed *et al.,* 2011)

Primers design: The primers used were uspA-F and uspA-R which targeted uspA; S139F and S141R for invA gene; flis-F and Typ04-R for fliC gene; A058-F and A01-R for sefA gene and gatDp5 and gatDp6 for gatD gene (Chen and Griffiths, 1998; Oliveira *et al.,* 2003; Lee *et al.,* 2009). The invA, fliC, sefA and gatD genes targeted *Salmonella* species. The invA encodes invasion proteins specific to *Salmonella* species, fliC encodes *Salmonella* serovar Typhimurium flagellin, gatD encodes galactitol-1-phosphate dehydrogenase for galactitol (dulcitol) metabolism which is specific to *Salmonella* species and sefA encodes fimbrial proteins in *S.*serovar Enteritidis. The uspA encodes universal stress proteins used to differentiate *E. coli* from other Gram negative bacteria. These primers were supplied by Zymo Research Corp, Irvine, California, USA.

Amplification of DNA and gel electrophoresis of PCR product: This was carried out using Master cycler Nexus Gradient (Eppendorf). A mixture of primer (20 µl), template DNA (20µl), water (72 µl) and master mix (108 µl), which comprises taq polymerase, DimethylSulfoxide (DMSO), magnesium chloride ($MgCl_2$) and nucleotides triphosphates (NdTPs), was prepared in 1.5 ml tube and homogenized using vortex mixer (Eppendorf). This was then placed in the block chamber of the master cycler and then programmed. The PCR program for invA, fliC and sefA genes conditions were as follows: initial incubation at 94°C for 5 mins, followed by 35 cycles of denaturantion at 94°C for 15 secs, annealing at 55°C for 15 secs, elongation at 72°C for 21 secs and final extension period for 10 mins at 72°C. The PCR program for uspA gene conditions were as follows: initial incubation at 94°C for 10 mins, followed by 40 cycles of denaturation at 94°C for 30 secs, annealing at 74°C for 15 secs, elongation at 72°C for 30 secs and final extension period for 20 mins at 72°C. The PCR program for gatD gene conditions were as follows: initial incubation at 95°C for 10 mins, followed by 40 cycles of denaturation at 95°C for 60 secs, annealing at 60°C for 60 secs, elongation at 72°C for 60 secs and final extension period for 10 mins. The amplified products were electrophoresed in 1.0% agarose gel and a1kb DNA ladder was used as a size reference. After staining with 3µl of nucleic acid stain (GR green), the gel was documented with gel documentation apparatus (Mohammed *et al.,* 2011).

DNA sequencing of 16s rRNA fragment: The 16s rRNA amplified PCR products generated from universal primer(16s), was used for the sequencing using ABI DNA sequencer (Applied Biosystem Inc) at International Institute of Tropical Agriculture (IITA), Ibadan using the method of Mohammed *et al.* (2011).

Computational analysis: This was carried out using the modified method of Mohammed *et al.* (2011). The chromatograms generated from the sequences were cleaned to obtain regions with normal sequences. The cleaned nucleotides were aligned using pair wise alignment tool. The consensus sequences formed by the alignment of the forward and reverse sequences were used to perform the Basic Local Alignment Search Tool (BLAST) using National Centre for Biotechnology Information BLAST over the internet. The sequences of the isolates with 95% and above similarities were accepted. Also the maximum scores, total scores and accession numbers of the isolates were assessed. The relatedness of the isolates was determined by tracing their phylogenetic tree using DNA distance neighbor phylogenetic tree tool.

Molecular Detection of Enterotoxins

This was carried out using Polymerase Chain Reaction (PCR) methods as described by Lorena *et al.* (2014). The primers used were: Lp30: CAGTTAATGTCGTGGCGAAGG and Lp31: CACCAGACAATGTTACCGCTG for enterotoxin-1 (Ltx1A) gene and Lp43: ATCCTATTCCCGGGAGTTTACG and Lp44: GCGTCATCGTATACACAGGAGC for enterotoxin-2 (Ltx2A) gene. The amplification was done in Master Cycler Nexus Gradient (Eppendorf). A mixture of primer (20µl), template DNA (20µl), water (72µl) and master mix (108µl), which comprises taq polymerase, Dimethyl Sulfoxide (DMSO), magnesium chloride ($MgCl_2$) and nucleotides triphosphates (NdTPs), was prepared in 1.5 ml tube and homogenized using vortex mixer (Eppendorf). This was then placed in the block chamber of the master cycler and then programmed. The PCR program for Ltx1 and Ltx2 genes conditions were as follows: initial incubation at 94°C for 3 mins, followed by 40 cycles of denaturation at 94°C for 30 secs, annealing at 53°C for 60 secs, elongation at 72°C for 30 secs and final extension period for 10 mins at 72°C. The amplified products were electrophoresed in 1.0% agarose gel and a 1kb DNA ladder was used as a size reference. After staining with 3µl of nucleic acid stain (GR green), the gel was documented with gel documentation apparatus.

Statistical Analysis: The results of the data generated were expressed as mean ± standard deviation (SD). The statistical analysis of data generated from protective study was carried out using chi-square at 95% confidence limit (Wafaa *et al.,* 2012). The data generated from this study were examined using SPSS package program version 20.0. Data were analyzed by one-way Analysis of Variance (ANOVA) to determine the significant difference of the mean values at 95% confidence limit. Pair wise comparison of mean was done by Least Significant Difference (LSD) (Wafaa *et al.,* 2012, Dashe *et al.,* 2013).

RESULTS

The morphological characteristics of the isolates are shown in Table 1. Isolates 5, 7 and 11 were isolated from *Salmonella-Shigella* agar (SSA) and they exhibited similar morphological characteristics on SSA plates. In addition, isolates E and G exhibited similar morphological characteristics on MacConkey agar (MA) plates. The biochemical characteristics and identities of the enteric bacterial isolates are shown in Table 2. The results of the present study reveal that isolates 5, 7 and 11 exhibited similar biochemical characteristics; they showed positive results to hydrogen sulphide production, catalase, and methyl red, utilize citrate as carbon source and able to ferment glucose, dulcitol, arabinose and maltose. Isolate 5 fermented inositol, showed slight positive reaction to xylose and was negative to mucate unlike isolates 7 and 11 that fermented xylose but negative to inositol. Isolates E and G exhibited similar biochemical properties; they showed positive results to Indole reaction, methyl red, catalase and able to ferment glucose, maltose, arabinose and lactose.

The results of the molecular characteristics showed that the quality of nucleic acids (DNA) extracted from the isolates were pure when the ratio of the absorbances A_{260}/A_{280} were determined (Table 3). The PCR characteristics of the isolates using primer sequences uspA (specific to *E. coli*), invA (specific to *Salmonella* species), fliC (specific to serovar Typhimurium), sefA (specific to serovar Enteritidis) and gatD for metabolism of galactocitol (dulcitol) are shown in Table 4.. The results of the sequencing of 16s rRNA using universal primer (16s) revealed the presence of *Escherichia coli* 0157:H7 strain SS52 (isolate E), *Escherichia coli* strain SEC 470 (isolate G), *Salmonella enterica* subspecies *enterica* serovar Typhimurium strain U288 (isolate 5), *Salmonella enterica* subspecies *enterica* serovar Enteritidis strain FM366 (isolate7) and *Salmonella enterica* subspecies *enterica* serovar Enteritidis strain YU39 (isolate11) (Table 5).

The primer sequences specific for enterotoxins; heat labile-1 (Ltx1) and heat labile-2 (Ltx2) is shown in Table 6. The agarose gel showing polymerase chain reaction (PCR) amplification products of heat labile-1 (LTx1) and heat labile-2 (LTx2) enterotoxins are shown in figure 1. The results revealed that *E. coli* 0157:H7 SS52, *S.* ser. Enteritidis FM366 and *S.* ser. Typhimurium U288 showed significant amplification of LTx1 and LTx2. There was no amplification of LTx1 and Ltx2 respectively from *S.*ser. Enteritidis YU39 and *E. coli* SEC470. Heat labile-1 (LTx1) was significantly amplified from *E. coli* 0157:H7 SS52 and *S.*ser. Enteritidis FM366 with 130 bp and 1100bp, the LTx1 of *S.* ser. Typhimurium U288 was amplified at 284bp and 1100 bp, while that of *E. coli* SEC472 was amplified at 1100bp. No amplification was detected from *S.* ser. Enteritidis YU39. Similar amplification of LTx2 was also observed for *E. coli* 0157:H7 SS52 and *S.*ser. Enteritidis FM366 at 346bp, 600bp and 1100bp. Amplification was observed at 700bp and 1100 bp for *S.* ser. Typhimurium U288, 400bp and 900bp for *S.*ser. Enteritidis YU39 and no amplification were observed for *E. coli* SEC470.

Table 1: Morphological characteristics of the isolates from chicken feed samples

Parameter \ Isolate	E	G	5	7	11
Appearance on agar plate	Red colony on MA	Red colony on MA	Colourless with black center on SSA	Colourless and dark at the center on SSA	Colourless and dark at the center on SSA
Edge	Entire	Entire	Entire	Entire	Entire
Size (mm)	1.00	1.20	2.20	1.40	1.60
Consistency	Soft	Soft	Soft	Soft	Soft
Optical property	Opaque	Opaque	Opaque	Opaque	Opaque
Elevation	Slightly raised	Convex	Slightly raised	Slightly raised	Slightly raised
Pigmentation	–	–	–	–	–
Gram Reaction	–	–	–	–	–
Shape	Rod	Rod	Rod	Rod	Rod
Motility	+	+	+	+	+

SSA = *Salmonella-Shigella* Agar, MA = MacConkey Agar, + = Positive, – = Negative

Table 2: Characteristics and identities of the enteric isolates from the chicken feed samples

Parameter \ Isolate	E	G	5	7	11
Indole production	+	+	–	–	–
Hydrogen Sulphide	–	–	+	+	+
Ornithine decarboxylase	–	–	–	–	–
Methyl Red	+	+	+	+	+
Voges-Proskauer	–	–	–	–	–
Citrate Utilization	–	–	+	+	+
Catalase	+	+	+	+	+
Urease	–	–	–	–	–
Glucose	+	+	+	+	+
Maltose	+	+	+	+	+
Dulcitol	–	–	+	+	+
Lactose	+	+	–	–	–
Xylose	+	+/–	+/–	+	+
Arabinose	+	+	+	+	–
Inositol	–	–	+	–	–
Mucate	–	–	–	+	+

E – *Escherichia coli*, G – *Escherichia coli*, 5 – *Salmonella* species, 7 –*Salmonella* species
11 – *Salmonella* species, + = Positive, – = Negative

Table 3: Quality of nucleic acid (DNA) used for the study

Sample	Concentration of Nucleic acid (ng/µL)	A_{260}	A_{280}	260/280
5	171.50	3.430	1.820	1.88
7	230.50	4.610	2.435	1.89
E	222.40	4.449	2.358	1.89
11	207.70	4.154	2.201	1.89
G	103.90	2.078	1.143	1.82

Table 4: Primer sequences used for polymerase chain reaction characterization of the isolates

Primer	Length	Sequences (5 – 3')	Target Amplification fragmnent (bp)
USpA-F	20	CCGATACGCTGCCAATCAGT	uspA
USpA-R	20	ACGCAGACCGTAGGCCAGAT	
S139F	26	GTGAAATTATCGCCACGTTCGGGCAA	invA
S141f	22	TCATCGCACCGTCCAAGGAACC	
Flis-F	22	CGGTGTTTGCCCAGGTTGGTAAT	FliC
Typ0$_4$-R	16	ACTGGTAAAGATGGCT	
A058-F	21	GATACTGCTGAACGTAGAAGG	SefA
A01-R	24	GCGTAAATCAGCATCTGCAGTAGC	
gatD-F	23	GGCGCCCATTATTATCCTATTAC	gatD
gat-R	23	CATTTCCCGGCTATTACAGGTAT	

Table 5: Molecular identities of the isolates

Isolate	Max score	Total score	Query Cover	Gap	Identity	Accession Number	Description
E	2856	2967	100%	0%	100%	CO010304.1	*Escherichia coli* strain 0157:H7 str SS52 Complete genome
G	1297	1297	100%	0%	96%	CP007594.1	*Escherichia coli* strain SEC470 Complete genome
5	2193	4386	100%	0%	98%	CP003836.1	*Salmonella enterica* subsp. enterica serovar Typhimurium str U288 Complete genome
7	660	660	100%	0%	96%	NG03836.1	*Salmonella enterica* subsp. enterica serovar Enteritidis str FM366 Complete genome
11	2844	2844	100%	0%	100%	CP011428.1	*Salmonella enterica* subsp. enterica serovar Enteritidis str YU39 Complete genome

Table 6: Primer sequences used for the detection of the presence of enterotoxins in the isolates

Primer	Length	Sequences (5 – 3')	Target
Lp30	21	CCGATACGCTGCCAATCAGT	Heat labile- 1 enterotoxin (Ltx1)
Lp31	21	ACGCAGACCGTAGGCCAGAT	
Lp43	22	GTGAAATTATCGCCACGTTCGGGCAA	Heat labile-2 enterotoxin (Ltx2)
Lp44	22	TCATCGCACCGTCCAAGGAACC	

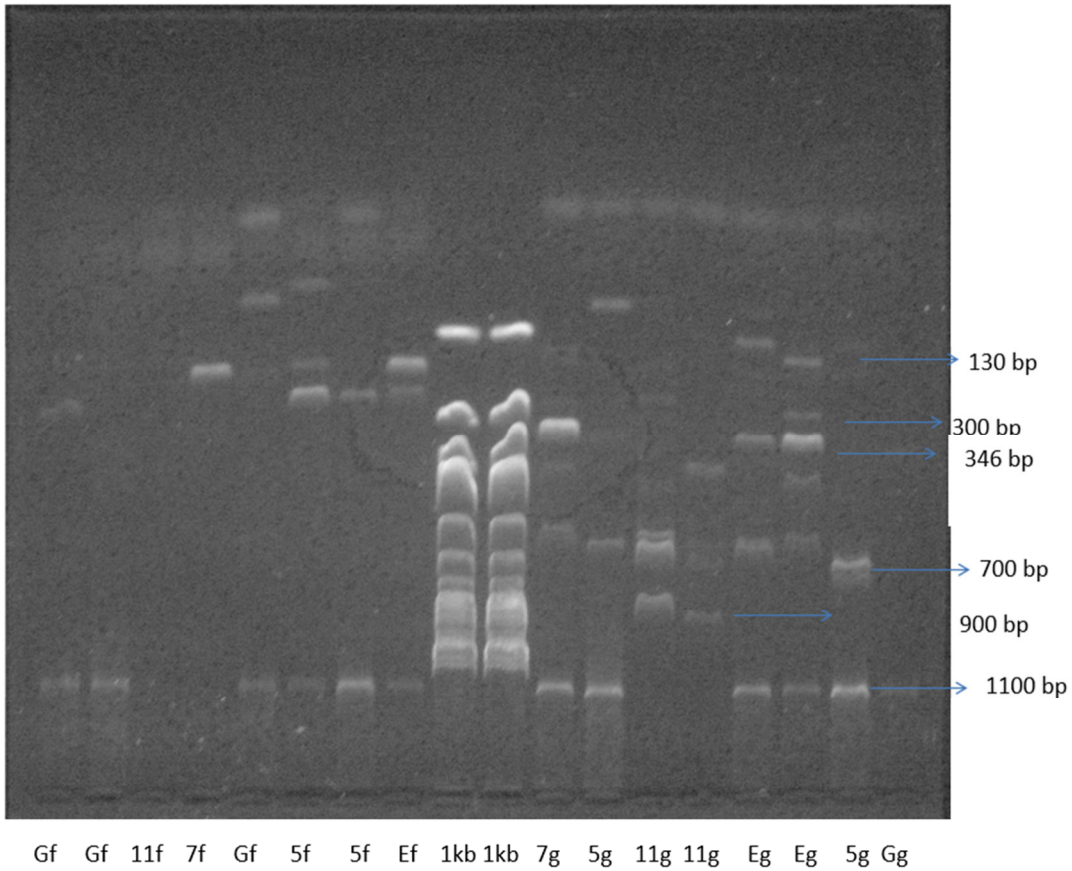

Figure 1: Agarose gel amplification of PCR products of heat labile-1 enterotoxin (Ltx1/f) and heat labile- 2 enteotoxin (Ltx 2/g) of the isolates

DISCUSSION

The presence of *Escherichia coli* O157:H7 SS52, *Escherichia coli* SEC470, *Salmonella enterica* subspecies *enterica* serovar Typhimurium U288, *Salmonella enterica* subspecies *enterica* serovar Enteritidis FM366 and *Salmonella enterica* subspecies *enterica* serovar Enteritidis YU39 from studied feed samples supported the occurrence enteric bacteria in the samples. Traditionally, the laboratory detection of *Escherichia* and *Salmonella* species has relied on non-selective and/or selective enrichment and subsequent culture on selective media. The introduction of molecular techniques provides a more sensitive and rapid technique for detecting these bacteria. The presence of *E. coli* SEC470 and *Salmonella* serovar Enteritidis YU39 in the chicken feed samples were negligible due to their very low counts in the samples.

Heat labile 1 (LTx1) and heat labile 2 (LTx2) enterotoxins were significantly detected from *E. coli* 0157:H7 SS52, *S.* serovar Enteritidis FM366 and *S.* serovar Typhimurium U288. The absence of LTx1 and LTx2 respectively from *S.* serovar Enteritidis YU39 and *E. coli* SEC470 could be attributed to their low occurrences in nature. Many studies have shown that chickens were majorly infected by *E. coli* O157:H7 and natural infection of chicken by *E. coli* O157:H7 had been attributed to differences in geographic areas (Zende *et al.,* 2013). Many studies have shown that *E. coli* O157:H7 is majorly associated with LT-1 and LT-2 which are responsible for several human diseases like haemolytic uremic syndrome (HUS) and haemorrhagic colitis (HC) (Zende *et al.,* 2013). The present study revealed that *E. coli* 0157:H7 SS52 isolated from the poultry feed samples had LTx1 and LTx2 with 130bp and 346bp respectively which was also reported by Zende *et al.* (2013).

Studies have shown that *Salmonella* species produce two major toxins; cholera toxin-like enterotoxins and Shigella toxin-like cytotoxins, and the production of these toxins is rare and depends on the geographical differences amongst *Salmonella* strains in their enterotoxigenicity (Zende *et al.* (2013). The detection of LTx1 and LTx2 from *S.* serovar Enteritidis FM366 and *S.* serovar Typhimurium U288 in this study, agrees with the findings of Ketyl *et al.* (2009), who detected the presence of *Shigella dysentriae* 1-like cytotoxic enterotoxins produced by different strains of *Salmonella* serovar Enteritidis. Molina and Peterson, reported the isolation of *Salmonella* toxins that closely resemble cholera toxins and *E. coli* heat-labile enterotoxin, but the toxin failed to induce fluid and electrolyte secretion in the rabbit intestinal loop Zende *et al.* (2013). Baloda and co workers, as reported by Zende *et al.* (2013), isolated heat-labile (LT) enterotoxins (Shiga-like toxins) from different Indian

strains of *S.* serovar Enteritidis and *S.* serovar Typhimurium that induced the fluid and electrolyte secretion in the rabbit loop. *Salmonella* serovar Enteritidis FM366 studied in this present work produced LTx1 and LTx2 with 130 bp and 346 bp respectively as reported by Zende *et al.* (2013). However, Gemmel (2004) concluded that the heat-labile toxins of Gram negative enteric bacteria have much in common in their structures, membrane receptors and biochemical modes of action as they induce nucleotide cyclase activation system.

CONCLUSION

This study revealed the presence of *Escherichia coli* O157:H7 SS52, *E. coli* SEC470, *Salmonella* serovar Typhimurium U288, *Salmonella* serovar Enteritidis FM366 and *Salmonella* Enteritidis YU39 in the chicken feed samples randomly collected from the major towns in twenty-one Local Government Areas of Anambra State, and there was presence of heat labile enterotoxins in the isolated enteric bacteria

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