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Molecular Characterization and Diversity of Enteric Bacteria Isolated from Chicken Feeds

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Abstract
This study focused on molecular characterization and diversity 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. The diversity of the enteric bacteria was determined by carefully recording the number of occurrences of each identified isolate from the studied feed samples. 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 significantly ($P<0.05$) isolated from the feed samples. The organisms were detected most from the samples collected from the consumers while the samples from the wholesalers showed the least isolates. EC (60.49%) was the most predominant isolate, followed by SE (22.13%) and ST (16.52%). The occurrences of ES (0.66%) and SY (0.21%) were non significant ($p>0.05$). This study has revealed that EC, ST, ES, SY and SE were the enteric bacteria detected from the studied feed samples, of which EC was recorded most.
Keywords: Molecular characterization, Enteric bacteria, Chicken Feeds, Diversity.

INTRODUCTION

Enteric bacteria belong to the family Enterobacteriaceae which is the largest of the medically important Gram-negative bacilli with more than 130 described species. These bacteria are found worldwide in soil, water and vegetation and are usually part of the normal flora of most animals and humans (Oguttu, *et al*., 2008).Many of the bacteria in this family can live in the gut without causing any health problems but some bacteria always cause infection with symptoms like vomiting, diarrhea and fever, People usually get infected with enteric bacteria as a result of poor unhygienic conditions, such as inadequate sanitation and contaminated food and drinking water, which is common in developing countries(Maciorowski *et al.,* 2004).

Infections with enteric bacteria are one of the major causes of childhood morbidity and mortality in the developing world today and acute infectious diarrhea is estimated to cause 2 million deaths each year (Maciorowski *et al.,* 2004).

Enteric pathogens can be disseminated to chicken through variety of sources. Several studies have linked contaminated feed to the occurrence of pathogens in chicken (Primm, 2008). Analysis of commercially manufactured feeds confirmed that both feed ingredients and dusts can be sources of *Salmonella* contamination in feed mills. Moreover, some pathogens such as *Salmonella* species can survive for long periods of time in feed of low water activity.

Feed producers have used a variety of treatment to reduce pathogens in feed, including chemicals such as formic, hydrochloric, nitric, phosphoric, propionic, and sulphuric acids; isopropyl alcohol; formate and propionate salts; and trisodium phosphate have been evaluated. In determining their antimicrobial activity, consideration must be given to the effect of (Maciorowski *et al.,* 2004). Despite different methods of control attributed to enteric bacterial infections, enteric bacteria mainly *E. coli* and *Salmonella* species remain the primary cause chicken diseases and death including human food poisoning worldwide.

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).

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 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 3).

The prevalence of enteric bacteria in the studied chicken feed samples are shown in Table 4. The results reveal that isolate E; 2307 (60.49%), isolate 5; 630 (16.52%) and isolate 7; 844(22.13%) were mostly encountered in the studied feed samples. The occurrences of isolate G; 25 (0.66%) and isolate11; 8 (0.21%) were negligible. The results of the study show that isolate E was the most predominant isolate among the feed samples collected from the wholesalers, retailers and consumers. Isolate 7 was higher than isolate 5 in the samples collected from the wholesalers, retailers and consumers. Also enteric bacteria were most predominant in the feed samples collected from the consumers, followed by the samples from the retailers and the samples from wholesalers showed the least isolates.

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: 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 4: Prevalence of enteric bacteria in the studied chiken feed samples

Type of feed	Sources of the feeds											
	Wholesaler (n)				Retailer (n)				Consumer (n)			
	X	Y	Z	Total	X	Y	Z	Toatal	X	Y	Z	Total
E (%)	5 (26.32)	6 (31.58)	8 (42.11)	19 (54.29)	194 (18.62)	206 (19.77)	642 (61.61)	1042 (63.81)	249 (19.98)	248 (19.90)	749 (60.11)	1246 (58.06)
G (%)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0)	3 (27.27)	3 (27.27)	5 (45.45)	11 (6.74)	3 (21.43)	4 (28.57)	7 (50.00)	14 (0.65)
5 (%)	1 (14.29)	3 (42.86)	3 (42.86)	7 (20.00)	46 (20.18)	39 (17.11)	143 (62.72)	228 (13.96)	73 (18.48)	64 (16.20)	258 (65.32)	395 (18.41)
7 (%)	3 (33.33)	2 (22.22)	4 (44.44)	9 (25.71)	55 (15.76)	83 (23.78)	211 (60.46)	349 (21.37)	95 (19.55)	85 (17.49)	306 (62.96)	486 (22.65)
11 (%)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (33.33)	2 (66.67)	3 (0.18)	1 (20.00)	1 (20.00)	3 (60.00)	5 (0.23)

DISCUSSION

The presence of enteric bacteria in the studied feed samples could be traced from the management practices, feed ingredients, and transportation of the feeds, poor handling and sanitary conditions attributed to the feed samples. Similar findings were reported by many researchers (Immerseel *et al.,* 2002; Jones and Richardson, 2004; Alshawabkeh, 2006; Maciorowski *et al.,* 2007). Researchers had shown that animal housing and transportation of equipments can also harbour enteric bacteria and this contributes to the contamination of chicken feeds (Primm, 2008). Maciorowski *et al.* (2007) also stated that the high prevalence and high populations of enteric bacteria in animal wastes was evidence that manure could be a principal source of enteric pathogens to chicken industry. Chicken feeds contaminated by enteric bacteria pathogenic to humans can contribute to human food-borne illness through the feed-food-human chain. This shows that the production of chicken feeds requires microbiological safety regulations to escape microbial contamination of the product. Similar deduction was drawn by different researchers (Davies and Wales, 2010; Chowdhuri *et al.,* 2011; Fredrick and Huda, 2011).

The variation of enteric bacteria from different brands of chicken feeds studied could be attributed to the nature, texture and composition of the feed materials. Maciorowski *et al.,* (2007) reported that variation in microbial counts in different feed samples depend on the water activity, oxygen tension, pH and nutrient composition of the feed material. Barakat, (2004) also reported that the vegetable protein sources, cereal grains and their by-products were among the factors that contributed to the variations in enteric bacterial counts in different brands of chicken feeds. 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 (Davies and Wales, 2010; Chowdhuri *et al.,* 2011; Fredrick and Huda, 2011).

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. The highest counts of *E. coli* O157:H7 SS52 in the feed samples could be attributed to human activities during processing, transportation and storage of the feeds. Ali *et al.* (2014) stated that the presence of pathogenic strain of *E. coli* in chicken meat and its by-products is not only a potential threat of cross contamination but can also lead to become an infectious dose for handlers and consumers. Sher *et al.* (2010) also stated that the presence of *E. coli* in food materials is considered to be an indicator for the presence of other pathogenic bacteria in the respective food items. Zhao *et al.* (2001) reported 38.7% prevalence of *E. coli* in chicken meat in similar study in Washington D.C., USA.

The higher occurrence of total mean viable plate counts of *S.* Enteritidis FM366 more than *S.* serovar Typhimurium U288 in studied feed samples collected from the wholesalers, retailers and consumers supported the findings of many researchers. Patrick *et al.* (2004) estimated that the survival time of *Salmonella* species in chicken feed is more than 98 days. Davies and Wales (2010) reported the viability of *S.* serovar Enteritidis strains in feed at room temperature is 78 weeks. Furthermore, at 7°C the organism may survive up to 79 weeks in chicken feed. Also, the data from the studies of Jones and Richardson (2004) confirmed that dust and feed ingredients can be a major source of *Salmonella* contamination during the feed milling process.

The highest counts of enteric bacteria recorded among different bands of chicken feeds collected from the consumers could be attributed to the poor handling, poor sanitation and series of distribution channels involved before reaching the consumers. Similar findings were stated by many researchers (Davies and Wales, 2010; Ali *et al.,* 2014).

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, of which *Escherichia coli* O157:H7 SS52 recorded the highest counts and the occurrences of E. coli SEC470 and S. serovar Enteritidis YU39 were negligible due to very low counts of the isolates from the studied samples, and the samples from the consumers were mostly contaminated.

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