

Investigation of the antibiotics susceptibility patterns and pathogenic potential of bacteria isolated from poultry wastes

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ABSTRACT: The antibiotic resistant patterns and pathogenic potential of bacteria isolated from poultry wastes were investigated in this study. A total of 20 samples of poultry wastes were collected from different sites of poultry houses in Uli, Ihiala Local Government Areas, Anambra State, Nigeria using sterile polythene bags. The pathogens were isolated and identified using streaking plating technique, biochemical characterization and API Identification System. The disc diffusion method and plasmid curing were used to determine the antibiotic resistant patterns and resistant gene location. The pathogenicity study involves orally or intraperitoneally inoculation of 0.5 mL of the different bacterial pathogens into three months' immuno - competent albino mice for two weeks; and rabbit ileal loop assay for enterotoxin production in five healthy adult rabbit for 18 to 24 hrs. The isolated pathogens were *Staphylococcus aureus*, *E. coli*, *Pseudomonas aeruginosa*, *Clostridium*, *Salmonella*, *Shigella*, *V. cholerae* and *V. parahaemolyticus*. The antibiotics sensitivity test revealed that the bacterial pathogens were most susceptible to ciprofloxacin, rifampicin, levofloxacin and most resistant to ceporex, amoxil, ampiclox and norfloxacin with significance in the Gram negative antibiotics sensitivity testing ($P < 0.05$), but no significance in the Gram antibiotics sensitivity positive testing ($P > 0.05$). 12.5% of the strains lost their antibiotic resistance plasmids marker after sodium dodecyl sulfate (SDS) mediated curing. The pathogenicity testing revealed that the intestine had the highest significant microbial count ($9 \log \text{CFU/g} \times 10^5$) and the liver recorded the least significant count ($7 \log \text{CFU/g} \times 10^5$) compare to the control (inoculated with water) that had no count. The enterotoxin testing revealed that *Staphylococcus aureus* recorded the highest enterotoxin production (0.45 mL/cm) while *Pseudomonas aeruginosa* recorded the least (0.16 mL/cm). Thus, the test isolates have shown reasonable pathogenic potentials and hence proper enlightenment and sensitization of the public health problems associated with these wastes should be encouraged.

Keywords: Antibiotic resistance, gastrointestinal tract, pathogenicity, poultry wastes, public health.

INTRODUCTION

Poultry waste includes a mixture of fecal and urinary excreta (manure), bedding material of litter (e.g. wood shavings or straw), waste feed, dead birds, broken eggs packing material and feathers removed from poultry houses. It also includes waste from cage, conveyer belt and water flushing systems (Kelleher et al., 2002). The major waste composition like poultry feathers, offal and litter has different field applications. Poultry feather being

rich sources of keratin, protein and amino acids, can be converted into valuable products such as feather meal, biodiesel, biodegradable plastic and fertilizer (Thyagarajan et al., 2013).

Issues related to the environment, human health and the quality of life of people living near to and distant from poultry production operations make waste management a critical consideration for the long-term growth and

sustainability of poultry production in larger bird facilities located near urban and rural areas (Nahm and Nahm, 2004). Poultry wastes have been identified as a waste management issue and a source of potential environmental risk (Ezekoye et al. 2017). Environmental problems such as eutrophication, odours and contamination of drinking waters can result from poor handling and storage of the manure (Ezekoye et al. 2017).

As these wastes are composed of tissues and blood, these deposited wastes may serve as a reservoir for the multiplication of several pathogenic microorganisms that can cause severe disease outbreaks to both man and animals. Pathogenic microbial organisms gain access to the animal body through contaminated feed and water (Ngodigha and Owen, 2009). The presence of these pathogenic microorganisms impact negatively on feed utilization and physiological functions within the animal system. *E. coli* is a Gram negative, facultative anaerobic, rod shaped coliform bacterium of the genus *Escherichia* that is commonly found in the lower intestine of warm blooded organisms. Some serotypes can cause serious food poisoning in their hosts, and are occasionally responsible for product recalls due to food contamination. *E. coli* infection which is responsible for major losses in the poultry industry, is commonly found in poultry litter and fecal material (Fontenot, 2000). The bacterium grows massively in freshly faecal matter under aerobic conditions for 3 days, but its number declines slowly afterwards. The virulent strains cause gastroenteritis, urinary tract infections, neonatal meningitides, hemorrhagic colitis, and Crohn's disease. *E. coli* for example O157: H7 can produce shiga toxin (Abakpa et al., 2015). The toxin causes premature destruction of the red blood cells, which then clog the body's filtering system, the kidney causing haemolyticuremic syndrome (HUS). *Salmonella* is closely related to the genus *Escherichia* and can be found in cold and warm-blooded animals (including humans), worldwide and in the environment (Abakpa et al., 2015). They cause illnesses such as typhoid fever, paratyphoid fever, and food borne illness. *Salmonella* infections can be zoonotic and can be transferred between humans and animals. Many infections are due to ingestion of contaminated food. *Salmonella* species are facultative intracellular pathogens that enter cells via macropinosomes. *Salmonella* bacteria can survive for weeks outside a living body and they can be destroyed by heat at about 55°C for 10 mins (Parker et al., 2010).

Nwankwegu et al. (2016b) reported the prevalence of pathogens in poultry wastes and isolated potentially pathogenic organisms notably; *Salmonella enteritidis*, *Shigella* sp., *Clostridium perfringens*, *Pseudomonas* sp., *E. coli*, *Staphylococcus aureus*, *Vibrio cholerae* and *Vibrio parahemolyticus*. At present, there is dearth of scientific information on the potential microbial pathogens capable of producing endotoxin associated with poultry environmental samples and so necessitated this study. Thus, the aim of this study is to determine the level of

susceptibility to antimicrobial drugs and to characterize the potential pathogens associated with poultry environmental samples.

MATERIALS AND METHODS

Study area

Ihiala Local Government Area is one of the twenty-one Local Government Area (LGA) in Anambra State of Nigeria. It is located at the southern part of Anambra State and at the southern end of the south senatorial zone of the State. Uli is a town in Ihiala Local Government Area.

Sample collection

Twenty poultry samples were collected from Calvo Poultry Farm in Uli and different poultry farms at Ihiala LGA Anambra State. It was collected with the aid of a sterilized bag and a scoop for the transferring of the waste to the sterile bag. The samples were pooled and mixed properly using sterile plastic spatula in order to obtain a composite sample. The sample was brought to the laboratory in a cooler maintaining low temperature (< 4%) using ice blocks. The collected samples were processed within 6 hrs of their collection. The samples were marked with respect to time of collection (Nwankwegu et al., 2016a).

Preparation of the media

The following media were used for culturing: Nutrient agar (total heterotrophic aerobic bacterial counts), MacConkey Agar (Total coliform counts), Eosin methylene blue agar, Brain Heart Infusion Agar, Mannitol Salt Agar, *Salmonella* – *Shigella* Agar, Cetrimide Agar and Thiocitrate Bile Salt Sucrose Agar. All the media were prepared according to the Manufacturer's specification.

Sterilization of materials

As stated in Willey et al. (2008), conical flasks (Pyrex), prepared media and other plastic materials were sterilized by autoclaving at 121°C for 15 minutes at a pressure of 15 Psi. Glass wares such as pipettes, glass spreader, Petri dishes, measuring cylinder, and other glass materials were sterilized in the laboratory hot air oven at a temperature of 160°C for 1 hr before use.

Isolation of the Bacteria

Isolation of Salmonella and Shigella spp.

Twenty-five grams (25 g) of poultry waste sample was

dissolved in 225 mL of peptone water and allowed to stand for about 18 hrs at room temperature. The broth culture was then plated out on SS agar using streak plate method and pure cultures of the isolates were obtained after 18 to 24 hrs of repeated sub-culturing and incubation at room temperature (Resende et al., 2014).

Isolation of *Escherichia coli*

Twenty-five grams (25 g) of the poultry waste was dissolved in 225 mL of peptone water and allowed to stand for about 18 hrs at room temperature. The broth culture was then plated out on EMB agar using a streak plate method and pure cultures of the isolates were obtained after 18 to 24 hrs of repeated sub-culturing and incubation at room temperature (Ghaderpour et al., 2014)

Isolation of *Staphylococcus aureus*

Twenty-five grams (25 g) of the poultry waste was dissolved in 225 mL of sterile peptone water and allowed to stand for about 18 hrs at room temperature. The broth culture was plated out on Mannitol Salt agar using streak plate method. The plates were incubated at room temperature for 18 to 24 hrs. White to deep yellow colonies that developed on the plates were sub-cultured to obtain pure colonies (Ghaderpour et al., 2014).

Isolation of *Clostridium* sp.

Twenty-five grams (25 g) of the poultry waste was dissolved in 225 mL of sterile peptone water, and allowed to stand for about 18 hrs at room temperature. The broth culture was then plated out on Brain Heart Infusion agar using streak plate method. The plates were incubated at 37°C for 24 hrs in anaerobic condition. Yellow colonies that developed on the plates were sub-cultured on fresh Brain Heart Infusion agar in anaerobic condition. Pure cultures of the isolates were obtained (Alfa et al., 2014).

Isolation of *Vibrio* spp.

Twenty-five grams (25 g) of the poultry waste was dissolved in 225 mL of sterile peptone water and allowed to stand for about 18 hrs at room temperature. The broth culture was thereafter plated out on T.C.B.S agar using streak plate method. The plates were incubated aerobically at room temperature for 18 to 24 hrs. Yellow and green colonies that developed were sub-cultured for *V. cholerae* and *V. parahaemolyticus* respectively on a fresh T.C.B.S agar (Ghaderpour et al., 2014).

Isolation of *Pseudomonas aeruginosa*

Twenty-five grams (25 g) of the poultry waste was

dissolved in 225 mL of sterile peptone water and allowed to stand for about 18 hrs at room temperature. The broth culture was then plated on EMB agar using streak plate method. The plates were incubated aerobically at room temperature for 18 to 24 hrs. Pinkish colonies that developed on EMB agar were sub-cultured on Cetrimide agar in order to screen for the presence of creamy to yellow colonies (Ghaderpour et al., 2014).

Characterization and identification of the bacterial isolates

Colonial morphology

The colonies were carefully examined for bacterial characteristics. The colour, shape, elevation, and other peculiar features of the colonies were observed according to the method described by Willey et al. (2008).

Biochemical testing

The following biochemical tests were carried: Citrate test, Indole test, Voges - Proskauer test, Gelatinase, 2-nitrophenyl-B-D-galactopyranoside test; Arginine dehydrolase test; Lysine decarboxylase test; Ornithine decarboxylase test; Urease test, Hydrogen sulfide production test; Tryptophan deaminase; VP = Voges – Proskauer Test, Glucose, Mannitol, Inositol, Sorbitol, Rhamnose, Saccharose, Melbiose, Amigdalina, Arabinose, Nitrite, Nitrogen, Oxidase and Catalase tests. These tests were carried out by following the instructions on the API kit manual and as described by Cheesbrough (2006). The isolates were identified using Analytical Profiling Index (API) Identification System.

Antibiotics sensitivity test

The Clinical and Laboratory Standard Institute (CLSI) disc diffusion method of (2005) was used for the antibiotic sensitivity test. The turbidity of the inocula of various isolates was made to be equivalent to 0.5 of McFarland standard and each of the isolates was inoculated onto the surface of Muller Hinton agar using sterile swab sticks. The antimicrobial agents tested were: ciproflaxin 10 µg, norfloxacin 10 µg, gentamycin 10 µg, tarivid 10 µg, reflacine 10 µg, ceporex 10 µg, amoxicillin 20 µg, rifampicin 20 µg, ampiclox 20 µg, levofloxacin 20 µg, erythromycin 20 µg, streptomycin 30 µg, chloramphenicol 30 µg, augmentin 30 µg, nalidixic acid, septrin 30 µg, ampicillin 30 µg) (Opton Disc, Nigeria). These were aseptically placed on the surface of the inoculated agar plates. After 30 mins of applying the discs, the agar plates were inverted and incubated for 24 hrs at room temperature (Nwankwegu et al., 2016b). The clear zones that developed around each disc were measured as the zones of inhibition on the basis of CLSI guidelines.

Curing experiment

Curing of the isolates was carried out using the modifications of the Olukoya and Oni (1990) method. There were overnight cultures in Nutrient broth and 1 mL inoculum was added to 30 mL of Nutrient broth (pH of 7.6). The cultures were then incubated for 24 hrs with 1 mL 10% of sodium dodecyl sulfate (SDS) solution. The overnight broth cultures were diluted with sterile distilled water. Each pure culture that have their turbidity adjusted to McFarland standard was swabbed on Mueller Hinton agar plates containing a combination of antibiotics (resistant marker antibiotics) to which the pathogens were resistant to test run for the respective antibiotic sensitivity patterns. Resistance markers were expressed after as being chromosome - mediated while those that were not expressed were regarded as plasmid - mediated (Yah et al., 2007).

Pathogenicity testing

Laboratory animal

Three months old immune - competent albino mice of equal sex (1:1) weighing between 30 and 33 g, bred in Nkwegu Farm Ihiala, Anambra State were used. They had free access to water and food and were in 8 different aluminium wire cage each containing ten mice/aluminium wire cage and maintained in a controlled room at $25 \pm 3^\circ\text{C}$ in a 12 hrs light: dark cycle, for one week (for acclimatization) prior to infection. Two of the mice in the cage served as control, four were inoculated intraperitoneally and the remaining four was also inoculated orally in each cage. The mice where allowed *ad libitum* to grower feed of poultry (TOP VITAL FEEDS, Kano State, Nigeria) and water. The animals were handled in accordance with the principle of laboratory animal care (Morcos et al., 2015; Nwankwegu et al., 2016b).

Inoculum preparation

The bacteria isolated from the poultry wastes: *E. coli*, *Salmonella* sp., *Pseudomonas* sp., *Staphylococcus aureus*, *Shigella* sp., *Clostridium* sp., *Vibrio cholerae* and *Vibrio parahaemolyticus* were used. They were cultured differently in conical flasks containing 80 mL of sterile nutrient broth, overnight at 37°C inside shaker. The next day at mid logarithmic growth phase, 5 mL of each suspension was transferred to 25 mL of another sterile nutrient broth. The bacterial suspensions were grown at 37°C in a shaker until an optical density of 1.0 at a wavelength of 620 nm was achieved. Subsequently, 4 mL of each suspension was washed (centrifuged at 3000 rpm for 30 mins) twice in 4 mL sterile isotonic saline, which corresponded to 3×10^8 CFU/mL. The inocula were used for animal inoculation (Nwankwegu et al., 2016b).

Animal inoculation

The mice were infected using oral and intraperitoneal routes. 0.1 mL saline suspension of the inocula of different isolates were inoculated orally on 4 mice in each cage (8 sets of mice) while the other 4 mice were inoculated intraperitoneally (8 sets of mice). The two control mice were inoculated with 0.1 mL saline, one orally and the other intraperitoneally. The mice were fed and observed for pathological signs for 14 days. At the end of 14 days, the survived mice were dissected and their kidney, intestine, lungs, and livers were harvested. The organs of the mice that died after inoculation were also harvested for analysis (Nwankwegu et al., 2016b).

Enumeration of the inoculated pathogens

Two grams of each intestine was weighed and ground in 2 mL of saline with mortar and pestle. The number of the infecting organisms in the intestine was determined by plating after 10^{-5} serial dilution. The organs were also homogenized in 1 mL of sterile distilled water. The homogenated organs were serially diluted using ten - fold dilution with sterile water and the number of organisms was determined by plating. The antibiotic sensitivity results of the isolates were used as the marker to noting if the organisms injected were the ones isolated from the organs (Nwankwegu et al., 2016b).

Preparation of enterotoxin supernatant

Preparation of enterotoxin from the pathogens was determined using the method of Abbas et al. (2013). The bacterial isolates were inoculated each into 5 mL of nutrient broth in 15×125 mm sterile screw cap test tubes. The cultures were incubated at 37°C for 24 hrs. Then 0.1 mL of each test organism was inoculated into 10 mL of liquefied milk at pH 8 and pasteurized by heating to 80°C for 30 mins (Abbas et al., 2013). The cultures were then incubated at 37°C for 48 hrs. The cell free supernatants were collected by centrifugation at 5000 rpm for 5 mins and then decanted into sterile test tubes. The cell free supernatant of the test organisms were then used as crude toxin preparations (Khudor et al., 2012).

Enterotoxin production using rabbit ileal loop assay

The method of Everest et al. (2017) was used to assay the pathogens for enterotoxin production. Five healthy adult rabbits were anaesthetized. The peritoneal cavity of each rabbit was opened aseptically by surgical technique and the bowel was carefully washed with pre- warmed sterile water. The length of the bowel making the loop was cut at both ends and all cut surfaces were clamped. The ileal loops were ligated into 5 cm segments with 2 cm intervals.

The ends of the isolated intestine were closed with sutures and the required number of loops was constructed with ligatures. Then, 0.5 mL of the crude toxin supernatant of the test organisms was injected into each of the ligated segments of the ileum. Some segments were injected with 0.5 mL of sterile water to serve as control segments. The loops were replaced in the peritoneal cavity in their original positions and the peritoneum closed. The animals were again anaesthetized 18 hrs after inoculation and the peritoneum cavity opened again (Idahosa et al., 2017). The volume of the fluid recovered using syringes was used to determine the dilatation index (DI) which is estimated as the ratio volume of the fluid accumulated in the intestinal loop to the length of the ileal segment of the loop. A DI greater than 0.2 mL/cm was then reported as positive (Abbas et al., 2013).

Statistical analysis

The data were analyzed using Graph-Pad Prism statistical software version 7.00 (GraphPad software Inc. San Diego, California). All values were expressed as mean \pm standard deviation (SD). Two-way analyses of variance (ANOVA) for comparisons of differences among means of groups of treatments followed by post Tukey's multiple comparison test were determined. The results were considered statistically significant at 95% confidence intervals ($p < 0.05$) (Nwankwegu et al. 2016a, b).

RESULTS AND DISCUSSION

The morphology and biochemical characteristics of isolated pathogens is presented in Table 1. From the results, the isolated pathogen include: *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* sp., *Shigella* sp., *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Clostridium* sp. Most of the pathogens are Gram negative and there was presence of spore while few others are Gram positive and spore positive. Most pathogens were positive to O-nitrophenyl-b-D-galactopyranoside, urease, mannose, indole, sorbitol, rhamnase, saccharose, melibiose, amygdalin, oxidase tests and negative to arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate; hydrogen sulfide production, tryptophan deaminase, Voges-Proskauer test; gelatinase; glucose, arabinose, nitrite and nitrogen tests. Similar findings were obtained by Nwankwegu et al. (2016b) and Idahosa et al. (2017).

The antibacterial susceptibility patterns of the isolated Gram negative and positive bacterial pathogens are presented in Tables 2 and 3. From the Gram negative pathogens, *Escherichia coli* was susceptible to streptomycin and ciprofloxacin (18.00 mm), intermediate to reflacine (13.00 mm) and resistant to tarivid, gentamycin, augmentin, septrin, ampicillin, ceporex and

nalidixic acid (< 8.00 mm); *Pseudomonas aeruginosa* was most susceptible to septrin and ciprofloxacin (21.00 mm), intermediate to tarivid (16.00 mm) and resistant to augmentin, ampicillin and ceporex (0.00 mm); *Salmonella* sp., was most susceptible to tarivid (21.00 mm), intermediate to reflacine, streptomycin, gentamycin, augmentin, ciprofloxacin (15.00 to 17.00 mm) and resistant to septrin, ampicillin, ceporex and nalidixic acid (0.00 mm); *Vibrio cholerae* was most susceptible to septrin, ampicillin and ciprofloxacin (23.00 mm) and resistant to ceporex, nalidixic acid and tarivid (0.00 mm); *Vibrio parahaemolyticus* was most susceptible to streptomycin and ciprofloxacin (24.00 mm) and resistant to augmentin, septrin, ampicillin, ceporex and nalidixic acid (0.00 mm); and *Shigella* sp. was most susceptible to tarivid and ciprofloxacin (22.00 mm), intermediate to augmentin (15.00 mm) and resistant to ampicillin, ceporex and nalidixic acid (0.00 mm). The findings of Gram positive pathogens revealed that *Staphylococcus aureus* was most susceptible to rifampicin (24.00 mm) and resistant to amoxicil, ampiclox and norfloxacin (0.00 mm) while *Clostridium* sp. was most susceptible to ciprofloxacin and levofloxacin (21.00 mm), intermediate to chloramphenicol (16.00 mm) and resistant to rifampicin, streptomycin, gentamycin, amoxicil, ampiclox and norfloxacin. There was significant differences detected in the Gram negative antibiotics sensitivity testing ($P < 0.05$), but not significant differently in the Gram antibiotics sensitivity positive testing ($P > 0.05$). The bacterial isolates may have been resistant to some of the antibiotics because of the production of enzymes which inactivate or modify antibiotics, cause changes in bacteria cell membrane, modification of target site and development of metabolic pathways by bacteria (Kim et al. 2006). Similar findings were obtained by Nwankwegu et al. (2016b) but contradicts Idahosa et al. (2017) and the possible reason could due to the nature of samples which were collected from public waste dump sites as the various wastes sources of the dump sites may contribute to the wide scale resistances obtained unlike the poultry waste with single source.

The plasmid curing analysis of the isolated bacterial pathogens is presented in Table 4. From the result, *Escherichia coli* possesses resistant gene markers to tarivid, gentamycin, augmentin, septrin, ampicillin, ceporex and nalidixic acid before curing but lost the gene markers after curing; *Pseudomonas aeruginosa* possesses resistant gene markers to augmentin, ampicillin and ceporex before curing but lost the gene markers after curing; *Salmonella* sp. possesses resistant gene markers to septrin, ampicillin, ceporex and nalidixic acid before curing but lost the gene markers after curing; *Vibrio cholerae* possesses resistant gene markers to ceporex, nalidixic acid and tarivid before curing but lost the gene markers after curing except the nalidixic acid gene marker; *Vibrio parahaemolyticus* possesses resistant gene markers to augmentin, septrin, ampicillin, ceporex and

Table 1. Morphology and biochemical characteristics of the isolated bacterial pathogens.

Organisms	<i>Staphylococcus aureus</i>	<i>E. coli</i>	<i>Clostridium</i> sp.	<i>Salmonella</i> sp.	<i>Pseudomonas aeruginosa</i>	<i>Shigella</i> sp.	<i>Vibrio cholerae</i>	<i>Vibrio parahaemolyticus</i>
Colony color	White to deep yellow on MSA	Pinkish on MA and green metallic sheen on EMB	Creamy on Brain Heart Infusion	Black pigment on SSA	White to deep pink on EMB	Pink on SSA	Yellow on TCBS	Green on TCBS
Colony shape	Cocci	Rod	Long rod	Short rod	Rod	Short rod	Curved rod	Curved rod
Gram stain	+	-	+	-	-	-	-	-
Spore stain	-	-	+	-	-	-	-	-
ONPG	+	+	+	-	+	-	-	+
ADH	-	+	-	-	-	-	-	-
LDC	-	-	-	-	-	-	+	-
ODC	-	+	-	-	-	-	+	-
CIT	-	+	+	+	-	-	-	-
H ₂ S	-	-	-	-	-	-	-	-
URE	+	-	+	+	+	+	+	+
TDA	-	-	+	+	-	-	-	+
IND	-	-	-	+	-	-	-	-
VP	-	-	-	+	-	-	+	+
GEL	-	-	-	-	-	+	+	+
GLU	-	+	+	+	-	-	-	+
MAN	-	+	+	+	-	+	-	+
IND	+	-	+	+	+	+	-	+
SOR	-	+	+	+	-	+	-	+
RHA	-	-	+	+	-	+	+	+
SAC	+	-	+	+	+	+	-	+
MEL	+	+	+	+	+	+	+	+
AMY	+	+	+	+	+	+	+	+
ARA	-	-	-	+	-	-	-	-
OX	+	+	+	+	+	-	+	+
NO ₂	-	+	-	+	-	+	+	+
N ₂	+	-	+	-	+	-	-	-
Oxidase	+	-	+	+	+	-	+	+

Key: ONPG: O-nitrophenyl-b-D-galactopyranoside; ADH: Arginine dihydrolase; LDC: Lysine decarboxylase; ODC: Ornithine decarboxylase; CIT: Citrate; H₂S: Hydrogen sulfide production; URE: Urease; TDA: Tryptophan deaminase; IND: Indole; VP: the Voges-Proskauer test; GEL: Gelatinase; GLU: Glucose; MAN: Mannose; INO: Inositol; SOR: Sorbitol; RHA: Rhamnose; SAC: Sucrose; MEL: Melibiose; AMY: Amygdalin; ARA: Arabinose.

Table 2. Antibacterial susceptibility patterns of the isolated Gram negative bacterial pathogens (mm).

Gram negative antibiotics	<i>E. coli</i>	<i>Pseudomonas aeruginosa</i>	<i>Salmonella</i> sp.	<i>Vibrio cholerae</i>	<i>Vibrio parahaemolyticus</i>	<i>Shigella</i> sp.
Streptomycin	18.00	19.00	16.00	21.00	24.00	18.00
Gentamycin	0.00	19.00	15.00	22.00	22.00	19.00
Augmentin	0.00	0.00	15.00	20.00	0.00	15.00
Seprin	0.00	21.00	0.00	23.00	0.00	19.00
Ampicillin	0.00	0.00	0.00	23.00	0.00	0.00
Ceporex	0.00	0.00	0.00	0.00	0.00	0.00
Nalidixic acid	0.00	20.00	0.00	0.00	0.00	0.00
Reflaxin	13.00	19.00	17.00	22.00	23.00	19.00
Tarivid	8.00	16.00	21.00	0.00	21.00	22.00
Ciprofloxacin	18.00	21.00	15.00	23.00	24.00	22.00

Table 3. Antibacterial susceptibility patterns of the isolated Gram positive bacterial pathogens (mm).

Gram positive antibiotics	<i>Staphylococcus aureus</i>	<i>Clostridium</i> sp.
Rifampicin	24.00	0.00
Streptomycin	23.00	0.00
Chloramphenicol	18.00	16.00
Ciprofloxacin	19.00	21.00
Erythromycin	18.00	20.00
Levofloxacin	18.00	21.00
Gentamycin	17.00	0.00
Amoxil	0.00	0.00
Ampiclox	0.00	0.00
Norfloxacin	0.00	0.00

Table 4. Plasmid curing analysis of the isolated bacterial pathogens.

Bacterial pathogens	Antibiotics gene maker before curing	Antibiotics gene maker after curing
<i>Escherichia coli</i>	Tarivid, Gentamycin, Augmentin, Seprin, Ampicillin, Ceporex, Nalidixic Acid	NAGM
<i>Pseudomonas</i> sp.	Augmentin, Ampicillin, Ceporex,	NAGM
<i>Salmonella</i> sp.	Seprin, Ampicillin, Ceporex, Nalidixic Acid	NAGM
<i>Vibrio cholerae</i>	Ceporex, Nalidixic Acid, Tarivid	Nalidixic Acid
<i>Vibrio parahaemolyticus</i>	Augmentin, Seprin, Ampicillin, Ceporex, Nalidixic acid	NAGM
<i>Shigella</i> sp.	Ampicillin, Ceporex, Nalidixic Acid	NAGM
<i>Staphylococcus aureus</i>	Amoxil, Ampiclox, Norfloxacin	NAGM
<i>Clostridium</i> sp.	Rifampicin, Streptomycin, Gentamycin, Amoxil, Ampiclox, Norfloxacin	NAGM

NAGM = No antibiotic gene marker.

nalidixic acid before curing but lost the gene markers after curing; *Shigella* sp. possesses resistant gene markers to ampicillin, ceporex and nalidixic acid before curing but lost the gene markers after curing. *Staphylococcus aureus* possesses resistant gene markers to amoxil, ampiclox and norfloxacin before curing but lost the gene markers after curing and *Clostridium* sp. possesses resistant gene

markers to rifampicin, streptomycin, gentamycin, amoxil, ampiclox and norfloxacin before curing but lost the gene markers after curing with sodium dodecyl sulfate (SDS). These findings indicate that all the antibiotics resistant gene markers were found plasmid – mediated except nalidixic acid resistant gene marker to *Vibrio cholerae* that was found to be chromosomally – mediated representing

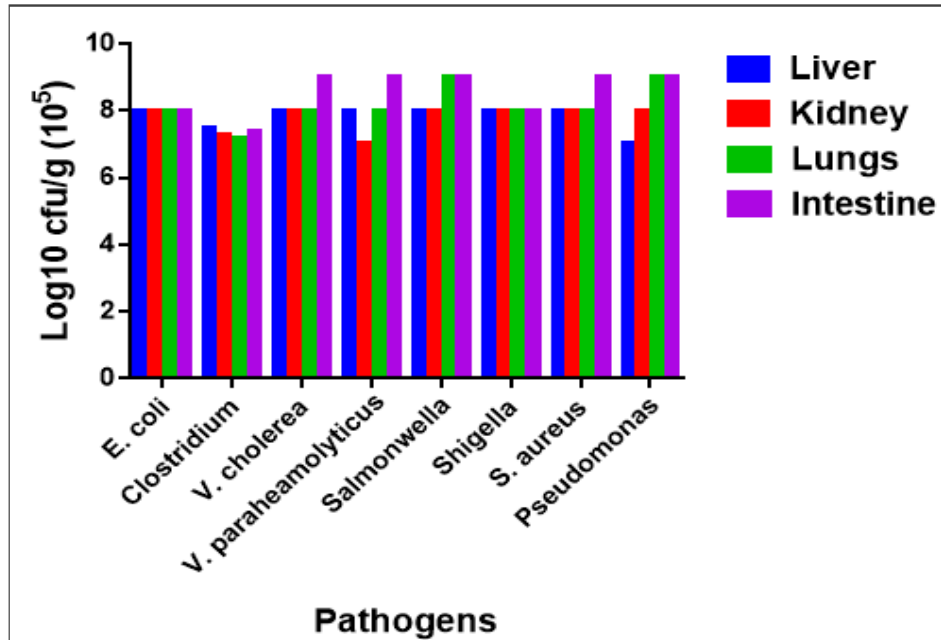


Figure 1. Number of organisms recovered after dissection of mice infected orally.

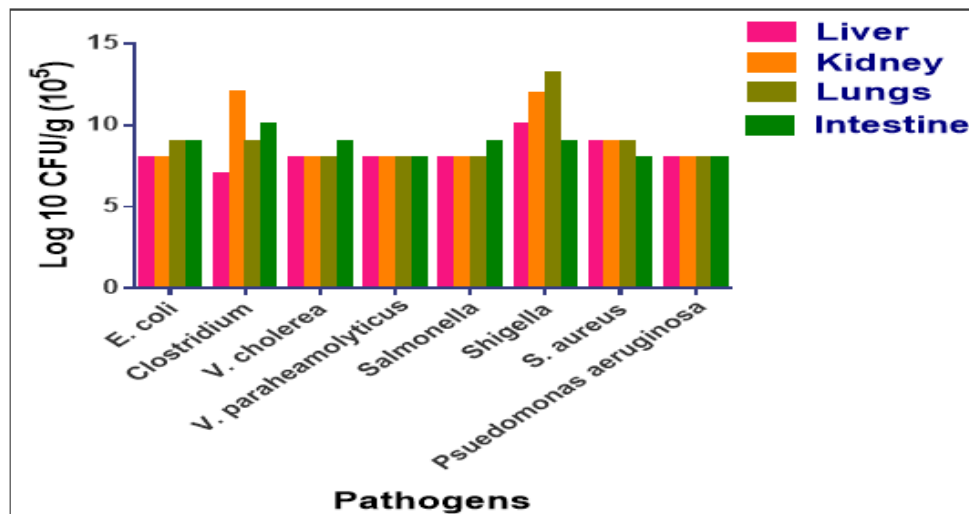


Figure 2. Number of organisms recovered after dissection of mice infected intraperitoneally.

87.5% of the bacterial pathogens that were plasmid-mediated and 12.5% that were chromosomally-mediated. Yah et al. (2007) reported that 34% of the strains of *Proteus* species lost their antibiotic resistance plasmids marker after sodium dodecyl sulfate (SDS) mediated curing and that plasmids have been known to play a major role in the dissemination of antibiotics resistance genes in a microbial population.

The microbial count of the harvested organs after the oral and intraperitoneal administrations are shown in Figure 1 and 2. From both results, the highest significant count (9 log CFU/g x 10⁵) was found in the intestine in all

the pathogens except *Shigella* sp. in intraperitoneal administration while the least significant count (7 log CFU/g x 10⁵) was found in the liver and kidney which were infected with *Pseudomonas* sp. and *V. parahaemolyticus*. These differences might be due to the high inhabitation of pathogens and the possible overgrowth of endogenous pathogens. Low level of the pathogens was recovered from the intestine of mice studied by Nwankwegu et al. (2016b) and is in disagreement with our finding.

The pathogens are known to cause Immunosuppression in hosts. Several cases caused by the pathogens have been reported and these have been traced to

Table 5. Enterotoxin production by the isolated bacterial pathogens.

Test organisms	Content of ileal loop	Length of segment (cm)	Measurement of fluid (mL)	Dilation index DI (mL/cm)	Enterotoxin response
<i>E. coli</i>	Slightly viscous yellow to very brownish and watery fluid	5	1.5	0.30	+
<i>Pseudomonas</i> sp.	Green brown, very viscous	5	1.0	0.20	+
<i>Salmonella</i> sp.	Trace red to brown and slightly viscous	5	1.3	0.26	+
<i>V. cholerae</i>	Brown, very viscous	5	1.5	0.30	+
<i>V. parahaemolyticus</i>	Slightly viscous ox-blood coloration	5	1.5	0.30	+
<i>Shigella</i> sp.	Trace of brown to red	5	1.0	0.20	+
<i>S. aureus</i>	Milk yellow to very brownish, watery fluid	5	2.7	0.54	+
<i>Clostridium</i> sp.	Chocolate brown, watery fluid	5	1.5	0.30	+
Control (0.5 mL water)	White and viscous fluid	5	0.08	0.02	-

contaminated food (Fontenot, 2000). They are known to produce toxins which enter the tissues, colonize and hijack nutrients (Ominisi and Oimage, 2006). The enterotoxin production by the isolated bacterial pathogens is presented Table 5. From the result, all the pathogens (*E. coli*, *Pseudomonas* sp., *Salmonella* sp., *V. cholerae*, *V. parahaemolyticus*, *Shigella* sp., *S. aureus* and *Clostridium* sp. produce enterotoxin (DI > 2.00 mL/cm) while control (0.02 mL/cm) were negative to enterotoxin production (DI < 2.00 mL/cm). This study showed that the isolated pathogens elicited positive response in ileal loops of the rabbit with DI values that ranged from 0.20 to 0.54 mL/cm. Similar observations was found by Idahosa et al. (2017) who reported that 62.5, 60 and 50% of the *E. coli*, *S. aureus* and *Salmonella* crude enterotoxins respectively elicited positive response in ileal loops of the rabbits with DI values that ranged from 0.29 to 0.42 mL/cm. Similar studies by Abbas et al. (2013) reported a DI of 0.2 to 0.48 mL/cm when enterotoxigenic *S. aureus* was introduced into rabbit ileal loops. The production of enterotoxins by these pathogenic bacteria species cause serious health problems when ingested and inhaled by humans. Staphylococcal enterotoxins are responsible for food poisoning, acute illness, fever, erythematous lesions and hypertension (Bhunia, 2008). Strains of *Salmonella* cause illnesses such as typhoid fever and salmonellosis due to ingestion of contaminated food (Ryan and Ray, 2004). *E. coli* toxins are responsible for numerous reports of contamination of foods and beverages causing diarrhea, urinary tract infections, respiratory illnesses and gastrointestinal infections (Idahosa et al. 2017). The colour and the viscosity of the accumulated fluids in the ileal loops after 24 hrs were pinkish (blood stained), yellowish, brownish and very watery when compared without the control loops that were white and viscous. The induced accumulated fluids showed destruction of the villus

structure with haemorrhage in the layer of the mucosa (Syngkon et al., 2010), observations consistent with the inflammatory type of illness seen in rabbit (Abbas et al., 2013). The release of enterotoxins from the organisms isolated from the poultry waste are capable of causing outbreak of food and water borne diseases through the contaminative route (Idahosa et al., 2016).

Conclusion

The whole study reveals that the poultry wastes are potential reservoirs of human pathogens. The antibiotics sensitivity test revealed that the bacterial pathogens were most susceptible to ciprofloxacin, rifampicin and levofloxacin and most resistant to ceporex, amoxil, ampiclox and norfloxacin. All the antibiotics resistant gene markers were found plasmid – mediated except nalidixic acid resistant gene marker to *Vibrio cholerae* that was found to be chromosomally – mediated representing 87.5% of the bacterial pathogens that were plasmid - mediated and 12.5% that were chromosomally – mediated. It also revealed that increase in microbial count/microbial index especially in the intestines either through oral or intraperitoneal administration. More so, all the bacterial pathogens were found to produce enterotoxin (DI > 2.00 mL/cm) thereby establishing the pathogenic characters of these organisms. Hence, enlightenment, public awareness and health education of daily clean up and proper disposal system of these wastes should be enhanced in order to get rid of their dangerous outcomes.

AUTHORS CONTRIBUTION

This work was carried out in collaboration between all

authors. Authors BOU and DKA designed the study, performed the statistical analysis, wrote the protocol and first draft of the manuscript. Author ELO and ISE managed the analyses of the study. Author SO managed the literature searches. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest in regard to the publication.

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