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Detection of *blaCTX*, *blaSHV* and *AAC-3-IV* resistance genes among selected *Klebsiella* sp, *Escherichia coli* and *Proteus mirabilis* isolated from feacal droppings of *Meleagris gallopavo f. domestica*

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Abstract: The study reported the detection of resistance genes among selected isolates from feacal droppings of *Meleagris gallopavo* in Ile-Ife, Nigeria; with the view to determining their antibiotic susceptibility profile and nature of resistance genes. Faecal droppings of turkey were collected at random from various poultries into sterile universal bottles and were cultured on MacConkey agar using pour plate technique. Antibiotic susceptibility of isolates was examined and detection of resistance genes was carried out by Polymerase Chain Reaction (PCR) using appropriate primers. Eight of the 258 samples of faecal droppings of turkey were devoid of any visible bacterial growth. Bacterial isolates identified include *Klebsiella* sp. (26%), *Escherichia coli* (22%), *Proteus* sp (8%), *Aeromonas* (8%), *Enterobacter* sp. (8%), *Citrobacter* sp. (7%), *Serratia* sp. (7%), *Yersinia* sp. (6.1%), *Shigella* sp. (4.1%), and *Providencia* sp. (3.3%). Resistance to antibiotic varied among the isolates. 32% of the isolates was resistant to ceftazidime, (43 %) to cefuroxime, gentamycin (23%), 42% to cefixime, and 100% to Augmentin. The isolates were 94 % susceptible to nitrofurantoin. Three of the twelve selected MAR bacterial harboured *bla*-CTX-M gene, *K. ornithinolytica* harboured *AAC*-3-IV gene. The study concluded that poultry litters could serve as an environmental reservoir of multiple antibiotic resistant bacteria. The detection of the resistance genes have great health and economic consequences. Therefore, effective hygiene of poultry environment should be emphasized. Treatment options for bacterial infections from this study include; nitrofurantoin, gentamicin, ofloxacin and ciprofloxacin, if properly administered.

[Agun Tosin Funmilayo, Oluduro Anthonia Olufunke, Thonda Oluwakemi Abike,. **Detection of *blaCTX*, *blaSHV* and *AAC-3-IV* resistance genes among selected *Klebsiella* sp, *Escherichia coli* and *Proteus mirabilis* isolated from feacal droppings of *Meleagris gallopavo f. domestica*.** *Rep Opinion* 2020;12(10):15-23]. ISSN 1553-9873 (print); ISSN 2375-7205 (online). http://www.sciencepub.net/report. 2. doi:10.7537/marsroj121020.02.

Keywords: Faecal droppings, Resistance genes, *AAC-3-IV*, *Meleagris gallopavo, Proteus mirabilis*

Introduction

Poultry is an essential component of the Nigerian economy, providing income for small scale farmers and a good source of high quality protein for the ever-growing population of Nigeria. In livestock production, poultry occupies a prominent position in the provision of animal protein and this accounts for about 25% of local meat production in Nigeria (Agbaje *et al*., 2010). Turkey occupies an important position next to chicken, duck, guinea fowl and quail in contributing the most evolving sector, which is playing a significant role in augmenting the economic and nutritional status of varied population. They form almost two percent of the total poultry population. They are reared for meat only and its meat is the leanest among other domestic avian species. Turkeys are classified based on seven standard varieties that are available, Bronze, White Holland, Bourbon red, Narragansett, Black, Slate and Beltsville small white (Majdood *et al*., 2012).

Turkey meat has nutritional and sensorial properties which make it almost ideal raw material for rational and curative nutrition. People prefer turkey meat because of its leanest nature. The protein, fat and energy value of turkey meat are 24%, 6.6%, 162 Calories per 100 gm of meat. Mineral such as potassium, calcium, magnesium, iron, selenium, zinc and sodium are present (Majdood *et al*., 2012). It is also rich inessential amino acids and vitamins like niacin, vitamin B6 and B12. It is rich in unsaturated fatty acids and essential fatty acids and low in cholesterol. Turkeys can be reared under free range or intensive system.

Food animals are increasingly recognized as a reservoir for ESBL-producing strains. These strains can be transmitted via the food chain. Faecal contamination might occur during animal slaughtering, milking, and/or processing, and the growth of the contaminating bacteria may occur

during the product transport and storage phases. Consequently, without good hygienic practices, foods may act as a vehicle of transfer of β-lactam resistant bacteria to the gastrointestinal tract of consumers (Overdevest *et al*., 2011).

Poultry faeces are waste products excreted by poultry fowls such as chickens, ducks, turkey and geese. It can also be defined as the by-product that resulted from the digestion of food intake by poultry birds. Faeces can be in form of semi-solid or water and the colour varies among the species of birds. Some are whitish, ashes and dark brown in colour. There are several billions of bacteria present in poultry faeces including pathogenic and non-pathogenic species, the normal flora and the opportunistic ones (Adegunloye, 2006).

Poultry faeces are a complete nuisance in an age where there is concern with pollution of the environment. It is moist and because of its nutrient and organic matter content, the manure is a suitable breeding ground for pestiferous flies like houseflies, flesh flies, black garbage flies and biting stable flies. The manure is also a source of odour, caused by the production of fatty acids such as butyric, valeric, capronic and caprylic acids (Adegunloye, 2006). The high nutrient content of bird excrement provides an excellent sanctuary for potentially harmful organisms. Bird droppings do pose a public health risk and cause illness. Humans become infected by inhaling dust containing dried faeces, urine, or respiratory secretions of infected birds (Chang *et al.,* 2004). Other sources of exposure include a bite from an infected bird and handling the plumage and tissues of infected birds. Poultry droppings cause corrosion on roofs around the dumpsite due to accumulation of droppings while the birds are resting on them (Horta *et al*., 2002).

In some developing countries like Nigeria, antibiotics are sold over the counter without a prescription which compounds the problem. In human medicine, the major problem of the emergence of resistant bacteria is due to misuse and overuse of antibiotics by doctors as well as patients (Witte, 1998). Other practices contributing towards resistance include the addition of antibiotics to feeds of livestock (Matthew *et al*., 2007). Antibiotic-resistant bacteria in domestic animals such as poultry, beef and swine are well documented and have been implicated as reservoirs for multidrug-resistant food borne pathogens (Prithwiraj *et al*., 2008). Also unsound practices in the pharmaceutical manufacturing industry such as production of counterfeit drugs can contribute towards the likelihood of creating antibiotic resistant strains (Larson *et al*., 2009). Emergence of bacteria resistant to antibiotics is common in areas where antibiotics are used, but occurrence of antibiotic resistance bacteria is also increasing in freshwater basins (Aibinu *et al*., 2004).

Methods

A total of 258 samples of faecal droppings of turkey (*Meleagris gallopavo f. domestica*) were collected at random from Obafemi Awolowo University Teaching and Agricultural Research Farm poultry, individual poultries, and commercial market places in Ile-Ife, Nigeria. The samples were collected by bringing out the birds into a well cleaned room where they were observed until they excreted, the droppings were carefully picked with the spatula attached to the sterile universal bottle and were immediately transported to the laboratory for analysis. The physical properties of the samples were observed. One gram of the faecal sample were weighed and serially diluted.

1 ml of the serially diluted culture were plated in duplicates on MacConkey agar using pour plate method, and incubated at 37^{o}C for 24 h. Each colony was isolated in a pure form by sub-culturing on freshly prepared culture agar plates incubated at 37^{o}C for 24 h. Distinct colonies were picked for further studies. Distinctive morphological properties of each pure culture on the agar plates were observed.

Preliminary identification of bacterial isolates was performed using colony and morphological characteristics of isolates. Bacterial isolates were further characterized on the basis of gram staining and biochemical reactions of the bacterial isolates to some reagents and media. Bacterial isolates were identified using the Bergey's Manual of Determinative Bacteriology (Holt *et al*., 2010). The identity of some of the isolates was further confirmed using Analytical Profile Index (API) kit (BioMerieux, Incorporation, France).

Antibiotic susceptibility of the isolates was examined using the Kirby-Bauer's disc diffusion method (Bauer *et al.,* 1966; Thonda *et al*., 2018) and interpreted according to the guidelines of Clinical Laboratory Standard (CLSI, 2013).

Extraction of DNA

A colony each of the selected multiple antibiotic resistant isolates was picked and an overnight nutrient broth of the colony was prepared at 37^{o}C for 24 h. The tubes containing an overnight broth culture of the isolates were vortexed to re-suspend the cells. One milliliter of the vortexed broth culture of the isolates was then transferred into an already labeled Eppendorf tube and centrifuged at 13,000 rpm for 10 min. The supernatant in the tubes was then discarded and blotted on paper towel. One milliliter of sterile distilled water was then added into each of the tubes. The tubes were then vortexed and centrifuged at 13,000 rpm for 10 min. The supernatants were again

discarded and blotted on the paper towel. After this, 200 µl of sterile distilled water was added and vortexed to homogenize the pellets. The tubes were then boiled at 100°C for 10 min. After boiling, the tubes were again vortexed and centrifuged at 13,000 rpm for 10 mins. The supernatants obtained were then transferred into another pre- labelled Eppendorf tubes by gentle aspiration using a micropipette and kept in the refrigerator 4°C until needed for Polymerase Chain Reaction (PCR).

Molecular Detection of Resistance Genes in the Selected Isolates

Multiple antibiotic resistant isolates that were resistant to Beta-lactam, aminoglycoside, nitrofurantoin and flouroquinolones antibiotics were selected for the detection of *bla*-SHV (393 bp), *aac*-3-IV (286) and *bla*-CTX-M (585 bp) resistance genes.

Amplification reaction were carried out in a volume of 25µl of a Polymerase Chain Reaction (PCR) mixture containing 1.5mM $MgCl_2$, 200µM each of dATP, dCTP, GTP and dTTP, 0.2 µl primer 2,1.5 µl of genomic DNA and 0.1 µl of Taq polymerase. The thermocycler (PRIME, UK) was programmed for optimum condition. The PCR reaction was performed as follows: an initial denaturation at 95°C for 3 min, 30 cycles at 95°C for 30 sec, annealing temperature at 54°Cfor *bla* (CTX), 55°C for *bla* (SHV) and 61°C for *aac (*-3-IV*)* genes detection for 60 sec, elongation at 72°C for 60 sec and final extension period at 72°C for 10 min. Gel electrophoresis was used to detect amplified DNA products. A volume of 10µl of amplified PCR products was subjected to electrophoresis at 80 volts in horizontal gel containing 1.5% agarose in Tris-borate buffer (45 mM Tris borate, 1 nM EDTA) for about 2 h. A 100 base pair marker was added to one of the lanes in the gel to serve as reference. The gel was stained with ethidium bromide (Promega, Madison, USA), exposed to Ultra violet light to visualize the amplified products and photographed in an Ultra violet-transilluminator. Significant differences and relationship between various data obtained were compared using T- test method.

Results

The breeds and age range of the breeds are represented in table 1. The age range of the local breed and foreign breeds was 6-19 months and 18-19 months respectively. Of the 258 faecal samples collected, 76.7% were from local turkeys and 23.2% from imported turkeys.

The physical properties observed on the faecal samples includes colour, consistency, mucoid and presence of blood as represented in table 2. 39.5% of the turkey were black in colour. However, 48.4% were formed in consistency while 62% of the faecal sample showed non mucoid and 100% of the faecal dropping had no blood stains.

A total number of 242 Gram-negative bacterial isolates were recovered from the faecal droppings of turkey as *Klebsiella* sp had the highest percentage of 26%, followed by *E. coli* (22%), *Enterobacter* sp. (8%), *Proteus* sp (8%), *Aeromonas* sp (8%), *Citrobacter* sp (7%), *Serratia* sp (7%), Y*ersinia* sp (6.4%), *Shigella* sp (4.1%) while the least was *Providencia stuartii* (3.3%) as depicted in table 3.

Table 1: Number of positive samples in respect to the breeds and age

Breeds	Age range	No of samples	No of samples positive for bacteria (%)
Local	6-19 months	198 (76.7%)	190 (79%)
Foreign	18-19 months	60 (23.2%)	52 (21%)
	Total	**258**	**242**

Table 2: Physical properties of the faecal droppings of *Meleagris gallopavo f. domestica* raised for consumption in Ile – Ife

Physical properties	Characteristics	Number positive	Percentage (%)
Colour	Black	102	39.5
	Brown	92	35.6
	White	64	24.8
Consistency	Formed	125	48.4
	Semi-formed	92	36.6
	Soft	41	15.8
Mucoid	Mucoid	98	37.9
	Non-mucoid	160	62.0
Presence of blood	Stained	0	0
	Unstained	258	100

Table 3: Occurrence of Bacterial Isolates Cultured from Faecal Droppings of Turkey

Bacterial Identity	No of occurrence	Percentage (%)
Klebsiella sp	63	26
Citrobacter sp	16	7
Escherichia coli	53	22
Serratia sp	18	7.4
Yersinia sp	15	6
Proteus sp	20	8
Shigella sp	10	4.1
Enterobacter sp	20	8
Aeromonas sp	19	8
Providencia stuartii	8	3.3
Total	**242**	**100**

The antibiotic susceptibility profile of 120 bacterial isolates to various antibiotic was tested. One hundred and twenty isolates were resistant to augmentin (100%), cefuroxime (43%), cefixime (42%), ceftazidime (32%), 23% resistant to gentamycin, 21% to ciprofloxacin, 16 % to ofloxacin, while only 1% was resistant to nitrofurantoin as depicted in figure 1.

The susceptibility of the isolates to the antibiotics were 78% to ofloxacin, 75% to gentamycin, 57% to ciprofloxacin, 36% to cefixime, and 32% were susceptible to ceftazidime, 24% to cefuroxime while none of the isolates were susceptible to Augmentin. (Figure 1)

Forty-seven percent of the isolates showed multiple resistance patterns to 3-4 different classes of antibiotics. The classes of antibiotics used included Beta-lactams (augmentin, ceftazidime, cefuroxime and cefixime), fluoroquinolones (ciprofloxacin and ofloxacin), Aminoglycosides (gentamycin) and nitrofurans (nitrofurantoin). Multiple antibiotic resistance was defined as resistance to at least 3 or more different classes of antibiotics.

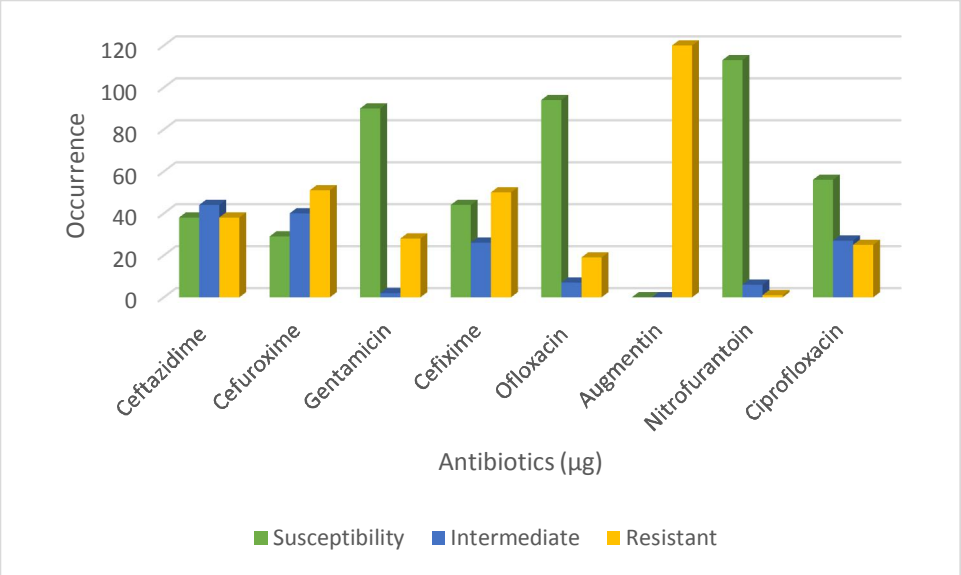

Figure 1: Antibiotic Susceptibility Profile of Bacterial Isolates Cultured from Faecal Droppings of Turkey

The agarose gel electrophoresis of AAC-3-IV (286 bp) and *bla*-CTX-M (585 bp) genes in selected multiple antibiotic resistant bacterial isolates is depicted in figure 2 and 3. The Lane designated 1- 6 were *Klebsiella* sp; 7- 9 were from *E. coli* and 10-12 were *P. mirabilis*. Only *K. ornithiolytica* in plate 1 on the lane designated as A showed AAC-3-IV (286bp) resistance gene.

In plate 2, only three of these isolates (designated as 3 – 5) showed *bla*-CTX-M (585 bp) resistance gene; 3 and 4 were *K. Oxytoca* while 5 was *K. ornithiolytica.*

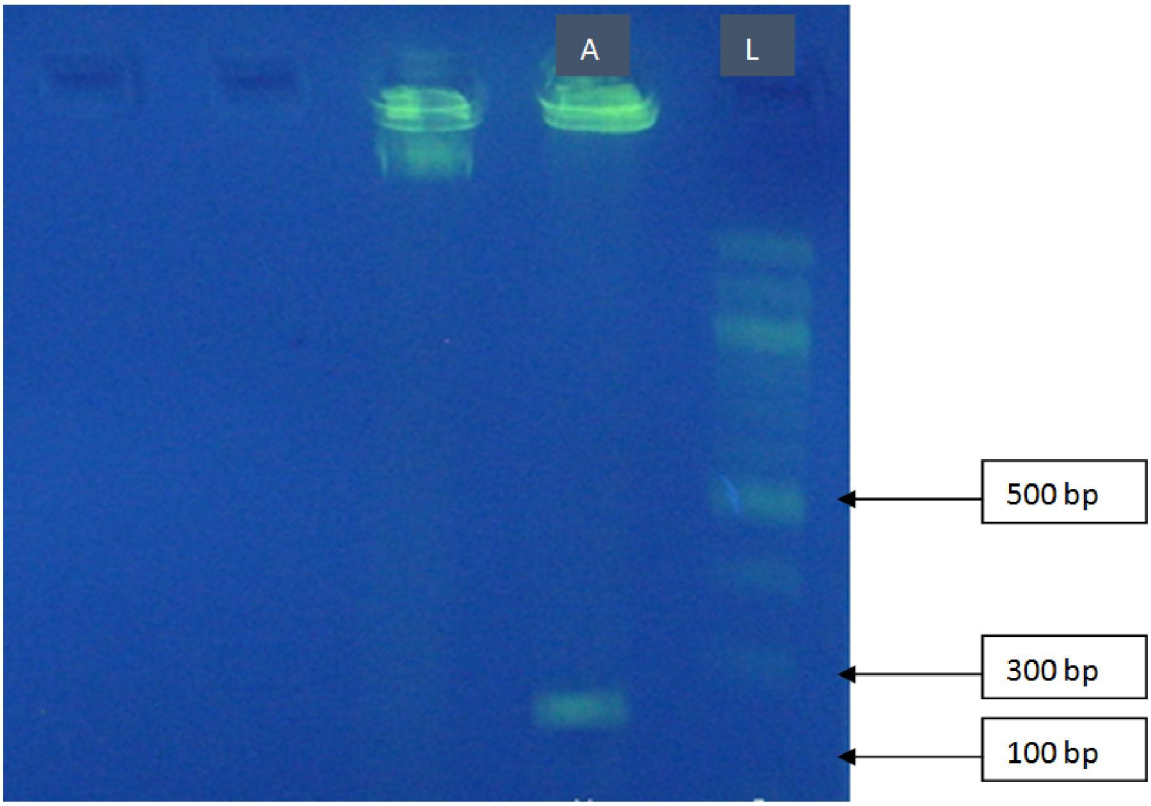

Plate 1: Agarose gel electrophoresis of the amplification product coding *AAC*-3-IV (286bp) L = 100 bp, A = (K*lebsiella ornithiolytica*)

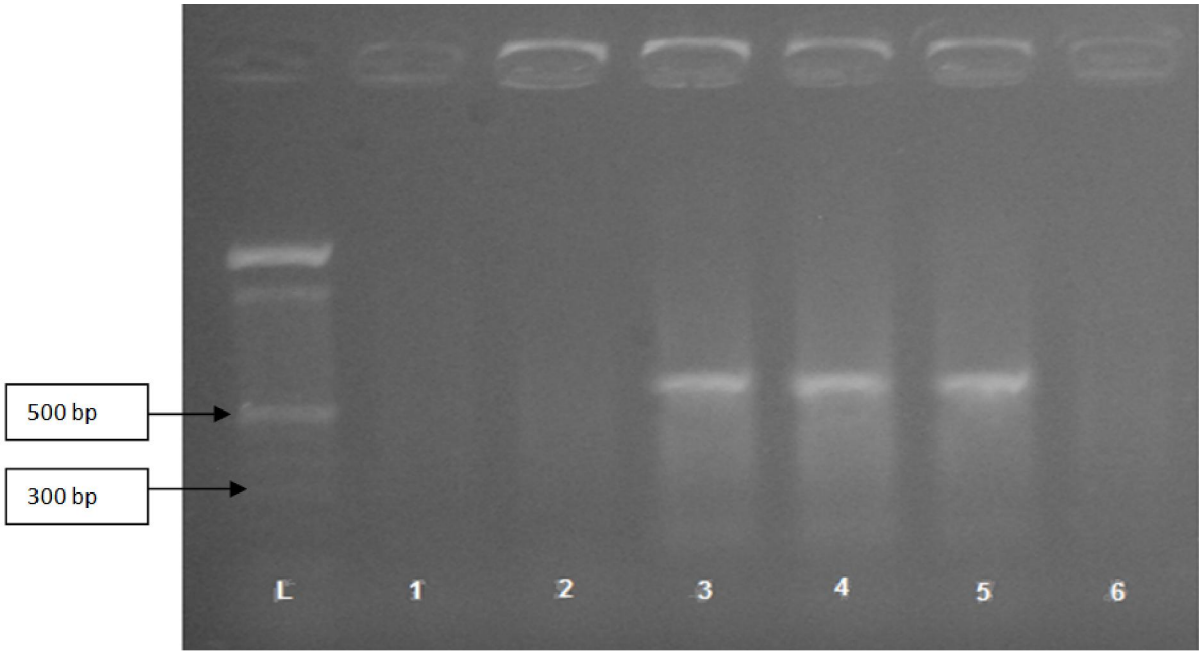

Plate 2: Agarose gel electrophoresis of the amplification product coding *bla*-CTX-M (585 bp) gene in selected MAR Isolates L (LADDER) = 100bp, 3 and 4 =*Klebsiella oxytoca*, 5 =*Klebsiella ornithiolytica*

Discussion

The detection of the organisms in this study agreed with the fact that the bacteria are part of the enteric flora of the birds. However, it was observed from the results obtained that there was a slight variation in the carriage of the organism isolated in this study. This could be due to environmental settings in which the birds are raised and water sources of the birds (Ezekiel *et al*., 2011).

In this study, *Klebsiella* species (26 %) was identified as the most prevalent organism, then followed *by E. coli* (22%) which is in contrast to Ojo *et al*., (2012), Okwori *et al*., (2013) and Kennedy and Wilbard (2015) who reported that *Escherichia coli* (50%) are the most prevalent pathogen in poultry droppings and followed by other organisms.

More importantly, all the organisms were tested for their susceptibility to selected antibiotics, an incidence of resistance against individual antibiotics was confirmed. The high level of resistance of isolates to some of the antibiotics tested in the study is of great concern. Studies have shown that ciprofloxacin, aminoglycosides, and β-lactam antibiotics are widely effective in the treatment of Gram-Negative bacteria. However, resistance to these antibiotics is already emerging (Thapa *et al.,* 2009). This finding is in consonance with other studies (Ajayi and Egbebi, 2011) that have also confirmed high incidence of antibiotic resistance among bacteria recovered from poultry droppings. As observed in this study, the isolates were particularly resistant to augmentin, cefixime and cefuroxime which a similar pattern was observed in the study of Van den Boggard, *et al*., (2001), Ezekiel *et al*., (2011) and Anthony *et al*., (2012) which were confirmed to be the most commonly used antibiotics in this study area.

Gentamicin is another commonly used antibiotic in this study area, but resistance against the antibiotics is one of the lowest. The low resistance against gentamicin despite its use could definitely be attributed to intrinsic factor, relatively high price that affects easy accessibility which is in agreement with the findings of Ajayi and Egbebi, (2011) and Kennedy and Wilbard (2015). However, the sensitivity to the fluoroquinolone (ofloxacin) was observed at 78% across all isolates, a trend similar to the report of Ezekiel *et al,* (2011) at 70%. This prevalence of resistance could be attributed to the heavy dependence on these antibiotics for therapeutic and sub-therapeutic uses which create a selective pressure for the emergence of antibiotic resistant bacteria in the fecal droppings that were sampled (kilonzo *et al*., 2008).

In this study, nitrofuratoin also showed high sensitivity at 94% which is in contrast to Ezekiel *et al*., and Oyinloye *et al*., (2015) which showed greater resistance at 56.17% and 80% respectively. The statistical analysis of percentage resistance in the bacterial isolates showed that there was no significant difference ($p<0.05$)

The presence of antibiotic resistant Gram-negative bacteria among poultry droppings presents serious implication in view of public health significance among poultry farms in the location.

Over the years, the consumption of turkey and poultry meat have increased and this carries with it the risk of infection of humans through direct contact with the poultry droppings and the possibility of cross contamination of food and drinking water sources with antibiotic-resistant bacteria according to Ajayi *et al,* (2011), it could also be through agricultural usage of poultry droppings as source of manure according to Orji *et al* (2005). This may lead to an increase in the prevalence of clinical illness associated with these enteric organisms.

Bacteremia, lower respiratory tract infections, skin and soft-tissue infections, urinary tract infections (UTIs), endocarditis, intra-abdominal infections, septic arthritis, meningitis, osteomyelitis, central nervous system (CNS) infections, ophthalmic infections and various forms of infection among immuno-compromised individuals and infants have been associated with Enterobacteriaceae (Singlton and Sainsbury, 2001; Ko *et al.,* 2002; Paterson, 2006; Jacobsen *et al*. 2008; Samonis *et al.,* 2009; Liu and Matsumura, 2010).

Klebsiella sp is one of the most common infectious bacteria affecting poultry, causing great economic losses. It also posses food safety and antimicrobial resistance threats, as it may act as a source of contamination of poultry meat and eggs. It also leads to weakness, gasping, pump handled respiraton, dyspnoea, mucous discharge, mortality, swelling of sinuses, facial oedema, trachetis, exuative pneumonia, pleuritis, air sacculitis, pericarditis, sinusitis, drop in egg production and poor egg quality (Zorman *et al*., 2000; Canal *et al*., 2005).

In this study, *Klebsiella* species harboured $bla_{\text{CTX-M}}$ (25%) and AAC-3-IV (8.3%). Detection of Cefotaximase-Munich *bla*-CTX-M (585bp) resistance genes in this study was similar to Paterson, (2003) who reported detection of *bla*-CTX (585 bp) in *Klebsiella* sp. and also in contrast to Flannery *et al* (2009) who detected same resistance genes pattern in *Proteus* species which could mean that they were from the same origin before spreading. There was a downward trend in the prevalence of *AAC*-3-IV gene which is similar to the findings of Wang *et al*, (2013). None of the representative isolates examined harboured bla_{TEM}, bla_{SHV} resistance genes.

The presence of these resistance genes shows that the isolates may produce toxins, chemical substances capable of causing infection in the host (consumers or handlers) which may lead to infection in the environment.

Conclusion

The present result provides evidences that poultry litter can serve as an environmental reservoir of multiple antibiotic resistance bacteria and hence a potential route for the entry of multiple antibiotic resistant zoonotic pathogens into human population which is detrimental to human health. It is very important to monitor the resistance to antibiotics not only in human bacterial pathogens but also in pathogenic and commensal bacteria of animal origin. Also, the carriage of antibiotic resistant enteric bacteria by flies in the poultry production environment increases the potential for human exposure to antibiotic-resistant bacteria.

In conclusion, antibiotics such as gentamicin, nitrofuratoin, ciprofloxacin and ofloxacin are good treatment options for bacterial infection from turkey. Proper hygiene of poultry environment and personal hygiene should be taken into consideration.

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Recommendation

1. Gentamicin, nitrofuratoin, ciprofloxacin, ofloxacin are still effective against *Klebsiella oxytoca* and other organisms isolated from faecal droppings of turkey in the study area.
2. Indiscriminate use of antibiotics in poultry farms should be discouraged.
3. Effective hygiene of poultry environment and personal hygiene should be emphasized.
4. Poultry handlers should consult veterinary doctors for proper care of the animals.
5. Continuous investigation or surveillance of resistance pattern, nature of the resistance and virulence genes in pathogenic microorganisms will reduce the incidence of multiple antibiotic resistance.

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10/14/2020