



***Enterobacter* species Obtained from Poultry Droppings of Selected Farms in Southwest Nigeria is a Hotspot of Antibiotic-Resistant Genes**

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Abstract

This study aimed to determine the antibiotic resistance patterns of *Enterobacter* spp. isolated from poultry droppings in the southwest of Nigeria. *Enterobacter* species were isolated from poultry droppings of selected farms in the southwest, Nigeria, between February and June, 2019. The isolates were identified by MALDITOF-MS and tested against 20 antibiotics using the Kirby-Bauer disk diffusion method. Genomic DNA and plasmids of the isolates were extracted using the sodium dodecyl sulfate protocol and alkaline lysis method, respectively. Antibiotic resistance genes were detected with specific primers using the polymerase chain reaction. Seventy-two *Enterobacter* spp. comprising *E. cloacae* (n=52), *E. asburiae* (n=12), *E. kobei* (n=7), and *E. ludwigii* (n=1) were isolated. Antibiotic resistance patterns revealed that all the isolates were resistant to amoxicillin, cefpodoxime, and cefixime, while high resistance to cefotaxime (98.6%), ceftriaxone (86.1%), and cefuroxime (84.7%) was observed. All *Enterobacter* species were multiple antibiotic-resistant, and plasmids were detected in 43.1% of the isolates, and 52.8% harbored at least one antibiotic resistance gene. This study showed that multidrug-resistant *Enterobacter* spp. are present in poultry droppings in southwest Nigeria, and also revealed that antibiotic resistance genes were plasmid and chromosomally borne in the *Enterobacter* species, thus, might be posing a serious health threat to humans and animals.

Keywords: Antibiotic resistance, *Enterobacter* species, Poultry waste, Resistance genes, Southwest Nigeria

1. Introduction

Poultry is one of the most consumed meats globally, and its production has increased over the years to meet the demand of the world's growing population [1]. Faecal waste from poultry can harbor pathogenic microorganisms, which can pose risks to the livestock's health and the environment. The presence of pathogenic bacteria in poultry droppings can cause a variety of diseases in birds. Diseases can spread through drinking water, feeds, and secretions from diseased birds [2]. The high rise in poultry production, combined with the lack of proper regulatory supervision, particularly in developing countries where antibiotic misuse is common, poses a significant challenge [3]. In many countries of the world, poultry is raised with a wide variety of antibiotics, most commonly administered orally as prevention for the treatment of infectious diseases, or as a diet to encourage growth and production. The overuse and misuse of antibiotics has led to an increase in the occurrence of antibiotic resistance in the environment, animals, and humans [4].

Enterobacter species are one of the ESKAPE (*Enterococcus faecium*- *Staphylococcus aureus*- *Klebsiella pneumoniae*- *Acinetobacter baumannii*- *Pseudomonas aeruginosa*- *Enterobacter* species) groups of pathogens, which are on the World Health Organization priority list for developing new antibiotics [5]. Members of the *Enterobacter cloacae* complex (ECC) are common nosocomial pathogens that have been implicated in a wide variety of infections, including septicemia, pneumonia, and urinary tract infections. The healthcare industry is typically linked to the risk factors for acquiring these multidrug-resistant bacteria. There have been reported potential environmental transmission routes (exposure to pet animals and livestock) of these bacteria to people

outside of healthcare facilities. Pathogens that can spread from animals to humans have been reported in poultry and poultry products [6]. Previous studies had documented the presence of *Enterobacter* spp. among different bacterial isolates in poultry droppings from a state in the North central part of Nigeria and Ethiopia [2, 4]. Antibiotic resistance is a global challenge due to the increasing resistance to antibiotics, which poses a major threat to the treatment of both human and animal diseases [7]. Globally, the prevalence of multidrug-resistant Gram-negative bacterial infections with limited treatment options in intensive care units has significantly increased. The recent development of resistance to last-resort antibiotics and broad-spectrum antibiotic resistance has increased the interest in this group of organisms [8].

Chromosomal resistance or acquisition of genetic material such as plasmids originating from bacterial populations of the same or different species are the major challenges of antibiotic resistance. Chromosomally borne antibiotic resistance genes have been reported in *Enterobacter* spp. of poultry origin [6]. Plasmids are extrachromosomal genetic entities that can replicate independently and are mainly found in bacteria. The ability of plasmids to replicate extensively and spread across bacterial populations made them act as genetic vehicles for the transfer of functional and accessory genes that give bacteria distinct characteristics across populations. Gene transfer mediated by plasmids and other genetic elements can occur within the same bacteria species and different bacteria species (horizontal transmission) [9].

Antibiotic resistance genes such as the *qnr* genes, which mediate quinolone resistance through plasmids, have been reported and may contribute rapidly to the increase of quinolones resistance in bacteria. In Enterobacteriaceae, different *qnr* gene variants (*qnrA*, *qnrB* and *qnrS*) have been reported [10]. Resistance to third generation cephalosporins in Enterobacteriaceae (group II Enterobacteriaceae, including *Enterobacter* spp., *Serratia* spp., *Providentia* spp., *Morganella morganii*, *Citrobacter freundii*, and *Hafnia alvei*) is mediated by *AmpC*, and it is mainly encoded on the chromosome [11]. *AmpC* can also be encoded on plasmids; these plasmid-mediated *AmpC* (*pAmpC*) genes are derived from chromosomal *AmpC* genes of group II Enterobacteriaceae and were primarily found in Enterobacteriaceae that lack or have low expression of chromosomally encoded *AmpC*, such as group I Enterobacteriaceae, including *Klebsiella* spp., *Proteus* spp., *Salmonella* spp., *Escherichia coli*, and *Shigella* species. Multidrug resistance is frequently linked to the presence of *pAmpC*; thus, infections caused by Enterobacteriaceae that produce *pAmpC* have significant rates of treatment failure and mortality [11]. Ribosomal methylation, which leads to less susceptibility to macrolides, is caused by methyltransferase encoded by *ermB*. Acquired resistance to lincosamides, streptogramins, and macrolide mediated by the *ermB* gene is mostly widespread in bacteria host such as the Enterobacteriaceae [9].

The use of untreated poultry litter as manure on agricultural lands in developing countries such as Nigeria can promote the proliferation of commensal and pathogenic bacteria carrying antibiotic resistance genes in water, vegetables, and crops. Thus, mobile genetic elements have the ability to spread antibiotic resistance genes to other bacteria. In Nigeria, multidrug resistance and antibiotic resistance genes in *Enterobacter* spp., as one of the ESKAPE bacteria from poultry droppings has not been well-studied. Therefore, the aim of the present study was to determine the antibiotic resistance patterns and presence of antibiotic resistance genes in *Enterobacter* spp. isolated from poultry droppings of some selected farms in the southwest, Nigeria.

2. Materials and Methods

2.1 Sample collection

Samples were collected between February and June 2019 from 27 selected farms in the six states of the southwest, Nigeria, including: Oyo (7), Osun (6), Lagos (5), Ondo (4), Ogun (3), and Ekiti (2). The different farms belonged to three categories of large (7), medium (9), and small (11) farms [12]. A total of 56 pooled poultry dropping samples were collected from layer chicken (24), broiler chicken (16), cockerel (8), Noiler (4), and combined litters from the dumpsites (4).

2.2 Isolation and identification of *Enterobacter* species

MacConkey agar was used for isolation using the standard pour plate method, and identification was done using Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF-MS) as previously described [3].

2.3 Antibiotic susceptibility testing of the isolates

The susceptibility of the isolates were tested against 20 different antibiotics using the disk diffusion Kirby–Bauer standard method. The antibiotics include: tetracycline 30 µg, streptomycin 10 µg, gentamicin 10 µg, netilin 30 µg, amikacin 30 µg, azithromycin 15 µg, chloramphenicol 30 µg, ampicillin 10 µg, amoxicillin 10 µg, amoxillin-clavulanic acid 20/10 µg, ciprofloxacin 5 µg, norfloxacin 10 µg, levofloxacin 5 µg, co-trimoxazole

25 µg, cefaclor 30 µg, cefuroxime 30 µg, ceftriaxone 30 µg, cefotaxime 30 µg, cefixime 5 µg and cefpodoxime 10 µg. The interpretation was according to CLSI guidelines [13].

2.4 Genomic DNA extraction

The genomic DNA of the isolates was extracted using the Sodium Dodecyl Sulfate (SDS) protocol [14]. Pellets of overnight cultures were inoculated into 200 µL of DNA extraction buffer, vortexed for five minutes, and incubated at 70 °C for 30 mins. At 10-minute intervals, vortexing was done and allowed to cool down at room temperature. Chilled absolute alcohol (500 µL) was added to the mixture, vortexed, centrifuged at 10000 rpm for five minutes, and the supernatant was discarded. The pellets were washed twice with 500 µL of chilled 70% alcohol and dried overnight. Forty (40) µL of Tris-EDTA (TE) buffer containing 10µg/mL RNase, an elution buffer was added and thoroughly mixed. The suspension was centrifuged at 1200 rpm for five minutes, and the supernatants were carefully pipetted into sterile labelled Eppendorf tubes. The concentration and purity of the extracted DNA were determined using a Nanodrop spectrophotometer (ND 1000 Nanodrop Tech D139) at 260nm and 280nm absorbance.

2.5 Plasmid extraction

The plasmid extraction was done as previously described [15]. Briefly, plasmids were extracted using the alkaline lysis method. Pure colonies of 24-hour-old culture were inoculated into 1.5 mL of Luria Bertani broth, and the suspension was incubated overnight and centrifuged for 1 min at 12000 rpm. The supernatant was decanted, and the pelleted cells were resuspended in 150 µL of buffer. The lysis solution (200µL) was added and mixed until the solution became viscous; 300 µL of neutralization solution was added and mixed. It was centrifuged at 14000 rpm for five minutes, and 300 µL of the supernatant was transferred into a new 1.5 mL tube, and isopropanol (300µL) was added to precipitate plasmid DNA. The mixture was mixed and incubated at -80 °C for 30 minutes, centrifuged at 14000 rpm for 5 minutes, the supernatant was decanted, and 600µL of 70% ethanol was added. Centrifugation was done at 14000 rpm for five minutes, and the precipitated plasmid DNA was allowed to dry for 30 mins. The pellets were dissolved in 40µL of TE buffer [15].

2.6 Detection of antibiotic resistance genes

Screening for the following antibiotic resistance genes was done using PCR: tetracycline efflux pump (*tetA*), chloramphenicol resistance protein (*cmlA*), erythromycin resistance methylase (*ermB*), beta-lactamase-ampicillin resistance genes (*ampC*), trimethoprim resistance genes (*dfrA1*), and quinolone resistance genes (*qnrB*). The extracted genomic DNA was used as a template for amplification of target genes using specific primers (Table 1). The PCR mix consisting of 2.5 µL of 10X buffer with 2.5M MgCl₂, 1 µL of 100mM dNTPs, 0.12 µL of Taq DNA polymerase, 1 µL each of (forward and reverse) specific antibiotic resistant genes primers, 1 µL of genomic DNA, and 18.38 µL of nuclease-free water was dispensed into well-labeled PCR tubes. The negative was the PCR mix and nuclease-free water (1 µL) without a DNA template. A final volume (25µL) was run on a DNA Thermal Cycler (Model 2720 Applied Biosystems). The PCR cycling conditions include: initial denaturation of five mins at 94°C (pre-heating), 35 cycles, one min at 94°C (denaturation), 45 sec at 56°C, and 30 sec at the annealing temperature for different primers, 1.5 min at 72°C (extension), and seven min at 72°C for final extension.

Table 1 Oligonucleotide primers used for detection of antibiotics resistant genes

Gene	Direction	Primer Sequence (5'- 3')	Annealing temperature	PCR product size (bp)	References
<i>tetA</i>	F	GCTACATCCTGCTTGCCCTTC	60°C	210	[16]
	R	CATAGATCGCCGTGAAGAGG			
<i>dfrA1</i>	F	TTCAGGTGGTGGGGAGATATAC	60°C	150	[17]
	R	TTAGAGGCGAAGTCTTGGGTAA			
<i>ermB</i>	F	GAAAAGGTACTCAACCAAATA	50°C	639	[18]
	R	AGTAACGGTACTTAAATTGTTTAC			
<i>cmlA</i>	F	CCGCCACGGTGTGTGTTATC	56°C	698	[16]
	R	CACCTTGCCCTGCCCATCATTAG			
<i>qnrB</i>	F	GGMATHGAAATTCGCCACTG	56°C	264	[10]
	R	TTTGCYGYCGCCAGTCGAA			
<i>ampC</i>	F	TTCTATCAAMACTGGCARCC	49°C	550	[19]
	R	CCYTTTATGTACCCAYGA			

Key: F- forward; R- reverse.

2.7 Statistical Analysis

Descriptive statistics and Fisher's Exact Test were used for analysis. The Fisher's Exact Test was used to determine the variation of antibiotic resistance across the different poultry types and states.

3. Results

3.1 Occurrence of *Enterobacter* species in poultry droppings from different poultry types

Out of the 218 bacterial isolates obtained, 72 were identified as *Enterobacter* species, including: *Enterobacter ludwigii* (1), *Enterobacter kobei* (7), *Enterobacter asburiae* (12), and *Enterobacter cloacae* (52). From the different poultry types, 50.0% were from layer chicken, while 27.8%, 9.7%, 6.9%, and 5.6% were from broiler chicken, dumpsites, noilers, and cockerels, respectively. The four species were obtained from the samples of layer chicken, while three (*E. cloacae*, *E. asburiae*, and *E. kobei*) were found in broiler chicken and cockerels. However, two (*Enterobacter cloacae* and *E. asburiae*) were found in Noilers (Table 2, Fig. 1).

3.2 Distribution pattern of *Enterobacter* species across different farm categories and states

The distribution pattern of the isolates showed that 21 (29.2%) were from the large farms, 19 (26.5%) from medium farms, and 32 (44.5%) from the small farms (Table 3). From Oyo state, 20 (27.8%) *E. cloacae*, 6 (8.3%) *E. asburiae*, 3 (4.2%) *E. kobei*, and 1 (1.4%) *E. ludwigii* were obtained, while from Osun state, *E. cloacae* 10 (13.9%), *E. kobei* 2 (2.8%), and *E. asburiae* 3 (4.2%) were recovered. Moreover, in Ondo state, *E. cloacae* 10 (13.9%) and *E. asburiae* 2 (2.8%) were found, while *Enterobacter cloacae* 4 (5.6%), *E. kobei* 1 (1.4%), and *E. asburiae* 1 (1.4%) were obtained from Lagos state (Table 4).

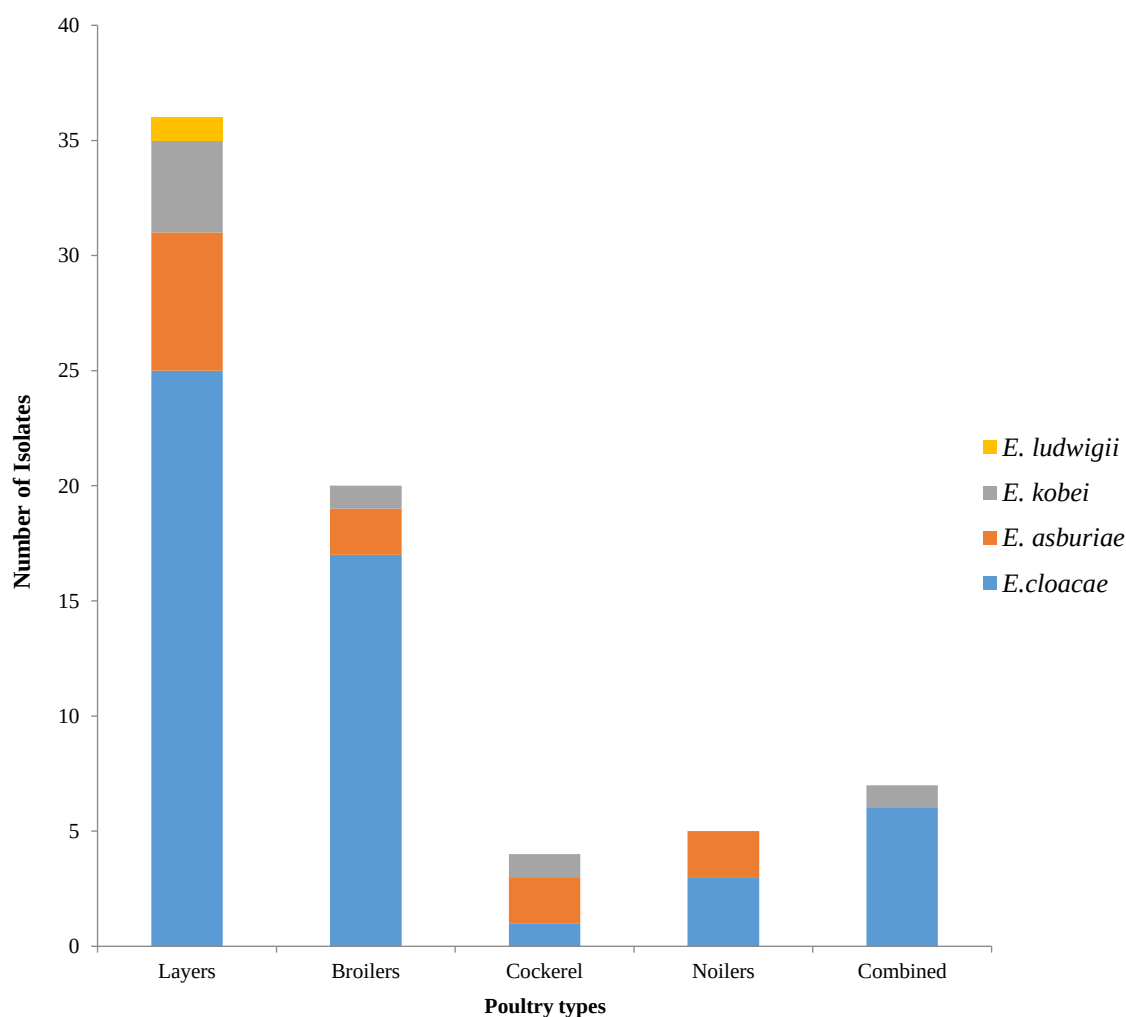


Figure 1 Occurrence of different *Enterobacter* species in poultry droppings from different poultry types

Table 2 Occurrence of Gram- negative bacteria isolated from poultry droppings

Bacterial isolates	Number and % of Isolates
<i>Enterobacter cloacae</i>	52 (23.9)
<i>Enterobacter asburiae</i>	12 (5.5)
<i>Enterobacter kobei</i>	7 (3.2)
<i>Enterobacter ludwigii</i>	1 (0.46)
<i>Klebsiella pneumonia</i>	45 (20.6)
<i>Klebsiella aerogenes</i>	31 (14.2)
<i>Citrobacter freundii</i>	27 (12.4)
<i>E. coli</i>	24 (11)
<i>Klyuvera ascorbata</i>	8 (3.7)
<i>Proteus alimentorum</i>	5 (2.3)
<i>Serratia marcescens</i>	1 (0.46)
<i>Pluralibacter gergoviae</i>	2 (0.92)
<i>Acinetobacter schindleri</i>	2 (0.92)
<i>Acinetobacter iwoffii</i>	1 (0.46)
Total	218 (100)

Table 3 Occurrence of *Enterobacter* species in poultry droppings from different farm sizes in different states n(%)

States	Small farm	Medium farm Number (%)	Large farm	Total
Oyo	13 (18.1)	6 (8.3)	11 (15.3)	30 (41.7)
Ogun	2 (2.8)	1 (1.4)	1 (1.4)	4 (5.6)
Osun	8 (11.1)	3 (4.2)	4 (5.6)	15 (20.9)
Lagos	2 (2.8)	2 (2.8)	2 (2.8)	6 (8.3)
Ondo	6 (8.3)	3 (4.2)	3 (4.2)	12 (16.7)
Ekiti	1(1.4)	4 (5.6)	0 (0.0)	5 (7.0)
Total	32 (44.5)	19 (26.5)	21 (29.2)	72 (100)

Table 4 Occurrence of different *Enterobacter* species in poultry droppings across the six states of southwest Nigeria n(%)

States	<i>E. cloacae</i>	<i>E. asburiae</i> Number (%)	<i>E. kobei</i>	<i>E. ludwigii</i>	Total
Oyo	20 (27.8)	6 (8.3)	3 (4.2)	1 (1.4)	30 (41.7)
Ogun	3 (4.2)	0 (0.0)	1 (1.4)	0 (0.0)	4 (5.6)
Osun	10 (13.9)	3 (4.2)	2 (2.8)	0 (0.0)	15 (20.9)
Lagos	4 (5.6)	1 (1.4)	1 (1.4)	0 (0.0)	6 (8.3)
Ondo	10 (13.9)	2 (2.8)	0 (0.0)	0 (0.0)	12 (16.7)
Ekiti	5 (6.9)	0 (0.0)	0 (0.0)	0 (0.0)	5 (6.9)
Total	52 (72.2)	12 (16.7)	7 (9.8)	1 (1.4)	72 (100)

3.3 Antibiotic resistance pattern of the *Enterobacter* species

The antibiotic susceptibility patterns of the isolate showed that all the isolates were resistant to cefpodoxime, cefixime, and amoxicillin, in addition to the only *E. ludwigii* that showed resistance to eight (Cefpodoxime, Cefuroxime, Cefotaxime, Cefaclor, Cefixime, Co-trimoxazole, Ampicillin, and Amoxicillin). Moreover, 9.6% and 8.3% of the *E. cloacae* and *E. asburiae* were resistant to ciprofloxacin, while none of the *E. kobei* and *E. ludwigii* showed resistance to ciprofloxacin (Table 5a).

Furthermore, among the isolates recovered from the layers, broilers, cockerels, and noilers, the combined sample, 50.0%, 27.8%, 5.6%, 6.9%, and 9.2%, respectively, were resistant to cefpodoxime, while it was 12.5%, 12.5%, 2.8%, 2.8%, and 0%, respectively, to amoxicillin-clavulanic. However, resistance to ciprofloxacin was 4.2%, 2.8%, 0%, 0%, and 2.8%. The Fisher's exact test statistic showed that the p- values of resistance to antibiotics belonging to different classes were greater than 0.05 (Ceftriaxone p = 0.678; Co-trimoxazole p = 0.117 Amoxicillin-clavulanic p = 0.165; Chloramphenicol p = 0.331; Ciprofloxacin p = 0.876; Gentamicin p = 0.616; Azithromycin p = 0.435) with the exception of tetracycline (p- value of 0.023), which has a p- value <0.05 (Table 5b).

Likewise, from the different states, resistance exhibited by the isolates to cefpodoxime were 41.7% (Oyo), 20.8% (Osun), 16.7% (Ondo), 8.3% (Lagos), 6.9% (Ekiti), and 5.6% (Ogun). The Fisher's exact test revealed that the p- values of resistance to Ciprofloxacin (0.000), Chloramphenicol (0.003), and Tetracycline (0.036) were less than 0.05, while the p-values of resistance to other antibiotics were greater than 0.05 (Table 5c).

The antibiotic resistance patterns of the isolates showed that the 20 from the broiler chickens exhibited resistance to five (cefpodoxime, cefixime, amoxicillin, cefotaxime, and co-trimoxazole) antibiotics, the seven *Enterobacter* spp. from the combined source showed resistance to five (cefpodoxime, cefotaxime, cefixime, cefuroxime, and amoxicillin) different antibiotics. This is in addition to all five isolates from the Noilers that were resistant to seven different antibiotics (cefpodoxime, cefuroxime, cefotaxime, cefaclor, cefixime, amoxicillin, and co-trimoxazole). Likewise, the four *Enterobacter* species from the cockerels showed resistance to eight different

antibiotics that included cefpodoxime, cefixime, ceftriaxone, cefotaxime, amoxicillin, ampicillin, streptomycin, and co-trimoxazole (Table 5b).

Considering the resistance of the *Enterobacter* species from the different states to antibiotics, the 30 isolated in Oyo state showed resistance to cefpodoxime, cefixime, cefotaxime, and amoxicillin. Moreover, the 12 isolated in Ondo state showed resistance to eight (cefpodoxime, cefuroxime, cefotaxime, ceftriaxone, cefixime, amoxicillin, netillin, and tetracycline) different antibiotics, while the 15 isolated in Osun state samples were resistant to four (cefpodoxime, cefixime, amoxicillin, and co-trimoxazole) antibiotics (Table 5c).

Table 5a Distribution of antibiotic-resistant *Enterobacter* species n(%)

Class of antibiotics	Antibiotics	<i>E. cloacae</i> 52	<i>E. asburiae</i> 12	<i>E. kobei</i> 7	<i>E. ludwigii</i> 1
Cephalosporins	Ceftriaxone	45 (86.5)	11 (91.7)	6 (85.7)	0 (0.0)
	Cefpodoxime	52 (100.0)	12 (100.0)	7 (100.0)	1 (100.0)
	Cefuroxime	45 (86.5)	10 (83.3)	5 (71.4)	1 (100.0)
	Cefotaxime	51 (98.1)	12 (100.0)	7 (100.0)	1 (100.0)
	Cefaclor	34 (65.4)	10 (83.3)	5 (71.4)	1 (100.0)
	Cefixime	52 (100.0)	12 (100.0)	7 (100.0)	1 (100.0)
Sulfonamide	Co-trimoxazole	44 (84.6)	11 (91.7)	7 (100.0)	1 (100.0)
Penicillin	Ampicillin	37 (71.1)	9 (75.0)	6 (85.7)	1 (100.0)
	Amoxicillin	52 (100.0)	12 (100.0)	7 (100.0)	1 (100.0)
	Amoxicillin-clavulanic	16 (30.8)	4 (33.3)	2 (28.6)	0 (0.0)
Phenicol	Chloramphenicol	19 (36.5)	5 (41.7)	4 (57.1)	0 (0.0)
Tetracyclines	Tetracycline	39 (75.0)	9 (75.0)	4 (57.1)	0 (0.0)
Aminoglycosides	Streptomycin	23 (44.2)	7 (58.3)	5 (71.4)	0 (0.0)
	Gentamicin	26 (50.0)	7 (58.3)	6 (85.7)	0 (0.0)
	Amikacin	23 (44.2)	8 (66.7)	5 (71.4)	0 (0.0)
	Netillin	38 (52.8)	9 (75.0)	6 (85.7)	0 (0.0)
	Ciprofloxacin	5 (9.6)	1 (8.3)	0 (0.0)	0 (0.0)
Quinolones	Levofloxacin	15 (28.8)	4 (33.3)	2 (28.6)	0 (0.0)
	Norfloxacin	14 (26.9)	7 (58.3)	1 (14.3)	0 (0.0)
Macrolide	Azithromycin	36 (69.2)	8 (66.7)	4 (57.1)	0 (0.0)

Table 5b The resistance patterns of *Enterobacter* species in different poultry types to different antibiotics n (%)

Class of antibiotics	Antibiotics	Layers 36	Broilers 20	Cockerel 4	Noilers 5	Combined 7	Total
Cephalosporins	Ceftriaxone	32 (44.4)	17 (23.6)	4 (5.6)	4 (5.6)	5 (6.9)	62 (86.1)
	Cefpodoxime	36 (50.0)	20 (27.8)	4 (5.6)	5 (6.9)	7 (9.2)	72 (100.0)
	Cefuroxime	31 (43.1)	16 (22.2)	2 (2.8)	5 (6.9)	7 (9.2)	61 (84.7)
	Cefaclor	24 (33.3)	14 (19.4)	3 (4.2)	5 (6.9)	5 (6.9)	50 (69.4)
	Cefotaxime	35 (48.6)	20 (27.8)	4 (5.6)	5 (6.9)	7 (9.2)	71 (98.6)
	Cefixime	36 (50.0)	20 (27.8)	4 (5.6)	5 (6.9)	7 (9.2)	72 (100.0)
Sulfonamide	Co-trimoxazole	29 (40.3)	20 (27.8)	4 (5.6)	5 (6.9)	5 (6.9)	63 (87.5)
Penicillin	Ampicillin	24 (33.3)	17 (23.6)	4 (5.6)	4 (5.6)	4 (5.6)	53 (73.6)
	Amoxicillin	36 (50.0)	20 (27.8)	4 (5.6)	5 (6.9)	7 (9.2)	72 (100.0)
	Amoxicillin-clavulanic	9 (12.5)	9 (12.5)	2 (2.8)	2 (2.8)	0 (0.0)	22 (30.6)
Phenicol	Chloramphenicol	16 (22.2)	9 (12.5)	1 (1.4)	0 (0.0)	2 (2.8)	28 (38.9)
Tetracyclines	Tetracycline	27 (37.5)	18 (25.0)	2 (2.8)	3 (4.2)	2 (2.8)	52 (75.0)
Aminoglycosides	Streptomycin	16 (22.2)	11 (15.3)	4 (5.6)	1 (1.4)	3 (4.2)	35 (48.6)
	Gentamicin	18 (25.0)	13 (18.1)	3 (4.2)	2 (2.8)	3 (4.2)	39 (54.2)
	Amikacin	19 (26.4)	11 (15.3)	3 (4.2)	0 (0.0)	3 (4.2)	36 (50.0)
	Netillin	26 (36.1)	17 (23.6)	3 (4.2)	1 (1.4)	6 (8.3)	53 (73.6)
	Ciprofloxacin	3 (4.2)	2 (2.8)	0 (0.0)	0 (0.0)	1 (1.4)	6 (8.3)
Quinolones	Levofloxacin	10 (13.9)	9 (12.5)	0 (0.0)	0 (0.0)	2 (2.8)	21 (29.2)
	Norfloxacin	9 (12.5)	8 (11.1)	2 (2.8)	1 (1.4)	2 (2.8)	22 (30.6)
Macrolide	Azithromycin	21 (29.2)	15 (20.8)	2 (2.8)	4 (5.6)	6 (8.3)	48 (66.7)

Table 5c The resistance patterns of *Enterobacter* species in different states to different antibiotics n(%)

Class of antibiotics	Antibiotics	Oyo 30	Osun 15	Ondo 12	Lagos 6	Ekiti 5	Ogun 4
Cephalosporins	Ceftriaxone	26 (36.1)	12 (16.7)	12 (16.7)	5 (6.9)	4 (5.6)	3 (4.2)
	Cefpodoxime	30 (41.7)	15 (20.8)	12 (16.7)	6 (8.3)	5 (6.9)	4 (5.6)
	Cefuroxime	26 (36.1)	11 (15.3)	12 (16.7)	5 (6.9)	4 (5.6)	3 (4.2)
	Cefotaxime	30 (41.7)	14 (19.4)	12 (16.7)	6 (8.3)	5 (6.9)	4 (5.6)
	Cefaclor	27 (37.5)	10 (13.9)	6 (8.3)	4 (5.6)	2 (2.8)	1 (1.4)
	Cefixime	30 (41.7)	15 (20.8)	12 (16.7)	6 (8.3)	5 (6.9)	4 (5.6)
Sulfonamide	Co-trimoxazole	25 (34.7)	15 (20.8)	10 (13.9)	6 (8.3)	5 (6.9)	2 (2.8)
Penicillin	Ampicillin	21 (29.2)	12 (16.7)	10 (13.9)	3 (4.2)	4 (5.6)	3 (4.2)
	Amoxicillin	30 (41.7)	15 (20.8)	12 (16.7)	6 (8.3)	5 (6.9)	4 (5.6)
	Amoxicillin-clavulanic	9 (12.5)	6 (8.3)	3 (4.2)	1 (1.4)	2 (2.8)	1 (1.4)
Phenicol	Chloramphenicol	6 (8.3)	8 (11.1)	8 (11.1)	4 (5.6)	2 (2.8)	0 (0.0)
Tetracyclines	Tetracycline	18 (25.0)	12 (16.7)	12 (16.7)	5 (6.9)	4 (5.6)	1 (1.4)
Aminoglycosides	Streptomycin	9 (12.5)	9 (12.5)	9 (12.5)	5 (6.9)	2 (2.8)	1 (1.4)
	Gentamicin	10 (13.9)	11 (15.3)	10 (13.9)	5 (6.9)	2 (2.8)	1 (1.4)
	Amikacin	12 (16.7)	9 (12.5)	9 (12.5)	5 (6.9)	1 (1.4)	0 (0.0)
	Netillin	15 (20.8)	14 (19.4)	12 (16.7)	6 (8.3)	4 (5.6)	2 (2.8)
Quinolones	Ciprofloxacin	4 (5.6)	0 (0.0)	0 (0.0)	0 (0.0)	2 (2.8)	0 (0.0)
	Levofloxacin	5 (6.9)	5 (6.9)	8 (11.1)	2 (2.8)	1 (1.4)	0 (0.0)
	Norfloxacin	6 (8.3)	6 (8.3)	8 (11.1)	0 (0.0)	1 (1.4)	1 (1.4)
Macrolide	Azithromycin	18 (25.0)	11 (15.3)	7 (9.7)	6 (8.3)	4 (5.6)	2 (2.8)

3.4 Detection of antibiotic resistance genes in the chromosomal DNA of the *Enterobacter* species

Out of the 72 *Enterobacter* species, the *ampC* resistance gene was detected in the chromosomal DNA of 10 isolates, including eight *E. cloacae* and two *E. kobei*. Also, the sulfamethoxazole-trimethoprim (*dfrA1*) resistance gene was detected in the chromosomal DNA of 12 of the *Enterobacter* species, including *E. cloacae* (10), *E. asburiae* (1), and *E. kobei* (1). Moreover, the *ermB* gene was detected in the chromosomal DNA of four *Enterobacter* species that included: *E. cloacae* (2), *E. kobei* (1), and *E. ludwigii* (1). Also, the *qnrB* resistance gene was found in the chromosomal DNA of seven *E. cloacae*, while tetracycline (*tetA*) and chloramphenicol (*cmlA*) resistance genes were not detected in any of the isolates (Table 6).

3.5 Plasmid DNA occurrence and antibiotic resistance genes detection in the plasmid of the *Enterobacter* species

Plasmid DNA was found in 31 (43.1%) out of the 72 *Enterobacter* species. The plasmid's molecular weight was 11kb and was detected in *E. cloacae* (17), *E. asburiae* (9), *E. kobei* (4), and *E. ludwigii* (1). These isolates cut across all the poultry types (Table 6). Furthermore, four (*ampC*, *dfrA1*, *ermB*, *qnrB*) out of the six antibiotic resistant genes were detected in the plasmid DNA of the *Enterobacter* species. The *ampC* gene was detected in the plasmid of two of the isolates, including *E. cloacae* and *E. kobei*, while the *dfrA1* gene was found in two *E. cloacae*. Among the 21 *Enterobacter* species that were found to harbor the *ermB* gene in their plasmid DNA, nine were *E. cloacae*, seven were *E. asburiae*, four were *E. kobei*, and one was *E. ludwigii*, while the 16 isolates that carried the *qnrB* gene in their plasmids were seven *E. cloacae*, six *E. asburiae*, and three *E. kobei* (Table 7).

Table 6 Occurrence of plasmids in different *Enterobacter* species from different poultry types (numbers)

<i>Enterobacter</i> spp.	Layer chickens 36	Broiler chickens 20	Combined 7	Noilers 5	Cockerels 4	Total 72
<i>E. cloacae</i>	7	3	5	1	1	17
<i>E. asburiae</i>	4	2	-	2	1	9
<i>E. kobei</i>	3	-	1	-	-	4
<i>E. ludwigii</i>	1	-	-	-	-	1
Total	15	5	6	3	2	31

Table 7 Relative abundance of antibiotic resistance genes in *Enterobacter* spp.

<i>Enterobacter</i> spp.	<i>ampC</i>		<i>dfrA1</i>		<i>qnrB</i>		<i>ermB</i>		<i>cmlA</i>		<i>TetA</i>	
	D	P	D	P	D	P	D	P	D	P	D	P
<i>E. cloacae</i>	8	1	10	2	7	7	2	9	-	-	-	-
<i>E. asburiae</i>	-	-	1	-	-	6	-	7	-	-	-	-
<i>E. kobei</i>	2	1	1	-	-	3	1	4	-	-	-	-
<i>E. ludwigii</i>	-	-	-	-	-	-	1	1	-	-	-	-
Total	10	2	12	2	7	16	4	21	-	-	-	-

Key: D-DNA; P- Plasmid; - -Absent

4. Discussion

The increasing demand for poultry products as a good source of protein worldwide has led to an increase in poultry farming and a large amount of waste being generated [1]. In southwest Nigeria, poultry production is more

predominant compared to the distribution of poultry production in other geopolitical zones in the country [32]. In this study, it was observed that there was a higher proportion of small farms compared to the medium and the large farms. This observation is in perfect agreement with the previous report, where it was noted that small-scale poultry farming contributes significantly to the poultry value chain in Nigeria, with over 80% of households practicing poultry in the rural areas [1]. It has also been observed that medium and large-scale chicken farms are rapidly emerging mainly in the southern part of the country, and chicken production is quite spatially concentrated in the urban and Peri-urban areas of the southern part of the country [21].

The observation that the different bacterial genera isolated from the poultry droppings were opportunistic or potentially pathogenic bacteria, which have been implicated in human infections, is similar to the presence of *Enterobacter* spp., *E. coli*, *Klebsiella* spp., and *Proteus* spp. reported in poultry droppings in Africa [4]. The recovery of *Enterobacter* spp., *Citrobacter* spp., *E. coli* and *Klebsiella* spp. in this study is in agreement with another study conducted on poultry droppings in a city in Egypt [3].

The presence of *Enterobacter* spp. from a small-scale poultry farm in this present study is similar to another report on poultry products from a small-scale poultry farm in a state in the United States [22]. It was observed in this study that the recovery of *Enterobacter* spp. from small farms was higher compared to that of the large and medium farms, and this implies that small-scale poultry production can greatly contribute to the spread of *Enterobacter* species in the environment. The small-scale farms that are in very close contact with residential areas can act as a reservoir for this pathogen and aid its rapid spread to humans directly or indirectly.

In addition, the highest number of sampled farms was in Oyo state, which has been reported to be the state with the highest poultry farms in the southwest part of the country and the second largest in Nigeria. The recovery of *Enterobacter* species in the present study corroborates the presence of this bacterial species in poultry, as was previously reported that the bacteria were isolated from cloacal swabs of poultry in Bangladesh [6]. In this study, *E. cloacae* were found to be the most predominant species in the poultry droppings; this observation agrees with the report from another study carried out on poultry with a similar report in Bangladesh [6].

The observation from this study which showed that the highest occurrence of *Enterobacter* spp. was from Oyo state followed by Osun state with the least being from Ogun state is similar to the report on the isolation of *Enterobacter* species from fishpond water samples where it was reported that the highest occurrence rate of the isolated was from Oyo state followed by Osun state and the least from Ogun state [23]. However, the recovery rate of the isolates in this study from the states might have been influenced by the number of samples collected.

The indiscriminate use of antibiotics in the poultry industry for therapeutic, growth, and prophylaxis purposes in order to meet the rise in demand for poultry products is a contributory factor to the emergence of antibiotic resistance in bacteria isolated from poultry [7]. Hence, screening of isolates from poultry for antibiotic resistance pattern determination is essential to assessing the level of antibiotic resistance spread in the environment for intervention to curtail the spread.

The observed resistance of the *Enterobacter* species in this study to sulfonamides (87.5%), tetracycline (72.2%), ampicillin (73.6%), streptomycin (48.6%) and gentamicin (54.2%) is in disparity with the report from a similar study with the reported resistance to sulfonamides (72.2%), tetracycline (33.3%), ampicillin (94.4%), streptomycin (55.6%) and gentamicin (5.6%) in Bangladesh [6]. These discrepancies might be as a result of the use and the regulation of the use of antibiotics on poultry farms, which may be different from one country to another. Similarly, the observed resistance of the *Enterobacter* species in this study to ciprofloxacin (8.3%), streptomycin (48.6%), and co-trimoxazole (87.5%) is not in agreement with the report from another study in Lagos state, Nigeria, from which none of the studied *Enterobacter* spp. showed any resistance to the listed antibiotics [24]. The reason for the noticeable difference might be due to the variation in the studied samples from which the isolates were obtained. While the present study was on chicken droppings, the latter study was on chicken meat.

The resistance of *E. cloacae* to amoxicillin-clavulanic (22.0%), streptomycin (44.2%) and chloramphenicol (36.5%) observed in this study showed some variations when compared with the resistance previously reported on *E. cloacae* from samples collected from healthy broiler chickens in Egypt with resistance to amoxicillin-clavulanic, streptomycin and chloramphenicol which were 80.0%, 80.0% and 20.0%, respectively [3]. Although it was observed that the resistance of *E. kobei* to cefuroxime (71.4%) and ceftriaxone (85.7%) in this present study is high, the observation is, however, not in agreement with the total resistance previously reported from a study on *E. kobei* isolated from food of animal origin, including eggs, to the antibiotics in Egypt [25]. Furthermore, 33.3% and 83.3% of the *E. asburiae* in this study were observed to be resistant to amoxicillin/clavulanic acid and cefuroxime, respectively; this is, however, not in line with the total resistance reported from a recent study on *E. asburiae* from fish pond water to these antibiotics [23]. Nevertheless, exhibition of total resistance was reported in both studies by *E. asburiae* to cefixime. Moreover, the resistance of *E. ludwigii* to ampicillin and amoxicillin in this study is in agreement with the resistance to ampicillin and amoxicillin reported in *E. ludwigii* from infected gold fish organs [26].

The antibiotic resistance pattern of *Enterobacter* spp. across the poultry types indicated that there is no variation across the poultry types at p-values > 0.05 to all the tested antibiotics, with the exception of tetracycline

(p -value < 0.05), which showed variation across the poultry types. However, the antibiotic resistance pattern observed in *Enterobacter* spp. isolated across the different states suggests that resistance to three tested antibiotics (ciprofloxacin, tetracycline, and chloramphenicol) varied geographically, while others were not significantly different. Tetracycline is the most widely used antibiotic in poultry production in Africa, while ciprofloxacin and chloramphenicol were also employed in poultry [27]. The variation in resistance to the antibiotics across the state may be influenced by the level of usage at different locations.

The occurrence of multiple antibiotic-resistant *Enterobacter* spp. obtained in the poultry droppings of this study, which showed that all the isolates were multiple drug resistant, which implied that these bacteria have the tendency to spread to the food chain, food crops, fish ponds, and finally to humans. This therefore suggests that there is a need for continuous monitoring of poultry waste for multi-resistant bacterial isolates. However, this observation disagreed with the 45.2% that was previously obtained from isolates from poultry cloacal swabs. The occurrence rate of the multiple antibiotic-resistant *Enterobacter* spp. is not in agreement with the 45.2% multiple antibiotic-resistant *Enterobacter* spp. recently reported from a study on poultry cloacal swabs in Cameroon [28].

The multiple-resistant *Enterobacter* spp. in this study that were found to harbor various antibiotic resistance genes responsible for the resistance nature of the bacteria are in line with the observation from a similar study on poultry production environment, where it was shown that *Enterobacter* isolates obtained carried several antibiotic resistant genes [29]. The detection of the *ermB* gene in the *Enterobacter* species in this study might signify the inactivation of macrolides due to methylation of the *erm* genes, which could have resulted in the observed resistance to macrolides such as azithromycin. Similarly, the fact that the gene detection rate was high is also in agreement with the report that showed the abundance of the *ermB* gene in Enterobacteriaceae recovered from poultry wastewater [30].

Furthermore, the detection of the *ampC* gene in the *Enterobacter* spp. of this study differs from the report of a study on *Enterobacter* spp. from a poultry production environment, where it was observed that the gene was not detected [29]. However, the plasmid-mediated *ampC* gene found in the *Enterobacter* isolates in this study correlates with the observation from another study that showed plasmid-mediated *ampC* in Enterobacteriaceae from human and retail meat, including poultry [11]. Likewise, the detection of the *dfrA1* resistant gene in this study corroborates the presence of this gene in isolates of poultry sources, because it has been previously reported that the gene was found in *E. coli* from a broiler farm in Egypt and *Enterobacter* species from poultry origin in China [3, 29]. The presence of the *qnrB* gene, as found in the isolates from this study, has also been reported to be widely encountered in *E. cloacae* and has been reported to be responsible for the decreased susceptibility to quinolone antibiotics [8]. The detection of plasmids in the *Enterobacter* spp. of this study differs from the report of a study on *Enterobacter* spp. of a poultry origin in Bangladesh [6]. The findings in this study highlight the need for antimicrobial stewardship in poultry to curtail the spread of multidrug-resistant bacteria and antibiotic resistance genes in potentially pathogenic bacteria of poultry origin, and also for implementing alternatives to antibiotics for growth promotion in poultry.

5. Conclusion

This study revealed the presence of multidrug-resistant *Enterobacter* spp. in poultry droppings in southwest Nigeria, in addition to the presence of several antibiotic resistance genes, which are both plasmid and chromosomally borne. This might constitute a serious health threat to the animals, fish, and humans, either by direct or indirect contact with poultry, their products, or the untreated poultry droppings. Hence, it is highly recommended that poultry waste be properly treated before it is discharged into the environment or used as fish feed.

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7. Author Contributions

M. J. O. and O. I. F. conceived and designed the experiments, M. J. O. performed the experiments, M. J. O. and O. I. F. did literature search and analyzed the data, O. I. F. and Y. S. S. supervised the work. M. J. O. wrote the first draft. O. I. F. revised the draft.

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