Occurrence of Antibiotic Resistant Bacteria (ARB) in Poultry Farms Located in Ilara-Epe, Lagos State, Nigeria

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Abstract: There is increasing concern about the public health risks associated with routine and indiscriminate use of antibacterial agents in raising livestock worldwide. Our study investigated the antibiotics resistance of bacterial isolates obtained from four poultry farms located in a commercial livestock farm settlement in Ilara-Epe, Lagos State, Nigeria. The antibiotic resistance pattern of readily available and commonly used antibiotics against 92 bacterial strains isolated from the selected farms was determined by Kirby-Bauer disc diffusion method and the minimum inhibitory concentrations (MICs) of antibiotics for the bacterial isolates were determined by a standard two-fold serial broth microdilution method using Mueller–Hinton broth. The results showed high resistance values to ampiclox (97.5%), amoxicillin and zinacef (95.1%), augmentin (94.1%), and streptomycin (82.2%). Multiple drug resistance (MDR) was observed for both Gram-negative and Gram-positive bacterial isolates at 86.3% and 90.2% respectively. Also, 24 (26.4%) of the bacterial isolates were completely resistant to all tested antibiotics in the study. The majority of identified bacterial isolates were *Staphylococcus aureus*, *Escherichia coli*, and *Micrococcus luteus*. All the antibiotics used in this study showed high MICs values against the test bacterial isolates. Our findings have added to existing evidence that poultry farms habour antibiotic resistance bacteria (ARB). These ARB may pose a public health risk as they may be pathogenic to humans and animals and also contribute to the flow of antibiotic resistance genes in the ecosystem. Hence, there is the need to avoid the indiscriminate use of antibiotics in poultry farming, especially as growth promoters.

Keywords: Poultry farms; Antibiotics; Antibiotic Resistance; Antibiotic Resistant Bacteria (ARB)

INTRODUCTION

The use of antibacterial compounds in veterinary practice started soon after they became available for the treatment of human diseases in mid 1940s (Aryal, 2001). Antibiotic use in livestock production has been implicated as a risk factor in the development and dissemination of drug resistance from livestock production farms (Bauer *et al*., 2006; Gosh and Lapara, 2007). The four main reasons for antibiotics administration are for the treatment of infections, as a prophylactic measure, as a metaphylactic treatment of diseases, as growth promoters, and to improve feed conversion efficiency (Viola and DeVincent, 2006). China is the largest consumer of antimicrobials in livestock worldwide, with nearly 100 million kg added to feed each year (Hvistendahl, 2012).

Over the past 50 years, the use of antibiotics combined with strict biosecurity and hygiene measures have helped the poultry industry to grow by preventing the negative impacts of many avian diseases (Bermudez, 2003). In Nigeria, antimicrobial agents are routinely used in livestock production especially as additives to feed and water (Adelowo *et al*., 2009). Chlortetracycline and streptomycin were the two most effective and frequently used antibiotics in poultry birds. Their routine use may result in a selective advantage and a consequent increase in the abundance of resistant bacteria in animals, their waste and the surrounding environment (Heuer and Smalla, 2007). Previous reports have demonstrated that antibiotic resistant bacteria are detected in poultry waste (Kelley *et al*., 1998; Khan *et al*., 2005; Adelowo *et al*., 2009; Furtula *et al*., 2010), commercial poultry production environments (Hayes *et al*., 2004; Nilsson *et al*., 2009) and poultry products (Girlich *et al*., 2007, Fortini *et al*., 2011).

However, concerns over food-borne pathogens acquiring and spreading antibiotic resistance have driven research for alternatives to antibiotic growth promoters. These concerns exist worldwide in areas where antibiotics are used for growth promoting purposes; the EU nations, however, banned the use of antibiotics at sub-therapeutic levels in 2006 (European Union, 2009). As poultry farming in Nigeria is gradually tending towards the commercial scale as a sub-sector in the nation's agriculture; there is a need for substantial and adequate knowledge on the possible effects of the use of antimicrobials on farms, particularly with the acquisition of antimicrobial resistant bacteria (Doyle, 2015). Hence, the need for surveillance data on the use of antimicrobials in Nigeria, which is scarce, but crucial if the problem is to be managed.

In this study, we investigated the antibiotic resistance patterns of bacterial isolates obtained from water, litters and sludge samples collected from four poultry farms located in a farm settlement in the Ilara, Epe axis of Lagos State, Nigeria. The selected farms' facilities are for commercial production of meat. There were reports from the various farms on the incorporation of antibiotics in the animal feed and drinking water especially for prophylactic purposes and also to promote growth in the birds. There were no waste treatment facilities before final disposal. In most cases, the farm litters are used as manure for crop farming or as feeds in fish ponds. In other situations, they are washed into the public drains, and finally to larger waterbodies. This practice has constituted a potential risk to the water sources available to end users. Hence, this study is aimed at evaluating the occurrence of antibiotic resistant bacteria in poultry farms.

MATERIALS AND METHODS

Sampling sites

Samples were directly obtained from four poultry farms located in a farm settlement in Ilara-Epe (6°34´59.99´´N Longitude 3.58´59.99´´E), Lagos State, Nigeria. The poultry farms practice the commercial production system in a large dedicated to only livestock farming. The battery cage system was the adopted practice with greater than a thousand birds within each farm. There was no free-range practice in any of the farms. All farm produce is intended for commercial consumption on a large scale.

Sample Collection

The samples obtained included faecal matter, soil and litter materials, feedstock, drinking water and sludge samples. The water samples were collected aseptically in sterile 2-L glass bottles. The samples were collected in duplicates and used as composite samples. They were kept at 4˚C in the refrigerator in the dark bottles and were processed within 24 hours of collection.

Isolation of Bacterial Community

The samples were serially diluted in 10-fold normal saline and 1.0 ml aliquots of appropriate dilutions (10^{-2} and 10^{-4}) were inoculated and plated using the pour plate method on non-selective agar media, Tryptone Soy Agar (TSA) and Plate Count Agar (PCA); selective agar, Eosin Methylene Blue (EMB) and MacConkey agar and differential media, Mannitol salt agar and Salmonella-shigella agar (Oxoid Ltd., Basingstoke, Hampshire, UK). Duplicate plates were placed in an incubator (Labnet, Model DNP) under aerobic conditions at 35˚C for 48 hours. Bacterial counts were taken at the 24-hour and 48-hour of incubation. The Colony Forming Unit per milliliter (CFU/mL) was calculated based on the categories of the samples collected. Macroscopical observation of the bacterial isolates on each culture plate was carried out. Morphologically distinct colonies are sub-cultured onto fresh plates of Nutrient Agar (NA) (Oxoid Ltd., Basingstoke, Hampshire, UK). Isolates were restreaked up to three times and purity was verified by Grams reaction and microscopy. Pure colony of each isolated bacteria strain was stored on Nutrient agar (Oxoid) slants at

4˚C, and for prolonged storage at -20˚C in Nutrient Broth (Biolab) containing 20% glycerol.

Identification of Bacterial Isolates

The bacterial isolates were identified using standard cultural, morphological and biochemical tests and the identity of the isolates were confirmed by comparing the standard test results to the taxonomic scheme of Bergey's Manual of Determinative Bacteriology (Krieg *et al*, 2010) and online Identification software (https://www.microrao.com/) (Sridhar-Rao, 2010) was used to identify the bacterial isolates. The biochemical and morphological tests employed in the identification and characterization of the bacterial isolates include Gram staining, motility test, catalase test, citrate utilization test, triple sugar Iron test, oxidase test, methyl red /voges-proskauer (MRVP) test, Indole test, urease test, starch hydrolysis test, gelatin Hydrolysis test, tryptophan deaminase test, tartrate utilization test, coagulase test, haemolysis test and sugar fermentation tests.

Antibiotic susceptibility test

Screening for Antimicrobial Susceptibility Testing using the Disk Diffusion Method

The disc diffusion method was used to determine the antibiotic susceptibility testing of 92 bacterial isolates to different antibiotics. Antibiotic sensitivity discs (Abtek) employed contained pefloxacin (10µg), gentamycin (10µg), ampiclox (30µg), zinacef (20µg), rocephin (25µg), ciprofloxacin (10µg), streptomycin (30µg), septrin (30µg), erythromycin (10µg) and amoxicillin (30µg) were used for Gram positive isolates. For the Gram-negative isolates, septrin (30µg), chloramphenicol (30µg), sparfloxacin (10µg), ciprofloxacin (10µg), amoxicillin (30µg), augmentin (30µg), gentamycin (10µg), pefloxacin (30µg), tarivid (10µg) and streptomycin (30µg) were used. It was carried out using the Clinical and Laboratory Standards Institute (CLSI, 2018) modified Kirby-Bauer disc diffusion technique as recommended by the World Health Organization (Bauer *et al*., 1966). An overnight culture of each isolate was prepared in Nutrient broth (Biolab, Canada) and incubated at 37°C for 18 hours. The culture was adjusted to a 0.5 McFarland standard. Dry sterile plates of Mueller-Hinton agar (Oxoid, UK) were prepared and each of the isolates was uniformly and aseptically inoculated into the Mueller-Hinton agar plates using sterile swab sticks. The sensitivity discs were carefully and aseptically layered on each plate and the plates incubated overnight at 37˚C, after which zones of growth inhibition around each disc were measured and interpreted by the zone breakpoint standards of the Clinical and Laboratory Standards Institute (Bauer *et al*., 1966; CLSI, 2018).

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) was determined for the commonly used antibiotics that are available on the counter. The MICs of antibiotics for the bacterial isolates were determined by a standard two-fold serial broth microdilution method using Mueller–Hinton broth according to the CLSI Standards guidelines (CLSI, 2018) with antibiotic concentrations ranging from 0.5 to 512 µg/mL. Four antibacterial agents were used per isolate depending on their Gram status and of different classes: β-lactams, aminoglycosides, macrolides, tetracyclines, fluoroquinolones. Antibiotics used on the Gram-positive isolates are erythromycin, septrin, ciprofloxacin and augmentin, while for the Gram negative this includes tetracycline, ciprofloxacin, augmentin and amoxicillin. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as reference strains (CLSI, 2018).

RESULTS

Plate Count of Bacterial Isolates from Collected Samples

The plate counts of the bacterial colonies were carried out within 24h and 48h. The counts are presented based on sample categories.

The different samples collected from the farms showed a relatively high number of colony forming units per milliliter (CFU/mL) except for the water samples with counts ranging from 4.0x10 - $1.6x10^4$ CFU/mL. At the end of the 48 h of incubation on Plate Count Agar (PCA), the total cell count of the samples for faecal matter, water samples, sludge and soil/ litter materials were: 2.4x10 - $4.0x10^6$ CFU/mL, 4.0x10 - $1.6x10^4$ CFU/mL, $5x10^6$ - $2.8x10^8$ CFU/mL, $4.0x10^6$ - $1.1x10^8$ CFU/mL respectively. The results are in the average values of triplicate plating for each of the samples.

Identification of Bacterial isolates

The morphological and biochemical test performed on 24 bacterial isolates that were resistant to all tested antibiotics in the preliminary antibiotic susceptibility test gave their most probable identities. The selected bacterial isolates were identified as *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus faecalis*, other *Streptococcus* spp, *Staphylococcus aureus*, *Micrococcus varians*, *Micrococcus luteus* and other *Micrococcus* spp (Table 1).

Table 1: Frequency Distribution and identities of Bacterial isolates resistant to all tested antibiotics

Genus or species	No of isolates from				Total number of isolates
	F1	F2	F3	F4	
Streptococcus faecalis	2	-		-	2
Streptococcus spp	-	1	1	-	2
Escherichia coli	-	1	1	3	5
Staphylococcus aureus	2	5	1	2	10
Micrococcus varians	-	-	1	-	1
Micrococcus luteus	-	-	1	2	3
Micrococcus spp	-	1	-	-	1
TOTAL	4	8	5	7	**24**

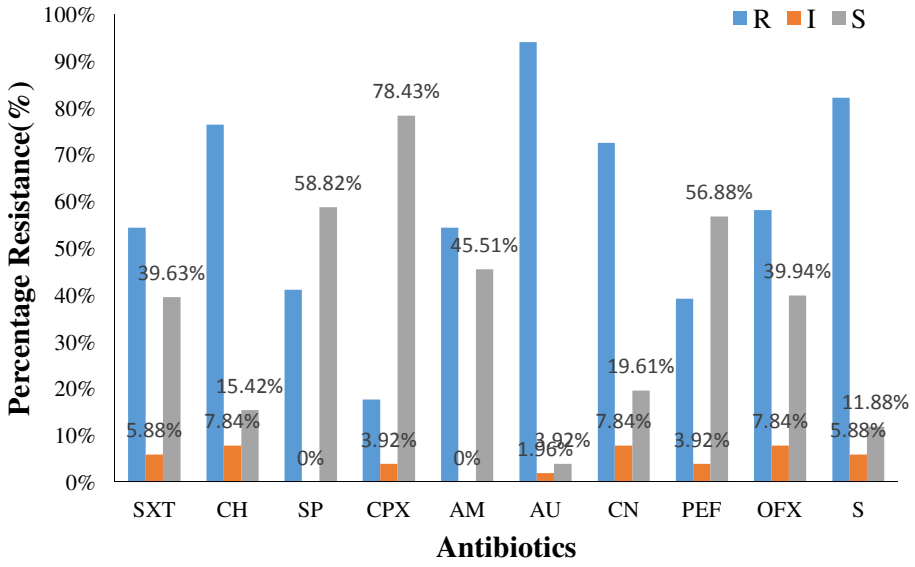
FI-F4, Poultry Farm 1-4

Antibiotics resistance prevalence and Multiple Drug Resistance

The distribution of multiple antibiotic resistance in both Gram-positive and Gram-negative isolates based on the antibacterial agents showed that, for Gram-negative isolates, the highest percentage resistance was to augmentin amongst the panel of antibiotics at 94.1%, while the Gram-positive isolates showed the highest resistance prevalence to ampiclox at 97.5%. The Gram-negative and Gram-positive isolates have the least percentage resistance to ciprofloxacin at 17.7% and 48.8% respectively (Figure 1 and 2).

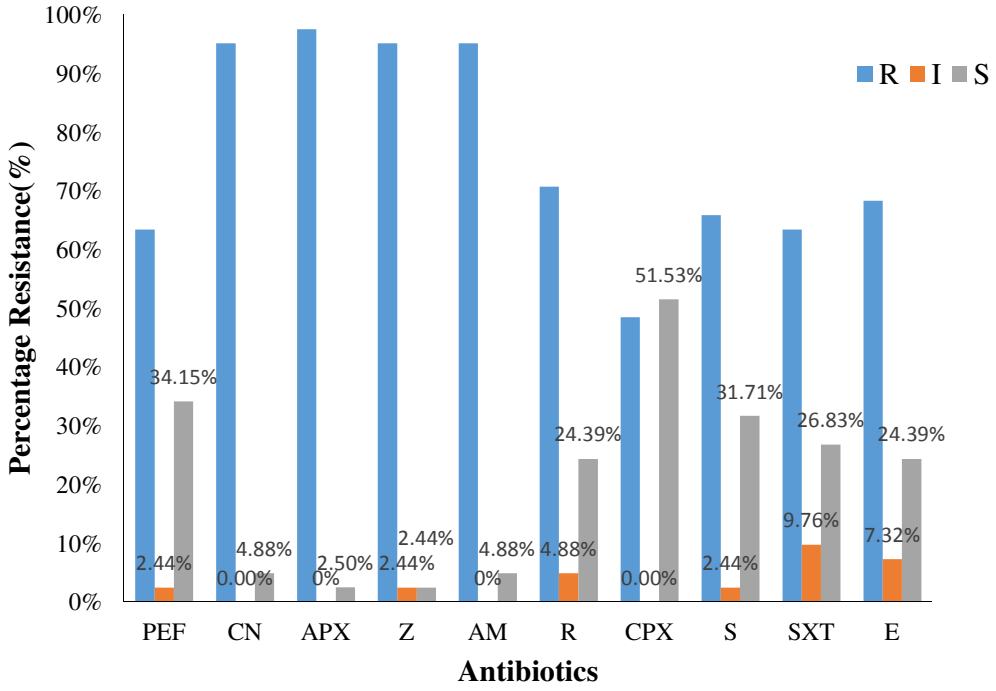
The antibiotic resistance profiles of the bacterial isolates showed that all the isolates were resistant to at least one or more antibacterial agents and most (≥86.3%) of the isolates were resistant to more than three antibacterial agents. From the results, resistance to three or more tested antibiotics was shown in 44(86.3%) of Gram-negative isolates and 37(90.2%) of the Gram-positive isolates.

The results also showed that of the 51 bacterial isolates screened, 43(84.3%) of the Gram-negative isolates and 37(90.2%) of the Gram-positive isolates had MAR index greater than 0.2, with 20(39.2%) of the Gram-negative isolates and 8(19.5%) of the Gram-positive isolates having considerably higher MAR index (0.6-0.9) (Table 2).



Key: CH-Chloramphenicol, SXT= Septrin, SP- Sparfloxacin, CPX- ciprofloxacin, AM-Amoxicillin, AU- Augmentin, CN- Gentamycin, OFX- Tarivid, S- Streptomycin, R-Resistance; I- Intermediate; S- Susceptible.

Figure 1. Sensitivities of Gram-negative isolates to a panel of 10 antibiotics



KEY: R- Resistant, S- Susceptible, I- Intermediate, PEF- Pefloxacin, CPX- Ciprofloxacin, CN- Gentamycin, S-Streptomycin, APX- Ampiclox, SXT- Septrin, Z- Zinacef, E-Erythromycin, R- Rocephin, AM- Amoxicillin

Figure 2. Sensitivities of Gram-positive isolates to a panel of 10 antibiotics

Table 2. Multiple Antibiotic Resistance Index of Gram-negative and Gram-positive isolates

MAR index	Percentage (%)
Gram Negative Isolates	
Less than or equal to 0.2	8 (15.7)
Greater than 0.2	43 (84.3)
Total	51(100)
Gram Positive Isolates	
Less than or equal to 0.2	4 (9.8)
Greater than 0.2	37 (90.2)
Total	41 (100)

Minimum Inhibitory Concentration (MIC) values of Selected Isolates

The minimum inhibitory concentration (MIC) result of the most commonly used antibiotics available on the counter, shows that the resistance prevalence for almost all antibiotics tested in this study was high in all the bacterial isolates. The resistance to septrin (trimethoprim/ sulfamethoxazole) was the highest amongst the Staphylococci and other Gram-positive cocci, while the resistance to amoxicillin was the highest amongst the Gram-negative isolates (Table 3). As shown in Table 3, the antibiotic levels of the bacterial communities in all bacterial isolates were reflected by the MIC50s and MIC90s, which represent MICs required for the inhibition of 50% and 90% of bacterial strains, respectively. The bacterial isolates had the lowest resistance prevalence (80%) to augmentin and ciprofloxacin with MIC50 and MIC90 values of 64 and 256 µg/ml respectively. Isolates expressed the highest resistance prevalence to septrin, with 512 µg/ml for MIC50 and MIC90 values (Table 3).

Table 3. Minimum inhibitory concentration (MIC) of 6 antibiotics against bacterial isolates with MIC50 and MIC90 values

Antibiotics	Resistance Prevalence (%)	MIC (µg/ml)		
		Range	50%	90%
Amoxicillin (AMO)	83.3%	16 to ≥512	128	512
Septrin (S)	100%	4 to ≥512	512	512
Tetracycline (TET)	83.3%	4 to ≥64	64	64
Erythromycin (ERY)	85.7%	1 to ≥512	128	512
Augmentin (AU)	80%	4 to ≥512	64	256
Ciprofloxacin (CIP)	80%	2 to ≥512	64	256

Key: MIC50 MIC90, the MICs for each antibiotic for the tested isolates which represent MICs required for the inhibition of 50% and 90% of bacterial strains respectively.

DISCUSSION

The development of resistance to antimicrobial agents is a global challenge that is increasingly frustrating efforts to treat infectious diseases. This has been attributed largely to human and agricultural use of antimicrobials.

Thus, this study tried to investigate and establish the occurrence of antibiotic resistance among bacterial isolates from selected poultry farms in Ilara- Epe, Lagos, Nigeria, an expanse of land for commercial husbandry where little is known about the contribution of antibiotics use to the development and dissemination of resistance to the drug.

The samples collected include faecal matter, soil/ litter materials, water samples from different sources and sludge. In faecal matter and sludge samples, antibiotic resistance phenotypes were very common, resistance levels (Table 5) were shown to be more than three classes of the tested antibiotics. The bacterial isolates resistant to all selected antibiotics which were selected for identification accounted for 24 (22.82%) of the isolates. The most probable phenotypic identity of the bacterial isolates includes *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus faecalis*, other *Streptococcus* spp, *Micrococcus varians*, *Micrococcus luteus* and other *Micrococcus* spp. (Table 3.1). The resistance of these organisms to the tested antibiotics may be due to the incessant use of antibiotics by most farmers in Nigeria. A similar observation has been reported by Ayandiran *et al*. (2018). High usage of quinolones, tetracycline and other antibiotics at sub- therapeutic level has been reported in Ekiti state, Nigeria by Alo and Ojo (2007). Ogunleye *et al*. (2008) also reported sub therapeutic use of antibiotics in many poultries in Abeokuta, Nigeria where enrofloxacin and tetracycline are on the majority list.

According to Jorgenson *et al*. (2009), the goal of antimicrobial susceptibility testing of significant bacterial isolates is to detect possible drug resistance in common pathogens and to assure susceptibility to drugs of choice for particular infections. A pathogen is multidrug resistant (MDR) when it is resistant to three or more antibiotics at any given time (Jan *et al*., 2004). The result of this study revealed the presence of multidrug resistant bacteria on the four poultry farms. The antibiotics susceptibility profile of the bacterial isolates in this study were observed to have high resistance against augmentin, streptomycin, chloramphenicol and gentamicin for the Gram-negative isolates while high resistance was observed against ampiclox, gentamicin, amoxicillin and zinacef for the Gram-positive isolates. The high resistance seen in the bacteria isolates is similar to results obtained by Daodu *et al*. (2017), who observed high resistance rates against ampiclox, amoxicillin and augmentin.

Overall, multi-drug resistance to at least 3 of the antibiotics tested was high in large proportion for both the Gram-positive bacteria and Gram-negative bacteria in this study (Tables 4 and 5). The large proportion of the MDR is in line with the work of Umaru *et al*. (2016), who reported multidrug resistance in a similar study at 91.7% on average. The lowest resistance for the Gram-negative isolates was shown towards ciprofloxacin and pefloxacin (Figure 1), while for the Gram-positive bacteria, the least resistance was shown towards ciprofloxacin (Figure 2). The high rate of susceptibility towards ciprofloxacin was also reported by Adelowo *et al*. (2009). Ciprofloxacin was typically the most effective drug against the bacterial isolates as the majority of them were susceptible to the drug (Tables 2 and 3). This work agrees with the assertion by Thomson (1999) who stated that ciprofloxacin is usually the last resort for the treatment of difficult infections because of its potency in treatment where other antibiotics have failed to treat.

The MIC performed on the identified isolates showed absolute resistance to septrin (trimethoprim/sulfamethoxazole, co-trimoxazole), and also to amoxicillin and tetracycline with high (Table 8). These findings are consistent with the research carried out by Bebora, *et al*. (1994), which showed MIC determinations of *E. coli* strains that demonstrated resistance to trimethoprim-sulfamethoxazole (septrin) (100%), ampicillin (62.2%) and tetracycline

(51.4%). The antibiotic resistance levels of the identified bacterial isolates were reflected by the MIC50s and MIC90s, which represent MICs required for the inhibition of 50% and 90% of bacterial strains, respectively. MIC50 and MIC90 values of tetracycline were lowest amongst the tested antibiotics, while the values for, amoxicillin, septrin and erythromycin are the highest with ≥512 µg/ml for and MIC90 values. This is in line with work of Obayiuwana *et al*. (2018) who reported similar MIC90 values. A serious limitation to this study was the difficulty encountered in sample collection which limited the number of isolates included in the study.

CONCLUSION

In conclusion, this research work has established that antibiotic resistant bacteria are present in the poultry farms that were sampled. Our findings showed that all the identified bacteria isolates were resistant to all tested antibiotics in the study. The work points to the fact that the indiscriminate use of antibiotics especially tetracycline, streptomycin, sulphonamides, and ampicillin in these farms, and the lack of proper waste disposal promote the dissemination of drug resistance amongst related bacterial species. The overall results showed that this newly inhabited land used for farming purposes in this locale is not free from antibiotic resistant bacteria and this could have implications in the environment with possible public health concerns especially if these organisms get into the waterways and food sources. Poultry farms may thus serve as a source for the spread of MDR bacteria and gene flow of antibiotic resistance genes.

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