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Poultry Environment as a Reservoir of Antimicrobial Resistant Bacteria – A Nigerian Story

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Authors' contributions

This work was carried out in collaboration between both authors. Author KO designed the study, managed the analyses of the study, managed the literature searches, and wrote the first draft of the manuscript. Author TIC wrote the protocol, was primarily responsible for the lab work, and performed the statistical analysis. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: This study set out to help define the role of the poultry environment as a reservoir of drug resistant bacteria in Nigeria.
Introduction: The poultry environment has been acclaimed as a potential source of antimicrobial resistant bacteria but information is lacking in Nigeria. Despite worldwide control strategies, a predominance of small-scale poultry farming poses a challenge to proper veterinary monitoring in Nigeria.
Methodology: Three commercial laying farms were sampled and total heterotrophic counts determined. Bacterial identification, susceptibility profile and multiple antibiotic resistance (MAR) index and diversity index were determined using standard methodologies.
Results: Higher bacterial counts were observed in litter than feed samples (6.7×10^7 to 1.6×10^9 CFU/g versus 2.2×10^5 to 3.5×10^6 CFU/g) and majority of isolates (73.2%) belonged to only 5 bacterial species (*Escherichia coli*, *Staphylococcus aureus, Enterobacter aerogenes*, *Klebsiella pneumoniae* and *Bacillus* sp). With respect to antibiotic resistance in general, both litter and faecal matter isolates exhibited similar average rates of 62.2% and 63.1% respectively. Feed

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samples however had a lower average rate of 46.8%. A similar trend was observed when considering rates of multidrug resistance (MDR). Litter and faecal isolates had MDR rates of 88% and 91% respectively, while feed isolate had a MDR rate of 73%. A focus on the antibiograms of *Bacillus cereus*, *Staphylococcus aureus* and *Escherichia coli* specifically revealed a wide diversity among these isolates with 31 antibiotic resistance patterns observed from 55 isolates and a diversity index of 0.88, 0.9 and 0.98 respectively.

Conclusion: These findings indicate that the Nigerian poultry environment may serve not only as a reservoir of antibiotic resistant organisms, but also as an environment for the development of this resistance. A continuous monitoring of the situation is of essence to form the basis of future intervention strategies.

Keywords: Antimicrobial resistance; poultry manure; reservoir; multidrug resistance; diversity.

1. INTRODUCTION

The poultry environment has long been acclaimed as a potential source of antimicrobial resistant bacteria, acting as a possible reservoir for the dissemination of these organisms to man via the food chain (poultry meat), person to person contact (handlers) and environment (poultry waste disposal, organic fertilizers). Initial concerns for the possible role this environment plays as a reservoir of antibiotic resistant bacteria stemmed from the uncontrolled use of sublethal doses of antibiotics in the poultry industry as "growth promoters". Over the years, this was thought to have caused the high levels of resistance in both commensals and pathogens associated with poultry. Several strict guidelines were therefore put in place limiting the use of medically important antibiotics as growth promoters [1] with the expectation that this would result in a reduction in risk to man. A 2007 European Union report still however noted nalidixic acid or flumequine resistance rates of up to 50% in broiler isolates from EU countries [2] highlighting the need for continuous surveillance and monitoring of the situation. In Nigeria specifically however, with poultry farming mainly characterised by small scale farming (<500 birds), there appears to be a lack of proper veterinary monitoring and a misuse of antibiotics both in prophylaxis and therapy, compounded with a lack of adhesion to "withdrawal" time prior to sale and consumption [3,4,5].

Numerous studies have been geared towards assessing the prevalence of antibacterial resistant isolates in various poultry samples in a bid to more accurately define the threat this poses to humans. In Nigeria, these studies have generally involved an assessment of drug resistance in bacteria isolated from several poultry related samples, such as living birds [6,7], carcasses of both healthy and diseased animals [8,9], poultry waste dumps, feed [10,11, 12,13,14], egg [15], faecal matter [16] and litter [17].

Of all these samples, a greater threat has been thought to arise from poultry litter. This litter is composed of faecal material, spilled feed, bedding material and feathers [18,19]. With the increase in the demand for poultry, there has been a subsequent increase in waste generated [20]. The two most common ways of poultry litter waste utilisation include its use as fish feed in aquaculture and as organic fertilizer [20,21]. Additionally, poultry litter has been explored as a tool of bioremediation [18,22]. Generally, these practices are safe if carried out according to published guidelines but most times they involve the contraindicated practice of direct application without relevant treatments. A 2015 study by Ogundiran and colleagues which assessed 150 farms in Lagos, Nigeria, noted that of the 104 who responded, 82.5% carried out no treatment on their poultry litter prior to disposal [21]. Therefore a combination of indiscriminate use of antibiotic in poultry farming combined with inappropriate use of poultry litter could pose a severe public health risk to man.

Studies in Nigeria which have focused on poultry litter have mainly either assessed for antibiotic resistance profile of specific microorganisms or explored the microbial load of the litter. Few of these studies have been holistic in their approach. This study therefore set out to explore the load, profile, diversity and multidrug resistant status of bacteria in poultry litter and ascertain how this compares to feed and faecal samples obtained from the same environment.

2. MATERIALS AND METHODS

2.1 Sample Collection

Sampling was carried out over a three month period from three commercial laying poultry farms in Port Harcourt metropolis, Rivers State, Nigeria. Samples comprising of poultry litter, faecal matter and poultry feed were collected aseptically into sterile polythene sample bags. Three independent samples were collected for each sample type resulting in a total of nine samples per farm and twenty-seven samples in total. These samples were immediately transported to the laboratory for bacteriological analysis.

2.2 Bacterial Enumeration, Isolation and Identification

Total heterotrophic counts were determined for each sample using the standard spread plate count method on nutrient agar. Plates were incubated at 37^{o}C for 24 hours. Isolates present were identified using standard conventional microbiological and biochemical methods [23,24].

2.3 Antibiotic Resistance Screening

Antimicrobial susceptibility testing was carried out on Mueller Hinton agar using the standard Kirby Bauer disc diffusion test [25]. A total of 12 antibiotics (Abtek Biologicals Ltd, USA) were used. Gram positive organisms were tested against 10 µg ampicillin (AMP), 10 µg chloramphenicol (CHL), 5 µg cloxacillin (CXC), 5 µg erythromycin (ERY), 10 µg gentamicin (GENT), 10 µg streptomycin (STREP), 1 unit penicillin (PEN) and 10 µg tetracycline (TET). Gram negative organisms were tested against 25 µg ampicillin (AMP), 25 µg cotrimoxazole (COT), 10 µg gentamicin (GEN), 30 µg nalidixic acid (NAL), 200 µg nitrofurantoin (NIT), 25 µg colistin (COL), 25 µg streptomycin (STREP) and 25 µg tetracycline (TET). Zones of inhibition were read and data interpretation carried out using the NCCLS (2000) criteria [26]. Additionally, the multiple antibiotic resistance (MAR) index was calculated for as described by Krumperman [27] using the formula a/b whereby “a” is the total number of antibiotic to resistance scored and “b” is the total number of antibiotics against which the isolates were tested.

2.4 Assessment of Isolate Diversity

Based on the antibiogram pattern generated for each isolate, the diversity index was determined per sample type, per farm and in total, using the Simpson’s index of diversity [28] using the formula below.

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j(n_j - 1)$$

Formula 1: Parameters used to ascertain discriminatory power.

(Where D is the index of discrimination, N is the population size; S is number of types and n is the distribution of strains within types).

Simpson’s index of diversity assesses both the number of types in a sample (richness) as well as the relative distribution within the types (evenness).

2.5 Statistics

In this study, the colony counts obtained were expressed as Log 10 values and were statistically compared using analysis of variance (ANOVA). Fisher’s exact test was used to analyse the composition of the isolates. The level of significance was set at $P \leq .05$.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Bacterial load

All 9 poultry litter samples obtained from the three farms in this study revealed total heterotrophic bacterial (THB) counts ranging from 6.7×10^7 to 1.6×10^9 CFU/g. This was similar to THB counts of the nine faecal samples (4.9×10^7 to 1.8×10^9 CFU/g) but much higher than the THB counts of the poultry feeds (2.2×10^5 to 3.5×10^6 CFU/g) (Fig. 1). These differences were significant in the bacterial loads obtained from the various samples ($F(2,18) = 17.35$, $P < .001$).

3.1.2 Microbial composition and susceptibility profile

In general, majority of the 112 unique isolates obtained were Gram negative (75, 66.4%).

Looking specifically at the different samples, a similar trend was observed for both the poultry litter and faecal samples (Fig. 2), where 71% of isolates obtained were Gram negative. The poultry feed samples had a different trend with a 53% to 47% Gram negative to Gram positive ratio noted. However, the association between the microbial composition and type of sample was found to be non-significant (P = .24).

In total, 20 bacterial genera were identified but 73.2% of isolates were however comprised of only 5 bacterial species (Fig. 3); *Escherichia coli*, *Staphylococcus aureus,* *Enterobacter aerogenes*, *Klebsiella pneumoniae* and *Bacillus* sp. (20.5%, 18.6 %, 11.6%, 11.6% and 10.7% occurrence respectively).

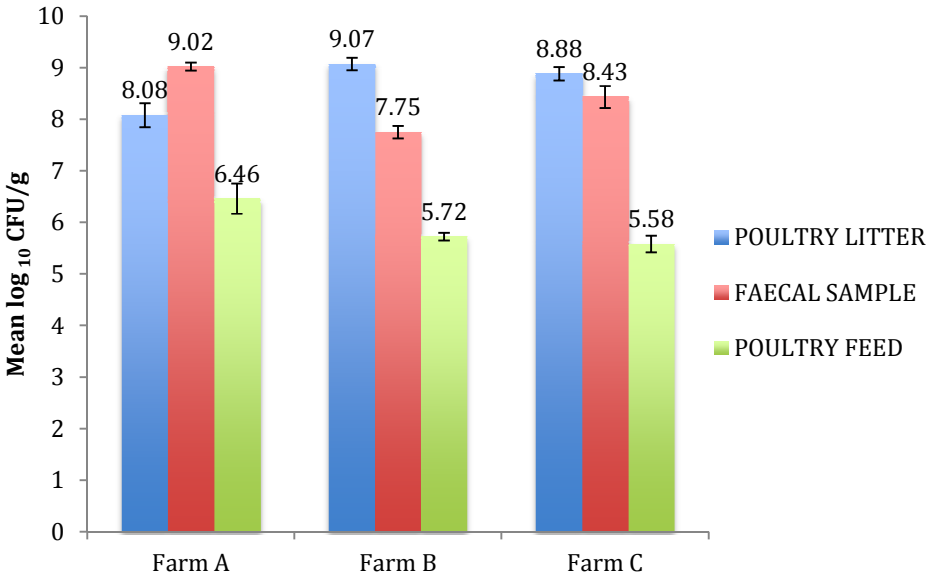

Fig. 1. Total heterotrophic bacterial counts associated with different samples obtained from poultry environment

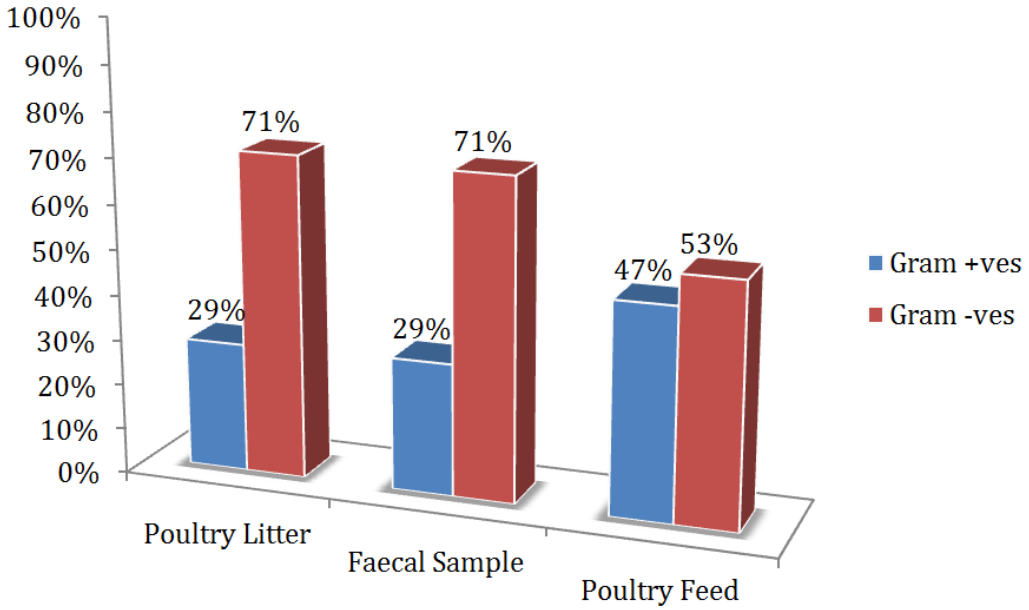

Fig. 2. Sample based variation in microbial composition

The resistance of these isolates as a whole, ranged from 16% for gentamicin to 100% for penicillin (Fig. 4). Less than 40% resistance was observed for streptomycin (29%) and chloramphenicol (32%), while more than 80% of tested isolates were resistant to tetracycline (81%), ampicillin (95%) and oxacillin (97%).

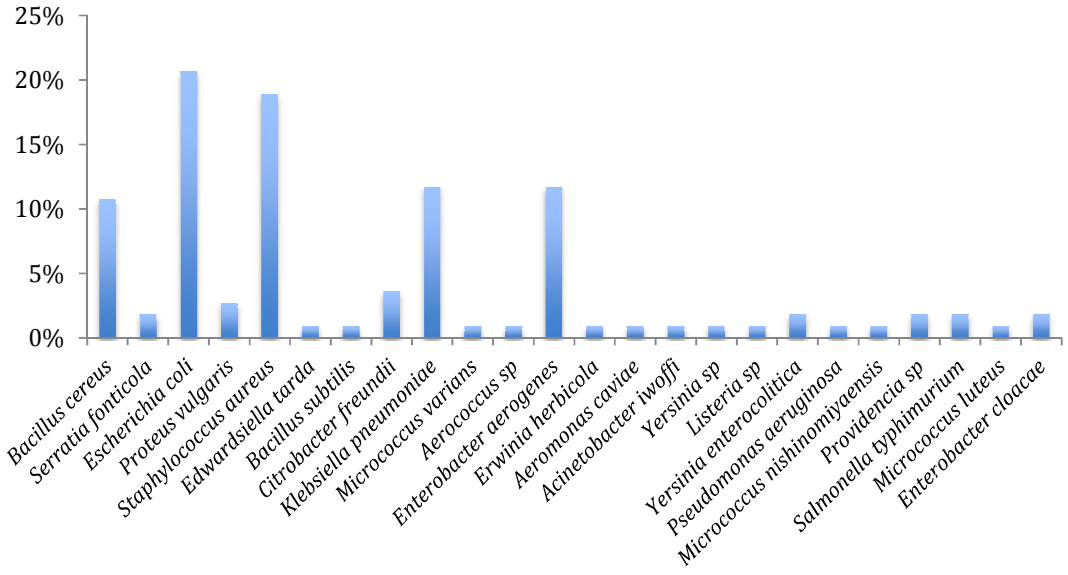

Fig. 3. Frequency of occurrence of bacterial isolates from different sample types associated with poultry environment

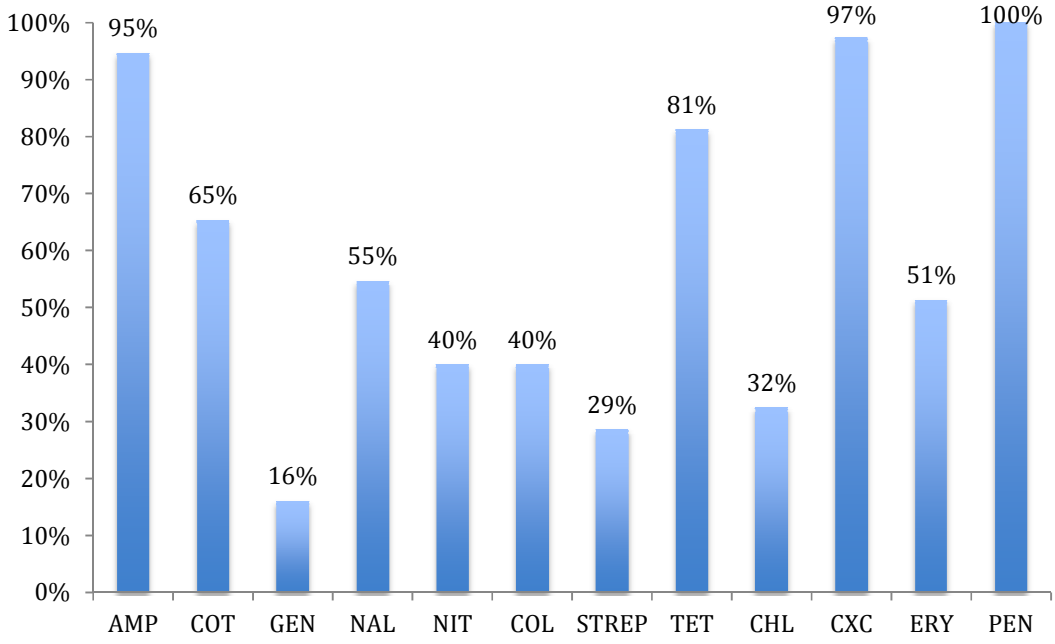

Fig. 4. Diversity of antimicrobial resistance associated with bacteria from poultry environment

3.1.3 Sample related variations in drug resistance patterns and MDR status

As a whole, there appeared to be a relationship between level of antibiotic resistance and source of isolates (Table 1). In 4 of 12 cases (Gentamicin, Nalidixic acid, Streptomycin and Tetracycline), isolates from poultry litter exhibited higher levels of resistance than the other isolates, while in 5 of 12 cases (Ampicillin, Chloramphenicol, Oxacillin, Erythromycin and Penicillin), these isolates had similar levels (<5% difference) of resistance as isolates from faecal matter. Isolates from faecal matter had a higher level of resistance against three antibiotics (Cotrimoxazole, Nitrofurantoin and Colistin). Isolates from poultry feed on the other hand, consistently exhibited a lower level of resistance than the other two categories.

3.1.4 Antibiogram/strain diversity (anti-microbial susceptibility patterns)

A total of thirty-one different antibiotic resistance patterns (Table 2) were noted among the 55 isolates of three of the largest groups of isolates (*B. cereus*, *S. aureus* and *E. coli*), with a total diversity index of 0.96. All farms had a similar diversity index (0.94 – 0.98), while for the each of group of isolate specifically, the diversity index ranged from 0.88 (*B. cereus* isolates) to 0.98 (*E. coli* isolates).

3.1.5 Multiple antibiotic resistance (MAR) index

An assessment of the multiple antibiotic resistance (MAR) index of these three groups of isolates (Fig. 5) revealed that isolates from poultry feed were predominant (47%) at the lower MAR index values (0 – 0.4) while isolates from faecal matter were predominant (64%) at higher MAR index values (0.6 – 1.0). In general, faecal matter isolates had similar rates of multidrug resistance (resistant to >3 antibiotics, MARI ≥ 0.4) as isolates from poultry litter (91% versus 88%), but higher rates than isolates from poultry feed (73%).

3.2 Discussion

As the scourge of antimicrobial drug resistance increases worldwide posing an ever-pressing public health problem, more and more research is geared towards reducing the development of drug resistant pathogens and halting this negative trend. One of such approaches has been to determine possible reservoirs of antibacterial drug resistance and assessing possible effects on man. One such environment with the potential to act as a reservoir of antimicrobial drug resistance due to the application of large amounts of antibiotics as growth promoters, prophylaxis and therapy, is the poultry environment. With the poultry environment noted to generate up to 6.69 kg of poultry litter per day [21], results from this study show that in the absence of adequate treatment, the poultry environment could serve as a source of introduction of large numbers of bacteria (6.7×10^7 to 1.6×10^9 CFU/g) into the environment. While similar to reports by other studies [29,30,31,32,33,34] noting bacterial loads ranging from 1.32×10^7 CFU/g to 7.2×10^9 CFU/g, this study had a lower load than a comprehensive 2000 study [35] involving 12 regions in the United States which reported with an average bacterial load of 2.54×10^{11} CFU/g. The bacterial load of litter has been found to be effected by several factors [36,29], with broilers, wood shaving and low litter replacement frequencies resulting in higher loads.

Table 1. Sample based variation in antimicrobial resistance

Antibiotics	Poultry litter % resistant	Faecal matter % resistant	Poultry feed % resistant
Ampicillin	95	90	100
Cotrimoxazole	67	79	41
Gentamicin	24	15	7
Nalidixic acid	63	57	35
Nitrofurantoin	37	54	24
Colistin	30	61	24
Streptomycin	44	24	13
Tetracycline	95	85	57
Chloramphenicol	36	38	23
Oxacillin	100	100	92
Erythromycin	55	54	46
Penicillin	100	100	100
Total	62.2	63.1	46.8

Table 2. Antibiotic resistance pattern of isolates

Isolate	Antibiotic resistance patterns (source)	No of isolates
Bacillus cereus		
FARM A	AMP-CXC-PEN (PF)	1
	AMP-CXC-PEN-TET (PL)	2
	AMP-CXC-PEN-TET-GEN (FS)	1
	AMP-CXC-PEN-CHL-ERY (FS)	1
FARM B	AMP-CXC-PEN-TET-CHL (FS)	1
	AMP-CXC-PEN-TET-CHL-GEN (1 PL, 2 PF)	3
FARM C	None (PL)	1
	AMP-CXC-PEN-TET (FS)	2
	AMP-CXC-PEN-TET-ERY (PF)	1
Staphylococcus aureus		
FARM A	AMP-CXC-ERY-PEN (PF)	1
	AMP-CXC-ERY-PEN-TET (PL)	1
	AMP-CXC-ERY-PEN-TET-CHL (PL, FS)	2
	AMP-CXC-ERY-PEN-TET-CHL-STREP (FS)	1
FARM B	AMP-CXC-PEN (FS)	1
	AMP-CXC-PEN-TET (2 PL, 1 FS)	3
	AMP-CXC-PEN-TET-ERY (PF, FS)	2
	AMP-PEN-TET-ERY (PF)	1
FARM C	AMP-CXC-PEN-TET (2 PF)	2
	AMP-CXC-PEN-TET-ERY (PL)	2
	AMP-CXC-PEN-TET-ERY-CHL (PL, FS)	2
	AMP-CXC-PEN-TET-ERY-STREP (PF, FS)	2
Escherichia coli		
FARM A	None (FS)	1
	COL-TET (2 FS)	2
	AMP-COT-TET-NAL (PL)	1
	AMP-COT-TET-NAL-STREP (PL)	1
	AMP-COT-TET-NIT-STREP (PL)	1
FARM B	AMP (FS)	1
	AMP-TET (2 PL)	2
	AMP-TET-COT (2 FS)	2
	AMP-TET-COT-STREP (PL, PF)	2
FARM C	AMP-STREP-TET-COT (PF)	1
	AMP-STREP-TET-NAL (PL)	1
	AMP-STREP-TET-NAL-COT (FS)	1
	AMP-STREP-TET-NAL-COT-NIT (PL)	1
	AMP-STREP-TET-NAL-COT-NIT-COL (PL)	1
	AMP-TET-NAL-COT-NIT (PF)	1
	AMP-TET-NAL-COT-COL (PF)	1
	AMP-TET-NAL-COT-NIT-COL (2 FS)	2

Table 3. Simpson's index of diversity

	Farm A	Farm B	Farm C	Diversity/Isolate
B. cereus	0.9	0.5	0.83	0.88
S. aureus	0.9	0.81	0.86	0.9
E. coli	0.93	0.81	0.97	0.98
Diversity/Farm	0.98	0.94	0.94	

In addition to the high bacterial load observed, the high rates of occurrence of bacteria belonging to known pathogenic genera (Fig. 3), further highlight the potential public health hazard posed by these poultry environments. *Escherichia coli* is the number one indicator organism for faecal contamination and its presence in poultry litter unsurprising. It however highlights a major risk of the utilisation of poultry litter as organic manure and points at one of the suspected routes of transmission of antibiotic resistant bacteria to man, via contaminated vegetables. Fruits and vegetables have been implicated as the leading foods associated with interstate foodborne outbreaks in the US [37] and organic manure noted as a possible source of contamination of these items [38,39,40,41].

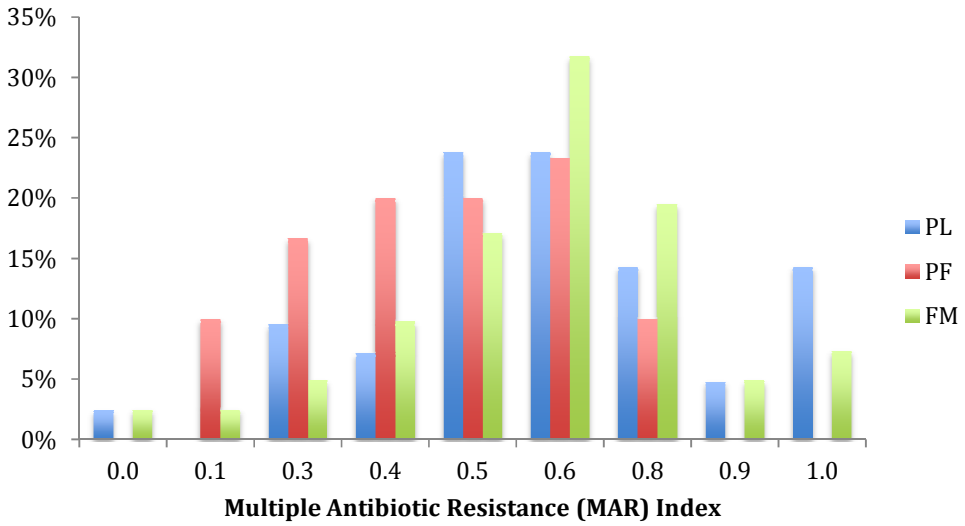

Fig. 5. Multiple antibiotic resistance index of poultry isolates

The poultry environment is expected to harbour a higher level of antibiotic resistant bacteria due to industry practices. Results of this study indicate such a similar trend of the role of the poultry environment as a reservoir of antibacterial resistant isolates with >50% resistance observed to majority (7 of 12) of the antibiotics tested. The rates of resistance noted in this study are similar to the majority of published reports from Nigeria and the rest of the world [42]. In this study, isolates were particularly resistant to ampicillin (and related drugs) and tetracycline. Exceptions to these are the low rates of resistance to streptomycin (29%) observed in this study. This is in contrast to the higher ranges reported by other studies 68% to 84.3% [43,44,45] but similar to 35.2% [46]. Though antibiotic usage was not monitored in this study, an assessment of published literature reveals that streptomycin is one of the more commonly used antibiotics in the Nigerian poultry industry [47,48,49]. This however does not explain the results observed in this study.

That the poultry environment serves not just as a reservoir of antibiotic resistant organisms but also possibly as the perfect environment for the development of such resistance may perhaps be seen in the consistently lower levels of resistance observed in poultry feed isolates opposed to faecal matter and litter isolates. Unlike the case of poultry feed whereby the isolates present do not originate from the chickens but depend on production and storage conditions, this is not so for isolates obtained from faecal matter and poultry litter. These isolates are rather, a reflection of poultry industry practices, with the higher levels of resistance in these isolates probably a reflection of the indiscriminate use of antibiotics in the poultry industry. More worrisome however is the high diversity index (0.96 in total) based on the antibiograms of three of the major isolate classes with 31 antibiotic resistance patterns observed from 55 isolates. This points at the possibility that the antibiotic resistant isolates present in these poultry environments, rather than being a result of the spread of a single drug resistant clone, may have resulted from multiple acquisitions of drug resistant genes by susceptible isolates. More profiles per isolates were noted in *E. coli* (16:22), than in *S. aureus* and *B. cereus* with profile to isolate ratios of 8:20 and 8:13 respectively. Additionally, the predominant occurrence of faecal and litter isolates at higher MAR index values of >0.5 (57% of PL isolates and 64% of FM) attest to a high-risk source involving the use of antibiotics [50]. This is in contrast to the 43% of feed isolates occurring at higher MAR index values, perhaps indicating a slightly reduced pressure from exposure to antibiotics.

4. CONCLUSION

These findings point to the fact that the Nigerian poultry environment may serve as a reservoir of antibiotic resistant organisms. A continuous monitoring of the situation is of essence to form the basis of future intervention strategies, which may include sensitisation of farmers and an

increase in policy implementation with respect to the guidelines governing the use of medically important antibiotics in this industry. While the presence and prevalence of antibiotic resistant bacteria in poultry environment could potentially pose a public health issue to man, such a link has not yet been concretely established [51], with some researchers even going as far as doubting even if such a link exists. A monitoring of the situation is however essential as it would form the basis of future intervention strategies.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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