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Multiple Antibiotic Resistant Index of Gram-Negative Bacteria from Bird Droppings in Two Commercial Poultries in Enugu, Nigeria

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Abstract

Antimicrobial resistance refers to the ability of microorganisms to grow in the presence of an antimicrobial agent at a concentration that will normally kill or inhibit their growth. Antimicrobial resistance has become a major global threat making treatment of infections tougher especially with high cost of treatment in humans and animals. This study was done to determine the Multiple Antibiotic Resistant Index (MARI) of Gram-negative bacteria from bird droppings in two commercial poultries in Enugu. Forty (40) samples were collected from each of the poultries. Isolates were identified by standard microbiological methods. The isolates identified were *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Citrobacter* spp, *Proteus* spp. and, *Enterobacter* spp. Antibiotic susceptibility testing was carried out using disc diffusion technique. The organisms were tested against pefloxacin, augmentin (amoxicillin and clavulanic acid), ceftazidime, streptomycin, ciprofloxacin, gentamycin, cephalothin, neomycin and ofloxacin. The result of the susceptibility test showed that *Proteus* spp had the highest resistance and MARI value of 0.5 and 1.0 respectively. The other MARI values were *Escherichia coli* (0.9), *Klebsiella pneumonia* (0.9), *Pseudomonas aeruginosa* (0.8), *Citrobacter* spp (0.8) and *Enterobacter* spp (0.7). These results suggest that bacterial organisms from poultry source can contribute significantly to the spread of multi-antibiotic resistant organisms. This could arise from the indiscriminate use of antibiotics in bird feeds in poultries.

1. Introduction

Antimicrobial Resistance (AMR) has become a big threat to global health. It has risen to dangerously high levels in all parts of the world, making it difficult to treat infectious diseases [1]. This has led to the use of expensive second or third-generation antibiotics and prolonged hospital stay which then translates to higher hospital bills [2]. The high rates of antimicrobial resistance occurrence have attracted the attention of international bodies such as the World Health Organization (WHO), Food and Agriculture Organization (FAO) and, the World Organization for Animal Health who have now forged a united approach to combat it as a common force [1]. It is estimated that worldwide, 700,000 patients die annually as a result of resistant infections and if nothing is done to combat antimicrobial resistance, the death rate is estimated to escalate to 10 million by the year 2050 [3].

Antimicrobials are also used in animals to treat animal diseases and as growth promoters to increase productivity [4]. These animals are important to the well-being of humans as they contribute to human nutrition and wealth especially in countries where the livestock are a major source of income. Any increase in the prevalence of antimicrobial resistance organisms will impact negatively directly and indirectly. To avoid economic loses, livestock farmers are tempted to use more effective and often more expensive antimicrobial in treating sick animals, thus ending-up infringing upon those that are last line options for use in humans; especially if they can easily be bought over the counter [2]. This ends-up in the development of resistance to the few antimicrobials that are relied upon for treating human infections. The microorganisms that are mainly involved in the infection process both in hospital and community are called ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and, *Enterobacteriaceae*) emphasizing their capacity to "escape" from common antibacterial treatments [5] [6].

The poultry industry is one of the largest and fastest-growing agro structures in Africa and the world. This can be attributed to increasing demand for poultry meat and egg products. These high demands of poultry products have also led to the large-scale accumulation of wastes including manure and litter which may pose disposal and pollution problems to man and the environment. Resistance bacteria of animal origin can be transmitted from animals to humans through the food supply, water or, through direct contact with animals. Sometimes resistant genes can be transferred from animals through human pathogens that are

normally human-specific. Contaminated food of animal origin is one source of human bacterial infections when consumed; therefore, the presence of antibiotic-resistant strains in the animal foods such as poultry has raised concerns that the treatment of human infections will be compromised [7] [8]. It has resulted in disease burden, high cost of treatment and, a continuous spread of resistant microbial pathogens [3] [9]. Multiple Antibiotic Resistance (MAR) index is a valid and useful method of tracking especially bacterial infections and drug resistance. MAR index is calculated as the ratio of the number of antibiotics to which an organism is resistant to the total number of antibiotics to which the organism is exposed. Bacteria having MAR index $\geq$ 0.2 originate from a high-risk source of contamination where several antibiotics are used [10] [11] [12]. Thus, it became imperative to investigate the significance of multi-antibiotic resistant bacteria of poultry source in the emergence and, spread of antibiotic resistance in Enugu metropolis, Nigeria and also evaluate the MAR indices of the isolates.

The limitation of this study was the inability of the authors to collect adequate representative samples for the study. This was due in part to frequent collection of the droppings as manure for their farms by the natives.

2. Materials and Methods

2.1. Study Area and Samples Collection

The study was carried out in Enugu State, Nigeria. Fecal droppings were obtained from two commercial poultry farms in Emene and Abakpa. A total of forty (40) samples of fresh bird droppings were collected from each of the two commercial poultry farms located in Enugu metropolis over a period of 4 weeks (September 20 - October 18, 2019). A 1 g poultry dropping sample was collected in sterilized Mac Cartney bottle that contained peptone water and was transported to the research laboratory for bacteriological analysis within an hour of collection. The two poultry farms are located in a densely populated area with frequent farming practices. The farmers use the bird droppings as manure for their crops to boost yield.

The sample size was based on the availability of fresh droppings on sample collection days within the sample collection duration of four weeks. Samples were randomly collected from poultry sites.

2.2. Isolation of Bacteria from Poultry Droppings

Bacteriological examinations were carried out using standard methods for aerobics bacteria [13]. Samples collected in Mac Cartney bottles were gently shaken and stirred with a sterile glass rod until mixed thoroughly, 1.0 ml was transferred into the test tube containing 9.0ml of distilled water and serial dilution was carried out. Aliquots of each suspension were evenly spread plating on nutrient agar, Mac Conkey agar. (They were grown on Mac Conkey as a selective and differential medium due to the preference for Gram-negative organisms in this study). The plates were incubated for 24 hours at 37˚C aerobically and growth of

the colony was characterized and representative colonies were selected and purified by successive sub-culturing. The identification of bacteria was done based on their morphology, Gram staining, and biochemical tests. Different biochemical tests used for Gram-negative include indole, urea, Simon's citrate agar, lysine iron agar, oxidase, sugar fermentation and motility [14].

2.3. Antibiotics Disc

Antibiotic discs used include Pefloxacin (30 μg), Ciprofloxacin (30 μg), Ceftazidime (30 μg), Augmentin® (Amoxicillin and clavulanic acid combination) (30 μg), Gentamicin (30 μg), Streptomycin (30 μg), Cephalotin (30 μg), Ofloxacin (30 μg), Neomycin (30 μg).

2.4. Determination of Susceptibility of Bacteria Isolates

2.4.1. Disc Diffusion Method

A 20 ml volume of Mueller Hinton agar was prepared and dispensed aseptically into 90 mm Petri dishes. A loopful of each isolate was inoculated into 100 ml of nutrient broth and cultured overnight. From a 100 μl of each of the isolates equivalent to 0.5 ml, MacFarland standard was aseptically seeded into the Mueller Hinton agar plate. This was allowed to dry. The antibiotic disc was aseptically placed on the surface of the Muller Hinton agar and allowed for 30 minutes to pre-diffuse. The set up was done in triplicate with a control containing no antibiotic disc. It was incubated for 24 hours at 37˚C and thereafter, the inhibition zone diameters were measured. Values obtained were interpreted according to the Clinical and Laboratory Standards Institute [15].

2.4.2. MARI Index

MAR index is calculated as the ratio of the number of antibiotics to which an organism is resistant to the total number of antibiotics to which the organism is exposed. MARI is calculated as a/b, where a = number of antibiotics isolate was resistant to b = the total number of antibiotic used Bacteria having MAR index ≥ 0.2 originate from a high-risk source of contamination where several antibiotics are used [11] [12].

3. Results

3.1. Phenotypic Identification of Isolates

A total of twenty (20) isolates were obtained from the bird droppings collected from the two poultries. The six Gram-negative bacteria were isolated from the sample using morphological, biochemical characteristics and include *Escherichia coli*, *Proteus* spp, *Klebsiella pneumoniae*, *Enterobacter* spp, *Citrobacter* spp and *Pseudomonas aeruginosa* (Table 1).

The frequency of each of the isolates as show on Table 2 explains the distribution of the different isolates from the poultries. *Escherichia coli* was (7), *Proteus* spp (4), *Klebsiella pneumonia* (4), *Enterobacter* spp (2), *Citrobacter* spp (2) and *Pseudomonas aeruginosa* (1). The above distribution shows that *E. coli* had the

Table 1. Phenotypic identification of isolates.

Isolates	Citrate	Indole	Urease	Oxidase	Motility	Lysine iron agar	Probable organism
1	-	+	-	-	Motile	-	*Escherichia coli*
2	+	+	+	-	Motile	-	*Proteus* spp
3	-	+	-	-	Motile	-	*Escherichia coli*
4	+	+	+	-	Motile	-	*Proteus* spp
5	-	+	-	-	Motile	-	*Escherichia coli*
6	+	-	+	-	Non Motile	-	*Klebsiella* spp
7	-	+	-	-	Motile	-	*Escherichia coli*
8	+	+	+	-	Motile	-	*Proteus* spp
9	-	+	-	-	Motile	-	*Escherichia coli*
10	+	-	-	-	Motile	-	*Enterobacter* spp
11	+	-	+	-	Non Motile	-	*Klebsiella* spp
12	-	+	-	-	Motile	-	*Escherichia coli*
13	+	+	+	-	Motile	-	*Proteus* spp
14	+	-	+	-	Non Motile	-	*Klebsiella* spp
15	+	-	-	-	Motile	-	*Enterobacter* spp
16	-	+	-	-	Motile	-	*Escherichia coli*
17		-		-	Motile	-	*Citrobacter* spp
18	+	-	+	-	Non Motile	-	*Klebsiella* spp
19		-		-	Motile	-	*Citrobacter* spp
20		-		-		-	*P. aeruginosa*

KEY: (-) = NEGATIVE, (+) = POSITIVE.

Table 2. Frequency of isolation identified from both poultry farms.

ISOLATES	POULTRY 1	POULTRY 2
E. coli	5	2
Proteus spp	3	1
Klebsiella spp	1	3
Enterobacter spp	1	1
Citrobacter spp	0	2
Pseudomonas aeruginosa	0	1
TOTAL	10	10

highest frequency which also tallies to the findings of Chika *et al.,* [16] who recoreded a total of 29 *E. coli* isolates from the 40 sample swabs collected.

The sensitivity results on Table 3 show that most of the isolates were resistant to the conventional antibiotics used. *Proteus* spp had the highest resistance to all the antibiotics tested while *Escherichia coli* and *Citrobacter* spp showed

Table 3. Susceptibility pattern (Inhibition zone diameter in millimeters (mm) of the Isolates).

Isolate	CT	OFX	N	PEF	GN	AU	CIP	ST	CF	AM
E. coli	0	0	5	6	14	15	6	3	2	0
Proteus spp	5	4	0	0	0	0	0	0	0	0
E. coli	2	6	12	14	40	20	6	8	1	1
Proteus spp	20	15	0	16	12	11	30	15	0	2
E. coli	0	12	4	30	0	23	15	0	15	0
Klebsiella pneumoniae	0	0	6	40	0	0	15	4	6	8
E. coli	0	0	7	15	0	23	14	0	0	2
Proteus spp	15	0	6	5	0	11	32	0	5	11
E. coli	3	0	1	4	14	0	2	4	12	8
Enterobacter	30	0	0	23	6	8	17	22	6	0
Klebsiella pneumoniae	0	0	3	6	5	0	34	0	8	8
E. coli	0	15	4	23	8	9	0	8	0	40
Proteus spp	0	0	6	4	7	32	5	4	0	3
Klebsiella pneumoniae	7	0	8	0	0	23	7	23	0	8
Enterobacter spp	0	4	25	1	5	9	12	15	0	17
E. coli	0	7	18	7	3	4	4	30	4	1
Citrobacter spp	15	8	0	8	8	7	7	19	6	0
Klebsiella pneumoniae	0	5	2	4	0	17	6	1	7	15
Citrobacter spp	0	30	7	0	4	19	6	0	30	7
Pseudomonas spp	0	5	0	15	1	4	2	0	20	8

PEF = Pefloxacin, AU = Augumentin (Amoxicillin/clavulanic acid), AM = Amoxicillin, CT = Ceftazidime, ST = Streptomycin, CIP = Ciprofloxacin, GN = Gentamycin, CF = Cephalotin, N = Neomycin, OFX = Ofloxacin.

intermediary response to the antibiotics. The high resistant pattern of the isolates suggests that most of the isolates have lost sensitivity to the conventional antibiotics used in this study. Similar studies [16] in the Eastern parts of the country identified high resistant pattern to the antibiotics used with *Escherichia coli* showing the highest resistance.

This measures the resistance index of the isolates. MAR index is calculated as the ratio of the number of antibiotics to which an organism is resistant to total the number of antibiotics to which the organism is exposed. Bacteria having MAR index ≥ 0.2 originate from a high-risk source of contamination where several antibiotics are used. Where 1) is the aggregate antibiotic resistance score of all isolates from the sample, 2) is the total number of antibiotics used. Proteus spp had the highest MARI index of 1.0 which confirms high resistance. This is in line with a study by [17], who identified the Enterobacteriaceae as the prevailing organisms identified from samples collected in poultry dropping.

3.2. Mean Distribution of MAR Index of Isolates between Poultries

The ratio of the MARI distribution shows that there is an insignificant difference in the MAR index from the two poultries. High resistance is observed though the poultries are not situated in the same area. Figure 1 shows the mean distribution of the MARI index in the two poultry. Poultry 1 had a MARI index value of 0.73 while poultry 2 had s value of 0.8.

4. Discussion

The bacteria pathogen isolated were mostly Gram-negative bacteria with *E. coli* having the highest prevalence. The result is consistent with earlier reports [18] [19]. Although the isolates are part of the enteric flora of the poultry birds, they are of public health importance since they are implicated in antibiotic resistance. Another interesting aspect of this study is the use of the droppings as manure to boost local crops, there is a high possibility of antibiotic resistance gene transfer when the crops are consumed by humans and further transfer between individuals within the community [20]. The variations observed in the carriage of organisms in both poultries may be due to the environmental settings in which the birds are raised, nutrition and, the physiological state of the gut of animals which influence the distribution of organisms found [21].

The Multiple Antibiotic Resistance Index (MARI) as shown on Table 4 is an important analysis to check antibiotic resistance and health risk factors. MAR index is calculated as the ratio of the number of antibiotics to which an organism is resistant to total the number of antibiotics to which the organism is exposed. Bacteria having MAR index ≥ 0.2 originate from a high-risk source of contamination where several antibiotics are used.

Organisms which have MAR indices of greater than ≥0.2 confirm the presence of multidrug-resistant genes originating from the environment where there is an abuse of these drugs and also that the plasmids contain one or more resistance genes, each encoding a single antibiotic resistance phenotype [12] [22]. Using the MARI analysis is also simple and does not require specialized training and expensive equipment [11] [12], while also providing the needed data. *Proteus* spp is a common cause of community-acquired infection, its prevalence in this study suggests a chance infection spread if its proliferation is not controlled.
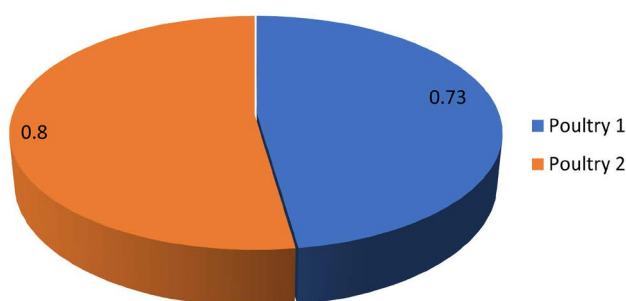

Figure 1. Mean distribution of mar index of isolates between poultry 1 and poultry 2.

Table 4. Multiple antibiotic resistance indices (MARI) of isolates.

S/N	Isolates	MAR Index
1	*Escherichia coli*	0.8
2	*Proteus* spp	1.0
3	*Escherichia coli*	0.7
4	*Proteus* spp	0.5
5	*Escherichia coli*	0.6
6	*Klebsiella pneumoniae*	0.8
7	*Escherichia coli*	0.7
8	*Proteus* spp	0.8
9	*Escherichia coli*	0.8
10	*Enterobacter* spp	0.6
11	*Klebsiella pneumoniae*	0.9
12	*Escherichia coli*	0.7
13	*Proteus* spp	0.9
14	*Klebsiella pneumoniae*	0.8
15	*Enterobacter* spp	0.7
16	*Escherichia coli*	0.9
17	*Citrobacter* spp	0.8
18	*Klebsiella pneumoniae*	0.8
19	*Citrobacter* spp	0.7
20	*Pseudomonas* spp	0.8

MAR index is calculated as the ratio of number of antibiotics to which organism is resistant to total number of antibiotics to which the organism is exposed. Where 1) is the aggregate antibiotic resistance score of all isolates from the sample, 2) is the total number of antibiotics used.

Pseudomonas and *Klebsiella pneumoniae* are also major pathogens in health-care-associated infections. They are associated with significant morbidity in humans and are also difficult to manage with first-line antibiotics [23] [24]. The susceptibility test on Table 3, suggests high resistance to the Co-amoxiclav, Ceftazidime, Neomycin and, Amoxicillin. This observation is consistent with previous reports of [15] and [25] whose findings identified multidrug-resistant bacteria in poultry droppings. These organisms have very important implications on human health as antibiotic- resistant infections are difficult to treat and often require expensive antibiotics, long term therapy, and in extreme cases may lead to mortality. Resistance to Gentamicin was significantly high; this is not unexpected because of the use of antibiotics on the bird feeds, particularly through agriculture-influenced and urban-influenced treated and untreated water. Such water samples have all been reported to harbour either bacteria, which carry (multiple) antibiotic-resistance genes, or plasmids carrying these genes [26]. The multidrug-resistance bacteria isolates identified in this study may be due to the

indiscriminate use of antimicrobial agents in poultry. The use of these antibiotics for infection control may ultimately favor the widespread of drug-resistant strains in the environment [27].

5. Conclusion

This study indicates that poultry dung is a carrier of pathogenic bacteria which is capable of transmitting antibiotic resistance genes to human. The most resistant organisms isolated were *E. coli* and *Proteus* spp with the latter having the highest frequency. The study calls for close monitoring of antibiotic resistance in our environment and controlled use of antibiotics in poultry since the birds serve as a source of protein and a staple in many homes. The use of the bird droppings for manure is also a call for concern because of the health hazard to the general populace when crops are consumed.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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