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INVESTIGATION OF HETERORESISTANCE IN MULTI DRUG RESISTANT BACTERIA ISOLATED FROM POULTRY DROPPINGS

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ABSTRACT

Antimicrobial resistance (AMR) is a growing global health challenge, with agricultural practices playing a significant role in its emergence and spread. This study investigated heteroresistance (the presence of resistant subpopulations within an otherwise susceptible bacterial population) in multidrug-resistant (MDR) bacteria from poultry droppings in Obio-Akpor, Rivers state, Nigeria. Bacteria were isolated using standard microbiological techniques, identified through biochemical tests, and subjected to antibiotic susceptibility testing over three days using the disk diffusion method following CLSI guidelines. Heteroresistance was detected through inconsistent results, the appearance of microcolonies, or haze within inhibition zones. Thirteen bacterial isolates were recovered, predominantly Gram-negative rods, including *Escherichia coli, Salmonella spp., Klebsiella spp.,* and *Proteus spp.* A high overall prevalence of heteroresistance was observed (74.6%), with notable variability in susceptibility patterns across testing days. Some isolates, such as *Klebsiella spp.* and *Proteus spp*., displayed heteroresistance to over 85% of antibiotics tested. A strong correlation was found between MDR status (which was reflected in high Multiple Antibiotic Resistance (MAR) indices) and heteroresistance. Bacterial isolates showed more heteroresistance to, cephalosporins (e.g., ceftazidime, ceporex) and fluoroquinolones. These findings indicate that heteroresistance represents a hidden reservoir of AMR in Nigerian poultry, likely fueled by uncontrolled antibiotic use. This phenomenon poses risks of treatment failure and complicates AMR surveillance. Heteroresistance is a critical factor in the dynamics of AMR in agricultural settings and highlights the need for its integration into national AMR monitoring programs.

KEYWORDS: Antimicrobial resistance, Heteroresistance, poultry droppings.

INTRODUCTION

According to estimates, drug-resistant infections might kill 10 million people a year by 2050 if current trends continue, making antimicrobial resistance (AMR) one of the most urgent global public health concerns of the twenty-first century (Murray et al., 2022). In agricultural settings, where antimicrobials are widely used for growth promotion, disease prevention, and treatment in food-producing animals, the emergence and spread of multidrug-resistant (MDR) bacteria which is defined as non-susceptibility to at least one agent in three or more antimicrobial categories, have been especially noticeable (Aworh et al., 2019). Over the last 20 years, poultry production in Nigeria has grown quickly to meet the protein needs of a growing population of more than 200 million people. However, this rapid growth has been accompanied by widespread and frequently uncontrolled use of antimicrobials, including crucial antibiotics such as fluoroquinolones, third-generation cephalosporins, and colistin (Ishaleku et al., 2020). Poultry droppings, which contain significant amounts of excreted antimicrobials, resistant bacteria, and resistance genes, are an important vehicle for the environmental spread of AMR determinants. Studies carried out in Nigeria have found disturbingly high rates of multi-drug resistant Enterobacterales in poultry droppings including *Escherichia coli*, *Klebsiella pneumoniae*, and *Salmonella species* with resistance rates frequently exceeding 70% for commonly used antibiotics (Ngbede et al., 2020). These findings highlight the urgent need for extensive monitoring of antimicrobial resistance patterns in Nigerian poultry systems within a One Health framework that acknowledges the interconnectedness of human, animal, and environmental health. Despite increasing awareness of AMR in poultry, a critical yet under studied phenomenon 'Heteroresistance' is frequently overlooked in veterinary and agricultural microbiology in sub-Saharan Africa. Heteroresistance refers to the presence of resistant subpopulations within an otherwise susceptible bacterial population, where a small fraction of cells (usually 10^{-6} to 10^{-7} of the total population) exhibits resistance to antimicrobial concentration significantly higher than the minimum inhibitory concentration (MIC) of the main population (Band et al., 2021). Unlike homogeneous resistance, which occurs when all bacterial cells in a population have similar resistance profiles, heteroresistance is distinguished by phenotypic diversity, which can result in treatment failures, persistent infections, and the selection of fully resistant mutants under antimicrobial pressure (El-Halfawy & Valvano, 2022).

The clinical and epidemiological importance of heteroresistance has grown in human medicine, particularly for carbapenem-resistant Enterobacterales, colistin-resistant *Acinetobacter baumannii*, and vancomycin-resistant *Staphylococcus aureus* (Nicoloff et al., 2019). Heteroresistance can develop through a variety of mechanisms, including adaptive mutations, gene amplification, efflux pump upregulation, and heterogeneous expression of resistance determinants, and it may help to select and stabilise fully resistant clones during antimicrobial therapy which can lead to treatment failure (Band et al., 2022). However, data on heteroresistance in bacteria from food-animal sources, particularly poultry, remain scarce globally and almost nonexistent for Nigeria and West Africa. Detecting heteroresistance in the laboratory can be difficult. This is because even while molecular methods like single-cell genomics, quantitative PCR, and whole-genome sequencing (WGS) offer conclusive characterisation, they are still too expensive and technically unavailable in many situations with limited resources (El-Halfawy & Valvano, 2022). Hence, in the majority of Nigerian labs, phenotypic techniques serve as the main detection strategy. However, when conducted in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2023), standard antimicrobial susceptibility testing (AST) using disc diffusion can detect heteroresistance under certain conditions: prolonged incubation periods (48–72 hours), careful examination of inhibition zones for microcolonies or haze, repeated testing to identify inconsistent results, and population analysis profiling (PAP) that involves plating bacterial suspensions on agar containing increasing antimicrobial concentrations to enumerate resistant subpopulations (Nicoloff et al., 2019).

MATERIALS AND METHOD

2.1 Study Location and Farm Description

Sampling was conducted in Choba, Obio/Akpor LGA, Rivers State, Nigeria. Two farms were carefully selected to represent different husbandry contexts:

(a) Farm R (Small-Scale Commercial): A small scale family owned commercial “backyard farm” with an estimated 50-100 birds. The farm had a history of routine antibiotic usage for the purpose of prophylaxis and growth promotion. GPS position: 4.87846°N, 6.94221°E.

(b) Farm C (An Educational Demonstration Farm): A university-affiliated farm used for teaching, stricter antibiotic usage and better regulation.GPS position: 4.8948081°N, 6.9095843°E.

Sample collection

Two poultry farms in Obio akpor LGA were carefully selected and fresh poultry droppings were randomly collected from each farm. On each farm, a randomized sampling method was used. The chicken coops were surveyed, and only fresh, visually intact droppings were sampled. Samples were placed in a storage box and immediately transported to the laboratory within 30 minutes of collection and quickly processed to prevent degradation and contamination.

Preliminary isolation of Bacteria from the poultry droppings

For each step, 1 mL of the previous dilution is transferred to 9 mL of sterile or distilled water and mixed properly for 10-15 seconds. A fresh sterile pipette tip is used for each transfer. After the 10^{-4}, 10^{-5}, and 10^{-6} dilutions, 0.1 mL was spread onto the surface of prepared Nutrient agar, Salmonella-Shigella agar(SSA), Thiosulphate-Citrate-Bile-Sucrose (TCBS) agar, MacConkey agar and Eosin Methylene Blue (EMB) agar using an L shaped glass rod and incubated at 37°C for 24 hours. 24 hours later, the total plate count was taken and the CFU/g was calculated using the formula below:

Formula: CFU/g = (Number of colonies counted × Dilution Factor) / Volume plated (mL)

After the plate count was done, 13 distinct colonies were picked at random. 8 from samples of Farm R (Ar) and 5 from samples of Farm C (ACh) respectively. Each colony was then streaked using quadrant method onto fresh duplicate plates of the following media: Nutrient agar, Salmonella-Shigella agar (SSA), Thiosulphate-Citrate-Bile-Sucrose (TCBS) agar, MacConkey agar and Eosin Methylene Blue (EMB) agar. Then, each plate was labeled with a laboratory code. Thereafter, all the plates were incubated at 37°C for 24 hours. After incubation, the plates were examined for distinct colony morphology and recorded accordingly. Each media was prepared according to manufacturer's specifications. Pure isolates of each colony was preserved on Nutrient Agar slants in Bijou bottles at 4°C for further testing.

Identification of bacterial isolates

In order to identify the isolates Gram staining was carried out for all isolated colonies according to standard procedure. Lactose Fermentation test, Glucose utilization test, Indole test, Citrate utilization test, Catalase test, Methyl Red (MR) and Voges-Proskauer, Triple sugar Iron agar test, Hydrogen sulphide and Gas Production and starch hydrolysis test was

done.

2.5 **Antimicrobial susceptibility testing (AST) and investigation of heteroresistance**

Primary AST (Kirby-Bauer Disk Diffusion method was Performed in triplicates) as described by Onwukwe et al., 2024 and interpreted according to CLSI M100 Performance Standards for Antimicrobial Susceptibility Testing. The process was repeated independently three times for each isolate on three different days to assess reproducibility and screen for heteroresistance. Mueller-Hinton Agar (MHA) was prepared and dried according to the manufacturer's specifications and isolates were plated on Petri dishes. Then swab sticks were dipped in each isolate (which were suspended in sterile saline) and swabbed on the surface of the Mueller-Hinton agar. Afterwards, forceps were flamed properly to ensure sterility and were used to carefully pick up multidisc panels containing discs of different antibiotics before placing and slightly pushing each disc to ensure appropriate contact with the surface of the agar. Each gram negative multidisc panel contained the following antibiotics: Ofloxacin (OFX), Augmentin (AU), Peflacine (PEF), Ceftazidime (CTZ), Gentamicin (CN), Ciprofloxacin (CPX), Ceporex (CEP), Ceftriaxone (TRX), Streptomycin (S), Cefuroxime (CEF). While each gram positive multidisc panel contained the following antibiotics: Ciprofloxacin (CPX), Streptomycin (S), Levofloxacin (LEV), Erythromycin (E), Cefuroxime (CEF), Gentamicin (CN), Azithromycin (AZM), Ceftazidime (CTZ), Amoxicillin (AXM), Rifampicin (RD). Each plate was incubated at 37°C for 24 hours and the diameters of the zones of inhibition were measured to the nearest mm. using CLSI guidelines, the diameters were recorded as susceptible (S), intermediate (I), or resistant (R) depending on their values in mm.

2.5.1 Heteroresistance Screening and Confirmation

An isolate was considered a candidate for heteroresistance if, in one or more of the triplicate AST tests, inner colonies or a "haze" of growth was observed within a clear inhibition zone, or if the zones of inhibition in all triplicates for three days provided varying results (susceptible in one test and resistant in another).

3.0 RESULTS

The result of the total bacterial count in CFU/g ranged from 1.2×10^5 to 9.5×10^6 CFU/g. The preliminary results revealed that a total of 13 bacteria were isolated from the poultry droppings including *Salmonella spp.* (3), *Vibrio spp.* (2), *Citrobacter spp.* (2), *Proteus spp.* (1), *Escherichia coli* (1), *Klebsiella spp.* (1), *Enterobacter spp.* (1), *Staphylococcus.* AST was done in triplicates with Day 1 seeing a considerable number of resistance. The MAR index

ranged from 0.1 to 0.9 on day 1. MAR index on Day 2 ranged from 0.2 to 1.00 showing high resistance

Table 1: Antibiotic Susceptibility Pattern Of Gram Negative Isolates Day 1

Organism					Antibiotics						MAR
Codes	CPX	CEP	TRX	S	CEF	OFX	AU	PEF	CTZ	CN	Index
A1	S	R	I	S	R	S	S	S	I	S	0.20
A2	S	I	I	S	R	R	I	R	I	S	0.30
A3	S	I	S	S	R	R	R	S	I	S	0.30
A5	I	R	S	S	I	R	S	S	S	S	0.20
A7	I	R	R	R	R	R	R	R	R	R	0.90
A8	S	R	I	S	I	R	I	S	R	S	0.30
A9	S	R	S	S	R	R	S	S	R	S	0.40
A10	S	S	S	S	I	S	I	R	I	S	0.10
A11	S	R	S	S	I	S	I	S	R	S	0.20
A12	S	I	S	S	R	R	S	S	I	S	0.20
A13	S	R	S	S	I	R	I	R	I	S	0.30

Table 2: Antibiotic Susceptibility Pattern Of Gram Positive Isolates Day 1

Organism					Antibiotics						MAR
Codes	CPX	S	LEV	E	CEF	CN	AZM	CTZ	AMX	RD	Index
A4	S	I	I	I	S	I	R	I	R	I	0.20
A6	I	S	S	S	S	S	I	I	R	I	0.10

Table 3: Antibiotic Susceptibility Pattern of Gram Negative Isolates Day 2

Organism Codes					Antibiotics						MAR Index
	CPX	CEP	TRX	S	CEF	OFX	AU	PEF	CTZ	CN	
A1	R	I	I	R	R	S	S	I	I	I	0.30
A2	S	I	I	S	R	S	S	R	I	R	0.30
A3	R	R	R	R	R	R	R	R	R	R	1.00
A5	S	R	S	S	R	S	R	S	R	S	0.40
A7	R	I	I	I	R	S	I	I	I	S	0.20
A8	R	R	I	I	I	S	I	I	I	S	0.20
A9	S	I	S	S	R	S	R	R	R	I	0.40
A10	S	R	S	S	R	S	I	I	S	I	0.20
A11	S	I	S	I	R	S	I	I	R	R	0.30
A12	S	I	S	I	R	S	R	R	R	R	0.50
A13	S	I	S	S	R	S	R	R	R	R	0.50

Table 4: Antibiotic Susceptibility Pattern Of Gram Positive Isolates Day 2

Organism Codes					Antibiotics						MAR Index
	CPX	S	LEV	E	CEF	CN	AZM	CTZ	AMX	RD	
A4	S	I	S	I	I	I	I	I	R	I	0.10
A6	S	I	S	R	I	I	I	I	I	S	0.10

On Day 3 of carrying out AST, the MAR index ranged from 0.1 to 0.8, the lowest range of all three days of AST but still very high.

Table 5: Antibiotic Susceptibility Pattern Of Gram Negative Isolates Day 3

Organism					Antibiotics						MAR
Codes	CPX	CEP	TRX	S	CEF	OFX	AU	PEF	CTZ	CN	Index
A1	S	R	R	R	R	I	R	R	R	R	0.80
A2	S	R	R	S	R	S	R	R	R	S	0.60
A3	S	R	S	S	R	R	R	S	R	S	0.50
A5	S	I	S	S	S	R	S	S	S	S	0.10
A7	S	R	R	R	R	S	R	I	R	I	0.60
A8	S	R	I	I	R	S	R	I	R	S	0.40
A9	S	I	R	I	R	S	R	I	R	I	0.40
A10	S	I	R	S	R	S	I	S	R	I	0.30
A11	S	R	S	R	R	R	I	R	I	I	0.50
A12	S	I	S	S	R	R	I	S	I	I	0.20
A13	R	I	S	S	R	S	I	S	I	S	0.20

Table 6: Antibiotic Susceptibility Pattern Of Gram Positive Isolates Day 3.

Organism Codes					Antibiotics						MAR Index
	CPX	S	LEV	E	CEF	CN	AZM	CTZ	AMX	RD	
A4	S	I	S	S	I	S	S	R	R	I	0.20
A6	I	I	I	R	R	R	I	R	I	I	0.40

MAR index= Number of antibiotics to which the isolate is resistant / Total number of antibiotics tested (Fajoyomi et al., 2020).

Average MAR index= Sum of all individual MAR indices / Number of isolates (or days)

Table 7: Overall Resistance Percentage Per Antibiotic

Antibiotic	Total R	Total Tests	% Resistance
CPX	5	33	15.15%
CEP	17	33	51.52%
TRX	7	33	21.21%
S	6	33	18.18%
CEF	26	33	78.79%
OFX	13	33	39.39%
AU	13	33	39.39%
PEF	12	33	36.36%
CTZ	17	33	51.52%
CN	7	33	21.21%

For gram negative organisms (11 isolates across 3 days = 33 tests/observations)

Table 8: Overall Resistance Percentage Per Antibiotic

Antibiotic	Total R	Total Tests	% Resistance
CPX	0	6	0.0
S	0	6	0.0
LEV	0	6	0.0
E	2	6	33.33
CEF	1	6	16.7
CN	1	6	16.7
AZM	1	6	16.7
CTZ	2	6	33.33
AMX	4	6	67.67
RD	0	6	0.0

For gram positive organisms (2 isolates across 3 days = 6 tests/observations)

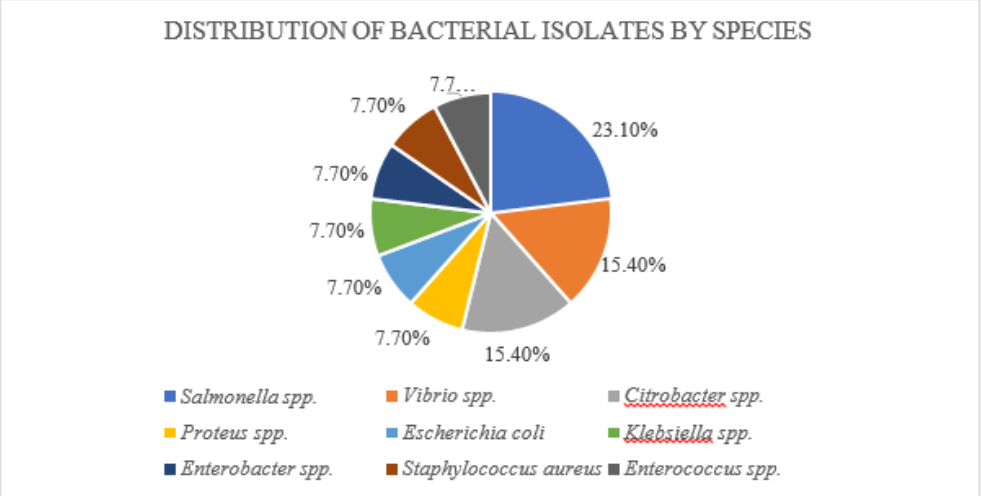

Fig1: Showing Distribution Of Bacterial Isolates By Species.

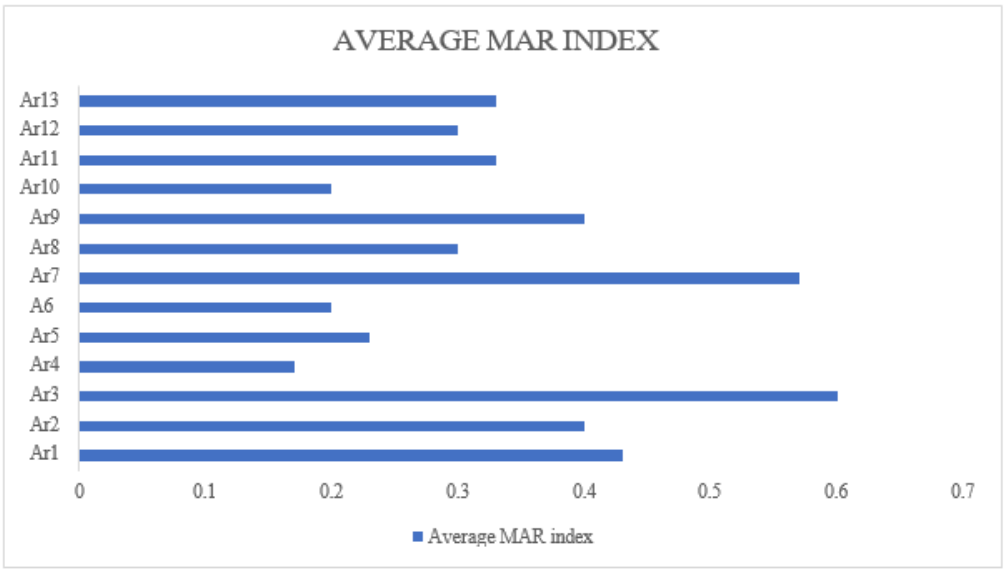

Fig 2: Average MAR Index of All Isolates Across Three Days Of Antibiotic Susceptibility Testing

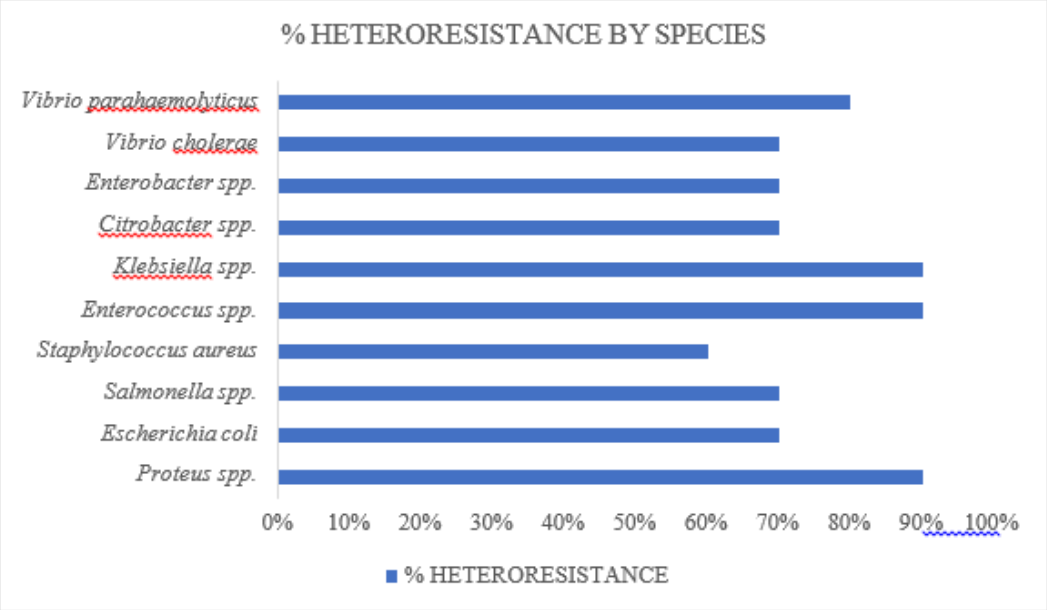

Fig 3: Percentage Heteroresistance By Specie.

DISCUSSION

This study investigated the prevalence and patterns of heteroresistance in bacteria isolated from poultry droppings in Obio-Akpor, Rivers State, Nigeria. The findings reveal a critical and underexplored phenomena of the antimicrobial resistance (AMR) burden in this agricultural setting, characterized by a high prevalence of multidrug resistance (MDR) and a strong association with phenotypic heteroresistance. A total of thirteen (13) bacterial organisms were isolated from poultry droppings with Gram- negative rods (84.6%) being the predominant species. This aligns with established literature on the gut flora of poultry with the exception of the *Vibrio spp.* (*Vibrio cholera* and *Vibrio parahaemolyticus*) which are usually found in aquatic environments. it is assumed that the *Vibrio spp.* observed were likely as a result of contaminated water source of the birds. The diversity of species, including *Escherichia coli*, *Salmonella spp.*, *Klebsiella spp.*, and *Proteus spp.*, confirms that poultry droppings act as a significant reservoir for a wide range of potential pathogens (Ohemu & Fajoyomi 2022). The antibiotic susceptibility testing that was carried out over the course of three (3) days revealed a very high level of multidrug resistance. The Multiple Antibiotic Resistance (MAR) index, a key indicator of antibiotic exposure, was high for the majority of isolates. With 69.2% (9 out of 13) of isolates having an average MAR index greater than 0.2, the study confirms that the sampled poultry farms represent high-risk environments where bacteria are under intense antibiotic pressure. This is consistent with the background of the study, which highlighted the widespread and frequently uncontrolled use of antimicrobials in

Nigerian poultry production (Oluwasemowo et al., 2023). Isolates such as *Klebsiella spp.* (A7, MAR=0.57) and *Proteus spp.* (A1, MAR=0.43) demonstrated extreme resistance, being non-susceptible to up to 90% of the tested antibiotics on some testing days. The analysis of resistance per antibiotic showed that cefuroxime was the least effective, with a 78.79% resistance rate among Gram-negative isolates. Other antibiotics, including Ceporex and Ceftazidime, also showed high resistance (51.52% each). This pattern points towards the extensive use (and likely misuse), of cephalosporins and other critical antibiotics within the studied poultry sector, creating a powerful selective pressure for resistance. High levels of heteroresistance were detected phenotypically in this study. After performing triplicate AST over three days (subculturing isolates after each AST test), inconsistent results were observed which is a key indicator of heteroresistance. The overall prevalence was 74.6%, meaning that nearly three-quarters of all antibiotic-isolate tests showed evidence of a resistant subpopulation that could be missed in a single, standard AST. Certain isolates showed extremely high heteroresistance rates. *Klebsiella spp.* (A7), *Proteus spp.* (A1), and Enterococcus spp. (A6) each displayed heteroresistance in 90% of the antibiotics they were tested against. For these bacteria, a standard AST report would have most likely categorized them as susceptible to most antibiotics, thereby masking the hidden reservoir of resistance and creating a high risk for treatment failure if those antibiotics were used. A notable correlation was observed between an isolate's MDR status and its risk for heteroresistance. Isolates with MDR (with an average MAR index ≥ 0.3) also exhibited high heteroresistance prevalence, ranging from 70% to 90%. This finding provides strong phenotypic evidence supporting the literature which suggests that heteroresistance acts as a "genetic reservoir" or a stepping stone to stable, high- level multidrug resistance. The constant, often sub-therapeutic, antibiotic pressure in the poultry environment possibly enriches these pre-existing heteroresistant subpopulations, providing a fertile ground for them to acquire additional, stable resistance mechanisms and evolve into fully MDR strains that are extremely difficult to treat. Certain antibiotics tested in this study were most prone to heteroresistance, which has direct implications for clinical and veterinary treatment decisions. Among cephalosporins, Ceftazidime (92.3%) and Ceporex (90.9%) showed the highest prevalence of heteroresistance. Among aminoglycosides, Gentamicin had an 84.6% heteroresistance rate. The high heteroresistance to these clinically relevant antibiotics is particularly worrisome. It implies that therapeutic decisions based on a susceptible AST report for these drugs are at a substantially elevated risk of failure, both in poultry veterinary medicine and in potential zoonotic infections in humans.

CONCLUSION

In conclusion, this study provides compelling evidence that heteroresistance is a common and significant feature of the antimicrobial resistance landscape in Nigerian poultry production. The high prevalence of multidrug-resistant bacteria, particularly Gram-negative rods, in the sampled droppings confirms the substantial selective pressure exerted by antibiotic use in this agricultural sector. More critically, the detection of phenotypic heteroresistance in a majority of the isolates reveals a hidden layer of complexity that standard antimicrobial susceptibility testing fails to capture. The strong correlation observed between multidrug resistance and heteroresistance suggests that this phenomenon is not an isolated event but is intrinsically linked to the development and persistence of MDR strains, acting as a crucial stepping stone in the evolution of resistance. Heteroresistance screening should be integrated into national AMR monitoring programs.

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