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# Molecular characterization of antibiotic-resistant bacteria associated with maggots obtained from chicken droppings

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## Abstract

**Background** The use of maggots, developed from chicken droppings as alternative fish meal might serve as a reservoir and vehicle for the widespread of single and multiple antibiotic resistant bacteria (MARB). This study aimed at investigating antibiotic resistant bacteria from chicken droppings and maggots developed from them, and the associated resistance genes and mobile genetic elements in their genomes. Freshly collected poultry droppings obtained from three commercial poultry farms, in Akure, Nigeria and maggots developed from their composting were plated on Luria–Bertani (LB) agar plates that had been incorporated with antibiotics. The emerged MARB were presumptively identified by conventional cultural microbiological methods and then authenticated using 16S rRNA gene sequence analysis. The antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) in the genome of MARB were detected by standard molecular tools.

**Results** Potential bacterial pathogens isolated include *Escherichia coli, Bacillus cereus, Salmonella enterica*, *Providencia stuartii*, *P. rettgeri*, *Pseudomonas aeruginosa*, *Staphylococcus aureus, Alcaligenes faecalis*, *Paelnalcaligenes hominis* and *Micrococcus luteus*. Seventeen ARGs were widely distributed among the 19 ARB/MARB. Tetracycline resistance gene was widely distributed among the ARB/MARB with prevalence of 62%, while macrolides and beta lactam resistance gene had 17% and 13%, respectively. At least six MARB isolates showed the presence of two or more class 1 integron, while five isolates was detected to carry two or more class 2 integron among the 19 MARB.

**Conclusions** The results highlight a much higher risk of ARGs spreading through maggots derived from chicken droppings to humans and environmental microbiota.

**Keywords** Maggots, Chicken feces, Antimicrobial susceptibility, Antibiotic resistance, Antibiotic resistance genes

## Background

The advancement of antibiotic resistance genes (ARGs), their spread among bacterial pathogens and their inevitable implications on human health has been getting a lot of attention in recent times. The genes are exchanged among bacterial species by horizontal gene transfer mechanisms (HGT) potentially leading to untreatable infections. Antibiotic-resistant bacteria (ARB) and their associated genes have also been found in animals due to the administration of antibiotics for growth, hormone and treatment purposes in animal husbandry [1]. The spread of ARGs in the environment is promoted by anthropogenic activities, such as waste/wastewater treatment [2], animal farming [3] and aquaculture [4].

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Heavy metals such as copper, zinc, arsenic and mercury added to animal feeds for growth promotion and disease control can also accumulate in the soil after consistent application of metal-containing fertilizers [5]. Due to the functional and structural characteristics commonly shared by many antibiotic and metal-resistance systems, there is an increasing concern that heavy metals such as zinc, copper, and arsenic may ultimately influence the proliferation of ARGs [6]. ARGs have been investigated in several environmental compartments, such as farmlands [7], rivers [8], and fish ponds [4, 9]. However, there is little or no knowledge of ARB/MARB and their associated genes implicated in *Musca domestica* larva commonly used as an alternative to commercial fish feed recognized for its quality protein with amino acids profile showing its superior biological value to groundnut cake and soybean [9, 10].

In addition, chicken droppings and urine are continuously discharged directly into fish ponds in animal farm-fish pond system, a traditional household-based integrated farming system which is now practiced in Nigeria. This practice often than not leads to the spread of ARGs and ARB via horizontal gene transfer [11] and this may also change the bacterial community composition in aquaculture environment since the chicken droppings often contains residues of antimicrobials used for the treatment and growth promotion of poultry [12]. Therefore, there is need for the molecular characterization of ARB/MARB associated with maggots obtained from chicken droppings. The study aimed at developing maggots from chicken droppings, isolating ARB from the maggots and droppings and analyzing them for ARGs and mobile genetic elements (MGEs).

## Materials and methods

### Sample collection and development of maggots

Approximately 30 g fresh chicken droppings were collected same day from three different poultry farms with a sterile shovel into sterile containers at the farms designated as FRR (located at the Federal University of Technology, Akure, Nigeria), ACF (located at Ipinsa, Akure, Nigeria) and BPF (located at Ondo Road, Akure, Nigeria) and transported to the laboratory within two hours of collection for immediate microbial analysis. The faeces were initially exposed to infestation by housefly for 8 h to culture their larva. The houseflies fed and laid eggs on the faeces. The faeces were placed in a shade to prevent direct rays from sunlight that could inhibit the growth of the maggots. After 3 days, the emerged maggots from the faeces were harvested [9, 13].

### Determination of heavy metal content of the chicken droppings and maggots

Using the approach outlined by Allen et al. [14], the presence of heavy metals in the samples were determined. To eliminate the organic content, samples of chicken droppings and maggots were put through an acid oxidation procedure. The heavy metals, copper, cadmium, chromium, manganese, lead, zinc, iron, and cobalt, were next evaluated using the atomic absorption spectrophotometer (Buck Scientific VGP 210 model, England) by adhering to the established procedures after the manure samples had been digested.

### Isolation, purification and preservation of single and MARB from chicken droppings and maggots

Routine bacterial isolation was carried out using spread plate method. One gram of chicken dropping from the sterile container was transferred into a test tube containing 9 ml sterile distilled water to form a stock solution. It was serially diluted until a dilution factor of $10^5$ was obtained. Six different types of antibiotics; gentamycin, ampicillin, chloramphenicol, tetracycline, erythromycin and ciprofloxacin, were purchased () and used for the isolation of single and MARB. Each antibiotic was added individually at working concentration as prescribed by Clinical and Laboratory Standards Institute [17] (Additional file 1: Table S1) to the Luria–Bertani agar and inoculated with one millilitre chicken dropping sample suspension (after serial dilution from $10^{-2}$ to $10^{-5}$) and incubated at 37 °C for 18–24 h. Luria–Bertani plates without antibiotics but with the same inoculum were used for the determination of total heterotrophic bacteria counts (THBC), and were considered as the control experiments. MARB were isolated in LB agar medium containing two to three classes of antibiotics. The isolates were sub-cultured repeatedly to obtain pure isolates. The emerged isolates on LB containing single antibiotic and multiple antibiotics were regarded as ARB and MARB, respectively. Pure isolates were inoculated in slant bottles containing sterilized double strength LB agar, incubated at 37 °C for 24 h and stored in the refrigerator for identification [9, 15]. The maggots were collected with sterile forceps and surface-sterilized with 70% ethanol for ~ 60 s to decontaminate the external surfaces. One gram of the maggots repeatedly rinsed with sterile normal saline (0.9%, w/v) was air dried and grinded with a mortar and pestle. Bacteria (ARB and MARB) were isolated as previously described for chicken droppings. The population of bacteria from the samples (chicken droppings and maggots) that were resistant to an individual or combined antibiotics was evaluated based on the ratio

of the colony number of ARB/MARB to total culturable bacteria (THBC) in each agar plate and multiplied by 100.

#### Antibiotics susceptibility assay

The Kirby–Bauer test, otherwise known as disc diffusion assay was adopted for the determination of antimicrobial activity of antibiotics on the bacterial isolates in line with the method described by Murray et al. [16]. Commercial discs coated with antibiotics were aseptically placed on Mueller–Hinton agar plates already streaked with the isolates using sterile forceps and then incubated at 37 °C for 24 h. Gram-negative bacterial pathogens were tested against the following antibiotics; ciprofloxacin (30 µg), amoxicillin (30 µg), chloramphenicol (30 µg), gentamycin (30 µg), streptomycin (30 µg) and pefloxacin (30 µg), while Gram-positive isolates were tested against pefloxacin (10 µg), ciprofloxacin (10 µg), streptomycin (30 µg), gentamycin (10 µg), erythromycin (10 µg) and amoxicillin (30 µg). The zones of inhibition around the antibiotics disc were measured in millimeters and recorded in triplicates. The result was interpreted as susceptible, intermediate and resistant following the prescription of Clinical and Laboratory Standards Institute [17]. *Escherichia coli* ATCC 25922 was considered as a reference bacterium for quality control purposes.

#### Genomic DNA extraction from cultivable bacterial isolates

Genomic DNA was extracted from pure bacterial isolates using the boiling-centrifugation method. Few colonies of each isolate were aseptically homogenized in 100 µl of sterile distilled water. The suspensions were separately boiled at 100 °C for 15 min and centrifuged at 11,200 g-force for 10 min. The supernatant was transferred to a new Eppendorf tube after centrifugation and was used as template DNA for Polymerase Chain Reaction (PCR) [18]. The 16S rRNA gene was amplified by Polymerase Chain Reaction (PCR) with two universal primers (F27:5′-AGAGTTTGATCCTGGCTCAG-3′ and R1492:5′TACGGTTACCTTGTTACGACTT3′). A total volume of 50 µl PCR mixtures was prepared to contain 25 µl 2×ExTaq PCR Master Mix, 1 µl of each primer, 22 µl double-distilled water and 1 µl DNA template. The standard PCR protocol that was adopted was as follows: primary denaturation of the DNA for 5 min at 95 °C; followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1 min and finally, an additional reaction for 10 min at 72 °C. The quality of PCR products was checked by agarose gel electrophoresis after which they were submitted to Inqaba Biotec West Africa for sequencing. The sequences obtained were compared with other 16S rRNA gene sequences already deposited in the GenBank using the BLASTN program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

#### Molecular detection of resistance genes by PCR amplification

The resistance genes specific to tetracycline, ampicillin, erythromycin, gentamycin, ciprofloxacin and chloramphenicol in the genomes of the isolates were amplified with PCR with specific primers. Primer sequence and PCR conditions for amplification are documented in the Additional file 1: Table S2). The PCR was carried out in a thermal cycler using a 25 µl reaction mixture containing 12.5 µl 2×master mix, 0.5 µl each of the forward and reverse primers, 8.5 µl of nuclease-free water and 3 µl of the DNA template at specified conditions following Yang et al. [15].

#### Detection of integrons (class 1 and 2)

All the isolates were screened for class 1 and 2 integrons by Polymerase Chain Reaction (PCR). Class 1 integron was detected and amplified by Levesque 5CS and 3CS primers which bind the 5′ and 3′ conserved ends, respectively. Class 2 integron was detected and amplified by White hep74 and White hep51 1 to hybridize *attI2* and *orfX*, respectively [18].

#### Data analysis

Data obtained were subjected to analysis of variance (ANOVA) and the means were separated using Duncan's multiple range test at 95% level of significance ($p \leq 0.05$). Results were expressed as mean ± standard error of the three replicates.

## Results

#### Organic and mineral content of chicken droppings

Additional file 1: Table S3 shows the organic and mineral composition of chicken droppings collected from different farms in Akure metropolis. nitrogen (N), phosphorus (P) and potassium (K) were present in all the samples in varying concentrations. Sample B collected from ACF had the highest organic matter and organic carbon values of 13.55% and 7.86%, respectively, while the least value of 10.87% occurred in the sample represented by A (FRF). Sample A had the highest nitrogen (6.125%) and potassium (32.30 mg/kg), while they were least in sample B with 4.38% and 28.50 mg/kg, respectively.

#### Quantity of heavy metals in chicken droppings

The quantity of different heavy metals in chicken droppings is presented in Additional file 1: Table S4. The chicken droppings revealed the presence of Zinc (Zn) in varying quantities (0.314, 0.192 and 0.246 mg/kg) for sample collected from a farm designated FRF, ACF and

BPF represented with A, B and C, respectively. Manganese (Mn), Lead (Pb) and Cobalt (Co) were below detectable limit for all the samples. There was significant reduction in the metals in all the samples after three (3) days of composting when compared with the freshly collected chicken droppings. Cu, Cd, Cr, Zn and Fe reduced in the sample collected from FRF by approximately 24.19%, 37.14%, 40%, 58% and 16.20%, respectively, while a reduction of ~ 22.92%, 29.55%, 38.75%, 32.81% and 23.81% was recorded, respectively, for the same metals in sample collected from Animal Concept. Furthermore, a reduction of approximately 30.40%, 33.33%, 39.31%, 18.29% and 20.74% was recorded for Cu, Cd, Cr, Zn and Fe, respectively, in sample collected from Bliz Poultry.

#### Total bacterial counts in chicken droppings and maggots

The total heterotrophic bacterial counts (THBC) in chicken droppings from different farms and maggots developed from them is presented in Table 1. In general, highest bacterial counts were recorded in sample (chicken droppings) represented by B (sample from ACF) in all the days (i.e. from day 1 to day 5). The THBC in the sample represented by A (sample form FRF) had the highest value of $2.08\times10^7$ cfu/g at day 3 of composting and declined to $1.60\times10^7$ cfu/g at day 5. Furthermore, THBC in sample represented as B (sample from ACF) decreased consistently from $3.32\times10^7$ cfu/g at day 1 to $2.04\times10^7$ at day 5 of composting. The THBC in maggots developed from the chicken droppings were significantly higher in samples represented as A (sample from FRF) and C (sample from BPF) with $3.90\times10^7$ cfu/g, respectively, than in sample represented as B (sample from ACF) with $1.90\times10^7$ cfu/g.

#### Percentage antibiotic-resistant bacteria in chicken droppings and maggots

In general, the inclusion of the antibiotics in Luria–Betani medium resulted in the decline in the population of ARB (Additional file 1: Figs. S1–S6) with the percentage of ARB from Luria–Betani medium containing gentamycin had the least (Additional file 1: Fig. S5).

The percentage of ARB in chicken droppings from FRF and BPF to erythromycin decreased from 83.13% and 52.22% at day 1 to 59.62% and 49.21% at day 3 and then increased by 15.38% and 66.46% at day 5 of composting, while percentage of ARB in sample B decreased consistently (Additional file 1: Fig. S1).

The percentage of ARB in all the samples (A, B and C) to tetracycline increased from 49.39%, 25.57% and 68.29% at day 1 to 75%, 56.25% and 76.67% at day 3, respectively (Additional file 1: Fig. S2). Meanwhile, at day 5, percentage of ARB decreased by approximately 7.5% and 13.71% in samples collected from FRF and BPF, except for sample B (ACF) where an increase of 10.42% occurred when compared with the sample composted for 3 days.

In Additional file 1: Fig. S3, the percentage of ARB in chicken droppings to ampicillin collected from different farms dropped exceptionally when compared with the control experiments. The percentage of ARB to ampicillin in sample A (sample from FRF) increased by ~ 2.30% and 7.80% on day 3 and day 5 of composting when compared with 19.32% at day 1.

The percentage of ciprofloxacin resistant bacteria was highest in sample A (collected from FRF) with a value of 83.13% at day 1, while lowest value of 53.33% was recorded from sample C (BPF) at day 3 of composting (Additional file 1: Fig. S4).

The percentage of gentamycin resistant bacteria in chicken droppings from different farms is shown in Additional file 1: Fig. S5. The population of gentamycin resistant bacteria was highest in sample C collected from BPF with 24.39%, and least in sample A from sample from FRF (7.69%) on day 1. Meanwhile, the percentage of ARB reduced significantly in sample A (FRF) and sample C (sample from BPF) after 3 days of composting, while sample B (sample from ACF) recorded 0% at day 3 and day 5. The percentage of chloramphenicol resistant bacteria in chicken droppings from different farms is presented in Additional file 1: Fig. S6. There were variations in the population of chloramphenicol resistant bacteria in the samples at different days of composting. At days 1, 3, and 5, the highest percentage of chloramphenicol resistant bacteria of 84.09%, 86.67% and 81.48% were found in

**Table 1** Total heterotrophic bacterial counts in chicken droppings and maggots

| Sample | Total heterotrophic bacterial counts (cfu/g) | | | |
|---|---|---|---|---|
| | Day 1 | Day 3 | Day 5 | Maggots |
| A | $1.76\times10^7\pm0.024^b$ | $2.08\times10^7\pm0.038^c$ | $1.60\times10^7\pm0.112^b$ | $3.90\times10^6\pm0.050^b$ |
| B | $3.32\times10^7\pm0.115^c$ | $2.56\times10^7\pm0.073^b$ | $2.04\times10^7\pm0.244^c$ | $1.90\times10^6\pm0.080^a$ |
| C | $1.28\times10^7\pm0.153^a$ | $1.20\times10^7\pm0.087^a$ | $1.08\times10^7\pm0.188^a$ | $3.90\times10^6\pm0.427^b$ |

Data represented as mean ± standard error ($n=2$) with the same superscript down the row are not significant different ($p\leq0.05$)

Key: A = FRR, B = ACF, C = BPF, cfu/g = colony forming unit per gram

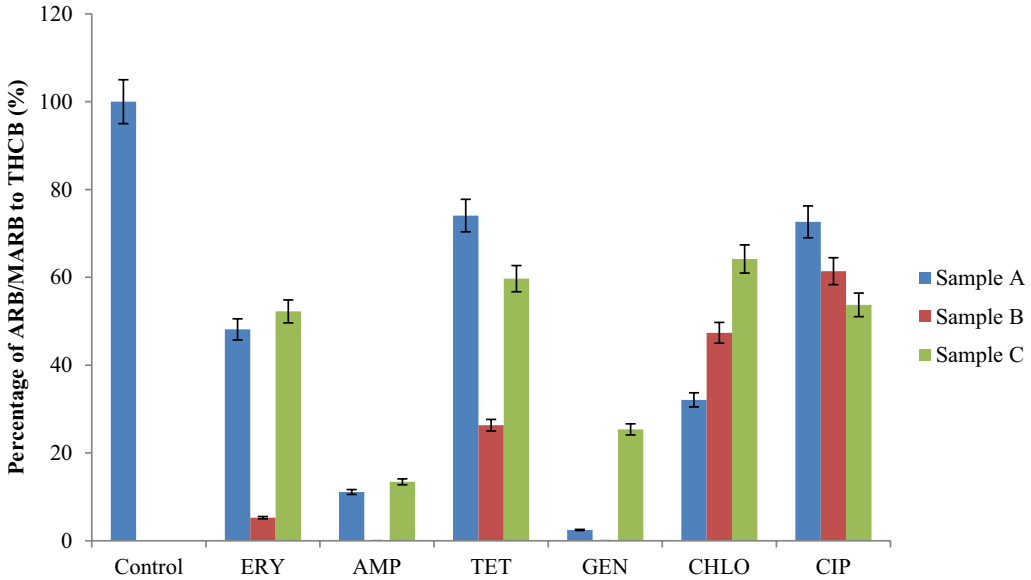


**Fig. 1** Percentage of ARB in maggots developed from chicken droppings to different antibiotics. *ERY* erythromycin, *AMP* ampicillin, *TET* tetracycline, *GEN* gentamycin, *CHLO* chloramphenicol, *CIP* ciprofloxacin, Sample A = FRF, Sample B = ACF, Sample C = BPF, THCB = Total Heterotrophic Cultivable Bacteria

the samples collected from ACF (Sample B), BPF (Sample C) and BPF (Sample C), respectively.

In Fig. 1, percentage of ARB in maggots developed from chicken droppings collected from different farms to the representative of different classes of antibiotics is presented. The percentage of tetracycline and ciprofloxacin resistant bacteria was highest in maggots developed from Sample A (sample collected from FRF). The percentage of erythromycin, ampicillin, gentamycin and chloramphenicol resistant bacteria were highest in maggots developed from Sample C (chicken droppings collected from BPF). Maggots developed from Sample B (chicken dropping collected from ACF) had no ampicillin and gentamycin resistant bacteria.

### Molecular identities of antibiotic-resistant bacteria (ARB)/multiple antibiotic-resistant bacteria (MARB)

The molecular identities of the ARB/MARB from the chicken droppings and maggots via 16SrRNA gene sequence analysis is shown in Table 2. The identities of the isolates were authenticated to be *Salmonella enterica, Escherichia coli, Alcaligenes faecalis, Bacillus cereus,*

**Table 2** Molecular identities of antibiotic-resistant bacteria/multiple antibiotic resistant bacteria

| S/N | Isolate code | Percentage identity (%) | Description of organism | Accession number of NCBI match | Assigned NCBI Accession number |
|---|---|---|---|---|---|
| 1 | 6 | 100 | *Salmonella enterica* | MH356699.1 | Not assigned |
| 2 | 1 | 93.86 | *Escherichia coli* | NR_114042.1 | OR793894 |
| 3 | 5 | 78.69 | *Bacillus cereus* | MH304266.1 | Not assigned |
| 4 | 19 | 73.52 | *Escherichia coli* | MH194190.1 | Not assigned |
| 5 | 2 | 94.31 | *Alcaligenes faecalis* | NR_113606.1 | OR778389 |
| 6 | 17 | 96 | *Paenalcaligenes hominis* | NR_116967.1 | OR794110 |
| 7 | 16 | 91.98 | *Providencia stuartii* | NR_024848.1 | OR783159 |
| 8 | 27 | 92.08 | *Providencia stuartii* | NR_114964.1 | OR783241 |
| 9 | 32 | 98.79 | *Providencia rettgeri* | NR_042413.1 | OR784045 |
| 1 | 26 | 90.46 | *Escherichia coli* | NR_112558.1 | OR777607 |
| 1 | 3 | 99.53 | *Providencia stuartii* | NR_114964.1 | OR793890 |
| 1 | 18 | 91.90 | *Escherichia coli* | NR_114042.1 | OR782924 |

*Providencia stuartii, P. rettgeri* and *Paenalcaligenes homini.*

### Antibiotic susceptibility pattern of antibiotic-resistant bacteria/multiple antibiotic-resistant bacteria

Tables 3 and 4 reveal the antibiotic susceptibility pattern of ARB from chicken droppings and maggots. All Gram-positive isolates (*Bacillus cereus, Staphylococcus aureus* and *Providencia stuartii*) were resistant gentamycin, amoxicillin, ciprofloxacin, erythromycin and streptomycin with the exception of *M. luteus* that showed an intermediate reaction to streptomycin and perfloxacin. All Gram-negative isolates were resistant to gentamycin, amoxicillin, ciprofloxacin, erythromycin and perfloxacin, while *Paenalcaligenes hominis* and *Escherichia coli* both showed intermediate reactions (11.13 mm and 11.27 mm) to streptomycin. *Providencia rettgeri* showed an intermediate reaction to amoxicillin (13.13 mm).

### Multiple antibiotic-resistant bacteria and their resistance to antibiotics

Table 5 shows the MARB and their resistance to various antibiotics. Ninety percent of the isolates were resistant to erythromycin, 85% and 75% were resistant to tetracycline and chloramphenicol, respectively. The least resistance was recorded for ciprofloxacin with 50% of the isolates showing resistant to the antibiotics. *Escherichia coli*, *Alcaligenes faecalis, Micrococcus luteus, Providencia stuatii* and *Providencia rettgeri* were resistant to all the antibiotics, while *Bacillus cereus*, *Pseudomonas aeruginosa, Providencia stuartii*, *Paenalcaligenes hominis*, *Escherichia coli*, and *Staphylococcus* sp. were susceptible to gentamycin.

### Prevalence of antibiotic resistance genes (ARG) and mobile genetic elements (MGE) in the genome of ARB/MARB

Percentage occurrence of ARG in the genome of MARB is presented in Fig. 2. A total of twenty-three (23) ARGs were selected to investigate the distribution of ARGs in the genome of the 19 MARB. Among the 19 isolates, 17 ARGs were detected to be present; these covered seven classes of antibiotics: tetracycline, trimethoprim, macrolides, aminoglycosides, beta lactam, quinolones and chloramphenicol. Tetracycline-resistance genes were the most diverse and prevalent ARGs detected in the 19 MARB with (65.21%), followed by genes encoding resistance to macrolides (30%), quinolones (20%) and aminoglycosides (13%). Four tetracycline-resistance genes; *tetA*,

**Table 3** Antibiotic susceptibility pattern of Gram-positive ARB/MARB from chicken droppings and maggots (mm)

| Antibiotics | CLSI zone diameter (mm) | | | *M. luteus* | *B. cereus* | *S. aureus* | *Providencia stuartii* |
|---|---|---|---|---|---|---|---|
| | S | I | R | | | | |
| CN | ≥ 15 | 13–14 | ≤ 12 | $11.13 \pm 0.08819^{bc}$ | $10.63 \pm 0.08819^{a}$ | $11.03 \pm 0.03333^{bc}$ | $11.43 \pm 0.29627^{c}$ |
| AM | ≥ 18 | 14–17 | ≤ 13 | $12.27 \pm 0.08819^{b}$ | $11.14 \pm 0.08819^{a}$ | $12.03 \pm 0.08819^{b}$ | $12.23 \pm 0.08819^{b}$ |
| CPX | ≥ 21 | 16–20 | ≤ 15 | $14.17 \pm 0.03333^{c}$ | $13.23 \pm 0.08819^{a}$ | $14.03 \pm 0.08819^{bc}$ | $13.87 \pm 0.08819^{b}$ |
| E | ≥ 23 | 14–22 | ≤ 13 | $11.67 \pm 0.17638^{c}$ | $10.77 \pm 0.08819^{a}$ | $11.33 \pm 0.03333^{bc}$ | $11.27 \pm 0.06667^{b}$ |
| ST | ≥ 15 | 12–14 | ≤ 11 | $11.17 \pm 0.08819^{c}$ | $10.27 \pm 0.08819^{a}$ | $10.73 \pm 0.14530^{b}$ | $10.43 \pm 0.12019^{ab}$ |
| PER | ≥ 17 | 13–16 | ≤ 12 | $12.07 \pm 0.12019^{c}$ | $10.47 \pm 0.08819^{a}$ | $11.27 \pm 0.14530^{b}$ | $11.27 \pm 0.14530^{b}$ |

Data represented as mean ± standard error ($n = 2$) with the same superscript across the column are not significant different ($p \leq 0.05$)

Keys: CN = Gentamycin, AM = Amoxicillin, CPX = Ciprofloxacin, E = Erythromycin, ST = Streptomycin, PER = Perfloxacin, S = susceptible, I = intermediate, R = resistance

**Table 4** Antibiotic susceptibility pattern of Gram-negative ARB/MARB from chicken droppings and maggots (mm)

| Antibiotics | CLSI Zone Diameter (mm) | | | *S. enterica* | *Paenalcaligene faecalis* | *Escherichia coli* | *E. coli* | *Providencia rettgeri* |
|---|---|---|---|---|---|---|---|---|
| | S | I | R | | | | | |
| CN | ≥ 15 | 13–14 | ≤ 12 | $11.13 \pm 133^{ab}$ | $11.23 \pm 033^{a}$ | $11.03 \pm 240^{ab}$ | $11.03 \pm 145^{a}$ | $11.03 \pm 240^{ab}$ |
| AM | ≥ 18 | 14–17 | ≤ 13 | $11.03 \pm 120^{ab}$ | $12.23 \pm 203^{bc}$ | $12.5 \pm 100^{c}$ | $12.37 \pm 067^{ab}$ | $13.13 \pm 088^{d}$ |
| CPX | ≥ 21 | 16–20 | ≤ 15 | $14.27 \pm 176^{e}$ | $13.97 \pm 240^{d}$ | $14.27 \pm 120^{d}$ | $14.03 \pm 203^{c}$ | $13.77 \pm 120^{e}$ |
| E | ≥ 23 | 14–22 | ≤ 13 | $12.9 \pm 173^{d}$ | $12.33 \pm 384^{c}$ | $12.5 \pm 153^{c}$ | $12.63 \pm 173^{d}$ | $12.5 \pm 186^{c}$ |
| ST | ≥ 15 | 12–14 | ≤ 11 | $10.74 \pm 240^{a}$ | $11.13 \pm 240^{a}$ | $10.63 \pm 318^{a}$ | $11.27 \pm 120^{a}$ | $10.7 \pm 231^{a}$ |
| PER | ≥ 17 | 13–16 | ≤ 12 | $11.53 \pm 240^{c}$ | $11.57 \pm 088^{ab}$ | $11.6 \pm 289^{b}$ | $11.37 \pm 088^{a}$ | $11.47 \pm 2120^{b}$ |

Data represented as mean ± standard error ($n = 2$) with the same superscript across the column are not significant different ($p \leq 0.05$)

Keys: CN = Gentamycin, AM = Amoxicillin, CPX = Ciprofloxacin, E = Erythromycin, ST = Streptomycin, PER = Perfloxacin, S = susceptible, I = intermediate, R = resistance

**Table 5** ARB/MARB isolates and their resistance to various antibiotics

| Antibiotics | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Isolates Code** | **Presumptive Identities** | **CIP** | **TET** | **AMP** | **GEN** | **ERY** | **CHLO** |
| 1 | *Escherichia coli* | R | R | R | R | R | S |
| 2 | *Alcaligenes faecalis* | R | R | R | R | R | R |
| 3 | *Providencia stuartii* | R | R | R | R | R | R |
| 4 | *Micrococcus luteus* | R | R | R | R | R | R |
| 5 | *Bacillus cereus* | I | R | R | R | R | R |
| 6 | *Salmonella enterica* | R | R | R | R | R | R |
| 7 | *Bacillius cereus* | S | R | I | S | R | R |
| 9 | *Alcaligenes faecalis* | I | R | I | R | I | R |
| 10 | *Staphylococcus hominis* | R | R | I | R | R | I |
| 11 | *Pseudomonas aeruginosa* | I | R | S | S | R | R |
| 16 | *Providencia stuartii* | R | R | R | S | R | R |
| 17 | *Paenalcaligenes hominis* | I | I | R | S | R | I |
| 18 | *E. coli* | R | R | R | R | R | R |
| 19 | *Escherichia coli* | I | R | R | S | R | R |
| 21 | *Staphylococcus* sp. | I | R | I | R | R | I |
| 26 | *E. coli* | R | I | R | R | R | R |
| 27 | *Providencia stuartii* | S | R | R | S | R | R |
| 32 | *Providencia rettgeri* | R | R | R | R | R | R |
| 34 | *Staphylococcus* sp. | S | R | R | S | R | R |
| Prevalence of resistance (%) | | 50 | 85 | 70 | 60 | 90 | 75 |

Key: S = susceptible, I = intermediate, R = resistant, CIP = ciprofloxacin, TET = tetracycline, AMP = ampicillin, GEN = gentamycin, ERY = erythromycin, CHLO = chloramphenicol

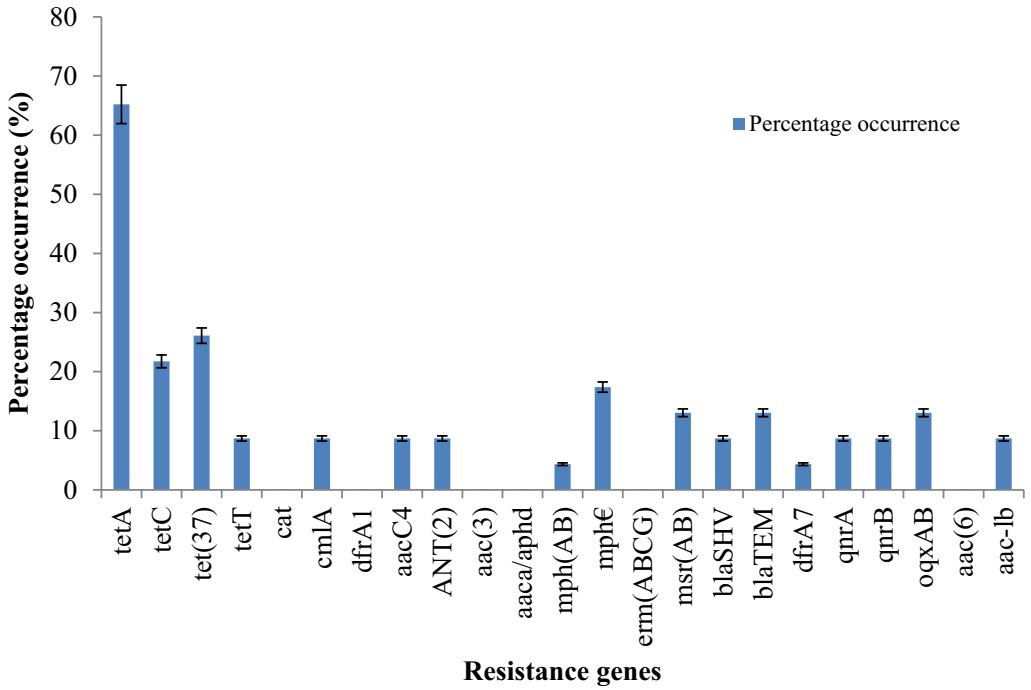


**Fig. 2** Percentage occurrence of ARGs in the genomes of ARB/MARB

*tetC*, *tetT* and *tet(37)*, three aminoglycoside-resistant genes; *aacC4*, *aac-lb* and *ANT(2)*, one sulphonamide–trimethoprim resistance genes (*drfA7*), three macrolides resistance genes (*mph(E)*, *mph(ABC)* and *msr(AB)*), two beta-lactam (*blaTEM* and *blaSHV*), one chloramphenicol resistance gene (*cmlA*) and three quinolones resistance genes (*OqxAB*, *qnrA* and *qnrB*) were the most frequently detected ARGs, while six other ARGs were not detected; *qnrC*, *aac(6')-lb-CR*, *dfrA1*, *AAC*, *cat* and *erm (ABCG)*.

Table 6 reveals ARGs and integrons in the genome of ARB/MARB. *Escherichia coli* harbors *tet(A)* and *mph(E)* resistance genes and class 2 integron in its genome, while *Pseudomonas aeruginosa* had *tet(A)* resistance gene and class 2 integrons. *Alcaligenes faecalis* had *tet(37)*, *msr(AB)* and *cmlA* resistance genes, and also class 1 and 2 integrons in its genome. *Paenalcaligenes hominis* had *tet(37)*, *mph(ABC)* and *blaTEM* resistance genes and class 2 integron in its genome. *E. coli* (isolate 18) had *cmlA*, *tet(A)*, *(C)* and *ANT(2)* resistance genes in its genome, however, no class 1 or 2 integron was detected.

### Polymerase chain reaction amplification of 16SrRNA of the ARB/MARB

Figure 3 shows the gel image of amplified 16SrRNA gene for the 12 ARB/MARB isolates. The base band of the amplified DNA was 1.5 kb in comparison to the DNA ladder. Figure 4 reveal the PCR amplification of the genomic DNA of MARB targeted to amplify and detect different ARGs. This image reveals the presence of sulphonamide–trimethoprim *(dfrA7)* and chloramphenicol resistance genes (*cmla*) in the genome of *Staphylococcus* sp. and *Alcaligenes feacalis*, respectively, and an aminoglycoside resistance gene (*ANT2*) in the genome of *Escherichia coli*. Figure 5 reveals the presence of tetracycline resistance genes *tet(37)* and *tet(A)* in the genome of *Alcaligenes faecalis*, *tet(37)* resistance gene in the genome of *Escherichia coli*, *tet(C)* resistance gene in the genome of *Micrococcus luteus*, beta-lactam resistance gene, *blaSHV* in the genome of *Staphylococcus* sp. and *tet(C)* resistance gene in the genome of *Providencia stuartii.* Figure 6 reveals the presence of *tet(T)* resistance gene in the genome of *Staphylococcus* sp. and an aminoglycoside resistance gene (*aacC4*) in the genome of *Alcaligenes feacalis* and *Micrococcus luteus.* Figure 7 reveals the presence of beta-lactam resistance genes (*blaSHV, blaTEM* and *blaSHV*) in the genomes of *Staphylococcus* sp., *Salmonella enterica* and *Providencia rettgeri*. Figure 8 reveals the presence of quinolone resistance gene (*oqxAB*) in the genome of *Pseudomonas aeruginosa*, *Providencia rettgeri* and *Staphylococcus* sp.

**Table 6** Resistance genes and integrons in the genome of ARB/MARB

| Isolate code | Probable identity | Resistance genes | Integrons |
|---|---|---|---|
| 1 | *Escherichia coli* | *tet(A)*, *mph(E)* | Class 2 (150 bp) |
| 2 | *Alcaligenes faecalis* | *tet(37)*, *(A)*, *mph(E)*, *aacC4*, *qnrB* | Class 1 (700 bp), Class 2 (500 bp, 600 bp) |
| 3 | *Providencia stuartii* | *tet(A)* | Class 2 (150 bp, 700 bp) |
| 4 | *Micrococcus luteus* | *tet(A)*, *(C)*<br>*ANT(2)*, *aacC4* | |
| 5 | *Bacillus cereus* | *msr(AB)* | Class 2 (450 bp, 500 bp) |
| 6 | *Salmonella enterica* | *tet(37)*, *blaTEM* | Class 2 (300 bp) |
| 7 | *Bacillus cereus* | *tet(A)* | |
| 9 | *Alcaligenes faecalis* | *tet(37)*, *msr(AB)*, *cmlA* | Class1 (150 bp, 300 bp) Class 2 (700 bp) |
| 10 | *Staphylococcus hominis* | *mph(E)* | |
| 11 | *Pseudomonas aeruginosa* | *tet(A)*, *aac-lb*, *OqxAB* | Class 1 (900 bp), Class 2 (1000 bp) |
| 16 | *Providencia stuartii* | *tet(A)*, *(C)* | Class 1 (150, 600 bp) Class 2 (600 bp) |
| 17 | *Paenalcaligenes hominis* | *tet(37)*, *mph(ABC)*, *blaTEM* | Class 1 (300 bp) Class 2 (200 bp) |
| 18 | *E. coli* | *cmlA*, *tet(A)*, *(C)*, *ANT(2)* | Class 2 (400) |
| 19 | *E. coli* | *tet(A)*, *(C)* | Class 1(300,600, 1000 bp)<br>Class 2 (350, 700 bp) |
| 21 | *Staphylococcus* sp. | *tet(A)*, *(37)*, *qnrA*, *blaSHV*, *drfA7*, *cmlA* | Class 1 (800 bp)<br>Class 2 (300, 400, 800 bp) |
| 26 | *E. coli* | *tet(37)* | Class 2(450 bp) |
| 27 | *Providencia stuartii* | *tet(A)*, *qnrB* | Class 1 (600 bp) |
| 32 | *Providencia rettgeri* | *blaTEM*, *aac-lb*<br>*OqxAB* | Class 1 (150, 200, 350, 600 bp) |
| 34 | *Staphylococcus* sp. | *tet(A)*, *tetT*, *blaSHV*, *OqxAB*, *mph(E)* | Class 1 (250, 600 bp) |

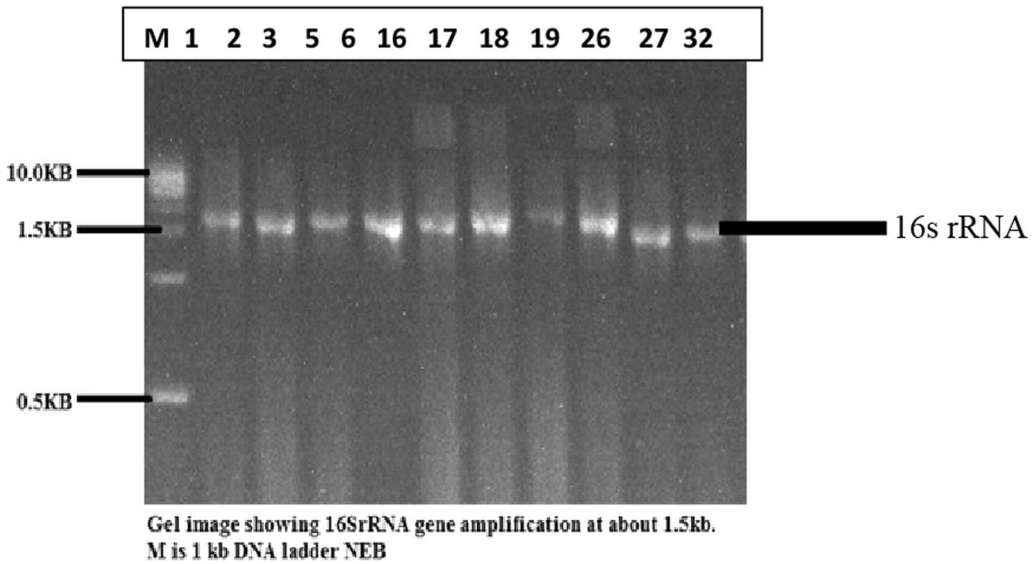


**Fig. 3** Gel image of amplified 16SrRNA gene amplification of the ARB/MARB. Key: 1 = *Escherichia coli*, 2 = *Alcaligenes feacalis*, 3 = *Providencia stuartii*, 5 = *Bacillus cereus*, 6 = *Salmonella enterica,* 16 = *Providencia stuartii,* 17 = *Paenalcaligenes hominis*, 18 = *E. coli*, 19 = *E. coli*, 26 = *E. coli*, 27 = *Providencia stuartii*, 32 = *Providencia rettgeri*, M = DNA ladder

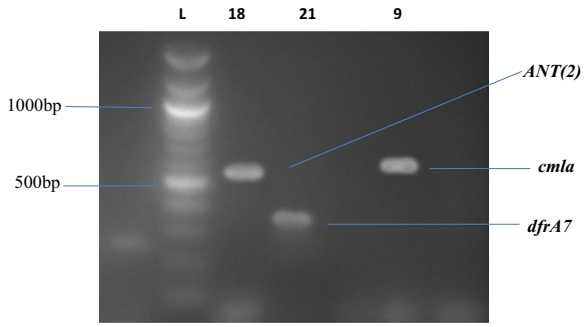


**Fig. 4** PCR amplification of *Ant(2)*, *cmla* and *dfrA7* in the genome of ARB/MARB. Key: L = DNA ladder, lane 18 = *Escherichia coli*, lane 21 = *Staphylococcus* sp., lane 9 = *Alcaligenes feacalis*

### Prevalence of class 1 and 2 integrons in the genome of ARB/MARB

Figures 9 and 10 reveal the PCR amplification of genomic DNA of ARB/MARB targeted to amplify and detect class 1 and 2 integrons. In Plate 7, gel image reveals the presence of class 1 integron in the genome of ARB/MARB. *Escherichia coli* revealed the presence of three (3) class 1 integron, while *Alcaligenes feacalis*, *Staphylococcus* sp. and *Pseudomonas* sp. revealed the presence of one (1) class 1 integron each. Plate 8 shows the gel image of class 2 integrons in the genome of ARB/MARB. Two (2) class 2integrons was detected in the genome of *Escherichia coli,* while *Paenalcaligenes hominis, Salmonella enterica, E. coli,* and *Pseudomonas aeruginosa* had one (1) class 2 integron in their genome.

## Discussion

The use of maggots; *Musca domestica* L. larva commonly developed from chicken droppings could serve as an alternative source of protein for fishes, accredited to its quality protein with amino acids profile showing its superior biological value to groundnut cake and soybean [10]. Also chicken droppings and urine are continuously discharged directly into fish ponds in animal farm-fish pond system, a traditional household-based integrated farming system which is now practiced in Nigeria. This practice often than not leads to the spread of ARGs and ARB via horizontal gene transfer [11] and this may also change the bacterial community composition in aquaculture environment since the chicken droppings often contains residues of antimicrobials used for the treatment and growth promotion of poultry [12]. Therefore, there is need for the molecular characterization of ARB associated with maggots produced from chicken droppings.

In this study, some heavy metals commonly implicated in the dissemination of ARGs to bacteria resident in environmental samples were detected in chicken droppings. Deng et al. [19] detected heavy metals such as copper, zinc and manganese in chicken droppings from chicken with previous history of antibiotics application and reported that the dissemination of ARGs is influenced by the presence of these metals. Tripathy et al. [20] reported elevation of copper and zinc in soil after prolong

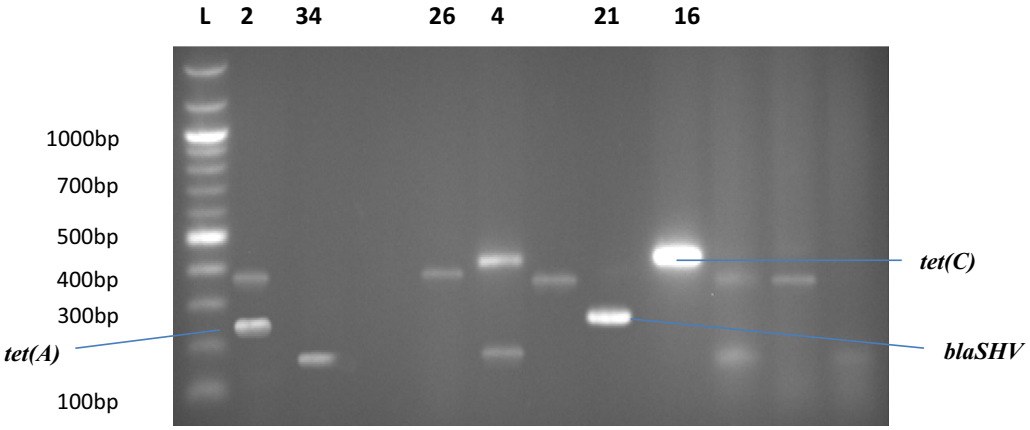


**Fig. 5** PCR amplification of *tet(A)*, *tet(37)*, *tet(C)* and *blaSHV* in the genome of ARB/MARB. Key: L = DNA ladder, lane 2 = *Alcaligenes faecalis*, lane 34 = *Staphylococcus* sp., lane 26 = *Escherichia coli*, 4 = *Micrococcus luteus*, lane 21 = *Staphylococcus* sp., lane 16 = *Providencia stuartii*

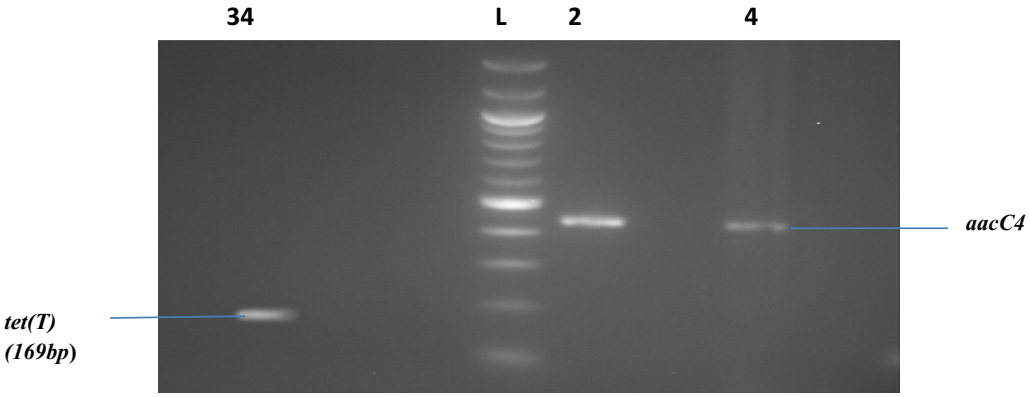


**Fig. 6** PCR amplification of *tet(T)* and *aacC4* resistance genes in the genome of ARB/MARB. Key: L = DNA ladder, lane 34 = *Staphylococcus* sp., lane 2 = *Alcaligenes faecalis*, lane 4 = *Micrococcus luteus*

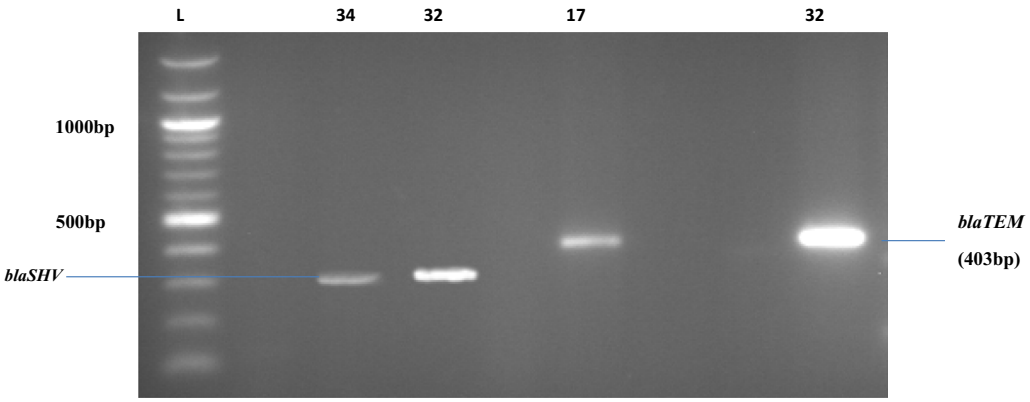


**Fig. 7** PCR amplification of *blaSHV* and *blaTEM* in the genome of ARB/MARB. Key: L-DNA ladder, lane 34 = *Staphylococcus* sp., lane 32 = *Providencia rettgeri*, lane 17 = *Paenalcaligenes hominis*, lane 32 = *Providencia rettgeri*

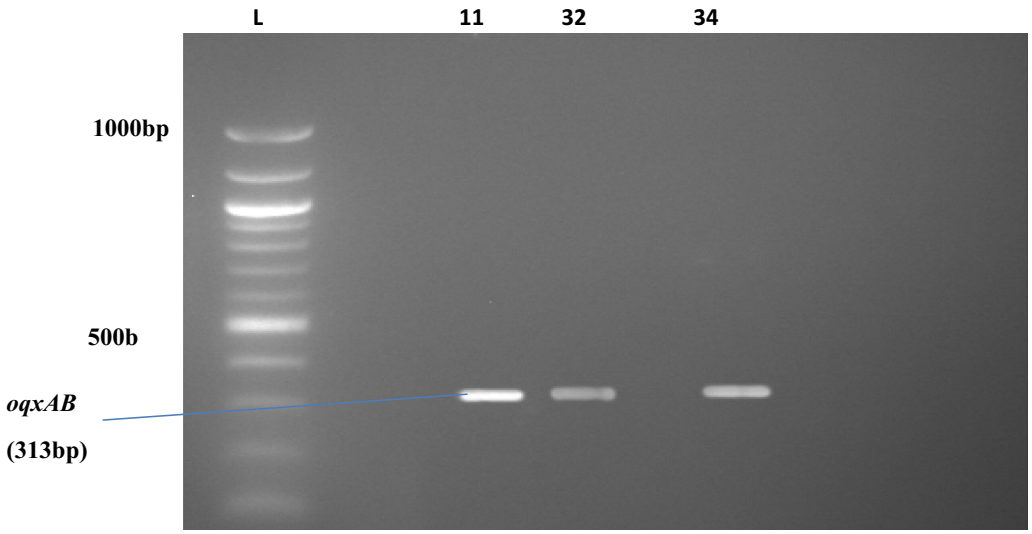


**Fig. 8** PCR amplification of *oqxAB* resistance gene in the genome of ARB/MARB. Key: L = DNA ladder, lane 11 = *Pseudomonas aeruginosa,* lane 32 = *Providencia rettgeri*, lane 34 = *Staphylococcus* sp

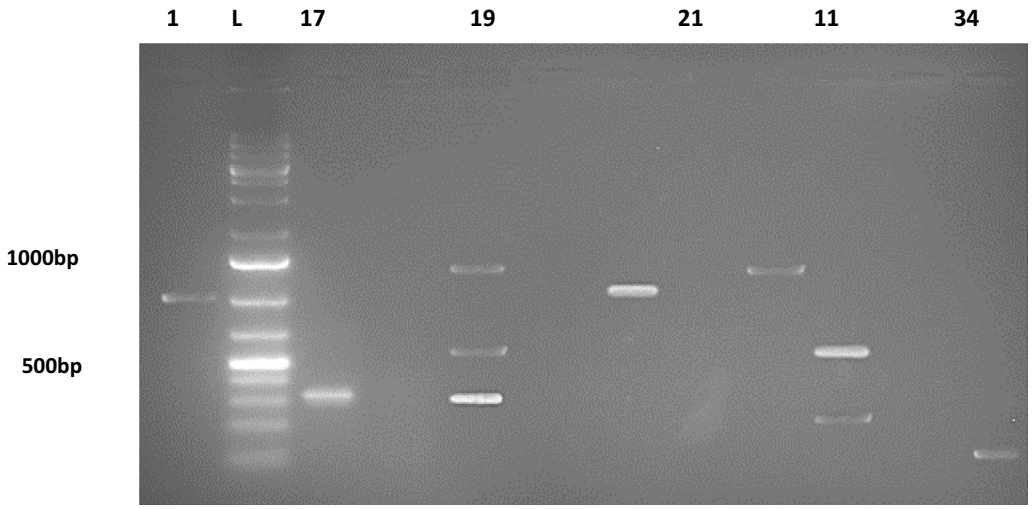


**Fig. 9** PCR amplification of class 1 integron in the genome of ARB/MARB. Key: L = DNA ladder, lane 1 = *Alcaligenes faecalis*, lane 17 = *Palnalcaligenes hominis,* lane 19 = *Escherichia coli*, lane 21 = *Staphylococcus* sp., lane 11 = *Pseudomonas aeruginosa*, lane 34 = *Staphylococcus* sp., lane 9 = *Alcaligenes faecalis*

application of livestock manure. In support of the claim of Tripathy et al. [20] and Luo et al. [21] found that more than half of cadmium, copper and zinc in agricultural soil were attributed to the utilization of poultry droppings as organic fertilizer. Zhang et al. [22] reported the positive correlations between ARGs and heavy metal concentrations. The presence of heavy metals in higher magnitude in receiving environments could exert significant selective pressure for ARGs that code for efflux pumps in ARB [22]. Also in support of the claim of Zhang et al. [22], Di Cesare et al. [23] unravelled the relationship between the abundance of ARGs, heavy metal resistance genes and a mobile genetic element (class 1 integron); which is efficient in the lateral transfer of resistance genes in environmental settings in a study on the co-occurrence of integrase 1, antibiotic and heavy metal resistance genes in municipal wastewater treatment plants. This correlation may be due to the fact that genes encoding resistance to antibiotics and metals are commonly found on the same plasmid or mobile genetic element [24].

In this study, bacteria isolated from chicken droppings and maggots were mostly enteric and of potential human

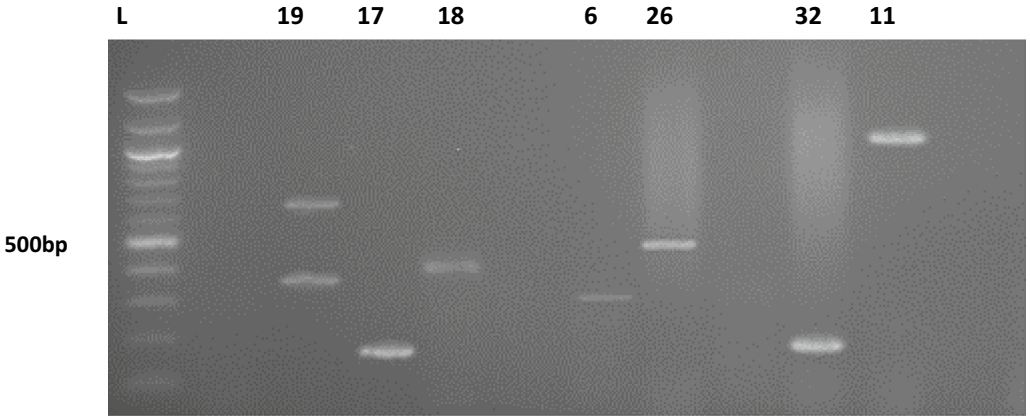


**Fig. 10** Amplification of class 2 integron in the genome of ARB/MARB. Key: L-DNA ladder, lane 19 = *Escherichia coli*, lane 17 = *Paenalcaligenes hominis,* lane 18 = *E. coli,* lane 6 = *Salmonella enterica*, lane 26 = *E. coli*, lane 32 = *Providencia rettgeri*, lane 11 = *Pseudomonas aeruginosa*

pathogens or opportunists such as *Escherichia coli, Paenalcaligenes hominis, Providencia stuartii, Alcaligenes faecalis* and *Staphylococcus aureus.* The findings from this study is in agreement with the report of Omojowo and Omojasola [25] who also reported the same enteric organisms from poultry droppings. Their high prevalence could be attributed to the fact that they have a slow die off rate in the presence of selective pressure like antibiotics [25]. *Staphylococcus* sp. the etiological agents of staphylococcosis, pododermatitis (bumblefoot) and septicaemia which affect mostly chicken and turkeys and also implicated in human and animal infections have also been reported in chicken droppings [26]. The presence of *Staphylococcus* species in the chicken droppings and maggots could be as a result of anthropogenic activities within the poultry house since they are known to be carried in the nasopharynx, throat, skin, cuts, boils, nails and as such can easily contribute to the normal microflora. Yang et al. [15] also reported the incidence of staphylococci in poultry droppings. The presence of *S. aureus* in poultry droppings can cause food poisoning in human when contaminated poultry meat is consumed [27]. *Pseudomonas aeruginosa*, a specie of considerable medical importance, isolated in this study is associated with serious illnesses, especially hospital acquired infections such as ventilator-associated pneumonia and sepsis syndromes. This same bacterium was also isolated from chicken droppings by Yang et al. [15], a study conducted in China. The presence of this bacterium in chicken droppings might be due to the fact that *P. aeruginosa* is characterized by its low antibiotic susceptibility and easy acquisition of resistance genes via horizontal gene transfer mechanisms [15]. *Salmonella* sp. is one of the widely distributed pathogens in chicken litter, with poultry and eggs remaining as the predominant reservoir. The detection of *S. enterica* in this study corroborates with a report documented by Fagbamila et al. [28] to further confirm the prevalence of *Salmonella* in chicken droppings. The presence of *Salmonella enterica* might be due to the fact that they are found relatively in the alimentary tract of animals. They are known to be the etiological agent responsible for salmonellosis in both humans and animals [29].

*Providencia* spp. were reported to cause a large outbreak of foodborne illnesses, pericarditis, meningitis, endocarditis and diarrhea [30]. The detection of multidrug resistant *Providencia* spp. in this study corresponds with the findings of Nahar et al. [31] who reported the prevalence of multidrug resistant *Providencia stuartii* in chicken droppings, its public health implications for poultry workers and associated communities in a study in Bangladesh. Sub-standard sanitation practice in densely populated countries could aggravate the antibiotic resistance lineage easily to population level. So, multidrug resistant *P. stuartii* in chicken droppings may aggravate spreading both by person-to-person transmission and via human to animal interface [30]. They may impose a threat of cross-resistance to other pathogens [31], by changing in ways that lead to the reduced effectiveness of antibiotics to cure or prevent infections.

There are no report about the recovery of potential bacterial pathogens carrying ARGs in maggots developed from poultry droppings. The isolation of *Bacillus cereus* and *E. coli* from maggots in this study corroborates with the findings of Banjo et al. [13] who reported the recovery of the same organisms as microflora of the maggots, and this shows that the practice of feeding fishes with maggots raises concerns about potential transmission of bacterial pathogens. *Bacillus cereus* is known to frequently contaminate clinical

environments, biotechnological processes, and food production [32]. Olaniyi et al. [9] also detected different potential bacterial pathogens of clinical importance that were predominant in ARB/MARB communities in chicken droppings and maggots in a report on the metabolic responses of indigenous bacteria in chicken faeces and maggots to multiple antibiotics which also indicates that the spread of the potential pathogens in environments via unethical agricultural practices poses a serious risk to human health [9].

All the isolates showed high multiple antibiotic-resistant patterns to the conventional antibiotics. All Gram-positive isolates were resistant to gentamycin, amoxicillin, ciprofloxacin, erythromycin and streptomycin with the exception of *Micrococcus luteus* that showed an intermediate reaction to streptomycin and perfloxacin. All Gram-negative isolates were resistant to gentamycin, amoxicillin, ciprofloxacin, erythromycin and perfloxacin, also *Paenalcaligenes hominis* and *Escherichia coli* both showed intermediate reactions to streptomycin while *Providencia rettgeri* showed an intermediate reaction to amoxicillin. This observation was consistent with previous report of Omoya et al. [33] showing that multidrug-resistant bacteria were isolated in poultry litters.

The *tet* genes, known to be responsible for tetracycline resistance was detected in some of the isolates in this study. The *tet* gene family, involved in the active efflux of tetracycline compounds, ribosomal protection or the enzymatic modification of antimicrobial agents, was reported as one of the most frequently detected ARGs in animal manures [34, 35], and this agrees with the findings from this study that shows that *tet* gene family has the highest occurrence of all the ARGs detected. This may be due to the fact that after administration, most tetracyclines are excreted as active compounds through faeces and urine, which are spread in the environment by manure fertilization practices and these compounds have a high affinity for soil organic matter [36]. Kumar et al. [37] reported that from the soil, some antibiotics such as chlortetracycline are adsorbed onto food crops such as vegetables (green onion and cabbage) and corn in levels increasing with soil contamination. Human consumption of antibiotics-contaminated foods can increase antibiotic resistance, leading to food poisoning or allergies [38].

The detection of macrolide resistance genes [*erm, mph(E)* and *mph(ABC)*] in some isolates in this study corroborates with the findings of Mu et al. [39] and Luby et al. [40] who reported that various erythromycin ribosome methylation (*erm*) genes have been found in livestock. This may be due to the fact that macrolide antibiotics, such as erythromycin and tylosin are not completely metabolized in the gut and up to three quarters of the antibiotic can be excreted in urine and faeces [40].

The detection of mobile quinolone resistance genes in this study, notably the *qnrA*, *qnrB* and *oqxAB*, is in agreement with the result of McIver et al. [41] who also detected quinolone resistance genes in a study on the molecular epidemiology of antibiotic resistant *E. coli* from farm to fork in intensive poultry production carried out in South Africa. The presence of quinolone resistance genes may be due to mutations to DNA gyrase and an increase in efflux or porin loss [41].

β-lactams are one of the most widely used antibiotics, in veterinary medicine and the resistance to these antibiotics is a severe threat to human and animals because they have low toxicity and are used to treat a broad range of infections. The detection of *blaTEM* in this study corroborates with the findings of Wang et al. [42] who reported that chicken manure could serve as a reservoir for the transfer of antibiotic resistance genes *blaTEM*. This result also corroborates with past reports on extended spectrum β-lactam-producing enterobacteria from clinical and environmental samples in different regions of the world [43]. The presence of these superbugs as normal microbiota in companion with farm animals indicates a potential source of acquired resistance for human pathogens and normal microbiota.

Integrons are gene exchange systems and are known to play a significant role in the acquisition and dissemination of antimicrobial resistance genes and to be selected by antimicrobial pressure [44]. While this study had the limitation of not being able to detect the gene cassette, we were able to detect the presence of class 1 and 2 integrons in ARB/MARB from chicken droppings and maggots that binds the 5'and 3'conserved ends of Levesque 5CS and 3CS of class 1 integron and also to the White hep74 and White hep51 to hybridize *attI2* and *orfX* of the class 2 integron were detected. The detection of class 1 and 2 integrons in this study corroborates with the findings of Yang et al. [15] who also detected these mobile genetic elements in poultry manure collected from different farms in China. These integrons are markers of multidrug resistance strains and the potential that integrons are physically linked to other resistance genes.

## Conclusion

Chicken droppings and maggots are reservoirs of diverse and abundant multidrug-resistant bacteria which is capable of transmitting pathogenic and opportunistic bacteria to human and environmental microbiota. The multiple antibiotic-resistant bacteria obtained in this study generally carried about 17 types of ARGs covering resistance to six classes of clinically important antibiotics, classes 1 and 2 integrons were also detected in the majority of the MARB which could lead to the evolution of antibiotic resistance. The traditional farming system of feeding

fish with chicken droppings and maggots emanated from them might enhance the spread of ARB and their ARGs to the surrounding environments, and might eventually enter into the food chain and human pathogens, imposing a serious threat to human health.

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12302-024-00840-w.

**Additional file 1: Table S1.** Primer sequence and PCR conditions. **Table S2.** Antibiotic working concentrations (CLSI standards). **Table S3.** Organic and mineral content of chicken droppings. **Table S4.** Quantity of heavy metals in chicken droppings. **Figure S1.** Percentage of ARB in chicken droppings to erythromycin. **Figure S2.** Percentage of ARB in chicken droppings to tetracycline. **Figure S3.** Percentage of ARB in chicken droppings to ampicillin. **Figure S4.** Percentage of ARB in chicken droppings to ciprofloxacin. **Figure S5.** Percentage of ARB in chicken droppings to gentamycin. **Figure S6.** Percentage of ARB in chicken droppings to chloramphenicol.

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**Author contributions**
OAA, AOA, GO and BO contributed reagents and materials, and carried out the experiment, OOO and BO analyzed and interpreted the data, OOO and OAA conceived and designed the experiments and wrote the paper, while OOO and BO edited the manuscript. All the authors approved the final version of the manuscript and agreed to be accountable for all the findings from this study.

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**Availability of data and materials**
All data generated or analysed during this study will be made available on request (not well understood).

## Declarations

**Ethics approval and consent to participate**
Not applicable.

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare that they have no competing interests.

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## Publisher's Note