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Antimicrobial Resistance In Poultry Bacterial Flora: A Comparative Analysis Of Two Farms In Thiruvananthapuram District

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ABSTRACT

The increasing demand for poultry products has led to the excessive use of antibiotics in the poultry industry, both as growth promoters and as disease preventives. The practice significantly contributed to the transformation of poultry farms as reservoirs of antimicrobial-resistant (AMR) bacteria and resistance genes that can jeopardize the global healthcare industry. In this study, poultry litter samples were collected from two farms located at distinct zones of Thiruvananthapuram district, Kerala, India. A total of 28 AMR bacterial strains were isolated, with selected potent strains further characterized by 16S rRNA sequencing. The draft assemblies were deposited in GenBank. Comparative evaluation of antimicrobial resistance patterns between the poultry farms located at Ayiroor and Vizhinjam revealed both similarities and site-specific differences, likely influenced by variations in management practices, antibiotic usage, environmental conditions, and biosecurity measures. Resistance to penicillin and ampicillin was consistently observed across both sites, underscoring the reduced efficacy of β-lactam antibiotics in poultry-associated isolates.

These findings highlight the urgent need for surveillance and detailed molecular characterization of resistant strains to mitigate the growing public health burden of antimicrobial resistance.

Keywords: Antimicrobial resistance, Poultry, 16S rRNA sequencing, β-lactam resistance, Pantoe sp., MAR index, PCR

1. INTRODUCTION

The steepened rise in human population globally raised persistent demand for animal food products. The scenario facilitated the widespread establishment of livestock industry, especially poultry farms with multitude of poultry rearing trends and significantly increased meat production. From a global point of view, the Food and Agriculture Organization (FAO) estimated that the poultry industry contributes to approximately 40% of the world's meat production [1]. Accordingly, intensive farming

techniques gained momentum worldwide, but unfortunately accelerated the spread of zoonotic diseases and compromised animal health and productivity [2].

It is well established that conventional antimicrobials have been extensively employed to prevent bacterial infections in the livestock sector, particularly in poultry production [3]. The use of antimicrobial growth promoters (AGPs) in poultry was first documented in 1946 [4][5]. Since then, intensive poultry farming has become highly dependent on antibiotics for both growth promotion and disease prophylaxis. In 1951, the United States Food and Drug Administration (FDA) formally authorized the inclusion of antimicrobial agents in animal feed without veterinary prescription [6]. Although initially implemented in large-scale production systems, the use of antimicrobials has increasingly permeated small- and medium-scale poultry enterprises, particularly in low-resource settings, owing to their capacity to enhance meat and egg yields [7]. The major classes of antibiotics commonly administered in poultry farms include β-lactams, sulfonamides, lincosamides, tetracyclines, fluoroquinolones, ionophores, and glycopeptides, each exerting distinct mechanisms of action [8].

Despite the substantial contributions of poultry production to food security, the widespread use and misuse of antimicrobials have precipitated a major global public health challenge: antimicrobial resistance (AMR) [9]. AMR arises when bacteria acquire the ability to withstand the inhibitory or bactericidal effects of antibiotics. Resistance can emerge through spontaneous genetic variation or via horizontal gene transfer, with mechanisms including alterations to cell wall structure, enzymatic degradation of antibiotics, and active efflux of antimicrobial compounds [10,11]. Preserving the efficacy of antimicrobials for treating bacterial infections has therefore become a critical concern for both veterinary and human medicine [12–14].

The challenge is particularly acute in low-resource settings, where the transition to more intensive poultry farming practices has heightened the risk of AMR emergence and dissemination. Poultry is recognized as a reservoir for multidrug-resistant (MDR) bacteria, including Escherichia coli, Clostridium perfringens, Klebsiella pneumoniae, Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa, and Bacteroides fragilis [15]. Mounting evidence confirmed the occurrence of MDR strains in poultry farms. For instance, Sebastian et al. investigated four sample types (fresh feces, litter inside the shed, litter outside the shed, and agricultural soil) and reported that E. coli isolates exhibited resistance to ampicillin, amoxicillin, meropenem, and tetracycline [16]. Similarly, evidence from central and southern India suggests a significant risk of AMR emergence, although the magnitude of this risk remains poorly characterized [15].

Further, the transmission of AMR strains from poultry to humans occurs through direct interaction with fowls, handling of fowl-derived products, and consumption of contaminated food. Environmental dissemination also contributes substantially, as poultry litter is commonly repurposed as agricultural fertilizer or aquaculture feed, thereby facilitating intersectoral spread of resistant bacteria. Recent reports describe a high prevalence of MDR and extended-spectrum β-lactamase (ESBL)-producing E. coli in intensive chicken farming systems in India, along with the detection of MDR avian pathogenic E. coli harboring virulence genes in backyard layer flocks. A cross-sectional survey of poultry farmers and veterinarians in Assam and Karnataka further revealed limited awareness among farmers regarding the link between antimicrobial use and resistance [17]. Importantly, exposure to AMR from commercial farms is often confined to occupational contact or environmental contamination of adjacent soil and water by animal waste [18]. Inadequate biosafety practices in such settings frequently prompt the indiscriminate use of additional antimicrobial agents, inadvertently exacerbating MDR bacterial burdens [19]. Moreover, these environments heighten the potential for anthropo-zoonotic AMR transmission, whereby resistance traits originating in humans are transferred to animals. Alarmingly, this can extend resistance even to antibiotics seldom used in poultry, such as colistin and fluoroquinolones [20]. The increasing convergence of humans and animals across diverse farming systems creates further opportunities for the interspecies transmission and amplification of resistant pathogens [21–25].

The sustained use of antimicrobials in poultry farms, coupled with the emergence of AMR in both pathogenic and commensal organisms, has consequently drawn significant attention from the global scientific community. Extensive research has been directed toward elucidating the mechanisms underlying AMR, identifying the factors contributing to its emergence, and assessing its profound impact on human health, animal welfare, and ecological sustainability. This growing body of evidence underscores the urgent need to develop and implement alternative approaches to reduce antimicrobial dependence in poultry production and to mitigate the escalating threat of AMR at the human–animal–environment interface.

This study provides insights into the isolation and identification of antimicrobial-resistant strains from poultry litter samples collected across two distinct zones in Thiruvananthapuram district, Kerala, India.

2. METHODOLOGY

2.1. Sample collection

Poultry droppings were collected from two poultry farms situated at two different zones of the Thiruvananthapuram district, Kerala, India that raise domestic fowls specifically for egg rearing. The fowls were kept in cages with a sawdust bed with limestone to prevent microbial infections. The fowls were given antibiotic injections during the initial stage of their growth. The most commonly used antibiotics include Sochrine and Sephalin. Azithromycin would be the choice in the case of respiratory issues. The collected droppings were kept in the laboratory under the desired conditions for further experiments.

2.2. Isolation of bacteria by Serial dilution

2.2.1. Materials

Poultry droppings

Sterile water

Nutrient Agar

Laminar Airflow

2.2.2. Method

The collected samples of poultry droppings were serially diluted to isolate pure bacterial colonies. Serial dilution is a widely used technique for the isolation of pure cultures that involves the systematic dilution of samples to reduce the concentration of microorganisms, allowing for the growth of individual colonies on agar plates.

9 ml distilled water was filled in ten test tubes labeled 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}. One test tube with sterile distilled water was kept as the master tube. The usual dilution factor was 10-fold, meaning each dilution was 1/10th the concentration of the previous dilution. The sample was weighed and transferred into the master tube containing 10 ml sterile distilled water and made into a heterogeneous suspension. 1 ml of the suspension was aseptically transferred into the test tube labeled 10^{-1}. Similarly, the suspension was transferred from 10^{-2} to 10^{-3}, 10^{-3} to 10^{-4}, and 10^{-4} to 10^{-5}. Finally, 1 ml was discarded. This procedure was repeated multiple times, which led to an array of dilutions with decreasing microbe concentrations. The inoculated samples were kept overnight at 37°C for incubation.

2.3. Identification of bacteria

2.3.1. Gram's staining

A thin smear was prepared on a clean grease-free slide. Air dried and fixed the smear with gentle bearable heat. The sample was flooded with Gram's Crystal Violet stain and kept for one minute. The mordant enhanced both the affinity and binding with the primary stain. The prepared smear was decolorized utilizing acetone or 95% absolute alcohol, followed by an after-wash in slow-running tap water for one minute. The smear was counterstained using Safranine and washed out with tap water. The smear was air-dried and subjected to microscopic examination for bacterial identification.

2.4. Sub culturing of bacteria

2.4.1. Preparation of nutrient broth and sub culturing of bacterial colonies

Three grams of nutrient broth powder were dissolved in 100millilitres of distilled water and autoclaved for fifteen minutes at 15 lbs pressure (121^0C) to sterilize it. Cotton plugs were used to keep the medium in sterile test tubes. The isolated bacterial colonies were transferred to nutrient broth and kept for overnight incubation. The turbid colonies were sub cultured in sterile petri plates. The procedure was repeated to obtain pure cultures.

2.5. Determination of multidrug resistance pattern by disc diffusion

The disc diffusion method was utilized to examine the antibiotic sensitivity of the isolated bacterial strains. The antibiotic discs (Penicillin, Tetracycline, Ampicillin, Ciprofloxacin, Gentamycin, Erythromycin and Kanamycin) were transferred to the agarose plates containing the bacterial suspension. These were kept overnight at 37^0C.

2.6. Multiple Antibiotic Resistance (MAR) Index

The Multiple Antibiotic Resistance (MAR) index was calculated to assess the extent of resistance among the isolates obtained from the two poultry farms under study using the formula mentioned below.

$MAR = a / (b \times c)$

Were

a = total "resistance incidences" (sum of resistant counts across all antibiotics),

b = number of antibiotics tested,

c = total isolates tested in that farm.

2.7. Biochemical identification of the multi drug resistant strains

The biochemical identification of the bacterial colonies that exhibited multi drug resistance was done utilizing the growth-based VITEK 2 automated microbiology technology.

A sterile swab or applicator stick was used to transfer adequate number of colonies of a pure culture and to suspend the microorganism in 3.0 mL of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) in a 12 x 75 mm clear plastic (polystyrene) test tube. The turbidity was adjusted and measured using the DensiChek Turbidity meter. Identification cards were inoculated with microorganism suspensions using an integrated vacuum apparatus. A test tube containing the microorganism suspension was placed into a cassette and the identification card was placed in the neighboring slot while inserting the transfer tube into the corresponding suspension tube. After the application of vacuum re-introduction of air into the station, the organism suspension is forced through the transfer tube into micro-channels that fill all the test wells.

Inoculated cards were incubated on-line at 35.5 + 1.0ºC. Each card was removed from the carousel incubator once every 15 minutes, transported to the optical system for reaction readings, and then returned to the incubator until the next read time. Data were collected at 15-minute intervals during the entire incubation period. The test reactions were interpreted using the transmittance optical system.

Test data from the unknown organism was compared to the respective database to determine a quantitative value for proximity to each of the database taxa.

2.8. Molecular identification of MDR strains using 16srRNA sequencing

Molecular identification was done for selected strains which express maximum number of resistances

2.8.1. Isolation of bacterial DNA

2ml overnight culture was taken and the cells were harvested by centrifugation at 10000rpm for 10 minutes. The supernatant was discarded. 875 µl of TE buffer was added to the cell pellet and the cells were resuspended in the buffer by gentle mixing. 100 µl of 10% SDS and 5 µl of Proteinase K were added to the cells. After thorough mixing, the mixture was incubated at 37^0 C for an hour. 1ml of Phenol-Chloroform-Isoamyl alcohol mixture was added to the contents, mixed well by inverting and incubated at room temperature for 5 minutes. The contents were centrifuged at 10000rpm for 10 minutes at 40 C. The highly viscous jelly like supernatant was collected using cut tips and was transferred to a fresh tube. The process was repeated with Phenol-Chloroform-Isoamyl alcohol mixture and the supernatant was collected in a fresh tube, 3/4th. 100 µl of 5M sodium acetate was added to the contents and mixed gently. 2ml of ice-cold Isopropanol was added and mixed gently by inversion till white strands of DNA precipitated. The contents were centrifuged at 5000rpm for 10 minutes. The supernatant was removed, followed by the addition of 1ml of ice cold 70% ethanol. The contents were centrifuged at 5000rpm for 10 minutes. The ethanol was discarded. The pellet was air dried for 1 to 2 hours till it dried completely. 50-100 µl of TE Buffer was added, mixed gently, and stored at – 200 C for further studies.

2.8.2. Quantification of bacterial DNA

The isolated bacterial DNA was estimated by the measurement of sample absorbance at 260 nm. The 260/280, 260/230, and 260/325 absorbance ratios were used to determine the DNA purity and the presence of contaminants in the biological samples during the DNA extraction process. The purity and concentration of the DNA obtained from the bacterial isolate was determined through 260/280 nm absorbance measures using the MultiskanSkyHigh Microplate Spectrophotometer (THERMOSCIENTIFIC). Table 1 represents the concentration and purity measures of microbial DNA.

Table 1. Concentration and purity measures of microbial DNA

Sample code	Concentration nµg/ml	Absorbance $A_{260/280}$
AT5A	229	1.8
AT5B	195	1.8
AT1	210	1.8
V1W2	230	1.8

2.8.3. Agarose Gel Electrophoresis

2.8.3.1. Preparation of agarose gel

400mg of agarose was weighed and dissolved in 40ml of 1X TAE buffer by heating and constant stirring in a water bath at 95ºc. After cooling, 2µl of (10mg/ml) ethidium bromide solution was added into it and the gel was cast. After solidifying, the comb was removed and transferred the gel into the electrophoretic apparatus containing 1X TAE buffer. The incubated DNA sample was mixed with DNA loading dye and loaded in the wells along with the molecular weight DNA marker. It was allowed to run at 50v for 1 hour followed by the gel analysis under UV transilluminator.

2.8.3.2. DNA amplification using PCR

The microbial DNA was amplified using 16srRNA primers. PCR reaction was carried out in SimpliAmp Thermal Cycler, Thermofischer (The Applied Biosystems). The reaction mixture (25 µl reaction volume) included 1.5µl of 10µM forward primer, 1.5µl of 10µM reverse primer, 12µl of Takara master mix; 5µl of sterile autoclaved water, and 5µl of template DNA samples. The determined conditions were mentioned in Table 2. The DNA was transferred to PCR tubes along with forward and reverse primers to perform PCR.

Table 2. Primer sequence for DNA amplification

27F	CGGCCCAGACTCCTACGGGAGGCAGCA
1492R	GCGTGGACTACCAGGGTATCTAATTC

2.8.3.3. Preparation of PCR Master mix

The PCR reaction was carried out in 20µl reaction mixture containing 10µl SsoAdvanced Universal SYBR Gr supermix (2X, Biorad), 1µl of 10µM forward and reverse primer, 3µl of template DNA and 5µl Nuclease free water. Table 3 represents 16s rRNA primers.

Table 3. Master Mix for 16S rRNA Universal Primers

PCR Components	Stock	Volume to be taken
Nuclease free water	-	5µl
Forward Primer	100 µM	1.5µl
Reverse Primer	100 µM	1.5µl
Takara Master Mix	2X	12µl
Template DNA	-	5µl
Total Volume	-	25µl

The template DNA was amplified on DNA thermocycler using the PCR conditions 94°C for 4 minutes, 94°C for 30 seconds, 64.5°C for 30 seconds, 72°C for 30 seconds. The total number of cycles were 35, with final extension at 72°C for 5 minutes were performed using the following programmer.

Table 4. Primer specification for DNA amplification

Primer	Bacteria 16srRNA primer
Tm	16sF 74.08ºC 16sR 64.80 ºC

Table 5. PCR Profile for 16S rRNA Universal Primers

Profile	Temperature	Time
Lid temperature	98^0 C	
Initial Denaturation	94^0 C	00:04:00
Denaturation	94^0 C	00:00:30
Annealing	64.5^0 C	00:00:30
Extension	72^0C	00:00:30
Go to step 2 Repeat 34 cycles		
Final Extension	72^0 C	00:05:00

2.8.4. Agarose gel electrophoresis of PCR products

Agarose gel electrophoresis was done for the qualitative analysis of PCR products. Horizontal gel electrophoresis unit was used to run the sample on the gel to determine the size of amplicons. The PCR products were electrophoresed on 2% agarose gel stained with Ethidium Bromide (1mg/ml), run at constant voltage of 50V in 1XTAE buffer. A 100bp DNA ladder was used for the comparative study. The gel documentation was carried out using Documentation Unit. The remaining PCR product was stored at -20°C for sequencing.

2.8.5. Sequencing of PCR products

PCR products of 16S rRNA of the isolate was obtained through amplification and were purified and sequenced.

3. RESULTS AND DISCUSSION

3.1. Identification of bacterial isolates

3.1.1. Bacterial isolates identified from poultry litter samples at Ayiroor

Samples collected from the Ayiroor poultry farm yielded 20 bacterial isolates. Gram staining confirmed that all isolates were Gram-negative bacilli. The majority of the isolates were identified as belonging to the Pantoea species, while additional strains included Aeromonas hydrophila, Morganella morganii, Sphingomonas paucimobilis, Aeromonas punctata, and Proteus mirabilis. The distribution of the identified organisms is presented in Table 6. Notably, the predominance of Pantoea species, along with the detection of opportunistic pathogens such as Aeromonas spp. and Proteus mirabilis, highlights the potential risk of zoonotic transmission and the need for continuous monitoring of microbial communities in poultry environments.

Table 6. Bacterial isolates identified from poultry litter samples at Ayiroor

S No	Culture Code	Gram Positive/ Gram Negative	Organism
1	A1C1	Negative bacilli	Aeromonas hydrophila
2	A1C2	Negative bacilli	Morganella morganii
3	A1C3	Negative bacilli	Pantoea species
4	A1C4	Negative bacilli	Sphingomonas paucimobilis
5	A1C5	Negative bacilli	Pantoea species
6	A1C6	Negative bacilli	Pantoea species

7	A1T2	Negative bacilli	Pantoea species
8	A1T8	Negative bacilli	Pantoea species
9	AT3	Negative bacilli	Pantoea species
10	AT4	Negative bacilli	Pantoea species
11	AT6	Negative bacilli	Pantoea species
12	AT7	Negative bacilli	Pantoea species
13	A1W1	Negative bacilli	Pantoea species
14	A1W2	Negative bacilli	Pantoea species
15	A1Y1	Negative bacilli	Pantoea species
16	A1Y2	Negative bacilli	Aeromonas punctata
17	A1Y3	Negative bacilli	Aeromonas punctata
18	AT5A	Negative bacilli	Proteus mirabilis
19	AT5B	Negative bacilli	Lysinibacillus sp.
20	AT1	Negative bacilli	Alcaligenes faecalis

3.1.2. Bacterial Isolates Identified from Poultry Litter Samples at Vizhinjam

Eight bacterial isolates were identified from poultry litter samples collected at a farm in Vizhinjam, all of which were characterized as Gram-negative bacilli. The isolates included Sphingomonas paucimobilis, Alcaligenes faecalis, Chryseobacterium indologenes, and Providencia stuartii. The distribution of the identified organisms is presented in Table 7. The detection of these opportunistic pathogens highlights the microbial diversity in poultry litter and points to their potential role in the dissemination of antimicrobial resistance and environmental contamination.

When compared with the isolates obtained from the Ayiroor farm, notable differences in microbial diversity were observed. While Pantoea species predominated in the Ayiroor samples, the Vizhinjam samples revealed a broader representation of opportunistic pathogens such as Alcaligenes, Chryseobacterium, and Providencia. These site-specific variations in bacterial populations may reflect differences in farm management practices, environmental conditions, or biosecurity measures, and highlight the potential role of poultry farms as reservoirs of diverse Gram-negative organisms with varying pathogenic and resistance potential.

Table 7. Bacterial isolates identified from poultry litter samples at Vizhinjam

S No	Culture Code	Gram Positive/ Gram Negative	Organism
1	V1W1	Negative bacilli	Shingomonas paucimobilis
2	V1W2	Negative bacilli	Alcaligenes faecalis
3	V1W3	Negative bacilli	Shingomonas paucimobilis
4	V1W4	Negative bacilli	Alcaligenes faecalis
5	V1C1	Negative bacilli	Shingomonas paucimobilis

6	V1C4	Negative bacilli	Chryseobacterium indologenes
7	V1T1	Negative bacilli	Shingomonas paucimobilis
8	V1T2	Negative bacilli	Providencia stuartii

3.2. Antimicrobial resistance pattern of isolated cultures

3.2.1. Antimicrobial resistance pattern of isolated cultures from Ayiroor farm

Among the isolates from the Ayiroor farm, Aeromonas showed resistance against the antibiotics penicillin and ampicillin. These strains were found to be exhibit varied sensitivity to gentamicin, ciprofloxacin, erythromycin and tetracycline. The resistance observed for kanamycin was moderate. Morganella morganii demonstrated resistance to penicillin, and ampicillin. This strain was susceptible to gentamicin, kanamycin, tetracycline, and erythromycin, although intermediate response was exhibited to ciprofloxacin. Various isolates of Pantoea species showed resistance to penicillin and ampicillin, while being susceptible to gentamicin, kanamycin, ciprofloxacin, and tetracycline. Variable resistance was also noticed for erythromycin. Sphingomonas paucimobilis demonstrated resistance to penicillin, and ampicillin, but was sensitive to gentamicin, kanamycin, ciprofloxacin, and tetracycline. An intermediate response was noted for erythromycin. Alcaligenes faecalis displayed resistance to penicillin, ampicillin, and tetracycline. It exhibited susceptibility to gentamicin, kanamycin, and ciprofloxacin, with an intermediate resistance pattern for erythromycin. Proteus mirabilis was resistant to penicillin, ampicillin, gentamycin, tetracycline, and erythromycin, while being susceptible to kanamycin, and ciprofloxacin.

Most isolates exhibited significant resistance to penicillin and ampicillin, which could be attributed to the production of β-lactamase, intrinsic resistance, or alterations in penicillin-binding proteins (PBPs) that lower the drug's binding affinity. The resistance seen against erythromycin may be linked to efflux pumps or impermeability of the outer membrane. Enzymatic alterations such as acetylation, phosphorylation, adenylation, efflux pumps, and occasionally modified ribosomal binding sites may account for resistance to gentamicin and kanamycin. Both Morganella and Aeromonas displayed intermediate resistance to ciprofloxacin, potentially due to mutations in the gyrA/parC genes, efflux pumps, or plasmid-mediated resistance mechanisms. The resistance observed in Proteus mirabilis and Alcaligenes to tetracycline could be attributed to Tet efflux pumps (tetA, tetB genes) or ribosomal protection proteins that prevent tetracycline's binding. Additionally, intrinsic factors contributing to resistance remain species specific. Figure 1, Table 8 shows the antimicrobial resistance pattern of isolated cultures from Ayiroor farm.

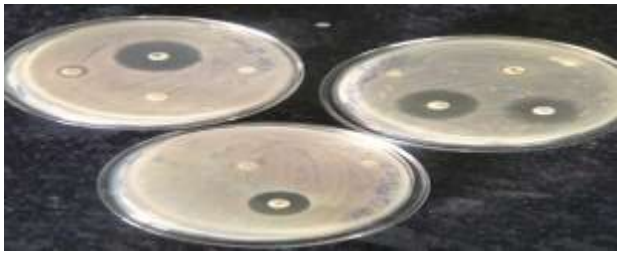
Table 8. Antimicrobial resistance pattern of isolated cultures from Ayiroor farm

Culture No	Organism	Pen	Amp	Gent	Kan	Cipro	Tet	Ery
A1C1	Aeromonas hydrophila	R	R	S	I	S	S	S
A1C2	Morganella morganii	R	R	S	S	I	S	S
A1C3	Pantoea species	S	S	S	S	S	S	S
A1C4	Sphingomona spaucimobilis	R	R	S	S	S	S	I
A1C5	Pantoea species	R	R	S	S	I	S	S
A1C6	Pantoea species	R	R	S	S	S	S	S
AT1	Alcaligenes faecalis	R	R	S	S	S	R	I

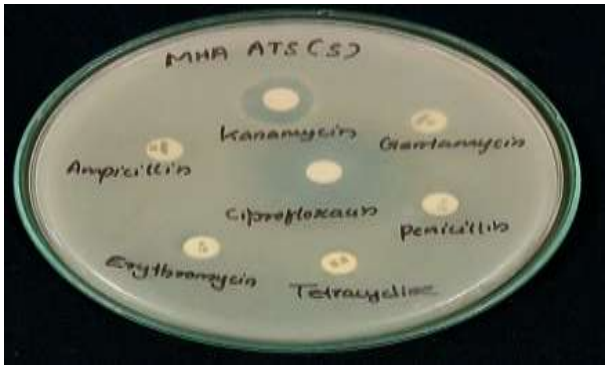
AT2	Pantoea species	R	R	S	S	S	I	I
AT3	Pantoea species	R	R	S	S	S	S	I
AT4	Pantoea species	R	R	S	S	S	S	S
AT5A	Proteus mirabilis	R	R	R	S	S	R	R
AT5B	Lysinibacillus sp.	R	R	R	S	S	R	R
AT6	Pantoea species	I	S	S	S	S	S	S
AT7	Pantoea species	R	R	S	S	S	S	I
AT8	Pantoea species	R	R	S	S	S	S	I
AW1	Pantoea species	R	R	S	S	S	S	I
AW2	Pantoea species	R	R	S	S	S	S	I
AY1	Pantoea species	R	R	S	S	S	S	S
AY2	Aeromonas punctata	R	R	S	I	S	S	S
AY3	Aeromonas punctata	R	R	S	S	S	I	I

R: Resistance, S: Sensitive & I: Intermediate
Pen: Penicillin, Amp: Ampicillin, Gent: Gentamycin, Kan: Kanamycin,
Cipro: Ciprofloxacin, Tet: Tetracycline & Ery: Erythromycin

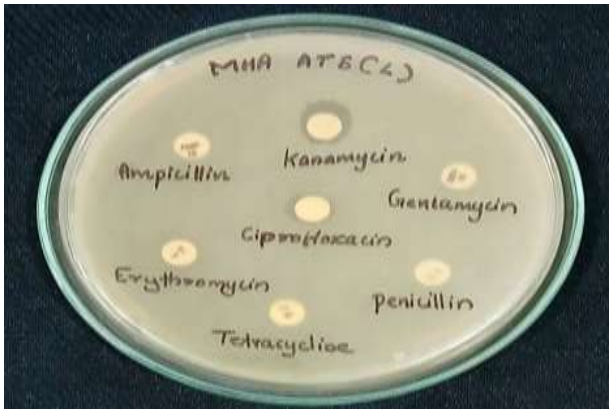
Figure 1. Multidrug resistance pattern of potent bacterial strain, Alcaligenes faecalis (AT1), Proteus mirabilis (AT5A) & Lysinibacillus sp (AT5B) isolated from Ayiroor farm



AT1



AT5A



AT5B

3.2.2. Antimicrobial resistance pattern of isolated cultures from Vizhinjam farm

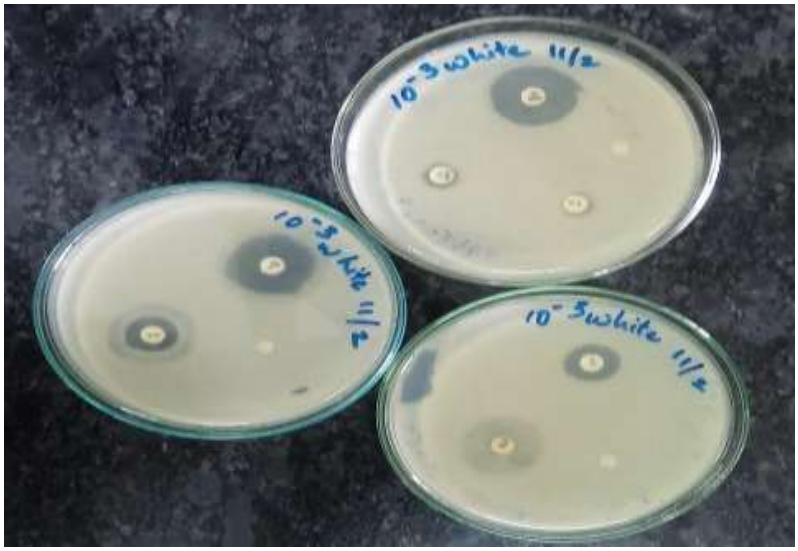
Sphingomonas paucimobilis demonstrated resistance to ampicillin and penicillin, as anticipated, but showed variability in resistance to kanamycin, gentamycin, tetraccyclin and ciprofloxacin. This variability may be attributed to the diversity of efflux pumps among isolates. Bothe the isolates of Alcaligenes faecalis, known for its multidrug resistance, exhibited resistance to ampicillin and pencillin. The strain V1W2 exhibited an additional resistance to kanamycin, tetracycline and erythromycin and sensitivity to gentamycin. The resistance to ciprofloxacin was moderate. Chryseobacterium indologenes is intrinsically resistant to various drugs, including tetracycline and ampicillin and pencillin and sensitivity to gentamycin, ciprofloxacin and kanamycin. Providencia stuartii showed resistance to ampicillin, penicillin, and kanamycin but was susceptible to gentamycin, ciprofloxacin and tetracycline, indicating the potential presence of a partial multidrug resistance plasmid.

Table 9. Antimicrobial resistance pattern of isolated cultures from Vizhinjam farm

Culture No	Organism	Amp	Pen	Gent	Kan	Cipro	Tetr	Ery
V1 W1	Shingomonas paucimobilis	R	R	S	S	S	S	I
V1W2	Alcaligenes faecalis	R	R	S	R	I	R	R
V1W3	Shingomonas paucimobilis	R	R	S	I	S	R	R
V1W4	Alcaligenes faecalis	R	R	S	S	S	S	I
V1C1	Shingomonas paucimobilis	R	R	S	I	I	S	S
V1C4	Chryseobacterium indologenes	R	R	S	S	S	R	I
V1T1	Shingomonas paucimobilis	R	R	S	R	S	S	I
V1T2	Providencia stuartii	R	R	S	R	S	S	I

R: Resistance, S: Sensitive & I: Intermediate
Pen: Penicillin, Amp: Ampicillin, Gent: Gentamycin, Kan: Kanamycin,
Cipro: Ciprofloxacin, Tet: Tetracycline & Ery: Erythromycin

Figure 2. Multidrug resistance pattern of potent bacterial strain, Alcaligenes faecalis (V1W2) isolated from Vizhinjam farm



A comparative evaluation of antimicrobial resistance patterns between the Ayiroor and Vizhinjam farms revealed both commonalities and notable differences. Across both farms, penicillin and ampicillin resistance was widespread, underscoring the diminished efficacy of β-lactam antibiotics in poultry-associated bacterial isolates. This observation is consistent with earlier reports attributing high resistance levels to extensive use of β-lactams in animal production and the widespread dissemination of β-lactamase encoding genes.

At the Ayiroor farm, Pantoea species predominated among the isolates, exhibiting consistent resistance to penicillin and ampicillin but susceptibility to aminoglycosides, fluoroquinolones, and tetracycline. Opportunistic pathogens such as Morganella morganii and Proteus mirabilis demonstrated broader resistance profiles, including resistance to cephalosporins and tetracycline, raising concerns about their potential role as reservoirs of multidrug resistance.

In contrast, the Vizhinjam farm exhibited a different resistance spectrum. Opportunistic pathogens such as Alcaligenes faecalis and Chryseobacterium indologenes demonstrated intrinsic or multidrug resistance traits, including resistance to tetracycline and erythromycin. Providencia stuartii showed partial multidrug resistance but retained susceptibility to ciprofloxacin and tetracycline, which may suggest plasmid-mediated resistance mechanisms rather than chromosomal resistance. Notably, Chryseobacterium isolates, absent in Ayiroor, and inherently display broad resistance, reflecting the microbial diversity and environmental adaptation of the Vizhinjam farm isolates.

The site-specific differences may be attributable to multiple factors, including variations in farm management practices, antimicrobial usage patterns, environmental conditions pertinent to rural and urban geography, and biosecurity measures. For instance, the predominance of Pantoea species in Ayiroor suggests a more uniform microbial community, while the higher diversity of opportunistic pathogens in Vizhinjam highlights the potential influence of environmental exposure and litter management practices on microbial colonization.

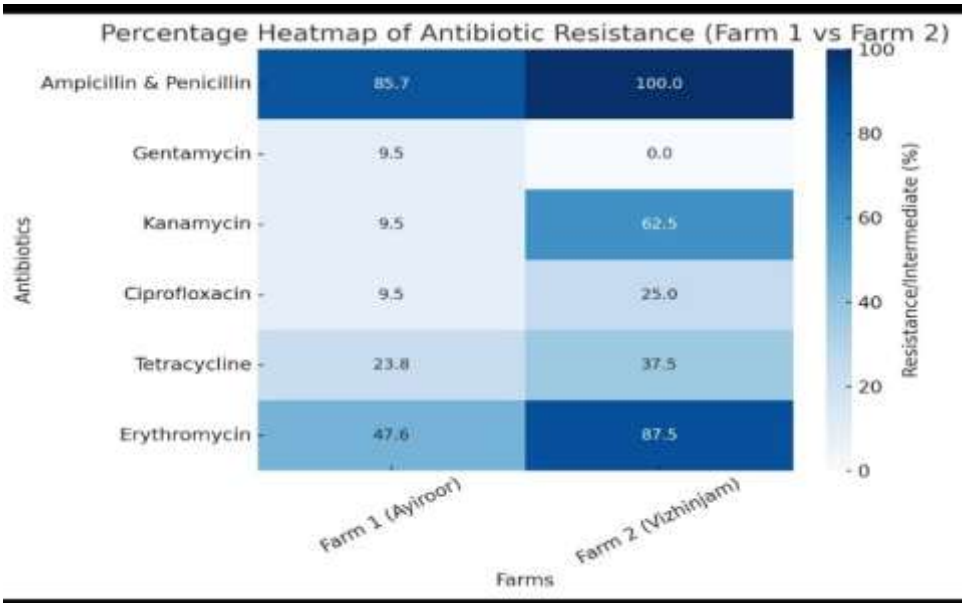


Figure 3. Percentage of antibiotic resistance exhibited by bacterial isolates from two farms towards the selected antibiotics

A comparative analysis of the antibiotic resistance exhibited by two farms through comparative analysis revealed that Vizhinjam farm has 100% resistance to ampicillin & penicillin, while Ayiroor farm has ~86%. Kanamycin & Erythromycin resistance percentages are notably higher in Vizhinjam farm compared to Ayiroor farm. Gentamycin remains completely effective in Vizhinjam farm but shows ~10% resistance in Ayiroor farm. Figure 3 shows the heat map representation of AMR in two farms.

In summary, both farms demonstrated resistance trends that reflect the growing challenge of AMR in poultry production. The persistence of β-lactam resistance, coupled with emerging resistance to cephalosporins, tetracycline, and erythromycin in certain isolates, indicates the ongoing risk of multidrug resistance dissemination.

The presence of AMR strains, particularly those identified from the Vizhinjam farm, highlights the potential for serious consequences if resistance is not addressed. The results suggest a high likelihood of horizontal gene transfer, allowing even non-pathogenic bacteria to act as reservoirs of resistance genes. Prolonged and repeated use of the same antibiotics further exacerbates this risk by selectively enriching resistant populations, ultimately leading to near-complete resistance within the farm microbiota. From a public health standpoint, the risk of transmission to humans through contaminated meat or eggs, occupational exposure among farm workers, and environmental dissemination is of particular concern, as such pathways can compromise treatment efficacy and restrict therapeutic options.

These findings highlight the significance of the study and emphasize the necessity of implementing stringent antimicrobial stewardship, improving farm-level biosafety practices, and monitoring site-specific resistance profiles to mitigate the risk of AMR spread to humans and the environment.

3.3. MAR Index

The Multiple Antibiotic Resistance (MAR) index revealed notable differences between the two farms. At Vizhinjam, the MAR value was approximately 0.43, designating the farm as high-risk, with substantial antimicrobial resistance despite a comparatively smaller number of isolates. In Ayiroor, the MAR value was around 0.31, which, although lower than that of Vizhinjam, still indicated a high-risk profile. Since a MAR index greater than 0.2 is widely recognized as a threshold for identifying

high-risk sources of antimicrobial contamination [26,27], both farms clearly exceeded this benchmark.

The comparatively higher MAR index in Vizhinjam highlights a greater proportional resistance burden than Ayiroor. This may be explained by stronger antibiotic selection pressures, potentially arising from more frequent or indiscriminate antibiotic use in poultry health management. Another plausible explanation is variation in farm-level practices, such as differences in biosecurity measures, hygiene maintenance, and waste management strategies. Previous studies have shown that inadequate biosecurity and uncontrolled antimicrobial use are major drivers of elevated MAR indices in poultry and livestock systems [28,29].

The fact that both farms demonstrated MAR values well above the 0.2 threshold underscores the urgent need for interventions to curb antimicrobial misuse and overuse in poultry farming. These findings align with reports from other regions of India, where poultry farms frequently act as reservoirs of multidrug-resistant bacteria with the potential for zoonotic transmission [30,31]. The data from Thiruvananthapuram not only point to localized management issues but also highlight the broader public health concern of antimicrobial resistance dissemination from agricultural environments to the community. Strengthening antimicrobial stewardship, improving farm biosecurity, and implementing routine surveillance are therefore critical to mitigating resistance risks.

Table 10. MAR Index of two poultry farms

Farm	Total isolates	Antibiotics tested	Sum resistant incidences	MAR index
Vizhijnam	8	7	24	0.429
Ayiroor	20	7	43	0.307

3.4. Molecular identification of multidrug resistant strains

The sequences derived from the 16S rRNA sequencing of the three MDR strains collected from Ayiroor farm, followed by a BLAST search, indicated that the consensus sequence of AT5A demonstrated a 99.79% similarity to Proteus mirabilis with a 100% query coverage in the NCBI nr database and AT5B demonstrated a 99.29% similarity to Lysinibacillus **sp.** with a 100% query cover in the nr database of NCBI. The consensus sequence of AT1 exhibited a 99.71% similarity to Alcaligenes faecalis, also with a 100% query coverage in the NCBI 16S rRNA database. Likewise, the consensus sequence from the MDR strain sourced from the poultry fecal matter of Vizhinjam farm revealed a 99.17% similarity to Alcaligenes faecalis with a 96% query coverage in the NCBI 1

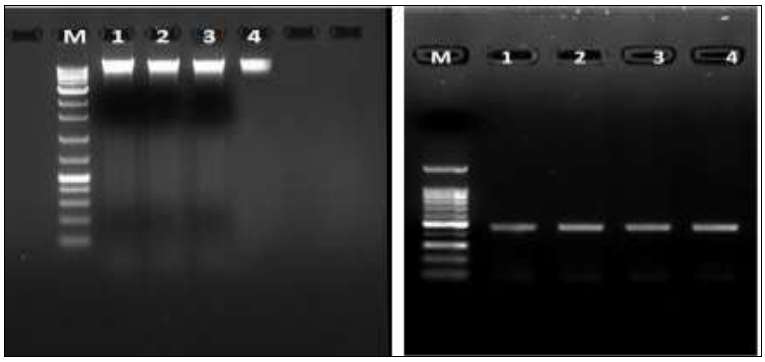


Figure 4. DNA images (a) lane M, DNA ladder 1Kb (Takara), lane 1, AT5A & lane 4, AT1; 16SrRNA gene images (B) lane M, DNA ladder 100-5000bp (Takara), lane 1, AT5A & lane 4, AT1

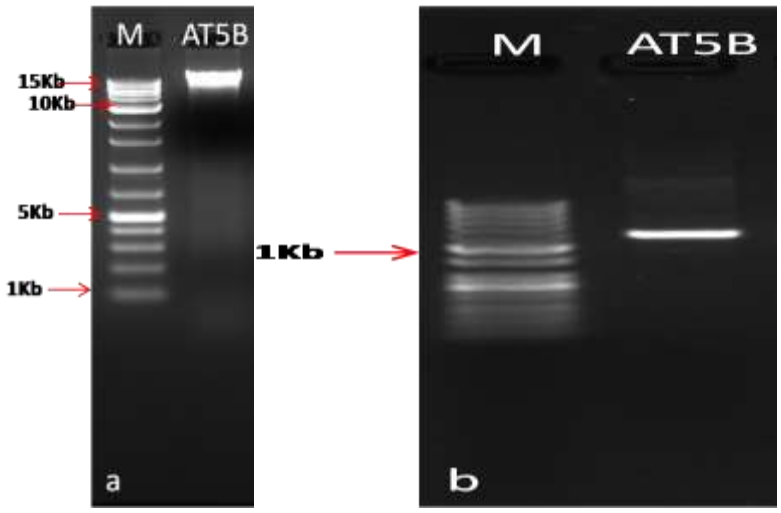


Figure 6. DNA images (a) lane M, DNA ladder 1Kb (Abclonal), 16S rRNA (b) lane M, DNA ladder 100-5000bp (Takara),

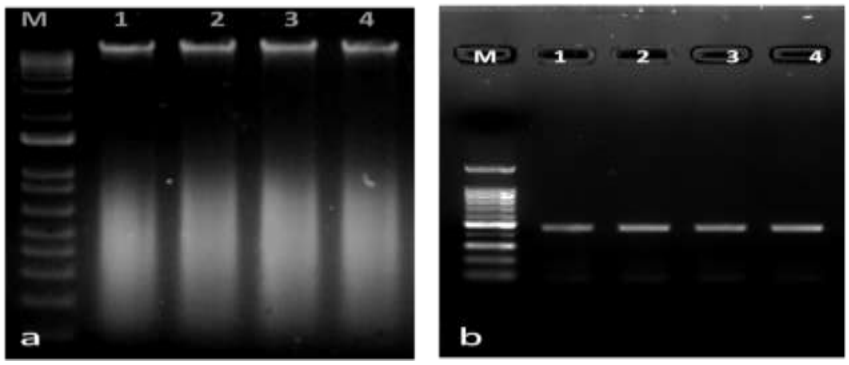


Figure 5. DNA images (a) lane M, DNA ladder 1Kb (Takara), lane 2, V1W2, 16SrRNA gene images (B) lane M, DNA ladder 100-5000bp (Takara), lane 2, V1W2

3.5. Genomic sequence of the identified MDR strains

>Consensus AT5A

TCGAGCGGTAACAGGAGAAAGCTTGCTTTCTTGCTGACGAGCGGCGGACGGGTAGTAAT
GTATGGGGATCTGCCCGATAGAGGGGGATAACTACTGGAAACGGTGGCAATACCGCATA
ATGTCTACGGACCAAAGCAGGGGCTCTTCGGACCTTGCACTATCGGATGAACCCATATGG
GATTAGCTAGTAGGTGGGGTAAAGGCTCACCTAGGCGACGATCTCTAGCTGGTCTGAGA
GGATGATCAGCCACACTGGGACTGAGACACGGCAGACTCCTACGGGAGGCAGCAGTGGG
GAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCT
TAGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGATAAGGTTAATACCCTTATCAATT
GACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGA
GGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCAATTAAGT
CAGATGTGAAAGCCCCGAGCTTAACTTGGGAATTGCATCTGAAACTGGTTGGCTAGAGTC
TTGTAGAGGGGGGTAGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAAT
ACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGG
GAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTAGAGGT
TGTGTCT

TGAACCCGTGACTTCTGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCA
AGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAA
TTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGCGAATCCTTTAGAGATAG
AGGAGTGCCTTCGGGAACGCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTG

AAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCACGTAAT
GGTGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCA
AGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCAGATACAAAGAG
AAGCGACCTCGCGAGAGCAAGCGGAACTCATAAAGTCTGTCGTAGTCCGGATTGGAGTC
TGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTG
AATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAG
AAGTAGGTAGCTTAACCTTCGGGAGGGCGC

>Consensus AT5B

ATGCAGTCGAGCGAACAGAAAAGGAGCTTGCTCCTTTGACGTTAGCGGCGGACGGGTGA
GTAACACGTGGGCAACCTACCCTATAGTTTGGGATAACTCCGGGAAACCGGGGCTAATA
CCGAATAATCTCTTTTGCTTCATGGCAAAAGACTGAAAGACGGTTTCGGCTGTCGCTATA
GGATGGGCCCGCGGCGCATTAACTAGTTGGGGAGGTAACGGCTCACCAAGGCGACGATG
CGTAACCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAAACACGGCCCAAACTCCT
ACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAACAACGCC
GCGTGAGTGAAGAAGGTTTTCCGATCGTAAAACTCTGTTGTAAGGGAAGAACAAGTACA
GTAGTAACTGGCTGTACCTTGACGGTACCTTATTAAAAAGCCACGGCTAACTACGTGCCA
GCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCG
CGCAGGCGGTCCTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTG
GAAACTGGGGGACTTGAGTGCAGAAGAAGAAAGTGGAATTCCAAGTGTAGCGGTGAAA
TGCGTAGAGATTTGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACG
CTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA
AACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAA
GCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCC
GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCT
TGACATCCCGTTGACCACTGTAGAGATATAGTTTCCCCTTCGGGGGCAACGGTGACAGGT
GGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGC
AACCCTTGATCTTAGTTGCCATCATTTAGTTGGGCACTCTAAGGTGACTGCCGGTGACAA
ACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACA
CGTGCTACAATGGACGATACAAACGGTTGCCAACTCGCGAGAGGGAGCTAATCCGATAA
AGTCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGT
AATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCA
CACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTGGAGCC

>Consensus AT1

CGGACGGGTGACTCCAATATCGGAACGTGCCCAGTAGCGGGGGATAACTACTCGAAAGA
GTGGCTAATACCGCATACGCCCTACGGGGGAAAGGGGGGGATCGCAAGACCTCTCACTA
TTGGAGCGGCCGATATCGGATTAGCTAGTTGGTGGGGTAAAGGCTCACCAAGGCAACGA
TCCGTAGCTGGTTTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTC
CTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGGGAAACCCTGATCCAGCCATCC
CGCGTGTATGATGAAGGCCTTCGGGTTGTAAAGTACTTTTGGCAGAGAAGAAAAGGTAC
CTCCTAATACGAGATACTGCTGACGGTATCTGCAGAATAAGCACCGGCTAACTACGTGCC
AGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGT
GTGTAGGCGGTTCGGAAAGAAAGATGTGAAATCCCAGGGCTCAACCTTGGAACTGCATT
TTTAACTGCCGAGCTAGAGTATGTCAGAGGGGGGGTAGAATTCCACGTGTAGCAGTGAA
ATGCGTAGATATGTGGAGGAATACCGATGGCGAAGGCAGCCCCCTGGGATAATACTGAC
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TACAATGGTCGGGACAGAGGGTCGCCAACCCGCGAGGGGGAGCCAATCTCAGAAACCCG
ATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCG
CGGATCAGAATGTCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCA
TGGGAGTGGGTTTCACCAGAAGTAGGTAGCCTAACCGTAAGGAGGGCGCTACCACGGTG
GATCAGAAGG

>Consensus V1W2

CGGGGGTTGGTAGCAGCAATACAAGTGATGATTCTGACCACGGCATCAAGAAAGCTTGC
TCCCATTGGCGGAGAGTGGCGGATGGGTGAGTAATATATCGGAACGTGCCCAGTAGCGG
GGGATAACTACTCGAAAGAGTGGCTAATACCGCATACGCCCTACGGGGGAAAGGGGGG
GATCGCAAGACCTCTCACTATTGGAGCGGCCGATATCGGATTAGCTAGTTGGTGGGGTAA
AGGCTCACCAAGGCAACGATCCGTAGCTGGTTTGAGAGGACGACCAGCCACACTGGGAC
TGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGGGA
AACCCTGATCCAGCCATCCCGCGTGTATGATGAAGGCCTTCGGGTTGTAAAGTACTTTTG
GCAGAGAAGAAAAGGTATCCCCTAATACGGGATACTGCTGACGGTATCTGCAGAATAAG
CACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGA
ATTACTGGGCGTAAAGCGTGTGTAGGCGGTTCGGAAAGAAAGATGTGAAATCCCGGGGC
TCACCCTTGGAACTGCATTTTTAACTGCCGAGCTAGAGTATGTCAGAGGGGGGTAGAATT
CCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAATACCGATGGCGAAGGCAGCCC
CCTGGGATAATACTGACGCTCAGACACGAAAGCGTGGGGAGCAAACAGGATTAGATACC
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GCAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAG
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GAACCGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA
GTCCCGCAACGAGCGCAACCCTTGTCATTAGTTGCTACGCAAGAGCACTCTAATGAGACT
GCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGGGT
AGGGCTTCACACGTCATACAATGGTCGGGACAGAGGGTCGCCAACCCGCGAGGGGGAGC
CAATCTCAGAAACCCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCG
GAATCGCTAGTAATCGCGGATCAGAATGTCGCGGTGAATACGTTCCCGGGTCTTGTACAC
ACCGC

Table 11. NCBI submission details of potent isolated bacterial isolates

Sl No	Culture code	Accession No
1	SUB15443715 AT5A	PV888700
2	SUB15443715 AT1	PV888703
3	SUB15563566 AT5B	PX219476
4	SUB15450022 V1W2	PV915542

CONCLUSION

This research highlights that poultry farms at Thiruvananthapuram, Kerala serve as significant reservoirs for antimicrobial resistant bacteria, exhibiting consistent resistance to β-lactam antibiotics such as penicillin and ampicillin. The identification of specific variations in resistance patterns emphasizes how farm practices, antibiotic use, and environmental factors contribute to the emergence and spread of resistant strains. By confirming the persistence of AMR in poultry litter and providing genomic data for potent isolates, this study offers a valuable resource for future surveillance and comparative investigations. Future research should focus on long-term monitoring of resistance trends, exploration of alternative non-antibiotic growth promoters, and evaluation of intervention strategies to minimize AMR dissemination. Integrating molecular epidemiology with farm-level

policy reforms will be crucial to mitigate the escalating public health burden of antimicrobial resistance.

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