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The Molecular Characterization of Antibiotic Resistant Genes Carrying Bacterial Species in Food Samples

Kahkasha Parveen[1], Deepak Bharati[2], Samrah Rehan[3], Rohit Kumar Vishwakarma[1] and Manisha Shukla[1]*

[1]Department of Biotechnology, Pandit S.N. Shukla University, Shahdol, Madhya Pradesh, India
[2]Centre for Molecular Biology Research (CMBR) Biotech. Pvt Ltd. Bhopal, Madhya Pradesh, India
[3]Department of Biochemistry, Ganesh Shankar Vidyarthi Memorial Medical College, Kanpur, Uttar Pradesh, India

**Corresponding author*

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A B S T R A C T

The findings from this investigation provide concrete evidence of the presence of the tetA resistance gene in certain chicken samples sourced from Bhopal District in Madhya Pradesh. This highlights a potential concern regarding the consumption of such contaminated poultry, as the resistance gene could render traditional tetA-targeting antibiotics ineffective in treating associated bacterial infections. These results underscore the need for vigilant monitoring and regulation of antibiotic use in poultry farming to mitigate the spread of antibiotic resistance.

Introduction

The increasing prevalence of antibiotic-resistant genes in bacterial species discovered in food samples endangers both public health and food safety. These resistance genes, which are frequently transferred through plasmids and other mobile genetic components, aid in the spread of antibiotic resistance among bacterial populations.

Studies have increasingly focused on the molecular characterization of these resistance genes in order to better understand the mechanisms driving their dissemination and discover therapeutic therapies. Such research is necessary for identifying measures to reduce the transmission of resistant bacteria via the food supply chain (Martinez, 2009; Van Hoek *et al.*, 2011). Antibiotic resistance poses a continuous threat to global health, necessitating urgent initiatives to counter this complex issue. The patterns of multidrug resistance in both Gram-positive and Gram-negative bacteria have resulted in infections that are increasingly difficult, if not impossible, to treat with conventional antimicrobials.

This situation is exacerbated by the lack of early identification of pathogenic bacteria and their antimicrobial susceptibility in many medical facilities (Marianne Frieri *et al.*, 2017). Recent studies have detected antibiotics and resistant bacteria in various environmental sources, including water and soil. Antibiotics have historically been critical in treating

infections in humans and animals, contributing significantly to improved healthcare and increased life expectancy over the past century (Duarte *et al.,* 2022). In particular, infections caused by bacteria such as *E. coli* and multidrug-resistant ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species*) pose substantial challenges due to their resistance to antibiotics (Duarte *et al.,* 2022).

Mechanisms of antimicrobial resistance are typically categorized into four types: (1) restricted drug absorption, (2) drug target modification, (3) drug inactivation, and (4) active drug efflux. Intrinsic resistance mechanisms include drug target modification, drug inactivation, and drug efflux, while acquired resistance mechanisms feature all four types, including the import of resistance genes from external sources. Structural differences between Gram-negative and

Gram-positive bacteria influence the specific resistance mechanisms they employ. For example, Gram-negative bacteria utilize all four major mechanisms due to their LPS outer membrane, while Gram-positive bacteria generally rely on restricted drug uptake and lack certain drug efflux capabilities (Reygaert, 2018).

Tetracycline resistance, often encoded in plasmids and transposons, highlights how resistance genes can transfer between species (Oppegaard *et al.,* 2001; Koo and Woo, 2011). Mechanisms of tetracycline resistance include efflux pumps, ribosome protection, and enzymatic deactivation, typically acquired through the acquisition of tet genes. Efflux pump-related genes such as tetA, tetB, tetC, tetD, and tetG are commonly found in Gram-negative bacteria (Skockova *et al.,* 2012; Schwaiger *et al.,* 2010). The presence and patterns of tetracycline resistance can serve as indicators of broader antibiotic resistance issues. Given the importance of poultry as a source of protein worldwide, the presence of antibiotic-resistant bacteria in chicken poses a significant public health hazard. Molecular characterization of these resistance genes provides critical insights into the mechanisms through which resistance is disseminated within bacterial populations and across the food chain. By employing advanced molecular techniques such as polymerase chain reaction (PCR), whole-genome sequencing, and metagenomic analysis, researchers can identify and catalog the specific genes responsible for resistance to various antibiotics.

This study aims to investigate the prevalence and genetic basis of antibiotic resistance in bacterial species isolated from chicken samples. In particular, it will focus on identifying key resistance genes and understanding their distribution and potential for horizontal transfer among different bacterial strains. The results will provide valuable data for developing targeted strategies to mitigate the spread of antibiotic resistance from poultry to humans. Ultimately, this research seeks to enhance food safety protocols and inform policy decisions regarding antibiotic use in agriculture, contributing to global efforts to combat antibiotic resistance.

Materials and Methods

Sample Collection

Chicken samples from different local vendors from various sites of Bhopal area were collected in an autoclaved collection tubes. The samples were then stored at 4^0C until further use to screen bacteria in NAM.

Enumeration of bacteria for screening in NAM media

Protocol for enumeration of bacteria

A total of 9 test tubes were autoclaved at 121^{o}C for 15 min. 10 ml distilled in first tube was added and 9ml in each tube except the first one.1 gm chicken sample was weighed and added in first test tube. It was mixed well. 1 ml of the mixed solution was transferred into 2nd tube containing 9 ml autoclaved distilled water and mix well. This would have the dilution factor of 10^{-1}. The process was continued till 9th tube by adding 1 ml mixture from the previous tube.100 µl from the 10^{-8} tube was plated in the NAM. The same process was performed with all the chicken samples. The NAM plates were incubated in incubator at 37^{o}C overnight for the desired bacterial growth, and PDA plates at 25^{o}C for 2-3 days for fungal growth. Total viable count (TVCs) was counted and reported. The isolated colonies were stored in pure cultures for further use.

Antibiotic susceptibility testing to isolate antibiotic resistant bacteria

The Antibiotic Susceptibility Testing is an *in vitro* test that uses the diffusion technique on agar media to determine how sensitive a bacteria is to one or more

antibiotics. NAM (Nutrient Agar Media) was prepared and poured in petri plates. The plates were kept for solidification. The bacterial colonies were selected from the pure culture and spread in the whole plate uniformly. One strain of bacteria was spread on one plate uniformly.

Different antibiotic discs were kept in each plate carefully at the appropriate distance from each other for the antibiotic susceptibility assay. Antibiotics used were Ciprofloxacin, Gentamicin, Ofloxacin, and Ampicillin. The plates were incubated at 37^0C for 24 hours. Zone of inhibition was visualized and measured for the antibiotic which were susceptible and the bacteria which were resistant to the antibiotic were further purified for further testing.

DNA extraction from bacterial cells

10 colonies were randomly selected for further investigation and were grown in LB broth liquid medium to extract DNA. The grown bacterial broth was centrifuged for 20 mins at 5000 RPM to extract bacterial pellet. The supernatant was discarded and 1 ml Solution B, 50 ul 20% SDS and 5 ul Proteinase K was added. The above mixture was mixed well and incubated at 56^oC for 60 mins. The supernatant was transferred in fresh Eppendorf tube and 250 ul of Sodium acetate and 500 ul of PCI mixture was added and the contents were incubated. The mixture was centrifuged at 10,000 rpm for 15 minutes. Three layers were formed. The upper layer which contains DNA was carefully transferred in a fresh tube. 500 ul of chilled IPA was added to precipitate the DNA. The mixture was incubated at -20 C overnight.

The mixture was centrifuged at 10000 RPM for 10 min. The supernatant was discarded and pellet was washed with 500ul 70% ethanol by centrifuging the content at 10000 RPM for 5 mins and then left for air dry. After air dry the DNA pellete was dissolved in 15ul of Nuclease free water. The extracted DNA was the visualized under UV in agarose gel electrophoresis.

qPCR amplification of antibiotic resistant gene present in isolated bacteria using specific primer

The isolated DNA was then converted to cDNA using the standard protocol after which the qPCR (Table 1) for the identification of tet A (164bp) resistant gene using species selected primers (Table 2) was performed as per the standard protocol.

Results and Discussion

Antibiotics play a crucial role in maintaining the health of food-producing animals, mirroring their significance in human healthcare. However, the escalating growth of antibiotic resistance (AR) poses a substantial threat to the efficacy of these indispensable treatments.

While antibiotic-resistant bacteria (ARB) and their corresponding resistance genes (ARG) have existed for millennia, their rapid proliferation is primarily attributed to the widespread production and utilization of antibiotics in both human and veterinary medicine (D'Costa *et al.,* 2011).

The emergence of antimicrobial resistance (AMR) has profound implications for public health, driving up medical costs by extending hospital stays and necessitating more intensive care, particularly as the efficacy of antibiotics wanes against common infections (Aarestrup, 2000).

Studies within the poultry industry have predominantly focused on the prevalence of tetracycline resistance, given the widespread use of tetracycline antibiotics in commercial chicken production worldwide (Chopra and Roberts, 2001; Koo and Woo, 2011; Skockova *et al.,* 2012; Schwaiger *et al.,* 2010). Tetracycline resistance is commonly triggered by the acquisition of tetracycline resistance (tet) genes, often harbored within transposons or mobile plasmids. To date, researchers have identified 11 mosaic tet genes and a minimum of 59 other tet genes, underscoring the genetic diversity contributing to tetracycline resistance (Miranda *et al.,* 2009; Shahbazi *et al.,* 2018).

The tet genes govern three primary resistance mechanisms: active efflux pumps that expel the drug from the cell, protection of ribosome binding sites to reduce drug binding, and enzymatic inactivation of the antibiotic's active component. In clinical settings, the former two mechanisms have gained prominence (Adesiyun *et al.,* 2007). Tetracycline stands as the predominant antibiotic for managing various poultry ailments due to its favorable risk profile, oral administration convenience, and cost-effectiveness (Chopra and Roberts, 2001; Miranda *et al.,* 2009). Its extensive usage renders poultry more susceptible to developing tetracycline resistance compared to other antibiotics.

Table.1 qPCR was performed using primers

S. No.	Reagents	Volume
1	RT PCR Master mix (2x)	5 µl
2	Primer forward	1 µl
3	Primer reverse	1 µl
4	cDNA	2 µl
5	Nuclease free distilled water	1 µl
	Total Volume	**10 µl**

Table.2 PCR was performed using primers

Primer	Sequence (5' to 3')
tet A Fw	GCGCGATCTGGTTCACTCG
tet A Rv	AGTCGACAGAAGCGCCGGC

Table.3 The table shows the presence of antibiotic resistant gene tetA in bacterial samples.

S. No.	tetA mutant gene
1	**-**
2	**-**
3	**+**
4	**-**
5	**+**
6	**-**
7	**-**
8	**-**
9	**-**
10	**-**

Figure.1 Antibiotic resistant bacteria showing resistance for tetracycline resistance

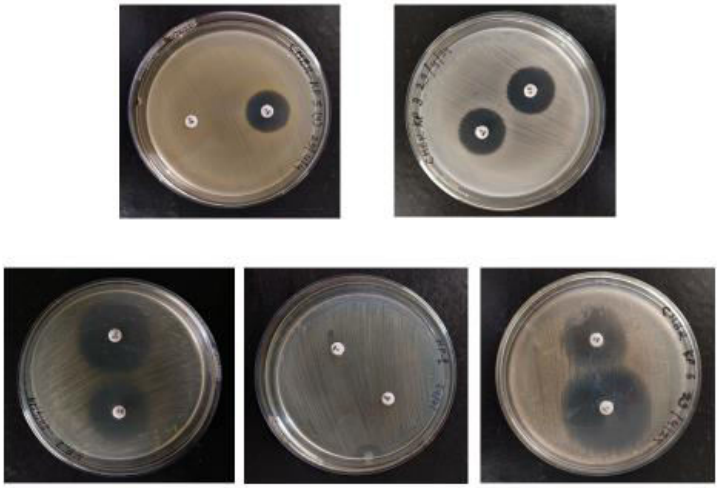


Figure.2 Visualization of extracted DNA under UV illumination in a Gel Doc

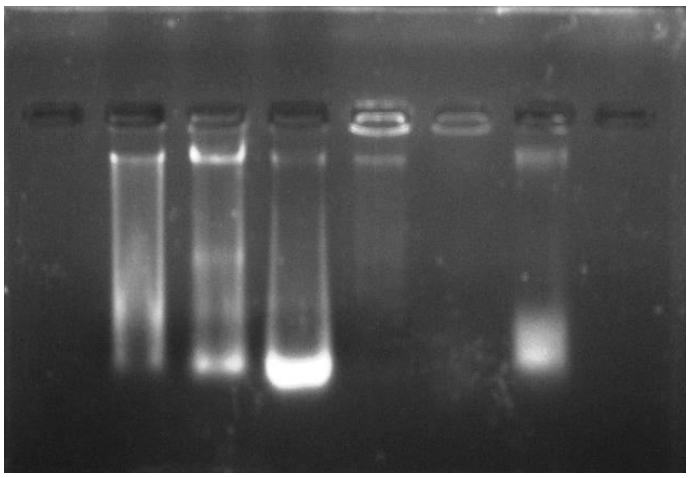
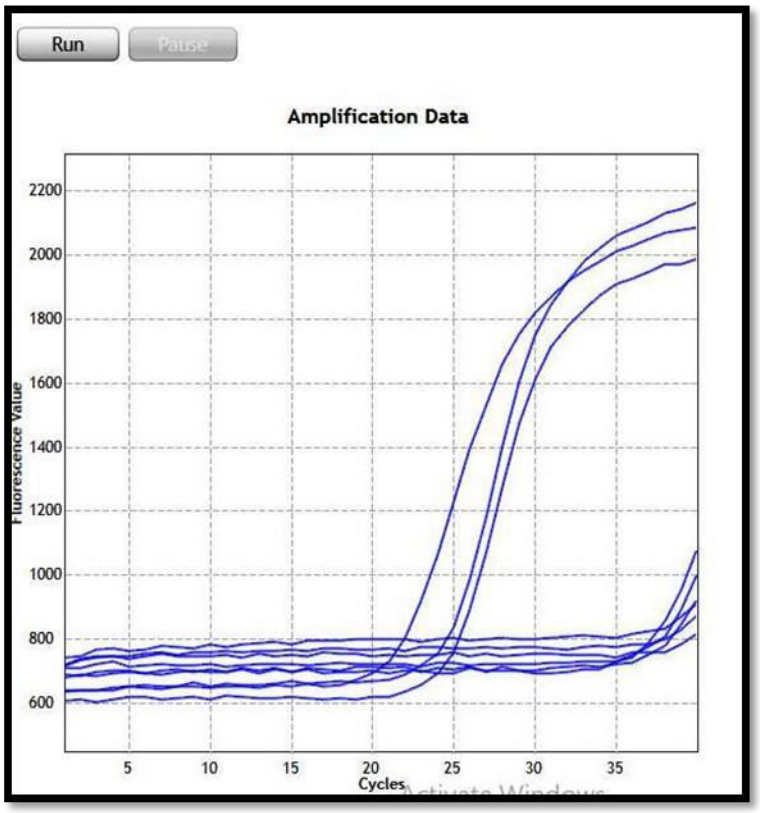


Figure.3 The figure shows the qPCR amplification of tet A gene.



Subsequently, we have isolated and purified the bacterial colonies obtained from the chicken samples, followed by conducting antibiotic susceptibility testing using a panel of different antibiotics. The antibiotics evaluated in the testing included Tetracycline, Ampicillin, Gentamicin, Ciprofloxacin, Ofloxacin, Streptomycin, and Norfloxacin (Figure 1).

Upon completion of the antibiotic susceptibility testing, specific bacteria from the chicken samples exhibited resistance to tetracycline. These tetracycline-resistant bacterial colonies were then subjected to DNA extraction and visualized on agarose gel (Figure 2), followed by quantitative polymerase chain reaction (qPCR) analysis (Figure 3) to identify and characterize the antibiotic-

resistant genes present within these bacteria. This detailed genetic analysis aimed to pinpoint the specific mechanisms conferring resistance and shed light on the genetic determinants driving antibiotic resistance in the bacterial population.

Table 3 showed the presence of the tetA resistant gene in chicken samples. In this study, it was observed that the chicken samples obtained from a local vendor in Bhopal contained the tetA resistant gene, specifically a 164 bp variant. These bacteria, if ingested in large quantities, have the potential to induce severe health issues that may not respond to antibiotics targeting the tetA gene.

The findings from this investigation provide concrete evidence of the presence of the tetA resistant gene in certain chicken samples sourced from Bhopal Districts in Madhya Pradesh. This highlights a potential concern regarding the consumption of such contaminated poultry, as the resistant gene could render traditional tetA-targeting antibiotics ineffective in treating associated bacterial infections. Additional references may provide further context and support for these conclusions.

Author Contributions

Kahkasha Parveen: Investigation, formal analysis, writing—original draft. Deepak Bharati: Validation, methodology, writing—reviewing. Samrah Rehan:—Formal analysis, writing—review and editing. Rohit Kumar Vishwakarma: Investigation, writing—reviewing. Manisha Shukla: Resources, investigation writing—reviewing.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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