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Research Article

Molecular Surveillance of *blaTEM* beta-Lactamase Gene in poultry droppings: A critical risk for antimicrobial resistance in Farm setting

Kakoli Akter[1,2], Marnusa Binte Habib[3] , Sarower Hossen Shuvo[1], Shomaia Yasmin Mitu[1] ,Shamsun Nahar[1] , and Salequl Islam 1,*

[1]Department of Microbiology, Jahangirnagar University, Savar, Dhaka 1342, Bangladesh

[2]Department of Microbiology, Khawaja Yunus Ali University, Sirajganj, Bangladesh.

[3]Department of Microbiology, Gono Bishwabidyalay, Savar, Dhaka 1342, Bangladesh.

* Corresponding author: salequl@juniv.edu

ABSTRACT

Antimicrobial resistance (AMR) is a growing global health concern, exacerbated by the overuse of antibiotics in animal husbandry, particularly in the poultry industry. A cross-sectional study was conducted between July 2021 and July 2022, collecting 80 samples from poultry farms in Savar, Hemayetpur, Manikganj, Gazipur, and Tangail. A total of 106 bacterial isolates were identified, where Escherichia coli became the most prevalent (47.2%), followed by Enterobacter cloacae (12.3%) and Citrobacter freundii (5.7%). Antimicrobial susceptibility testing revealed high resistance to beta-lactam antibiotics, including carbapenems, with 73.6% of isolates resistant to imipenem and 61.3% resistant to amoxicillin-clavulanic acid. The blaTEM gene was detected in 33% of the isolates. A weak association between phenotypic resistance and the presence of blaTEM was observed. The high prevalence of multidrug-resistant bacteria and ESBL genes in poultry feces indicates a significant risk of transmission to humans, posing a threat to public health. This study aimed to investigate the prevalence of one prominent Extended Spectrum Beta-Lactamase (ESBL) gene, blaTEM in bacterial isolates from poultry feces in Bangladesh.

Keywords: Antimicrobial Resistance, Husbandry, Cross-sectional, Phenotypic, Susceptibility, Prevalence

Introduction

Antimicrobial resistance (AMR) has become a rapidly growing public health concern worldwide (Al Asad, Shanta *et al*. 2024). It is believed that the nonjudgmental use of antibiotics is mostly to blame for the emergence, selection for, and spread of antibiotic-resistant microorganisms in both humans and animals (Saha and Sarkar 2021). In the poultry business, antibiotics are widely utilized for treatment, prophylaxis,

and growth booster supplements to poultry feed. (Upadhayay and Vishwa 2014). The growth of antibiotic resistance, mostly in gram-negative bacteria globally, is largely caused by the aggressive use of antibiotics, which also significantly reduces the available treatment alternatives (Upadhayay and Vishwa 2014). According to recent research, improper antibiotic usage in animal husbandry contributes to the emergence and spread of multidrug-resistant bacteria and is thought to be an important factor in resistant bacteria (Economou and Gousia 2015). Different resistant bacteria may arise and spread as a result of antibiotic use and abuses, and humans may contract these bacteria through the food cycle or close contact with diseased animals (Teuber 1999).

The most often used antibiotics are beta-lactams, which include both synthetic and natural penicillins as well as their byproducts, such as carbapenems, monobactams, cephalosporins, and cephamycins. (Vilvanathan 2021). Since the vital role that beta-lactam antibiotics play in both human and veterinary medicine, resistance to these drugs is particularly concerning (Nóbrega and Brocchi 2014). Extended-spectrum beta-lactamase (ESBL)-producing bacteria have been linked to food-producing animals, particularly poultry. Humans may contract the bacteria from these animals through ingestion of contaminated meat products or direct contact, which can colonize the intestinal tract and ultimately cause serious infections (Ribeiro, Nespolo *et al.* 2024). Human infections caused by bacteria that produce ESBLs are linked to higher rates of death, morbidity, and expensive hospital stays, as well as a delay in receiving the necessary treatment. ESBLs are most typically detected in Enterobacteriaceae (Pitout 2010). *Salmonella* species and *Escherichia coli are* frequent ESBL producers found in poultry and their surroundings. (Blaak, van Hoek *et al*. 2015). These bacteria produce CTX-M-, TEM-, and SHV-beta-lactamases, which are encoded by the *blaCTX-M, blaSHV*, and *blaTEM* genes, respectively, and are the primary cause of their resistance to penicillins, cephalosporins, and aztreonam (Shahid, Singh *et al*. 2011). The ESBL gene transport in Bangladeshi animal husbandry, namely in poultry-gut bacteria, is not commonly known (Islam, Urmi *et al.* 2020). One of the most popular sources for examining ESBL genes and antibiotic-resistant bacteria is poultry excrement (Gazal, Medeiros *et al.* 2021). Finding the type of bacteria in poultry feces, their level of antibiotic resistance, and the presence of the ESBL gene, specifically *blaTEM*, was the primary goal of the current investigation (Yiğin 2021). Commensal bacteria serve as a reservoir for these genes that confer resistance to antibiotics, and they can gradually spread to other recipient bacteria (Marshall, Ochieng *et al.* 2009). This study unveils the local distribution of the one particular ESBL gene, *blaTEM,* among isolates from poultry feces in Bangladesh. As a result, the study helps to provide some evidence-based knowledge regarding the antimicrobial resistance reservoir in the food animal business.

Materials and methods

Study Design and Specimen Collection:

Between July 2021 and July 2022, a cross-sectional study was carried out to investigate the prevalence of the ESBL gene in bacterial isolates from chicken droppings. The qualified team of microbiologists, veterinarians, medics, public health experts, statisticians, and postgraduate students conveniently chose poultry farms (PFs) for the collection of chicken excrement and subsequent microbiology analyses. We gathered 80 samples in all from Bangladesh's main poultry-producing regions of Savar, Hemayetpur, Manikganj, Gazipur, and Tangail.

Study approval:

Farm owners were asked to complete a structured questionnaire on the kinds of chickens they raised, any recent illnesses they had, and any antibiotics they had used for prophylaxis or treatment. Additionally, the inquiry asked the farm owners about their educational backgrounds and animal husbandry training. This study project received ethical approval from Jahangirnagar University's Biosafety, Biosecurity, and Ethical Review Committee. Homeowners and owners of poultry farms gave their verbal approval for the collection of their particular chicken droppings.

Bacterial isolation and identification*:*

Using aseptic techniques and other necessary safety precautions, samples of chicken excrement were promptly taken and placed in a special specimen collecting tube. Samples were immediately transferred to the Department of Microbiology at Jahangirnagar University in Bangladesh, where they were stored in insulated ice boxes at the One Health Laboratory. The associated microbiological and molecular biology analyses were finished there. Long-distance excrement samples were soaked in Cary Blair transit medium (Oxoid, UK) prior to being transported into the laboratory.

A loopful of the diluted sample was streaked on a differential culture medium, cysteine-, lactose-, and electrolyte-deficient (CLED) agar (Liophilchem, Italy), for the growth of Gram-negative enteric Bacilli, after about one gram of chicken feces was combined with four milliliters of phosphate-buffered saline (PBS) for bacterial isolation. The diluted chick droppings were streaked individually on Salmonella-Shigella (SS) agar (Oxoid, UK) media after being enhanced with Rappaport Vassiliadis Soya Broth (RVS Broth, Oxoid, UK) for the purpose of detecting Shigella and Salmonella. Each variety of bacterium was first distinguished based on its colony features during an overnight incubation period at 37°C. To create a pure culture repository and conduct additional tests, a unique single colony was removed and cultivated once more on tryptone soya agar (Liophilchem, Italy). Following the identification of the isolated bacterial colonies using standard biochemical techniques, a fast biochemical test kit (API 20E, BioMérieux, Durham, NC) comprising an enzymatic carbohydrate battery and chromogenic panel was used. 16s rDNA sequencing was done for further identification. Bacterial cells were boiled in order to extract their DNA. The 16S rRNA gene of the isolates was amplified using the 16SUni primer. When exposed to UV light, the amplified products in 1% agarose gel electrophoresis showed an amplification of approximately 1466 bp.

Antimicrobial susceptibility testing*:*

Antimicrobial susceptibility testing was performed by the disc diffusion method (Kirby-Bauer disc diffusion method) on Mueller-Hinton agar (MHA) plates, and the zone diameter for individual antimicrobial agents was interpreted according to Clinical Laboratory Standards Institute recommendations (CLSI 2018) and then translated into sensitive, moderate, or resistant categories (Vu, Choisy *et al*. 2021). The strain of Bacillus cereus ATCC 14579 was employed as the quality control. For the test, seven distinct commercially available antibiotic discs from the beta-lactam group (Oxoid, Basingstoke, UK) were employed. Amoxicillin + Clavulanic acid (20 + 10 ug), Aztreonam (30 ug), Cefuroxime (30 ug), Cefixime (5 ug), Cefepime (30 ug), Imipenem (10 ug), and Meropenem (10 ug) were among the antimicrobials used.

Detection of ESBL-specific genes*:*

The conventional polymerase chain reaction (PCR) method was applied for screening of all isolates for the presence of *blaTEM*. The sequences of primers used in this study and specific for *blaTEM are* mentioned in Table 1. For PCR, freshly cultured isolates of bacteria were used to prepare template deoxyribonucleic

acid (DNA) by the boiling method. A final volume of 24 µL was created by adding 2.0 µL of prepared bacterial DNA to a 12 µL 2X PCR pre-mixture (GeneON, Germany) and five pmol of each primer (1 µL). The reactions were then subjected to 32 cycles of amplification (Applied Biosystems 2720 Thermal Cycler, Singapore), which included denaturation (30s at 94°C), annealing (30s at 52°C), extension (1 min at 72°C), and a final 7 min extension at 72°C. Amplicons (857bp for *blaTEM*) were visualized under UV light following electrophoresis through 1.2% agarose gel at 100 volts for 30 minutes, followed by staining with ethidium bromide, the standard molecular weight marker (Gene Ruler, ThermoFisher Scientific, MA). To measure particular amplicon sizes, the standard molecular weight marker (Gene Ruler, ThermoFisher Scientific, MA) was run in parallel.

Table 1. Primer used for PCR amplifications.

Target Gene	Primer	Sequences (5'-3')	Amplicon size	Reference
blaTEM	Forward	GAGTATTCAACATTTTCGT	857bp	(Nahar, Urmi *et al*. 2021)
	Reverse	ACCAATGCTTAATCAGTGA		

Statistical Analysis: Data were verified, entered, and subsequently analyzed using IBM SPSS statistics data editor. Missing data were omitted from the bivariate analysis.

Results

In Bangladesh, small and medium-sized poultry farms (PFs) have grown significantly on both a traditional and commercial scale. Eighty samples in all were gathered from various locations throughout four districts of Bangladesh and examined for several enteric bacterial species.

Isolation and Identification of Chicken Feces Bacteria:

Out of the 80 poultry feces that were analyzed, 106 isolates were found. At least one or more species of bacteria were found in all of the poultry excrement. In order to confirm earlier findings, we repeated the process the following day using preserved materials because very few culture plates showed no growth. The analysis did not include any results that were in conflict with the two culture results. Among the 106 isolates from Chicken samples, 50 *Escherichia coli* (47.2%), 13 *Enterobacter cloacae* (12.3%), 6 *Citrobacter freundi* (5.7%), 3 *Escherichia albertii* (2.8%), 5 *Escherichia fergusonii* (4.7%), 6 *Serratia spp* (5.7), 6 *Proteus mirabilus* (5.7), 2 *Shigella dysentery* (1.9%), 2 *Solibacillus spp* (1.9%) and 11 others bacteria (10.3%) shown in **Figure 1**

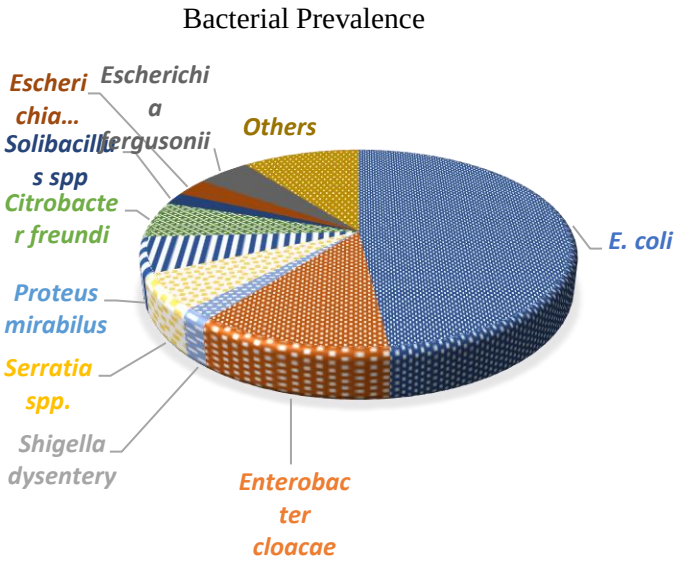

Figure 1: Percentage of different bacteria from poultry samples

Antibiotic Susceptibility Profiles of Isolates*:*

Among 106 isolates obtained from poultry samples, resistance against beta-lactam group antibiotics was observed. Among the isolates 61.3% showed resistance to amoxicillin + clavulanic acid, 31.1% were resistant against aztreonam, 51.9% against cefuroxime, 40.6% against cefixime, 25.5% against cefepime, 73.6% against imipenem, and 35.8% against meropenem shown in Figure 2.

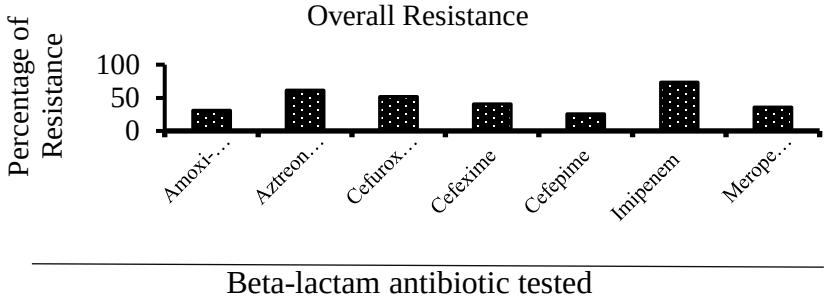

Figure 2: Phenotypic antibiotic resistance among isolates from poultry samples

***Molecular Detection of* blaTEM** *Gene:*

Simplex PCR was used to detect *the blaTEM* gene. Among 106 isolates, the *blaTEM gene* was detected positive in 35 (33%) isolates.

Association of Phenotypic and Genotypic Resistance:

Of the 106 isolates of chicken, 35 were *blaTEM* positive and 71 were *blaTEM* negative. 33.8%, 15.2%, and 36.4% of the 35 *blaTEM*-positive isolates were resistant to amoxicillin-clavulanic acid, cefuroxime, cefixime, 18.5%, imipenem, and 47.4%, respectively. Nonetheless, 66.2% of the 71 isolates that tested

negative for *blaTEM* were resistant to amoxicillin-clavulanic acid, 84.8% to aztreonam, 65.1% to cefexime, 63.6% to cefuroxime, 81.5% to cefepime, 61.5% to imipenem, and 52.6% to meropenem.

We detected a very weak association between the phenotypic resistance of some beta-lactam antibiotics and the presence of *blaTEM*. All negative isolates showed a higher percentage of resistance than positive isolates shown in **Figure 3.** The associations of *bla TEM* to the beta-lactam group antibiotics appeared marginally non-significant to highly significant with two-tailed Chi-square p = 0.004 for amoxicillin-clavulanate, p = 0.005 for aztreonam, p = 0.030 for cefuroxime, p = 0.090 for cefexime, p = 0.017 for cefepime, p = 0.052 for imipenem, and p = 0.015 for meropenem.

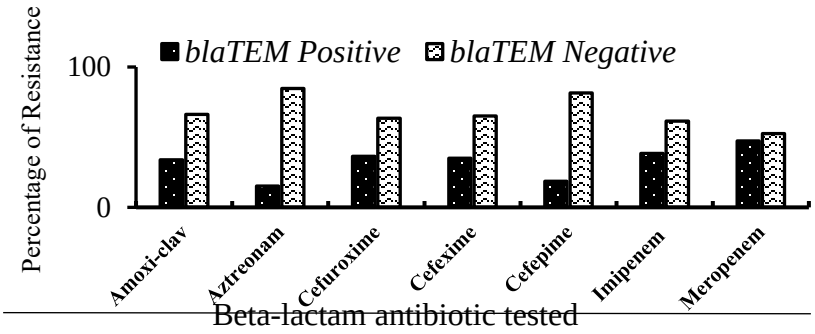

Figure 3: Comparative phenotypic resistance of beta-lactam antibiotics among *blaTEM* positive and *blaTEM* negative chicken pathogens

Discussion

Poultry dung is the source of a diverse range of microorganisms (Męcik, Buta-Hubeny *et al.* 2023). Numerous harmful bacteria have also been found in poultry excrement in addition to gram-positive bacteria (Nandi, Maurer *et al*. 2004). In the poultry excrement analyzed for this investigation, we found the highest prevalence of *E. coli*. The increased *E. coli* abundance in chicken gut material was consistent with a related earlier study (Van den Bogaard, London *et al.* 2001). Unlikely, some other studies had identified Proteus spp. Enterobacteria cloacae (Van den Bogaard, London *et al.* 2001, Jho, Park *et al*. 2011) as the most frequent isolates from poultry droppings; we identified these bacteria as the next most abundance isolates after *E. coli*. This investigation also found endemic poultry bacteria, *Shigella dysentery*, *Citrobacter freundi*, and *Serratia spp*., which may contaminate fresh produce or the environment. For the first time, this study has identified some bacteria in Bangladeshi fowl stomach material. Among these rare bacteria are *Bacillus koreensis*, *Priestia flexa*, and *Priestia aryabhattai*. Antibiotic resistance and the growth of multidrug-resistant ESBL producers have become worldwide problems. The indiscriminate use of antibiotics in livestock husbandry is predicted to accelerate antimicrobial resistance (AMR) among commensal microbes and pathogens. The antimicrobial susceptibility patterns of all the isolates in the current study show that they were all resistant to the majority of the antibiotics that were used in the investigation. These days, carbapenem antibiotics like meropenem and imipenem are commonly used as last resort medications. It is really concerning to us that our study revealed the highest percentage of imipenem resistance, approximately 73.6%. At the same time, another study revealed that the largest percentages of bacteria will likewise be resistant to imipenem (Tama, Ngwai *et al.* 2021).

The third-generation cephalosporin antibiotic cefuroxime primarily fights many bacteria, but regrettably, we discovered a sizable proportion of phenotypic resistance to it that is consistent with another study

(Bhushan, Khurana *et al*. 2017). On the contrary, several antibiotics of the cephalosporin group show the highest resistance, rather than cefuroxime found in a particular study (Sharma, Galav *et al.* 2017, Nahar, Urmi *et al.* 2021). Antimicrobial-resistant bacteria are becoming more prevalent worldwide, which has an impact on ecosystems that support both humans and animals. According to the study, there were bacteria in poultry feces that produced a lot of ESBL genes. 35 (33%) of the poultry isolates have *blaTEM,* which is largely consistent with previous investigations (Gundran, Cardenio *et al*. 2019). But another study found that other ESBLs are in significant numbers and are positive (Gundran, Cardenio *et al.* 2019); collectively, the ESBL genes revealed higher than previously published studies from the poultry sector. This study's phenotypic and genotypic associations with *blaTEM* were rather moderate, however a different published study found a high correlation between *blaTEM* and phenotypic resistances (Shahid, Singh *et al.* 2011). Some notable causes that lead to the use of irrational antibiotics in chicken flocks include the availability of antibiotics over-the-counter, lax or nonexistent regulations regarding their use in agriculture and animal husbandry, and a shortage of educated human resources. The phenotypic and genotypic AMR may eventually be acquired and disseminated in humans, poultry, and the environment as a result of antibiotic overuse. To find out more about these increasing inherited hazards, more comprehensive research is necessary. Other ESBL genes or variables not examined in this study may account for the discrepancy in the genotype-phenotype correlation. Thus, over 200 different types of ESBL genes give resistance to beta-lactam antibiotics. Variations of ESBL genes from the blaTEM lineage may have contributed to the different phenotypic resistance phenomena. According to earlier studies, different geographic areas could have different distributions of ESBL genotypes. Therefore, studies and observations covering a sizable portion of Bangladesh could validate our current findings about the ESBL gene and associated AMR symptoms.

Conclusion

In Bangladesh's chicken industry, ESBL gene-producing bacteria are becoming more widely known. Humans may be more susceptible to the spread of multidrug-resistant bacteria found in poultry habitats, especially if they work near poultry farms and the excretory products they produce. Bangladeshi poultry excrement has a high level of antibiotic resistance, which suggests a larger concentration of ESBL genes that may have negative consequences for the health of people, animals, and the environment.

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