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Molecular detection of blaTEM-encoding genes in multidrug-resistant *Escherichia coli* from cloacal swabs of ducks in Indonesia farms

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ABSTRACT

Background: Globally, there is an increasing frequency of community-acquired illnesses caused by extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli*. The presence of ESBL-producing *E. coli* in livestock is a concern, considering its transmission potential to humans, effects on animal health, risks to food safety, and the widespread spread of antibiotic resistance in both human and animal populations.

Aim: This study investigated the prevalence and characterization of ESBL-producing *E. coli* in cloacal swab samples collected from duck farms in Jombang, Indonesia.

Methods: In total, 125 cloacal swab samples of ducks were collected from farms. Samples were processed and analyzed for *E. coli* isolation using standard microbiology techniques. Isolated *E. coli* strains were further subjected to antimicrobial susceptibility testing and ESBL phenotypic detection using disc diffusion and double-disk synergy test techniques, respectively. Identified multidrug-resistant (MDR) *E. coli* strains were thereafter screened for the detection of *bla*TEM ESBL gene by PCR.

Results: A total of 94 (52.2%) out of the collected 180 swab samples were positive for *E. coli*. Twenty-five (39.1%) out of the recovered *E. coli* isolates were generally noted to exhibit MDR traits. Exactly 24 (96%) out of the 25 MDR *E. coli* strains that were selected for molecular studies harbored the *bla*TEM gene.

Conclusion: The detection of MDR *E. coli* harboring *bla*TEM ESBL gene in ducks in our study area is a significant public health problem. Therefore, strong and impactful preventive measures, which would curtail the increasing dissemination of MDR bacterial pathogens in agricultural settings, are urgently needed.

Keywords: *bla*TEM gene, ducks, *E. coli*, multidrug resistance, public health, antibiotics.

Introduction

The Indonesian population is increasing every year, causing the need for food to increase. Duck, as a source of animal protein, has become a food choice for people because not only does it taste delicious but also it is quite affordable; therefore, more duck farms have emerged to meet market needs (Kuźniacka *et al*., 2020). There are many openings of new farms that have a positive impact on meeting people's food needs, but livestock also have a negative impact on the form of

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waste, which has the potential to cause environmental pollution (Godde *et al*., 2021; Riwu *et al*., 2022).

Based on data from the National Statistics Agency (CBS, 2019), the duck population in Jombang Regency in 2019 was 2,85,176, which has increased to 3,15,822 in 2020; this number is above the average number of duck populations in other cities and districts in East Java. The number of ducks in Jombang Regency continues to increase, indicating that many new farms have opened in this area, where it could be said that the duck farming business in Jombang Regency has the potential to grow even bigger. One of the livestock wastes is livestock manure. Coliform bacteria are commonly found in feces (Alegbeleye and Sant'Ana, 2020).

Coliform bacteria consist of four major genera, namely *Enterobacter*, *Klebsiella*, *Citrobacter*, and *Escherichia*. *Escherichia coli* is one of the most commonly found groups of coliform bacteria. There are *E. coli* strains that are commensal, harmless, and some that are pathogenic in humans and animals (Ramos *et al*., 2020). *E. coli* in poultry can cause diseases, such as colibacillosis, omphalitis, air sacculitis, peritonitis, and salphingitis (Effendi *et al*., 2022a). The use of antibiotics in commercial livestock can be said to be unavoidable because the large population of livestock in a limited space makes the possibility of disease occurring in a pen greater and giving antibiotics is one of the preventive measures that is usually taken (Tyasningsih *et al*., 2022). Inappropriate administration of antibiotics can result in antibiotic resistance, which can be detrimental to life. Gram-negative bacteria worldwide continue to develop resistance to β-lactam antibiotics due to the widespread use of these antibiotics as the first-line treatment for bacterial illnesses (Putri *et al*., 2023).

The persistent exposure of β-lactams to bacteria leads to continuous production of β-lactamase mutations that expand activity against even the newest β-lactam antibiotics. This enzyme is known as extended-spectrum β-lactamase (ESBL) (Wibisono *et al*., 2021). Resistance resulting from ESBL often coexists with resistance to various antibiotic families that are often employed in human treatment. Since ESBL-producing *E. coli* bacteria can be transmitted to people, they pose a risk to animal health, food safety, and the spread of antibiotic resistance in both human and animal populations (Yanestria *et al*., 2022). ESBL-producing bacteria in humans cause limited treatment options because the bacteria usually exhibit resistance to many classes of antibiotics. Urinary tract infection (UTI) in humans is a bacterial infection that does not have many antibiotic options for treatment because it is mostly caused by ESBL-producing *E. coli* (Zhou *et al*., 2023). Research on ESBL-producing bacteria is needed within the scope of veterinary medicine, which concerns public health. Knowing the spread of ESBL-producing bacteria in animals is necessary to develop strategies to mitigate its spread.

Although there are a series of reports on the spread of ESBL-producing *E. coli* in livestock, especially chickens, and cattle, among others; however, studies conducted on ducks, which are also one of the major sources of animal protein in Indonesia, are scarce, hence, this study.

Materials and Methods

Ethical approval

Ethical clearance for this study was approved by the Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia's ethical committee on the use of animals in research (Ethics no: 1.KEH.073.05.2024).

Collection of samples

A total of 180 cloacal swab samples of ducks were aseptically collected in three distinct duck farms located in Jombang, East Java, Indonesia, between March 2024 and July 2024. Collected cloacal swab samples were labeled accordingly and transported to the laboratory within two hours of collection for bacteriological analysis.

Bacteriological analysis of samples

Collected duck cloacal swabs were first enriched in MacConkey broth (Merck, Germany) and incubated at 37˚C for 24 hours. A loopful from the inoculated MacConkey broth was then transferred to eosine methylene blue (EMB) agar (Merck, Germany) plates and incubated at 37˚C for 24 hours. Colonies typical of *E. coli* (greenish-metallic sheen on EMB agar) were subcultured through successive streaking in order to obtain pure colonies, which were then subjected to Gram staining and further biochemical characterization such as IMViC (indole, methyl red, Voges–Proskauer, and citrate tests). Pure colonies of identified isolates were then preserved at 4°C for further tests.

Antimicrobial susceptibility testing and phenotypic ESBL detection

This was done by the disc diffusion method on Mueller–Hinton agar (MHA) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2020). Mueller–Hinton (MH) agar (Merck, Germany) was prepared according to the manufacturer's instructions. An 18–24-hour-old broth culture of the test isolate was standardized to 0.5 McFarland's standard. A sterile swab stick was inserted into the standardized inoculums and inoculated by spreading on the surface of the prepared MHA plate. The inoculated MHA plate was allowed to dry for a few minutes at room temperature with the lid closed. The following antimicrobials (Oxoid, UK) were tested against the isolates: ampicillin (10 µg), erythromycin (15 µg), tetracycline (30 µg), ciprofloxacin (5 µg), and aztreonam (30 µg). All antibiotic discs were carefully placed on the inoculated MHA plates using sterile forceps. The inoculated MHA plates were then incubated at 37°C for 18–24 h. After incubation, the CLSI recommendations were followed in measuring, recording, and interpreting inhibition zone diameters

as resistant or susceptible (CLSI, 2020). *E. coli* ATCC 25923 and *Klebsiella pneumoniae* ATCC 700603 were used as quality control strains. Phenotypic detection of ESBL-producing *E. coli* was done using the double-disk synergy test as previously described (Wibisono *et al*., 2021).

blaTEM gene detection

All the 25 isolated strains that were identified as MDR were further subjected to molecular characterization for *bla*TEM gene using PCR. First, bacterial DNA was isolated using the QIAamp® DNA Mini Kit (QIAGEN, Germany) according to the manufacturer's protocol. The primers used in this study were *bla*TEM-*Forward* 5'-ATGAGTATTCAACATTTCCG-3' and *bla*TEM-*Reverse* 5'-CTGACAGTTACCAATGCTTA-3' (Ballhausen *et al*., 2014). The PCR reaction conditions were performed as previously described (Fouladi *et al*., 2011) in a thermocycler with slight modifications, which included pre-denaturation for one minute at 95°C, 40 denaturation cycles for one minute at 95°C, annealing for one minute at 55°C, and extension for one minute at 72°C. A final extension lasting two minutes at 72°C marked the end of the amplification step. Electrophoresis was run in a 2% agarose gel with SYBR® safe DNA gel stain at 100 V and 250 mA for 35 minutes. A UV transilluminator was used to view the amplified PCR products.

Results

Results showed that 94 (52.2%) out of the 180 cloacal swab samples collected from ducks in duck farms located in Jombang, East Java, Indonesia were positive for *E. coli.* Isolates were noted to be green metallic sheen colonies in EMB agar. They were Gram-negative and were positive for motility, indole, and methyl red tests but were negative for hydrogen sulfide production, Voges–Proskauer (VP), and citrate tests.

Isolates were also observed to be resistant to aztreonam (98.9%), erythromycin (94.7%), tetracycline (57.4%), ampicillin (41.5%), and ciprofloxacin (12.8%) being the least (Table 1, Fig. 1). Twenty-five (39.1 %) out of the recovered *E. coli* isolates were generally noted to exhibit MDR traits. None of the *E. coli* strains displayed ESBL production via the double-disk synergy test (DDST); however, 24 (96%) out of the 25 MDR *E. coli* strains that were selected for molecular studies harbored *bla*TEM ESBL gene (Figs. 2 and 3).

Table 1. Antimicrobial susceptibility profiles of *E. coli* recovered from cloacal swabs of ducks

Antibiotics	Resistance frequency *n* (%)	Susceptibility frequency *n* (%)
Ampicillin	39 (41.5%)	55 (58.5%)
Aztreonam	93 (98.9%)	1 (1.1%)
Ciprofloxacin	12 (12.8%)	82 (87.2%)
Tetracycline	54 (57.4%)	40 (42.6%)
Erythromycin	89 (94.7%)	5 (5.3%)

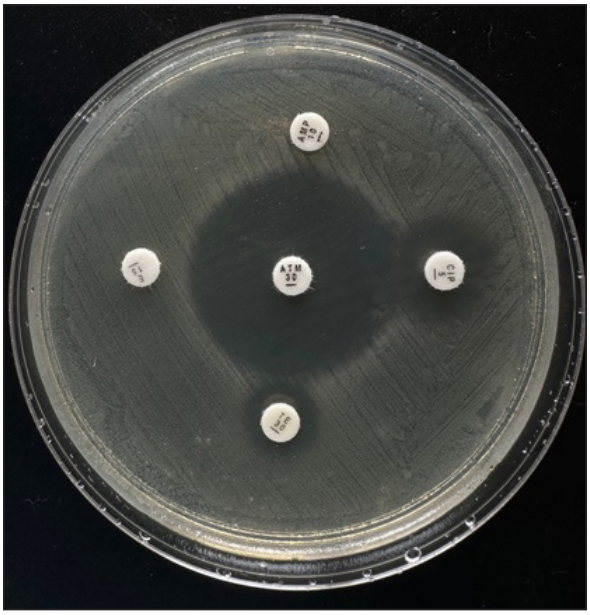

Fig. 1. Representative picture of antimicrobial susceptibility testing of *E. coli* isolates.

Discussion

Bacterial pathogens, such as *E. coli*, which belongs to the *Enterobacteriaceae* family and harbors the extended-spectrum beta-lactamase (ESBL)-encoding genes, are one of the main contributors to the challenging public health problems in the world today (WHO, 2016). Bacteria that harbor the ESBL genes can produce ESBL enzymes, which can hydrolyze beta-lactam antibiotics, including third- and fourth-generation cephalosporins. Many of the identified ESBL gene variants, including *bla*TEM, *bla*SHV, and *bla*CTX-M, have been identified to be plasmid-mediated (Husna *et al*., 2023).

In this study, 94 (52.2%) *E. coli* were recovered from 180 cloacal swab samples of ducks in various duck farms in Jombang, East Java, Indonesia, with only 25 being identified as exhibiting MDR strains. All the isolates were negative for the phenotypic detection of ESBL using the DDST technique; however, 24 out of the 25 MDR *E. coli* strains that were further molecularly characterized harbored the *bla*TEM gene. DDST is a simple and easy method for detecting ESBL, but DDST does not detect all ESBL-producing isolates. Additionally, the CLSI recommends a phenotypic confirmatory disk diffusion test (PCDDT) as a confirmation test for ESBL. However, we suspect that the non-detection of ESBL enzymes in the *E. coli* strains in this study by the DDST test might possibly be due to the production of Chromosomal

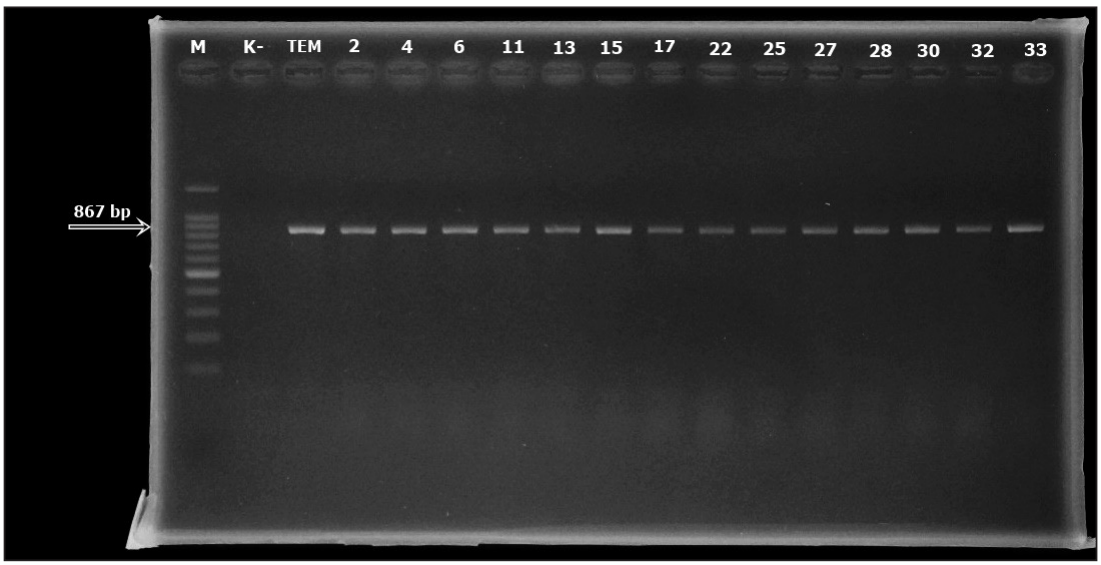

Fig. 2. PCR result for *bla*TEM detection in *E. coli* isolates (1/2).

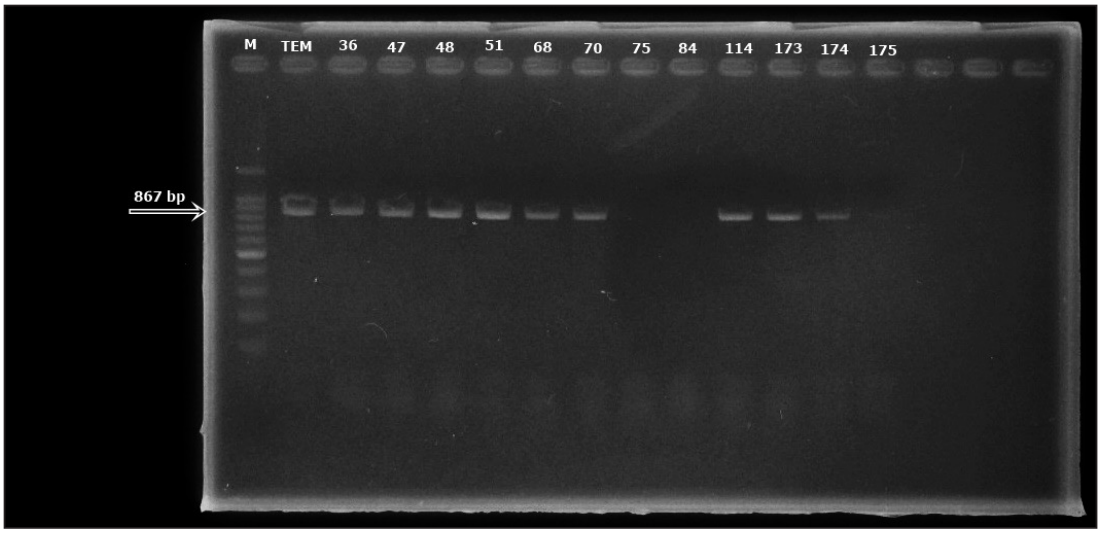

Fig. 3. PCR result for *bla*TEM detection in *E. coli* isolates (2/2).

cephalosporinase (Kaur *et al*., 2013). The results of our study have further reinforced that DDST needs to be followed up with a PCR for the genetic confirmation of ESBL-production, thus, making PCR a gold standard for ESBL detection.

The frequency of *bla*TEM gene (96%) among the 25 MDR *E. coli* isolates that were molecularly characterized in this study is higher than the study of Sarker *et al*. (2023) who also reported *bla*TEM gene in 72.4% of duck cloacal swab samples.

Inappropriate and excessive use of antibiotics cause selective pressure that favors the growth of resistant bacteria. Through conjugative plasmid-mediated horizontal gene transfer, the colonization of resistant bacteria in the intestines of people and animals results in the transmission of bacterial resistance genes in the intestinal flora. The majority of plasmid genes encode ESBLs. It was discovered that *Enterobacteriaceae* possessed plasmids containing resistance genes. The *bla*TEM gene is located on a plasmid (Gulfraz *et al*., 2014). The IncFII plasmid group is known to be a group that encodes ESBL, is widely distributed in *Enterobacteriaceae*, and is called the epidemic resistance plasmid group. *bla*TEM is one of the dominant ESBL genes in the world and is found in human, animal, and environmental isolates. The *bla*TEM gene was found mainly in *E. coli* and *Klebsiella pneumoniae* bacterial isolates (Soekoyo *et al*., 2020). The *bla*TEM

gene is a beta-lactamase most often found in Gram-negative bacteria. Most ampicillin resistance in *E. coli* is caused by the presence of the *bla*TEM gene (Effendi *et al*., 2022b). The existence of resistant bacteria in food-producing animals is caused by the continuous use of antibiotics for therapeutic and non-therapeutic purposes in production systems, which may be found in products of animal origin.

It is known that the main mechanism by which microbial populations can survive in threatened situations is through genetic mutation, expression of a latent resistance gene, or genes that have resistance determinants. Some bacteria have extrachromosomal genes in the form of plasmids or bacteriophages. The resistance factor that is transferred from a chromosome to a plasmid or on a transposon or integron is an R factor plasmid, which is called an infectious plasmid. This transfer can occur through conjugation, transformation, or transduction. Transposons containing resistance genes carry out inversion into plasmids so that resistance genes can be transferred to other cells (Partridge *et al*., 2018).

The *bla*TEM resistance gene harbored by the *E. coli* strains in this study may be plasmid-mediated or chromosomal-mediated. However, a limitation of our study was our inability to carry out further test to determine the location of the *bla*TEM resistance gene in the recovered *E. coli* isolates from duck cloacal swabs.

In a study conducted by Oberoi *et al*. (2013), 96 (35.16%) ESBL-producing Gram-negative bacterial pathogens were also reported, out of which 45.9% were implicated as culprits in UTIs. Additionally, the largest ESBL producers in the study of Oberoi *et al*. (2013) were *Escherichia coli* (56.25%), *Pseudomonas aeruginosa* (18.75%), and *Klebsiella pneumoniae* (15.62%).

Infections caused by MDR Gram-negative bacteria, which produce various types of β-lactamase enzymes, affect the length of stay in the ICU, as they are associated with patient morbidity and mortality. Many β-lactamases are encoded by chromosomal or transferable genes, located on plasmids or transposons. Initially, the β-lactamase enzyme was most often found in *Klebsiella sp.* and *E. coli*, but now, this enzyme is produced by all members of the *Enterobacteriaceae* and other Gram-negative bacilli (Al-Sheboul *et al*., 2023).

*bla*TEM is one of the common beta-lactamase genes found in ESBL-producing *Escherichia coli*. It was one of the earliest beta-lactamase genes identified and is widely distributed among *Enterobacteriaceae*, including *E. coli*. The presence of *E. coli* encoding ESBL genes in poultry, including ducks, has been documented globally, including in Southeast Asia where poultry farming is prevalent.

Conclusion

Our study has shown that ducks, which are important sources of animal protein in East Java, Indonesia, are colonized by MDR *E. coli*, which also harbors *bla*TEM ESBL gene. The detection of these pathogenic MDR *E. coli* strains in duck farms in our study area is a significant public health problem, which requires urgent attention. It is therefore imperative to establish strong guidelines and control measures, which would curtail the increasing spread of MDR bacterial pathogens in agricultural settings, especially in duck farms.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Author's contributions

SNA and WT: Conceptualization and design. IF, LL, and MKJK: Acquisition of data. MHE and IBM: Formal analysis and interpretation of data. ARK and KAF: Writing-original draft preparation. SMY, DAAK, and SW: Writing-review and editing. All authors have read and agreed to the published version of the manuscript.

Data availability

All data are available in the manuscript.

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