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Prevalence of the *bla*$_{CTX-M}$ and *bla*$_{TEM}$ genes among extended-spectrum beta lactamase–producing *Escherichia coli* isolated from broiler chickens in Indonesia

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Abstract

Introduction: Infections of humans and animals by multidrug resistant bacteria are increasing because of the inappropriate use of antibiotics. Disease management may be more challenging if *Escherichia coli* produce extended-spectrum beta-lactamase (ESBL), which could cause resistance to aztreonam and third-generation cephalosporins. This study was aimed at determining the prevalence of the *bla*$_{CTX-M}$ and *bla*$_{TEM}$ genes among ESBL-producing *E. coli* isolated from broiler chickens in Indonesia. **Material and Methods:** A total of 115 broiler cloacal swab samples were obtained from 22 farms and studied for the presence of *E. coli*. The isolates were identified using approved standard methods and were purified on eosin methylene blue agar media. The *E. coli* isolates were subjected to sensitivity testing using beta-lactam antibiotics, and ESBL production was confirmed by a double-disc synergy test. The presence of the *bla*$_{CTX-M}$ and *bla*$_{TEM}$ genes was identified using a PCR. **Results:** It was found that 99/115 (86.1%) of the isolated *E. coli* were resistant to beta-lactam antibiotics and 34/115 (29.6%) of them were phenotypically detected to be ESBL producers. Of the 34 isolates that were confirmed ESBL producers, 32/34 (94.1%) of them harboured the *bla*$_{CTX-M}$ and 13/34 (38.2%) the *bla*$_{TEM}$ genes. The *bla*$_{CTX-M}$ and *bla*$_{TEM}$ genes were detected together in 12/34 (35.3%) isolates. **Conclusion:** This study discovered that broiler chickens are possible reservoirs of ESBL-producing *E. coli* that may infect humans. Thus, a committed public health education campaign is recommended in order to mitigate the potential threat to human health.

Keywords: *bla*$_{CTX}$, *bla*$_{TEM}$, ESBL-producing *E. coli,* broiler chickens, human health.

Introduction

Antibiotic resistance was recognised more than fifty years ago, is now the biggest global health challenge, and has grave social and economic impacts (15). Deaths from antibiotic-resistant pathogens are increasing. In addition, resistance also causes an increase in patient treatment time in hospitals. Since

antibiotics are no longer as effective, treating many infections such as pneumonia, salmonellosis, gonorrhoea and tuberculosis is becoming more and more challenging. The misuse of antibiotics in animals and humans has contributed greatly to the increase in cases of resistance (9). Public knowledge about antibiotic resistance is still low, but the level of administration of antibiotics is high (15). Other factors that influence the rise in antibiotic resistance include their use without a doctor's prescription, their excessive use in humans and animals, and inappropriate prescribing. Antibiotics can be found in application in several sectors such as primary healthcare, livestock, agriculture and fisheries (26). In the livestock sector, antibiotics are often used as prophylactics and growth promoters (AGP) to accelerate the growth of livestock. The antibiotics most widely used as AGPs include tetracyclines, penicillin, macrolides, lincomycin, and virginiamycin (31).

Various attempts have been made to address the problem of antibiotic resistance. The European Medicines Agency (EMA) and the European Food Safety Authority (EFSA) recommended measures to reduce the use of antibiotics such as colistin on animal farms in the European Union (11). France also issued a policy in the form of a ban on the unwarranted use of antibiotics in chickens (5). Meanwhile in Indonesia, through the Ministry of Agriculture, the government prohibits the use of AGP in animal feed. Although the use of AGP has been banned since 2019 in Indonesia, its impact is still felt today. Animal feed must be ensured to be free from certain hormones and/or feed additive antibiotics (2).

The demand for food of animal origin will increase in step with the substantial increase in the size of the global human population (1). Broiler chicken farming has become the livelihood of a large number of Indonesians. The main readily enterable poultry business is broiler raising, and in East Java alone 250 million head were produced in 2019. Blitar district in East Java is one of the centres for chicken farming and large quantities of eggs are produced there every year to meet the demand of the Indonesian people (28).

The chicken is one of the major sources of meat for human consumption, and a protein of animal origin that has been noted to be prone to bacterial contamination and of which *E. coli* is one of the microorganisms most frequently implicated in health risks (17). *Escherichia coli* that has naturally acquired resistance genes has the ability to produce enzymes such as beta-lactamase for self-defence (3). In addition to beta-lactamase enzymes with a normal spectrum, *E. coli* can also produce extended-spectrum beta-lactamases (ESBL) (6), which derive from beta-lactamase enzymes as a result of a point mutation resulting in an increase in enzymatic activity. Because of this, almost all beta-lactam antibiotics, including third-generation cephalosporins and aztreonam can be hydrolysed by ESBL. The genes responsible for ESBL production include bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M}}$ (22). Microorganisms resist antibiotics through four major mechanisms, the first of which is production of the enzyme beta-lactamase. The second is the efflux pump, which pumps out of the *E. coli* cells the antibiotics that can enter the bacteria. The third is the impermeability of the membrane, achieved by changing the transport protein. The fourth and last is modification of the molecular target so that the antibiotic cannot recognise the appropriate receptor and cannot bind (21). Detection of the presence of ESBL can be undertaken in several ways, one of which is the PCR (18). The PCR technique has a high level of sensitivity, is easy to perform, and produces fast results. Therefore, the purpose of this study was to exploit this technique to evaluate the prevalence of the $bla_{\text{CTX-M}}$ and bla_{TEM} genes among ESBL-producing *E. coli* isolated from broiler chickens in the East Java Province of Indonesia.

Material and Methods

Isolation and identification of *E. coli.* The samples used in this study were broiler cloacal swabs. Amies agar gel transport swabs were used to collect 115 samples from 22 farms in the East Java Province. The samples were labelled and then stored in a coolbox and brought to the laboratory in the Department of Veterinary Public Health, Airlangga University for analysis. Samples on Amies transport medium were inoculated on eosin methylene blue agar (EMBA) for the isolation of *E. coli*. The inoculated plates were incubated at 37°C for 24 h. The samples that had *E. coli* growth on EMBA were indicated by a metallic green colour. Gram staining and the IMViC test, which consists of the indole test, methyl red test, Voges–Proskauer test and citrate test, were used to further identify and confirm the isolates. The ability of microorganisms to ferment carbohydrates was also evaluated using the triple-sugar iron agar test.

Antibiotic sensitivity test. The susceptibility of the *E. coli* isolates to antibiotics was tested using the Kirby–Bauer disc diffusion method on Mueller–Hinton (MH) agar (Oxoid, Basingstoke, UK). The antibiotic discs used were from the beta-lactam group, which included ampicillin (10 µg), ceftazidime (30 µg), cefotaxime (30 µg) and amoxicillin/clavulanic acid (30 µg). Suspensions of 2 mL volume containing fresh culture of the isolates were prepared to the equivalent of 0.5 McFarland standards. The surfaces of the agar plates were carefully swabbed with the suspension of the isolates and allowed to air dry before the antibiotic discs were placed and the plates and discs were incubated for 24 h. The results were interpreted using the Clinical and Laboratory Standards Institute Performance Standards for Antimicrobial Susceptibility (7). Beta-lactam-resistant *E. coli* isolates were further subjected to ESBL confirmatory assays.

Table 1. The list of primers used in this study

Gene	Primer	Sequences	Amplicon (bp)	Annealing	References
bla_{CTX-M}	Forward	CGC TTT GCG ATG TGC AG	550	54°C	(27)
	Reverse	ACC GCG ATA TCG TTG GT			
bla_{TEM}	Forward	ATAAAATTCTTGAAGACGAAA	1,080	59°C	(18)
	Reverse	GACAGTTACCAATGCTTAATC			

bp – base pairs

Table 2. The occurrences of *E. coli* in broiler cloacal swabs in Blitar district, East Java, Indonesia

Subdistrict	Farm number	Sample size	*E. coli* isolates
Ponggok	5	28	28 (100%)
Garum	5	25	25 (100%)
Selopuro	7	36	36 (100%)
Selorejo	5	26	26 (100%)
Total	22	115	115 (100%)

Double-disc synergy test. The pure cultures of potential ESBL-producing *E. coli* isolates were standardised to 0.5 McFarland standards. The isolates were inoculated on the surface of MH agar plates. The antibiotic discs that contained ceftazidime (30 µg), cefotaxime (30 µg), and amoxicillin/clavulanic acid (30 µg) were placed parallel to the MH agar media at a distance of 15 mm centre-to-centre and incubated for 24 h at 37°C. Positive ESBL-producing *E. coli* were confirmed by observing the inhibition zones of the ceftazidime and cefotaxime antibiotic discs extending towards the amoxicillin/clavulanic acid disc. All the isolates that were confirmed as ESBL producers were further studied using a PCR.

DNA extraction and detection of bla_{CTX-M} and bla_{TEM} genes by PCR. Phenotypically identified ESBL-positive *E. coli* isolates were examined for the presence of bla_{TEM} and bla_{CTX-M} genes in a PCR. The DNA extractions were carried out from overnight bacterial cultures of *E. coli* isolates using a GeneJET Genomic DNA Purification Kit (Thermo Scientific, Waltham, MA, USA). The purity and concentration of DNA were calculated using a spectrophotometer at wavelengths of 260 nm and 280 nm. The extracted DNA was stored at −20°C until use. The products of the PCR were visualised by 1% agarose gel electrophoresis using UV light after staining with ethidium bromide, and positive results were indicated by the formation of a band of 550 base pairs (bp) for the bla_{CTX-M} gene and a band of 1,080 bp for the bla_{TEM} gene. The ATCC 25922 *E. coli* isolate was used as the control. The primers used for the amplification are shown in Table 1.

Results

Escherichia coli was isolated from all the 115 (100%) broiler cloacal swab samples obtained, and therefore all the broiler cloacal samples were *E. coli* positive from all four subdistricts in East Java sampled. From Ponggok 28 (100%) samples yielded *E. coli* isolates, from Garum 25 (100%), from Selopuro 36 (100%), and from Selorejo 26 (100%) (Table 2). Samples of broiler cloacal swabs cultured on EMBA media showing a metallic green colour are shown in Fig. 1, and the rod-shaped *E. coli* isolates are presented in Fig. 2.

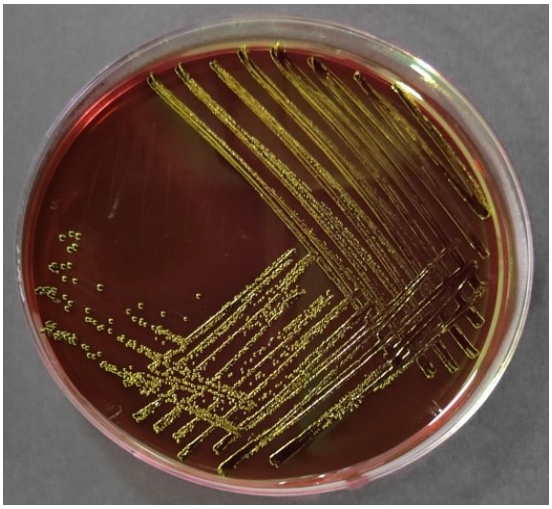

Fig. 1. Appearance of *E. coli* on eosin methylene blue agar media

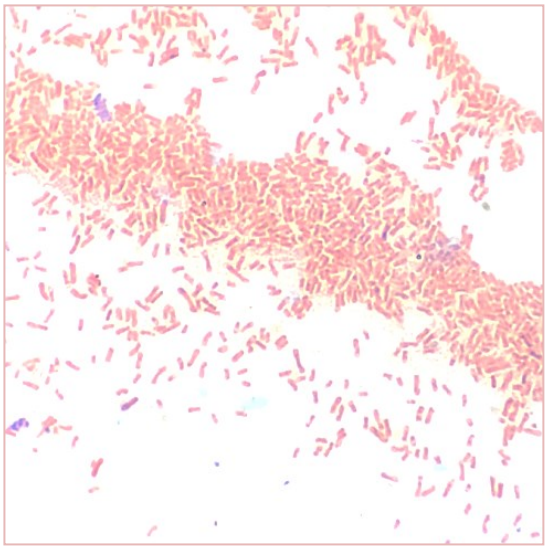

Fig. 2. The Gram staining appearance of *E. coli* in microscopy (1000×)

Fig. 3. Double-disc synergy test showing the keyhole effect of extended-spectrum beta lactamase–producing *E. coli*
CTX – cefotaxime; AMC – amoxicillin/clavulanic acid; CAZ – ceftazidime

The results of the antibiotic sensitivity test showed that 99 (86.1%) of the isolated *E. coli* were resistant to beta-lactam antibiotics and 34 (34.3%) of them were ESBL producers. Figure 3 depicts the inhibition zone for the ESBL-producing *E. coli* extending from the discs of cefotaxime and ceftazidime towards the disc of amoxicillin. Regarding the incidence of ESBL-producing *E. coli* by geographical area, farms in the Selorejo subdistrict had the highest (46.2%), followed by those of the Ponggok (35.7%) and Selopuro (22.2%) subdistricts, and farms in the Garum subdistrict (16%) had the lowest prevalence (Fig. 4).

Of the 34 isolates that were confirmed to be ESBL producers phenotypically, 13 (38.2%) isolates had the bla_{TEM} gene with binding size of 1,080 bp (Fig. 5). This bla_{TEM} gene was found in all subdistricts in Blitar District; 4 (11.8%) isolates came from Ponggok, 4 (11.8%) from Selopuro, 3 (8.8%) from Selorejo, and the last 2 (5.9%) from Garum. Out of 32 *E. coli* isolates carrying the $bla_{\text{CTX-M}}$ gene, 11 (32.4%) isolates originated from the Selorejo subdistrict, 9 (26.5%) from Ponggok, 8 (23.5%) from Selopuro, and the final 4 (11.8%) from Garum. The results of this study also showed that all the 34 ESBL-producing isolates harboured the $bla_{\text{CTX-M}}$ gene with molecular weight of 550 bp (Fig. 6) and 12 (40.0%) isolates had the presence of both bla_{TEM} and $bla_{\text{CTX-M}}$ genes (Table 3).

Table 3. The extended-spectrum beta lactamase–producing *E. coli* harbouring $bla_{\text{CTX-M}}$ and bla_{TEM} genes

Subdistrict location	Encoding gene	
	$bla_{\text{CTX-M}}$	bla_{TEM}
Ponggok	+	−
	−	−
	+	+
	+	+
	+	−
	+	+
	+	−
	+	+
	+	−
	+	−
Total	9/10; 9/34 (26.5%)	4/10; 4/34 (11.8%)
Garum	+	−
	+	−
	+	+
	+	+
Total	4/4; 4/34 (11.8%)	2/4; 2/34 (5.9%)
Selopuro	+	−
	+	+
	+	−
	+	+
	+	+
	+	−
	+	+
	+	−
Total	8/8; 8/34 (23.5%)	4/8; 4/34 (11.8%)
Selorejo	+	+
	+	−
	+	−
	+	−
	+	−
	+	+
	+	−
	+	−
	+	−
	−	+
	+	−
	+	−
Total	11/12; 11/34 (32.4%)	3/12; 3/34 (8.8%)
Overall total	32/34 (94.1%)	13/34 (38.2%)

+ - sample positive for the extended-spectrum beta lactamase gene; − - sample negative for this gene

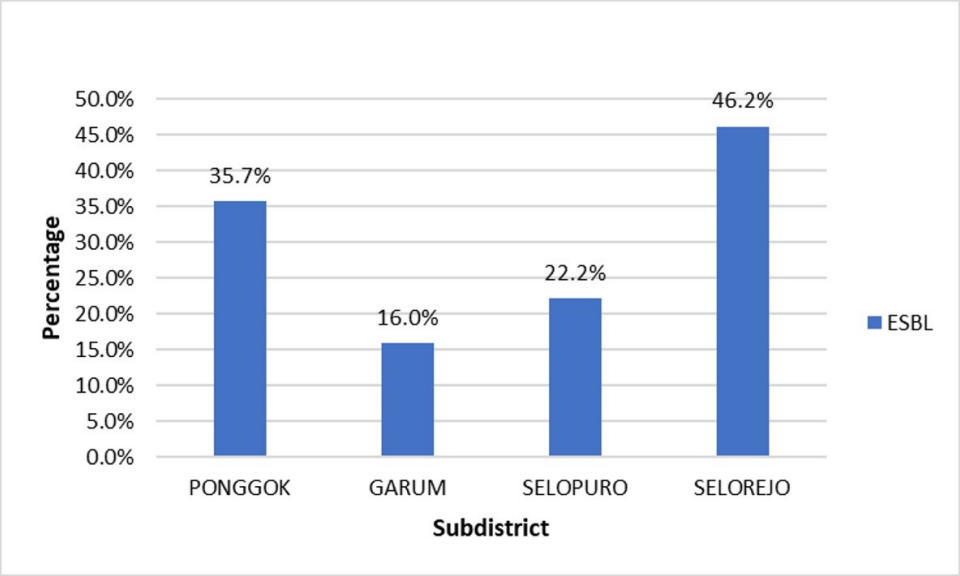

Fig. 4. Distribution of extended-spectrum beta lactamase (ESBL)–producing *E. coli* from broilers in East Java Province, Indonesia

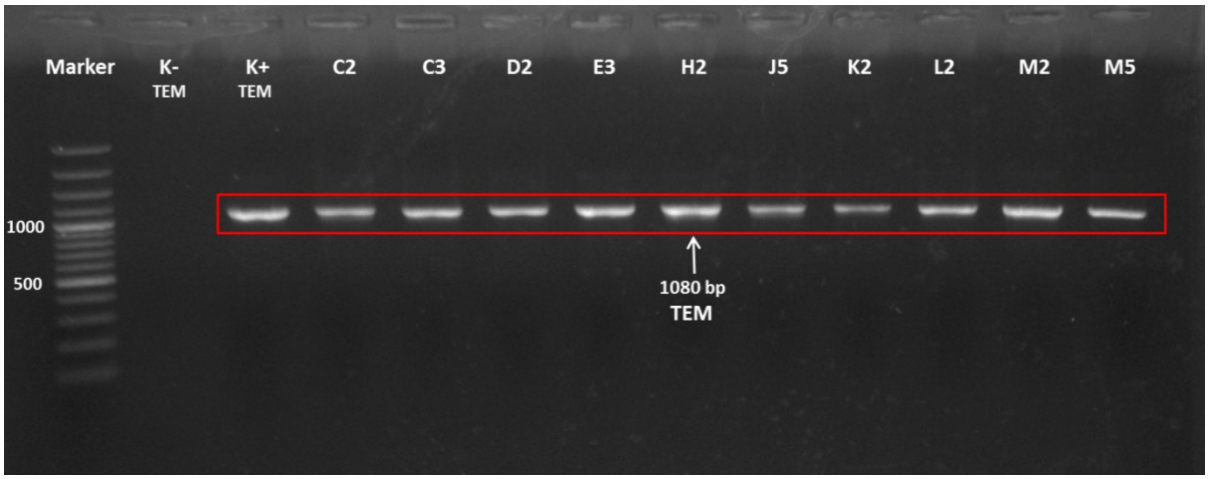

Fig. 5. Gel picture showing the presence of the bla_{TEM} gene in extended-spectrum beta lactamase–producing *E. coli*
bp – base pairs

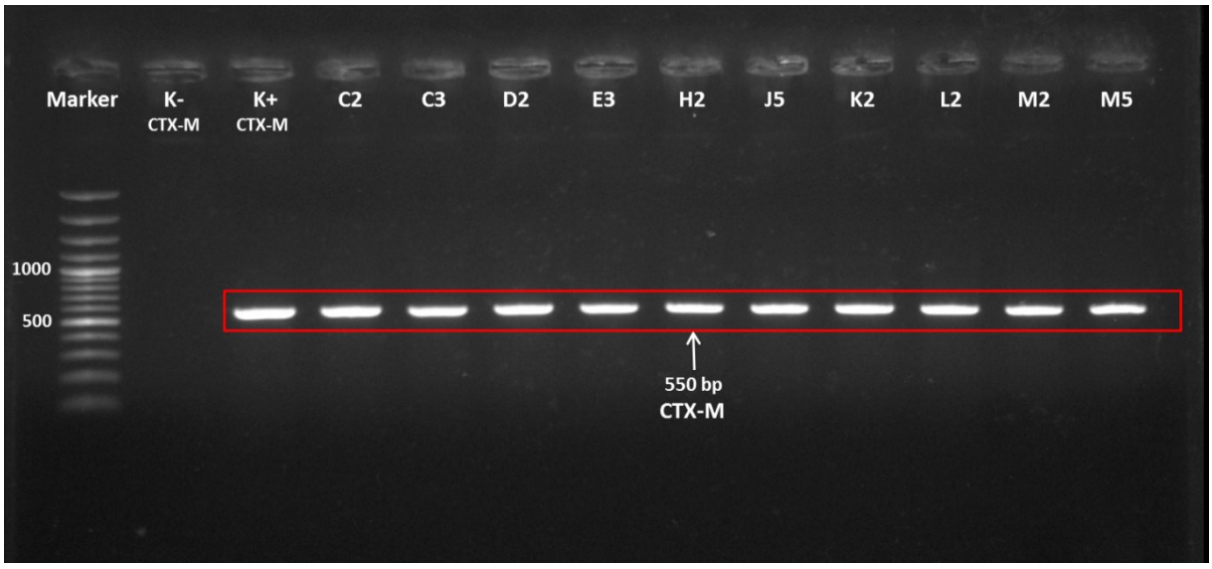

Fig. 6. Gel picture showing the presence of the bla_{CTX-M} gene in extended-spectrum beta lactamase–producing *E. coli*
bp – base pairs

Discussion

The discovery of beta-lactam–resistant and ESBL-producing *E. coli* in animals and foods derived from animals, such as chicken meat, has prompted researchers to further investigate their incidence and spread in animals used for food production. According to this study, *E. coli* isolates were present in all 115 (100%) of the broiler chicken cloacal samples evaluated. The different farms from the four subdistricts of Ponggok, Garum, Selopuro and Selorejo were found to have high levels of *E. coli* contamination. This finding is in agreement with the work of a researcher who reported the presence of *E. coli* from chicken farms in Nsukka, Nigeria (6). Other research also stated that *E. coli* is common on broiler farms because it appears to be a normal component of the flora of the intestinal tract of humans and most animals, and reported the presence of *E. coli* from faecal material of chicks in Iran (22). These microbes help to break down food in the large intestine and suppress the growth of other infectious bacteria in animals and humans. On the other hand, they can cause infectious diseases and intestinal disorders (19), for example the colibacillosis that often attacks poultry (11). Being inhibitory compounds, antibiotics are the drugs required to treat this disease so that it will not cause significant economic losses to poultry farmers (8). This study observed that 99 (86.1%) of the isolated *E. coli* were resistant to beta-lactam antibiotics and 34 (29.6%) of the isolates were phenotypically confirmed to be ESBL producers. The overuse of beta-lactam antibiotics has created the possibility of acquisition of beta-lactamase production–encoding genes by bacteria, which could complicate the treatment of bacterial infection because of resistance. This is very likely to be one of the main reasons why we observed a large proportion of the *E. coli* isolates showing resistance to beta-lactam antibiotics. Extended-spectrum beta lactamase–producing *E. coli* was reported from both humans and animals in Ghana (13). Other studies reported a high prevalence of ESBL-producing *E. coli* in samples from chickens in France (5) and Indonesia (10). These bacilli are able to produce beta-lactamase enzymes for self-defence and adaptation in the presence of antibiotics, and these enzymes have the ability to hydrolyse the beta-lactam ring on antibiotics (30).

Escherichia coli can also acquire resistance through horizontal transfer or mutation. Researchers reported the presence of ESBL-producing *E. coli* in chickens in South Korea (17) and stated that horizontal gene transfer is one of the major pathways of resistance gene acquisition by the microorganism (20). The observation of ESBL-producing *E. coli* as reported by other researchers in their studies is matched by the findings of this study. A resistance gene can be transferred from a donor cell to a recipient cell in a process known as conjugation, which occurs when two bacteria are in physical contact. The genetic element that facilitates this gene transfer event is the transposon (4). Genetic material can also be transferred in a process known as transformation, which takes place without direct contact between bacteria. Genetic material is obtained from donor cells by lysis or chemical extraction in the presence of plasmids (14). The third way in which resistance genes may be transferred is by transduction, the process by which genes are transferred from one bacterium to another by means of a bacteriophage. When a bacteriophage attacks a bacterium, its genetic material is injected into the bacterial cell (16). Bacteriophages not only insert their own DNA but also include DNA from other bacteria that were once their hosts. Mutation can also endow bacteria with genes for resistance. Resistance may be the consequence of mutations causing changes in nucleotides that code for gene expression. A mutant strain can become dominant in the population and render a large part of the population resistant to specific antibiotics, for example on the scale observed in this study.

This study found that 34.3% of broiler cloacal swabs contained the $bla_{\text{CTX-M}}$ and bla_{TEM} genes. These genes are two of the set of genes encoding enzyme production in *E. coli* (17), and this ascribed activity of $bla_{\text{CTX-M}}$ and bla_{TEM} is in accordance with the observations of this study. These genes are responsible for producing the ESBL enzyme that causes *E. coli* to be resistant to beta-lactam antibiotics, third-generation cephalosporins and monobactams (30). When *E. coli* produces ESBL, it also tends to be multidrug resistant, and as such, resistant to several classes of antibiotics at the same time. The most common ESBL subtype was $bla_{\text{CTX-M}}$, with a prevalence rate of 94.1% in our research. This study confirmed previous findings that stated that broiler chickens carry ESBL-producing *E. coli* isolates that harbour the $bla_{\text{CTX-M}}$ gene at a high frequency of 96.0% (12). A similar finding was also reported by Casella *et al.* (5), who discovered that up to 91.9% of raw chicken samples had the $bla_{\text{CTX-M}}$ gene. Another study by Ramadan *et al.* (24) detected 82% of ESBL-producing *E. coli* isolates in human samples to harbour the $bla_{\text{CTX-M}}$ gene. The presence of $bla_{\text{CTX-M}}$ genes was noted in layer chickens from Blitar, Indonesia by Wibisono *et al.* (27). The prevalence of the bla_{TEM} gene was identified to be 38.2% in this study. The frequency of the bla_{TEM} gene in *E. coli* isolates in this study was lower than that of the $bla_{\text{CTX-M}}$ gene. The studies of Casella *et al.* (5) also reported a low prevalence of the bla_{TEM} gene, with a frequency of 6.7% in broiler chicken samples. Similar results showed that the frequency of the bla_{TEM} gene was lower than that the frequency of the $bla_{\text{CTX-M}}$ gene. In this study, multiple ESBL-producing *E. coli* strains were identified to harbour $bla_{\text{CTX-M}}$ and bla_{TEM} with a prevalence rate of 12 (40.0%). This is in line with the finding of other research in Nigeria, where ESBL-producing *E. coli* were discovered to harbour $bla_{\text{CTX-M}}$ and bla_{TEM} in multiple instances, being prevalent at a rate of 15.3% (25).

According to the literature, ESBL bacteria were found in the soil and water near farms and markets and

in vegetables grown on the farms or sold at the markets (10). As a result, it is possible for ESBL-production genes to spread quickly from animals to humans or *vice versa* through genetic elements in bacteria such as transposons, insertion sequences, and integrons (29). Evaluation was made of some of the farms' standard hygiene practices, and while most were adequate, some were poor. As a result of this poor hygiene and sanitation, *e.g.* unsatisfactory waste dumping regimes, contaminants such as resistant bacteria can spread from the poultry farm to the surrounding area and cause damage to the farm's land and water bodies (13). The existence of *E. coli* that produces ESBL enzymes must be understood and treated as a public health threat (23). In an intensive production system, waste and manure from chicken farms must be appropriately managed to prevent contamination of the air, soil and water as well as adverse effects on human health (27).

In conclusion, this study reported the prevalence in broiler cloacal swabs of ESBL-producing *E. coli* to be 29.6%, that of the $bla_{\text{CTX-M}}$ gene to be 94.1% and that of the bla_{TEM} gene to be 38.2%. Isolates harbouring both genes were detected with a prevalence of 35.3%. The presence of multiple ESBL genes in the isolates as reported indicates a serious public health challenge since these microorganisms harbouring these genes can easily cause infection in humans and animals. The genetic evidence of ESBL genes reported in this study can be utilised as a guide when measures are designed to prevent the spread of ESBL-encoding genes on poultry farms and in other livestock industries. We recommend that governmental bodies or supra-governmental organisations should enhance their oversight of the application of antibiotics to prevent future abuse, misuse, underuse, or excess use in the human population and on poultry farms.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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Animal Rights Statement: Cloacal swabs were used in this study, hence ethical approval was not necessary. Cloacal swabs samples were collected from Blitar broiler farms in East Java province, Indonesia**.**

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