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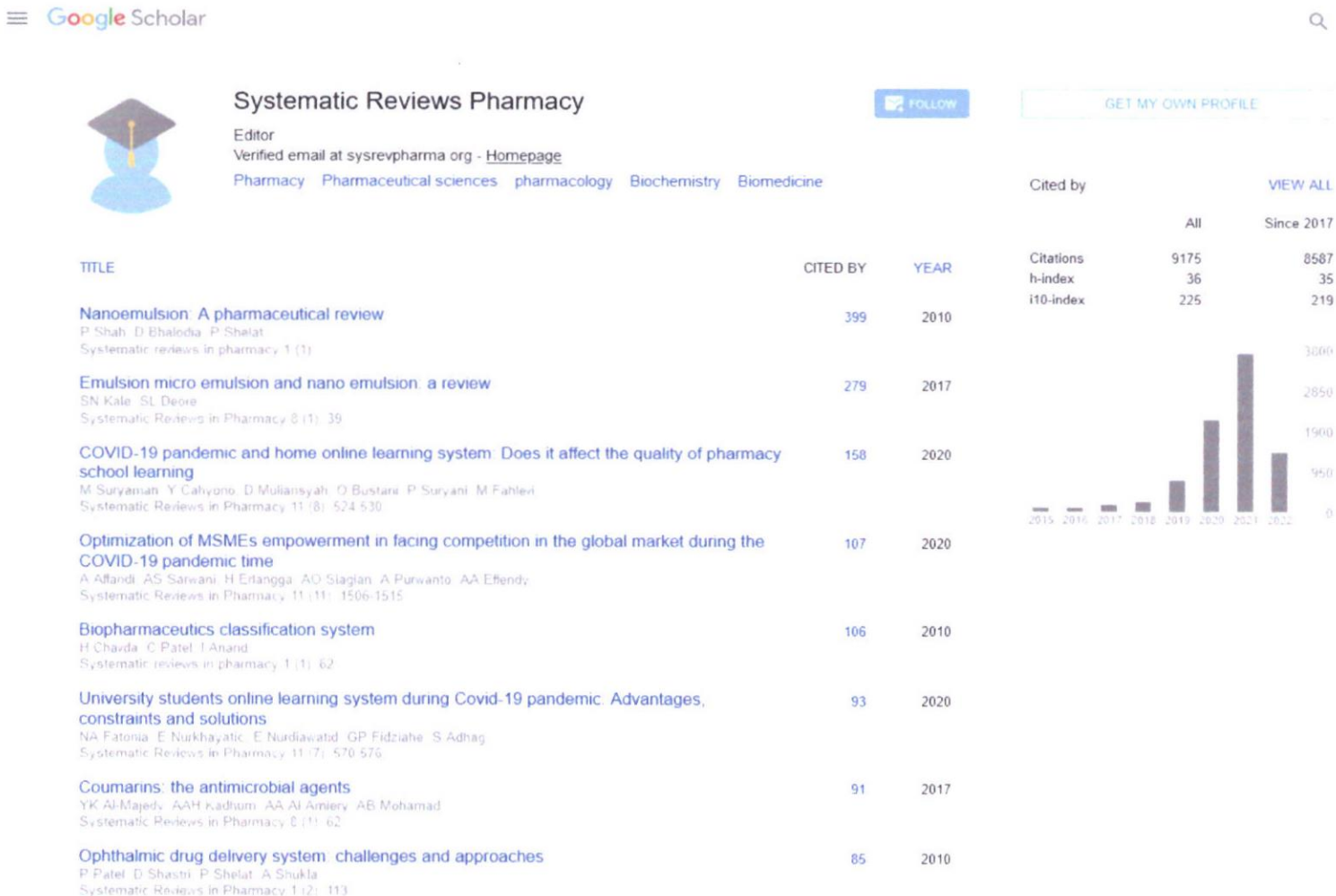

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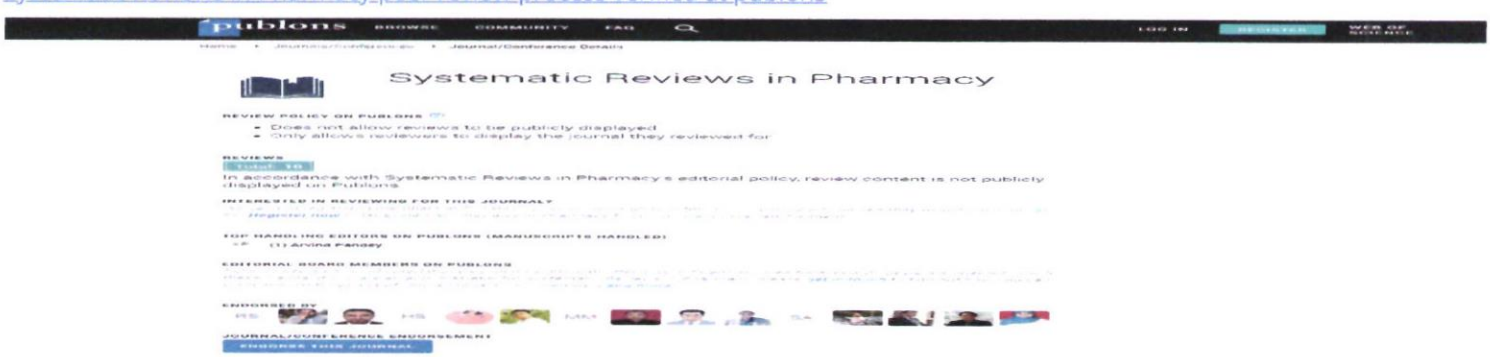

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Identification of Bla_{TEM} and Bla_{SHV} Genes of Extended Spectrum Beta Lactamase (ESBL) Producing *Escherichia coli* from Broilers Chicken in Blitar, Indonesia

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ABSTRACT

The purpose of this research was to identify bla_{TEM} and bla_{SHV} genes of extended spectrum beta lactamase (ESBL) of *Escherichia coli* from cloacal swab of broiler chicken in several broiler farms in Blitar. This study used 95 broiler chicken samples, with cloacal swab method. The samples were isolated and identified to find Escherichia coli with several procedures, MacConckey Agar (MCA), Eosin Methylene Blue Agar (EMBA), Gram staining, indole test, Methyl Red-Voges Proskauer (MR-VP), citrate, and Triple Sugar Iron Agar (TSIA). ESBL-producing *Escherichia coli* bacteria isolated from cloacal swabs of broiler chicken were confirmed by the Double Disc Synergy Test (DDST). This confirmation test with DDST was conducted to evaluate the presence of inhibitory zones of ESBL activity with clavulanic acid using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar (Merck, Germany). Double Disc Synergy Test uses the antibiotic disc Amoxycillin-clavulanic 30µg (Oxoid, England), Cefotaxime 30µg (Oxoid, England), Ceftazidime 30µg (Becton Dickinson, USA and Aztreonam 30µg (Oxoid, England). ESBL producing E. coli were 10 isolates and bla_{TEM} gene from 7 isolates and bla_{SHV} gene was no isolate. It can be explained that broiler chicken should be considered as a source of transmission for ESBL of E. coli to the public health.

Keywords: Escherichia coli, bla_{TEM}, bla_{SHV}, ESBL, Broiler chicken, Public health

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INTRODUCTION

The production of meat from broilers cannot be separated from the use of antibiotics. The main reason for the use of antibiotics is used as a treatment and growth promoter (1). The main reason for using antibiotics is that chickens are very susceptible to pathogenic diseases due to the high density of the cage which causes the chickens to become stressed. Many uncontrolled use of antibiotics will leave residues and cause pathogenic microbes to become resistant to antibiotics (2, 3). Escherichia coli, besides being an indicator of the level of sanitation in farms, can also act as a reservoir for the spread of antibiotic resistance because it can easily transfer genes for resistance to other bacteria. One of the characteristics of *E. coli* is that it has the potential to produce an extended spectrum beta-lactamase (ESBL) enzyme (4, 5). *E. coli* that produces ESBL has been isolated from food from animals, hospital environments, plants and feces. Several studies have also reported a high prevalence of ESBL producing *E. coli* in food-producing animals, food products, and the environment (6 - 8). *E. coli* is a polluting bacteria commonly found in meat. Meat contaminated with resistant *E. coli* bacteria can result in the transfer of bacteria from animals to humans via the food chain or direct contact. The long-term use of antibiotics can change the resistance of bacteria, both pathogens and normal microflora in living things (9, 10).

This study was conducted to obtain an overview of the molecular identification of bla_{TEM} and bla_{SHV} genes encoding ESBL producing E. coli from broiler chickens related to biosafety based on the high cases of resistance of E. coli in humans that can be transmitted from food product of animal origin. Broiler chickens for research were taken from several broiler farms in Blitar by cloacal swab. The broiler farms were chosen because of the high supply of broiler chickens and the large number of purchases by consumers in the East Java province.

MATERIALS AND METHODS

Samples

The sample consisted of 95 cloacal swabs taken from broiler chickens, in Blitar, Indonesia.

Isolation and Identification

Ninety five samples taken by the cloacal swab method were then put into a vacutainer tube containing Buffered Peptone Water (BPW) and put into a cool box. Samples were cultured on Mac Conkey Agar (MCA) media for 24 hours at 37ºC. *E. coli* bacterial colonies on MCA media grew with red, convex characteristics, and clear boundaries (2). Then suspected colonies of *E. coli* cultured on EMBA media, as shown on Figure 1. Colonies suspected of being *E. coli* bacteria on EMBA media were again stained with Gram to confirm the morphology and nature of the bacteria. Separate colonies that had been tested for Gram staining were followed by biochemical IMViC tests (Indol, MR-VP, citrate) and TSIA (11, 12). *E. coli* bacteria showed positive indole results and there was motility on the SIM media. In the Methyl-Red (MR) test, E. coli bacteria showed positive results and Voges-Proskauer (VP) with negative results. In the citrate test, E. coli bacteria showed negative results. TSIA test results showed Acid / Acid results, negative H_2S, and positive gas (2).

Confirmation for ESBL producing *Escherichia coli*

ESBL-producing *Escherichia coli* bacteria isolated from cloacal swabs of broilers chicken were confirmed by the Double Disc Synergy Test (DDST). This confirmation test with DDST was conducted to evaluate the presence of inhibitory zones of ESBL activity with clavulanic acid using the Kirby-Bauer disk diffusion method on Mueller-

Hinton agar (Merck, Germany). Double Disc Synergy Test uses the antibiotic disc Amoxycillin-clavulanic 30µg (Oxoid, England), Cefotaxime 30µg (Oxoid, England), Ceftazidime 30µg (Becton Dickinson, USA), and Aztreonam 30µg (Oxoid, England). Culture was incubated at 35-37 ºC for 18-24 hours (13, 14). The results of the evaluation after incubation showed that the inhibition zone that appeared in the plate was measured based on CLSI 2018 guidelines (13) as shown on Figure 2.

Identification of bla_{TEM} and bla_{SHV} genes by Polymerase Chain Reaction (PCR)

The Extended spectrum beta-lactamase-producing *E. coli* bacteria which has been phenotypically confirmed by DDST method, then genotypically confirmed by further analyzing the presence of bla_{TEM}, and bla_{SHV} genes encoding ESBL producing *E. coli* by using molecular identification of PCR. Bacterial DNA was isolated with the QIAamp® DNA mini kit (QIAGEN, Germany). *E. coli* ATCC 35218 was used as a positive control standard for strains of ESBL-producing bacteria and *E. coli* ATCC 25922 was used as a negative control or non-ESBL-producing bacteria (14). The PCR results was visualized by electrophoresis using 2% agarose gel (Invitrogen, USA) (15). The primers used to encode the bla_{TEM} and bla_{SHV} encoding genes refer to Kurekci et al. (2017) (16), as shown in Table 1.

Table 1. Nucleotide sequence of the primers used in PCR

Targets	Sequence (amplicon sizes)	Annealing temperature	Reference
bla_{TEM} gene	F: ATAAAATTCTTGAAGACGAAA R: GACAGTTACCAATGCTTAATC (Amplicon: 1080 bp)	59 ◦ C	16
bla_{SHV} gene	F: CGGCCTTCACTCAAGGATGTA R: GTGCTGCGGGCCGGATAAC (Amplicon: 927 bp)	59 ◦ C	16

RESULTS

This study was conducted for the identification of bla_{TEM} and bla_{SHV} genes among ESBL-producing *E. coli* strains isolated from 95 cloacal swab samples in broilers poultry. The results 10 isolates were confirmation positive of ESBL-producing *E.coli* on broilers chicken cloacal swab by Double Disc Synergy Test (DDST), shown on Figure 2. The presence of ESBLs-producing bacteria by the DDST to detect ESBL producing bacteria and then confirmed by polymerase chin reaction (PCR) and indicated 70% ESBL producing *E.coli* contain bla_{TEM} gene as shown in Table 2. For the identification of bla_{TEM} and bla_{SHV} genes present in ESBL producing *E. coli* PCR was used (16, 17), as shown on Figure 3.

Tabel 2. Extended Spectrum Beta Lactamase (ESBL) producing *E. coli* from Broilers in Blitar

Location	Sample size	*Escherichia coli*	ESBL DDST Test	PCR Product	
				***bla*TEM Gene**	***bla*SHV Gene**
Ponggok	18	18	1	1	Negative
Srengat	30	30	5	4	Negative
Kademangan	7	7	4	2	Negative
Talun	20	20	nil	Not tested	Not tested
Garum	20	20	nil	Not tested	Not tested
Total	**95**	**95**	**10**	**7**	**0**

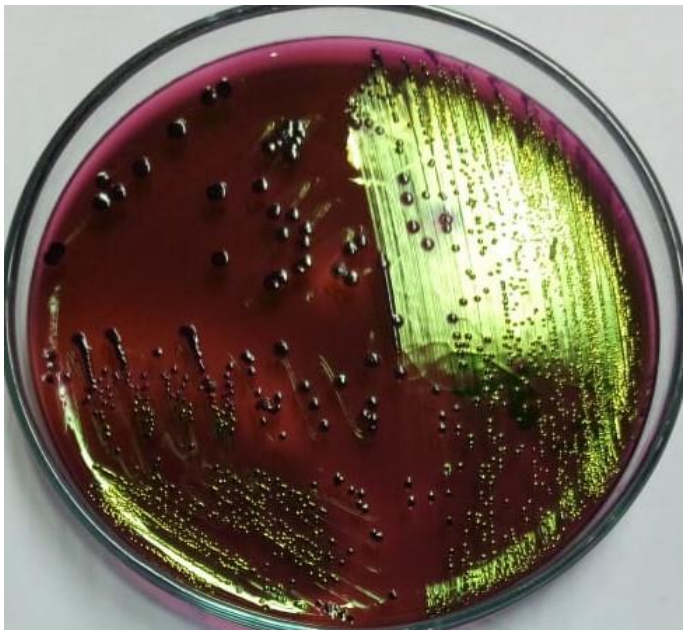

Figure 1. *Escherichia coli* on Eosin Methylen Blue Agar (green metalic color)

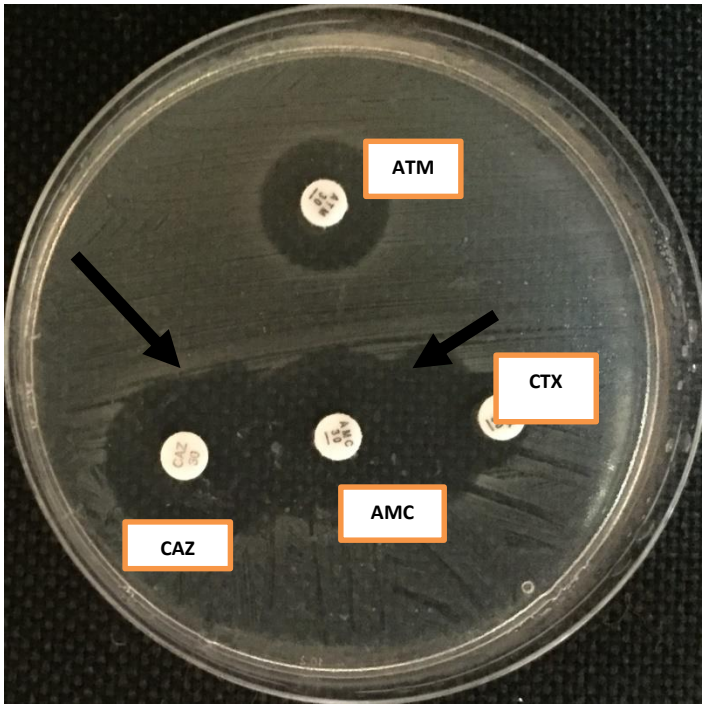

Figure 2. ESBL-producing *Escherichia coli* confirmation test with Double Disc Synergy Test (DDST) (indicator by black arrows)

Note: ATM: Aztreonam, CAZ: Ceftasidime, AMC: Amoxycillin clavulanic, and CTX: Cefotaxime.

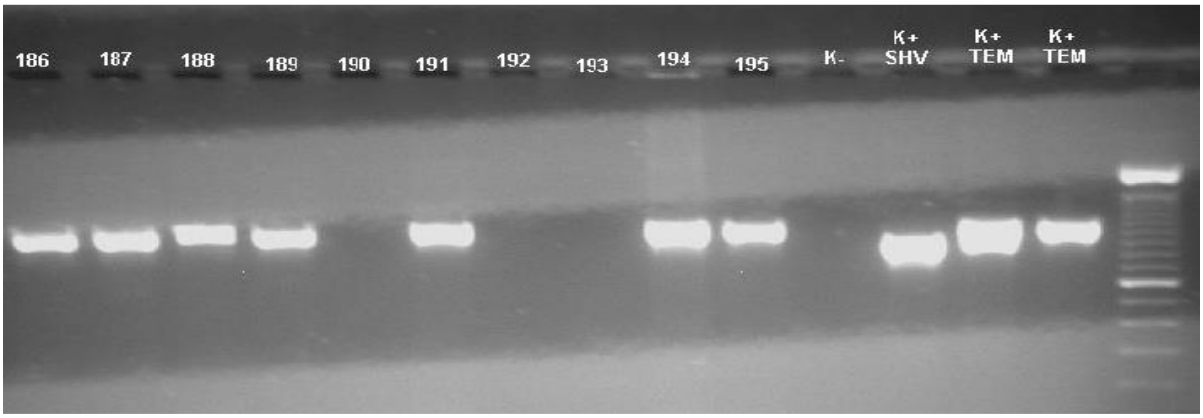

Figure 3. Molecular identification of bla_{TEM}, and bla_{SHV} genes by PCR (PCR product for bla_{TEM} gene = 1080 bp and PCR product for bla_{SHV} gene = 927 bp)

Legend: Code sample 190, 192 and 193 there were no bla_{TEM} and bla_{SHV} genes.

DISCUSSION

Table 2. showed the spread of ESBL-producing *Escherichia coli* in 3 districts from 5 sub-districts. Srengat sub-district has 5 samples, one sample from Ponggok sub-district and Kademangan district was 4 samples from 10 ESBL-producing *Escherichia coli* samples, while Talum and Garum sub-districts were not found any ESBL producing *Escherichia coli*. Several other studies have examined the number of *E. coli* isolates that isolated from animal and animal products, showing concordance results between studies as shown on Table 2. (18). The relative abundance of the ESBL producing *E. coli* in samples from cattle, dogs and poultry has been shown to vary with geographic location (7, 8, 19). In this study, isolates including ESBL producing *E. coli* were dominated by encoding bla_{TEM} gene. Molecular identification as shown in Table 2. that 70% (7/10) samples of ESBL producing *E. coli* encoding bla_{TEM} gene. The bla_{TEM} encoding gene is most commonly found in *E. coli*. Molecular identification shown in Figure 3 that visualization of the bla_{TEM} gene fragment band. Electrophoresis results of bla_{TEM} gene represent samples describing the same fragments as positive controls with a gene length of 1080 bp, however there was not bla_{SHV} gene on represent samples (16), as shown on Figure 3.

The presence of the bla_{TEM} gene in ESBL producing *E. coli* showed that there has been the spread of bacteria that have ESBL enzymes. Based on this study, it was found that the prevalence of the bla_{TEM} gene ESBL producing *E. coli* from cloacal broiler swab samples with the highest number of bla_{TEM} gene was detected compared to other ESBL gene, namely bla_{SHV} gene. These results indicate that the genotype prevalence of ESBL is quite high in Blitar. Of the 10 ESBL isolates producing E. coli, only 3 isolates (30%) did not have the bla_{TEM} and bla_{SHV} genes encoding ESBL. However, it does not rule out that the negative sample has other ESBL genes that were not examined in

this study, considering that ESBL has several classes and each class has several genes. This was in accordance with Wibisono et al., (2020) stated that the bla_{CTX-M} gene is a high ESBL-producing *E. coli* gene that 45 isolates from 46 isolates (97.8%) of ESBL producing ESBL were bla_{CTX-M} encoding gene in from broilers (14). The incidence of ESBL producing *E. coli* from cloaca swabs on broilers chicken was consistent with the incidence of *E. coli* on slaughterhouses in Bogor by 8.6% (20), but smaller compared to the incidence of *E. coli* as ESBL producing *E. coli* from feces of broiler chickens in Bogor ESBL by 25% (21) and the incidence of ESBL producing *E. coli* in India on chicken was around 42% (22). ESBL bacteria can be identified by detecting the presence of ESBL encoding genes (23). This research showed that the bla_{TEM} gene was found in 70% ESBL samples. In this study, the ESBL encoding bla_{TEM} gene was detected dominance of ESBL producing *E. coli* samples from broilers chicken. The bla_{TEM} gene is the most prevalent ESBL type among animals product samples (24). In many countries bla_{TEM} gene is one of the most frequent ESBL types in ESBL-producing bacteria, causing human infections, therefore the evidence of bla_{TEM} gene in this study should be used as reference in controlling the spread of ESBL encoding gene in poultry farms (24). The presence of bla_{TEM} and bla_{SHV} genes are often reported in food from animal origin. In this study the findings of ESBL producing *E. coli* isolates were dominated by the presence of the bla_{TEM} gene. Similar to the research of Hinthong et al. (2017), it was mentioned that *E. coli* contamination found in animal product has a high tendency to be found in ESBL producing *E. coli* bacteria that have the bla_{TEM} gene. This showed that pathogenic *E. coli* sourced from milk is also exposed to antibiotics and has the potential to transfer these genes to other pathogenic bacteria under certain conditions (25).

The spreading of genetic elements such as transposons, insertion and integrons in the bacteria cause ESBL genes move quickly from animals to humans or vice versa. Genetic factors can also spread the virus nature of resistance to other bacteria in animals digestive tract. The bacteria then spread from cage to the surrounding environment through facilitated waste by poor hygiene and sanitation, which pollutes land and water around agriculture. ESBL bacteria are also detected in vegetables, soil and surrounding water agriculture and markets (Wu *et al.,* 2016). The spreading genetic elements can also occur in Gram positive bacteria moving rapidly from animals to humans or vice versa such as in livestock (26 - 30) or pets (31 -33). The presence of ESBL producing *E. coli* is threat to the public health and animal health (34, 35). This condition can occur in limited maintenance options. The steps that can be done is to build supervision program, supervising feed and poultry. Farmers also need to improve biosecurity practice. Garbage and chicken manure must be correct managed in an intensive production system, to prevent air, soil and water contamination, as well negative consequences for human health (36, 37).

CONCLUSION

Ninety-five *E. coli* samples were isolated from cloacal swabs broilers chicken from broiler farms in Blitar, East Java, Indonesia. Ten *E. coli* were classified as ESBL producing bacteria. Through PCR testing, ESBL encoding gene of bla_{TEM} gene was identified in seven samples, and no one sample have bla_{SHV} gene. The presence of ESBL encoding gene in bacteria has potential to spread its resistance to the other bacteria in the gastrointestinal tract of broilers chickens as well as in the poultry environment.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial, personal, or other relationships with other people or organization related to the material discussed in the manuscript.

ACKNOWLEDGEMENT

This research was funded by the Direktorat Riset dan Pengabdian Masyarakat, Deputi Bidang Penguatan Riset dan Pengembangan Kementerian Riset dan Teknologi/ Badan Riset dan Inovasi Nasional, Indonesia. This article is part of the research.

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