




ORIGINAL ARTICLE

## Isolation and detection of antibiotics resistance genes of *Escherichia coli* from broiler farms in Sukabumi, Indonesia

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### ABSTRACT

**Objective:** This study aimed to isolate and identify *Escherichia coli* from broiler samples from Sukabumi, Indonesia. Also, antibiogram studies of the isolated bacteria were carried out considering the detection of the antibiotic resistance genes.

**Materials and Methods:** Cloaca swabs ( $n = 45$ ) were collected from broilers in Sukabumi, Indonesia. Isolation and identification of *E. coli* were carried out according to standard bacteriological techniques and biochemical tests, followed by confirmation of the polymerase chain reaction targeting the *uspA* gene. Antibiotic sensitivity test, using several antibiotics [tetracycline (TE), oxytetracycline (OT), ampicillin (AMP), gentamicin (CN), nalidixic acid (NA), ciprofloxacin (CIP), enrofloxacin (ENR), chloramphenicol, and erythromycin] was carried out following the Kirby–Bauer disk diffusion method. Detection of antibiotic resistance coding genes was carried out by PCR using specific oligonucleotide primers. Statistical analysis was carried out with one-way analysis of variance.

**Results:** The results showed that 55.6% (25/45) of the samples were associated with the presence of *E. coli*. Antibiotic sensitivity test showed that the *E. coli* isolates were resistant to TE (88%; 22/25), OT (88%; 22/25), AMP (100%; 25/25), CN (64%; 16/25), NA (100%; 22/25), CIP (88%; 22/25), ENR (72%; 18/25), chloramphenicol (0%; 0/25), and erythromycin (92%; 23/25). On the other hand, the antibiotic resistance coding genes were *tetA* (86.4%; 19/22), *blaTEM* (100%; 25/25), *aac(3)-IV* (0%; 0/16), *gyrA* (100%; 25/25), and *ermB* (13%; 3/23). It was found that chloramphenicol is markedly different from other antibiotic treatment groups.

**Conclusion:** *Escherichia coli* was successfully isolated from cloacal swabs of broiler in Sukabumi, Indonesia. The bacteria were resistant to TE, OT, AMP, CN, NA, CIP, ENR, and erythromycin. Chloramphenicol was more sensitive and effective than other antibiotics in inhibiting the growth of *E. coli*. The antibiotic resistance genes detected were *tetA*, *blaTEM*, *gyrA*, and *ermB*.

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### KEYWORDS

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### Introduction

Sukabumi district of Indonesia has many chicken farms, mostly broiler farms, having an estimation of 10.096.368 broiler chickens [1]. A high population without adequate management can cause an increase in the incidence of disease or even emerging microbial diseases. Antibiotics are randomly used to treat bacterial infections nowadays [2].

Antibiotics are mostly produced by microorganisms (bacteria and/or fungi), functioning as inhibitors of bacterial growth or killers of other microorganisms [3]. In both humans and livestock, antibiotics have an essential role

in the minimizing disease [4]. Antibiotics are generally given to treat diseases caused by bacterial infections [5]. However, on chicken farms, antibiotics are used for treatment, disease prevention, and growth triggers [6]. The unauthorized use of antibiotics is one of the risk factors influencing growing antibiotic resistance [5].

*Escherichia coli* acts as normal flora present in the digestive tract of humans, animals, and birds [7]. *Escherichia coli* resistance to various antibiotics was recorded at 88.2% in chickens. It is a massive concern for animal health and the veterinary communities [8]. *Escherichia coli* resistance

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to antibiotics in chickens can be transferred directly or indirectly through foods and other chickens or animals. Resistant bacteria colonize and can share their resistance properties to normal flora. Resistance genes are transferred vertically between genera and family or horizontally between bacteria in the genus and family [9]. Therefore, this research aims to isolate and identify *E. coli* and assessing antibiotic sensitivity patterns considering the antibiotic resistance genes.

## Materials and methods

### Ethical statement

Written or verbal permission was taken from the farm owner/manager/chicken handler before sample collection. A professional veterinarian did the sample collection. No animals were used for many experiments in this study.

### Sample collection

Sample collection used a random sampling method. Cloacal swab samples ( $n = 45$ ) were collected in January 2019 from healthy broilers in Sukabumi, Indonesia. The samples were stored in buffer peptone water in an ice box at 4°C during transportation. The samples were stored in a refrigerator at 4°C until further analysis.

### Microbiological analysis

Samples were cultured by direct streaking method on Eosin Methylene Blue and MacConkey agars, and then incubated for 18–24 h at 37°C [10]. The tests were carried out on triple sugar iron agar media, urea, and indole, methyl-red, Voges–Proskauer, and citrate (IMViC) media. Bacterial incubation in the triple sugar iron agar and urea media was carried out at 37°C for 18–24 h. IMViC test was carried out at 37°C for 48 h. Test results referring to *E. coli* were then stained with Gram stain to determine bacterial cell morphology [7].

According to the manufacturer's instructions, the Presto™ Mini gDNA bacteria kit (Geneaid) was used to extract bacterial DNA. Confirmation of *E. coli* isolates was carried out using the PCR method with MyTaq™ HS Red Mix (Bioline). The *uspA* gene amplification was carried out using primers reported by Mishra et al. [11]. A volume of 25 µl was prepared for conducting PCR reactions containing 4 µl template, 2 µl forward primer (20 µM), 2 µl reverse primer (20 µM), 12 µl MyTaq™ Red Mix (2×), and added ddH<sub>2</sub>O to 25 µl. Thermal Cycler T100™ (Bio-Rad) was used to carry out PCR amplification.

Pre-denaturation was carried out at 95°C for 1 min. Amplification of 30 cycles consisted of denaturation at 95°C for 15 sec, annealing at 58°C for 15 sec, and extension

at 72°C for 10 sec. A final extension step was carried out at 72°C for 5 min. Electrophoresis for PCR products was carried out on 1% agarose gel in TAE buffer (1×). The staining for the amplified DNA fragments was carried out by using 2 µl FloroSafe DNA Stain (1st BASE). A DNA marker (100 bp) was used as a standard. The positive samples for the *uspA* gene will show DNA bands at an amplicon length of 884 bp. The positive control bacterium was *E. coli* ATCC 25922.

### Antibiotics sensitivity testing

The Kirby–Bauer's disk diffusion method was used in this study to identify antibiotic resistance patterns in *E. coli* [9]. Bacterial suspension from Tryptic Soy agar was diluted with sterile physiological NaCl to make the standard equivalent of  $1.5 \times 10^8$  colony forming units/ml with the 0.5 McFarland standard. The suspension was cultured on Mueller–Hinton agar media using a sterile cotton bud then antibiotic disks were put on the agar surface. Incubation was carried out for 16–18 h at 35°C. Antibiotics used in this study included tetracycline (TE) 30 µg/disk, oxytetracycline (OT) 30 µg/disk, ampicillin (AMP) 10 µg/disk, gentamicin (CN) 10 µg/disk, nalidixic acid (NA) 30 µg/disk, ciprofloxacin (CIP) 5 µg/disk, enrofloxacin (ENR) 5 µg/disk, chloramphenicol (C) 15 µg/disk, and erythromycin (E) 30 µg/disk. Antibiotic inhibition zones formed on the Mueller–Hinton agar medium were then measured and adjusted to the standards set by Clinical and Laboratory Standards Institute [12].

### Detection of antibiotic resistance genes

According to the manufacturing procedures, the PCR detection of antibiotic resistance genes using the MyTaq™ HS Red Mix (Bioline) was carried out. The specific primer pairs were prepared for *tetA* [13], *blaTEM* [14], *aac(3)-IV* [15], *gyrA* [16], and *ermB* gene [17]. The 25 µl PCR component consisted of 4 µl template, 2 µl forward primer (20 µM), 2 µl reverse primer (20 µM), 12 µl MyTaq™ Red Mix (2×), and then added ddH<sub>2</sub>O to 25 µl. The PCR process was carried out with Thermal Cycler T100™ (Bio-Rad) and then visualized by electrophoresis.

### Statistical analysis

Statistical analysis for each antibiotic group was carried out using the one-way analysis of variance method using Statistical Package for the Social Sciences 21 software to see its difference. Follow-up tests were carried out to see which group was different. First, the homogeneity test of variance; if the homogeneous variety is continued with Scheffe's test, if the variance is not homogeneous, then the Dunnett C test is used. A  $p$ -value < 0.05 was considered significant.

## Results and Discussion

Overall, the prevalence of *E. coli* was 55.6% (25/45). Confirmation of *E. coli* was carried out by targeting the *uspA* gene. The *uspA* gene plays an essential role for *E. coli* to survive during growth and helps in adhesion and motility. As long as the bacteria are growing normally, *uspA* does not affect, but when the conditions are not sufficient, for example, lack of carbon, the *uspA* will be expressed [18]. In this study, the *uspA* gene in *E. coli* could be amplified using specific primers. The amplification product by PCR was 884-bp (Fig. 2a).

The antibiotic sensitivity of *E. coli* isolates was conducted against nine antibiotics. The antibiotics used in this study were often used in Indonesian chicken farms. Based on the data from the Directorate General and Animal Health

of Indonesia [6], the widely used antibiotics included: ENR (49.4%), CIP (5.1%), OT (4.8%), AMP (3.9%), and erythromycin (3.1%). Zalizar et al. [19] stated that TE (1.83%) and aminoglycoside (0.46%) antibiotics were also widely used in Indonesian chicken farms. Table 1 shows the patterns of antibiotic resistance in *E. coli*; eight antibiotic resistance patterns were recorded. Each pattern shows *E. coli* resistance to AMP and NA. All isolates tested showed multidrug-resistant (MDR) properties. Multidrug resistance is a condition of resistance to three or more antibiotics groups in an isolate [20]. Two main mechanisms cause the nature of MDR in bacteria; they are (1) accumulation of several genes in plasmid R (resistance), each of which encodes the resistance to one antibiotic in a single cell, and (2) due to the increased gene expression that encodes the efflux pump against several antibiotics [21].

Antibiotic resistance in this study (Fig. 1) followed several studies conducted in Indonesia. According to Edityandari [22], *E. coli* from chickens were resistant to erythromycin (100%), AMP (100%), ENR (80%), OT (20%), and still sensitive to CN. The utilization of antibiotics, while not an oversight, will cause cases of antibiotic resistance. According to Arief et al. [23], 72.3% of the farmers used antibiotics without veterinary supervision. Antibiotics are recommended to treat bacterial infections. However, only 30.2% of the farmers in Indonesia used antibiotics for treatment purposes. The majority of breeders in Indonesia (81.4%) use antibiotics as disease prevention. Besides, a small proportion of farmers (0.3%) use antibiotics as a growth promoter [6].

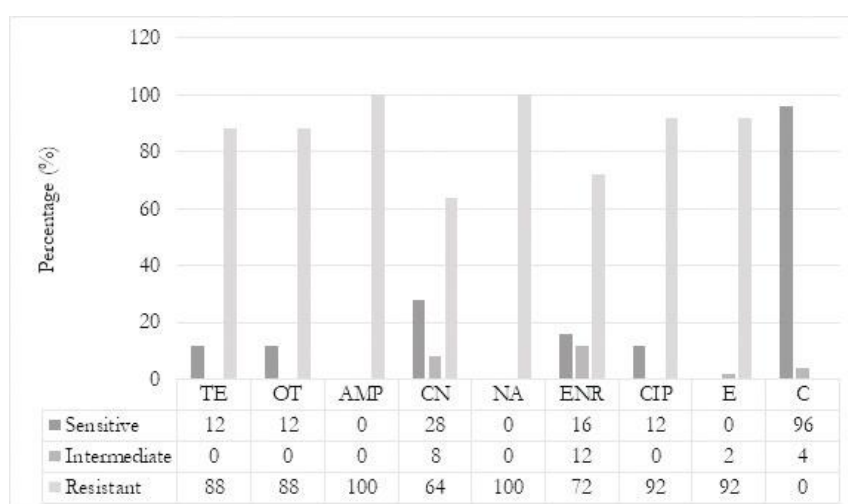
Antibiotics added to feed as antibiotics growth promoters are considered to trigger livestock growth significantly in a relatively short time. Furthermore, it increases feed

**Table 1.** Antibiotics resistance patterns in *E. coli*.

No	Antibiotic groups	Resistance Patterns	Resistant isolates
1	6	AMP <sup>1</sup> -NA <sup>2</sup> -E <sup>3</sup> -TE <sup>4</sup> -OT <sup>4</sup> -CIP <sup>5</sup> -ENR <sup>5</sup> -CN <sup>6</sup>	10
2	5	AMP <sup>1</sup> -NA <sup>2</sup> -E <sup>3</sup> -TE <sup>4</sup> -OT <sup>4</sup> -CIP <sup>5</sup> -ENR <sup>5</sup>	6
3	5	AMP <sup>1</sup> -NA <sup>2</sup> -TE <sup>4</sup> -OT <sup>4</sup> -CIP <sup>5</sup> -ENR <sup>5</sup> -CN <sup>6</sup>	3
4	5	AMP <sup>1</sup> -NA <sup>2</sup> -E <sup>3</sup> -CIP <sup>5</sup> -ENR <sup>5</sup> -CN <sup>6</sup>	1
5	5	AMP <sup>1</sup> -NA <sup>2</sup> -E <sup>3</sup> -CIP <sup>5</sup> -CN <sup>6</sup>	1
6	4	AMP <sup>1</sup> -NA <sup>2</sup> -TE <sup>4</sup> -OT <sup>4</sup> -CIP <sup>5</sup> -ENR <sup>5</sup>	1
7	4	AMP <sup>1</sup> -NA <sup>2</sup> -E <sup>3</sup> -TE <sup>4</sup> -OT <sup>4</sup>	2
8	3	AMP <sup>1</sup> -NA <sup>2</sup> -CN <sup>6</sup>	1

<sup>1</sup>β-lactamase group, <sup>2</sup>Quinolone group, <sup>3</sup>Macrolide group, <sup>4</sup>Tetracycline group, <sup>5</sup>Fluoroquinolone group, <sup>6</sup>Phenocoll group.

AMP = ampicillin, NA = nalidixic acid, E = erythromycin, TE = tetracycline, OT = oxytetracycline, CIP = ciprofloxacin, ENR = enrofloxacin, CN = gentamicin.



**Figure 1.** Antibiogram profile of *E. coli* from the poultry farm in Sukabumi. TE = tetracycline, OT = oxytetracycline, AMP = ampicillin, CN = gentamicin, NA = nalidixic acid, ENR = enrofloxacin, CIP = ciprofloxacin, E = erythromycin, C = chloramphenicol.

efficiency. The addition of antibiotics to feed in Indonesia has been carried out since 1970 when broiler chicken farms began to develop [24]. Since the 1990s, some countries have been using antibiotics as food additives, like Sweden (1986), Denmark (1995), German (1996), and Switzerland (1996) [25]. Prohibition of the addition of antibiotics in feed in Indonesia has existed since 2009, which is regulated by Law Number 18 of 2009 Article 22 Paragraph 4c. However, the law has not been effectively implemented. The Indonesian Ministry of Agriculture has reiterated the ban since January 2018, with Permentan Number 14 of 2017 [26].

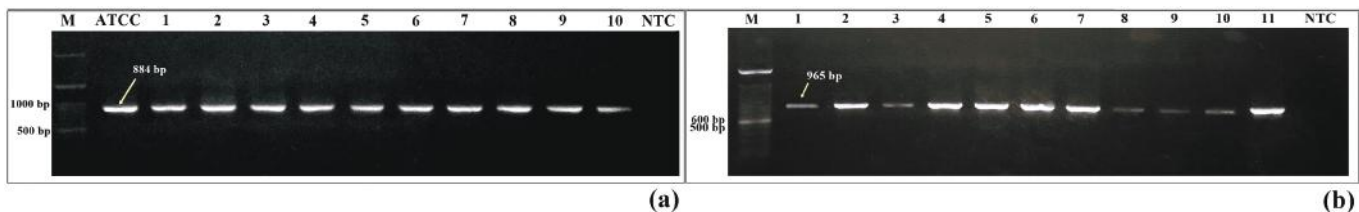
The efflux pump activity in antibiotic-resistant Gram-negative bacteria was encoded by *tet*, one of which is *tetA*. Efflux pump activity removes TE from cells using protons as an energy source [27]. The results of *tetA* gene amplification (Fig. 2b) are shown with 965-bp amplification products. Isolates of *E. coli* are TE and OT-resistant, which has 86.4% *tetA* gene (19/22). These findings supported the previous report of Ibrahim et al. [28], who described that 78.4% of *E. coli* isolates possessed the *tetA* gene. According to van Hoek et al. [29], in addition to the *tetA* gene, other *tet* genes could encode bacterial resistance to the TE group. The other *tet* genes included *tetB*, *tetC*, *tetD*, and *tetE* [30].

The  $\beta$ -lactamase enzymes induce the  $\beta$ -lactam group's resistance in many bacteria, especially the enzyme extended-spectrum  $\beta$ -lactamases (ESBLs). ESBL will hydrolyze the AMP  $\beta$ -lactam ring in the periplasm of bacteria. Broken bonds cause antibiotics to fail, so antibiotic reactions do not occur with Penicillin Binding Proteins PBPs [31]. One of the ESBLs encoding genes is *blaTEM*. The TEM  $\beta$ -lactamase produced by clinical *E. coli* strains was firstly reported in

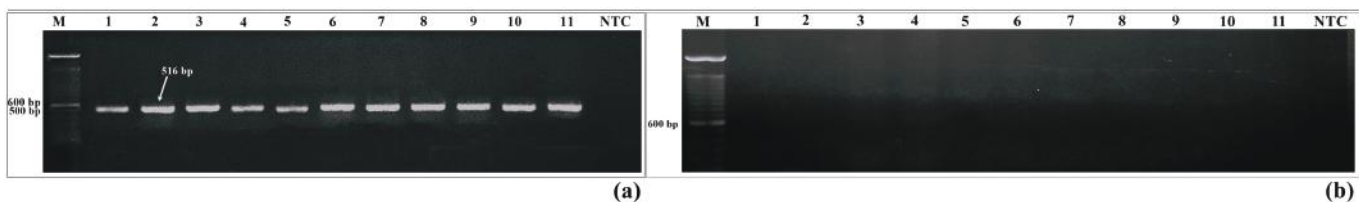
1965 [32]. In this research, the *blaTEM* gene was present in all isolates (100%; 25/25) with a 516-bp PCR product (Fig. 3a), as reported by Hayati et al. [33].

Bacterial resistance to aminoglycosides is primarily occurred by modifying the chemical composition of antibiotics by aminoglycoside modifying enzymes. The enzyme consists of three subclasses based on chemical modifications made to aminoglycosides, namely AG N-acetyltransferases, AG O-nucleotidyltransferases, and AG O-phosphotransferases. Every subclass modifies certain specific positions [34]. The *aac(3)-IV* gene was not present (0%, 0/16) in this study (Fig. 3b). In contrast to the research by Amer et al. [35], eight (40%; 8/20) isolates of *E. coli* were detected having the *aac(3)-IV* gene. *E. coli* might have other genes related to CN resistance, namely *aac(3)-I*, *aac(3)-II*, *aac(3)-III*, *aac(3)-VIII*, *aac(6)-I*, and *aac(6)-II* [36].

In this study, all the isolates showed (100%; 25/25) the presence of the *gyrA* gene. The *gyrA* gene amplification product was 626-bp (Fig. 4a). A high percentage (98.3%) of *gyrA* gene detection was also reported by Ogbolu et al. [37]. The resistance to CIP, NA, and ENR found in this study probably resulted from mutations in the *gyrA*. According to Hopkins et al. [38], quinolone and fluoroquinolone resistance are mostly caused by changes in the *gyrA* gene. The target protein change occurs in the terminal amino acid domain, the quinolone resistance determining region. The amino acid substitution will change the target protein structure, thus decreasing the affinity by enzyme [39]. Based on the report of Ogbolu et al. [37], Gram-negative bacteria having resistance property to CIP and NA undergo amino acid substitution in *gyrA* gene codon 83 protein

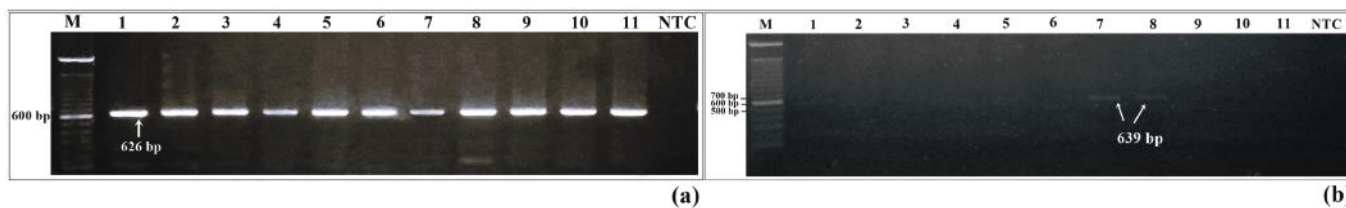


**Figure 2.** Amplification *uspA* gene 884-bp (a) and *tetA* gene 965-bp (b) from *E. coli*. M = DNA marker 100 bp, ATCC = *E. coli* ATCC 25922 as a positive control, 1–11 = *E. coli* isolates, NTC = non-template control.



**Figure 3.** Amplification *blaTEM* gene 516-bp (a) and *aac(3)-IV* gene 286-bp (b) from *E. coli*. The *aac(3)-IV* gene was not detected. M = DNA marker 100 bp, 1–11 = *E. coli* isolates, NTC = non-template control.





**Figure 4.** Amplification *gyrA* gene 626-bp (a) and *ermB* gene 639-bp (b) from *E. coli*. M = DNA marker 100 bp, 1-11 = *E. coli* isolates, NTC = non-template control.

(Serin → Leucine). The *gyrA* gene of ENR-resistant *E. coli* changed the codon 83 (Serin → Leucine) and 87 (Aspartate → Glycine) [40].

The *ermB* gene amplification results in erythromycin-resistant *E. coli* isolates showed 13% (3/23) positive isolates of the *ermB* gene with 639-bp amplification products (Fig. 4b). According to Cesur and Demiroz [41], erythromycin-resistant isolates are caused by Gram-negative bacteria's impermeable nature to macrolide that is hydrophobic. The action of macrolide antibiotics is to inhibit protein synthesis. Erythromycin binds 50s ribosome subunit in bacteria so that protein synthesis is inhibited [42]. The *erm* gene (erythromycin ribosome methylase) encodes the Erm methyltransferase enzyme group. The Erm methyltransferase enzyme modifies a single 23s rRNA in the 50s ribosome subunit. For this reason, it causes a decrease in the affinity of the antibiotics bond [43].

The mean test results for group differences showed a mean difference in each antibiotic group with a value of  $p = 0.00$  ( $p < 0.05$ ). The Dunnett C test was chosen for the follow-up test because there were differences in each group's mean. Chloramphenicol had a significant difference in mean difference with all groups. The TE treatment group had a significantly different mean difference with CN, ENR, and CIP, in addition to the chloramphenicol group. In addition to having differences with the chloramphenicol treatment group, AMP had a significantly different mean difference with CN, ENR, CIP, erythromycin, and chloramphenicol. The CN treatment group also had a significantly different mean difference with NA and erythromycin. The NA treatment group had a significantly different mean difference with ENR, CIP, and erythromycin. AMP and TE antibiotics are indeed the antibiotics that are often used in the world of chicken farming [6,14].

The findings of this study enrich the data of current antibiotic resistance conditions. Antibiotic-resistant bacteria in animals multiply and become the dominant bacterial population. They transmit their antibiotics resistance genes to offspring via vertical gene transfer, called innate or natural or intrinsic resistance. Antibiotic-resistant bacteria, called acquired resistance, can horizontally transfer their resistance genes within and between bacterial species [44].

Veterinarians, farmers, abattoir workers, and food handlers can be contaminated by resistant bacteria directly, where the exchange and acquisition of resistance mechanisms occur [45]. The indirect transmission along the food chain is a complex pathway. By contact or ingestion of infected food items, humans may be exposed to resistant bacteria. The presence of resistant bacteria in various food products from different animal sources (poultry, cattle, pigs, goats, and sheep) and diverse food production stages has been identified [46,47]. Many antibiotic resistance consequences include inadequate treatment of pathogenic bacterial infection, increased patient-level morbidity and mortality, increased resource use, higher costs, and decreased hospital operation at the healthcare level [48].

## Conclusion

MDR *E. coli* have been isolated from poultry farms in Sukabumi, Indonesia. The *E. coli* were resistant to TE, OT, AMP, CN, NA, CIP, ENR, and erythromycin. The genes, namely *tetA*, *blaTEM*, *gyrA*, and *ermB*, were present in the *E. coli* isolates.

## List of abbreviations

°C, degree CelciusCelsius; PCR, polymerase chain reaction; TAE, Tris Acetic EDTA; ATCC, American Type Culture Collection; ml, milliliter;  $\mu$ l, microliter; h, hours; min, minutes; sec, seconds; bp, base pair.

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## Conflict of interest

The authors declare that they have no conflict of interests.

## Authors' contribution

FHP and S designed the study. AH was involved in laboratory work, interpreted the data, and drafted the manuscript. S, IWTW, and AI took part in the critical checking of this manuscript. All the authors read and approved the publication of this article.

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