

Research

## Phenotypic and Genotypic Study of Antibiotics Resistance Profile in *Escherichia coli* Isolated from Broilers Farm in Cianjur, Indonesia

### Kajian Fenotip dan Genotip Profil Resistensi Antibiotik pada *Escherichia coli* yang Diisolasi dari Peternakan Ayam Broiler di Cianjur, Indonesia

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#### ABSTRACT

This study aimed to investigate the phenotypic and genotypic of antibiotics resistance profile in *Escherichia coli*. The 30 samples come from cloacal swab of broilers in Cianjur, Indonesia. Isolation and identification of *E. coli* was performed by culturing in McConkey agar, eosin methylene blue agar, Gram staining and five essential biochemical tests (indole, methyl red, Voges-Proskauer, and citrate). In this study, 10 isolates (33.3%) were confirmed *E. coli* positive. Phenotypic profile was performed by screening all isolates with 8 antibiotics of 6 antibiotic groups. The screening was carried by Kirby-Bauer disk diffusion method based on the standard of CLSI. For genotypic profile, each resistant isolate was detected antibiotic resistance-encoding gene. The result showed all isolates (100%) resistant against tetracyclin, oxytetracycline and erythromycin. Nine isolates (90%) detected nalidixic acid and enrofloxacin-resistant. The ciprofloxacin and gentamicin-resistant isolates were 70% and 40%, respectively. There was no resistant isolate for chloramphenicol. Multi drug-resistant was detected on 90% isolates. Only *gyrA* (100%) and *tetA* (80%) genes were detected. This study showed high rate of occurrence of antibiotic resistance in *E. coli*. Not all resistant isolates were detected to have target genes encoding antibiotic resistance in this study.

**Keywords:** *Escherichia coli*, broiler, antibiotic resistance, phenotypic, genotypic

#### ABSTRAK

Penelitian ini bertujuan untuk mengetahui fenotip dan genotip profil resistensi antibiotik pada *Escherichia coli*. Sampel yang digunakan diambil dari swab kloaka ayam pedaging di Cianjur, Indonesia. Isolasi dan identifikasi *E. coli* dilakukan dengan membiakkan pada agar McConkey, agar eosin metilen biru, pewarnaan Gram dan uji biokimia esensial (indole, methyl red, Voges-Proskauer, dan sitrat). Sepuluh isolat (33,3%) dikonfirmasi positif *E. coli*. Identifikasi profil fenotip dilakukan dengan melakukan penyaringan semua isolat dengan 8 antibiotik dari 6 kelompok antibiotik. Penyaringan dilakukan dengan metode difusi cakram Kirby-Bauer berdasarkan standar CLSI. Identifikasi profil genotip masing-masing isolat dilakukan dengan deteksi gen penyandi resistensi antibiotik. Hasil penelitian menunjukkan semua isolat (100%) resisten terhadap tetrasiklin, oksitetrasiklin dan eritromisin. Sembilan isolat (90%) mendeteksi asam nalidixat dan resisten terhadap enrofloksasin. Isolat yang resisten terhadap ciprofloxacin dan gentamisin adalah 70% dan 40%. Tidak ada isolat yang resisten terhadap kloramfenikol. Resistensi karena beberapa jenis obat (*multi drug-resistant*) terdeteksi pada 90% isolat. Gen yang terdeteksi hanya *gyrA* (100%) dan *tetA* (80%). Penelitian ini menunjukkan tingginya angka kejadian resistensi antibiotik pada *E. coli*. Tidak semua isolat resisten terdeteksi memiliki target gen penyandi resistensi antibiotik dalam penelitian ini.

**Kata kunci:** *Escherichia coli*, broiler, resistensi antibiotik, fenotip, genotip

## INTRODUCTION

Antibiotic resistance has become an increasingly uncontrollable health problem in recent years. Global awareness of the emergence of multi drug-resistant bacteria (MDR) has increased but new MDR strains have also increased (Solomon & Oliver, 2014). Therefore, the health threat due to MDR bacterial infection is increasing (Mohammad et al., 2015). Antibiotic resistance can lead to ineffectiveness of standard treatment, persistent infection, and can spread to other individuals (WHO, 2019). Patients with these infections need a more complicated treatment plan because of the ability of bacterial cells to fight off antibiotics, resulting in higher morbidity and mortality rates (Bonomo, 2000). The United States Centers for Disease Control and Prevention (CDC) estimated in 2013 that at least 2 million people acquire antibiotic-resistant bacterial infections each year and more than 23 thousand people die from these infections (Mohammad et al., 2015).

Improper use of antibiotics in livestock, farms, domestic animals, plants and humans causes antibiotic resistance in agricultural and clinical biomes. Antibiotic resistance to pathogenic bacteria is caused by the widespread and uncontrolled use of antibiotics in agricultural and medical (Cameron & McAllister, 2016). Many countries in the world have made substantial efforts to reduce the use of antibiotics in food-producing animals. These efforts include establishing national reduction targets, implementing a ban on antibiotics in animal feed for food-producing animals, comparing the use of antibiotics at the farm level, and encouraging antibiotic stewardship such as requiring susceptibility testing prior to the use of high priority antibiotics. This kind of intervention is expected to reduce the rate of antibiotic resistance in animals, the environment, and in humans (Karen et al., 2017).

*Escherichia coli*, including the family *Enterobacteriaceae*, acts as commensal bacteria and pathogens. They can be found in the environment, food, animal intestines, and human intestines (Markey et al., 2013). It is known that *E. coli* is a bacterium that has antibiotic resistance properties (Kurnia et al., 2018). According to WHO (2017), *E. coli* is the main bacterial cause of the spread of antibiotic resistance. It expresses the MDR mechanism by multiple and varied mechanisms i.e. changing the target site to prevent antibiotic binding, stopping the active pump of the antibiotic entry pathway into cells and impaired absorption of antibiotics due to reduced outer membrane permeability (Tilak, 2011). Expression of antibiotic resistance mechanisms encoded by certain

genes. Bacteria that are resistant to more than one antibiotic class also have more than one resistance coding gene. Even bacteria that are resistant to one antibiotic can have more than one resistance coding gene. Tetracycline-resistant *E. coli* was detected to have the *tetA* and *tetB* genes; quinolone resistance was detected to have the *qnrA*, *qnrB*, and *qnrS* genes (Kurnia et al., 2018).

Among Gram negative bacteria, *E. coli* has the highest resistance pattern (Sukhumungoon et al., 2011). Antibiotic resistance testing is important to understand and monitor the pattern of antibiotic resistance conditions in *E. coli*, especially those from production animals. Therefore, the main objective of this study was to investigate the phenotype and genotype phenotypic and genotypic profile of antibiotic resistance in *E. coli* isolated from cloacal swab samples of broilers.

## MATERIALS AND METHODS

### Materials

Materials used in this study were thirty cloacal swab samples from broilers, 0.1% buffered peptone water (Oxoid CM1049, Thermo Scientific, England), tryptone soya agar (Oxoid CM0131, Thermo Scientific, England), MacConkey agar (Oxoid CM0007, Thermo Scientific, England), eosin methylene blue agar (Levine) (L-EMB) (Oxoid CM0069, Thermo Scientific, England), methyl red-Voges Proskauer (MR-VP) medium (Oxoid CM0043, Thermo Scientific, England), Kovacs indole reagent (1.09293.0100 Milipore, Merck, Germany), KOH 40%, Mueller Hinton agar (MHA) (Oxoid CM0337, Thermo Scientific, England), tryptone soya broth (Oxoid CM0129, Thermo Scientific, England), antibiotic disks, i.e. tetracycline (TE) 30 µg (Oxoid OT 30 CT0054B, Thermo Scientific, England), oxytetracycline (OT) 30 µg (Oxoid OT 30 CT0041B, Thermo Scientific, England), gentamicin (CN) 10 µg (Oxoid CN 10 CT0024B, Thermo Scientific, England), nalidixic acid (NA) 30 µg (Oxoid NA 30 CT0031B, Thermo Scientific, England), ciprofloxacin (CIP) 5 µg (Oxoid CIP 5 CT0425B, Thermo Scientific, England), enrofloxacin (ENR) 5 µg (Oxoid ENR 5 CT0639B, Thermo Scientific, England), chloramphenicol (CL) 30 µg (Oxoid CL 30 CT0013B, Thermo Scientific, England), and erythromycin (E) 15 µg (Oxoid E 15 CT0020B, Thermo Scientific, England), Presto™ Mini gDNA bacteria kit (GBB300/301, Geneaid, Taiwan), MyTaq™ HS Red Mix (Bioline BIO-25048, England), FloroSafe DNA Stain (BIO-5130, 1st

BASE, Malaysia), 100 bp DNA ladder (Invitrogen 15628019, Thermo Scientific, USA), BlueJuice™ Gel Loading Buffer (10X) (Invitrogen 10816015, Thermo Scientific, USA), Tris-Acetate-EDTA (TAE) buffer (1×) (T9650, Sigma-Aldrich, Germany) was carried at 70 Volt 50 minutes by electrophoresis power supply Bio-Rad Power Pac 300 (15008 C24, Bio-Rad, California).

### Samples Collection

Thirty cloacal swab samples were collected from broilers at a farm in Cianjur, West Java, Indonesia. Each sample was placed into tube containing 5 ml of 0.1% buffered peptone water (Oxoid CM1049, Thermo Scientific, England), and labelled with the identity of the sample. All of samples were stored in a cool box with a temperature of 4-10 °C and immediately taken to the Medical Microbiology Laboratory, Faculty of Veterinary Medicine, IPB University.

### Isolation and Identification of *Escherichia coli*

The samples were cultured into MacConkey agar (Oxoid CM0007, Thermo Scientific, England) and incubated at 37 °C for 18-24 hours. The pink colonies surrounded by pink on the media because of acidic conditions resulting from lactose fermentation were transferred into eosin methylene blue agar (Levine) (L-EMB) (Oxoid CM0069, Thermo Scientific, England) and incubated at 37 °C for 18-24 hours. The expected *E. coli* colonies were shown as methalic sheen color. A suspicious colony from each eosin methylene blue agar (Levine) (L-EMB) (Oxoid CM0069, England) plate inoculated to tryptone soya agar (Oxoid CM0131, England) slant and incubated for 18-24 hours at 37 °C for further test. Gram staining of suspicious colonies was carried out to determine bacterial cell morphology. The five essential biochemical tests were carried out for confirmation consisting of indole, methyl red, Voges-Proskauer, and citrate (IMViC). All cultures that produced IMViC patterns of ++- were considered to be *E. coli* (Markey *et al.*, 2013).

### Antibiotics Resistance Test

Antibiotic resistance test was conducted to all *E. coli* colonies isolated from cloacal swab samples. Test referred Clinical and Laboratory Standards Institute guidelines (CLSI, 2018) using the Kirby-Bauer disk diffusion method. Nine antibiotics were used in this test, i.e. . tetracycline (TE) 30 µg (Oxoid OT 30

CT0054B, Thermo Scientific, England), oxytetracycline (OT) 30 µg (Oxoid OT 30 CT0041B, Thermo Scientific, England), gentamicin (CN) 10 µg (Oxoid CN 10 CT0024B, Thermo Scientific, England), nalidixic acid (NA) 30 µg (Oxoid NA 30 CT0031B, Thermo Scientific, England), ciprofloxacin (CIP) 5 µg (Oxoid CIP 5 CT0425B, Thermo Scientific, England), enrofloxacin (ENR) 5 µg (Oxoid ENR 5 CT0639B, Thermo Scientific, England), chloramphenicol (CL) 30 µg (Oxoid CL 30 CT0013B, Thermo Scientific, England), and erythromycin (E) 15 µg (Oxoid E 15 CT0020B Thermo Scientific, England). Bacterial colonies that had been grown on tryptone soya agar for 24 hours at 37 °C were suspended in physiological NaCl and then matched with 0.5 McFarland standard ( $1-2 \times 10^8$  CFU/ml). Bacterial was cultured spread on Mueller Hinton Agar (Oxoid CM0337, Thermo Scientific, England) surface and left for ± 5 minutes. The disks contained antibiotic were put on Mueller Hinton Agar (Oxoid CM0337, Thermo Scientific, England). The cultured bacteria were incubated at 35 °C for 16-18 hours. Inhibition zones formed were then measured. The categories of susceptible, intermediate, and resistant were based on the size of the according to the standard of CLSI 2018.

### Preparation DNA Template

*Escherichia coli* isolates were inoculated into tryptone soya broth (Oxoid CM0129, Thermo Scientific England) and incubated for 18-24 hours at 37 °C. Bacterial suspension were centrifuged for 1 minute  $14.000-16.000 \times g$  and the pellets were taken for DNA extraction. DNA extraction was using Presto™ Mini gDNA bacteria kit (GBB300/301, Geneaid, Taiwan) and stored at -20 °C for further use.

### Detection Antibiotics Resistance Genes

Detection *tetA*, *aac3-IV*, *gyrA*, and *ermB* as antibiotics resistance genes in *E. coli* isolates used polymerase chain reaction (PCR) method (Kurnia *et al.*, 2018). This reaction was carried out by MyTaq™ HS Red Mix (Bioline BIO-25048, England) according manufacturer instruction. Annealing temperature was set according to each of the target genes (Table 1). Electrophoresis for PCR products on 1% agarose gel and Tris-Acetate-EDTA (TAE) buffer (1×) (T9650, Sigma-Aldrich, Germany) was carried at 70 Volt 50 minutes by electrophoresis power supply Bio-Rad Power Pac 300 (15008 C24, Bio-Rad, California). DNA staining used 2 µl FloroSafe DNA Stain (BIO-5130, 1st BASE, Malaysia). 100 bp DNA ladder (Invitrogen

Table 1 Primer sequence used for detection antibiotics resistance genes of *E. coli*

Gene target	Base sequence	Amplicon	Annealing temperature	Reference
<i>tetA</i>	(F)5'-GTA ATT CTG AGC ACT GTC GC-3' (R)5'-CTG CCT GGA CAA CAT TGC TT-3'	965 bp	62 °C	Chuah et al., 2018
<i>aac(3)-IV</i>	(F)5'-CTT CAG GAT GGC AAG TTG GT-3' (R)5'-TCA TCT CGT TCT CCG CTC AT-3'	286 bp	55 °C	Van et al., 2008
<i>gyrA</i>	(F)5'-CGA CCT TGC GAG AGA AAT-3' (R)5'-GTT CCA TCA GCC CTT CAA-3'	626 bp	62 °C	Nawaz et al., 2012
<i>ermB</i>	(F)5'-GAA AAG GTAC TCA ACC AAA TA-3' (R)5'-GTA ACG GTA CTT AAA TTG TTT AC-3'	639 bp	54 °C	Song et al., 2004

Notes : *tetA*: for tetracycline and oxytetracycline resistant; *aac(3)-IV*: gentamicin resistant; *gyrA*: for nalidixic acid, ciprofloxacin or enrofloxacin resistant; and *ermB*: for erythromycin resistant

15628019, Thermo Scientific, USA) was used as standard. The gene bands formed were adjusted to DNA marker to determine the approximate base length. The size of the DNA bands that have estimated base lengths were matched with references in Table 1. The detection antibiotics resistance genes were only for resistant isolates.

### Data Analysis

Data were presented in tables and analyzed descriptively.

## RESULTS

On the basis of colonies morphology, biochemical and staining properties, 30 the samples (33.3%) were *E. coli* positive. Among 10 *E. coli* isolates, all (100%) isolates were resistant to tetracycline, oxytetracycline, and erythromycin. Nine isolates (90%) were resistant to nalidixic acid and enrofloxacin. There were no isolates susceptible to chloramphenicol (20% intermediate; 80% susceptible). Seven isolates (70%) showed resistant against ciprofloxacin and only 4 (40%) isolates were resistant to gentamicin. Nine isolates (90%) samples showed resistant to more three antibiotics groups (MDR). MDR isolates

more three antibiotics groups (MDR). MDR isolates were resistant to TE-OT-CN-NA-ENR-CIP-E or TE-OT-NA-ENR-CIP-E. Eight tetracycline and oxytetracycline-resistant isolates (80%) had *tetA* gene. All quinolone and fluoroquinolone-resistant isolates showed positive for *gyrA* gene. The *aac(3)-IV* were not detected on all gentamicin-resistant isolates. Erythromycin-resistant isolates were negative for *ermB* gene (Table 2).

## DISCUSSION

The emergence of antibiotics resistance agents had become a public health threat. Caution was necessary when there were few or no effective alternative antibiotic agents for the treatment of infections caused by *E. coli*. Although most strains of *E. coli* were harmless and commonly found in the intestines of humans and warm-blooded animals, some strains could cause severe foodborne disease in humans.

In this study, we studied the phenotype and genotype profile of antibiotic resistance in *E. coli* from cloacal swabs of broilers in Cianjur, Indonesia. The 33.3% prevalence of *E. coli* isolates from samples collected in this study was lower than some previous studies (Masruroh et al., 2016; Niasono et al., 2019; Al

Table 2 Phenotypic and genotypic *E. coli* isolates

ISO-lates	Antibiotics									No. of Resistant Groups	Genes			
	TE	OT	CN	NA	ENR	CIP	E	C	tetA		aac(3)-IV	gyrA	ermB	
1	R	R	S	R	R	R	R	I	4	+	*	+	-	
2	R	R	S	R	R	S	R	S	4	+	*	+	-	
3	R	R	S	S	S	S	R	S	2	+	*	*	-	
4	R	R	R	R	R	R	R	I	5	-	-	+	-	
5	R	R	R	R	R	I	R	I	5	+	-	+	-	
6	R	R	R	R	R	R	R	S	5	-	-	+	-	
7	R	R	R	R	R	R	R	S	5	+	-	+	-	
8	R	R	S	R	R	R	R	S	4	+	*	+	-	
9	R	R	S	R	R	R	R	S	4	+	*	+	-	
10	R	R	S	R	R	R	R	S	4	+	*	+	-	

Notes : TE: tetracycline; OT: oxytetracycline; CN: gentamicin; NA: nalidixic acid; CIP: ciprofloxacin; E: erythromycin; C: chloramphenicol; tetA for tetracycline and oxytetracycline resistant; aac(3)-IV gentamicin resistant; gyrA for nalidixic acid, ciprofloxacin or enrofloxacin resistant; and ermB for erythromycin resistant; S: susceptible; I: intermediate; R: resistant; "+":antibiotics resistance-encoding gene was present in sample; "-": antibiotics resistance-encoding gene was not present in sample; "\*": not performed detection antibiotics resistance-encoding genes because it was not resistant

Azad et al., 2019). However, the results of isolation and identification of *E. coli* in our study were not much different Andriyani et al. (2020) that is 26.7%.

Antibiotic resistance test results showed that the number of isolates resistant to three or more antibiotic classes was quite high. Multi drug-resistant of tetracycline, quinolone, fluoroquinolone and macrolides was most frequently observed. The presence of resistant *E. coli* on samples was a matter of concern. In general, when antibiotics were used, they would eliminate the susceptible bacteria strains and remain resistant bacteria. Then the resistant bacteria multiplied and become a dominant population which could transfer resistance genes (either horizontally or vertically) to other bacteria (Madigan et al., 2014). Resistance traits could be obtained from mutations, gene transfer by conjugation or transformation, transposons, integron, and bacteriophages (Cogliani et al., 2011). In fact, *E. coli* was the main bacteria that causes the spread of antibiotic resistance (WHO, 2017).

Tetracycline resistance was frequently reported from chicken samples (Kurnia et al., 2018; Al Azad et

al., 2019). In this study, 100% of the isolates showed phenotypic resistance to tetracyclines and oxytetracyclines. These results were consistent with previous studies which reported tetracycline resistance in 97.3% of isolates (Niasono et al., 2019). Tetracycline resistance occurred in chicken isolates because it was widely used as growth promoters in poultry (Kolar et al., 2002).

The tetA and tetB is the most common gene responsible for resistance to tetracyclines (Skockova et al., 2012). In this study, we performed the detection of tetA gene. As many as 80% of isolates that were phenotypically resistant showed the presence of tetA gene. Although the remaining 20% were also phenotypically resistant but did not carry tetA gene. This resistance could be caused by other mechanisms such as enzymatic inactivation or target modification (Chopra & Roberts, 2001).

All isolates (100%) showed erythromycin resistant. Similarly, (Talebiyan et al., 2014) reported erythromycin resistant in *E. coli* was 71.7%. The high resistance of erythromycin was related to the impact of *Mycoplasma* treatment. Erythromycin was

highly used in breeders against *Mycoplasma synoviae* and *Mycoplasma gallisepticum*.

Erythromycin-resistant *E. coli* was screened for the *ermB* gene. Although phenotypically resistant to erythromycin, genotypically no *ermB* gene was detected in all *E. coli* isolates. These results differ from detection of the *ermB* gene by Kappell et al. (2015), there were 38.9% of the *ermB* gene detected in *E. coli* isolates from urban waterways. It is possible that another erythromycin resistance gene encodes this phenotype.

The prevalence of gentamicin resistance phenotype in *E. coli* isolates was 40%. The result was similar to the study conducted by Al Azad et al. (2019) showed 49% *E. coli* resistance to gentamicin. Gentamicin was used widely in the poultry industry with or without a combination of other antibiotics.

Genotypically, all gentamicin resistant isolates were not detected by the *aac(3)-IV* gene. Nevertheless, the study conducted by Choi et al. (2011) showed 46.2% and 50.7% of the *aac(3)-IV* genes in *E. coli* indicator and *E. coli* pathogenic isolates, respectively. Zarate et al. (2018) stated that there were other genes capable of coding resistance to gentamicin and other aminoglycoside antibiotics, i.e. *aac(6)-I*, *aac(6)-II*, *aac(3)-I*, *aac(3)-II*, *aac(3)-III* and *aac(3)-VII*.

The resistance test for quinolone and fluoroquinolones in this study was carried out against nalidixic acid, enrofloxacin, and ciprofloxacin. Phenotypically, the resistance of this group is quite high (70-90%). According to the study Al Azad et al. (2019), *E. coli* isolates from broilers experienced high resistance (100%) to ciprofloxacin and enrofloxacin. However, different report from Niasono et al. (2019) showed lower resistance to nalidixic acid, enrofloxacin and ciprofloxacin (40.5-64.9%). The diversity in the use of antibiotics, geographic differences, and different poultry production systems gave different patterns of resistance (Bywater et al., 2004).

The *gyrA* gene represented quinolone and fluoroquinolone resistance coding genes. All resistant isolates of this group were phenotypically (100%) detected by the *gyrA* gene. Quinolone and fluoroquinolone resistance usually occurs due to *gyrA* gene mutation. Kotb et al. (2019) showed that 100% of the *gyrA* gene had a mutation in codon 83 (serine → leucine) in 71.3% of the quinolone and fluoroquinolone-resistant *E. coli*. In addition, the *gyrA* gene in the high resistance phenotype underwent a codon mutation in 87 (Asp87Asn) (4%).

There were no isolates detected chloramphenicol resistance. Similar to the finding of Hailu & Tefera

(2016) had reported no resistant *E. coli* isolates to chloramphenicol. The current study finding was distantly related to the finding of Shecho et al. (2017) who found only 3.85% of healthy broilers resistant to chloramphenicol. The different finding was reported by Halfaoui et al. (2017), the chloramphenicol resistance was medium level (39.22%) in *E. coli* from broiler chickens in Algeria. Chloramphenicol was prohibited in breeding. The medium level resistance might due to the persistence of previous resistances or exposes illegal usage.

In conclusion, occurrence of resistant antibiotics in *E. coli* isolated from broiler chickens in Cianjur, Indonesia, was high. Multi drug-resistant was found among isolates. The high level of multi drug-resistant among bacterial isolates indicates the indiscriminate use of antimicrobials in poultry sectors. In this study, not all resistant phenotypes were detected their resistance genes according to the target primers. For future studies, it was advisable to use more varied target primers.

“All authors declare that there are no conflicts of Interest”.

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