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Detection of Tetracycline Resistance Genes among *Escherichia coli* Isolated from Layer and Broiler Breeders in West Java, Indonesia

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ABSTRACT

A major current problem in public health is the issue of antimicrobial resistance of *Escherichia coli* in humans and poultry. In Indonesia, multidrug-resistant *E. coli* are of specific concern since such *E. coli* may cause public health problems in humans. The prevalence of multidrug-resistant chicken *E. coli* strains and the *E. coli* resistance genes, which are *tet(A)* and *tet(B)* genes, were investigated in the present study. A total of 57 swabs were collected from layer and broiler breeder farms in West Java, Indonesia, and used in the experiment. Eighteen isolates were identified as *E. coli* by the disk diffusion method. The isolates classified as drug-resistant and intermediate were then identified using PCR for the antimicrobial resistance genes. The results showed that 18 isolates of *E. coli* from layer-breeder and broiler-breeder farms in West Java were resistant to ampicillin (100%), nalidixic acid (94%), tetracycline (88%), oxytetracycline (83%), gentamicin (27%), and chloramphenicol (22%). PCR identification of *E. coli* antimicrobial-resistant genes in 18 isolates showed tet(A) and tet(B) genes. This study reports antimicrobial resistance genes among *E. coli* on layer and broiler breeder farms in West Java. This present study showed that *E. coli* isolated from layers-breeder and broiler-breeder farms in West Java of Indonesia carried tet(A) and tet(B) genes, the multidrug-resistance genes.

Keywords: livestock; multidrug-resistant E. coli; layer-breeder farm; broiler-breeder farm

INTRODUCTION

Escherichia coli is a normal human inhabitant of warm-blooded intestinal flora but may also be a possible pathogenic agent. *E. coli* causes colibacillosis in poultry which causes significant economic losses (Amer *et al.,* 2018). In addition, some important *Enterobacteriaceae* such as *E. coli* and *Salmonella* spp. can also bear antimicrobial-resistant genes, leading to multidrug resistance and the risk of spreading it to the other bacterial species, including those pathogenic to humans. Several studies revealed a high genetic similarity between *E. coli* in humans and poultry (Vounba *et al.,* 2019), indicating that the chicken could serve as a reservoir of *E. coli*, a human pathogen. Multidrug resistance has become quite widespread in isolates of *E. coli* of livestock-origin (Alonso *et al.,* 2017) in recent years. Since multidrug resistance is a global problem and emerging antimicrobial resistance has become a thread for public health (Zaman *et al.,* 2017), the discovery of multidrug-resistant *E. coli* and the antimicrobial-resistant gene is a major public health concern.

Even though Indonesia has stopped using antimicrobial as a growth promoter, the practice of antimicrobials application in the treatment of infectious disease is widely applied (Shecho *et al.,* 2017) in the poultry farm. Therefore, antimicrobial resistance is often caused by the use of a high dosage of antibiotics. Surprisingly, the tetracycline resistance gene was observed in some strains (Liljebjelke *et al.,* 2017) even though tetracycline is rarely used in Indonesia. This fact indicates that there is a contamination of antimicrobial to the environment by bacteria carrying the antimicrobial-resistant gene.

A continuous monitoring system is required to assess the levels of baseline resistance and the impact of the different targeted interventions (De Kraker *et al.,* 2017). However, there is a lack of information about multidrug-resistant *E. coli* in layer and broiler breeder farms in West Java, Indonesia. An investigation of multidrug-resistant *E. coli* is needed at poultry farms located in West Java. It is fundamental to update the latest information about multidrug-resistant *E. coli* since *E. coli* is abundant in the environment, animals, and humans.. Therefore, the present study aims to assess the trends

of antimicrobial resistance to *E. coli* isolates and identify the gene resistance to *E. coli* in poultry.

MATERIALS AND METHODS

Ethical Approval

This study had no ethical approval because all methods did not harm chickens, including sample collection.

Sample Collection

A total of 57 samples (23 cloacal swabs, 14 drinking water swabs, and 20 litter swabs) were obtained from two-layer and two broiler breeder farms in West Java, Indonesia (Table 1). All of 57 samples were obtained through the use of an aseptic technique. The samples were preserved at 4°C and sent to the Microbiology Laboratory at the Faculty of Veterinary Medicine, IPB University, within 24 h, for examination.

Bacterial Isolation and Identification

All specimens were tested and dispersed on *Eosin Methylene Blue Agar* (EMBA) (Oxoid-UK). Colonies suspected of being *E. coli* were isolated to Gram staining and IMViC test. A confirmation test for *E. coli* was performed by PCR. Genomic DNA was extracted by boiling technique (Qabajah *et al.,* 2014). All the extracted sample isolates were examined using PCR for molecular typing of the *usp*A target gene using *E. coli* specific primers, having the following nucleotide sequences 5'-CCGATACGCCTGCCAATCAGT-'3 and 5'-ACGCAGACCGTAGGCCAGAT-3' (Chen & Griffiths, 1998). Amplification of *usp*A gene was done in a total volume of 25 µL with 50 ng/mL DNA template, 0.5 unit KAPA2G Fast Hotstart Readymix (KAPA Biosystems, Cape Town, South Africa), 0.5 pmol primers, and dH_2O (DNAse, RNAse free). Temperature conditions were an initial 95°C denaturation step for 3 minutes followed by 35 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute. In the thermal cycling system, the final cycle was followed by one cycle at 75°C for 5 minutes. The amplified fragments using standard PCR markers (VC 100 bp Plus BNA Ladder Vivantis) were evaluated using agarose gel electrophoresis.

Antimicrobial Susceptibility Test

Each *E. coli* isolate had completed antimicrobial resistance procedures according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2018). The antibiotic susceptibility was evaluated by the disk diffusion method of Kirby Bauer using the following disks (Oxoid, UK): ampicillin (30 µg/disk), tetracycline (30 µg/disk), oxytetracycline (30 µg/disk), nalidixic acid (30 µg/disk), enrofloxacin (15 µg/disk), gentamicin (10 µg/disk), and chloramphenicol (30 µg/disk). The disk diffusion method of Kirby Bauer tested the susceptibility of a target microorganism to a specific antibiotic. The disks of antibiotics were placed on an agar plate containing bacteria. After incubation, the susceptibility of bacteria against antibiotics was determined by the diameter of the ring formed on the disk. A wide ring without bacterial growth indicated that the bacteria were susceptible to the antibiotic. On the other side, a resistant bacteria was indicated by no change in the surrounding of the disk. While the intermediate one was indicated by a small inhibition zone.

Multidrug Resistance Genes Detection by PCR

All *E. coli* (18) and non-*E. coli* (2) isolates showing resistance to antimicrobial were isolated to perform DNA amplification to detect drug resistance based on the *tet(A)* and *tet(B)* genes using PCR machine. The non-*E. coli* isolates were used in this study to determine the presence of the *tet(A)* and *tet(B)* genes in non-*E. coli.* The sequences of oligonucleotide primer and PCR conditions are listed in Table 2. PCR was performed in a total volume of 10 µL with 50 ng/mL DNA template, 0.5 unit KAPA2G Fast Hotstart Readymix (KAPA Biosystems, Cape Town, South Africa), 0.5 pmol primers, and dH2O (DNAse, RNAse free).

RESULTS

Identification of *E. coli* Isolates Based on *usp*A Gene

The biochemical tests were performed for all isolates of presumptive *E. coli*. Only 18 (13.5%) identified as *E. coli* based on the presence of *usp*A gene by PCR (Figure 1). The information of positive samples for *E. coli* is shown in Table 3. In layer farm, 17.5% of *E. coli* were isolated only from cloacal swabs (14%) and litter swabs (3.5%). On the other side, the remain of *E. coli* (14%) was obtained from cloacal swabs (7%), litter swabs (2%), and drinking water swabs (3.5%).

Multidrug Resistance

Table 4 shows the antibiotic resistance profiles of the *E. coli* isolates against six antimicrobials,

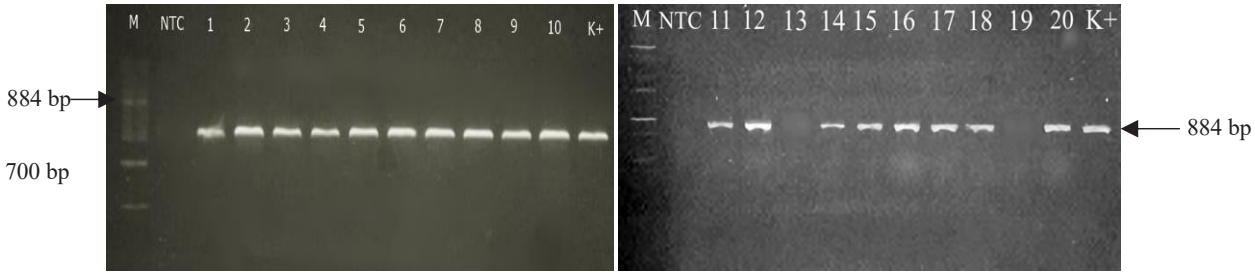
Table 1. Sample collection

Farm	N	Cloacal swabs	Litter swabs	Drinking water swabs
Layer (1)	15	5	5	5
Layer (2)	14	5	5	4
Broiler (1)	14	5	5	4
Broiler (2)	14	5	5	4
Total n	57	20	20	17

Table 2. Oligonucleotide primer sequences and PCR conditions

Primer name	Sequences	Program*	Size of PCR product (bp)	References
tet(A)-F	5'GGTTCACTCGAACGACGTCA-3'	1	577	(Randall *et al*., 2004)
tet(A)-R	5'-CTGTCCGACAAGTTGCATGA-3'	1	577	(Randall *et al*., 2004)
tet(B)-F	5'-CCTCAGCTTCTCAACGCGTG-3'	1	634	(Randall *et al*., 2004)
tet(B)-R	5'-GCACCTTGCTCATGACTCTTT-3'	1	634	(Randall *et al*., 2004)
uspA-F	5'-CCGATACGCCTGCCAATCAGT-'3	2	884	(Chen & Griffiths, 1998)
uspA-R	5'-ACGCAGACCGTAGGCCAGAT-3'	2	884	(Chen & Griffiths, 1998)

Note: PCR program 1= x1 (3 min at 95°C), x30 (60 s at 95°C, 30 s at 55 °C, 30 s at 73°C), x1 (5 min at 75°C); PCR program 2= x1 (3 min at 95°C), x35 (30 s at 95°C, 30 s 58°C, 1 min at 72°C), x1 (5 min at 75°C).



Figures 1. Amplification of the *usp*A gene (884 bp) encoding *Escherichia coli* universal stress protein A gene. A total of 18 isolates showed positive results on *E. coli*. M= 100 bp (marker); NTC= non-template control; K+= *E. coli*.

Table 3. *Escherichia coli* identification based on *usp*A gene

Farm	No. sample positive of *Escherichia coli*				Prevalence
	N	Cloacal swabs	Litter swabs	Drinking water swabs	
Layer (1)	7	5	2	0	38.9%
Layer (2)	3	3	0	0	16.7%
Broiler (1)	3	1	2	0	16.7%
Broiler (2)	5	3	0	2	27.8%
Total	18	12 (66.7%)	4 (22.2%)	2 (11.1%)	100%

respectively. The results indicated that *E. coli* was resistant to over three of the seven antimicrobials, which were ampicillin (100%), nalidixic acid (94%), tetracycline (88%), oxytetracycline (83%), gentamicin (27%), and chloramphenicol (22%), in declining order.

Detection of Drug Resistance Genes

The distributions of drug-resistance genes are listed in Table 4. Figure 2 and Figure 3 show the detections of *tet(A)* and *tet(B)* genes based on PCR, respectively. Among the genes detected for drug resistance, the *tet(A)* gene detection rate was the most prevalent, 94% (17/18), followed by the *tet(B)* gene detection rate of 56% (10/18) for *E. coli*. In addition, two strains of non-*E. coli* showed a positive reaction for *tet(B)* gene (Figure 3).

DISCUSSION

Despite the fact that *E. coli* can be found as a commensal bacterium in various animals, including humans in the intestinal microflora (Zhang *et al*., 2017), not all the strains are harmless. *E. coli* is known as the most common Gram-negative bacteria that can cause fatal diseases for humans and birds and also has antimicrobial resistance (Pumipuntu & Pumipuntu, 2019). The presence of multidrug-resistant *E. coli* in the environment-swab samples, therefore, signals a warning for human health. A total of 18 isolates (31.5%) from the cloacal swabs, litter, and drinking water samples were positive for the presence of the flanking regions *usp*A gene (Figure-1). The *usp*A gene has been used as a DNA marker for the identification of *E. coli* by Chen & Griffiths (1998), and it is considered by another study (Godambe *et al*., 2017). Another report about the prevalence of *E. coli* in poultry from Egypt showed that 35% of the samples were positive for *E. coli* (Amer *et al*., 2018). There is a possibility that non-pathogenic *E. coli* will acquire virulence genes through horizontal gene transfer and causes virulent strains to emerge (Juhas, 2015).

Genes with tetracycline resistance [*tet(A)* and *tet(B)*] were confirmed at the prevalence of 94% and 56%, respectively, the *E. coli* isolates (Figure-2, Figure-3). In fact, tetracycline usage for the animal is prohibited but very common in human. Based on the investigation, it indicates that there is a distribution of tetracycline resistance genes in layer-breeder and broiler-breeder farms.

Table 4. The antibiotic resistance profiles of the isolates against six antimicrobial agents

No.	Isolates	Antimicrobial agents						*Escherichia coli* specific gene	Drug resistance genes	
		A	B	C	D	E	F	uspA	tet(A)	tet(B)
1	BLc.10	R	I	I	I	S	S	+	+	+
2	BLl.13	R	R	R	R	S	S	+	+	-
3	PLc.12	R	R	R	R	I	S	+	+	+
4	PLc.16	R	R	R	R	R	R	+	+	+
5	PBa.47	R	R	R	R	R	R	+	+	-
6	BLc.4	R	R	R	R	S	I	+	+	-
7	BLc.3	R	R	R	R	S	S	+	-	+
8	PBc.10	R	R	I	R	I	I	+	+	-
9	PBc.8	R	R	R	R	S	S	+	+	+
10	BBc.18	R	R	R	R	S	S	+	+	-
11	BLc.5	R	I	R	R	I	S	+	+	-
12	BLc.40	R	R	R	R	I	I	+	+	-
13	PLc.15	R	R	R	R	S	I	+	+	+
14	PBl.26	R	R	R	R	S	S	+	+	+
15	Bl.l.49	R	R	R	R	S	S	+	+	+
16	BBl.23	R	R	R	R	S	S	+	+	-
17	PBa.42	R	R	R	R	S	S	+	+	+
18	PBc.4	R	R	R	R	I	R	+	+	-
19	PLc.11	R	R	I	R	I	I	-	-	+
20	BBc.22	R	R	R	R	S	I	-	+	+
Total of *E. coli*		100%	89%	89%	94%	11%	17%	31.5%	94%	56%

Note: A= ampicillin; B= oxytetracycline; C= tetracycline; D= nalidixic acid; E= chloramphenicol; F= gentamicin; R= resistant; I= intermediate; S= susceptible.

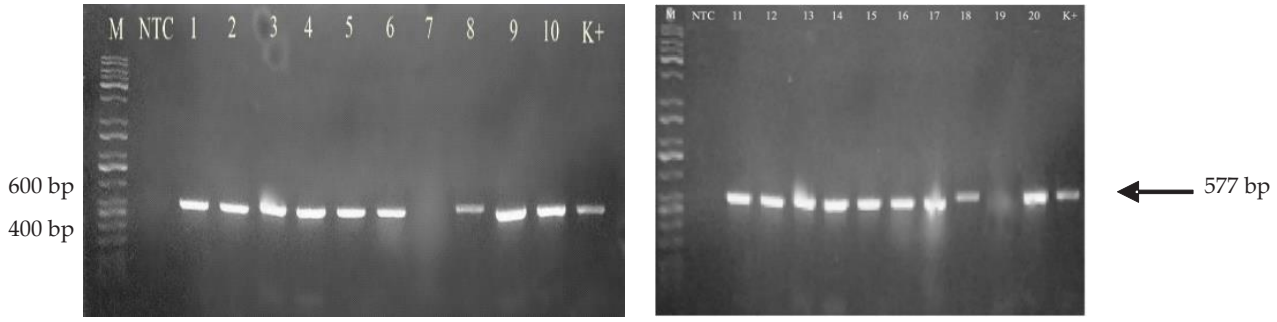


Figure 2. Amplification of the *tet(A)* gene (577 bp) encoding tetracycline resistance in *Escherichia coli*. A total of 17 isolates of *E. coli* (1-18) and 1 of non-*E. coli* isolate (19-20) showed positive results on *tet(A)*. M= 100 bp (marker); NTC= non-template control; K+= positive control of *tet(A)* gene.

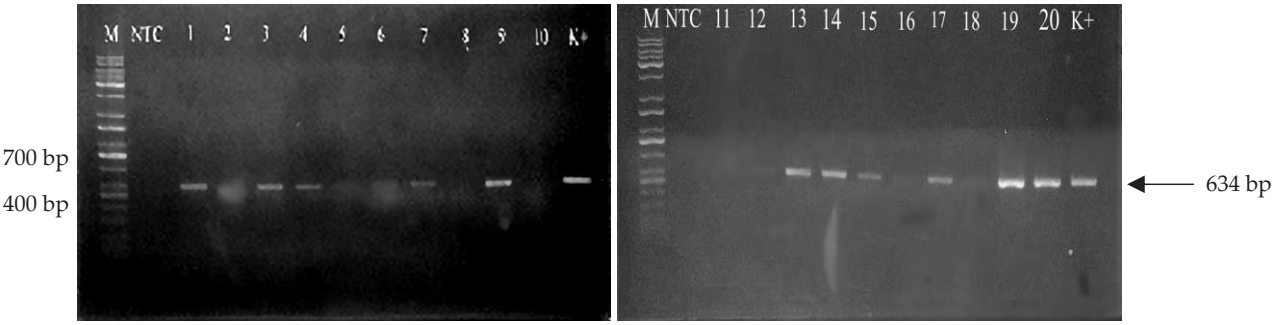


Figure 2. Amplification of the *tet(B)* gene (634 bp) encoding tetracycline resistance in *Escherichia coli*. A total of 10 isolates of *E. coli* (1-18) and 2 of non-*E. coli* isolates (19-20) showed positive results on *tet(B)*. M= 100 bp (marker); NTC= non-template control; K+= positive control of *tet(B)* gene.

About 56% of the isolated strains harbored one or more tet resistance genes, and that these strains were most frequently isolated from animals living in close contact with humans (Gargano *et al*., 2021). In this analysis, the percentage of tetracycline resistance among *E. coli* [*tet*(*A*) and *tet(B)*] is lower than *E. coli* resistant to tetracycline in Saudi Arabia (95%) (Abo-Amer *et al.*, 2018). The most frequently used tetracycline-resistant gene in this study was *tet(A*) gene (94%). In addition, *tet(A)* gene is the most widespread determinant of Gram-positive and Gram-negative bacteria (Hedayatianfard *et al.*, 2014) and has a wide variety of host compounds, and is often borne by specific environmental genera (Zhang *et al*., 2009). Besides, the present study shows that *tet*(*A*) and *tet(B)* genes are present in non-*E. coli* isolates. Recent studies have shown that *tet*-genes are often found on the plasmids and can be transferred horizontally between bacterial strains (Osińska *et al*., 2016). Meanwhile, *tet(A)* gene has been commonly observed in various environments (Ling *et al*., 2013).

The high levels of resistance determined by genotyping methods and reported in the current study confirm the need for close monitoring of the use of tetracycline in poultry in West Java. This investigation might help to develop an alternative method to minimize the development and proliferation of resistant bacteria in the future, such as bacteriophage. Moreover, the high percentages of plasmid-mediated tetracycline resistance in animals may be a possible horizontal transfer of the resistance genes between strains (Veldman *et al*., 2011). Therefore, it is a great thread for human health.

CONCLUSION

The identification of multidrug-resistant of *E. coli* in layer and broiler breeder farms in West Java is still rarely done. The present study showed that *E. coli* isolated from layers and broiler breeder farms in West Java of Indonesia carried *tet*(*A*) and *tet(B)* genes, the multidrug resistance genes.

CONFLICT OF INTEREST

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

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