



Detection of Ampicillin Resistance Encoding Gene of *Escherichia coli* from Chickens in Bandung and Purwakarta

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Abstract

The purpose of this study was to test the resistance and to detect antibiotic resistance encoding gene in E. coli bacteria from chickens in Bandung and Purwakarta livestock. 18 E. coli isolates were tested for antibiotics resistance using the disk diffusion method. Isolates that were categorized as resistance and intermediate to antibiotics, then polymerase chain reaction was utilized to detect the resistance coding gene. The test results showed that all E. coli isolates from chickens in Bandung and Purwakarta were resistance to ampicillin (100%). E. coli isolates were still sensitive to chloramphenicol (11.1%) and gentamicin (22.2%). The gene encoding for ampC resistance were found 77.7%. Sensitivity test results and detection of resistance coding gene showed that almost all isolates were resistance to ampicillin antibiotics and E. coli isolates were still sensitive to chloramphenicol and gentamicin.

Key words: antibiotics, *E. coli*, genes, resistance

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Introduction

Escherichia coli in chickens is naturally a commensal and harmless bacteria found in the gastrointestinal tract, and there are some that are disease-causing pathogens and are used as indicators to monitor the incidence of antibiotic resistance in livestock as well as products of animal origin (Ajayi *et al.* 2011). Recently, pathogenic *E. coli* bacteria are increasingly found in the field that cause diseases such as cholibasilosis, which is very detrimental to the world of poultry.

The threat to antibiotic resistance has become an important concern in the field of public health. According to Laxminarayan *et al.* (2013), due to antibiotic-resistant bacterial infections each year, nearly 58 391 infants in India and estimated 25 000 ones in Europe die. The use of antibiotics on farms usually aims to treat disease or therapy, to prevent the occurrence of disease by inhibiting the growth of pathogenic microorganisms, and mixing light-dose antibiotics in feed is done in order to increase feed efficiency (Barton 2000). The use of antibiotic doses continues to be the cause of the emergence of resistance in bacteria, and because of its resistance to antibiotics, it can cause the least selection of drugs for the treatment of infections, then this

affects on treatment failure and requires repeated use of antibiotics with various classes of antibiotics.

E. coli is known to show resistance to several types of antibiotics. Bacteria that naturally have antibiotic resistant genes can act as the reservoir by transferring these genes to other bacteria (Ibrahim *et al.* 2016). The nature of resistance can be transferred through a mechanism called horizontal gene transfer to other bacteria including pathogenic bacteria, and it ultimately can affect on human health.

Materials and Methods

Research Sample

Samples were in the form of cloaca swabs, litters, and drinking water taken in aspiration from breeder and layer chicken farms in Bandung and Purwakarta districts. Samples were carried with ice flasks for laboratory analysis at the Laboratory of the Department of Veterinary Sciences and Veterinary Public Health, Faculty of Veterinary Health, Bogor Agricultural University. Based on the estimated observation, prevalence of disease incidence in the amount of 3.7% with a 5% degree of error, the number of samples needed for testing with a 95% confidence level is minimum in the amount of 57 samples (Martin *et al.*, 1987).

Isolation and Identification Of Escherichia Coli

Samples were plated in eosin methylene blue agar (EMBA) media with spread method. Culture was incubated at 37°C for approximately 18-24 hours. Identified as *E. coli*, further confirmed by Gram staining procedure and biochemical tests. Furthermore, they were tested by motile-indole sulfide (SIM) test, Methyl Red (MR) test, Voges-Proskauer test (VP), citrate test, and incubated at 37°C for 24 hours, after media color changes were observed (SNI 2008).

Antibiotic Sensitivity Test

The antibiotic sensitivity test was carried out based on the disk diffusion method according to the Clinical and Laboratory Institute (CLSI) guidelines (CLSI 2018). Each of 2-5 groups of *E. coli* isolates from each sample was suspended in a physiological NaCl solution. Turbidity of bacterial suspension was diluted to reach McFarland standard in the amount of 0.5 or equivalent to 1.5×10^8 CFU / ml, then in the amount of 1 mL of each bacterial suspension was poured and then flattened on Mueller-Hinton agar in a petri dish, using sterile cotton swab. Diffusion disks that contain antibiotics were placed on top of the Mueller-Hinton using sterile tweezers with the same distance, and then incubated at 37°C for 18-24 hours, and measurement of antibiotic inhibition zones was done based on predetermined standards. Standards in the form of categories included susceptible (S), susceptible dose dependent (SDD), intermediate (I), and resistant (R). The categories were determined by the range of diameter of the antibiotic inhibitory zone which was formed on Muller-Hilton agar based on the Clinical and Laboratory standards Institute guidelines (CLSI 2018).

Detection of ampC Antibiotic Resistance Encoding Gene

Extraction of *E. coli* DNA that was resistant to antibiotics was carried out by the boiling method. The presence of antibiotic resistance coding genes was detected by genes primer of ampC (ampicillin resistant gene) using single PCR. Primer sequences of the ampC gene were 5'-AATGGGTTTTCTACGGTCTG-3' for forward sequence and 5'-GGGCAGCAAATGTGGAGCAA-3' for reverse sequence (Brinas *et al.* 2002) with amplicon target in the number of 191 bp. The amplification process used KAPA2G Fast Readymix PCR Kit®. The total volume of PCR

reactions was 25 μ L, consisting of 1 μ L DNA template, 12.5 μ L mastermix, 1.25 μ L forward primer 10 μ M, 1.25 μ L reverse primer 10 μ M, and 9 μ L H₂O. The amplification process began with pre-denaturation at 95 °C for 3 minutes, then 35 cycles of the amplification process with denaturation temperature at 95 °C for 30 seconds, annealing at 58 °C, extension at 72 °C for 1 minute and at the end the amplification was the final extension at 75 °C for 5 minutes. The amplified samples were visualized by electrophoresis using 1% agarose gel and stained using ethidium bromide 0.5 μ g / mL. A molecular weight marker with 100 bp (VC 100 bp Plus DNA Ladder Vivantis, Selangor, Malaysia) was used as a Standard size.

Results and Discussions

Isolation and Identification of Escherichia coli

The results of *E. coli* growth from 57 samples on the EMBA media showed as many as 18 metallic green colonies with black spots in the middle of the colony. The Gram staining procedure showed rod-shaped cell in Gram staining. The confirmation of *E. coli* colonies with the IMVIC test showed positive results on the indole test and methyl red test, while the negative results in the voges proskauer test and citrat test. With the results of the biochemical test, the growing colonies were confirmed as colonies of *E. coli* bacteria. (Soemarno 2000) states the groups that include fecal bacteria show positive results for the indole and methyl red test whereas bacteria that show positive results on the voges proskauer test and citrat test are included in the non-fecal group.

According to OIE (2013), *E. coli* as a commensal bacterium is a bacterium that is commonly used as an indicator in surveillance programs and monitoring antibiotic resistance. *E. coli* can act as a reservoir of genes that have been resistant to antibiotics. This gene can be transferred to other pathogenic bacteria. *E. coli* is chosen because it is an indicator of Gram negative bacteria that is often found in animal feces and is related to treatment in humans. Furthermore, it frequently contains plasmids that can move between enteric bacteria. The presence of commensal *E. coli* in livestock gut acts as a reservoir of resistant genes that can move horizontally to pathogenic *E. coli* and other bacteria in the food chain.

Antibiotic Sensitivity Test

Antibiotic sensitivity testing of the 18 *E. coli* isolates showed that all isolates are resistance to ampicillin (100%), 11.1% resistant to chloramphenicol, and 22.2% resistant to gentamicin (Table 1).

Table 1: Results of antibiotic sensitivity test for E. coli bacteria

Group	Antibiotic	Resistant	Intermediate	Susceptible
β -laktam	Ampisilin	100 %	0 %	0 %
Kloramphenicol	Kloramphenicol	11.1 %	66.6 %	22.2 %
Aminoglycoside	Gentamicin	22.2 %	72.2 %	5.5.%

The results of the antibiotic sensitivity test showed 100% resistance to ampicillin, followed by 11.1% resistant to chloramphenicol, and 22.2% resistant to gentamicin (Table 1). The used of antibiotics as Antibiotic Growth Promoter (AGP) mixed in feed or drinking water was thought to be one of the causes of the high incidence of resistance to ampicillin. Addition of AGP plays a role in the emergence of this resistance event. The addition causes normal flora and pathogenic bacteria to be eliminated, stock farmers did this probably to anticipate the losses caused by bacterial diseases declines, then livestock production is going to increase. The antibiotic concentration added to animal feed in low dose around 2.5-12.5 mg / kg (ppm) was

proven to be able to spur the resistance of pathogenic bacteria and commensal bacteria in the digestive tract (Noor and Poeloengan 2005).

Prohibition of Hormones and AGP use written in Law No. 18 in 2009 concerning Animal Husbandry and Animal Health which is then clarified by Minister of Agriculture Regulation No. 14/Permentan/ PK.350 /5/2017 about Classification of Antibiotic Animal Medicine as AGP. This regulation needs to be followed by routine surveillance and monitoring. The ease of obtaining antibiotics and the low price of antibiotics makes it difficult to control antibiotic use on farms in Indonesia (Amalia and Adisasmito, 2017). Detection of ampC Antibiotic Resistance Encoding Gene.

Molecular detection of resistance encoding genes was conducted on *E. coli* isolates with antibiotic resistance which categorized as intermediate by using PCR. Detection of the ampC gene showed positive results in 14 *E. coli* isolates (77.7%) that were resistant to ampicillin with a 191 bp amplicon (Figure 1).

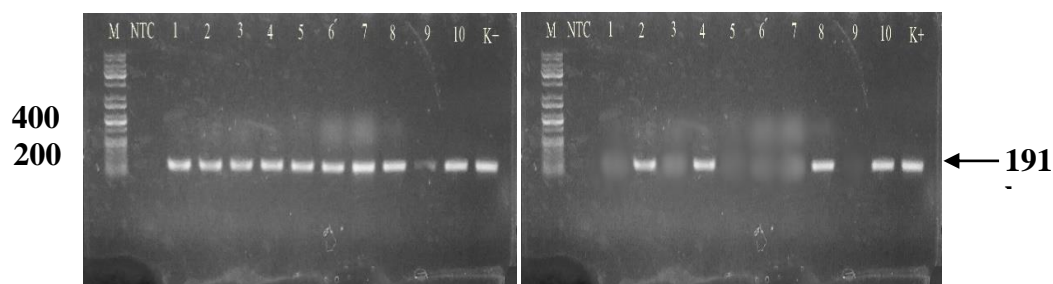


Figure 2 Amplification of genes ampC (191 bp) encoding resistance to ampicillin in *E. coli*. A total of 14 isolates showed positive results for ampC and 4 isolates showed negative results against ampC. M: 100 bp marker; NTC: non template control.

The presence of the ampC gene in bacteria cells is related to resistance to ampicillin. Many Enterobacteriaceae expressing ampC have a low affinity, but they are induced in response to beta-lactam exposure. The induction mechanism is complex. Interference with biosynthesis of murein by beta-lactam agent causes accumulation of N-acetylglucosamine-1,6-anhydro-N-acetylmuramic acid oligopeptide. The N-acetylglucosamine group is removed to produce a series of 1,6-anhydro-N-acetylmuramic acid tri-, tetra, and pentapeptides. These oligopeptides compete with UDP-N-acetylmuramic oligopeptides for binding sites in ampR, those which are parts of the LysR regulator. Transfer of UDP-N-acetylmuramic acid peptides indicates a conformational change in ampR, which activates the transcription of ampC (Jacoby 2009).

Conclusion

Eighteen *E. coli* isolates from cloacal swabs, litters, and drinking water in chicken farms in Bandung and Purwakarta. All *E. coli* isolates this present study revealed a high rate of resistance AMP. In addition, 11.1% of isolates are resistant to chloramphenicol, and 22.2% of isolates are resistance to gentamicin. Resistance encoding gene that can be detected include *ampC* in the amount of 77.7%. Further studies need to be conducted to detect antibiotics encoding genes other than *ampC* so that the pattern of spread can be identified.

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References

- Ajayi AO, Oluyeye AO, Olowe OA, Famurewa O. 2011. *Antibiotik resistance among commensal Escherichia coli isolated from faeces of cattle in Ado-Ekiti, Nigeria. J Anim Vet Adv.* 10(2):174-175.
- Amalia Z, Adisasmito W. 2017. Analysis of Policy making Factors on The Prohibition of Hormones and Antibiotics Use for Feed as a public health Protection. *JHHPA.* 2(@): 14-19.
- Barton MD. 2000. *Antibiotic use in animal feed and its impact on human health. Nuutr Res Rev.* 13(2):279-299.
- Brinas L, Zarazaga M, Sa'enz Y, Larrea FR, Torres C. 2002. β -Lactamases in ampicillin-resistant *Escherichia coli* isolates from foods, humans, and healthy animals. *Antimicrob Agents Chemother.* 46(10):3156-3163.
- [CLSI] Clinical and Laboratory Standards Institute. 2018. *Performance Standards for Antimicrobial Susceptibility Testing* 28th ed. CLSI Supplement M100. Wayne, PA : Clinical and Laboratory Standards Institute ; 2018.
- Ibrahim DR, Dodd CER, Stekel DJ, Ramsden SJ, Hobman JL. 2016. Multidrug resistant, Extended Spectrum β -Lactamase (ESBL)-producing *Escherichia coli* from a dairy farm. *FEMS Microbiol Ecol.* 92(4):1-13.
- Jacoby GA. 2009. *AmpC β -Lactamases.* *Clinical Microbiology Reviews*, Jan. 2009, p. 161–182
- Laxminarayan R, Duse A, Wattal C, Zaidi AKM, Wertheim HFL, Sumradit N, Vlieghe E, Hara GL, Gould IM, Goossens H, Greko C, So AD, Bigdeli M, Tomson G, Woodhouse W, Ombaka E, Peralta AQ, Qamar FN, Mir F, Kariuki S, Bhutta ZA, Coates A, Bergstrom R, Wright GD, Brown ED, Cars O. 2013. *Antibiotic resistance- The need for global solutions. Lancet infect. Di.* 13: 1057-1098
- Martin, S.W., Week, A.H. and Willenberg, P., 1987. *Veterinary Epidemiology Principles and Methods*, 1st ed. Iowa State University Press, Ames: 22-175.
- Noor SM, Poeloengan M. 2005. Pemakaian antibiotik pada ternak dan dampaknya pada kesehatan manusia. *Prosiding lokakarya nasional keamanan pangan produk peternakan.* Bogor (ID): Puslitbang Peternakan. 18-22.
- [OIE] Office Internationale des Epizooties. 2013. Harmonisation of national antimicrobial resistance surveillance and monitoring programmes chapter 6.7. [www.oie.int/fileadmin/Home/eng/Health_standards/tahc/2010/en_chaptire_1.6.7.htm.]
- [SNI]. Standar Nasional Indonesia. 2008. *Metode Pengujian Cemaran Mikroba dalam daging, telur, dan Susu serta Hasil Olahannya.* SNI 2897. ICS 67.050. Jakarta, Indonesia (ID): SNI.
- Soemarno. 2000. *Isolasi dan identifikasi bakteri klinik.* Yogyakarta. Akademi Analisis Kesehatan Yogyakarta Departemen Kesehatan RI.