Deteksi Dan Isolasi *Escherichia coli* Resisten Ampisilin Yang Diisolasi Dari Peternakan Ayam Di Surabaya, Indonesia

Detection Of Ampicillin Resistance of *Escherichia coli* Isolated From Chicken Livestock Farming In Surabaya, Indonesia

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INTISARI

Tujuan penelitian ini yaitu untuk mengisolasi dan mendeteksi bakteri E.coli resisten dari empat peternakan ayam di Surabaya, Indonesia.

Penelitian ini menggunakan 40 sampel daging paha ayam. *Escherichia coli* diisolasi dari sampel dengan menggunakan media BGBB (*Brillilliant Green Bile Broth*), selanjutnya digunakan media EMBA (*Eosin Methylen Blue Agar*) untuk uji konfirmasi, dan dilakukan uji IMViC (Indole, Methyl Red, Voges Praskauer, dan Citrat) untuk mengidentifikasi bakteri *enterobacteriaceae*, kemudian dilakukan pewarnaan gram. Sensitivitas *Escherichia coli* terhadap antibiotik ditentukan dengan metode diffuse disc.

Uji resistensi dengan metode MHA menghasilkan persentase resistensi E.coli terhadap ampisilin sebesar 100% pada semua sampel. Dalam penelitian ini 13 dari 40 sampel daging ayam positif mengandung E.coli. Hasil dari produk elektroforesis PCR menunjukkan bahwa resistensi *Escherichia coli* memiliki band spesifik. Total 13 sampel positif *Escherichia coli* yang resisten ampisilin memiliki band hasil amplifikasi primer DNA spesifik dengan panjang 768 bp.

Kata kunci: *Escherichia coli*; uji sensitivitas antibiotik.

ABSTRACT

The study was carried out to isolate and detect the resistance of E. coli bacteria from four livestock in Surabaya, Indonesia.

This research used 40 samples of chicken thigh meat. The first step is isolated Escherichia coli bacteria using BGBB media (Brillilliant Green Bile Broth), and then used EMBA (Eosin Methylen Blue Agar) media for confirmatory tests, IMViC tests (Indole, Methyl Red, Voges Praskauer, and Citrat) to identify the enterobacteriaceae bacteria, and performed the gram staining test. Determined the Escherichia coli sensitivity to antibiotic by using disk diffusion method.

Resistance test has resulted for ampicillin against E.coli is 100% resistance in all farms. In this research 13 of 40 samples from chicken meat had positive E.coli. The result from electrophoresis product of PCR said that resistance *Escherichia coli* had a specific band. Total 13 samples of positive *Escherichia coli* resistance ampicillin has specific DNA band primary shv with *768* bp in length.

Keywords: *Escherichia coli*; antibiotic sensitivity test.

1. INTRODUCTION

Escherichia coli is a bacterium that can normally grow in the digestive tract, but in certain condition can be pathogenic and able to attack animals and humans.

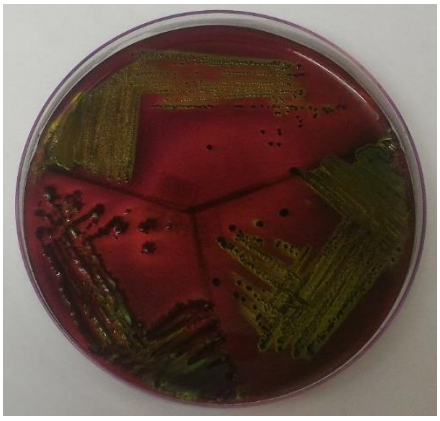

Figure 1. *Escherichia coli* on EMBA agar

The bacteria is classified as an environmental contaminant caused by lack of proper sanitation. On the farm, the bacteria often contaminate animal product including chicken meat.[1]

The use of antibiotics to treat diseases in poultry is still the best choice for chicken farmers. Mixing mild doses of antibiotics in feed has been done in livestock farming to increase feed efficiency. [2]

So far, prevention and treatment of bacterial attacks are generally carried out by administering antibiotics and chemicals, but the use of antibiotics can actually cause side effects for the pathogenic bacteria itself and for livestock that are kept. Microorganisms are able to develop resistance to drugs through various mechanisms, namely microorganisms are able to produce enzymes that can damage the active drug, change its permeability to the drug, develop structural targets that are different from the original target, develop metabolic shortcuts that cannot be inhibited by drugs, and forming an enzyme that has undergone a change but the enzyme can still carry out its metabolic function but is not influenced by drugs such as enzymes in sensitive bacteria. [3]

Antibiotic resistance to pathogenic bacteria has become a problem worldwide. The occurrence of antibiotic resistance is caused by inappropriate use of antibiotics for treatment in humans and the use of antibiotics in animals as a growth booster that has contributed to the occurrence of antibiotic resistance in both humans and animals. [3]

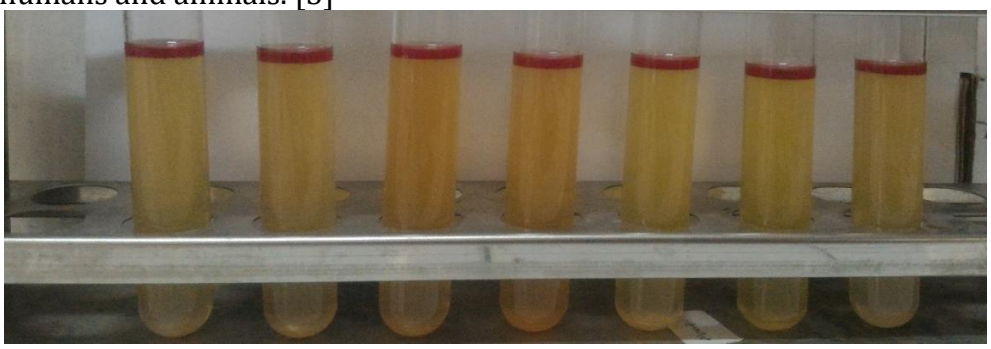

Figure 2. Positive indol test at 1% peptone water.

In 1944, ampicillin antibiotics were found which include the member of the beta lactam antibiotic. Unfortunately, in the past 5 years, 90% strains have been resistant to this antibiotic positively. The increasing of ampicillin antibiotics use also increased bacterial resistance to these antibiotics. The primary mechanism of Gram-positive and Gram-negative bacteria resistance to ampicillin antibiotics is by producing betalactamase enzyme which acts to cut the betalactam ring. This enzyme opens the betalactam ring from ampicillin and removes its antimicrobial ability. [4]

The encode betalactamase gene located on the chromosome of the Escherichia coli. This gene encodes betalactamase enzyme which inactivates the ampicillin betalactam ring by hydrolyzing the betalactam ring, thus becoming resistant to ampicillin. Ampicillin resistance can also be caused by the expression of the betalactamase-encodase gene, the shv gene which found in plasmids.

This study used ampicillin antibiotics to determine the sensitivity of *Escherichia coli*. The ampicillin-resistant coding genes *for Escherichia coli*, can be detected using specific primer, namely shv primers, which produce 768 bp PCR ampliconts. [5]

The shv gene detection from chicken meat can be used to monitor the potential transmission of *Escherichia coli* that is resistant to ampicillin antibiotics from animals to humans so that preventive action can be taken.

2. MATERIALS AND METHODS

2.1. Equipments and Materials

Equipments used for *Escherichia coli* identification are Polymerase Chain Reaction machine, petri dishes, erlenmeyers, test tubes, cotton, stove, ose, bunsen, durham tubes, micropippets (Nichipet), eppendorf tubes, microtubes, white tip, yellow tip, PCR (eppendorf) UV-transluminator, vortex, electrophoresis apparatus gel (Biorad), incubator and autoclave.

The samples used in this study are chicken meat taken from four farms and four chicken collectors in Surabaya. The media used in the identification of *Escherichia coli* is Brilliant Green Bile Broth (BGBB), Eosin Methylen Blue Agar (EMBA), Buffer Pepton Water (BPW) 1%, aquadest, kovach reagents. Materials used for antibiotic sensitivity test include Mc. Farland 1, physiological NaCl, Mueller Hinton Agar (MHA), ampicillin antibiotics are ready to use in disc form.

Materials for DNA extraction are 200 µl Tris Edta (TE) buffer (Vivantix) and some E.coli germicidal colonies. Materials for gene amplification include: 12.5 µl master mix, 0.5 µl destilated water, shv forward primer (1 µl), reverse shv primer (1 µl) and DNA template 5 µl. Primers used for the amplification of 768 bp fragments from the shv gene include forward primers (5'-TCGCCTGTGTATTATCTCCC-3'), reverse primers (5'-CGCAGATAAATCACCACAATG-3') [6]. Materials for electrophoresis include using agarose gel and DNA marker 100 bp 4 µl.

Chicken meat samples were taken from four farms and four chicken collector in Surabaya, Indonesia. Chicken meat samples which were taken from several farms and chicken collectors in Surabaya with some criteria including: a). Livestock cages that are not really clean, b). Never been given formalin before. Sampling of chicken

meat in the morning at 05.00-06.00 WIB. On each farm 10 samples of chicken thigh meat were taken. Total chicken meat used as the sample is 40 samples.

2.2. Research Procedure

Isolation and Identification of *Escherichia coli* Bacteria

a. Isolation of Bacteria *Escherichia coli* [6]

Each suspension of 10% chicken meat was inoculated on the Brilliant Green Bile Broth (BGBB) media and then incubated at 37ºC for 24 hours. The positive results are indicated by the presence of gas bubbles on the durham tube and the change from green to cloudy green color.

b. Identification of *Escherichia coli*

Pure culture isolates plating results that Single colony that has been stored in glycerol 3% was inoculated on EMBA medium and incubated at 37°C for 18-24 hours prior to identification, The positive result shown by the presence of colonies in shiny greenish red color. Identification of bacteria was performed using the morphological approach, and biochemistry. Biochemical tests include Indole test, Methyl Red, Vagos-Pasteur, and simon Citrate (IMViC) to determine the level of genus.

Antibiotic Sensitivity Test

The antibiotic sensitivity test used Kirby-Baurer method that uses *agar disk diffusion* to produce qualitative categories with sensitive, intermediate and resistant assessments [6]. The workings in the sensitivity test are explained below;

a. Germ Culture

Germ cultures obtained from colonies contained in the EMBA media were suspended in 8 ml of *Nacl physiologist*, and homogenized using vortex to obtain the same turbidity as standard of McFarland 1.

b. Sensitivity Test

Planting on the plate by taking 1-2 colonies of *Escherichia coli* in the EMBA media using ose and then put into the *Nacl physiologist* which had been tested for turbidity with standard of McFarland 1, then taken 0.2 ml and gently rub the entire surface of *Mueller Hinton Agar* (MHA) medium. The germs were left to stick to the media for 15 minutes, then the ampicillin antibiotic disc was placed on top of the Mueller Hinton Agar (MHA) medium. The discs are slightly pressed on the surface so that the drug can absorb properly. Culture of bacteria was incubated at 37°C for 24 hours. The area of antibiotic resistance to germ growth was measured by using a ruler in mm. [7]

DNA extraction

Escherichia coli DNA extraction used the boiling method. A sterile 200 µl Tris-Edta buffer is inserted into a 1.5 ml tube. Add 1-2 bacterial colonies and then vortex so homogeneous. Then incubated at 90°C for 10 minutes. Let stand at room temperature 1 minute. After that put it into the 10,000 rpm centrifuge for 5 minutes. Supernatant is used to process PCR. The obtained template DNA is stored at -30 °C.

Amplification with Polymerase Chain Reaction

The extracted suspension, 5µl of volume was directly used as a template for PCR amplification of shv gene fragments. The 20 µl PCR reaction consists of 12.5 µl master mix, 0.5 µl destilated water, reverse primers (1 µl), forward primers (1 µl) and DNA templates 5 µl.

The PCR reagent mixture was then put into a thermocycler with an initial incubation of 95°C for 3 minutes, 35 cycles each consisting of 94°C for 1 minute, annealing at 55°C for 90 seconds, and elongation of 72°C for 1 minute. Followed by a final extension at 72°C for 10 minutes [6].

Electrophoresis

Each 5 µl of the amplification product was put into a 2% agarose gel well submerged in a tank containing TE buffer. The marker is also inserted into the agarose gel well to determine the DNA size of the PCR product, then electrophoresis is run for 30 minutes with a constant voltage of 110 volts. The electrophoresis is stopped and the gel is removed to be observed under Ultra Violet (UV) light after 30 minutes. The results obtained in the form of DNA bands with a size of 768 bp were tested positive for the shv gene. [8]

3. RESULTS AND DISCUSSION

This study has successfully isolated 13 *Escherichia coli* from 40 chicken meat samples isolated from 4 farms in Surabaya. The existence of *Escherichia coli* in chicken meat indicated that the quality of the meat was not accordance with the SNI 7388-2009 standard.

In this study, the presence of E.coli shown by metallic green colonies. On indole test, the pink indole ring was obtained by adding kovach reagents. The presence of *Escherichia coli* bacteria shows that chicken meat is contaminated by chicken feces during slaughtering on a farm because *Escherichia coli* is an indicator of fecal contamination. Generally, the causes of the large number of *Escherichia coli* bacteria found can be due to several factors. [9]

The first factor of contamination is the lack of cleanliness of the farm environment. Livestock farmers have less concerned about environmental hygiene conditions. The livestock environment consist of feed, water, and soil which is contaminated with animal waste. The second factor is the cleanliness condition of the farmers themselves. [10]

Less clean equipment is the third contamination factor. The poor sanitation is due to less clean tools and careless storage of the equipment. The tools are only washed with water which should be washed with hot water or with chemicals. Contamination is often caused by the equipment at the milking time and using the dirty water when cleaning it. [10]

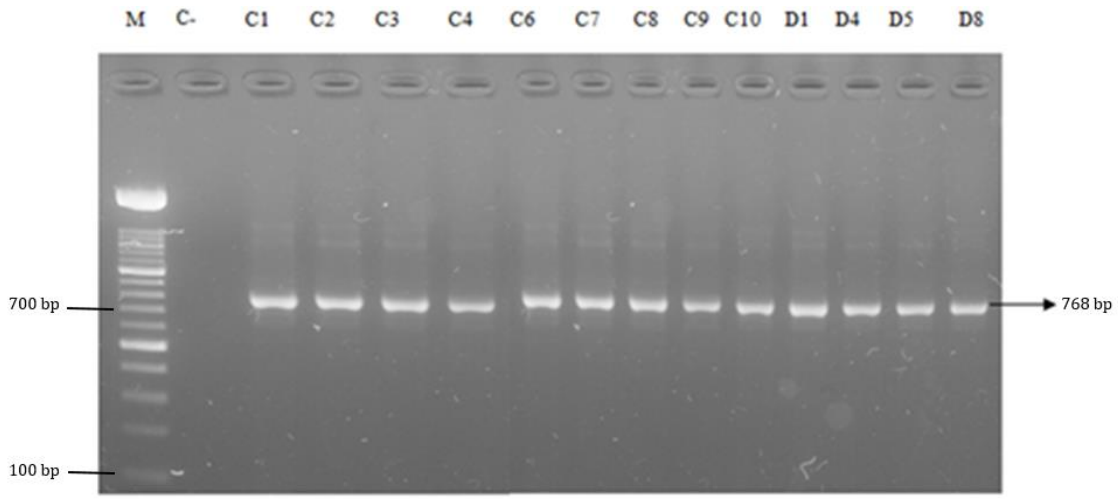

Figure 3. PCR product electrophoresis results, the shv gene was indicated by the band at 768 bp.

Information :
M = Marker
C- = Negative Control
C1 = The first sample from tandes
C2 = Second samples from tandes
C3 = The third sample from tandes
C4 = The fourth sample from tandes
C6 = The sixth sample from tandes
C7 = The seventh sample from tandes
C8 = The eighth sample from tandes
C9 = The ninth sample from tandes
C10 = The tenth sample from tandes
D1 = The first sample from tidar
D4 = The fourth sample from tidar
D5 = The fifth sample from tidar
D8 = The eighth sample from tidar

The fourth factor is poor cage sanitation. A good cage sanitation can minimize the presence of contamination, for example by cleaning the cage regularly during slaughtering. Dirty cage conditions such as the remaining dirt of chicken feed and unclean chicken litter can make the flies come to the cage [11]. *Escherichia coli* has high potential to cause disease even as a normal bacteria, because *Escherichia coli* bacteria have more virulence factors than other.

Table 1 Source of chicken farms and collectors in Surabaya

No.	Sample	***Resistant Escherichia coli***	Source
1.	Chicken Meat A1 - A10	0	Kebraon Barat
2.	Chicken Meat B1 - B10	0	Rungkut
3.	Chicken Meat C1 – C10	9	Tandes
4.	Chicken Meat D1 – D10	4	Tidar

Ampicillin resistant E coli

Based on the results of isolation and identification of 40 samples, 13 isolates were stated as positive for *Escherichia coli* or 32.5%. The isolates were then tested for sensitivity to ampicillin. Resistance test performed using the agar diffusion method, this method is based on the diffusion of antibiotics from paper disks in a petri dish so that the microbes grown are inhibited in circle areas or zones around

the paper disk containing antibiotic solution. This method produces qualitative categories with sensitive, intermediate and resistant assessments. Measurements in Sensitive clear zone meters ≤ 17 mm, intermediate ≤ 14 mm and resistances ≤ 12 mm [7].

Resistance to antibiotics in bacteria can be differentiated into innate (primary) resistance, acquired resistance (secondary) and episomal resistance. Innate resistance (primary) is caused by the presence of antibiotic decomposing enzymes of bacteria, so that bacteria can decompose antibiotics naturally, resistance can be obtained (secondary) due to mutations in bacteria that can occur quickly and can also occur over a long period of time and episomal resistance where bacteria has an R factor on the plasmid which can be transmitted to other bacteria that have related species through conjugate and transduction cell contact. [12]

The development of antibiotic resistance in bacteria is due to two important things, that is the excessive use of antibiotics and the presence of resistant genes. There is a very close relationship between the development of antibiotic resistance and the amount of antibiotic use [13]. When bacteria get a resistance, treatment will be difficult, a large amount of money is required and the results are not necessarily successful.

Basically, there are three mechanisms of how antibiotics work [14]; that is inhibition of cell wall synthesis, inhibition of protein synthesis and inhibition of nucleic acid synthesis. The bacterial cell wall containing peptidoglycan, is a peptide and glucic chain covalently *cross linked*. This relationship requires the transpeptidase enzyme to close it. Betalactam antibiotics (Penicillin, ampicillin, cephalosporin) bind the transpeptidase enzyme and inhibit cell wall synthesis. Transpeptid enzymes are also called penicillin binding proteins. [15]

Table 2 Antibiotic sensitivity test results

No.	Sample	Ampicillin 10 µg
1	Chicken Meat C1	6 (R)
2	Chicken Meat C2	5 (R)
3	Chicken Meat C3	7 (R)
4	Chicken Meat C4	8 (R)
5	Chicken Meat C6	6 (R)
6	Chicken Meat C7	4 (R)
7	Chicken Meat C8	5 (R)
8	Chicken Meat C9	9 (R)
9	Chicken Meat C10	6 (R)
10	Chicken Meat D1	8 (R)
11	Chicken Meat D4	8 (R)
12	Chicken Meat D5	7 (R)
13	Chicken Meat D8	6 (R)

Information: (R) for resistant

Antibiotic resistance can occur through four main mechanisms: transfer of antibiotic target point (such as changes in *penicillin binding proteins*), drug breakdown and enzymatic inactivation of antibiotics *(penicillinase)*, permeability changes in cell wall that prevent antibiotics to enter, and increasing pressure

activity in cells prevent the accumulation of antibiotics in cells. [16]

Ampicillin includes bactericidal antibiotics and have a working mechanism causing damage to bacterial cell walls [17]. The ampicillin working mechanism consists of: 1). Inhibition of bacterial cell wall synthesis by inhibiting transpeptidation of peptidoglycan synthesis in bacterial transpeptidase enzyme reactions. Transpeptidase is an enzyme that works in the cross-linking process of the peptide chain in forming peptidoglycan compounds that occur in the final stages of cell wall formation. The cross linking process is used in the structural integrity of bacterial cell walls. 2). Drug attachment to the specific proteins that bind penicillin or Penicillin-Binding Protein (PBP) which applies as a drug receptor in bacteria. 3). Activation of autolytic enzymes on cell walls due to drug attachment to PBP. This activation causes lysis of bacterial cell walls. [18]

Electrophoresis Result of Polymerase Chain Reaction Products

The results of *Polymerase Chain Reaction* electrophoresis products showed that from positive *Escherichia coli* samples that were resistant to ampicillin antibiotics showed the existence of primary amplification bands that had a size of 768 bp. [6]

This study has succeeded in isolating 13 *Escherichia coli* bacteria that are resistant to ampicillin antibiotics and found 13 *Escherichia coli* bacteria that have a band size of 768 bp. It proves that genotically there are *Escherichia coli* bacteria which are resistant to ampicillin antibiotics in the chicken meat samples.

The possibility of *Escherichia coli* that is resistant to ampicillin antibiotics will increase because the shv gene can be removed through a mechanism of horizontal gene transfer. The mechanism of antibiotic resistance also causes changes in the bacterial genome. Permanent genetic changes in the sequence of base pairs are called mutations. This can occur naturally and is called spontaneous mutation, or it can also be caused by environmental factors, such as exposure to physical agents or chemicals. Regardless of the cause of the mutation, changes in the number and type of genes are known as genotypes and as a result there is a changes in the physical characteristics or phenotypes of bacteria. [19]

These changes can be caused due to permanent changes in genes at one point (mutation point); there are deletions, additions, or replacements of other base pairs, gene deletions, changes in gene pair order and gene insertion. [20]

A process when the genetic material of *Escherichia coli* can be transferred from one bacterium to another is when conjugation mechanism happens. In this situation two compatible bacterial cells form a temporary connection through the conjugate bridge. The bridges function is as a line or channels that transfer genetic material from one bacteria (donor) to other bacteria (recipient). [21]

4. CONCLUSION

This study succeeded in isolating 13 *Escherichia coli* bacteria from 40 chicken meat samples isolated from four farms and chicken collectors in Surabaya. A total of 13 samples of *Escherichia coli* were resistant to ampicillin 10 µg with the size of the inhibitory zone ≤ 12 mm and it was found that there were shv gene fragments in *Escherichia coli* that were resistant to ampicillin antibiotics which had a size of 768

bp. The results can be concluded that the quality of microbes and the safeness of chicken meat isolated from several farms and chicken collectors in Surabaya is not safe for consumption. The existence of *Escherichia coli* bacteria shows that chicken meat is contaminated by chicken feces during the chicken slaughtering process that causes pathogens for human health. Continuous efforts are needed to reduce resistance in humans by close monitoring of the resistance of E. coli antibiotics from chicken meat.

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