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# Incidence of Multidrug Resistance *Escherichia Coli* Bacteria in Broiler Chickens in Malang Regency

Fidi Nur Aini Eka Puji Dameanti[1*], Jeffrys Salsabila[1], HarioPuntodewo Siswanto[1], Indah Amalia Amri[1], Sruti Listra Adrenalin[1], Mira Fatmawati[1], Taty Aryanti[2]

[1]Faculty of Veterinary Medicine Universitas Brawijaya, Indonesia
[2]Center for Veterinary Research Bogor, Indonesia

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*Escherichia coli*

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Antibiotic Sensitivity Test

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**ABSTRACT**

Chicken meat is an important source of protein but the presence of bacterial infections such as colibacillosis is a major concern for the chicken producers. Further, colibacillosis is a major cause of mortality, morbidity, and economic loss for the poultry industry. Various efforts including the use of antibiotics have been carried out to treat colibacillosis. Recently, inappropriate use of antibiotics not only induced antibiotic resistance, but sometimes it might change into multidrug resistance due to a large number of antibiotic uses. This study aimed to identify the incidence of multidrug resistance in broiler chickens on 4 farms of Malang Regency. For this, samples of 40 chicken jejunum swabs that had a history of colibacillosis with clinical symptoms of lethargy, drooping, dwarfism, hair loss, depression, thinness, diarrhea, abdominal swelling, and osteoarthritis were used in this study. Testing begins with a microscopic examination, followed by the isolation of *E. coli* on Nutrient broth (NB) and Eosin Methylene Blue Agar (EMBA) media, and finally, antibiotic sensitivity was tested against eight antibiotics namely Gentamicin, Bacitracin, Enrofloxacin, Doxycycline, Oxytetracycline, Erythromycin, Colistin, and Amoxicillin on Mueller-Hinton Agar (MHA) media. The microscopic observations showed that the chickens had a hemorrhage in the proventriculus and intestines, pericarditis, and fibrinous exudate in the air sacs and heart. Among the tested samples, 72.5% (29 samples) were found positive for *E. coli*. Further, in case of antibiotic resistance, 100% of *E. coli* positive samples were found resistant to Erythromycin, Bacitracin, and Amoxicillin, 96.6% to Enrofloxacin, 92.6% to Oxytetracycline, 37.9% to Colistin and Doxycycline, 10.3% to Gentamicin. Results of the study can be concluded that most of the E.coli positive samples have antibiotic resistance and the maximum samples are showing multidrug resistance against four or more antibiotics.

* Corresponding author
E-mail: drhfidi@ub.ac.id (Fidi Nur Aini Eka Puji Dameanti)

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## 1 Introduction

Chicken is a source of fulfillment of animal protein needs in Indonesia. Broiler farms of Malang regency are the second largest producer of broiler chickens with 28,929,203 chickens in East Java. These days' chicken industries are facing many challenges including the meet of growing community demand and strict supervision of the chicken's health. Further, pathogenic microorganisms such as viruses, bacteria, and various other parasites are hazardous to the health of broilers because these are consumed by humans. Among the broiler, colibacillosis also known as mushy chick disease and cellulitis is the most common one and is caused by the localized or systemic infection of *Escherichia coli* bacteria. This disease caused the highest morbidity, mortality rates, and economic loss, and it has a terrible impact on broiler production (Ibrahim et al. 2019). In this disease, *E. coli* colonized in various organs of broiler chicken such as omphalitis, perihepatitis, pericarditis, mesenteries, and others (Suryani et al. 2014). Frequent or uncontrolled use of antibiotics is the most common method used by the Indonesian chicken producer for the treatment of colibacillosis. However, as time goes on, more and more antibiotics become ineffective in treating *E. coli* infections.

Shecho et al. (2017) reported antibiotic resistance in E. coli against various antibiotics such as erythromycin, clindamycin, spectinomycin, ciprofloxacin, ampicillin, and amoxicillin, and suggested that among the tested antibiotics, E. coli had highest antibiotic resistance against the ampicillin (92.3%) while had lowest resistance against the amoxicillin (34.61%). If it continues, *E. coli* might develop a significant resistance against the various groups of antibiotics which are considered multidrug resistance. Information regarding the colibacillosis infection and multidrug resistance in Malang regency, Indonesia chicken farms are in scanty; therefore this study was carried out to identify the incidence of multidrug resistance in four broiler chicken farms of Malang Regency.

## 2 Materials and Methods

This research was conducted in July - August 2020 at the Laboratory of Anatomical Pathology, Faculty of Veterinary Medicine, Universitas Brawijaya, which includes the process of necropsy and swab of broiler chicken intestines. Identification and antibiotic sensitivity testing was carried out at the Laboratory of Microbiology and Immunology of the Faculty of Veterinary Medicine, Universitas Brawijaya.

### 2.1 Used Media and Materials

The tissue culture media and other materials used for the isolation and multiplication of *E. coli* isolates from broiler chicken intestine swabs COBB strain are phosphate-buffered saline (PBS), Nutrient Borth (NB) media (Merck® no 105443), Eosin Methylene Blue Agar (EMBA) media (Oxoid® CM0069), Triple- Sugar Iron Agar (TSIA), Simmon Citrate Agar (SCA), Sulfide Indole Motility (SIM) media, Urease media, Methyl Red (MR), Voges-Proskauer (VP) media, media catalyze, Glucose medium, Lactose medium, Sucrose medium, and Gram stain set (crystal violet, Lugol, acetone alcohol, safranin). Media Mueller-Hinton Agar (Oxoid® CM0337) supplemented with various group of antibiotics i.e. Erythromycin (Oxoid® CT0019B @ 10 µg concentration), Colistin (Oxoid® CTOO17B @ 10 µg concentration), Bacitracin (Oxoid® DD0002 @ 10 µg concentration), Enrofloxacin (Oxoid® CT0639B @ 5 µg concentration), Doxycycline (Oxoid® CT0018B @ 30 µg concentration), Oxytetracycline (Oxoid® CT0041B @ 30 µg concentration), Gentamycin (Oxoid® CT0024B @ 10 µg concentration), and Amoxicillin (Oxoid® CT0061B @ 10 µg concentration).

### 2.2 *E. coli* Isolation and Identification

This research used a descriptive laboratory method carried out by qualitative laboratory examination. Samples were collected from the broiler chickens aged between 14-17 days from the 4 farms of Malang Regency, East Java, Indonesia. Determination of the sample using 10% disease prevalence, sampling error rate (5%), and sampling confidence level (95%) using the Win Epi application (Thrusfield et al. 2001). The sample was selected by purposive sampling by considering the symptoms of suspected chickens suffering from colibacillosis.

The first stage of this study was to examine the chicken body as a whole to see clinical symptoms of chickens suspected of colibacillosis, followed by euthanasia by the decapitation method, and dissection of the carcass and examination of each organ to find out the pathological changes. Wahyuwardan et al. (2014) studied the results of necropsy regarding the anatomical modifications and identified *E. coli* from the jejunum swab of broiler chickens and recommended that *E. coli* can be isolated from the jejunal intestine by swab technique.

In this study, the swab sample was taken using sterile cotton and inoculated on Nutrient Borth (NB) (Merck® no 105443), and incubated at 37ºC for ± 24 hours. After incubation, it continued with primary isolation on Eosin Methylene Blue Agar (EMBA) (Oxoid® CM0069) media using the streak method and incubated at 37ºC for 24 hours. The metallic green colonies from EMBA were subject to Gram staining to see the microscopic morphology of the isolated bacteria, and biochemical tests consisting of IMViC test (Indole test, Methyl Red test, Voges Proskauer test, and Citrate test), sugar test ( glucose, lactose, and sucrose), catalase test, and urease test was carried out to identify the biochemical properties of *E. coli* (Fardiaz 1992).

### 2.3 Antibiotic Sensitivity

The isolated *E. coli* strains were tested for antibiotic sensitivity. A total of eight types of antibiotics belonging to six groups of antibiotics were tested against the isolated *E.coli* strain. The selection of used antibiotic types was based on interviews with farmers regarding the often used antibiotics.

The disc diffusion method including the Kirby-Bauer method or filter paper disk was used for estimating the antibiotic sensitivity. The diameter of the inhibition zone was measured and analyzed for all the tested antibiotics. The standard used to determine the level of antibiotic sensitivity is based on the Clinical and Laboratory Standards Institute (CLSI) standards.

### 2.4 Statistical Analysis

The research results analysis was descriptive, describing the data from the isolation and identification of *E. coli* from broiler chicken intestine swabs and quantitatively analyzing the data from the diameter of the antibiotic inhibition zone obtained.

## 3 Results and Discussion

### 3.1 Necropsy

Samples that meet the criteria of inclusion are systematically operated on starting by examining the poultry body as a whole, followed by euthanasia using the decapitation method and necropsy according to the poultry necropsy protocol. Each organ of the euthanized chickens has been examined for the finding out the pathological changes (Figure 1). Examination of the carcass condition includes fluid that comes out of natural holes, nutritional status, abnormal formations, skin, wattles, cloaca, and the presence of external parasites (Poultry Industry Council of Canada 2016).

Figure 1 Chickens with Clinical Symptoms of Colibacillosis

According to Hastarinda (2016), clinical symptoms of chickens suffering from colibacillosis are generally characterized by thinness, dull fur, decreased appetite, depression, disturbed growth, diarrhea, and dirty feathers in the cloaca area. According to Mahari (2014), other clinical signs that can be found are skeletal lesions and continued systemic infection. This is following the results of the pre-euthanasian examination carried out in this study. All the collected 40 samples showed similar symptoms such as lethargy, drooping, and stunted growth. Other symptoms that appeared

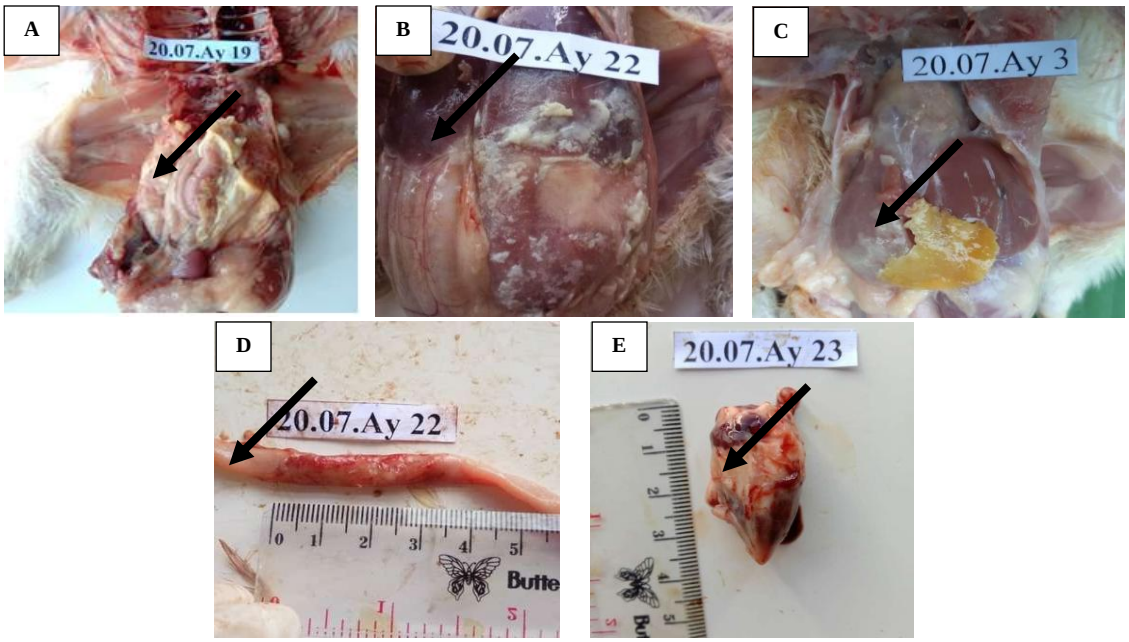


Figure 2 Appearance of macroscopic lesions resulting from the necropsy of poultry (A - fibrinous exudate on the air sac; B - Fibrinous exudate on the peritoneum; C - Chewy exudate on the liver; D - Intestinal hemorrhage; E - Pericarditis

during the study were hair loss (11 samples), depression (29 samples), thinness (28 samples), diarrhea (12 samples), and swelling in the abdominal area (7 samples).

The most common pathological changes reported from the collected broiler chicken samples after necropsy were bleeding in the proventriculus and intestines, pericarditis, and fibrinous exudate in the intestines, liver, air sacs, and heart (Figure 2). According to Mahari (2014), E. coli that infects the intestine can enter the bloodstream and infect other organs in systemic cases. *E. coli* that infects tissue will cause an inflammatory response to increase the cytokine IL-1, IL-6, and tumor necrosis factor-alpha. This inflammatory response increases vascular permeability and increases the accumulation of fluid and protein in the tissues to form a gelatinous exudate. The thick exudate becomes a visible exudate that accumulated and, in the end, caseation occurs to form a compact mass like cheese, dry and yellow.

### 3.2 Isolation of *E. coli*

The purpose of the necropsy is to see pathological changes and treat the swab site namely the jejunum swab. Isolation of *E.coli* was carried out on the EMBA media to obtain metallic green colonies for *E. coli* (Prawesthirini et al. 2009). The results of colony growth can be seen in Figure 3. EMBA media is a differential selective media for *E.coli* (Lindquist and John 2004), and it formed metallic green colonies on this medium by the end product of lactose fermentation by acidic *E. coli* resulting in the absorption of methylene blue color to form metachromatic, which gives the absorption of methylene blue a metallic green sheen. This medium can also inhibit the growth of Gram-positive bacteria (Lal and Cheeptham 2007). Isolation of *E. coli* from the jejunum swab of broiler chickens in the jejunum section showed that the total samples suspected to be positive for *E. coli* were 33, with a percentage of 82.5%.

### 3.3 Identification of *E. coli*

#### 3.3.1 Gram stain

Metallic green colonies on EMBA media were confirmed by using Gram stain and revealed the presence of Gram-negative *E. coli* in the form of colibacillosis (Figure 4). According to Ulfah et al. (2017), *E. coli* produced metallic green colonies on EMBA, and due to the absence of a thick peptidoglycan wall; it absorbs pink-red staining in Gram staining and the shape of bacteria is rod-shaped and fimbriae. Based on the identification stage with Gram staining, it was found that all 33 samples were colibacillosis and Gram-negative bacteria.

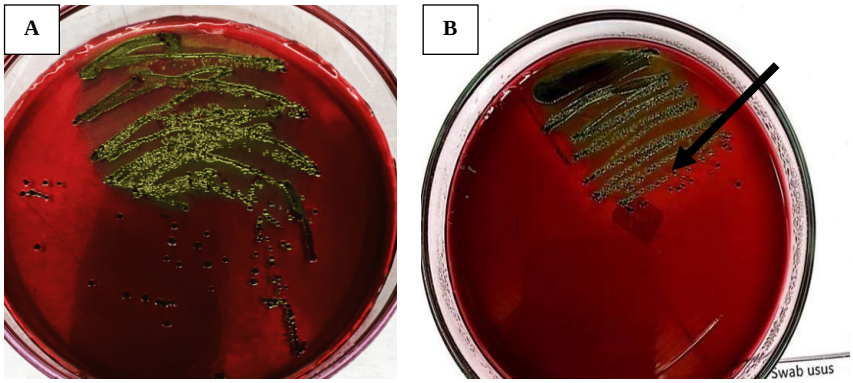


Figure 3 E. coli colony on EMBA media (A - Primary isolation; B - Secondary isolation contained separate E. coli colonies)

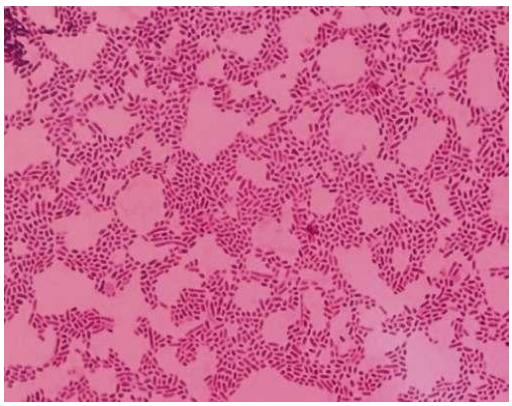

Figure 4 Gram stain results *in Escherichia coli* with 1000 x magnification

### 3.3.2 Biochemical Test

Samples that have been identified through Gram staining are subjected to biochemical tests consisting of the IMViC test (Indole test, Methyl Red test, Voges Proskauer test, and Citrate test), sugar test (glucose, lactose, and sucrose), catalase test, TSIA, and urease test (Figure 5- 12). Biochemical tests were carried out for differentiation based on differences in the biochemical properties of bacteria. Speciation between bacteria can be distinguished based on sugar fermentation, metabolic materials, and food ingredients based on biochemical properties. The biochemical test results showed that there were 29 samples (72.5%) were positive for *E. coli*. Elements of chemical requirements between various bacteria are also different in carbon, nitrogen, sulfur, phosphorus, metal compounds, oxygen, growth factors, and nutrient uptake (Dzen et al. 2010).

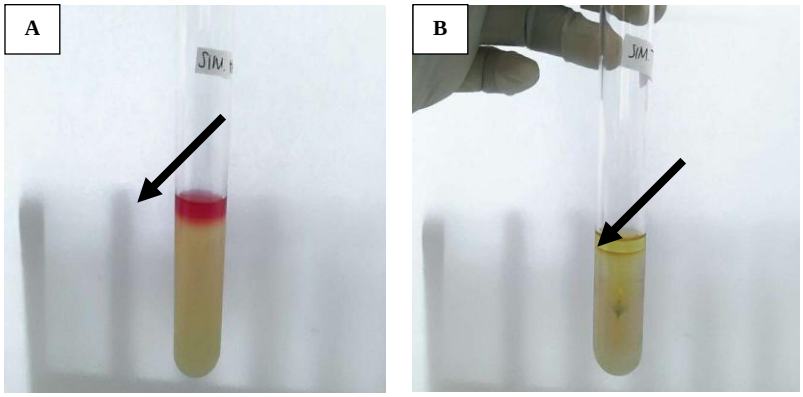


Figure 5 Results of Indole test (A - Positive indole forms a red ring; B - Negative indole does not form a red ring)

Figure 6 Results of Methyl Red - Positively marked media color change

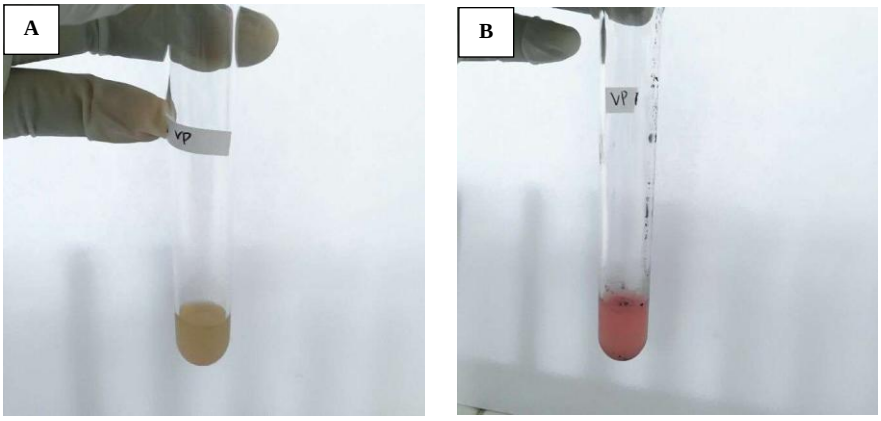


Figure 7 Results of Voges Proskauer (A - Voges Proskauer negative; B - Voges Proskauer positive)

Figure 8 Results of Citrate negative

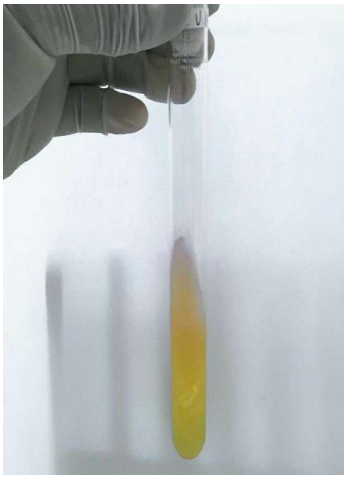

Figure 9 Results of Urease test as a negative result, media remains Yellow

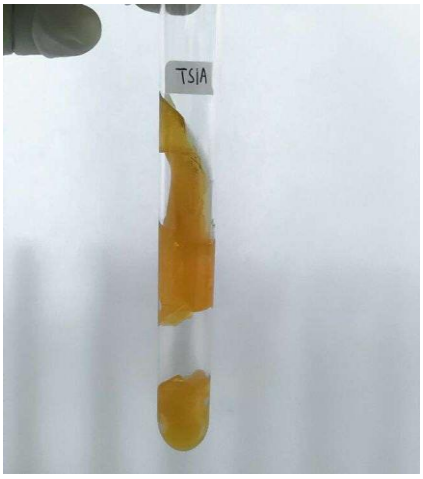


Figure 10 Results of TSIA test; Slant and Butt on the media changes color to yellow, and gas is formed

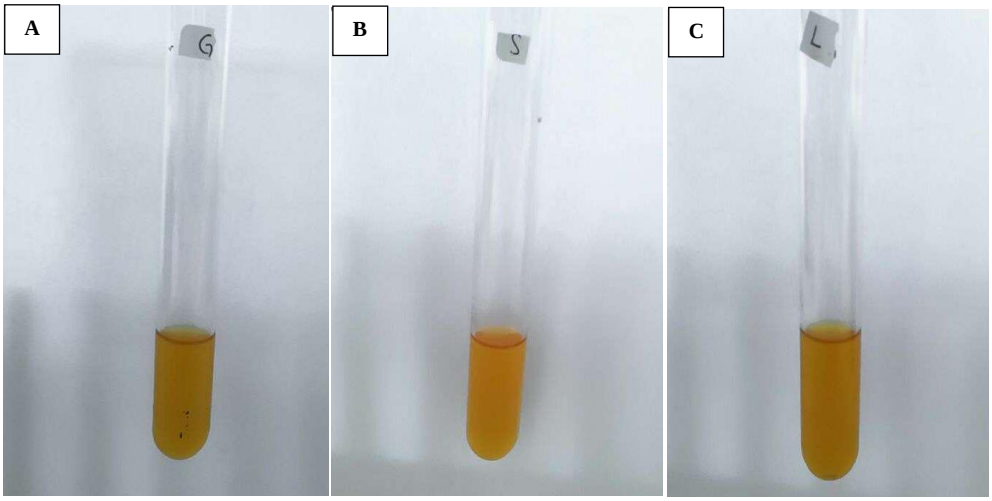


Figure 11 Results of Confectionery test (A) Glucose positive, (B) Sucrose positive, (C) Lactose positive

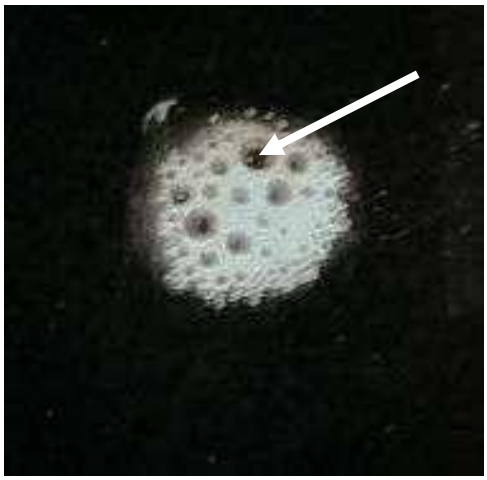

Figure 12 Results of Positive catalase showed bubble formation

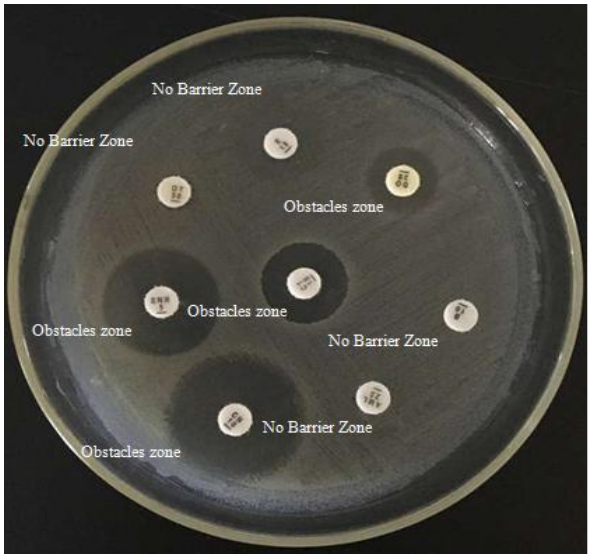


Figure 13 Observation of the diameter of the antibiotic inhibition zone

Table 1 Antibiotic sensitivity test results

| No. | Sample Code | | AB Susceptibility Test (mm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | E (15µg) | CT (10µg) | ENR (5µg) | DO (30µg) | OT (30µg) | CN (10µg) | B (10µg) | AML (25µg) |
| Amount and Percentage | | S | 0 | 18 | 1 | 11 | 2 | 22 | 0 | 0 |
| | | | 0.00 | 62.10 | 3.40 | 37.90 | 7.40 | 75.80 | 0 | 0 |
| | | I | 0 | 0 | 0 | 7 | 0 | 4 | 0 | 0 |
| | | | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 13.70 | 0 | 0 |
| | | R | 29 | 11 | 28 | 11 | 27 | 3 | 29 | 29 |
| | | | 100.00 | 37.90 | 96.60 | 37.90 | 92.60 | 10.30 | 100 | 100 |

S (Susceptible), I (Intermediate), R (Resistant), E (Erythromycin), CT (Colistin), ENR (Enrofloxacin), DO (Doxycycline), OT (Oxytetracycline), CN (Gentamicin), B (Bacitracin), AML (Amoxicillin)

Table 2 Pattern of Multi-Drug Resistance in *E. coli* Isolates

| Total Group | Antibiotics group | n | % |
|---|---|---|---|
| 4 | Macrolides + Peptides + Fluoroquinolones + Penicillins | 1 | 3.45 |
| | Macrolides + Peptides + Tetracyclines + Penicillins | 1 | 3.45 |
| 5 | Macrolides + Peptides + Fluoroquinolones + Aminoglycosides + Penicillins | 1 | 3.45 |
| | Macrolides + Peptides + Fluoroquinolones + Tetracyclines + Penicillins | 24 | 82.76 |
| 6 | Macrolides + Peptides + Fluoroquinolones + Tetracyclines + Aminoglycosides + Penicillins | 2 | 6.90 |
| | Amount | 29 | 100 |

### 3.3.3 Antibiotic Sensitivity Test

Twenty-nine samples with confirmed *E. coli* were continued with the antimicrobial susceptibility test. The disc diffusion method including the Kirby-Bauer method or filter paper disk was used for estimating the antibiotic sensitivity and as a result of this study, the inhibition zone was measured which indicated the absence of bacteria growth in the area around the antibiotic disk (Figure 13). The diameter produced by this test indicates the nature of the bacteria to antibiotics based on the standards of each antibiotic, namely sensitive (S), intermediate (I), and resistance (R) (CLSI 2018). The results of the study showed that 100.00% of *E. coli* isolates were resistant to Erythromycin, Bacitracin, and Amoxicillin while 96.60% were resistant to Enrofloxacin, 92.60% to Oxytetracycline, 37.90% to Colistin and Doxycycline, and lowest resistance of 10.30% was reported for the Gentamicin (Table 1).

Table 2 shows that all samples of *E. coli* isolate experienced MDR and 6.90% of the samples were resistant to 4 and 6 classes of antibiotics while 86.21% were resistant to 5 classes of antibiotics. The results of the current study are in line with the findings of Bushen et al. (2021) those who reported a high incidence of MDR (52.5%) in healthy chickens taken from Southwest Ethiopian farms. Noor and Poeloengan (2005) suggested that inappropriate use of antibiotics due to misdiagnosis, too harsh treatment, wrong doses, and continuous use of antibiotics as growth promoters are some important causes of multi-drug resistance. According to Utami (2011), the use of antibiotics in livestock as growth promoters agents or as growth stimulators of livestock in subtherapeutic doses will increase the risk of multi-drug resistance.

**Conclusion**

There are 29 of 40 samples (72.50%) collected from 4 farms in Malang Regency, East Java was found positive for *E. coli*. The results of antibiotic sensitivity testing showed that 100% *E. coli* isolates were resistant to Erythromycin, Bacitracin, and Amoxicillin, while 96.6% to Enrofloxacin, 92.6% to Oxytetracycline, 37.9% to Colistin and Doxycycline and 10.3% to Gentamicin. All isolates of *E. coli* showed the occurrence of MDR against four or more classes of antibiotics.

**Acknowledgments**

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