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Webb CO, Cannon CH, Davies SJ. 2008. Ecological organization, biogeography, and the phylogenetic structure of rainforest tree communities. In: Carson W, Schnitzer S (eds) Tropical Forest Community Ecology. Wiley-Blackwell, New York.

Abstract:

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Proceeding:

Alikodra HS. 2000. Biodiversity for development of local autonomous government. In: Setyawan AD, Sutarno (eds.) Toward Mount Lawu National Park; Proceeding of National Seminary and Workshop on Biodiversity Conservation to Protect and Save Germplasm in Java Island. Universitas Sebelas Maret, Surakarta, 17-20 July 2000. [Indonesian]

Thesis, Dissertation:

Sugiyarto. 2004. Soil Macro-invertebrates Diversity and Inter-Cropping Plants Productivity in Agroforestry System based on Sengon. [Dissertation]. Universitas Brawijaya, Malang. [Indonesian]

Information from internet: Balagadde FK, Song H, Ozaki J, Collins CH, Barnet M, Arnold FH, Quake SR, You L. 2008. A synthetic Escherichia coli predator-prey ecosystem. Mol Syst Biol 4:187. www.molecularsystembiology.com

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Detection of virulence factor encoding genes on *Escherichia coli* isolated from broiler chicken in Blitar District, Indonesia

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Abstract. *Efendi MH, Faridah HD, Wibisono FM, Wibisono FJ, Nisa N, Fatimah F, Ugbo EN. 2022. Detection of virulence factor encoding genes on Escherichia coli isolated from broiler chicken in Blitar District, Indonesia. Biodiversitas 23: 3437-3442.* Broiler chicken is a source of protein that is widely consumed by the public. However, broiler chicken production sometimes decreases due to infectious diseases such as colibacillosis caused by pathogenic *Escherichia coli* possessing virulence genes. Virulence factors function to facilitate colonization and invasion of host cells to cause disease. The presence of these virulence factors is encoded by various genes such as the increased serum survival gene and P fimbriae gene which plays a role in surface adhesion. The present study aims to detect the presence of virulence genes from extended-spectrum beta-lactamase (ESBL) producing *E. coli* isolated from broiler chickens in the Blitar District. A total of 110 cloacal swabs collected by systematic random sampling from broiler poultry farms in four different sub-districts were screened for ESBL-producing *E. coli* and virulence genes by phenotypic and molecular methods, respectively. Out of 110 *E. coli* recovered, 95 (86.4%) were observed to show a high level of resistance to the tested antibiotics, and 34 (35.7%) were ESBL-producers. Among ESBL producing *E. coli* isolates, 22 (73.5%) and 1 (2.9%) were found to have the *iss* and *papC* gene virulence factors, respectively using the polymerase chain reaction (PCR) method. The results of this study indicate that virulence genes can be found in *E. coli* from poultry farms. The *iss* gene is the most predominant virulence gene. The report of these virulence factors in *E. coli* isolated from broiler could impose a serious potential public health problem.

Keywords: Broiler chickens, ESBL, *Escherichia coli*, public health, virulence genes

INTRODUCTION

The main sector of the national economy is strongly supported by the success of the poultry industry. The largest food supplier for the entire human population in the world, especially animal protein, is highly dependent on poultry production. Evidence that poultry production has advantages over other types of animal food products because it has a relatively cheaper price and relatively high animal protein (Aryani and Jember 2019). The high market demand for poultry commodities can cause the development of poultry in Indonesia to increase. Prices that can meet purchasing power are the reason for most Indonesian people to fulfill animal protein nutrition at all socio-economic levels. Maintenance management, environmental sanitation, and poultry health are factors that support the success of poultry farms in Indonesia (Kabir 2010; Wiedosari and Wahyuwardani 2015; Wibisono et al. 2020a). The relationship of these related factors will appear a balance, if there is an imbalance of one of these factors it will cause a disease. Infectious diseases involve the causative agent and host, as well as environmental factors. Diseases caused by *Escherichia coli* are disease agents that are often faced by all livestock farms, especially poultry farms, therefore knowledge, and information about disease incidence and prevention, control, and eradication efforts are needed (Putra et al. 2020; Wibisono et al. 2020b; Ansharieta et al. 2021a).

Escherichia coli is a bacterium of the Enterobacteriaceae family that has morphological characteristics in the form of a rod, has a flagellum, and is a Gram-negative commensal bacteria (Jang et al. 2017). Naturally, *E. coli* is a normal flora that lives in the digestive tract of animals and humans (Daga et al. 2019). However, some *E. coli* acquire virulence properties so that they can adapt to a new environment. This factor causes *E. coli* to be able to invade the host to cause disease (Doxey et al. 2019). Several virulence factors function to facilitate colonization and invasion of host cells (Leitão 2020). These virulence properties can be categorized as adhesion, toxin

production, hemolysis, iron acquisition, and protection from host bactericidal, including those that produce the enzyme extended-spectrum beta-lactamase (ESBL) (Mohamed et al. 2014; Wibisono et al. 2020b; Widodo et al. 2020). Virulence factors present in pathogenic *E. coli* strains include the P fimbriae gene (*pap*C), increased serum survival protein (ISS), aerobactin (IUCD), temperature-sensitive haemagglutinin (TSH), iron repressible protein (irp2), vacuolating autotransporter protein (vat), and colicin plasmid operon genes (*cva*/*cvi*) (Ewers et al. 2005; Biran et al. 2021).

Surface virulence factors (adhesins) are one of the important virulence factors in *E. coli*. The main host attachment factor, P fimbriae, has been associated with pyelonephritis (Hossain et al. 2020). The *papC* gene is responsible for attachment to internal organs (Mahmoud et al. 2020). In addition, there is also an *iss* gene that is associated with the occurrence of colibacillosis in poultry (Bonjar et al. 2017). The *iss* gene was first described in the ColV plasmid and plays a role in resistance to serum complement (Gibbs et al. 2003; Biran et al. 2021). The gene encodes an iss protein that has a characteristic outer membrane proteins (OMP) signal sequence and encodes a lipoprotein 9 to 10 KDa from the bacterial outer membrane (Badouei et al. 2015).

Research on virulence genes from ESBL-producing *E. coli* is useful for increasing understanding of the pathogenesis of *E. coli* strains. Besides that, it can also minimize the complications of disease caused by infection with *E. coli* (Firoozeh et al. 2014). Therefore, the present study was aim to detect virulence factors associated with the *iss* gene that plays a role in developing the immune system by increasing survival serum and the *papC* gene that encodes the adhesin virulence factor of ESBL producing *E. coli.*

MATERIALS AND METHODS

Sample collection

A total of 110 cloacal swabs from broiler chickens were taken using Amies transport swab by inserting a swab stick into the vent, and by gently swabbing the mucosal wall till the swab was stained with fecal material. Samples were taken randomly in four different subdistricts. A total of 110 samples were collected from four different sub-districts including 28, 25, 31 and 26 samples from Ponggok, Garum, Selopuro and Selorejo sub-district, respectively. All samples were transported in specimen transport coolbox containers with ice packs to the Department of Veterinary Public Health, Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia. The samples were processed within a maximum of 5 h of collection.

Bacterial isolation and identification

Cloacal swab samples were cultured on eosin methylene blue agar (EMBA) plates by streaking using a loop. After that, EMBA plates were incubated for 24 h at 37°C in an incubator. A single colony showing a green color with a metallic flash was taken for purification. The pure culture was obtained by re-streaking the colony on EMBA plates. After that, a biochemical test was carried out consisting of the indole test, methyl red (MR) test, Voges Proskauer (VP) test, citrate test, and triple sugar iron agar (TSIA) test to identify the isolates. In addition, Gram staining was also performed for microscopic observation.

Antimicrobial susceptibility testing

The identified *E. coli* were then tested for antimicrobial sensitivity to several classes of antibiotics. The sensitivity test to antibiotics was carried out using the Kirby-Bauer disc diffusion method and the interpretation of the results was referred to the Clinical and Laboratory Standard Institute. The antibiotics used were ampicillin (10 μg), gentamicin (10 μg), tetracycline (30 μg), chloramphenicol (30 μg), and enrofloxacin (5 μg) antibiotics. Samples were cultured on Mueller-Hinton agar (MHA) media and flattened over the entire surface of the media. After that, the antibiotic disc was placed on top of the microbial culture in a petri dish using a sterile needle and incubation for 24 h. The inhibition zone diameter was measured using a caliper. Samples that were resistant to ampicillin were then further tested using Double Disc Synergy Testing (DDST) to determine their ability to produce ESBL.

Phenotypic confirmations of ESBL

The presumptive ESBL-positive isolates were retested for ESBL production by the Double Disc Synergy Test (DDST). A set of two discs containing extended-spectrum cephalosporin [cefotaxime (30 μg)] or ceftazidime (30 μg) alone and with a clavulanic acid combination (10 μg) were placed on-center spacing 15 mm apart on a MHA plates inoculated with a bacterial suspension compared with 0.5 McFarland turbidity standard. Zone diameters were measured after overnight incubation at 37°C. Strains resistant to cefotaxime (zone diameter ≤27 mm) or ceftazidime (zone diameter ≤22 mm) and an increase in zone diameter ≥5 mm with the discs containing clavulanic acid was defined as ESBL-producing isolates according to Clinical and Laboratory Standard Institute (CLSI 2020).

Virulence genotyping

The detection of virulence genes was carried out using polymerase chain reaction (PCR). The genes observed were *papC* and *iss*. *E. coli* were first cultured and harvested when they were 24 h old. The *E.coli* isolate was then centrifuged to take the pellets as DNA extraction material. *E. coli* genomic DNA was extracted using GenJET Genomic DNA Purification Kit. The specific primers were used to assess the virulence genes (Table 1). The PCR conditions used for the *papC* virulence gene were done by conditioning the denaturation temperature at 94°C for 60 sec, annealing at 59°C for 60 sec, and elongation at 72°C for 90 sec. Meanwhile, the *iss* virulence gene was detected by conditioning the denaturation temperature at 94°C for 30 sec, annealing at 52°C for 30 sec, and elongation at 72°C for 40 sec. The PCR was run for 30 cycles and then visualized by electrophoresis using 1% agarose gel and the results were documented using the Gel-Doc system (Mohamed et al. 2014).

RESULTS AND DISCUSSION

Isolation and identification of 110 broiler cloacal swabs on EMBA media showed that 100% of the samples contained *E. coli*. Positive results are indicated by a change in the color of the medium from red to metallic green (Figure 1). When observed under a microscope, *E. coli* is rod-shaped bacterium and appears pink.

The results of the antibiotic sensitivity test against several classes of antibiotics (Figure 2). Antimicrobial sensitivity test was performed, *E. coli* showed resistance to ampicillin (86.4%), enrofloxacin (79.1%), tetracycline (78.2%), gentamicin (43.6%), and chloramphenicol (14.5%). Meanwhile, antibiotics that still have a fairly high level of sensitivity to *E. coli* are chloramphenicol (78.2%) and gentamicin (52.7%) (Figure 3).

Of the 95 isolates of *E. coli* that were resistant to ampicillin, 35.7% were ESBL producing *E. coli* based on the DDST test (Figure 4). Then, the *iss* and *papC* virulence genes were detected using. A total of 73.5% of *E. coli* isolates had the *iss* gene, which was indicated by the presence of a DNA band at 290 bp (Figure 5). Meanwhile, the *papC* gene was detected in 2.9% of *E. coli* isolates with a product size of 500 bp (Figure 6).

Discussion

The ESBL-producing *E. coli* were analyzed for virulence genes using specific primers from 4 sub-districts (Table 2). Several other studies have examined the number of *E. coli* isolates that isolated from animal and animal products, showing concordance results between studies (Wibisono et al. 2020c). The relative abundance of the ESBL producing *E. coli* in samples poultry has been shown to vary with geographic location (Wibisono et al. 2020d). In this study, isolates including ESBL producing *E. coli* were detected by DDST (Anshariet et al. 2021a). Molecular identification showed that 25 (73,5%) samples of ESBL producing *E. coli* encoding *iss* gene, and 1 (2,9%) sample of ESBL producing *E. coli* encoding *papC* gene (Table 2). Electrophoresis results of *iss* gene represent samples describing the same fragments as positive controls with a gene length of 290 bp (Figure 5), and *papC* gene represents sample describing the same fragments as positive controls with a gene length of 500 bp (Figure 6) (Mohamed et al. 2014).

Treatment failure and the risk of resistance or side effects are often caused by inappropriate use of antimicrobials. Antibiotics have been used not only in human medicine but also in animal care. Initially, antibiotics were used to treat sick animals, with intensification of agriculture, expanding the use of antibiotics to include disease prevention and use as growth promoters (Witaningrum et al. 2021). Overuse of antimicrobials in livestock will pollute the environment and contribute to the increase in resistance of microorganisms that threaten not only human health but also animal health, animal welfare and sustainable poultry production and this has implications for food security (Effendi et al. 2021). The misuse of antimicrobials makes the use of these drugs ineffective for animal and human health because they cause antimicrobial resistance (AMR) to develop and appear in disease-causing microorganisms, and the Enterobacteriaceae group can develop by producing ESBL (Ibrahim et al. 2019; Wibisono et al. 2020c).

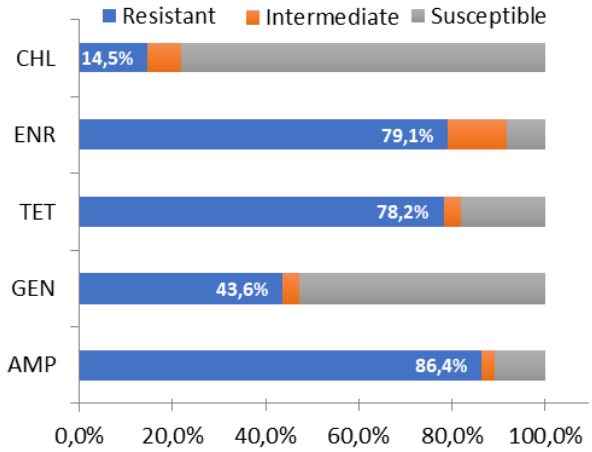
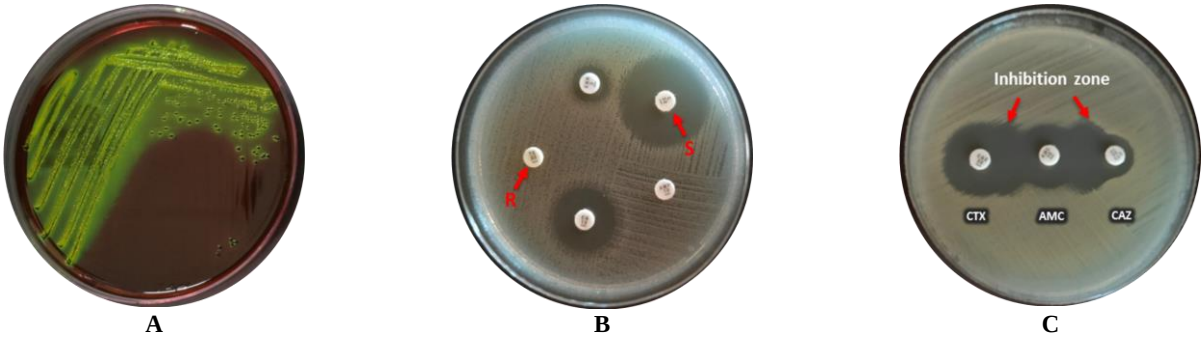


Figure 3. Graph of sensitivity test results to 5 antibiotics. ampicillin (AMP), enrofloxacin (ENR), tetracycline (TET), gentamicin (GEN), and chloramphenicol (CHL)



A **B** **C**

Figure 1. A. Growth of *E. coli* on eosin methylene blue agar media. B. *E. coli* sensitivity test to antibiotics (R: resistant, S: susceptible). C. Double disc synergy testing test on *E. coli.* cefotaxime (CTX), ceftazidime (CAZ), and clavulanic acid combination (AMC)

Tabel 1. Primers of virulence genes

Gene	Primer Sequence	Product size	Reference
iss-F	ATG CAG GAT AAT AAG ATG AAA	290 bp	Mohamed et al. (2014)
iss-R	CTA TTG TGA GCA ATA TAC A		
papC-F	TGA TAT CAC GCA GTC AGT AGC	500 bp	
papC-R	CCG GCC ATA TTC ACA TAA		

Table 1. Results of detection of ESBL producing *E. coli* and virulence genes (*iss* and *papC*)

Sub-district	Sample size	Resistant to ampicillin	ESBL	Genes detection	
				iss	papC
Garum	25	22	4	4	1
Selorejo	26	19	12	5	0
Ponggok	28	27	10	10	0
Selopuro	31	27	8	6	0
Total	110	95 (86,4%)	34 (35,7%)	25 (73,5%)	1 (2,9%)

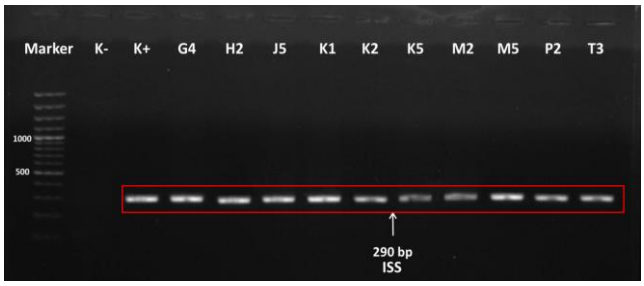


Figure 5. An agarose gel image showing the *iss* gene amplified from *E. coli*. Lane M: 100 bp DNA Ladder, Lane K+, G4, H2, J5, K1, K5, M1, M5, P2, T3: *iss* gene fragments amplified from *E. coli*. Lane K-: Negative control

Figure 6 An agarose gel image showing the *papC* gene amplified from *E. coli*. Lane M: 100 bp DNA Ladder, Lane K^+, G4: *papC* gene fragments amplified from *E. coli*. Lane K-: Negative control

Poultry has been identified as a reservoir of ESBL-producing *E. coli* (Kolenda et al. 2015; Effendi et al. 2021). Normally *E. coli* can be found in chicken cloaca, both pathogenic and non-pathogenic serotypes (Harijani et al. 2020; Wibisono et al. 2020c). The presence of virulence factors, pathogenic microbial strains will be able to defend themselves in host cells and increase the potential for causing disease. *E. coli* produces various types of virulence factors so the incidence of disease by *E. coli* infection can also occur in various ways (Effendi et al. 2018; Ansharieta et al. 2021b). The results of the expression of virulence genes allow non-pathogenic *E. coli* to turn into pathogenic *E. coli*, for example avian pathogenic *E. coli* (APEC) (Ievy et al. 2020).

The main classifications of pathogenic *E. coli* strains are extra-intestinal pathogenic *E. coli* (ExPEC) and diarrheagenic *E. coli* (DEC) (Paramita et al. 2021). ExPEC is often the cause of urinary tract infections and ultimately causes bloodstream infections (Cunha et al. 2017). *E. coli* in the bloodstream induces a strong host inflammatory response, resulting in sepsis. In addition ExPEC can also cause neonatal meningitis infection. Meanwhile, DEC strains are known to be a common cause of diarrheal disease (Kagambèga et al. 2012). This strain has six pathotypes including ETEC, EPEC, EAEC, STEC, EIEC, and DAEC (Paramita et al. 2021). Horizontal transfer is one way to get the character of pathogenicity, multi-drug resistant properties (Permatasari et al. 2020; Rahmahani et al. 2020), and also virulence that causes changes in the properties of *E. coli* (Sonda et al. 2018).

E. coli virulence factors that cause infectious diseases include fimbria virulence factors, capsule polysaccharides, O-antigen capsules, lipopolysaccharides, aerobactins, hemolysins, and other cytotoxins (Prihtiyantoro et al. 2014). When an infection occurs, the host's immune system will respond in an effort to defend itself. If the bacteria causing the infection are able to survive, then the host will experience further infection. The nature of virulence greatly affects the severity and level of infection (Garibyan and Avahia 2013). Virulence factors are encoded by genes located on chromosomes, more precisely on pathogenicity islands (PAIs) or located on bacterial plasmids (Dale and Woodford 2015).

Two kinds of virulence genes *iss* and *papC* were detected in this study. As many as 73.5% of *E. coli* producing ESBL have the iss gene. This gene was first identified from *E. coli* present in humans with septicemia (Biran et al. 2021). Its presence is associated with a 20-fold increase in complement resistance and also a 100-fold increase in virulence in day-old chicks (Johnson et al. 2008). In the United States, 85.4% of APEC strains isolated

from avian lesions diagnosed with colibacillosis were positive for the *iss* gene (80.5%) (Dissanayake et al. 2014). A total of 86.9% of *E. coli* isolates isolated from chickens with colibacillosis in Iran contained the *iss* gene (Bonjar et al. 2017). Meanwhile, in a study in Indonesia, as many as 68.2% of *E. coli* isolates contained the iss gene which is a component for developing the immune system by increasing serum survival (Paramita et al. 2021).

In the present study, the presence of the *papC* gene in *E. coli* isolates was low at 2.9%. The *papC* gene is one of the gene encoding the adhesin virulence factor and is the cause of urinary tract infections and bacteremia (Baby et al. 2020; Mahmoud et al. 2020). Research on *E. coli* in broiler samples in Portugal showed that isolates containing the *papC* gene were 14.96%, lower than the presence of the *iss* gene (33.07%) (Paixão et al. 2016). While in Bangladesh it was 33.3% (Ievy et al. 2020). *E. coli* isolated from farms in Brazil contains 25% of the *papC* gene (Ferreira et al. 2018). The *iss* and *papC* genes are the genes that encode the presence of virulence factors in APEC that cause colibacillosis in poultry. This disease can have an economic impact by decreasing the productivity of infected poultry, mortality, and medical costs throughout the livestock sector (Ibrahim et al. 2019). Poultry can act as a source of the spread of pathogenic *E. coli* (EL-Sawah et al. 2018). The spread of *E. coli* can be through feces from the cage and then into the environment. This pathogenic *E. coli* strain can be transferred to humans through food and drink contaminated with feces (Luna-Guevara et al. 2019). Control and prevention of infectious diseases in animals can be done by giving antibiotics to infected birds (Schwarz et al. 2004). However, inappropriate administration of antibiotics can also cause bacteria to become resistant (Enne et al. 2014; Wibisono et al. 2021).

In conclusion, it showed that the detection of two genes *iss* and *papC* encoding virulence factors illustrates that ESBL-producing *E. coli* has the potential to infect the host and cause disease. The *iss* gene plays a role in developing the immune system by increasing survival serum and the *papC* gene that encodes the adhesin virulence factor. The existing virulence factors also have the potential to increase the resistance of microbes to several types of antibiotics.

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