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MOLECULAR IDENTIFICATION OF CTX GENE OF EXTENDED SPECTRUM BETA-LACTAMASES (ESBL) PRODUCING *ESCHERICHIA COLI* ON LAYER CHICKEN IN BLITAR, INDONESIA

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ABSTRACT

Escherichia coli is one of the ESBL-producing bacteria responsible for the rise of antibiotic resistance. The most common type of ESBL-encoding gene is cefotaximase (CTX). The aim of study was to identify CTX-encoding gene among *Escherichia coli* on Layer Chicken in Blitar, Indonesia by using DNA-based techniques. To do this, a total of 130 swab cloacal samples were randomly collected from layer chicken in 4 districts of Blitar. Samples was initially cultured on MacConkey agar and Eosin Methyllen blue agar. Subsequently, the suspected isolates were identified in TSIA test and IMVIC biochemistry test. The method of Confirmation test ESBL-producing *Escherichia coli* was performed by Double Disc Synergy Test (DDST), and the final characterization of the isolates were conducted using VITEK® 2 Compact. Following that, the identified isolates were exposed to PCR amplification for the presence of CTX gene in ESBL-producing *Escherichia coli*, followed by the visualization of the amplicons in the electrophoresis. Overall, the results was showed that 80% of the ESBL-positive isolates contained CTX gene. In conclusion, this report the high frequency of CTX gene in ESBL producing *Escherichia coli*, and thereby posing a significant threat for the animal and human health.

Key words: CTX gene; ESBL; *Escherichia coli*; Human health; PCR; VITEK®

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INTRODUCTION

Antibiotic resistance is a serious problem worldwide, especially center of layer poultry in Blitar, Indonesia (Wibisono *et al.,* 2020a). *Escherichia coli* is one of the causes responsible for infection and antibiotic resistance in humans and animals through ESBL-mechanism (Paterson and Bonomo, 2005; Amelia *et al.,* 2016). The presence of pathogenic *Escherichia coli* infections in poultry can adversely affect economy (Wibisono *et al.,* 2018). The usage antibiotics can pose a risk of antibiotic resistance in poultry (Santos *et al.,* 2013). There is restricted data related to the prevalence of antibiotic resistance in developing countries, in particular, Indonesia. Surveillance of antibiotic resistance is needed to monitor the emergence of antibiotic resistance (Kurniawati *et al.,* 2015).

Antibiotic resistance which produced from *Escherichia coli* is Extended Spectrum β- lactamase (ESBL) (Santos *et al.,* 2013; Hammerum *et al.,* 2014). Extended-spectrum beta lactamase is an enzyme that is characterized by the ability to hydrolyze third generation cephalosporins and aztreonam but are inhibited by clavulanic acid (Paterson and Bonomo, 2005; Public Health England, 2014). Exposure large amounts of beta-lactam antibiotics can induces production and mutation of beta-lactamase type enzyme.

This mutation causes an increase in the enzymatic activity of beta-lactamase so that this enzyme can hydrolyze third generation cephalosporins and aztreonam (Paterson and Bonomo, 2005; Lim *et al.,* 2013). ESBL-producing bacteria can also be resistant to antibiotics from aminoglycoside, fluoroquinolone, tetracycline, chloramphenicol, and sulfamethoxazole-trimethoprim (Brower *et al.,* 2017; Sudarwanto *et al.,* 2017). Extended Spectrum Beta-Lactamase genes are often found in mutated genes, namely cefotaximase (CTX-M), temoneira (TEM) and variable sulfhydryl (SHV). The CTX-M gene in ESBL-producing *Escherichia coli* is a gene that codes and produces enzymes that can hydrolyze beta lactam rings from third-generation beta lactam antibiotics and cephalosporins (Biutifasari, 2018). The molecular detection is a genotypic confirmatory test to screen the ESBL encoding gene in *Escherichia coli* bacteria using PCR (Bradford, 2001). The highest prevalence of ESBL-producing bacteria with CTX-M-1 is the most common type of ESBL in poultry (Saliu *et al*., 2017; Upadhyay *et al.,* 2015; Rao *et al.,* 2014).

ESBL-producing *Escherichia coli* in India was about 42% in layer chicken (Brower *et al.,* 2017). In this study, we aimed to identify CTX gene for encoding ESBL producing *Escherichia coli* from cloacal swabs of layer chicken in Blitar, Indonesia.

MATERIALS AND METHODS

Isolation and Identification of Escherichia coli: A total of 130 cloacal swab samples was randomly taken from 4 districts in Blitar. The samples were kept in Amies Swab Viscosa (deltalab, Spain) transport medium at 4 ºC, and immediately taken to the laboratory for further analyses (Seni *et al.,* 2016). Isolation of *Escherichia coli* bacteria using selective media Mac Conkey Agar no. 3 CM0115 (Oxoid, England) and differential media Eosin Methylene Blue Agar CM0069 (Oxoid, England), incubated at of Escherichia coli were identified by biochemical testing of IMVIC (Indol-Motility, Methyl Red, Voges Proskauer, Citrate) and TSIA (Triple Sugar Iron Agar) (Effendi *et al.,* 2018a; Effendi *et al.,* 2019). The identification of *Escherichia coli* bacteria was confirmed by VITEK® 2 compact using a VITEK® 2 GN card (Biomerieux, 2017).

Confirmation for ESBL producing *Escherichia coli*: ESBL-producing *Escherichia coli* bacteria isolated from cloacal swabs of layer chicken were confirmed by the Double Disc Synergy Test (DDST) and VITEK® 2 compact. This confirmation test with DDST was conducted to evaluate the presence of inhibitory zones of ESBL activity with clavulanic acid using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar (Merck, Germany). Double Disc Synergy Test uses the antibiotic disc Amoxycillin-clavulanic 30µg (Oxoid, England), Cefotaxime 30µg (Oxoid, England), Ceftazidime 30µg (Becton Dickinson, USA), and Aztreonam 30µg (Oxoid, England).

Culture was incubated at 35-37 ºC for 18-24 hours (CLSI, 2017; Effendi *et al.,* 2018b). The results of the evaluation after incubation showed that the inhibition zone that appeared in the plate was measured based on CLSI 2017 guidelines (CLSI, 2017) as shown on Figure 2.

The antibiotic sensitivity 102 test of ESBL-producing Escherichia coli bacteria by the DDST was then confirmed by the VITEK® 2 compact. The bacterial suspension was homogenized and a bacterial turbidity of 0.50 to 0.63 Mc Farland was made using VITEK® 2 DensiCHEK (Biomerieux, 2017). Antimicrobial susceptibility and phenotypic detection of ESBL producers using AST N280 cards (bioMérieux, Marcy-L'Étoile, France). These results are analyzed automatically by the system and interpreted as sensitive, intermediate, and resistant (Biomerieux, 2017; Brower *et al.,* 2017).

Characterization of CTX gene by Polymerase Chain Reaction (PCR): The ESBL-positive strains were then subjected to molecular screening of CTX-gene. To do this, DNAs were extracted according to the instructions by a mini QIAamp® DNA kit (QIAGEN, Germany). *Escherichia coli* ATCC™ 35218 was used as positive control standard for strains of ESBL-producing bacteria, and *Escherichia coli* ATCC™ 25922 is used as negative control or non-ESBL-producing bacteria. The primers designed for screening CTX-M gene were used to encode CTX encoding genes using CTX-MA primers (CGCTTTGCGATGTGCAG), CTX-MB (ACCGCGATATCGTTGGT), respectively and the amplicon is 550-bp. The PCR conditions were given as with denaturation temperatures 94ºC, 2 minutes; extended denaturation 94ºC, 1 minute; annealing 54 ºC, 30 seconds; extension 72ºC), 45 seconds; extended extension 72 ºC, 5 minutes, this reaction is carried out for 30 cycles (Ali *et al.,* 2016), and the amplification was carried out by PCR (Blue-Ray Biotech Turbo Cycler, TST-9620). After that, the amplicons were visualized by electrophoresis using 2% agarose gel (Invitrogen, USA) (Yanestria *et al.,* 2019).

RESULTS

This study was conducted for the characterization of CTX gene among ESBL-positive E. oli strains isolated from 130 cloacal swab samples in layer poultry. The results were 8.69% (10) confirmation positive of ESBL-producing *Escherichia coli* on layer chicken cloacal swab by the Double Disc Synergy Test (DDST), shown on Figure 2. The presence of ESBLs-producing bacteria by double discs synergy test (DDST) to detect ESBL producing bacteria and then confirmed by the automatic VITEK® 2 compact and indicated 100% ESBL producing Escherichia coli as shown in Table 1. For the identification of CTX-encoding gene present in ESBL producing *E. coli* PCR was used (Putra *et al,* 2019), as shown on Figure 3.

Tabel 1. Data ESBL producing *Escherichia coli* from cloacal swabs layer chicken in Blitar.

Subdistric Location	Sample size	No of *Escherichia coli* isolates	No of ESBL positive strain		PCR
			DDST	VITEK®2 compact	No of CTX gene positive strain
Ponggok	40	31	-	-	-
Srengat	45	43	6	6	6
Talun	20	18	1	1	-
Kademangan	25	23	3	3	2
Total in Blitar	**130**	**115 (115/130; 88,5%)**	**10 (10/115; 8.69%)**	**10 (10/10; 100%)**	**8 (8/10; 80%)**

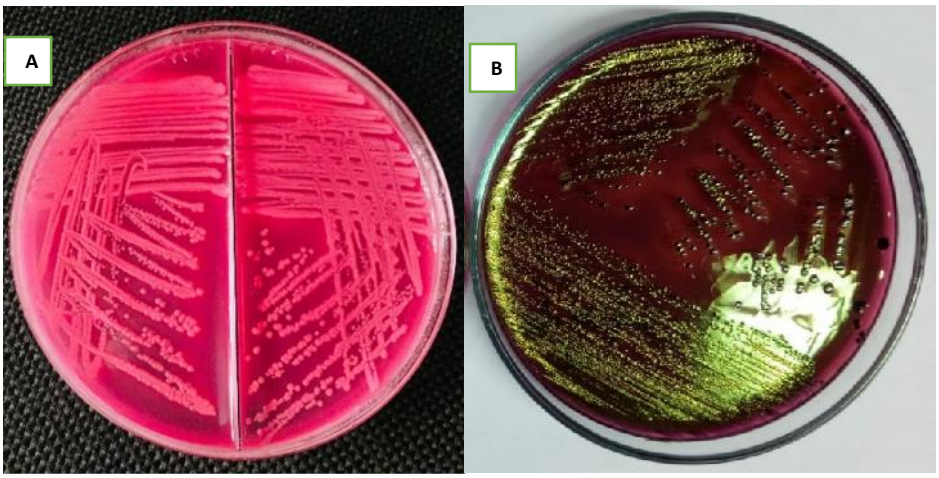

Figure 1. *Escherichia coli* on MacConkey Agar (A) and on Eosin Methylen Blue Agar (B)

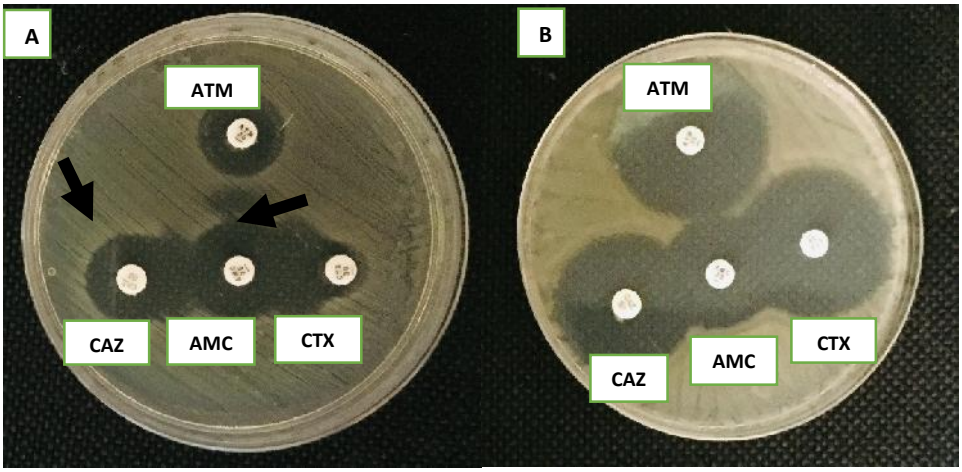

Figure 2. ESBL-producing *Escherichia coli* confirmation test with Double Disc Synergy Test (DDST) (A) positive result (B) negative result. Note: ATM: Aztreonam, CAZ: Ceftasidime, AMC: Amoxycillin clavulanic, and CTX: Cefotaxime.

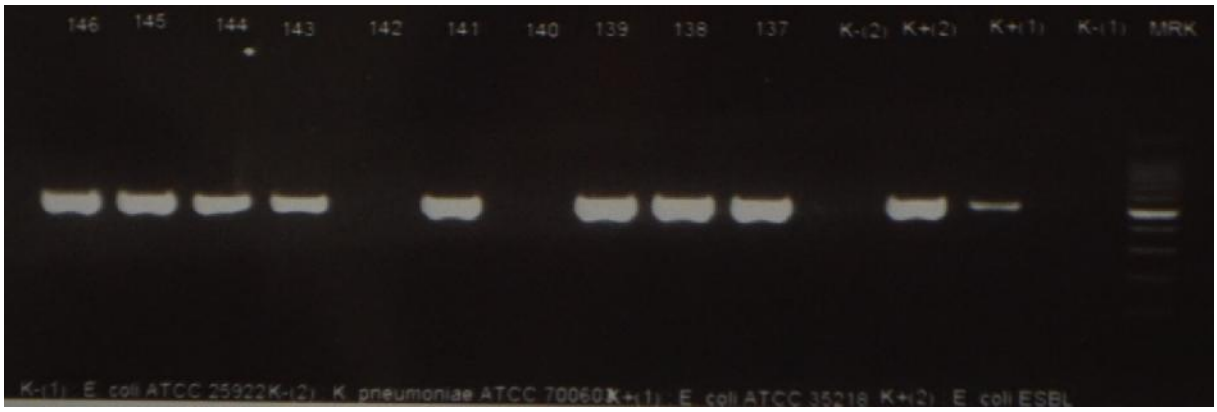

Figure 3. Molecular identification CTX gene of ESBL-producing *Escherichia coli* by PCR on 550 bp.
Legend: Code sample 140 and 142 there were no CTX gene.

DISCUSSION

Several other studies have examined the number of *E. coli* isolates that isolated from animal and animal products, showing concordance results between studies as shown on Table 1 (Saliu *et al.,* 2017; Upadhyay *et al.,* 2015; Rao *et al.,* 2014). The relative abundance of the ESBL producing *E. coli* in samples from cattle and dogs has been shown to vary with geographic location (Putra *et al.,* 2019; Kristianingtyas *et al.,* 2020). In this study,

isolates including ESBL producing *E. coli* were dominated by encoding CTX gene.

Table 1 showed the spread of ESBL-producing *Escherichia coli* in 3 districts from 4 districts. Srengat District has 6 samples, one sample from Talun district and Kademangan district was 3 samples from 10 ESBL-producing *Escherichia coli* samples, while Ponggok district was not found any ESBL producing *Escherichia coli*.

Cefotaxime synergy with the combination of amoxilin-clavulanate in the form of an expansion inhibition zone between the two discs represent that the bacteria is positive ESBL, synergy of third generation cephalosporins with combination of cephalosporin-clavulanic acid in the form of an expansion inhibition zone between the two discs. Positive results on ESBL-producing bacteria confirmed that there was increase in inhibition zone ≥ 5 mm between diameter of cephalosporin disc and cephalosporin-clavulanate disc combination revealed an ESBL positive (CLSI, 2017). The incidence of ESBL producing *Escherichia coli* from cloaca swabs on layer chicken was consistent with the incidence of *Escherichia coli* on slaughterhouses in Bogor by 8.6% (Sudarwanto *et al.,* 2016), but smaller compared to the incidence of *Escherichia coli* as ESBL producing *Escherichia coli* from feces of broiler chickens in Bogor ESBL by 25% (Masruroh *et al.,* 2016) and the incidence of ESBL producing *Escherichia coli* in India on layer chicken was around 42% (Brower *et al.,* 2017).

Molecular identification as shown in Table 1 that 80% (8/10) samples of ESBL producing *Escherichia coli* encoding CTX gene. The CTX encoding gene is most commonly found in *Escherichia coli*. CTX enzymes have hydrophilic ability against cephalosporins, especially cefotaxime, so called CTX (Bradford, 2001). Molecular identification shown in Figure 3 that visualization of the CTX gene fragment band. Electrophoresis results of CTX gene represent samples describing the same fragments as positive controls with a gene length of 550 bp (Ali *et al.,* 2016) as shown on Figure 3.

ESBL bacteria can be identified by detecting the presence of ESBL encoding genes (Bhoomika *et al.,* 2016, Surgers *et al.,* 2019). This research showed that the CTX gene was found in 80% ESBL samples. Other studies have been carried out mainly ESBL producing Kleibsiella pneumoniae (Hayati *et al.,* 2019). Some investigations show that the dominant genotype found was CTX gene (Zarfel *et al.,* 2014; Ibrahim and Hameed, 2015). This type of ESBL is often seen as single or combination. In this study, the ESBL encoding CTX gene was detected dominance of ESBL producing *Escherichia coli* samples from layer chicken. The CTX beta lactamase is the most prevalent ESBL type among chicken (96%) samples (Valentin *et al,* 2014). In many countries CTX gene is one of the most frequent ESBL types in ESBL-producing bacteria, causing human infections (Alonso *et al.,* 2017), therefore the evidence of CTX gene in this study should be used as reference in controlling the spread of ESBL encoding gene in poultry farms (Wibisono *et al.,* 2020b).

The spreading of genetic elements such as transposons, insertion and integrons in the bacteria cause ESBL genes move quickly from animals to humans or vice versa. Genetic factors can also spread the virus nature of resistance to other bacteria in animals digestive tract. The bacteria then spread from cage to the surrounding environment through facilitated waste by poor hygiene and sanitation, which pollutes land and water around agriculture. ESBL bacteria are also detected in vegetables, soil and surrounding water agriculture and markets (Wu *et al.,* 2016).

The presence of ESBL producing *Escherichia coli* is threat to the public health and animal health (Kristianingtyas *et al.,* 2020). This condition can occur in limited maintenance options. The steps that can be done is to build supervision program, supervising feed and poultry. Farmers also need to improve biosecurity practice. Garbage and laying chicken manure must be correct managed in an intensive production system, to prevent air, soil and water contamination, as well negative consequences for human health (Thyagarajan *et al.,* 2014).

Conclusion: One hundred and fifteen *Escherichia coli* samples were isolated from cloacal swabs layer chicken in layer farms Blitar, East Java, Indonesia. Ten E. coli were classified as ESBL bacteria. Through PCR testing, ESBL encoding gene of CTX gene was identified in eight samples. The presence of ESBL encoding gene in bacteria has potential to spread its resistance to the other bacteria in the gastrointestinal tract of layer chickens as well as in the poultry environment.

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

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