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***Front cover: Nycticebus javanicus* É. Geoffroy, 1812**
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Abstract:

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Proceeding:

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Thesis, Dissertation:

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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.molecularsystemsbiology.com

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Molecular identification of *bla$_{CTX-M}$* and *bla$_{TEM}$* genes encoding extended-spectrum ß-lactamase (ESBL) producing *Escherichia coli* isolated from raw cow's milk in East Java, Indonesia

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Abstract. *Ansharieta R, Ramandinianto SC, Effendi MH, Plumeriastuti H. 2021. Molecular identification of bla$_{CTX-M}$ and bla$_{TEM}$ genes encoding extended-spectrum ß-lactamase (ESBL) producing Escherichia coli isolated from raw cow's milk in East Java, Indonesia. Biodiversitas 22: 1600-1605.* The emergence of extended-spectrum β-lactamase (ESBL) producing bacteria and its increasing level has become public health issue. The presence of these bacteria in food of animal origin is quite alarming. The objective of this study was to detect and characterize *Escherichia coli* producing ESBL encoding genes, isolated from 200 raw cow milk samples in East Java, Indonesia. The results of this study showed that 70.5% of isolates were confirmed as *E. coli*, based on the morphological growth of colonies on the EMB Agar and biochemical IMViC tests. In this study, the double-disc synergy test (DDST) method was used to confirm the ESBL, and previously sorted out presumptively by using Aztreonam antibiotic disc. The antibiotics used were amoxicillin-clavulanate, ceftazidime, and cefotaxime for DDST. In addition, ESBL confirmation with Multiplex PCR method for bla$_{CTX-M}$ and bla$_{TEM}$ genes were done. The presence of ESBL-producing by *E. coli* isolated from raw cow's milk in East Java were 2.12% (3/141). The PCR results showed that the double bla$_{CTX-M}$ and bla$_{TEM}$ gene harbored by 2 ESBL isolates and one bla$_{TEM}$ gene as many as 1 ESBL isolate. Thus, the findings of our study indicate that milk can be a good reservoir of bacteria carrying bla$_{CTX-M}$ and bla$_{TEM}$ ESBL resistance genes with the potential to affect human health.

Keywords: bla$_{CTX-M}$ gene, bla$_{TEM}$ gene, ESBL, *Escherichia coli*, human health, raw cow's milk

INTRODUCTION

Extended-spectrum β-lactamase (ESBL) producing bacteria are a major threat to public health today. The presence of ESBL bacteria is often caused by inappropriate use of antibiotics in handling infections and their irrational administration (Abayneh et al. 2018). *Escherichia coli* (*E. coli*) is a major pollutant in the environment which is often associated with ESBL encoding genes, namely bla$_{CTX-M}$ and bla$_{TEM}$ (Jena et al. 2017). Milk is a food source of animal origin that can act as a reservoir in transmitting infectious bacterial diseases. The presence of *E. coli* bacteria in raw milk is often reported with regard to sources of food-borne disease (Odenthal et al. 2016).

The prevalence of ESBL-producing *E. coli* in animal origin food products is very high (Geser et al. 2012; Wibisono et al. 2020a). The existence of livestock and livestock products such as milk can be a mode of transmission and circulation of ESBL producing bacteria and act as a potential new threat because they are directly related to the food chain in humans. Livestock and livestock products are one of the main sources of animal protein, including the most commonly consumed sources of meat and milk and being one of the main elements in the food chain in humans. Livestock manure in the form of large-capacity feces has the potential to transmit bacterial infectious agents to humans through contamination. Most intestinal diseases in humans come from animals that are transmitted directly from animals to humans or indirectly through food of animal origin or water contaminated with feces (Widodo et al. 2020).

The distribution pattern of ESBL enzymes globally in humans and animals can be a reference and consideration in efforts to prevent and treat ESBL bacterial infections. The type of CTX-M enzyme is a variant of the enzyme found in isolates from humans, animals and the environment. The CTX-M-15 strain has been widely reported on all continents and has been detected in all major ecological aspects including humans, animals and the environment. Treatment of ESBL-producing bacterial infections in clinical trials has rarely been successful (Widodo et al. 2020). This study aimed to detect the presence of ESBL-producing bacteria from dairy cows milk by double-disc synergy test (DDST) and molecular tests to identify the encoding bla$_{CTX-M}$ and bla$_{TEM}$ genes for ESBL producing *E. coli*.

MATERIALS AND METHODS

Research design, location and sampling

Raw milk was taken from dairy farm with a total of 200 samples, located in Probolinggo, Pasuruan, Batu, and Blitar districts, East Java Province, Indonesia, in the period between September 2019-January 2020. The farms selected to take as research samples have belonged to farmers who had an average of 3 to 5 cows. About 10 mL of milk sample from dairy cows milk was taken and placed in a sterile tube. Raw milk samples contained in wrapped sterile plastic were taken to the laboratory using a cool box container at 4°C.

Isolation and identification of *E.coli*

Each 100 µL milk sample was cultured into 10 mL enrichment media of Brilliant Green Bile Lactose Broth (Merck, 105454) and incubated at 37 ºC for 18-24 hours. The positive results are characterized by change in media from green color to cloudy green and presence of gas in Durham tube (Putra et al. 2019). Then 100 µL of the resulted samples were streaked onto Eosin Methylene Blue (EMB) agar (Merck, 101347) and incubated at 37 °C for 18-24 hours (Effendi et al. 2018). Presumptive 3-5 colonies of *E. coli* that showed metallic green color was purified and continued to identification with the IMViC test (Wibisono et al. 2020b).

Antibiotic sensitivity test

Antibiotic sensitivity testing was done using Kirby-Bauer disc diffusion assay on Mueller-Hinton agar medium (Oxoid, CM0337). Firstly, screened for presumptive ESBL using Aztreonam and other antibiotic discs for checking the multidrug resistance profile. Antibiotics discs used were Tetracycline 30 µg (Oxoid, CT0054), Streptomycin 10 µg (Oxoid, CT0047), Chloramphenicol 30 µg (Oxoid, CT0013), Trimethoprim 5 µg (Oxoid, CT0057), Aztreonam 30 µg (Oxoid, CToid02). Antibiotics discs used for Double Disc Synergy Test (DDST) were Amoxicillin-Clavulanate 20/10 µg (Oxoid, CT0223B), Cefotaxime 30 µg (Oxoid, CT0166), Ceftazidime 30 µg (Oxoid, CT0412). Interpretation of results was done by measuring the diameter of the inhibitory zone formed, after overnight incubation at 37 °C, based on Clinical and Laboratory Standards Institutions (CLSI 2018, Putra et al. 2020).

Preparation for polymerase chain reaction

The initial step of DNA extraction from bacterial culture was referred to Kristianingtyas et al. (2020) and tested by specific primers for the bla_{CTX-M} and bla_{TEM} genes (Table 1) as described in Ali et al. (2016), with slight modifications in cycling conditions. Taq DNA polymerase enzyme, deoxyribonucleotide triphosphates and buffers used in the PCR mixture were obtained from Thermo Fisher Scientific Inc. (Massachusetts, USA). Thermocycling reaction was conducted for initial denaturation at 94ºC for 2 minutes followed by 30 cycles of: denaturation at 94ºC for 1 minute, annealing for 52ºC for 30 sec, extended at 72ºC at 45 sec, and final extension at 72ºC for 5 minutes. PCR products were visualized in mini gel electrophoresis and documented in the UV Reader/ Gel Documentation System.

RESULTS AND DISCUSSION

Isolation and identification of *Escherichia coli*

The results of the isolation and identification of 200 raw milk samples obtained as many as 200 (100%) isolate suspected of *E. coli* on the basis of macroscopic characters, on the Brilliant Green Lactose Broth medium, the color is cloudy green and all of them cause gas in the Durham tube after incubation at 37°C for 24 hours. A total of 141 (70.5%) samples on EMB Agar medium with two times purification showed the growth of convex colonies in metallic green with a round black center (Figure 1) after incubation at 37°C for 24 hours, can be seen in Table 2.

Table 1. Details of primers used in this study

Primers	Sequences (5' to 3')	Target gene	Amplicons size	References
CTX-MA	CGC TTT GCG ATG TGC AG	bla_{CTX-M}	550-bp	Villegas et al. (2004)
CTX-MB	ACC GCG ATA TCG TTG GT			
TEM-F	ATA AAA TTC TTG AAG ACG AAA	bla_{TEM}	1086-bp	Yao et al. (2007)
TEM-R	GAC AGT TAC CAA TGC TTA ATC			

Table 2. Results for detection multidrug-resistant (MDR) and ESBL producing *Escherichia coli*

Location	Sample size	Confirmed *E. coli*	Multidrug-resistant	Presumptive ESBL (Aztreonam disc)	DDST positive	bla_{CTX-M} gene	bla_{TEM} gene
Probolinggo (A)	50	36	2	0	0	0	0
Pasuruan (G)	50	30	4	1	1	1	1
Batu (H)	50	37	6	0	0	0	0
Blitar (S)	50	38	2	2	1	1	2
Total	200	141	14	3	2	2	3

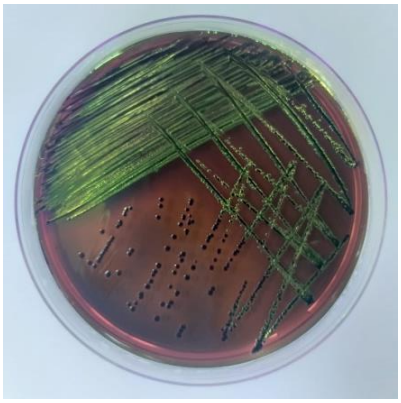

Figure 1. EMBA with *Escherichia coli* colonies in metallic green

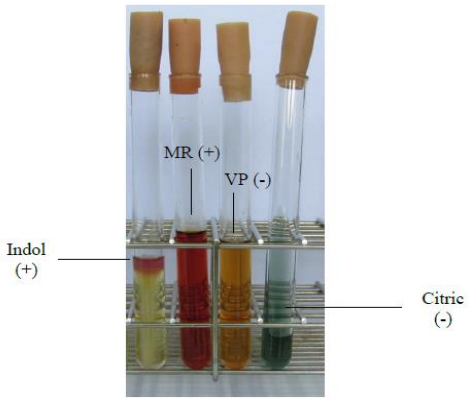

Figure 2. Positive biochemical test for *Escherichia coli*

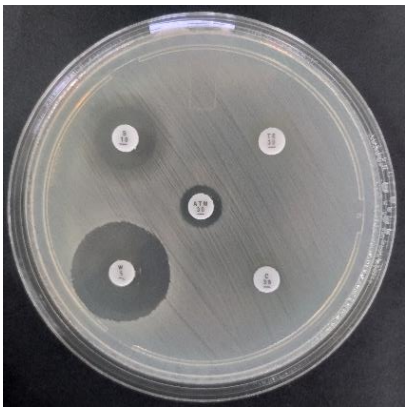

Figure 3. Sample code of S-25 showed Aztreonam resistant isolate as presumptive ESBL

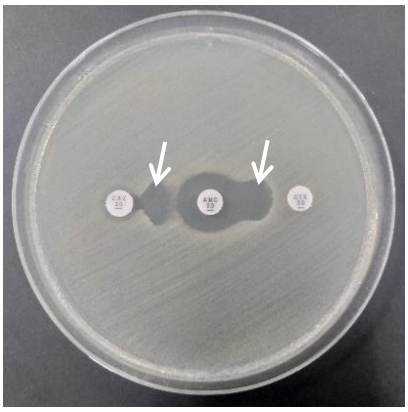

Figure 4. Sample code of S-25 DDST positive result (white arrow shows positive synergy)

Gram staining was performed and stained bacterial smear was viewed under a microscope with a magnification of 1000x. to ensure that the bacteria are Gram-negative rod-shaped bacteria. Microscopically, *E. coli* bacterial cells appeared in the form of short rods and were red in Gram stain. The next test was a biochemical test with IMViC and then incubated at 37 °C for 24 hours. Isolates used for the IMViC test came from separate colonies on EMBA media. It was found that 141(70.5%) isolates tested positive for *E. coli*. Isolates were stated as *E. coli* with negative Sulphide test results, positive indole, positive motile, positive MR, negative VP, and negative citrate (Figure 2).

The study has been carried out on 200 milk samples obtained from Probolinggo, Pasuruan, Batu, and Blitar, showed that 70.5% were contaminated with *E. coli* bacteria. From each area, *E. coli* bacteria were found which were multidrug-resistant to various antibiotics that had been tested (Table 2 and Table 3), and shown in Figure 3.

Identification for bla_{CTX-M} and bla_{TEM} Genes in ESBL-Producing *E.coli*

A total of two ESBL producer isolates showed the presence of 'keyhole' in DDST testing (Figure 4.) The three isolates were tested by multiplex PCR method to find out encoded ESBL genes. The two positive DDST isolates produced 2 double bands for the bla_{CTX-M} and bla_{TEM} genes. Whereas, one negative DDST isolate gave 1 single band for bla_{TEM}, due to resistant to Aztreonam (Figure 5).

Discussion

Based on this study, antibiotics Trimethoprim, Chloramphenicol, and Aztreonam may still be used to treat multidrug-resistant (MDR) bacteria found because there are still some MDR of *E. coli* isolates that are sensitive to it. Trimethoprim is usually combined with sulfonamides which work synergistically with a broad spectrum of activities. Chloramphenicol is also a broad-spectrum antibiotic. In the case of urinary tract infections, Trimethoprim is proven to be effective in killing infections caused by *E. coli* bacteria (AlRabiah et al. 2018), while in the case of gastrointestinal tract and reproductive tract infections due to *E. coli*, Chloramphenicol is a good antibiotic (Santos et al. 2010). The antibiotic monobactam (Aztreonam) is rarely used in veterinary practice and human medicine today (Kennedy et al. 2015). The discovery of isolates that are resistant to Aztreonam, increases the possibility of finding the ESBL of *E. coli* bacteria in raw milk.

Table 3. Detail samples of MDR and ESBL of *Escherichia coli* isolates

Sample code	Resistant to					Multidrug resistant (MDR)	ESBL producer
	TE	S	W	C	ATM		
	30 µg	10 µg	5 µg	30 µg	30 µg		
A-26	+	+	+	+	-	+	-
A-44	-	+	+	+	-	+	-
G-12	+	+	+	-	-	+	-
G-31	-	+	+	-	+	+	+
G-35	+	+	+	-	-	+	-
G-43	+	+	-	+	-	+	-
H-11	+	+	+	+	-	+	-
H-15	+	+	+	-	-	+	-
H-28	+	+	-	+	-	+	-
H-37	+	+	-	+	-	+	-
H-44	+	+	-	+	-	+	-
H-45	+	+	-	+	-	+	-
S-25	+	-	-	+	+	+	+
S-38	-	+	-	-	+	-	+

Note: TE: Tetracycline, S: Streptomycin, W: Trimethoprim, C: Chloramphenicol, ATM: Aztreonam, MDR is resistant to three or more classes, ESBL producer is resistant to ATM, +: positive resistant

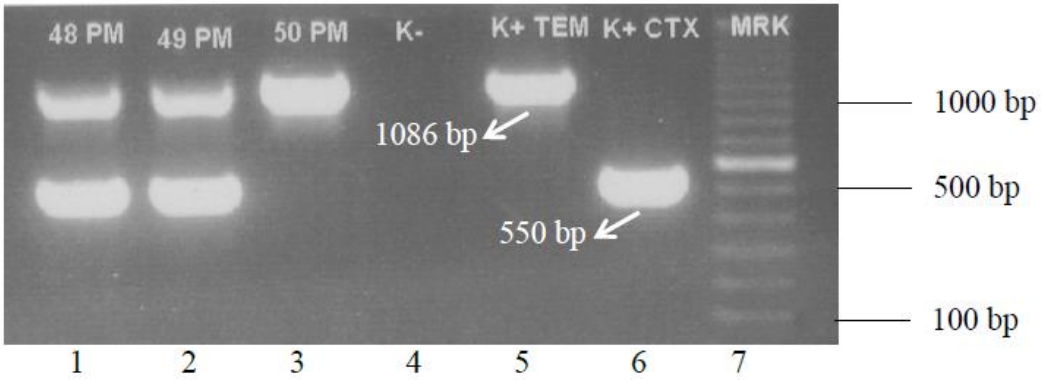

Figure 5. Electrophoresis result for 2 ESBL producer isolates and one contain blaTEM Isolate with negative DDST. Lane 1: G-31 (From Pasuruan), 2: S-25 (From Blitar), 3: S-38 (From Blitar), 4: Negative Control, 5: Positive Control for bla_{TEM} gene, 6: Positive Control for bla_{CTX-M} gene, 7: Marker

The occurrence of antibiotic resistance is known to originate from bacterial plasmids that are able to accommodate resistance genes and spread them to other bacteria (Ramírez-Castillo et al. 2018). Various resistance genes can accumulate in bacterial plasmids, usually in R (resistant) plasmids which is the reason for finding bacterial isolates that are resistant to various kinds of antibiotics and are able to create new gene sequences (Nikaido 2009).

Multidrug-resistant *E. coli* isolates are very common in many countries and are responsible for a series of infections with high severity and difficulty to treat. In Canada, a study of cases of urinary tract infection due to *E. coli* bacteria, 60% of which were the cases of *E. coli* infection with resistance to more than 3 classes of antibiotics. Consumption of food from raw undercooked animals, travel habits between regions, and contact with reservoir animals are associated with an increased risk of urinary tract infections caused by MDR of *E. coli* (Ukah et al. 2017).

Escherichia coli is a bacterium that can be a reservoir of various antibiotic resistance genes, including genes encoding beta-lactam resistance β-lactamase encoding genes (Effendi et al. 2021). ESBLs enzymes are produced several strains belonging to the Enterobacteriaceae family. They can hydrolyze penicillin and third-generation cephalosporins, monobactams, and other antibiotics, except carbapenems (meropenem, imipenem, and ertapenem) (Pitout 2012). These enzymes are mainly encoded by several specific genes, namely the bla_{SHV}, bla_{CTX-M} and bla_{TEM} genes (Bush 2013, Wibisono et al. 2020).

The presence of bla_{CTX-M} and bla_{TEM} genes is often reported in food of animal origin. In this study, the findings of ESBL producing *E. coli* isolates were dominated by the bla_{TEM} gene. Similar to the research of Hinthong et al. (2017) stated that *E. coli* contamination found in milk from

dairy farms tends to find the bla_{TEM} gene in ESBL-producing *E. coli* bacteria. This showed that pathogenic *E. coli* sourced from milk is also exposed to antibiotics and has the potential to transfer these genes to other pathogenic bacteria under certain conditions (Effendi et al. 2019; Rahmahani et al. 2020). This study was detected ESBL and bla_{CTX-M} gene in milk since it is rarely reported worldwide to find ESBL in milk. ESBL is mainly reported in feces of poultry and porcine (Wibisono et al. 2020c). The presence of ESBL bacteria is quite dangerous if found in food of animal origin. ESBL-producing *E. coli* strains obtained from cow's milk samples are of particular concern because these pathogens can affect human consumers and calves and lead to the spread of these antibiotic-resistant pathogens to humans and animals (Batabyal et al. 2018).

In dairy cows during lactation, ESBL producing *E. coli* can also be found in raw milk with and or without symptoms of mastitis, this indicates that the cleanliness of the cage that contaminates milk cages is also a risk factor for contamination of ESBL producing *E. coli* into raw milk products. (Su et al. 2014). In cases of mastitis due to infection with ESBL-producing *E. coli* bacteria infection, it is often associated with several antibiotics that have also been inactive against the bacteria causing it, making it difficult to find other replacement antibiotics to treat it (Ali et al. 2016).

Many sources of exposure have the potential to transmit ESBL-producing *E. coli*, making epidemiological investigations extremely difficult. Interactions at the microbial level in humans and animals, especially between commensal bacteria and pathogenic bacteria, facultative bacteria and obligate bacteria in the same environment and horizontal gene transfer from bacteria make the distribution of ESBL encoding genes between various bacterial species becomes wider. In order to understand and identify the possibility of preventing the spread of the ESBL encoding genes and infection in humans, an integrative approach such as 'One Health' is required (Calistri et al. 2013). The application of the concept of One Health integration is assumed to accelerate disease prevention and prediction as an effort to control ESBL-producing *E. coli* (Wendt et al. 2014).

Food-borne diseases are a major concern throughout the world. This is an important problem in developing countries that lack the application of high sanitation management during the collection and processing of cow's milk. *E. coli* contamination found in raw milk may be caused by cross-contamination of milk with impurities or the lack of hygienic measures during milk collection and processing (Tanzin et al. 2016). According to Ukah et al. (2017), one of the factors causing the occurrence of antibiotic resistance in humans is due to consuming food of animal origin in raw or undercooked form. A multi-sectoral approach to medical treatment in the field of veterinary medicine, animal food production, can realize global cooperation in controlling the ecological development of antibiotic-resistant *E. coli*, for public health (Landers et al. 2012).

In conclusion, molecular identification showed the bla_{CTX-M} gene found in raw cow milk collected from several regions in East Java, Indonesia which was used to identify ESBL producing *E.coli,* and the bla_{TEM} for molecular identification of penicillinase-producing *E. coli*. These results showed that ESBL producing *E. coli* from raw cow's milk has a relatively low prevalence. However, ESBL producing *E. coli* showed the potential for spreading and poses a threat to public health from *E. coli* isolates.

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