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Molecular Identification of Extended Spectrum Beta-Lactamase (ESBL) Producing *Escherichia coli* Isolated From Dairy Cows in East Java Province, Indonesia

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Abstract

Occurence of extended spectrum beta-lactamases (ESBL) in *Escherichia coli* (*E. coli*) from animal origin is a growing health concern of global significance. The objective of this study was to determine the occurrence of ESBL producing *E. coli* and the characteristics of their encoding genes from 115 rectal swab samples of dairy cows from Tulungagung and Surabaya farms. All samples were positive for *Escherica coli* as per indole test. To confirm the ESBL the Double Disc Synergy Test (DDST). Betalactam antibiotic disk namely Amoxicylyn-clavulanate, Ceftazidime and Cefotaxime were used for DDST. Molecular identification for bla_{CTX-M} and bla_{TEM} ESBL encoding genes was done by used Polymerase Chain Reaction. The *Escherichia coli* bacteria isolated from rectal swabs of dairy cows was 5.21% (6/115). PCR results showed that bla_{CTX-M} gene was 6 ESBL isolates and bla_{TEM} gene was 2 ESBL isolates. It can be concluded that dairy cows can be potential as reservoir for spreading ESBL isolates to human health.

Key words: *Eschericia coli*, ESBL, DDST, bla_{CTX-M} gene, bla_{TEM} gene, PCR

Producing bacteria is a major threat to public health. The existence of this Extended-Spectrum Beta-Lactamases (ESBLs) is due to an improper use of antibiotics in infections and treatment failure, (Effendi *et al.,* 2018).

ESBLs producing bacteria which induces resistance to the expanded spectrum of cephalosporins and monobaktam but do not affect cephamycins or carbapenem and is inhibited by beta-lactamase inhibitors such as clavulanate, sulbactam, and tazobactam (Peterson and Bonomo, 2005). Widespread third generation cephalosporins and aztreonam are believed to be the main cause for the mutations, which have led to the emergence of ESBL (Al-Jasser, 2006; Bradford, 2001).

The prevalence of ESBL-producing E. coli in food-producing animals and food products is very high. The study was to detect the presence of ESBLs-producing bacteria from dairy cows by double discs synergy test (DDST) and phenotypic confirmation test to detect ESBL producing bacteria and to identify encoding genes for ESBL producing *E. coli*.

Materials and Methods

During the period July 2018 to March 2019, a total of 115 samples from rectal swabs from dairy cows (50 samples from Tulungagung dairy farm and 65 samples from Surabaya dairy farm) were collected, using a sterile swab inserted into the eppendorf tube containing the Pepton water buffer media (Safitri *et al.,* 2017). Samples were taken to the laboratory in a thermobox container at 4◦C.

Each swab sample was innoculated on Briliant Green Bile Broth (BGBB) media and then incubated at 37°C for 24 hours. The positive results are characterized by the presence of gas bubbles in the Durham tube and the change of green colour to cloudy green. They were grown on Eosin Methylene Blue Agar (EMBA) media by streaking and incubated at 37°C for 18-24 hours. Typical *E. coli* colonies on EMBA media was metallic green, and it was planted again in Pepton Water and incubated at 37°C for 24 hours. The incubated Pepton Water media is dripped with Kovach reagents in two or three drops. A positive *E. coli* test is characterized by

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Table I. Data of ESBL isolates in this study

Location	Number of samples	Positive *E. coli*	ESBL Confimation by DDST	bla_{CTX-M} gene	bla_{TEM} gene
Tulungagung farm	50	50	3	3	1
Surabaya farm	65	65	3	3	1
Total	**115**	**115**	**6**	**6**	**2**

the formation of a red ring on the surface of the Pepton Water media (Effendi *et al.,* 2019).

Confirmation test for ESBL producing E. coli by using disk antibiotic (OXOID, Basingstoke, United Kingdom) amoxycilin-clavulanate 30 µg (CT0223B), Cefotaxime 30 µg (CT0166), Ceftazidime 30 µg (CT0412), as per Clinical and Laboratory Standards Institutions (CLSI, 2016).

A total of 115 positive samples of *E. coli* were tested with Double disc synergy test (DDST) to detect synergy between a disc of augmentin (Amoxycillin and clavulanic acid) and third generation cephalosporins. The clavulanate in augmentin disc diffuses through the agar and inhibits the beta-lactamases, surrounding third generation cephalosporin disc. Discs containing 30µg of ceftazidime, and cefotaxime were placed over inoculated Mueller-Hinton agar plates 20 mm apart from centrally placed amoxicillin-clavulanic acid disc (20/10 µg). Following overnight incubation at 37°C, diameter of zone of inhibition was measured.

The initial step of PCR DNA extraction of bacterial culture in EMBA media was made as per (Yanestria *et al.,* 2019) and tested by specific primers for the bla_{TEM}, and bla_{CTX-M} genes as described Ali *et al*., (2016) with slight modifications in cycling conditions, Table I. *Taq* DNA polymerase enzyme, deoxyribonucleotide triphosphates and buffers used in the PCR mixture were obtained from Thermo Fisher Scientific Inc. (Massachusetts, USA). Thermo-cycling reaction was conducted for denaturation at 94°C for 2 minutes, extended denaturation at 94°C for 1 minutes, annealing for 52°C for 30 sec, extended at 72°C at 45 sec, and extended at 72°C for 5 minutes, this reaction is 30 cycles. PCR product was visualized in mini gel electrophoresis and documented in UV Reader/Gel Documentation System.

Results and Discussion

The isolation and identification results of 115 samples showed the changes in green colour to cloudy green is presented in table I. All the 115 samples were positive for *E. coli* (100%). These results are in accordance with the finding of Rasheed *et al*. (2014) who reported that the level of ESBL producing *E.coli* contamination in cattle was found to be 3.3%, however the study conducted by Wasinki *et al*. (2013) reporte that the level of *E coli* contamination was 13.5% in food animals. Enterobacteriaciae bacteria in faecal samples can cause carcass contamination and as a potential for pollution in meat products (Geser *et al.,* 2011).

Extension of the edge of the inhibition zone of ceftazidime, and cefotaxime disc on the side exposed to the disc containing amoxicillinclavulanic acid was positive for ESBL. DDST showed that ESBL producing *E. coli* were 6 (5.21%) isolates (Table I). The test image results of the DDST is shown in Fig 1.

The prevalence of bacteriae. ESBL-producing *coli* and their evolution are due to

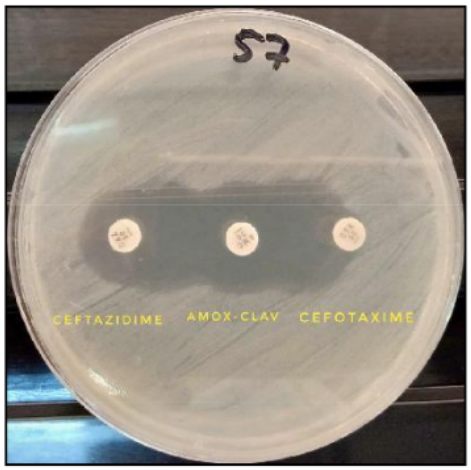

Fig 1. Double Disc Synergy Test (DDST) for ESBL confirmation

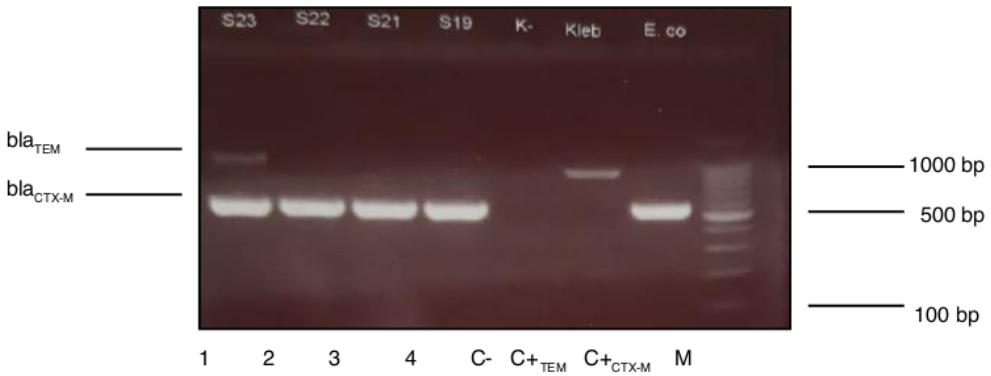

Fig 2. PCR profiles of molecular detection bla_{CTX-M} gene and bla_{TEM} gene of ESBLs
Lane 1-4 (ESBL isolates), C- (Non-ESBLs), C+TEM (+ bla_{TEM}), C+CTX-M (+ bla_{CTX-M}), M (marker 100 bp)

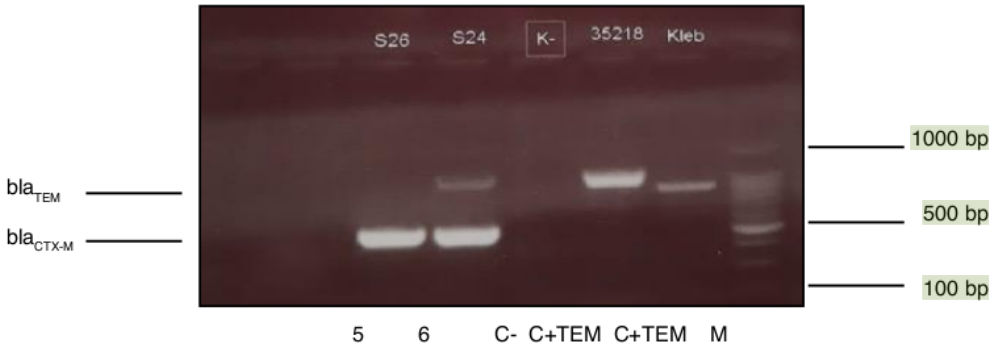

Fig 3. PCR profiles of molecular detection bla_{CTX-M} gene and bla_{TEM} gene of ESBLs
Lane 5-6 (ESBL isolates), C- (Non-ESBLs), C+TEM (+ bla_{TEM}), M (marker 100 bp)

the frequent administration of drugs such as penicillin, cephalosporin, monobactam, and carbapenem (Cheaito and Matar, 2014), which is associated with resistance to other types of antibiotics leading to Multidrug resistance. Haldorsen (2011) who has reported that genes coding for resistance enzymes such as AME (Aminoglycoside modifying enzyme) and ESBL are often found in bacterial plasmids. One of the main causes of the increasing prevalence of bacteria that are resistant to both beta-lactam and aminoglycoside group antibiotics is the presence of gene transfer that occurs in plasmid, integron, and transposon (Halderson *loc. cit*; Allocati *et al.*, 2013). Furthermore, the combination of several resistant genes causes bacteria to be resistant to most classes of antibiotics (Allocati *et al.*, *loc. cit*).

Our findings from molecular identification illustrate that bla_{CTX-M} gene is the most common genotype, followed by $_{TEM}$ gene (Table I) revealed on Figure 2 and 3. This finding is similar to in Turkey (Tekiner & Özpınar, 2016) and other studies from China (Liu *et al.*, 2015) and around the world that also report b$_{CTX-M}$ as the dominant ESBL genotype (Geser *et al.*, 2012; Kar *et al.*, 2015). This is in line with recent detection of bla_{CTX-M} produced by E. coli from cattle and other food anima$s in Egypt (Braun *et al.*, 2016), East Asia (Yu *et al.*, 2015), India (Upadhyay *et al.*, 2015), United Kingdom (Timofte *et al* ., 2014), and Tanzan$ (Seni *et al.*, 2016). Similarly, in animals, the prevalence of ESBL-producing *E. coli* in China has increased rapidly in years with bla_{CTX-M} being the main gene coding that applies to ESBL (Rao *et al.*, 2014). It is known that, in general, ESBL genes are located on plasmids which can spread easily

between commensal and pathogenic bacteria in flocks and the environment.

Summary

There is a high incidence of ESBL-producing *E. coli* in dairy cows. Molecular identification showed the dominance of the bla_{CTX-M} gene compared to the bla_{TEM} gene. The ESBL producing *E. coli* showed the potential for rapid and wider dissemination and poses a threat to animal health and public health. There is an alarming high prevalence of bla_{CTX-M} in ESBL producing *E. coli* from dairy cows in Tulungagung and Surabaya farms in East Java Province, Indonesia.

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