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CTX-M-15 and CTX-M-55 Producing *Escherichia coli* in Milk from Dairy Farms in West Java, Indonesia

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Abstract

This study was aimed to determine the occurrence of CTX-M-15 and CTX-M-55 producing *Escherichia coli* isolated from milk samples in West Java, Indonesia. A total of 129 milk samples were collected from diary farms from March to April 2016. Presence of extended-spectrum beta-lactamase (ESBL) producing *E. coli* was detected by disc diffusion test based on the recommendation from Clinical and Laboratory Standards Institute. Bacterial strains which were confirmed as producing ESBLs were further analyzed for the presence of *bla* genes of the ESBL by polymerase chain reaction (PCR). The results showed that CTX-M producing *E. coli* isolates were detected in 4 samples from 129 samples (3.1%). The β-lactamase genes detected included CTX-M-15 (n = 3) and CTX-M-55 (n = 1). All of the CTX-M producing *E. coli* isolates showed multidrug resistance phenotypes to at least three antibiotics. The highest incidence of antibiotics resistance was against penicillin G (100.0%), followed by streptomycin (75.0%), gentamicin (75.0%), tetracycline (75.0%), trimethoprim-sulfamethoxazole (50.0%), polymyxin B (25.0%), ciprofloxacin (0.0%), and enrofloxacin (0.0%). The occurance of CTX-M ESBL in milk could be a threat towards public health because its ability to spread resistant gene to the environment, food, human, animal and other pathogenic bacteria.

Keywords: CTX-M; diary farms; *Escherichia coli*; milk.

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1. Introduction

Extended spectrum β-lactamases (ESBLs) are the plasmid mediated enzymes that confer resistance to 3rd and 4th generation cephalosporins (oxy-imino β-lactam) and monobactam (aztreonam) groups of drugs except carbapenems and cephamycins [1]. Production of ESBLs is the most common mechanism of resistance to 3rd generation cephalosporins among Enterobacteriaceae including *Klebsiella pneumoniae* and *Escherichia coli* [2]. The CTX-M family of ESBLs have become prominent and are common in *Escherichia coli*, with many infections occurring in human patients in the community. The first CTX-M ESBL *E. coli* recovered from food producing animals in the UK was an *E. coli* isolated from diarrhoeic calves on a Welsh dairy farm in 2004 [3]. CTX-M β-lactamases form a large and still growing family of enzymes which is divided into several groups, with several members in each group [4]. Until now more than 600 ESBL variants are known. Among them, the over 100 CTX-M enzymes so far reported may be grouped into five main subgroups (CTX-M-1, M-2, M-8, M-9 and M-25) based on amino acid sequence similarities [5,6].

The occurrence of ESBL-producing bacteria has been broadly recognized as causative agents for mastitis in dairy cattle [7]. *Escherichia coli* is one of the most important causative agents for clinical mastitis in lactating cows, together with *K. pneumoniae*, and β-lactam antibiotics are the drugs of choice for treatment. Improper and excessive use of intramammary or parenteral β-lactam antibiotics may provoke bacterial adaptation [8]. Use of third and fourth generation cephalosporins in livestock is likely to provide a selective pressure for the maintenance of resistance or emergence of resistance to these compounds [9]. The emergence of resistant bacteria and the transmission of these bacteria from foods to humans have become serious public health problems in treating their infection [10,11]. This study was aim to determine the occurrence of CTX-M producing *E. coli* from milk from dairy farms in West Java, Indonesia.

2. Material and Methods

2.1. Isolation and Identification of ESBL Producing Escherichia coli

Isolation and identification of ESBL producing *E. coli* was done by refering to Sudarwanto and his colleagues [12]. A total of 129 milk samples from 8 districts in West Java, Indonesia were collected from March to April 2016. Milk samples were put in sterile tubes and transported to the laboratory using cooling box. Milk samples (10 mL) were enriched for 24 h at 37 °C supplemented with 20 µL cefotaxime (1 µg/mL). There after the enrichment was streaked onto MacConkay agar (Merck 1.05465.0500, Germany) containing 1 mg/L cefotaxime, and incubated at 37 °C for 24 h under aerobic condition. The colonies that ware presumed as *E. coli* will appear as red colonies in the media, and surrounded by turbid zone. Further works were continued by KOH test, Gram staining, oxidase test (Oxoid MB0266A, England), sulfide, indole, and motility (SIM) test, and biochemical test [indole, methyl red, Voges-Proskauer, and citrate (IMViC)]. The colonies that were presumed as *E. coli* were selected and sub cultured onto tryptic soy broth (Merck 1.05458.0500, Germany) at 37 °C for 24 h. The identification of the *E. coli*-like colonies were then confirmed using API 32E (Biomerieux). Isolates were stored in tryptic soy broth containing 20% glycerol at -20 °C until further workup.

2.2. ESBL confirmation and antibiotic susceptibility testing

All cefotaxime-resistant, and Oxidase-negative, isolates were confirmed for ESBL production by the disk diffusion method according to the Clinical Laboratory Standards Institute (CLSI) guidelines [13]. The inhibition zones were determined for each isolate, using antibiotic disks, each containing 30 mg of cefotaxime, ceftazidime, or cefpodoxime, either alone or in combination with 10 mg of clavulanic acid (MAST Group Ltd., Reinfeld, Germany). *E. coli* isolates which produced ESBL were subjected to susceptibility testing against 8 antimicrobial agents (penicillin G, streptomycin, gentamycin, ciprofloxacin, enrofloxacine, tetracycline, trimethoprim-sulfamethoxazole, and polymyxin B) with disk diffusion method according to CLSI protocols and evaluated with CLSI criteria. *E. coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 (*K. pneumoniae*) were used as a control strain.

2.3. Characterization of β-lactamase by polymerase chain reaction (PCR)

Bacterial strains which were confirmed as producing ESBLs were further analyzed for the presence of *bla* genes of the ESBL subtypes TEM, SHV, and CTX-M (group 1, 2, 8, 9, or 25) by PCR using primers and conditions as previously reported [12]. Bacterial DNA was isolated with the DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Two strains, *K. pneumoniae* ATCC 700603 was used as standard ESBL-positive strains and a non-ESBLproducing organism (*E. coli* ATCC 25922) was used as negative control. PCR products were determined by electrophoresis in a 2% agarose gel (Biozym, Hessisch-Oldendorf, Germany). The molecular marker GeneRuler 100-bp DNA ladder (MBI Fermentas, St. Leon-Roth, Germany) was used.

2.4. Sequencing of bla genes

The ESBL-encoding genes bla_{TEM}, bla_{SHV}, and $bla_{\text{CTX-M}}$ of the ESBL-positive isolates were amplified with primers and PCR conditions as described previously [12]. Resulting amplicons were purified using the PCR purification kit (Qiagen). Sequencing was performed at SeqLab (Goettingen, Germany). Results were evaluated using the BLAST algorithm available at http://blast.ncbi.nlm.nih.gov/Blast.cgi.

2.5. Data Analysis

Data were descriptively analyzed to describe occurrence of CTX-M producing *E. coli* isolated from milk from dairy farms in West Java, Indonesia.

3. Results

In this present study, 4 ESBL-producing *E. coli* isolated from milk samples produced CTX-M type ESBL. The $bla_{\text{CTX-M}}$ types were identified CTX-M-15 (n = 3) and CTX-M-55 (n = 1). PCR analysis followed by partial sequencing revealed the presence of $bla_{\text{TEM-1}}$ in all of CTX-M type ESBL isolates. All of CTX-M-15 and CTX-M-55 producing *E. coli* isolates showed multidrug resistance phenotypes against at least three antibiotics. The highest incidence of antibiotics resistance was against penicillin G (100.0%), followed by streptomycin (75.0%),

gentamicin (75.0%), tetracycline (75.0%), trimethoprim-sulfamethoxazole (50.0%), polymyxin B (25.0%), ciprofloxacin (0.0%), and enrofloxacin (0.0%). Detail results on characteristics and antibiotic susceptibilities of multidrug resistant of 4 CTX-M producing *E. coli* isolates were described in Table 1.

Table 1 Molecular characterization and antibiotic susceptibilities of ESBL producing *E. coli* isolates

No	Code	*bla* gene product		Antibiotic resistance[a]								Total[b]		
				β-lactam	Aminoglycosides		Fluoroquinolones		Tetracycline	Folic acid inhibitor	Lipopeptide			
		CTX-M	Other	P	S	CN	CIP	ENR	TE	STX	PB	R	I	S
1	15	CTX-M-55	TEM-1	R	R	S	S	I	S	R	R	4	1	3
2	22	CTX-M-15	TEM-1	R	I	R	S	S	R	S	S	3	1	4
3	26	CTX-M-15	TEM-1	R	R	R	S	S	R	I	S	4	1	3
4	27	CTX-M-15	TEM-1	R	R	R	S	S	R	R	S	5	0	3

[a] P: penicillin G; S: streptomycin; CN: gentamicin; CIP: ciprofloxacin; ENR: enrofloxacin; TE: tetracycline; STX: trimethoprim-sulfamethoxazole; PB: polymyxin B
[b] R: resistance; I: Intermediate; S: sensitive

4. Discussion

CTX-M producing *E. coli* isolates were detected in 4 of the 129 (3.1%) milk samples. In this work, the β-lactamase genes were detected in these isolates were $bla_{\text{CTX-M-15}}$ (75.0%) and $bla_{\text{CTX-M-55}}$ (25.0%). All of them harbored an additional $bla_{\text{TEM-1}}$ gene but none of them was positive for the bla_{SHV} gene.

In previous study was analyzed for the presence of ESBL producing Enterobacteriaceae isolated from bulk tank milk from diary farms in Indonesia. The result showed that $bla_{\text{CTX-M-15}}$ genes were detected in 2 of the 7 *K. pneumoniae* isolates. The relatively high frequency (8.75% (7/80)) of ESBL-producing *K. pneumoniae* in raw bulk tank milk from West Java imply the risk that milk is both a source of local exposure and a vector contributing to the supraregional spread of antibiotic-resistant bacteria by trade [8]. Another study in Germany showed that ESBL producing *E. coli* isolates were detected 5.9% (51/866) from bulk tank milk samples. In this study reported that CTX-M was also predominant sequence type among ESBL producing *E. coli* isolated from milk samples. The results indicated that the ESBL-positive isolates possibly carried the genes for $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-15}}$. Both enzymes are commonly found in clinical isolates from humans, while $bla_{\text{CTX-M-15}}$ so far has only sporadically been reported for isolates from dairy cattle and milk [4]. CTX-M producing *E. coli* also have been isolated from cattle in Japan (CTX-M-2; [14]), Switzerland (CTX-M-14; [6]), Bavarian, Germany (CTX-M-1, CTX-M-2, and CTX-M-9; [15]), Turkey (CTX-M-1, CTX-M-3 and CTX-M-15; [16]), and Egypt (CTX-M-15 and CTX-M-9; [17]).

A possible role for cephalosporin use (ceftiofur, cefoperazone and cefquinome) in the widespread occurrence of CTX-M-1 producing *E. coli* at a dairy farm has recently been suggested. The original isolation of a CTX-M-15 *E. coli* was made in 2006, and since then many cattle, at different stages of production, particularly milking cows, had received intra-mammary and injectable veterinary medicines containing third and fourth generation cephalosporins [18]. CTX-M-55 differs from CTX-M-15 only by a single amino acid substitution (valine for

alanine) at position 80 (Ala80Val). Therefore, CTX-M-55 is expected to have similar hydrolytic activity as CTX-M-15 and to exhibit increased catalytic efficiency against ceftazidime as well as cefotaxime. Previous studies have reported that CTX-M-55-producing isolates generally show high resistance to ceftazidime (MIC range from 32 to > 256 µg/mL) [19]. The results may suggest that the injudicious use of antimicrobial agents among livestock and hospitals may result in mutation and subsequent epidemiological change of major ESBL genotypes circulated (e.g. CTX-M-3 to CTX-M-15 and CTX-M-55) [20].

Multidrug resistance (MDR) to other classes of antibiotics was also described in this study. Multidrug resistance was defined as resistance to three or more different classes of antimicrobials [21]. This study showed that all of CTX-M producing *E. coli* displayed MDR to at least three antibiotics. A similar finding was also reported by the author in [16], who found that 84.6% (22/26) ESBL/AmpC producing *E. coli* isolated from cattle were also resistant to other class of antimicrobials agents. This could partly be explained by the fact that the plasmids harboring ESBL genes frequently carry other resistance genes that are responsible for other class antimicrobials, such as fluoroquinolones, aminoglycosides and trimethoprim-sulphamethoxazole [6, 16].

In Indonesia, there are few restrictions concerning the use of antibiotics, neither in human medicine nor in veterinary medicine. Thus, it may be suspected that the rapidly increasing incidence of multidrug-resistant bacteria could be a result of the unconscious and extensive use of antibiotics in this country [8]. In this country, waste milk cannot be sold for human consumption, but is widely used to feed calves. Waste milk is milk unfit for human consumption and may be designated unfit for various reasons. It includes milk from cows immediately post-calving (colostrum), milk from cows with udder infections (mastitis) and milk from cows undergoing treatment with antibiotics or other medicines in which drug residues in excess of Maximum Residue Limits may be present [9]. Feeding waste milk containing antibiotic resiudues to calves can increase the prevalence of CTX-M-positive *E. coli* in the farm environment due to increased shedding [22]. Another major problem raises up in this context, the resistance genes are usually found on plasmids and such mobile elements can be easily transferred to and between environmental bacteria as well as to other human pathogens. This poses a high risk to the environment, and the human population [17].

The rapid proliferation and worldwide spread of CTX-M-type ESBL in *E. coli* is a matter of concern both in human and veterinary medicine. Furthermore, it has been reported that plasmids carrying CTX-M enzymes can transfer these determinants to other commensal Enterobacteriaceae, such as *K. pneumoniae*, or to pathogens like *Shigella* or *Salmonella* spp. [12]. Their occurrence in milk can be a significant risk to consumers and animal handlers who may get such infection easily. On the other hand, persistence of such pathogen in udder or milk may complicate the therapeutic regimen of intra-mammary infection and clinical recovery of the animals. The pathogens may also disseminate to other in-contact animals via infected milk and contaminate the surrounding environment aggravating the situation further, if adequate precautionary measures are not taken [1].

Organism producing ESBL is estimated to increase in the future, both in animals and humans. The use of antibiotics requires the policy as issued internationally towards control of zoonotic pathogens [23]. To prevent the spreading of these isolates, the use of cephalosporins and fluoroquinolones should be limited in treating cow infection isolates, and enhanced sanitation of milk processing and transportation is needed [10]. Heat treatment

of milk, either by pasteurization or by ultrahigh temperature treatment safely inactivates Enterobacteriaceae [4].

5. Conclusion

CTX-M-15 and CTX-M-55 producing *E. coli* are the most prevalent type of our CTX-M ESBL-positive isolates and all of CTX-M producing *E. coli* isolates showed multidrug resistance phenotypes to at least three antibiotics. The existence of CTX-M producing *E. coli* could be a threat towards public health because its ability to spread resistant gene to the environment, food, human, animal and other pathogenic bacteria.

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