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Research Report

The Epidemiological Pattern and Risk Factor of ESBL (*Extended Spectrum B-Lactamase)* Producing *Enterobacteriaceae* in Gut Bacterial Flora of Dairy Cows and People Surrounding in Rural Area, Indonesia

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ABSTRACT

Livestock would be a risk factor of resistant bacteria that impact on human health. Rural area with farms as major economic source has become a risk of the spread of the ESBL producing Enterobacteriaceae The aim of the study was to explore the distribution and risk factor of ESBL (extended-spectrum β-lactamase) producing Enterobacteriaceae in the gut bacterial flora of dairy cows and people surrounding farming area. Total of 204 fecal swab samples were collected, 102 from dairy cows and 102 from farmers. Samples were sub-cultured by streaking on MacConkey agar supplemented with 2 mg/L cefotaxime. The growing colonies were confirmed of the ESBL producer by Modified Double Disk Test (M-DDST) and identification of Enterobacteriaceae by biochemical test. ESBL genes were identified by PCR. ESBL producing bacteria were found 13.7% in dairy cows and 34.3% in farmers. ESBL producing Enterobacteriaceae in dairy cows were 6.9% and in farmers of 33.3%. Statistical analysis showed: Distribution of ESBL producing Enterobacteriaceae strain were insignificant among dairy cows and farmers while bla_{TEM} distribution was significantly different (p= 0,035) and use of antibiotic was identified as a risk factor of colonization of ESBL producing Enterobacteriaceae in farmers (p= 0,007). Farmers had suspected as the source of ESBL producing Enterobacteriaceae based on higher prevalence. Further education of appropriate use of antibiotic need to enhance to control risk factor and prevent the colonization of ESBL producing Enterobacteriaceae.

Keywords: *Enterobacteriaceae, ESBL, gut flora, dairy cow, farmer, rural*

ABSTRAK

Hewan ternak diduga sebagai faktor risiko kejadian bakteri resisten yang berdampak terhadap kesehatan manusia. Area rural dengan potensi ekonomi di sektor peternakan merupakan area yang berisiko terhadap penyebaran Enterobacteriaceae penghasil ESBL. Penelitian bertujuan mengeksplorasi pola distribusi dan faktor risiko Enterobacteriaceae penghasil ESBL pada bakteri flora usus sapi perah dan penduduk sekitarnya. Total 204 sampel swab feses, terdiri dari 102 swab feses sapi perah dan 102 swab feses peternak. Swab feses ditanam pada media MacConkey yang ditambahkan 2 mg/L cefotaxime. Koloni yang tumbuh dikonfirmasi sebagai penghasil ESBL dengan metode Modified Double Disk Test (M-DDST) and diidentifikasi dengan uji biokimia. Identifikasi gen ESBL menggunakan metode PCR. Prevalensi bakteri penghasil ESBL di sapi perah sebesar 13.7% dan di peternak sebesar 34.3%. Distribusi Enterobacteriaceae penghasil ESBL pada sapi perah 6.9% dan pada peternak 33.3%. Analisis statistik menunjukkan: Tidak ada perbedaan signifikan antara distribusi bakteri Enterobacteriaceae penghasil ESBL pada sapi perah dan peternak, distribusi bla_{TEM} pada sapi perah dan peternak berbeda signifikan (p = 0,035), dan penggunaan antibiotik sebagai faktor risiko kolonisasi Enterobacteriaceae penghasil ESBL pada peternak (p= 0,007). Peternak diduga sebagai sumber Enterobacteriaceae penghasil ESBL. Penyuluhan

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tentang penggunaan antibiotik secara tepat perlu ditingkatkan untuk mengendalikan faktor risiko dan mencegah kolonisasi Enterobacteriaceae penghasil ESBL.

Kata kunci: *Enterobacteriaceae, ESBL, flora usus, sapi perah, peternak, rural*

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INTRODUCTION

The inappropriate use of antibiotics in human and animal health is a major cause of pathogenic bacterial resistance.[1] Resistant pathogenic bacteria that have increased significantly over the past few decades are ESBL-producing bacteria (extended-spectrum β-lactamase).[2] ESBL mainly distributed among gram-negative bacilli of the *Enterobacteriaceae* group.[3]

The use of antibiotics as growth promotion and the prevention of disease in veterinary [4] was correlated with the increase of ESBL producing Gram-negative bacteria.[5] It thus, the livestock are identified as a risk factor for ESBL producing *Enterobacteriaceae* (ESBL-E).[6] In 2018, East Java Province was identified has the highest population of dairy cows in Indonesia, about 283,311 cows.[7] Most of the dairy farms in East Java Province are located in rural areas to empowering the community's economy. Kalipucang Village in District of Pasuruan, East Java was established as the first center of public dairy farming in Indonesia at 2016.[8]

ESBL producing *Enterobacteriaceae* (ESBL-E) bacteria cause various infections in humans, such as: bacteremia, gastroenteritis, respiratory infections, urinary tract infections, and infections of the central nervous system.[9] In dairy cows, *Escherichia coli* and *Klebsiella spp* are identified as an agent that causing inflammation of mammary gland and udder tissue (mastitis) which impact on decreasing quantity and quality of milk production, increasing the rejected prematurely, and death.[10] ESBL-E becomes a serious challenge in therapy for infection includes prolong of diagnosis and expensive, a longer duration of treatment, limited antibiotic choices that impact on higher cost of therapy for an infection, as well as increased morbidity and mortality.[11] Multiple resistance to fluoroquinolones, aminoglycosides, and trimethoprim are commonly found in ESBL-E.[3] It also causes carrier in both humans[12] and livestock.[6]

Since ESBL-E has been identified as one of the causes of mastitis in dairy cows in 2000,[6] dairy farming was suspected to be at risk as a source of ESBL-E transmission. It thus the epidemiological profile of ESBL-E in farm needs to be explored. This study is the first study to analyze the epidemiological patterns of ESBL producing *Enterobacteriaceae* in livestock and humans in rural areas in Indonesia.

The aim of the study was to identify and analyzed the distribution and risk factor of ESBL producing *Enterobacteriaceae* in gut bacterial flora of dairy cows and people/farmer who have close contact with dairy cows.

MATERIALS AND METHODS

Design

This study was conducted a cross-sectional design. This study was approved by Research Ethics Committee in Faculty of Medicine of Universitas Airlangga, no: 82/EC/KEPK/FKUA/2019.

Samples Collection

Fecal samples were collected from April until July 2019 from dairy cows and farmers. Samples collected using Amies transport medium (Deltalab, Spanyol). The total dairy farming in the District of Pasuruan, East Java, are 648 clusters, of which as many as 102 were randomly included as the samples in this study, consisting

of 102 samples from dairy cows and 102 from humans that living around and have close contact with dairy cows. These clusters were located in Kalipucang village. The samples transportation were using a cool box and ice pack (4-8˚C). Samples were processed within 24 hours after taken from the sample source.

Bacterial and ESBL Identification

The isolation, confirmation, and identification of ESBL-E were conducted in The Clinical Microbiology Laboratory of Dr. Soetomo Hospital, Surabaya. Amies swab was streaked on MacConkey selective medium supplemented by cefotaxime 2 mg/L and incubated for 18-24 h at 37˚C. The growing colonies were ESBL confirmed by Modified Double Disk Synergy Test (M-DDST). Colonies which grow were inoculated in Mueller Hinton medium (0,5 MacFarland) with five (5) antibiotics disk : amoxicillin/clavulanic (AMC) 30 ug, ceftazidime (CAZ) 30 ug, cefotaxime (CTX) 30 ug, ceftriaxone (CRO) 30 ug, and aztreonam (ATM) 30 ug which placed within 15 mm of distance from edge to edge of AMC disk.[13] Incubated for 18-24 hours at 37˚C. The inhibition zone which show synergy zone between one of cephalosporin disk or aztreonam disk with amoxicillin/clavulanic disk was confirmed as ESBL producer. ESBL positive strain were bacteriologically identified using the biochemical test: Triple Sugar Iron (TSI) test, Indol test, Methyl Red (MR) test, Voges Proskauer (VP) test, Urease test, and Motility test. All bacterial isolates were then stored in deep freeze minus 80˚C.

Genotypic Examination

Genotypic examination was held in Institute of Tropical Diseases, Universitas Airlangga, Surabaya.

DNA Extraction

DNA extraction was conducted by boiling method. The identified ESBL producing bacteria were re-cultured on Mueller Hinton medium, incubated at 37˚C for 18 – 24 h. Four to five colonies were taken and suspended in sterile distilled water in 1,5 ml Eppendorf tube. The suspension was homogenized with vortex for 15 seconds and immersed in a thermostat at 95˚C for 10 minutes, then centrifuged at 14.000 rpm for 1 minutes. The supernatant was used as DNA template in PCR and stored in -20˚C.[14]

DNA Amplification

Three ESBL gene primers are used to amplify and identify the ESBL gene, as follow (Table 1) : [15]

PCR reaction was run in volume of 20 µl: 10 ul of GoTaq Green Master Mix 2x (Promega),1 ul for each of forward and reverse primers, 3 µl nuclease free water, and 5 µl DNA template. PCR was run as follow: for $bla_{\text{CTX-M}}$: denaturation on 94ºC for 7 minutes and the following 35 cycles on 94ºC for 50 seconds, annealing on 50ºC for 40 seconds, extension on 72ºC for 1 minute, and final extension on 72ºC for 5 minutes; for bla_{SHV}: denaturation on 96ºC for 5 minutes and the following 35 cycles on 96ºC for 1 minute, annealing on 60ºC for 1 minute, extension on 72ºC for 1 minute, and final extension on 72ºC

Table 1. Primers of ESBL Genes

Gen	Sekuens Primer (5'-3')	Amplicon size (bp/base pair)
$bla_{\text{CTX-M}}$	F : 5' ATGTGCAGYACCAGTAARGT 3' R : 5' TGGGTRAARTARCTSACCAGA 3'	593
bla_{SHV}	F : 5' GGTTATGCGTTATATTCGCC 3' R : 5' TTAGGTTGCCAGTGCTC 3'	867
bla_{TEM}	F : 5' ATGAGTATTCAACATTTCCG 3' R : 5' CTGACAGTTACCAATGCTTA 3'	867

for 10 minutes; and for bla_{TEM}: denaturation on 96ºC for 5 minutes and the following 35 cycles on 96ºC for 1 minute, annealing on 58ºC for1 minute, extension on 72ºC for 1 minute, and final extension on 72ºC for 10 minutes. PCR amplicon were visualized in 2% gel electrophoresis.

Questionnaire to Find Risk Factors

Information about risk factor of ESBL producing *Enterobacteriaceae* in dairy cows and farmers were obtained through interview and questionnaires. *Enterobacteriaceae* strain and genotype distribution among dairy cows and farmers and risk factors were analyzed by Chi Square/Fisher Exact Test on SPSS 22 version program.

RESULTS

Distribution of ESBL Producing *Enterobacteriaceae* in Dairy Cows and Farmers

Prevalence of ESBL producing bacteria in dairy cows was 13.7% (14/102) and in farmers 34.3% (35/102). ESBL producing *Enterobacteriaceae* in dairy cows were 6.9% (7/102) and in farmers 33.3% (34/102) (Table 2). The ESBL producing *Enterobacteriaceae* in dairy cows were mostly: *Escherichia coli* 85.7% (6/7) and *Enterobacter spp* 14.3% (1/7), whereas among 34 ESBL-E in human were *Escherichia coli* 82.4% (28/34), *Enterobacter spp* 14.3% (3/34), *Klebsiella pneumoniae* 5.9% (2/34), and *Klebsiella oxytoca* 2.9% (1/34). There were no significant differences in distribution of ESBL producing *Enterobacteriaceae* strain in dairy cows and in farmers (Table 2) and Fig.1.

Table 2. Distribution of ESBL producing *Enterobacteriaceae* strain in dairy cows and farmers

ESBL Producer	Dairy Cows (n=102)	Farmers (n=102)	*p* value
ESBL producing bacteria	14 (13.7%)	35 (34.3%)	
Non-*Enterobacteriaceae*	7 (6.9%)	1 (0.9%)	
Enterobacteriaceae	7 (6.9%)	34 (33.3%)	
Escherichia coli	6 (85.7)	28 (82.4)	$p = 1{,}000$
Enterobacter spp	1 (14.3)	3 (8.8)	$p = 0{,}542$
Klebsiella pneumoniae	0 / 0	2 (5.9)	$p = 1{,}000$
Klebsiella oxytoca	0 / 0	1 (2.9)	$p = 1{,}000$

Note: ESBL-E: ESBL producing *Enterobacteriaceae*

Figure 1. The Double Disk Synergy Test (DDST) for identifying ESBL producer bacteria.
Note: The increasing of inhibition zone in area between cephalosporin disk and clavulanic acid disk was marked as positive ESBL producer.

Escherichia coli were identified as dominant strain of ESBL producing *Enterobacteriaceae* in dairy cows and farmers (85.7% vs. 82.4%).

Distribution of ESBL Gene Among Dairy Cows and Human (Farmers)

Among seven ESBL producing *Enterobacteriaceae* in dairy cows, six isolates were harbored $bla_{CTX\text{-}M}$ (85.7%) and one an unidentified gene (14.3%). Among 34 isolates of ESBL producer in farmers, 26 isolates harbored $bla_{CTX\text{-}M}$ (76.5%), 15 isolates $bla_{TEM,}$ and three

Table 3. ESBL genes distribution of ESBL producing *Enterobacteriaceae* in dairy cows and farmers

ESBL Gene	Dairy Cows (n=7)	Farmers (n=34)	*p* value
$bla_{CTX\text{-}M}$	6 (85.7)	26 (76.5)	$p = 1{,}000$
bla_{SHV}	0 (0)	3 (8.8)	$p = 1{,}000$
bla_{TEM}	0 (0)	15 (44.1)	$p = 0{,}035$
Unidentified gene	1(14.3)	0 (0)	$p = 0{,}171$
$bla_{CTX\text{-}M,}$ bla_{TEM}	0 (0)	8 (23.5)	-
$bla_{CTX\text{-}M}$, bla_{SHV}, bla_{TEM}	0 (0)	1 (2.9)	-

Note: *Unidentified gene*: gene of ESBL producing *Enterobacteriaceae* which couldn't detected with specific primer used in this study

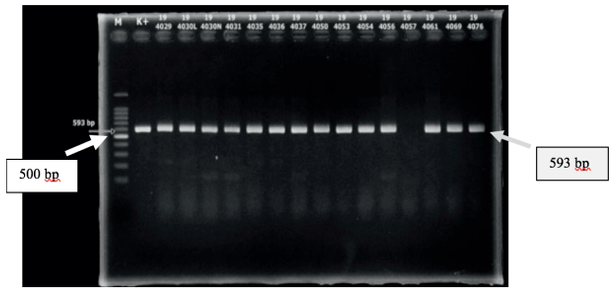

Figure 2. Electrophoresis of amplified gene of bla_{CTX-M} (593 bp)

isolates bla_{SHV} (8.8%), respectively. There was a significant difference of bla_{TEM} distribution in dairy cows and in farmers (p = 0, 035) (Table 3).

Combination of two and three of ESBL genes were found in *Enterobacteriaceae* producing ESBL isolates in farmers. Eight isolates harbored bla_{CTX-M} dan bla_{TEM} (23.5%) and one isolate harbored bla_{CTX-M}, bla_{SHV}, bla_{TEM} (2.9%) (Table 3).

Risk Factor for Colonization of ESBL Producing *Enterobacteriaceae*

Age, origin of dairy cows, history of illness during the last 3 months, history of drug use, type of drug given last 3 month, and type of feed were not risk factors for colonization of ESBL-producing *Enterobacteriaceae* in dairy cows. Risk factor for ESBL producing *Enterobacteriaceae* colonization in farmers was the use of antibiotics (p = 0.007). Gender, age, education level, household, hygiene sanitation of environment (location of dairy cow shed and type of toilet), personal hygiene sanitation (frequency and how to wash hands), and frequency of going out of the city during the last 3 months were not a risk factor.

DISCUSSION

Colonization of ESBL producing *Enterobacteriaceae* in dairy cows by 6,9% in this study is similar with study in healthy ruminant (cows and buffaloes) in rural areas in Cambodia by 7%[16] and lower than in cattle farm in German by 54.5%.[6] Colonization of ESBL producing *Enterobacteriaceae* in farmers by 33,33% lower than the colonization of ESBL-producing bacteria in healthy individuals in rural areas in Thailand by 65.7%,[17] in China by 73.9%,[18] and workers in cattle farms in Germany: 12.5%.[6]

Human and animal gut were the natural habitat of many bacterial especially *Enterobacteriaceae* and become a reservoir of various infections.[12] Non-appropriate and overuse of antibiotic caused selective pressure that supports the growth of resistance bacteria.[9] Colonization of resistance bacteria in human and animal gut causing transmission of resistance genes in gut flora bacterial through horizontal gene transfer by conjugative plasmid.[12] ESBL mostly encoded by genes in plasmids.[19] *Enterobacteriaceae* was identified as having plasmid carrying resistant genes. IncFII plasmid group known as a plasmid group that encoded ESBL genes and it widely distributed in *Enterobacteriaceae*. It called epidemic resistant plasmid group.[20]

This study identified *Escherichia coli* as the dominant ESBL producing bacteria in dairy cows (85.7%) and farmers (82.4%). Distribution of *Escherichia coli* as an ESBL producer in dairy cows in this study was 85.7%, higher than in cattle farms in Mecklenburg-Western Pomerania, Germany by 54.5%.[6] At farmers, distribution of ESBL producing *Escherichia coli* by 82.4% is lower than the distribution of *Escherichia coli* in healthy individuals in rural areas in Thailand by 85.4% [17] and in China 88%,[18] but higher than workers in cattle farms in Germany: 12.5%.[6]

Escherichia coli is the main organism that produces ESBL in communities[21] and associated with urinary tract infections (UTI). It is related to their role as gut bacterial flora and are pathogenic to humans and animals.[12] The resistance of commensal *Escherichia coli* to antimicrobial agents has been found in healthy individuals.[22] This bacterium also acts as an indicator of 'acquired antibiotic resistance genes' in the community.[23]

Distribution of bla_{CTX-M} in the ESBL producing *Enterobacteriaceae* in dairy cows by 85.7% is higher than the distribution of bla_{CTX-M} in cattle farms in Germany 80%.[6] At farmers,

distribution of bla_{CTX-M} in the ESBL-producing *Enterobacteriaceae* by 76.5% higher than in healthy individuals in rural areas in China by 68.1%[18] and in Thailand by 65.7%.[17] bla_{CTX-M} that mostly integrated with conjugative plasmid, and conjoint with the other resistant gene, has a higher transferability among bacteria, and impact on higher prevalence epidemiologically.[24]

bla_{CTX-M}, bla_{SHV}, and bla_{TEM} are dominant ESBL genes in various regions worldwide and found from isolates of humans, animals and the environment. bla_{TEM} and bla_{SHV} mainly found in *Escherichia coli* and *Klebsiella pneumoniae*.[25] The study identified bla_{TEM} in *Escherichia coli* and *Enterobacter spp* isolates and bla_{SHV} in *Klebsiella pneumoniae* and *Escherichia coli* isolates. bla_{CTX-M} is the dominant ESBL gene worldwide, especially in community and has increased in incidence since 2000.[12] It were identified in livestock and pets with *Escherichia coli* as the main producing bacteria.[26] Our study showed that bla_{CTX-M} was identified in *Escherichia coli*, *Enterobacter spp*, and *Klebsiella oxytoca*.

Dissemination of bla_{CTX-M} occurs rapidly, extensive, and significantly. Plasmids are known to carry genes which encode resistance for antibiotic.[27] Conjugative plasmids play an important role in facilitating the horizontal dissemination of bla_{CTX-M} among bacteria.[28] bla_{CTX-M} was identified in various epidemic resistant plasmid groups, including: groups IncF, IncN, IncI1, IncL / M, and IncHI2.[24] These plasmid group are able to capture and transfer resistant genes among bacteria.[29] The IncFII Group is the largest plasmid group encoding bla_{CTX-M} and widely found in *Enterobacteriaceae*[24] and isolates from human and animal.[20]

IS*Ecp*1 is the genetic element which associated with all variants of bla_{CTX-M}, play a role incoding transposase and inducing bla_{CTX-M} expression. Transposase is an enzyme that mobilizes bla_{CTX-M} in certain plasmids.[28] Other types of IS include: IS*CR*1 plays a role in bla_{CTX-M} group 2 and 9 expression, IS*10* in bla_{CTX-M} group 8 expression,[24] and IS*26* in bla_{CTX-M} group 1 and 9 expression. IS*Ecp*1 and IS*CR*1 play a role in the mobilization of class 1 integron that encodes various types of resistant genes (MDR cassettes).[28]

Clones of *Escherichia coli* were identified having a significant role in the dissemination of bla_{CTX-M}, among others, such as ST131, ST38, ST393, ST405. ST131 serotype 025: H4 phylogenetic group B2 is an extra-intestinal pathogenic *E. coli* strain and was mainly involved in bla_{CTX-M} dissemination especially $bla_{CTX-M-15}$ in worldwide.[24] It identified having IncFII plasmid group and found in isolates originated from the animal, environment, and especially human.[28]

Combination of two or three ESBL genes in one bacterial isolate is due to integron and plasmid that carry several resistant genes. *Enterobacteriaceae* would be harboring of 5 to 6 plasmids in one isolate.[30] Class 1 integron which related to bla_{CTX-M} were identified encoding several types of resistant genes (MDR cassettes).[28] The unidentified gene is thought to be an ESBL gene in addition to bla_{CTX-M} (group 1), bla_{SHV}, and $bla_{TEM.}$

The finding of antibiotic use as risk factor of ESBL producing *Enterobacteriaceae* in farmers in this study ($p= 0,007$) related according to the study of Luvsansharav et al,[17] which identified the use of antibiotics in the last 3 months (OR 1,883; 95% CI 1,221-2,903) as a risk factor for colonization of ESBL producing bacteria in healthy individuals in rural area in Thailand and Zang et al[18] that identified antibotic use in the previous 6 months (OR 1,892; 95% CI 1,242–2,903; $p = 0.034$) as a risk factor for colonization of ESBL-producing bacteria in healthy individuals in rural area in China.

In dairy cows in this study, there was not any antibiotic use detected based on data on questionnaires. The total of 52% of dairy cows were given anthelmintic every three months. Risk factors for colonization of ESBL producing *Enterobacteriaceae* in dairy cows was not identified. Dissemination of ESBL producing bacteria occurs from animals to humans or vice versa.[6] ESBL producing Gram negative in dairy cows have the potential as a zoonotic risk,[31] especially through close contact during daily care.[6]

The results of the study showed that the rural community could act as a reservoir of ESBL-producing *Enterobacteriaceae*. The finding of *Escherichia coli* and bla_{CTX-M} as the dominant strain and ESBL gene epidemiologically indicated alarming sign. *E. coli* ST131 consider as virulent strain,[24] multiple resistance, easily colonize and spread between humans, animals and environment isolates.[27] It contributes to the spread of bla_{CTX-M} globally through horizontal gene transfer.[24] ESBL-producing *E. coli* and bla_{CTX-M} have driven the spread of ESBL gene in the community. Colonization of ESBL-producing *Enterobacteriaceae* is a risk factor for infection of ESBL-E.[3] The colonization of ESBL-producing *Enterobacteriaceae* in community were predicted increasingby about 5% annually.[24] This certainly becomes a challenge in therapy of infectious disease.

CONCLUSION

Farmers had suspected as the source of ESBL producing *Enterobacteriaceae* based on higher prevalence. The use of antibiotic in human, was identified as risk factor for colonization of ESBL producing *Enterobacteriaceae* while not identified in dairy cows.

CONFLICT OF INTEREST

There is no conflict of interest of this study.

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