Research article

Antimicrobial resistance characteristics of Extended Spectrum Beta Lactamase (ESBL)-producing Escherichia coli from dairy farms in the Sleman district of Yogyakarta province, Indonesia

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Abstract

Extended Spectrum Beta Lactamase (ESBL)-producing Escherichia coli (E. coli) infections are a global health challenge resulting from human contact with infected animals and contaminated farm environments. This study aims to identify antimicrobial resistance patterns of ESBL-producing E. coli isolated from dairy farms in the Sleman District of Yogyakarta Province, Indonesia. Ninety-three dairy farms with a history of antibiotic use in the previous 6 months were identified. Samples were collected from 6 different sources (feces, milk, wastewater, animal drinking water, feed and rinses of workers’ hands) on each farm during August through November 2020. These samples were cultured with conventional microbiological methods for the isolation of ESBL-producing E. coli. ESBL-producing E. coli was identified in one or more of the sources in 54% (50/93) of the dairy farms sampled. Fecal samples were the most commonly positive (25%) while wastewater, animal drinking water, feed and rinses of workers’ hands were positive at 16%, 10%, 5%, 4% and 3% respectively. Colonies from each positive sample were screened for antibiotic susceptibility test using the Vitek-2 system. Resistance to trimethoprim/sulfamethoxazole, tetracycline and gentamicin were found in 74%, 63% and 48% of the isolates, respectively. Multidrug resistant (MDR) was identified in 50% (63/127) of the isolates. In conclusion, ESBL-producing E. coli appears widespread in dairy farms using antibiotics and antimicrobial resistance among these bacteria is common in this study area. Further study of the risk of human transmission from contaminated cattle and their environments could benefit the national antimicrobial resistance strategic plans.

Keywords: Antimicrobial resistance, Dairy farm, ESBL-producing E. coli, Sleman of Yogyakarta

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INTRODUCTION

The demand of milk consumption in Indonesia is increasing about 1.5% per year. Increasing dairy production is one of the Indonesian government’s agricultural development goals (Priyanti and Soedjana, 2015). Total fresh milk production in Indonesia was 947,685 tons/year in 2020. Yogyakarta Province had the fourth largest fresh milk production in Indonesia with supply 6% of total milk production (DGLAHS, 2020).

Escherichia coli (E. coli) is Gram-negative bacteria which is a commensal organism of the digestive tract in dairy cattle as well as other animals (Thepmanee et al., 2018; Prapasawat and Intarapuk, 2021; Rodroo et al., 2021; Saekhow and Sriphannam, 2021). E. coli is the most likely of the Enterobacteriaceae to produce the Extended Spectrum Beta Lactamase (ESBL) enzyme which is an indicator of antimicrobial resistance. The ESBL enzyme can hydrolyze beta lactam antibiotics along with 2nd and 3rd generation cephalosporines. Several types of ESBL arise by mutations and then can transmit resistance genes for many different antimicrobials (Ghafourian et al., 2015; Lee et al., 2012). ESBL-producing E. coli is most frequently detected resistant microbe within ESBL-producing Enterobacteriaceae worldwide and causes high human mortality (Russo et al., 2021).

The growing number of ESBL in livestock is a global public health concern (El-Mokadem et al., 2020). This challenge is associated with Multidrug resistance and failure of antibiotic treatment (Bassetti et al., 2017). The occurrence of ESBL-Producing E. coli in dairy farms has been reported in many countries such as Germany (Dahms et al., 2015), China (Zheng et al., 2019), New Zealand (Burgess et al., 2021) and Malaysia (Kamaruzzaman et al., 2020). In Indonesia, a few studies of ESBL-Producing E. coli found in feces from dairy farms in East Java (Putra et al., 2020; Putra et al., 2019) and in milk from dairy farms in West Java (Sudarwantoa et al., 2017). The distribution and antimicrobial resistance characteristics of ESBL-Producing E. coli represent key surveillance data required in order to understand global situation and tackle the problem (Rousham et al., 2018). Consequently, the World Health Organization implemented the Tricyle Antimicrobial Resistance Surveillance project for ESBL-producing E. coli using a One Health as part of a global program for monitoring antimicrobial resistance. Indonesia is one of the countries participating along with Pakistan, Ghana, Madagascar and Malaysia (WHO, 2021).

The imprudent use of antimicrobial agents on dairy farms may increase the risk of antimicrobial resistance. Resistant microbes on the farm can spread into communities through contaminated animals, food from animal origin (e.g., milk and meat) as well as the water supply, environment and farm workers. In order to provide information for the national surveillance system, this study aims to detect ESBL-producing E. coli contamination on dairy farms and its antimicrobial resistance patterns.
MATERIALS and METHODS

Study site and Sample collection
The Sleman District, of Yogyakarta Province, Indonesia was selected for the study (Figure 1). The Sleman District is recognized by the local veterinary department as having the most dairy farms and the majority of the dairy cattle in the Province.

Local veterinary officers were contacted to create a list of farms in the region. Farm records were reviewed in order to identify those dairy farms with a history of antibiotic usage in the previous 6 months. One hundred twenty-five (125) farms met these inclusion criteria and were contacted about the study. A total of 93 farmers (74%) agreed to participate in the on-farm sample collection.

A cross-sectional study was conducted from August to November 2020. Six samples were collected on each farm for a total of 558 samples from 93 farms. The samples including 10 g pooled fecal sample per rectums, 30 ml pooled milk sample, 1000 ml of the animal drinking water, 200 ml of hand rinse water collected from workers with close contact with the cows, 200 g feed (roughage and concentrates), and 100 ml wastewater collected from a drainage ditch. All samples except the feed were stored in a cool box and transported to the laboratory to be analyzed within 24 h.
Isolation of *E. coli*

All samples were diluted 1 part sample to 9 parts of Buffered peptone water/ BPW (Oxoid, UK) and incubated at 37°C for 18-22 h. One loop full (ca. 10 µL) of the resulting incubated sample was streaked on MacConkey agar/ MAC (Oxoid, UK) containing of 1 mg/L cefotaxime (Himedia, India) and incubated at 44°C for 18-22 h (Hasman et al., 2019). Three typical colonies were selected and subcultured on Eosin methylene blue agar (Modified) Levine (Oxoid, UK), and then incubated at 37°C for 20-24 h. The suspected colonies which appeared purple with green metallic sheen were isolated on Nutrient agar (Oxoid, UK). Samples of each of these colonies were examined by Gram stained and examined by microscope. Colonies resembling the typical *E. coli* profile of Gram-negative non-spore forming rods were confirmed by indole test, methyl red test, Voges-Proskauer test, and citrate utilization test (the IMViC reaction) followed the Bacteriological Analytic Manual (Feng et al., 2020). The *E. coli* ATCC 25922 was used for quality control.

Antimicrobial susceptibility test and ESBL identification

The Vitek-2 system (BioMérieux, France) was applied to a sample of each confirmed ESBL-producing *E. coli* colony to identify antimicrobial susceptibility and ESBL strains by MIC/ broth microdilution according to the manufacturer’s instructions. The Vitek-2 system uses cefotaxime, ceftazidime, cefepime/clavulanic acid, cefotaxime/clavulanic acid ceftazidime/clavulanic acid for the confirmation of ESBL-producing strains which will interpret from at least 3 two-fold concentration decrease in an MIC for either antimicrobial agent tested in combination with clavulanic acid vs the MIC of the agent alone (CLSI, 2020a) (CLSI, 2020b). Antimicrobial sensitivity to 12 antimicrobial drugs were screened: imipenem, amikacin, gentamicin, neomycin, enrofloxacin, marbofloxacin, prodoxofloxacin, doxycycline, tetracycline, nitrofurantoin, chloramphenicol, and trimethoprim/sulfamethoxazole. Interpretation of the antimicrobial susceptibility testing was performed automatically by the Vitek-2 software version 9 based on the Clinical & Laboratory Standards Institute (CLSI), the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Global European.

RESULTS

Occurrence of ESBL-producing *E. coli*

A total of 558 samples were collected; 6 samples from each of 93 farms. Fifty-four percent of the farms (50/93) were identified with ESBL-producing *E. coli*. Positive farms were defined as having at least one positive sample detected. A total of 59 positive samples were identified among the 558 samples collected. Most of the farms have a single positive sample except 7 farms. Five farms had two positives and two farms had three positive samples.

ESBL-producing *E. coli* was most frequently isolated from fecal samples 25% (23/93), followed by wastewater 16% (15/93), and animal drinking water 10% (9/93). ESBL-producing *E. coli* was least frequently isolated from feed 5% (5/93), milk 4% (4/93) and hand rinsing 3% (3/93) as shown in Table 1.
Table 1 Presence of ESBL-producing *E. coli* by sample types

| Sample type               | n   | Positive samples |
|---------------------------|-----|------------------|
| Feces                     | 93  | 23 (25%)         |
| Wastewater                | 93  | 15 (16%)         |
| Animal drinking water     | 93  | 9 (10%)          |
| Feed                      | 93  | 5 (5%)           |
| Milk                      | 93  | 4 (4%)           |
| Hand rinsing              | 93  | 3 (3%)           |
| Total                     | 558 | 59 (11%)         |

**Antimicrobial susceptibility test**

The antimicrobial susceptibility test categorized ESBL-producing *E. coli* isolates as resistant, intermediate, or susceptible to each antibiotic. The most common antimicrobial resistance identified among the 127 ESBL-producing *E. coli* isolates was trimethoprim/sulfamethoxazole (74%), followed by tetracycline (63%), and gentamicin (48%) (Figure 2). The isolates were most susceptible to the antibiotics marbofloxacin, chloramphenicol and neomycin (4%).

*Figure 2* Antimicrobial susceptibility test of ESBL-producing *E. coli* isolated from dairy farms. trimethoprim/sulfamethoxazole (SXT), tetracycline (TET), gentamicin (GEN), enrofloxacin (ERO), marbofloxacin (MBX), prodofloxacin (PRA), doxycycline (DOX), chloramphenicol (CHL), neomycin (NEO), imipenem (IPM), amikacin (AMK), nitrofurantoin (NIT)
Almost all of the ESBL-producing *E. coli* isolates (118/127, 93%) were resistant to at least one of twelve antimicrobial agents (Table 2). Half (63/127) of the ESBL-producing *E. coli* isolates were resistant to three or more antimicrobial agent classes. A total of 25 different antimicrobial resistance patterns were observed as described in Table 3. Tetracycline-trimethoprim/sulfamethoxazole as the most common resistant pattern, accounting for 14% (16) of the isolates.

### Table 2 Multidrug resistance to antimicrobial agents of ESBL-producing *E. coli* isolates

| Antimicrobial agent (classes) | No of isolates | Percentage (%) |
|------------------------------|---------------|----------------|
| 3 or more classes            | 63            | 50             |
| 2 classes                    | 33            | 26             |
| 1 class                      | 22            | 17             |
| No resistance                | 9             | 7              |
| Total                        | 127           | 100            |

### Table 3 Antimicrobial drug resistance pattern of ESBL-producing *E. coli*

| No  | Antibiotic pattern | Frequency (n=118) | Percentage (%) |
|-----|--------------------|-------------------|----------------|
| 1   | CHL                | 5                 | 4              |
| 2   | GEN                | 2                 | 2              |
| 3   | SXT                | 8                 | 7              |
| 4   | DOX-TET            | 5                 | 4              |
| 5   | GEN-SXT            | 7                 | 6              |
| 6   | GEN-TET            | 7                 | 6              |
| 7   | TET-CHL            | 3                 | 3              |
| 8   | TET-SXT            | 16                | 14             |
| 9   | ERO-MBX-PRA        | 2                 | 2              |
| 10  | GEN-DOX-SXT        | 1                 | 1              |
| 11  | GEN-TET-SXT        | 5                 | 4              |
| 12  | TET-CHL-SXT        | 12                | 10             |
| 13  | DOX-TET-CHL-SXT    | 1                 | 1              |
| 14  | GEN-DOX-TET-SXT    | 14                | 12             |
| 15  | GEN-NEO-TET-SXT    | 1                 | 1              |
| 16  | ERO-MBX-PRA-DOX-SXT| 1                 | 1              |
| 17  | ERO-MBX-PRA-TET-SXT| 2                 | 2              |
| 18  | ERO-MBX-TET-CHL-SXT| 1                 | 1              |
| 19  | GEN-ERO-MBX-PRA-SXT| 11                | 9              |
| 20  | GEN-ERO-MBX-PRA-TET-SXT| 6 | 5              |
| 21  | GEN-NEO-ERO-MBX-PRA-SXT| 2 | 2              |
| 22  | ERO-MBX-PRA-DOX-TET-CHL-SXT| 1 | 1              |
| 23  | GEN-ERO-MBX-PRA-DOX-TET-SXT| 2 | 2              |
| 24  | GEN-ERO-MBX-PRA-TET-CHL-SXT| 1 | 1              |
| 25  | GEN-NEO-ERO-MBX-PRA-DOX-TET-SXT| 2 | 2              |

trimethoprim/sulfamethoxazole (SXT), tetracycline (TET), gentamicin (GEN), enrofloxacin (ERO), marbofloxacin (MBX), prodofloxacin (PRA), doxycycline (DOX), chloramphenicol (CHL), neomycin (NEO), imipenem (IPM), amikacin (AMK), nitrofurantoin (NIT), Minimum Inhibitory Concentrations (MICs), Not applicable (NA).
A total 12 antimicrobial agents were tested to find the minimum inhibitory concentration (MIC) of antibiotics as an indicator of the level of resistance. The concentration of antimicrobial agents tested ranged from 0.12-320 µg/ml as demonstrated in Table 4. The greatest percentage of isolates exceeding the MIC breakpoints for resistance were observed for trimethoprim/sulfamethoxazole, tetracycline and gentamicin.

### Table 4 Distribution of Minimum Inhibitory Concentration MIC for ESBL-producing E. coli isolates (n=127)

| Antimicrobial agents            | ≤0.12 | 0.25 | 0.5 | 1    | 2    | 4    | 8    | 16   | 20** | 32   | 40** | 64   | 80** | 128  | >320** |
|---------------------------------|-------|------|-----|------|------|------|------|------|------|------|------|------|------|------|-------|
| trimethoprim/sulfamethoxazole   | NA    | NA   | NA  | NA   | NA   | NA   | NA   | 34   | NA   | 0    | NA   | 0*   | NA   | 93   |       |
| tetracycline                    | NA    | NA   | NA  | 43   | 4    | 0    | 0    | 80*  | NA   | NA   | NA   | NA   | NA   | NA   |       |
| gentamicin                      | NA    | NA   | NA  | 66   | 0    | 0    | 0    | 0*   | 61   | NA   | NA   | NA   | NA   | NA   |       |
| enrofloxacin                    | 5     | 0    | 10  | 82   | 0    | 30*  | NA   | NA   | NA   | NA   | NA   | NA   | NA   | NA   |       |
| marbofloxacin                   | NA    | NA   | 0   | 23   | 7    | 30*  | NA   | NA   | NA   | NA   | NA   | NA   | NA   | NA   |       |
| pradofloxacin                   | 5     | 39   | 20  | 34   | 0*   | 29   | NA   | NA   | NA   | NA   | NA   | NA   | NA   | NA   |       |
| doxycycline                     | NA    | NA   | 0   | 30   | 8    | 12   | 42   | 27*  | NA   | NA   | NA   | NA   | NA   | NA   |       |
| chloramphenicol                 | NA    | NA   | NA  | NA   | 26   | 50   | 12   | 16   | 0    | 0*   | NA   | 23   | NA   | NA   |       |
| neomycin                        | NA    | NA   | NA  | NA   | 121  | 1    | 0    | 1    | 0    | 0*   | NA   | 5    | NA   | NA   |       |
| imipenem                        | NA    | 126  | 0   | 0    | 0    | 0    | 0    | 0    | 0    | 0*   | 0    | 0    | 0    | 0    | NA    |
| amikacin                        | NA    | NA   | NA  | NA   | 127  | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | NA   | NA    |
| nitrofurantoin                  | NA    | NA   | NA  | NA   | NA   | NA   | NA   | 100  | 0    | 20   | NA   | 7    | NA   | 0*   | 0     |

*, MIC breakpoints for resistance according to the Clinical and Laboratory Standards Institute (CLSI, 2020a, b) and Comite de L’Antibiogramme de la Société Française de Microbiologie (CA-SFM); **trimethoprim/sulfamethoxazole concentration range is 20 (1/19) – 320 (16/304); ; MICs, Minimum Inhibitory Concentrations; NA, not applicable.

### DISCUSSION

This study affirms that ESBL-producing E. coli can be found on Indonesian dairy farms. The results are similar to a study in Malaysia that identified ESBL producing E. coli on many farms (Kamaruzzaman et al., 2020). ESBL above 50% have been documented in Greek (Filioussis et al., 2020) and Netherland (Heuvelink et al., 2019).

The study focused only on farms with a history of recent antibiotic usage so there is the evidence of ESBL-producing E. coli in dairy farms in this region. However, the sampling plan cannot represent the population as other prevalence study. Further, the study results may not reflect the true magnitude of the problem in the farms sampled due to the limited number of samples taken. Collection of more fecal samples on each farm might identify more positives. Nevertheless, the contamination of feces and wastewater found in this study illustrate the potential for spreading this ESBL in the environment. In agriculture, feces have the potential to be used as fertilizer as practiced by farmers in Yogyakarta. One of the distribution vectors for resistance bacteria through human is the use of feces as organic fertilizer in agriculture (Ben Said et al., 2015). Furthermore, research has shown that ESBL can be transmitted to
people through the food chain by eating vegetables (Blaak et al., 2014). The finding of positive ESBL-producing E. coli in rinse water from worker’s hands demonstrates the risk of cross-contamination between cattle and worker (Dahms et al., 2015) and the potential that workers may carry ESBL-producing E. coli back to their homes and families. However, the presence of the bacteria does not automatically demonstrate zoonotic transfer between cattle and worker as environmental contamination, such as by wastewater may represent an even greater risk (Friese et al., 2013).

The hand-milking of cows practiced by most farmers in this study likely increases the milk contamination with ESBL by increasing the exposure of the milk to ESBL-producing E. coli on the milkers’ hands. The study confirms others’ findings that milk represents a risk for transferring ESBL to the consumer by food consumption (Grami et al., 2014). The presence of positive ESBL-producing E. coli in cattle feed could be linked to cross contamination by feces or to soil contamination caused by the use of excrement as fertilizer.

Overall, the ESBL-producing E. coli isolates in this study had high susceptibility rates to amikacin, neomycin, and nitrofurantoin and high resistance to trimethoprim/sulfamethoxazole followed tetracycline and gentamicin. A previous study found that tetracycline and sulfonamide were the most commonly used antimicrobial agents in Indonesia for cattle treatment (Yusuf et al., 2017). This study also found the most common pattern of tetracycline-trimethoprim/sulfamethoxazole 14%. The most common resistance pattern reflects the drugs used most by Indonesian dairy farmers. According to national data in Indonesia, antibiotic treatment of livestock, especially cattle, increased from 2014 to 2016, with Oxytetracycline being the most commonly used antibiotic, followed by sulfonamides, penicillin aminoglycosides, penicillin, fluoroquinolone, and others (Havan et al., 2017).

In this study, approximately 50% of the ESBL-producing E. coli isolates were Multidrug resistance (MDR). The MDR referred to the strains that is resistant to at least three different antimicrobial classes. The most frequent MDR pattern in this study was aminoglycoside-tetracycline-sulfonamide which is not surprising as diverse resistance genes in same plasmid can be found (Wichmann et al., 2014). The majority of antibiotic classes used in human treatment are also used in animals. Multidrug resistance is becoming more frequent, especially among Gram negative bacteria. Moreover, many strains are developing resistance to important antibiotics used to treat humans including cephalosporin, aminoglycoside, trimethoprim-sulfamethoxazole, and carbapenems (Bajpai et al., 2014; Nigussie and Amsalu, 2017). Humans infected E. coli exhibit a high resistance to trimethoprim-sulfamethoxazole in Indonesia (Parathon et al., 2017).

Our results showed that the presence of ESBL-producing E. coli and AMR in various types of samples reflect the complexity of this situation in terms of potential routes for zoonotic transmission. Our study affirms the need for a One Health approach to antimicrobial resistance, working with veterinarians as well as physicians to encourage prudent use of antimicrobial agents as recommended by the WHO in the ESBL-producing E. coli Tricycle AMR Surveillance program (WHO, 2021).
CONCLUSIONS

Presence of ESBL-producing *E. coli* in dairy farms and all types of samples indicate the presence of antimicrobial resistance on many dairy farms in this area (the Sleman district of Yogyakarta province, Indonesia). This finding is similar to surveys in other countries, demonstrated the global nature of this public health challenge. The findings reiterate the importance of public health and veterinary officers learning from each other. Moreover, dissemination of this information is critical for raising awareness in the community and may be useful in developing a national antimicrobial resistance surveillance system in Indonesia.

CONFLICT of INTERESTS

The authors whose names are listed in this article certify that they are not involved in any organization or entity with any financial interest, nonfinancial interest in the subject matter or materials discussed in this article.

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AUTHOR CONTRIBUTIONS

**Kusnul Yuli Maulana**: Conception or design of the work, sample collection, perform experiment, data analysis and interpretation, and drafting the manuscript.

**Duangporn Pichpol**: Conception or design of the work, perform experiment, data analysis and interpretation, final approval of the version to be published.

**Nur Rohmi Farhani**: Provide the reference strain.

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**Tongkorn Meeyam**: Conception or design of the work, perform experiment, data analysis and interpretation, critical revision of the manuscript, final approval of the version to be published.
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