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Antibiotic sensitivity profile of multidrug-resistant (MDR) *Escherichia coli* isolated from dairy cow's milk in Probolinggo, Indonesia

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Abstract. *Widodo A, Lamid M, Effendi MH, Khairullah AR, Riwu KHP, Yustinasari LR, Kurniawan SC, Ansori ANM, Silaen OSM, Dameanti FNAEP. 2022. Antibiotic sensitivity profile of multidrug-resistant (MDR)* Escherichia coli *isolated from dairy cow's milk in Probolinggo, Indonesia. Biodiversitas 23: 4971-4976.* The presence of resistant bacteria in animal products such as milk can be a new threat because it is directly related to the human food chain. Resistant *Escherichia coli* has been widely studied and detected in farms in developing countries. The aim of present study was to determine the antibiotic resistance profile of *E. coli* bacteria from dairy cows taken during the milking process from several dairy farms in Probolinggo district, Indonesia. A total of 150 milk samples were obtained from farms and *E. coli* was isolated and identified on Eosin methylene blue (EMB) media and biochemical test, such as Triple Sugar Iron Agar (TSIA) and indole test, methyl red test, Voges-Proskauer test, and citrate test (IMViC) were also performed. The antibiotic sensitivity profile was screened using the Kirby-Bauer test and results were interpreted according to the CLSI standard. The results showed that 124/150 (82.67%) *E. coli* bacteria exhibited highest percentage of antibiotic resistance to tetracycline (13.71%), streptomycin (9.68%), trimethoprim (8.87%), chloramphenicol (0.87%), and aztreonam (1.61%). A total of 9/124 (7.26%) *E. coli* isolates were detected as multidrug-resistant (MDR) and 1/9 (0.81%) *E. coli* isolate was suspected as extended spectrum β-lactamase (ESBL) bacteria which was resistant to aztreonam antibiotic. Thus, the threat of multidrug-resistant (MDR) *E. coli* can come from milk which can affect public health.

Keywords: Dairy cows, *Escherichia coli*, MDR, milk, multidrug-resistant, public health

INTRODUCTION

The infection of human pathogenic *Escherichia coli* from milk has been reported in several cases in developing countries (Tanzin et al. 2016). Milk contamination with resistant *E. coli* has been widely studied. Currently, a public health focus is being developed regarding antibiotic resistance due to its use in the treatment of livestock, causing human diseases in developing countries (Robinson et al. 2016). Antibiotics are used for the prevention and treatment of various cases of *E. coli* infections that attack livestock (Ghimpeteanu et al. 2022). However, inappropriate and irrational administration of antibiotics has resulted in the emergence of *E. coli* antibiotic resistance (Hinthong et al. 2017). *E. coli* can be a reservoir of various antibiotic resistance genes, which can be transmitted to other bacteria, especially in the case of the spread of multidrug-resistant bacteria. *E. coli* is a major pollutant in the environment that is often associated with Extended Spectrum β-Lactamase (ESBL) encoding genes, namely $bla_{\text{CTX-M}}$ and bla_{TEM} (Jena et al. 2017).

The main potential source of harmful bacteria is raw milk. Outbreaks of foodborne illnesses have been reported due to the consumption of contaminated raw milk and raw milk products in Indonesia (Suwito 2010). *E. coli* is one of the most important pathogenic bacteria, a normal inhabitant of the large intestine in humans and animals (Hassan et al. 2014). It can be transmitted to raw milk and dairy products due to fecal contamination and poor hygienic practices during the milking process. Potential sources of milk contamination are milk cans, pens, farm environment, cow hair, manure, feed, milking equipment, and workers.

Contamination can also occur during storage, transportation, distribution, marketing, and sales (Effendi et al. 2018a). The presence of *E. coli* bacteria in raw milk has often been reported as a source of foodborne illness (Odenthal et al. 2016). Most intestinal diseases in humans originate from animals, which are transmitted directly from animals to humans or indirectly through the food of animal origin or water contaminated with feces (Tewari et al. 2022). *E. coli* can cause diarrhea in humans (Cahyani et al. 2019).

Human diseases from food products of animal origin can be categorized as foodborne diseases. One of these diseases is the consumption of raw milk. Raw milk contaminated with resistant *E. coli* bacteria can transfer it from animals to humans via a food chain pathway or direct contact. The use of antibiotics in the long term can affect the resistance of bacteria, both pathogens and normal microflora in the body of living things (Effendi et al. 2019; Wibisono et al. 2020a; Putra et al. 2020). The global distribution pattern of resistant bacteria in humans and animals can be a reference and consideration in efforts to prevent and treat resistant bacterial infections.

This study was conducted to obtain an overview of the antibiotic resistance profile of *E. coli* isolated from dairy cow's milk. This is based on the high cases of *E. coli* antibiotic resistance in humans that can be transmitted from raw milk.

MATERIALS AND METHODS

Sample collection and location

Milk samples were taken from the nipples of dairy cows during the milking process from several dairy farms located in Probolinggo district. A total of 150 samples were collected in the period from May 2021 to September 2021. The livestock selected for research was breeders who had an average 3 or more cows. A total of 10 mL of milk samples were taken into sterile tubes. Milk samples were kept in sterile plastic bags and brought to the laboratory using a cool box at 4°C.

Isolation and identification of *Escherichia coli*

Each 1 mL of milk sample was cultured into 10 mL of Brilliant Green Bile Lactose Broth (BGLB) enrichment media (Merck, 105544) and incubated at 37°C for 18-24 h. Positive results were indicated by a change in the media from green to cloudy green and the presence of gas in the Durham tube (Putra et al. 2020). Then the positive samples were streaked on Eosin Methylene Blue (EMB) media (Merck, 101347) and incubated at 37 °C for 18-24 h (Meghla et al. 2021; Ansharieста et al. 2021). 3-5 colonies of *E. coli* showing a metallic green color were purified and further biochemically identified by Triple Sugar Iron Agar (TSIA) and indole test, methyl red test, Voges-Proskauer test, and citrate test (IMViC) (Wibisono et al. 2020b).

Antibiotic sensitivity test

Antibiotic sensitivity of the isolates was investigated using the Kirby-Bauer disc diffusion test on Mueller-Hinton Agar (Oxoid, CM0337). Examination of the multidrug resistance profile using antibiotic discs tetracycline 30 µg (Oxoid, CT0054), streptomycin 10 µg (Oxoid, CT0047), chloramphenicol 30 µg (Oxoid, CT0013), trimethoprim 5 µg (Oxoid, CT0057), and aztreonam 30 µg (Oxoid, CT0057). Estimation of suspected ESBL based on the results of resistance tests for more than 3 antibiotics including aztreonam. The results were interpreted by measuring the diameter of inhibition zone formed, after overnight incubation at 37°C, according to Clinical and Laboratory Standards Institutions (CLSI 2020; Effendi et al. 2021).

RESULTS AND DISCUSSION

Results

A total of 150 suspected *E. coli* colonies were isolated from 150 cow's milk samples with 100% percentage according to macroscopic characters. On Brilliant Green Lactose Broth media, the colony color was cloudy green and all they all produced gas in Durham tube after incubation at 37°C for 24 h. A total of 124 (82.67%) isolates on EMB medium showed growth of metallic green convex colonies with black rounded centers (Figure 1) after incubation at 37°C for 24 h.

Bacterial smears were stained with Gram stain and viewed under a microscope at 1000x magnification to identify the isolates. Gram staining results showed that *E. coli* was Gram-negative rod shaped bacteria (pink color). Isolates used for biochemical tests were grown on EMBA. Biochemical test result revealed that 124 (82.67%) isolates tested positive for *E. coli* with TSIA showed yellow color in the slant and butt media, gas appeared in the media but did not show black color (H_2S). The IMViC test with negative sulfide test result, positive indole, positive motile, positive MR, negative VP, and negative citrate is shown in Figure 2.

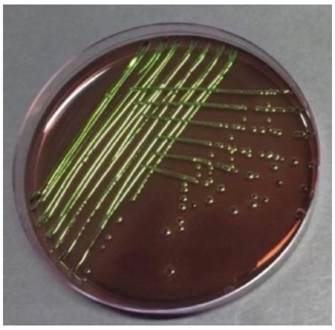

Figure 1. *Escherichia coli* isolates on EMB agar

A **B** **C** **D** **E**

Figure 2. Positive biochemical test for *Escherichia coli*. A. Acid/H2S (-), B. Indol (+), C. MR (+), D. VP (-), E. Citric (-)

Table 1. Antibiotic sensitivity profile of *Escherichia coli* isolates

Locations	Sample size	Confirmed *Escherichia coli*	Resistant to					MDR	Presumptive ESBL
			TE	S	C	W	ATM		
Farm 1	3	2	1	1	-	1	-	1	-
Farm 2	4	2	1	1	-	1	-	1	-
Farm 3	3	2	-	-	-	-	-	-	-
Farm 4	3	3	1	1	-	1	-	1	-
Farm 5	5	3	1	1	-	1	-	1	-
Farm 6	3	2	1	-	-	-	-	-	-
Farm 7	3	3	-	-	-	-	-	-	-
Farm 8	3	2	1	1	-	-	1	1	1
Farm 9	4	2	-	-	-	-	-	-	-
Farm 10	3	3	-	-	-	-	-	-	-
Farm 11	5	3	1	-	-	-	-	-	-
Farm 12	3	2	1	1	-	1	-	1	-
Farm 13	4	2	-	-	-	-	-	-	-
Farm 14	3	3	-	-	-	-	-	-	-
Farm 15	3	2	-	-	-	-	-	-	-
Farm 16	6	5	-	-	-	-	-	-	-
Farm 17	4	4	1	2	-	2	-	1	-
Farm 18	3	3	-	-	-	-	-	-	-
Farm 19	5	5	-	1	-	-	-	-	-
Farm 20	3	2	-	-	-	-	-	-	-
Farm 21	4	4	-	-	-	-	-	-	-
Farm 22	3	2	-	-	-	-	-	-	-
Farm 23	3	2	-	-	-	-	-	-	-
Farm 24	5	5	1	-	-	-	1	-	-
Farm 25	4	3	1	1	-	1	-	1	-
Farm 26	6	6	-	-	-	-	-	-	-
Farm 27	3	3	-	-	-	-	-	-	-
Farm 28	3	2	-	-	-	-	-	-	-
Farm 29	3	3	-	-	-	-	-	-	-
Farm 30	5	3	-	-	-	-	-	-	-
Farm 31	3	3	-	-	-	-	-	-	-
Farm 32	3	2	-	-	-	-	-	-	-
Farm 33	4	4	-	-	-	-	-	-	-
Farm 34	5	5	1	-	-	-	-	-	-
Farm 35	3	3	1	1	-	1	-	-	-
Farm 36	3	3	-	-	-	-	-	-	-
Farm 37	4	3	1	-	-	-	-	-	-
Farm 38	3	3	1	1	1	1	-	1	-
Farm 39	3	3	1	-	-	-	-	-	-
Farm 40	4	4	-	-	-	-	-	-	-
Farm 41	3	3	1	-	-	1	-	-	-
Total	150	124	17	12	1	11	2	9	1
Percentage (%)	100	82.67	13.71	9.68	0.81	8.87	1.61	7.26	0.81

Note: TE: Tetracycline; S: Streptomycin; W: Trimethoprim; C: Chloramphenicol; ATM: Aztreonam

Table 2. Measurement of inhibition zone of antibiotic sensitivity test of multidrug-resistant and presumptive ESBL *Escherichia coli*

Sampels code	Diameter of inhibition zone of antibiotic (mm)					MDR	Presumptive ESBL
	TE 30 µg	S 10 µg	C 30 µg	W 5 µg	ATM 30 µg		
AS 1	7 (R)	11 (R)	22 (S)	7 (R)	31 (S)	+	-
AS 5	7 (R)	7 (R)	26 (S)	7 (R)	26 (S)	+	-
AS 12	7 (R)	7 (R)	25 (S)	7 (R)	29 (S)	+	-
AS 15	8 (R)	7 (R)	28 (S)	7 (R)	29 (S)	+	-
AS 27	8 (R)	6 (R)	22 (S)	6 (R)	10 (R)	+	+
AS 40	8 (R)	7 (R)	21 (S)	7 (R)	28 (S)	+	-
AS 61	10 (R)	8 (R)	27 (S)	6 (R)	30 (S)	+	-
AS 91	10 (R)	8 (R)	22 (S)	7 (R)	23 (S)	+	-
AS 109	7 (R)	7 (R)	8 (R)	7 (R)	25 (S)	+	-

Note: R: Resistant; S: Sensitive; +: positive result; -: negative result; TE: Tetracycline; S: Streptomycin; W: Trimethoprim; C: Chloramphenicol; ATM: Aztreonam

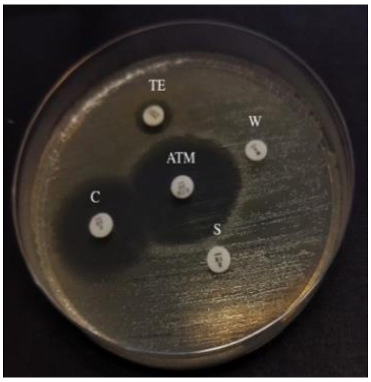

Figure 3. Isolate resistant to 3 antibiotic discs (AS-61)

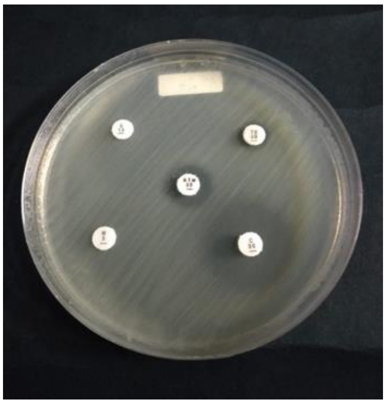

Figure 4. ESBL suspect isolates showed resistance to aztreonam (AS-27)

The antimicrobial resistance profile of the obtained bacterial isolates is shown in Table 1. *E. coli* bacteria showed resistance to antibiotics, such as tetracycline (13.71%), streptomycin (9.68%), trimethoprim (8.87%), chloramphenicol (0.87%), and aztreonam (1.61%). A total of 9 multidrug-resistant (MDR) isolates were found (7.26%) with three different patterns (Figure 3).

The multidrug-resistance (MDR) pattern can be seen in Table 2. The highest prevalence of multidrug-resistance (MDR) pattern was recorded with tetracycline, streptomycin, and trimethoprim in 7 out of 9 MDR isolates. One isolate was suspected to *E. coli* ESBL bacteria was resistant to Aztreonam disc (Figure 4).

Discussion

Foodborne disease is an important challenge to public health and cause significant economic problems in many countries (Kiambi et al. 2022). Public health problems not only have an impact on human health but also have an impact on the health of dairy cows (Berhe et al. 2020). Food safety programs aim to prevent food products from being contaminated by potential pathogens. Milk is an excellent medium for bacterial growth, which not only impairs milk quality but also become an infectious medium for humans (Tadesse et al. 2018). A large number of pathogenic microbes can gain access to milk and various dairy products (Virpari et al. 2013). Improper and unhygienic handling of milk, especially during the milking process, plays an important role in milk contamination (Amenu et al. 2019). High level of *E. coli* in milk samples indicates poor sanitation practices of farmers during the milking process (Munera-Bedoya et al. 2017). *E. coli*, a normal inhabitant of the intestines of animals and humans, can be harmful in and pathogenic to humans.

In this study, 124 samples (82.67%) were found to be contaminated with *E. coli*, this percentage is higher than test conducted by Ahmadi et al. (2020) in Iran, who isolated *E. coli* from 173 out of 400 milk samples (43.25%). In another study conducted by Hassani et al. (2022), 78 out of 100 milk samples (78%) were contaminated with *E. coli*. In research conducted by Liu et al. (2021), 195 raw milk samples were collected from 195 dairy farms located in China, of which 67 (34.4%) were *E. coli* positive samples and in developing countries such as Bangladesh the prevalence rate was 75% (Islam et al. 2016). The emergence of bacterial resistance to antibiotics is a natural mechanism for survival of bacteria, so their presence can have a negative impact on society (Fair and Tor 2014). In the present study, several isolates of *E. coli* were found multidrug-resistant (MDR) against more than 3 groups of antibiotics, namely 9 isolates (7.26%) using the disc diffusion method, this percentage is lower than the research conducted by Ansharieta et al. (2020) who found 16 out of 250 milk samples (9.1%) contaminated with *E. coli* resistant to more than 3 groups of antibiotics. Another study in Ethiopia by Dejene et al. (2022) screened 27 milk samples, of which 27 isolates (100%) were *E. coli* (O157:H7) resistant to more than 3 groups of antibiotics. MDR *E. coli* isolates are very common in many countries and are responsible for a range of infections of high severity and difficult to treat. In Canada, studies have been conducted on urinary tract infections caused by *E. coli* bacteria, of which 60% are cases of *E. coli* infections that are resistant to more than 3 classes of antibiotics. Consumption of undercooked food, travel habits between regions, and contact with reservoir animals are associated with an increased risk of urinary tract infections caused by MDR *E. coli* (Ukah et al. 2018). Multidrug resistance reported in this study is due to the high irrational use of antimicrobials in individual cows to treat various diseases affecting milk quality. The incidence of multidrug resistance is a public health problem. Foodborne outbreaks can be difficult to detect and treat. The presence of MDR *E. coli* in food is a source of resistant genes that can spread rapidly (Founou et al. 2016).

This study found a pattern of multidrug-resistance (MDR) combined of tetracycline, streptomycin, and trimethoprim in 7 of 9 total MDR isolates. One isolate was suspected to contain *E. coli* ESBL bacteria that were resistant to aztreonam discs. The percentage values of *E. coli* resistance to antibiotics in this study were tetracycline (13.71%), streptomycin (9.68%), trimethoprim (8.87%), chloramphenicol (0.87%), and aztreonam (1.61%). The percentage value is almost similar to the results of Ansharieta et al. (2020), such as tetracycline (17.05%), streptomycin (14.2%), trimethoprim (9.66%), chloramphenicol (7.95%), and aztreonam (1.7%). Tetracyclines have the highest antibiotic resistance because they are often used in veterinary medicine (Ilbeigi et al. 2021). The use of broad-spectrum antibiotics such as tetracycline and β-lactams is more common in cases of clinical mastitis in dairy cattle in Europe, because of their effective results. For respiratory and digestive tract problems, tetracycline and aminoglycoside groups are the first choice of antibiotics, while the second choice is a combination of sulfonamide-trimethoprim drugs which significantly affect rumen microbial activity, and the last choice is the third cephalosporin group antibiotics (Economou et al. 2015). In this study, eight MDR isolates and one suspected ESBL *E. coli* were identified showing the same pattern of resistance to several antibiotics, namely tetracycline, streptomycin, and trimethoprim, except for AS 109 which was also resistant to chloramphenicol. Although, the level of drug resistance of tetracycline, streptomycin, and trimethoprim was still relatively low, evaluation of the use of antibiotics in dairy cows should continue to be carried out routinely. Based on the findings of aztreonam antibiotic resistance and confirmation of *E. coli* ESBL from milk, it is necessary to pay attention to the potential of milk as a source of bacterial zoonotic infections that can manifest and attack humans. Zoonotic bacterial diseases can be transmitted from animals to humans in various ways, including contaminated animal products such as milk (Effendi et al. 2018b; Tyasningsih et al. 2019), direct fecal-oral route, improper food handling, and cooking (Harijani et al. 2020; Permatasari et al. 2020). Thus, in the one health concept, humans who are close to animals are able to contract zoonotic pathogenic bacteria that can spread to other humans in the community (Decline et al. 2020).

In conclusion, multidrug-resistant (MDR) *E. coli* is a public health threat resulting from the consumption of food of animal origin including milk. In this study, it was found that *E. coli* isolates were multidrug-resistant (MDR) and among these isolates, one isolate was suspected that ESBL producing *E. coli* was resistant to the antibiotic aztreonam. The risk factors for MDR *E. coli* contaminants in dairy cows, especially in milk or dairy products, need to be investigated and these data are very important for the development of specific guidelines for the control of MDR *E. coli* in veterinary practice support public health. The application of sanitation and monitoring of the use of antibiotics is needed as a prevention and control effort to suppress the spread of multidrug-resistant (MDR) *E. coli* in dairy farms in Probolinggo, East Java, Indonesia.

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