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Incidence of Extended Spectrum Beta-Lactamase (ESBL) of *Escherichia Coli* Isolated from the Feces of Dairy Cattles in Blitar

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KEYWORDS

Antibiotic resistance;
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Escherichia coli;
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Abstract Antibiotic resistance (AMR) has caused a serious global threat to human health, animal health and food security. The antibiotic resistance problem, especially in Gram-negative bacteria, is increasingly widespread in various areas and has increased mortality, morbidity, and incidence. This study aims to determine the incidence of ESBL *E. coli* and the AMR and MDR properties of *E.coli* isolated from the feces of dairy cattles in Blitar, which have not been reported. This research was conducted from June to September 2022 with 60 samples of dairy cattle feces in Blitar. Samples were isolated and identified until confirmed that 55 (91.6%) samples were *E. coli*. All samples showed AMR character with 100% streptomycin, 87.27% chloramphenicol, 85.45% ampicillin, 72.73% cefotaxime, 40% tetracycline, 21.82% ciprofloxacin, and 14.55% sulfamethoxazole-trimethoprim. Forty-nine samples were MDR, with 10.9% resistant in three groups, 34.5% in four groups, 29.1% in five groups, 10.9% in six groups, and 3.6% in seven groups. MDR *E. coli* isolates were then confirmed for ESBL using the DDST test. The incidence of ESBL-producing *E. coli* from dairy cattle feces in Blitar was 40% (22 isolates). The results of this study can be a warning in the human health, animal health and food security.

Introduction

Antibiotic resistance has caused a serious global threat to human, animal, and food security (Wetzker *et al*., 2019). The antibiotic resistance problam, especially in Gram-negative bacteria, is increasingly widespread in various areas and has increased mortality, morbidity, and incidence (Nobrega and Brochii, 2014; Munawir, 2017). Antimicrobial Resistance Collaborators (2022) reported that AMR-related deaths in 2019 were estimated at 4.95 million cases. Of these incidents, 70% were caused by cases of Extended Spectrum Beta-Lactamase (ESBL) causing resistance to penicillin antibiotics, first, second, third generation cephalosporins and aztreonam (except cefamycin and carbapenem) (Antimicrobial Resistance Collaborators, 2022; Yanestria *et al*., 2022).

Naturally, bacteria can produce genes with enzymes that cause antibiotic resistance and be transferred to other bacteria in self-defense (Masruroh *et al*., 2016). Enzymes in the ESBL gene can hydrolyze and inactivate beta-lactam antibiotics (Biutifasari, 2018). The multidrug-resistant bacteria can cause infections that are harder to treat, limited treatment choices, and can cause death

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(Doumith *et al*., 2012; Masruruoh *et al*., 2016). The incidence of ESBL continues to increase because of the unwise and ineffective antibiotic use in humans and animals (Ben Said *et al*., 2015).

The ESBL gen is found mainly in *Escherichia coli* (*E.coli*) bacteria. *E. coli* is an enteric bacterium that is used as an indicator of antimicrobial resistance and poor sanitation. *E. coli* is also the main reservoir of ESBL-producing bacteria (Normaliska *et al*., 2019). This is because *E. coli* is highly prevalent in the environment and in healthy animals. On the other hand, *E. coli* has the potency to spread resistance factors. *E. coli* ESBL gene mutations such as CTX-M, TEM, and SHV can change one or more amino acid sequences and are transferred to other bacteria by plasmids. This condition is responsible for the increased prevalence of ESBL (Masruroh *et al*., 2016; Imasari *et al*., 2016).

The incidence of ESBL-producing *E.coli* in dairy cattles has been reported in various regions in Indonesia, such as 72% in Surabaya, 6% in Tulungagung Regency, and 59% in Pasuruan Regency (Imasari *et al*., 2018; Putra *et al*., 2019; Soekoyo *et al*., 2020). Around 578,579 dairy cattles in Indonesia were spreading ESBL. East Java is the highest contributor to the dairy cattle population in Indonesia, which is 301,780 cattles. One of the regencies with the largest dairy cattle population in East Java is Blitar (BPS, 2021). The results of previous studies revealed that the number of dairy cattle population, the antibiotics used, and the sanitation of dairy cattle stalls played an important role in increasing the incidence of ESBL-producing *E. coli* (Santos *et al*., 2013).

Based on this background, it is important to research the incidence of ESBL-producing *E. coli*, especially in Blitar dairy farms. This study aims to determine the incidence of ESBL *E. coli* and the AMR and MDR properties of *E.coli* isolated from the feces of dairy cattles in Blitar, which have not been reported. The results of this study can be used as the basis for public awareness to evaluate antibiotics used in dairy farms, human health, animal health and food security .

Materials and methods

Sampling

Sampling was carried out on 26 July 2022. Feces per rectum of around ten grams was collected at 60 dairy farms in KUD Semen, Blitar using sterile flacon. The sample was stored in an icebox at 4^{o}C during sample transportation. The sampling was accompanied by the Department of Food Security and Agriculture of Blitar City and Village Unit Cooperatives (KUD) Semen Area in Blitar Regency

Isolation and Identification of E. coli

Dairy cattle feces samples were isolated in buffered peptone water media (BPW; Oxoid, UK). with a concentration of 2% with a ratio of 1:9 and incubated at 37°C for 18-22 hours. The isolation results on BPW media were streaked on Eosin Methylene Blue Agar Media (EMBA; Oxoid, UK) and incubated at 37^{o}C for 24 hours (Maulana *et al*., 2021). The suspected *E. coli* colonies appeared metallic green on EMBA media and were then identified using gram staining and biochemical tests. The biochemical tests used were IMViC (Indol motility (SIM; HIMEDIA, India), Methyl Red & Voges Proskauer (HiMedia, India), citrate (HiMedia, India)), Triple sugar iron agar (TSIA; HiMedia, India), and urease. (HiMedia, India) (Yanestria *et al*., 2022).

Antimicrobial Resistance (AMR) Testing

After *E. coli* isolates were confirmed, the samples were tested for antibiotic sensitivity with the Kirby-Bauer disc diffusion test to

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determine the AMR properties of *E. coli* bacteria. The antibiotic disc used in this test was based on the literature and the types of antibiotics frequently used in dairy farming, such as streptomycin, chloramphenicol, ampicillin, cefotaxime, tetracycline, ciprofloxacin, sulfamethoxazole-trimethoprim (Blaak *et al*., 2015). *E. coli* was isolated on Brain Hearth Infusion Agar (BHIA) media and incubated for 24 hours at 37^{o}C. The isolates were prepared in 100µl suspension or 0.5 McFarland equivalent turbidity (1.5x108 CFU/ml) and were streaking to Mueller Hinton Agar (MHA; Oxoid, UK). The antibiotic disc was placed on the surface of the media and incubated at 37^{o}C for 18 hours. The inhibition zone diameters' results were compared with the interpretation standards of the Clinical Laboratory Standard Institute (CLSI) (CLSI, 2020; Effendi *et al*., 2019). The number of diameters produced by this test was divided into three categories: sensitive (S), intermediate (I), and resistant (R). The interpretation results were shown as multidrug resistance if the sample was resistant to 3 antibiotics (Wibisono *et al*., 2020).

Confirmation of ESBL using Double Disc Synergy Test (DDST)

DDST is a phenotype confirmation test of ESBL-producing bacteria by evaluating the inhibitory zone of ESBL activity with clavulanic acid. DDST testing was confirmed on *E. coli* with MDR. This method was carried out using the Kirby-Bauer disc diffusion method on MHA media according to the recommendations of CLSI (Wibisono *et al*., 2020; Zhang *et al*., 2016). The bacterial isolate was resuspended with the standard 0.5 McFarland. Bacterial isolates were taken using a sterile cotton swab and swabbed evenly on the surface of the MHA media. After 15 min, an amoxicillin-clavulanic acid (AMC; 30/10 g) antibiotic disc was placed in the center of the agar plate and followed by a ceftazidime (CAZ; 30 mg) disc, and cefotaxime (CTX; 30 mg) 15 mm apart from the AMC. The culture was then incubated at a temperature of 35-37^{o}C for 18-24 hours (Imasari *et al*., 2018). Positive ESBL was confirmed when the synergy of Cefotaxime/Ceftazidime with the combination of amoxicillin-clavulanate acid was increased in the inhibition zone of 5 mm between the inhibition zone of cephalosporins and amoxicillin-clavulanic acid (Wibisono *et al*., 2020; Chukhunwejim *et al*., 2018; Masruroh *et al*., 2016)

Results and discussion

The results of the isolation and identification of dairy cattle feces samples were 91.6% (55/60) confirmed by *E. coli*. *E. coli* colonies appear a metallic green color with a black center of the colony on EMBA Media. The metallic green result was caused by the strong lactose fermentation of *E. coli* that made the methylene blue color of the adsorbed media becomes a metallic green sheen, as shown in Figure 1 (A) (Effendi *et al*., 2019: Lal and Cheeptham, 2007).

The results of the identification of *E. coli* showed the characteristics of gram-negative rods. It does not form spores in Figure 1 (B), changing the TSIA medium to yellow and producing gas, positive for the Indole and Methyl Red tests, negative for the VP, Citrate, and Urease tests, as shown in Figure 2 (Maulana *et al*., 2021; Chu *et al*., 2012; Leboffe & Pierce, 2011).

The results of the incidence of Antimicrobial Resistance (AMR) of *E. coli* isolates in the feces of dairy cattles in Blitar showed 100% resistance. AMR results for each class of antibiotics, namely, 100% in streptomycin, 87.27% in chloramphenicol, 85.45% in ampicillin, 72.73% in cefotaxime,

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40% in tetracycline, 21.82% in ciprofloxacin, and 14.55% in sulfamethoxazole-trimethoprim. AMR results in details can be seen in Figure 3. AMR showed significant resistance to the antibiotics streptomycin, chloramphenicol, ampicillin, and cefotaxime. This result should be a warning in considering the use of antibiotics ampicillin (penicillin) and streptomycin, as well as broad-spectrum antibiotics such as chloramphenicol, trimethoprim-sulfamethoxazole, and ampicillin, are often used as mastitis therapy in dairy cattle (Meutia *et al*., 2016; Haftu *et al*., 2012). Antibiotics Over the Counter (OTC) are thought to generate the development of *E. coli* resistance, so their adverse effects contribute to the problem of infection in humans (Feliatra *et al*., 2022). The antibiotics of tetracycline, ciprofloxacin, and sulfamethoxazole-trimethoprim showed significant sensitive results. These results can be the basis for the appropriate use of antibiotics in dairy farms in Blitar.

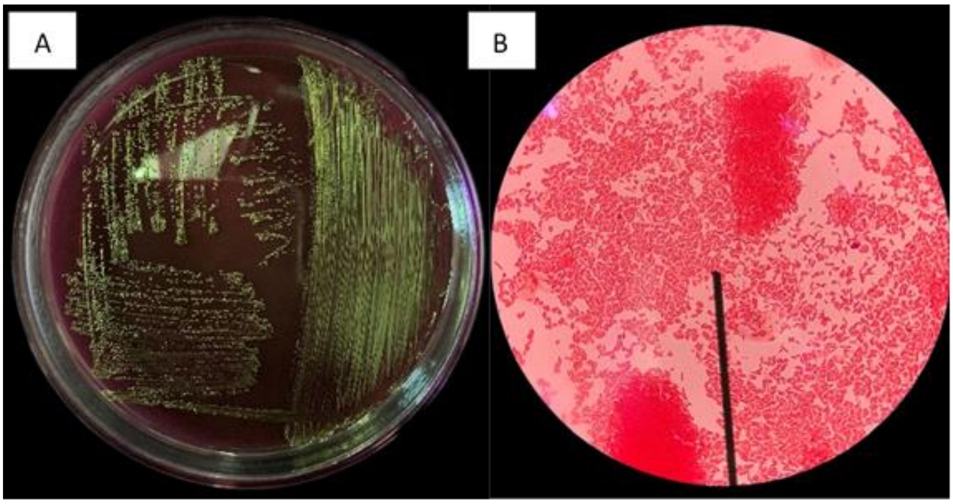

Figure 1. The results of the isolation and identification of *E. coli*. (A) EMBA results; (B) Gram. staining results

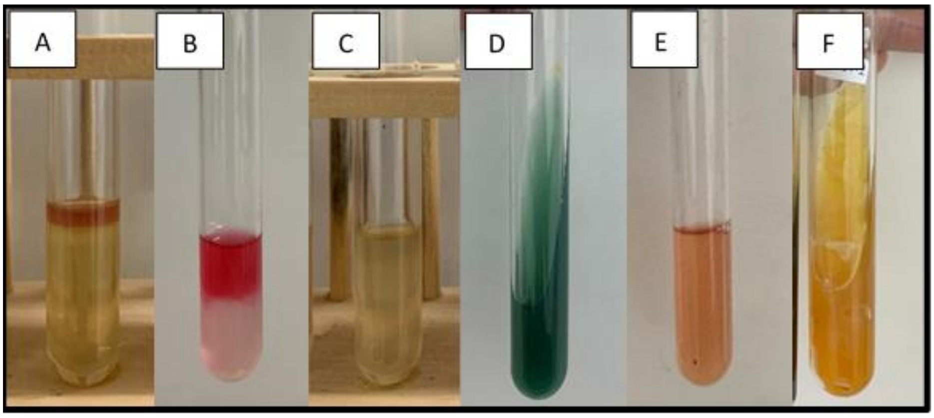

Figure 2. Results of *E. coli* biochemical tests. (A) Indole test result, (B) MR result, (C) VP result, (D) Citrate result, (E) Urease result, and (F) TSIA result.

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Multidrug resistance (MDR) incidence showed 49 or 89%. Around 10.9% resistance in three groups, 34.5% in four groups, 29.1% in five groups, 10.9% in six groups, and 3.6% in 7 groups, as shown in Table 1. The highest MDR was seen in 4 classes of antibiotics. In addition, isolates were found that were resistant to 7 classes of antibiotics. The incidence of MDR bacterial in the community indicates a problem in the health sector. Bacterial gen can move horizontally, which can occur in three ways: plasmid, phage, and transformation. Bacteria can engulf the free DNA nearby. Plasmids, as extra-chromosomal DNA, can independently replicate and play an important role in resistance to various types of antibiotics and the spread of antibiotic-resistant genes (Aminah and Jamilatun, 2016). A previous study reported that antibiotic resistance (ARG) genes in the R plasmid were transferred between pathogenic and nonpathogenic Gram-negative bacteria in the environment (Feliatra *et al.*, 2021).

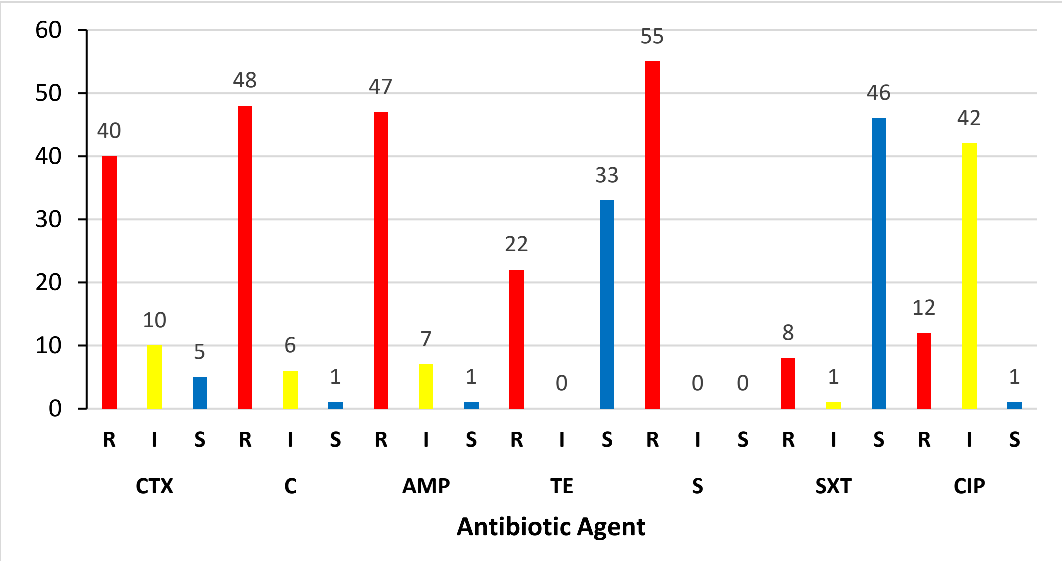

Figure 3. Results of AMR *E. coli* in the feces of dairy cattles in Blitar

Note, Red: resistant; yellow: Intermediate; Blue: sensitive; CTX: Cefotaxime; C: chloramphenicol; AMP: ampicillin; TE: tetracycline; S: streptomycin; SXT: sulfamethoxazole-trimethoprim; CIP: ciprofloxacin.

Table 1. Results of MDR *E. coli* in the feces of dairy cattles in Blitar

Number of Antibiotic Groups	3	4	5	6	7
n=49	6	19	16	6	2
%	10.9	34.5	29.1	10.9	3.6

Table 2. Detection results of ESBL-producing *E. coli* in dairy cattle feces in Blitar

Sample	Sample size	*E. coli*	AMR	MDR	DDST
Feces	60	55	55	49	22

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The results of the ESBL-producing *E. coli* in the feces of dairy cattle in Blitar was 40% (22/55) which can be seen in Table 2. These results are based on DDST confirmation by looking at the synergism produced between cephalosporin antibiotics and amoxicillin-clavulanate acid. This synergy was observed with an increase in the diameter of the inhibition zone observed in Figure 4. (Chukwunwejim *et al*., 2018). ESBLs are often found in bacterial plasmids. One of the main causes of the increasing prevalence of bacteria resistant to beta-lactam and aminoglycoside antibiotics is gene transfer that occurs in plasmids, integrons, and transposons. Furthermore, the combination of several resistant genes causes bacteria to become resistant to most antibiotics (MDR) (Putra *et al*., 2019).

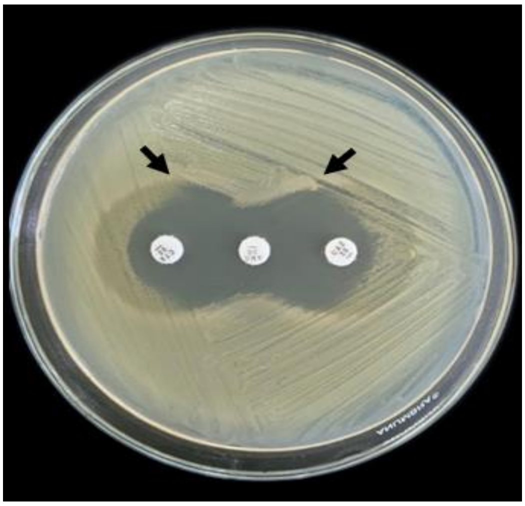

Figure 4. Results of ESBL confirmation against *E. coli* using the DDST test. (AMC) amoxicillin-clavulanate acid, (CTX) Cefotaxime, (CAZ) Ceftazidime, the arrow represents the synergy formed.

The incidence of ESBL-producing *E. coli* in dairy cattle feces has been reported in various regions in Indonesia, 72% Surabaya, 6% Tulungagung Regency, and 59% Pasuruan Regency (Imasari *et al*., 2018; Putra *et al*., 2019; Soekoyo *et al*., 2020). The incidence of ESBL-producing *E. coli* in food-producing animals is particular concern because it can become a problem for spreading from animals to the community (Santos *et al*., 2013). Most Gram-negative bacteria, including *E. coli*, are pathogenic to animals and humans. Feces released from animals can be a source of disease transmission by polluting the environment through the soil as fertilizer and surface water to rivers (He *et al*., 2020; Ma *et al*., 2012). One of the vectors for spreading bacterial resistance through humans is using livestock feces as organic fertilizer in agriculture (Ben Said *et al*., 2015). In addition, rivers are important reservoirs against spreading resistant genes to bacteria from various sources (Zurfluh *et al*. 2013).

Conclusions and Suggestion

In conclusion, the incidence of *E. coli* AMR-MDR and ESBL-producing in the feces of dairy cattle in Blitar by 40% can be a public health warning. This incident can be a risk factor for increasing the incidence of ESBL-producing *E. coli* in Blitar, considering that feces can contaminate the soil around farms and rivers. The presence of ESBL-producing *E. coli* can warn a public health threat because of its ability to spread resistant genes to the environment, food, humans, animals, and other bacteria.

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