

## ANTIBIOTIC RESISTANCE IN *Escherichia coli* ISOLATED FROM FECES OF BALI CATTLE WITH REPRODUCTIVE DISORDERS

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**Abstract.** Antimicrobial resistance (AMR) has become a global issue in animal, human and environmental health. The AMR profile of *Escherichia coli* reflects the use of antibiotics in production animals. The purpose of this study was to determine the antibiotic resistance of *Escherichia coli* bacteria isolated from the feces of female Bali cattle with reproductive disorders. Feces samples were taken purposively using a swab on 4 rectums from 7 Bali cattle with reproductive disorders in June 2021 in Lando Village, East Lombok Regency. *Escherichia coli* samples were isolated on Eosin Methylene Blue Agar media and identified by biochemical tests. An antibiotic resistance test against *Escherichia coli* was carried out by the disk diffusion method. The antibiotics used in the test were Penicillin G 10 U, Oxytetracycline 30 g, Gentamicin 10 g, and Tetracycline 30 g, and Cefotaxime 30 g. The results of planting on Eosin Methylene Blue Agar media obtained 4 *Escherichia coli* which were successfully isolated from 4 samples of Bali cattle feces. Data on the level of *Escherichia coli* susceptibility level to various antibiotics were analyzed using the chi-square test. The results of the susceptibility test to antibiotics showed that 4 samples of *Escherichia coli* (100%) were resistant to Penicillin G, (25%) were resistant to Oxytetracycline, (25%) were resistant to Cefotaxime, and (100%) samples of *Escherichia coli* were sensitive to Gentamicin and Tetracycline. The chi-square test on the level of *Escherichia coli* susceptibility to various antibiotics was significant with  $p < 0.05$  ( $p$ -value = 0.012). The results of this study have shown that *Escherichia coli* from Bali cattle feces experience multidrug resistance which later on might have an impact on human health and the environment.

**Keywords:** antibiotic, Bali cattle, *Escherichia coli*, reproductio,

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### INTRODUCTION

Reproductive disorders in female cows are still the main obstacle in achieving the need for protein from animal origin. Bacterial

infection is one of the causes of reproductive disorders in cattle. This bacterial infection in female cows is mainly caused by repeated mating, metritis, and is related to the use of antibiotics. Unmanageable use of antibiotics

for reproductive disorders in female cows on cattle causes bacteria to mutate leading to antibacterial resistance. Antimicrobial resistance (AMR) has become a global issue in both animal and human health. AMR has killed about 700,000 people, and by 2050 this number is expected to increase to about 10 million deaths each year (O'Neill, 2016). One of the bacteria that cause antibiotic resistance that widely discussed is *Escherichia coli* as an indicator bacterium of animal origin.

*Escherichia coli* is used as an indicator because the AMR profile of *E. coli* almost reflects the use of antimicrobials in animals for food production (EFSA, 2011). Previous research data on the island of Lombok documented that *E. coli* had been isolated from Bali cattle with reproductive disorders in Central Lombok (Aminuddi et al., 2020). Gunawan et al. (2018) have reported that among 94.4% of *E. coli* were isolated from 20 samples of Bali cattle feces in smallholder farms in Central Lombok, 61% of *E. coli* samples were resistant to Erythromycin and Tetracycline, and 16.7% of *E. coli* samples were resistant to Ciprofloxacin. *E. coli* that cause multidrug resistance has been detected in 8.6% of 220 samples of cow feces at slaughterhouses in Bogor (Sudarwanto et al., 2016). *E. coli* which produces TEM-type Extended-spectrum  $\beta$ -lactamase (ESBL) and CTX-M gene have also been found in cattle feces and the environment in Peninsular Malaysia (Kamaruzzaman et al., 2020).

The island of Lombok, West Nusa Tenggara Province, is rich in traditional livestock groups that keep Bali cattle as beef cattle for meat production, which is still constrained by cases of reproductive disorders. Dibia et al. (2015) stated that the number of cases of reproductive disorders found was 2,127 cases, namely endometritis as many as 533 cases (25.1%), repeat breeders

530 cases (24.9%), ovarian hypofunction 517 cases (24.3%), silent heat 368 cases (17.3%), persistent corpus luteum 89 cases (4.2%), cystic follicles 72 cases (3.3%), and pyometra 18 cases (0.8%). Cases of high reproductive disorders certainly cannot be separated from the use of antibiotics in their treatment. Administration of antibiotics such as penicillin and oxytetracycline for 3 to 5 days has been done to reduce bacterial infections in cases of retained placenta (Yusuf, 2016). Intra-uterine administration of Gentamicin, Flumequine, and PGF $2\alpha$  in cases of endometritis has also been carried out (Melia et al., 2014). The future impact that poses a threat is the excretion of *E. coli* producing Extended-spectrum  $\beta$ -lactamase (ESBL) through feces due to uncontrolled use of antibiotics that can contaminate the environment, food ingredients that can interfere with human health and the environment. FAO (2016) states that the use of antimicrobial drugs in health care, agriculture, or industry creates selection pressures that can support the survival of resistant bacterial strains thereby increasing the presence of antimicrobial-resistant bacteria in their communities.

Based on the facts of cases of high reproductive disorders in Bali cattle in West Nusa Tenggara, research is needed to determine antibiotic resistance in *E. coli* bacteria in traditional livestock groups that keep Bali cattle as beef cattle as a reflection of the use of antimicrobials in animals and as initial data about the presence of *E. coli* producing antibiotic-resistant genes that can threaten human health and the environment.

## MATERIALS AND METHODS

This research was an observational descriptive with a cross-sectional study and was conducted in June 2021 at the Pade Angen

Livestock Farmer Group in Lando Village, East Lombok Regency. Feces samples were taken purposively using a swab on 4 rectums of Bali cattle from 7 female Bali cattle with reproductive disorders based on the inseminator report. Samples were put in Brain Heart Infusion (BHI) media and incubated for 24 hours at 37°C at the Health Testing and Calibration Laboratory of West Nusa Tenggara Province. The samples were then cultured on Eosin Methylene Blue Agar (EMBA) media and incubated for 24 hours at 37°C to grow *E. coli*. The growing *E. coli* were then identified by gram staining and biochemical tests before being tested for resistance. Assessment of the results of biochemical tests including Triple sugar iron (TSI), Sulfide Indole Motility (SIM), and fermentation of lactose, glucose, fructose & mannitol refers to Basic laboratory procedures in clinical bacteriology (Vandepitte et al., 2003)

*Escherichia coli* resistance to antibiotics was tested by disk diffusion on Mueller Hinton Agar using the Kirby Bauer method. *E. coli* colonies took from EMBA media and then put into a test tube containing 0.9% NaCl and homogenized to reach the Mc Farland standard of 0.5. The *E. coli* suspension which had reached the standard Mc Farland 0.5 was then swabbed on Mueller Hinton Agar media evenly and allowed to stand for 5 minutes and then 5 types of antibiotics were planted, including Penicillin G 10 U, Oxytetracycline 30 g, Gentamicin 10 g, and Tetracycline 30 g and Cefotaxime 30 g and incubated for 24 hours at 37°C. Penicillin and Cefotaxime antibiotics were used in this study because they are beta-lactam antibiotics, specifically, Cefotaxime is a third-generation cephalosporin antibiotic used as an indication of the presence of ESBL-producing bacteria. Oxytetracycline which is a tetracycline group was used because this antibiotic has been used in the treatment of large animals, either short-

acting or long-acting. Tetracycline was used as a comparison to Oxytetracycline because commonly used of tetracycline by humans. While Gentamicin was used because it has been used for reproductive disorders such as metritis in combination with Prostaglandin F2 alpha (PGF2 $\alpha$ ) (Melia et al., 2014). Susceptibility to antibiotics was carried out by measuring the diameter of the inhibition zone formed by using a caliper. Sensitive (S), intermediate (I), and resistant (R) assessments were determined by the size of the inhibition zone formed based on the Clinical and Laboratory Standards Institute standards (CLSI, 2012). *E. coli* isolate of American Type Culture Collection (ATCC) type 25922 was used as a control in resistance testing in this study.

The data from the identification of *E. coli* based on gram staining and biochemical tests, as well as the test of *E. coli* resistance to antibiotics, will be presented in the form of figures and tables that are compared with the value of antibiotic susceptibility to bacteria based on the Clinical and Laboratory Standards Institute standards (CLSI, 2012). Data on the level of susceptibility of *E. coli* to various antibiotics will be analyzed using the chi-square test.

## RESULTS AND DISCUSSION

The results of planting 4 fecal samples of female Bali cattle with reproductive disorders on Eosin Methylene Blue Agar (EMBA) obtained 4 samples of *E. coli* which were successfully isolated. Morphological images of *E. coli* in EMBA can be seen in Figure (1). *E. coli* colonies grown in Eosin Methylene Blue Agar (EMBA) media (Figure 1) showed that the growing *E. coli* was metallic green, mucoid in shape with a dark center. These results are similar to the results of the isolation of *E. coli* in Bali cattle feces on local

farms in Central Lombok Regency (Gunawan et al., 2018).

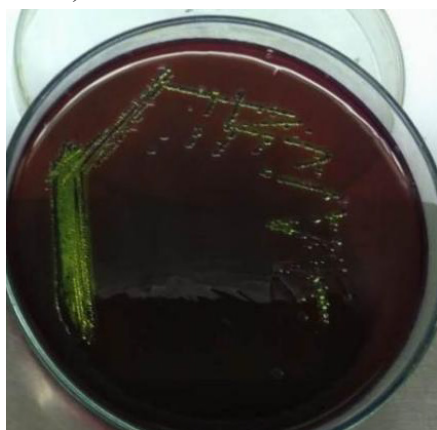


Figure 1. *Escherichia coli* colonies on Eosin Methylene Blue Agar (EMBA)

The same results were also shown in the study by Saputra et al. (2018) who succeeded in isolating *E. coli* from bat droppings on the island of Sumbawa, Nusa Tenggara Barat Province, Indonesia. This indicates that *E. coli* colonies derived from feces from both domestic and wild animals showed almost the same morphology on EMBA media with a metallic green color with a dark center.

The results of the gram staining of *E. coli* isolates from the feces of female Bali cattle with reproductive disorders are presented in (Figure 2).

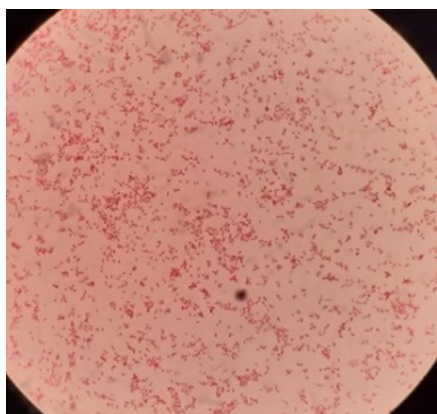


Figure 2. *Escherichia coli* Morphology by Gram Staining (1000x Magnification)

The results of the gram staining showed that *E. coli* has the shape of a short rod and is gram-negative (Figure 2). Cornelisse et al. (2013) stated that *E. coli* is a gram-negative bacterium, facultative anaerobic with morphology in the form of a short rod with a length of about 2  $\mu\text{m}$ , a diameter of 0.7  $\mu\text{m}$ , a width of 0.4-0.7  $\mu\text{m}$ . The results of the biochemical test of *E. coli* isolated from Bali cattle feces are presented in Table (1). The results of the biochemical test state that *E. coli* isolated from Bali cattle feces were catalase-negative and oxidase-negative, fermented glucose, lactose, sucrose, maltose, mannitol, and positive for Indole test. The *E. coli* also did not use citrate in Simon Citrate Agar. *E. coli* was also motile in Sulfide Indole Motility (SIM) media and produced acid and  $\text{H}_2\text{S}$  in *Triple Sugar Iron Agar* (TSIA).

This biochemical test results are almost the same as previous studies which stated that *E. coli* isolates from Bali cattle feces by fermenting Glucose, sucrose, Indole was positive and did not produce urea (Gunawan et al., 2018). The results are still in line with the characteristics of *E. coli* which produces gas during carbohydrate fermentation and indole shows positive results. Cornelissen et al. (2013) stated that *E. coli* produces acid and gas during carbohydrate fermentation. *E. coli* in methyl red and indole tests showed positive results and negative reactions for the Voges-Proskauer test and citrate, the results of these tests that distinguish them from other digestive tract bacteria (Cornelissen et al., 2013). This statement is corroborated by the research of Aminuddi et al. (2020) who documented that *E. coli* isolated from the reproductive tract of Bali cattle ferment carbohydrates and produce gas. *E. coli* with the same biochemical results have also been isolated from anal swabs in bats, where *E. coli* ferments glucose and lactose (Kholik et al., 2019).

The results of the susceptibility test of 4 samples of *Escherichia coli* bacteria from Bali cattle feces to antibiotics that are often used in livestock, namely Penicillin G 10 U, Oxytetracycline 30 g, Gentamicin 10 g, and Tetracycline 30 g, and Cefotaxime 30 g disc diffusion method on MHA (Mueller Hinton Agar) media are presented in Figure 3.

Figure 3 shows that all samples of *E. coli* from the feces of female Bali cattle with reproductive disorders did not form an inhibitory zone against Penicillin G, while for

Oxytetracycline, Gentamicin, and Tetracycline and Cefotaxime varied. Table 2 shows the results of the resistance test of 4 samples of *E. coli* that were isolated from the feces of Bali cattle with reproductive disorders. It was found that 4 samples of *Escherichia coli* (100%) were resistant to Penicillin G, (25%) were resistant to Oxytetracycline, (25%) resistance to Cefotaxime, and 4 (100%) *Escherichia coli* samples were sensitive to Gentamicin and Tetracycline.

Table 1. The Result of the Biochemical test of *Escherichia coli* from Bali cattle feces

Biochemical Test	Results
Catalase	+
Oxidase	-
Glucose Fermentation	+
Lactose Fermentation	+
Sucrose Fermentation	+
Maltose Fermentation	+
Mannitol Fermentation	+
Indole Test	+
Simon Citrate Agar	-
Sulfide Indole Motility (SIM)	Motil
Triple Sugar Iron Agar (TSIA)	Acid/Acid+ H <sub>2</sub> S

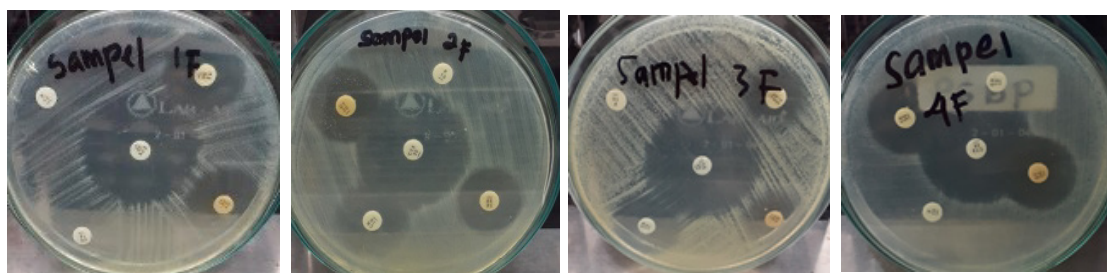


Figure 3. The results of the Susceptibility Test of *Escherichia coli* to MHA (Mueller Hinton Agar). Description: a= sample 1, b= sample 2, c= sample 3, d= sample 4; (black arrow) = zone of inhibition

Table 2. Diameter of inhibition zone of *Escherichia coli* isolates against various antibiotics

Diameter of Inhibition Zone of <i>Escherichia coli</i> Isolates Against Various Antibiotics					
Sample	Penicillin G (S >17, I= 14-16, R≤13)	Oxytetracycline (S>15, I=12-14, R≤11)	Gentamicin (S>15, I=13-14, R≤12)	Tetracycline (S>15, I=12-14, R≤11)	Cefotaxime (S>26, I= 23-25, R≤22)
1	0 R	25 S	25 S	25 S	30 S
2	0 R	0 R	25 S	20 S	30 S
3	0 R	20 S	28 S	18 S	20 R
4	0 R	25 S	18 S	24 S	36 S

Table 3. Category on *Escherichia coli* susceptibility level to various Antibiotics

<i>Escherichia coli</i>				
Type of Antibiotics	Susceptibility		Total	P-value
	Susceptible	Resistant		
Penicillin G	0	4	4	0.012
Oxytetracycline	3	1	4	
Gentamicin	4	0	4	
Tetracycline	4	0	4	
Cefotaxime	3	1	4	
Total	14	6	20	

Table 3 of chi-square analysis shows that the level of susceptibility of *E. coli* to antibiotics is strongly related to the type of antibiotic with  $p < 0.05$  ( $p$ -value = 0.012). The results showed that *E. coli* was resistant to Penicillin G, Oxytetracycline, and Cefotaxime with Penicillin G having the highest resistance value. These results are in line with Mustika et al. (2015) who stated that 100 % of *E. coli* from the feces of Bali cattle at South Kuta district Badung Bali samples were resistant to penicillin G.

*Escherichia coli* in this research was resistant to Penicillin G because Penicillin G is a class of antibiotics beta-lactams. It is well-known that Gram-negative bacteria such as *E. coli* have beta-lactamase enzymes, which capable of inactivating beta-lactam antibiotics (Bradford, 2001). *E. coli* resistance to penicillin can be also caused by the frequent use of Penicillin in the treatment of cattle so

that the bacteria have adapted. This statement is supported by Siswanto & Sulabda, (2018) which stated that the residue of Penicillin G in Bali beef is 1.66% of the 60 samples collected from five markets throughout Bali. *E. coli* in this research is also resistant to Oxytetracycline because these antibiotics have been used since ancient times for various cases of a disease in livestock. Reig & Toldra (2008) stated that the type of antibiotic that is most often found in livestock products, especially meat is Tetracycline antibiotics including Oxytetracycline.

This fact make it possible that *E. coli* has been exposed to antibiotics such as Penicillin G and Oxytetracycline so this bacteria adapted, by changing metabolic pathway that can fight antibiotics or by producing enzymes that can inhibit the action of antibiotics. Van Hoek et al. (2011) stated that bacterial resistance to antibiotics can occur by enzymatic

modification or the bacteria has an alternative metabolic pathway for antimicrobials.

*Escherichia coli* in this research is also resistant to Cefotaxime. Cefotaxime is a third-generation cephalosporin antibiotic of antibiotics beta-lactams class. In this case, *E. coli* can produce the Extended-spectrum  $\beta$ -lactamase (ESBL) enzyme to inactivating beta-lactam antibiotics such as Cefotaxime. Extended-spectrum beta-lactamase is an enzyme produced by gram-negative bacteria of the Enterobacteriaceae family including *E. coli* and has increased activity in hydrolyzing  $\beta$ -lactam antibiotics, especially oxyimino-cephalosporins (Bradford, 2001).

*Escherichia coli* samples that were isolated from the feces of Bali cattle with reproductive disorders in Lando Village, East Lombok Regency showed multidrug resistance with resistance to Penicillin G, Oxytetracycline, and Cefotaxime. Bacteria that are resistant to Cefotaxime can be used as an indication of the production of the Extended-spectrum  $\beta$ -lactamase (ESBL) enzyme. *E. coli* with multidrug resistance occurs due to the adaptation of bacteria to antibiotic stress by modifying the production of  $\beta$ -lactamase enzymes. Enzyme modification has been proven by the emergence of Extended-spectrum  $\beta$ -lactamase (ESBL) produced by *E. coli* which causes multidrug resistance which has been detected in 8.6% of 220 samples of cow feces at slaughterhouses in Bogor (Sudarwanto et al., 2016).

*Escherichia coli* with multidrug resistance can occur because the gene encoding the Extended-spectrum  $\beta$ -lactamase (ESBL) produced by *E. coli* can be transferred vertically or horizontally. Vertical transfer can occur in bacterial offspring when they propagate, while horizontally there can be an exchange of plasmids carrying these genes in the bacterial community, either in

the reproductive tract or the environment (Colodner & Raz, 2005). The resistant *E. coli* is of course very easy to be excreted through feces into the environment around people's farms. Farmers who lack knowledge about sanitation management will trigger bacterial contamination in the environment which will endanger the health of farmers and the environment.

This study has succeeded in isolating *E. coli* from 4 fecal samples of female Bali cattle with reproductive disorders in Lando Village, East Lombok Regency. The results of this study have shown that *E. coli* from Bali cattle feces has experienced multidrug resistance to the antibiotics Penicillin G, Oxytetracycline, and Cefotaxime and indicates the presence of *E. coli* which produces *Extended-spectrum  $\beta$ -lactamase* (ESBL) enzymes that can harm human health and the environment.

Based on the results of this study, it can be concluded that *E. coli* isolated from 4 fecal samples of female Bali cattle with reproductive disorders had resistance to the antibiotics Penicillin G, Oxytetracycline, and Cefotaxime and indicated the presence of *E. coli* which produces the Extended-spectrum  $\beta$ -lactamase (ESBL) enzyme which can endanger human health and the environment. The research on *E. coli* encoding antibiotic resistance genes with one health approach is very necessary to anticipate the spread of these bacteria to humans and the environment.

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