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DETECTION OF ANTIBIOTIC RESISTANT IN Escherichia coli FROM THE REPRODUCTIVE TRACT OF BALI CATTLE ON SMALLHOLDER FARM

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Abstract

Escherichia coli originating from animals, humans, and the environment can spread antibiotic-resistant genes and can encourage antimicrobial resistance (AMR) which is a global health problem. The purpose of this study was to detect the presence of *Escherichia coli* which is resistant to several antibiotics from the reproductive tract of Bali cattle on smallholder farms on the island of Lombok. This research is a cross-sectional study conducted from March to June 2021 using 8 female Bali cattle that experience reproductive disorders on community farms in Lando Village, East Lombok Regency to collect their reproductive tract fluids using an artificial insemination plastic sheet gun. Reproductive fluid samples are placed on BHIB (Brain Heart Infusion Broth) medium. *Escherichia* coli cultures were carried out on Eosin Methylene Blue Agar (EMBA) and identified by Gram staining and biochemical tests conducted at the Public Health and Calibration Laboratory, West Nusa Tenggara Province. Antibiotic sensitivity test on isolated *Escherichia coli* was carried out by disc diffusion method using 5 antibiotics including Penicillin G 10U, Oxytetracycline 30 µg, Gentamicin 10 µg, Tetracycline 30 µg, and Cefotaxime 30 µg. The results showed that 2 (two) 25% *Escherichia coli* bacteria were successfully cultured on EMBA media and isolated from 8 samples of the reproductive fluids of Bali cattle that were collected. The results of the *Escherichia coli* sensitivity test to antibiotics found that *Escherichia coli* samples were 100% resistant to Penicillin G, 100% resistant to Oxytetracycline, 100% resistant to Gentamicin, and 50% resistant to Tetracycline, and 100% resistant to Cefotaxime from 2 isolated *Escherichia coli*. This explains that Bali cattle in community farms have the potential to spread *Escherichia coli* which has an impact on the emergence of AMR.

***Keywords**: Bali cattle, Escherichia coli, reproductive tract, resistance, smallholder farming*

Abstrak

Escherichia coli yang berasal dari hewan, manusia, dan lingkungan telah dinyatakan dapat menyebarkan gen resisten terhadap antibiotik dan dapat mendorong kejadian antimicrobial resistance (AMR) yang menjadi masalah kesehatan global. Tujuan penelitian ini adalah untuk mendeteksi adanya Escherichia coli yang resisten terhadap beberapa antibiotik dari saluran reproduksi Sapi Bali pada peternakan rakyat di Pulau Lombok. Penelitian ini merupakan studi cross-sectional yang dilakukan pada Bulan Maret sampai Juni 2021 dengan menggunakan 8 ekor sapi Bali betina yang mengalami gangguan reproduksi pada peternakan rakyat di Desa Lando Kabupaten Lombok Timur untuk diambil cairan saluran reproduksinya menggunakan plastic sheet gun inseminasi buatan. Sampel cairan reproduksi diletakkan pada medium BHIB (Brain Heart Infusion Broth). Kultur Escherichia coli dilakukan pada Eosin Methylene Blue Agar (EMBA) dan diidentifikasi dengan pewarnaan Gram dan uji biokimia yang dilakukan di Balai Laboratorium Kesehatan Masyarakat dan Kalibrasi Provinsi Nusa Tenggara Barat. Uji kepekaan terhadap antibiotik pada Escherichia coli yang diisolasi dilakukan dengan metode difusi cakram menggunakan 5 antibiotik antara lain Penicillin G 10U, *Oxytetracycline* 30 µg, *Gentamicin*10 µg, *Tetracycline* 30 µg, *dan Cefotaxime* 30 µg. *Hasil penelitian mendapatkan 2 (dua) 25% bakteri Escherichia coli berhasil dikultur pada media EMBA dan diisolasi dari 8 sampel cairan reproduksi sapi Bali yang dikoleksi. Hasil uji kepekaan Escherichia coli terhadap antibiotik didapatkan bahwa sampel Escherichia coli 100% resisten terhadap Penicillin G, 100% resisten terhadap Oxytetracycline, 100% resisten terhadap Gentamicin, dan 50% resisten terhadap Tetracycline, serta 100% resisten terhadap Cefotaxime dari 2 Escherichia coli yang diisolasi. Hal ini menjelaskan bahwa sapi Bali di peternakan rakyat dapat berpotensi dalam penyebaran Escherichia coli yang berdampak pada timbulnya AMR.*

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***Kata Kunci**: Escherichia coli, resistensi, saluran reproduksi, sapi bali, peternakan rakyat*

1. INTRODUCTION

The province of West Nusa Tenggara (NTB), which consists of the islands of Lombok and Sumbawa, is one of the provinces rich in smallholder farms and a national supplier of beef cattle. Statistics of Nusa Tenggara Barat Province data for Nusa Tenggara Barat Province Province until 2019 stated that the total cattle population in Nusa Tenggara Barat Province was 1,234,357 heads (BPS, 2020). The problem that is often found in smallholder farms is cases of reproductive disorders which are treated using antibiotics that can potentially cause antimicrobial resistance (AMR). Dibia et al. (2015) reported that 2,127 cases of reproductive disorders were reported in Bali cattle in West Nusa Tenggara. Aminuddi et al. (2020) also found that *Escherichia coli* from Bali cattle experienced reproductive disorders on Lombok Island, while FAO (2016) stated that the intensive use of antibiotics in livestock and humans can encourage antimicrobial resistance (AMR). One of the things that can encourage the spread of AMR cases is the presence of *Escherichia coli* bacteria that are resistant to various antibiotics which can spread antibiotic-resistant genes to humans, animals, and the environment.

Several studies in Indonesia and abroad have found *Escherichia coli* that is resistant to several antibiotics and has the potential to produce Extended-spectrum β-lactamase (ESBL) in cattle. Kholik et al. (2021) isolated *Escherichia coli* which was resistant to β-lactam class antibiotics such as Penicillin G, Cefotaxime, and Oxytetracycline from Bali cattle feces on community farms on Lombok Island. Another study stated that 94.4% of Escherichia coli isolates were resistant to Erythromycin, 61.1% resistant to Tetracycline, and 16.7% resistant to Ciprofloxacin from 20 *Escherichia coli* isolated from Bali cattle feces. *Escherichia coli* which encodes the ESBL gene has also been isolated in 8.6% of 220 samples of cow feces at abattoirs in Bogor (Sudarwanto et al., 2016). *Escherichia coli*-producing ESBLs encoding the TEM and CTX-M genes have been found in cow feces and the environment in Peninsular Malaysia (Kamaruzzaman et al., 2020). *Escherichia coli*-producing ESBLs encoding CTX-M (Cefotaxime) gen was reported from Thailand at 65.7% which was identified from 417 stool samples obtained from adults living in rural areas (Luvsansharav et al. 2012).

Data stating that there are high cases of reproductive disorders in Bali cattle reared by smallholder farms if treated with poorly controlled antibiotics will cause the spread of *Escherichia coli* encoding Extended-spectrum β-lactamase (ESBL) to animals and humans. When the *Escherichia coli* which produces ESBLs from the cow's reproductive tract is excreted, the resistance gene from *Escherichia coli* has the potential to be disseminated into the environment and transfer the resistance gene to other bacteria. FAO (2016) states that the selection of antibiotics for treatment in animals may not necessarily solve the problem of antimicrobial resistance (AMR) because resistance genes can move between bacteria, hosts, and the environment and can mutate spontaneously, so a focus on biosecurity and food hygiene is important in reducing the spread of *Escherichia coli* bacteria encoding resistant genes in animals, the environment and humans.

Based on research data which states *Escherichia coli* which encodes the ESBL gene can spread to humans and the environment, it is necessary to research the detection of antibiotic-resistant *Escherichia coli* that has the potential to encode Extended-spectrum β-lactamase (ESBL) gene from the reproductive tract of Bali cattle in smallholder farms. The results of this study can be used as a reference in

anticipating the spread of antibiotic-resistant *Escherichia coli* in humans and the environment, given that antimicrobial resistance (AMR) has caused the death of around 700,000 people, and by 2050 this number is expected to increase to around 10 million deaths every year (O'Neill, 2016).

2. RESEARCH METHOD

2.1 Design Study

This research is a cross-sectional study. The study referred to in this research is a descriptive observational study regarding the detection of antibiotic-resistant *Escherichia coli* in the reproductive tract of Bali cattle. The research was conducted from March to June 2021 in the Pade Angen smallholder farm which is located in Lando Village, Terara District, East Lombok Regency, Nusa Tenggara Barat Province.

The samples in this study were 8 female Bali cattle in the stables of the Pade Angen smallholder farm in collaboration with the Faculty of Veterinary Medicine, Mandalika University of Education. Reproductive fluid samples were taken from 8 female Bali cattle with reproductive disorders based on reports from inseminators, alumni, and heads of livestock herds which were confirmed by rectal palpation.

Sampling was carried out by purposive sampling based on the criteria of having been inseminated or inseminated 3 times or more, there was no pregnancy with a normal estrus cycle, and had undergone antibiotics and after being confirmed by rectal palpation there were no abnormalities in the reproductive organs. These criteria are based on the definition of repeated mating which states that cows that have normal menstrual cycles and periods have been mated 2 or more times with fertile males or inseminated with fertile male semen but are still not pregnant (Wodaje and Mekuria, 2016).

2.2 Sample Collection

Samples of reproductive fluids of Bali cattle with reproductive disorders based on reports from breeders and artificial insemination officers as well as confirmation of examination by researchers. The reproductive fluids of Bali cattle were carried out in a sterile manner using an artificial insemination gun covered with a plastic sheet that was inserted into the reproductive tract of Bali cattle.

The tip of the plastic sheet was cut by 2-3 cm and immediately put in the BHIB (Brain Heart Infusion Broth) enrichment medium and taken to the Public Health Laboratory and Calibration Center for Lombok Island, Nusa Tenggara Barat Province using a cooling box. Fluid collection in the reproductive tract of Bali cattle is based on research conducted by Andriani et al. (2021).

2.3 Isolation and Identification of Bacteria

Isolation bacteria of reproductive fluid of Bali cattle from BHIB (Brain Heart Infusion Broth) were streaked onto Eosin Methylene Blue Agar (EMBA) after BHIB were incubated for 24 hours in an incubator at 37°C. The samples were streaked onto Eosin Methylene Blue Agar (EMBA) and incubated for 24 hours. Pure colonies of growing bacteria were gram-stained and subjected to biochemical tests to identify *Escherichia coli* identified by biochemical tests according to the Bergey's manual of determinative bacteriology (Holt et al. 1994).

Identification of bacteria in the reproductive fluids of Bali cattle was carried out by gram staining and biochemical tests. Gram staining was carried out by taking bacteria colonies with oose, then examining them on slides and fixing them with Bunsen. The fixed bacterial slides were dripped with crystal violet dye and allowed to stand for 1 minute, then washed with water and dried.

The bacterial smear slide was then dripped with Lugol's iodine solution and left for 1 minute and washed with water, then given 96% alcohol for 10-20 seconds, then washed with water, and then dried. After drying, the bacterial slides were given a safranin dye solution for 1 minute and washed with water and dried, and observed under a microscope. Gram-positive bacteria appear purplish-blue and Gram-negative are red (Vandepitte et al., 2003). The biochemical tests of bacteria carried out included: catalase, glucose, sorbitol, arabinose, lactose, sucrose, mannitol, urea, maltose, triple sugar iron agar (TSIA), sulfide indole motility (SIM), indole, and citrate which refer to Bergey's manual of determinative bacteriology (Holt et al., 1994). The control isolate used in the test was *Escherichia coli* ATCC 25922

2.4 Antimicrobial Susceptibility Test

The susceptibility test of *Escherichia coli* to antibiotics using the Kirby-Bauer disk diffusion test. The susceptibility test was carried out by taking *Escherichia coli* bacteria colonies on EMBA and then putting them into a test tube containing 0.9% NaCl and homogenizing using a vortex to reach the McFarland standard of 0.5. The *Escherichia coli* suspension which had reached the Mc Farland standard of 0.5 was then swabbed evenly on MHA (Mueller Hinton Agar) media and allowed to stand for 5 minutes and then planted with 5 types of antibiotics Penicillin 10 U, Oxytetracycline 30 µg, Gentamicin 10 µg, and Tetracycline 30 µg and Cefotaxime (CTX) 30 µg and incubated for 24 hours at 37°C.

Penicillin and Cefotaxime antibiotics were used in this study because these antibiotics belong to the beta-lactam class of antibiotics, specifically for Cefotaxime which is a third-generation cephalosporin class of antibiotics that is used as an indication of the presence of ESBL-producing bacteria. Oxytetracycline which is a tetracycline group was used in this study because this antibiotic has been used in the treatment of large animals either the form of short-acting or long-acting, Tetracycline was used in this study as a comparison to Oxytetracycline because tetracycline is commonly used in humans. Gentamicin was used in this study because Gentamicin has been used for reproductive disorders such as metritis in combination with Prostaglandin F2 alpha (PGF2α) (Melia et al., 2014).

The susceptibility of *Escherichia coli* bacteria to antibiotics interpretation was determined by measuring the diameter of the inhibition zone formed using a caliper. susceptible (S), intermediate (I), and resistant (R) ratings were determined by the size of the inhibition zone formed based on the Clinical and Laboratory Standards Institute standards (CLSI, 2014).

3. RESULT

This study has succeeded in collecting 8 (eight) fluid samples from the reproductive tract of female Bali cattle with reproductive disorders which were taken using an artificial insemination gun (IB) coated with a plastic sheet. All fluid samples from the reproductive tract were cultured on Eosin Methylene Blue Agar (EMBA) for bacteria isolation. Identification of the presence of *Escherichia coli* was carried out by gram staining and biochemical tests. Isolation and Identification of *Escherichia coli* were carried out at the Public Health and Calibration Laboratory of Lombok Island, Nusa Tenggara Barat Province.

Bacterial culture results from 8 (eight) fluid samples from the reproductive tract of Bali cattle on Eosin Methylene Blue Agar (EMBA) obtained 2 (25%) samples showing the characteristics of *Escherichia coli* colonies. The colonies of *Escherichia coli* on Methylene Blue Agar (EMBA) media can be seen in Figure 1.

Escherichia coli colonies in EMBA media in Figure 1 show that *Escherichia coli* growing is metallic green, shiny with a dark center. *Escherichia coli* culture results on

EMBA media, then stained with Gram staining. *Escherichia coli* morphology by Gram staining can be seen in Figure 2.

The results of the gram stain of *Escherichia coli* bacteria under microscopic observations in Figure 2 show that *Escherichia coli* is pink in color, gram-negative, and has a short rod shape.

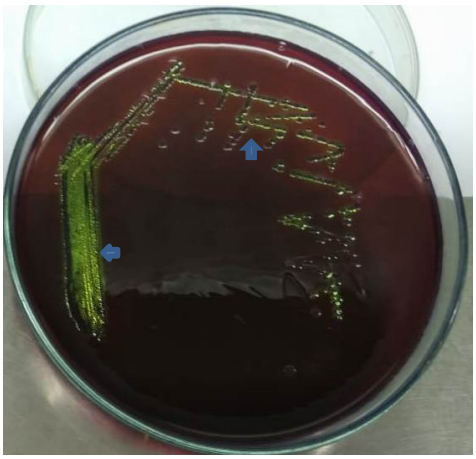

Figure 1. *Escherichia coli* colonies in Eosin Methylene Blue Agar (head arrow).

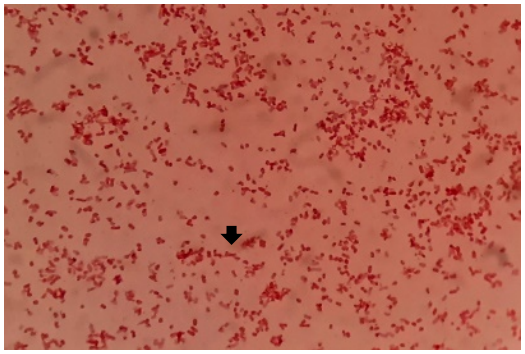

Figure 2. Morphology of *Escherichia coli* by gram staining (head arrow).

Two *Escherichia coli* from the reproductive tract fluids of Bali cattle which were successfully cultured on Eosin Methylene Blue Agar (EMBA) were then subjected to biochemical tests which included catalase and oxidase tests and sugar tests for identification. The results of biochemical tests of *Escherichia coli* from the reproductive fluids of Bali cattle with reproductive disorders can be seen in Table 1.

The biochemical test results in Table 1 show that the two *Escherichia coli* isolated from the reproductive fluids of Bali cattle had positive catalase test results, while the oxidase test was negative. The Escherichia coli showed positive indole tests and positive Sulfide Indole Motility (SIM), fermenting sugars (Maltose, Glucose, Lactose, and Mannitol). The isolated *Escherichia coli* was also able to hydrolyze urea with a positive urea test result. *Escherichia coli* in this study did not use citrate to produce carbon, as shown by the negative Cimon Citrate test results.

Table 1. Biochemical test results of *Escherichia coli* isolates

Biochemical test	Interpretation
Catalase	+
Oksidase	-
Glucose	+
Lactose	+
Sucrose	+
Maltose	+
Mannitol	+
Indole	+
Sorbitol	+
Sulfide indole motility (SIM)	+
Urea	+
Cimon Citrate	-
Tripel Sugar Iron Agar (TSIA)	Acid/Acid (+) gas

Antibiotic susceptibility test results on 5 antibiotics (Penicillin, Oxytetracycline, Gentamicin, Tetracycline, and Cefotaxime (CTX)). Penicillin and Cefotaxime were used in this study because these antibiotics belong to the beta-lactam group, where Cefotaxime is a cephalosporin which is the 3rd generation of beta-lactam antibiotic that can cause bacteria to produce Extended-spectrum β-lactamase (ESBL). Oxytetracycline, Gentamicin, and Tetracycline were used in this study because these antibiotics are often used in animal husbandry.

The results of the susceptibility test of five *Escherichia coli* bacterial isolates from the reproductive tract fluids of Bali to Penicillin 10 U, Oxytetracycline 30 µg, Gentamicin 10 µg, and Tetracycline 30 µg and added with Cefotaxime (CTX) 30 µg as an indication of the presence of bacteria can produce Extended-spectrum β-lactamase (ESBL) using the disc diffusion method on MHA (Mueller Hinton Agar) media can be seen in Figure 3.

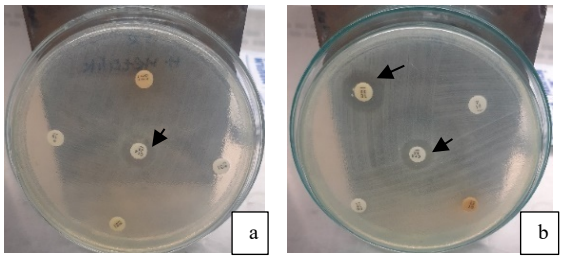

Figure 3. Antibiotic susceptibility test of *Escherichia coli* with disc diffusion method: (a= sample 1, b= Sample 2; (←) = inhibition zone).

Figure 3 states that all *Escherichia coli* samples (samples 1 and 2) appeared to be resistant to the antibiotics Penicillin G 10 U, Oxytetracycline 30 µg, and Gentamicin 10 µg with no inhibition zones formed around the antibiotic discs, whereas for Tetracycline 30 µg and Cefotaxime (CTX) 30 µg they still produced inhibition zone. The Susceptibility to Penicillin G, Oxytetracycline, Gentamicin, Tetracycline, and Cefotaxime (CTX) of *Escherichia coli* Isolates by measuring the inhibition zone formed around the antibiotic disc and its interpretation can be seen in Table 2.

Table 2. The susceptibility to antibiotics of *Escherichia coli* isolates

Antibiotik	Sample 1 (mm)	Sample 2 (mm)
Cefotaxime 30 µg	10 (R)	12 (R)
Penicillin G 10 U	0 (R)	0 (R)
Oxytetracycline 30 µg	0 (R)	0 (R)
Gentamicin 10 µg	0 (R)	0 (R)
Tetracycline 30 µg	0 (R)	12 (I)

S=susceptible, I= Intermediate, R= Resistant

Table 2 shows that *Escherichia coli* was 100% resistant to Penicillin G, 100% resistant to Oxytetracycline, 100% resistant to Gentamicin, and 50% resistant to Tetracycline (one sample was intermediate), and 100% resistant to Cefotaxime from 2 samples of *Escherichia coli* successfully isolated.

4. DISCUSSION

The results of bacterial culture on Eosin Methylene Blue Agar (EMBA) found only 2 bacterial isolates showing the characteristics of *Escherichia coli* from 8 samples of the reproductive fluid of female Bali cattle with reproductive disorders. This is due to the possibility of the reproductive tract being contaminated with *Escherichia coli*. Contamination of the reproductive tract by *Escherichia coli* can be caused by poor postpartum handling or unhygienic handling in cases of dystocia and retained placenta. Contamination can also occur during the implementation of artificial Insemination which is less aseptic or also due to poor sanitation management. Noakes et al. (2001) stated that a bad environment, especially during the postpartum period, will facilitate the entry of microbes into the uterine lumen, disrupting embryonic life which can lead to early embryonic death.

The results of observations of *Escherichia coli* colonies in EMBA media showed that the colonies were metallic green, and shiny with a dark center. The metallic green color occurs due to a reaction

between bacteria and Methylene blue. This colony morphology agrees with Cornelissen et al. (2013) who stated that *Escherichia coli* on Eosin Methylene Blue Agar (EMBA) medium was shiny metallic green like metal.

The results of microscopic observation of the morphology of *Escherichia coli* showed that *Escherichia coli* is pink in color and has a short rod shape because *Escherichia coli* is a gram-negative bacterium that has thinner walls, these bacteria are unable to bind crystal violet color and are only stained by safranin because ribonucleic acids in the cytoplasm of gram-negative cells do not form stronger bonds with the crystal violet complex, so they are only stained with safranin. Gram-negative bacteria are marked with a pink color indicating that these bacteria are unable to bind crystal violet color and are only stained by safranin.

The biochemical test results of *Escherichia coli* that were successfully isolated in this study produced positive and motile indole tests on Sulfide Indole Motility (SIM) media, as well as fermenting sugars, and showed positive catalase tests and negative oxidase tests. The results of this biochemical test are in line with the statement which states that *Escherichia coli* in the indole test showed positive results and a negative reaction for the citrate test. Negative indole and catalase test results can be used as a basis for distinguishing *Escherichia coli* from other digestive tract bacteria (Cornelissen et al. 2013).

The results of the research on the sensitivity test of *Escherichia coli* to antibiotics from the reproductive tract of Bali cattle found that 2 (100%) *Escherichia* coli were resistant to Penicillin G, 2 (100%) *Escherichia coli* were resistant to Oxytetracycline, 2 (100%) *Escherichia coli* were resistant to Gentamicin, 2 (100%) *Escherichia coli* to Cefotaxime (CTX) resistant, and one (25%) *Escherichia coli resistant* to Tetracycline from 2 *Escherichia coli* isolated.

This fact can be assumed that resistance to *Escherichia coli* has occurred to various antibiotics that have been used in Bali cattle farms on Lombok Island, including those with reproductive disorders. Poorly controlled administration of antibiotics will cause Escherichia coli bacteria to adapt and mutate so that they will produce resistance genes. These results are in line with statements about the AMR profile of *Escherichia coli* almost reflecting the use of antimicrobials in animal production (EFSA, 2011). This fact is reinforced by research which states that *Escherichia coli* isolated from Bali cattle in community farms on Lombok Island is resistant to Erythromycin, Tetracycline, and Ciprofloxacin (Gunawan et al., 2018).

The results regarding *Escherichia coli* that are resistant to non-beta-lactam antibiotics such as the Tetracycline and Gentamicin groups are because this class of antibiotics is often used to treat reproductive disorders. Resistance to Tetracycline and Gentamicin can also be due to the transfer of resistant genes between bacteria in the bacterial microenvironment. One mechanism for the occurrence of resistance occurs due to changes in enzymatic production due to mutations in the coding genes in *Escherichia coli*, while these genes can be transferred in the bacterial microenvironment.

The results regarding *Escherichia coli* that are resistant to beta-lactam class antibiotics such as the Penicillin and Cefotaxzime groups, this may occur because Penicillin is often used which results in gene mutations, resulting in resistant gene coding that produces Extended Spectrum Beta Lactamases (ESBLs). This is in line with the statement which states that Penicillin, which is a β-lactam antibiotic class, is one of the most frequently used antibiotics in animal husbandry and has caused resistance genes (Huang, L. et al., 2019; Qian, X. et al., 2019). Research data shows that *Escherichia coli* encoding the CTX-M-1 and CTX-M-9 genes

can cause multidrug resistance (Sudarwanto et al. 2016).

Escherichia coli that produce Extended-spectrum β-lactamases (ESBLs) with TEM and CTX-M encoding genes have also been found in cow feces and the environment in Peninsular Malaysia (Kamaruzzaman et al. 2020). Extended-spectrum β-lactamases (ESBLs) are enzymes that can be produced by *Escherichia coli* and other gram-negative bacteria that function to hydrolyze β-lactam class antibiotics, especially in oxyimino-cephalosporins (Bradford, 2001). *Escherichia coli* which can produce Extended-spectrum β-lactamases (ESBLs) will potentially increase cases of AMR in animals and humans because AMR coding genes can be spread horizontally (horizontal gene transfer) to other bacteria in their microenvironment (Le Roux et al. 2018).

5. CONCLUSIONS AND SUGGESTIONS

Based on the results of a study on the detection of Escherichia coli which was resistant to several antibiotics from the reproductive tract of Bali cattle in smallholder farms on Lombok Island, it was found that 25% of Escherichia coli bacteria had been isolated from 8 samples of the reproductive fluid of Bali cattle that were collected. The two isolated *Escherichia coli* bacteria were detected to be resistant to the antibiotics Penicillin, Oxytetracycline, Gentamicin, and Cefotaxime, and only one (25%) *Escherichia coli* was resistant to Tetracycline.

Phenotypic and genotypic research of *Escherichia coli* that produce Extended-spectrum β-lactamases (ESBLs) is urgently needed because it can have an impact on the emergence of antimicrobial resistance (AMR) in both animals and humans.

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