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Original article

Antibiotic sensitivity test of *Escherichia coli* and *Staphylococcus aureus* isolated from the reproductive tract of dairy cows

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ABSTRACT

The purpose of this study was to determine the sensitivity of *Staphylococcus aureus* and *Escherichia coli* isolated from the reproductive tract of dairy cows during estrus to tetracycline, gentamicin, amoxicillin, and ciprofloxacin. Isolation and identification of *E. coli* and *S. aureus* from 24 cervical mucus samples resulted 15 samples containing *E. coli* and one sample containing *S. aureus*. Sensitivity test was performed using Kirby-Bauer (disc diffusion) method and interpreted using the Clinical and Laboratory Standards Institute (CLSI, 2017) guidelines. The sensitivity test of 15 isolates of *E. coli* showed 100% sensitivity to ciprofloxacin and tetracycline. For gentamicin, 14 (93.3%) isolates were sensitive, and 1 (6.7%) isolate was resistant, while for amoxicillin, 14 (93.3%) isolates were sensitive, and 1 (6.7%) isolate were intermediate. The sensitivity test of one isolate of *S. aureus* showed 100% sensitivity to ciprofloxacin, gentamicin, and amoxicillin; as for tetracycline 100% isolates were declared resistant.

Keywords: antibiotic sensitivity, dairy cattle, reproduction tract

INTRODUCTION

Dairy cows were the main type of milk-producing livestock to meet milk needs, so that their maintenance, was always directed at increasing milk production (Buckley *et al*., 2014). Efforts to increase milk production could be done by increasing the population of dairy cows, improving management, and reproductive efficiency (Prendiville *et al*., 2011). Reproductive efficiency was one of the most important factors affecting cattle farming, where good reproductive conditions were attempted to get one calf every year (Rosadi *et al*., 2018). Reproductive efficiency in dairy cows was challenged when there was reproductive disorders caused by infectious agents (Kustanti, 2016). Reproductive disorders were caused by infectious diseases caused by microorganisms, for example fungi, bacteria and viruses (Campos *et al*., 2020). Reproductive tract infections by bacteria could be caused by specific and non-specific bacteria (Compton *et al*., 2017). Non-specific bacteria were normal bacteria that exist in nature and can enter the reproductive tract of cows, these bacteria would become pathogenic if there were wounds that could cause inflammation (Molina and Lucy, 2018). The

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non-specific bacteria most commonly involved were *Escherichia coli*, *Staphylococcus*, *Streptococcus*, *Corynebacterium*, *Bacillus*, *Pseudomonas*, *Micrococcus and Klebsiella* (Bhat *et al*., 2014). The problems that arised due to non-specific bacterial infections in the reproductive tract were a decrease in the calving rate, decreased body weight, and the occurrence of repeat breeding, pyometra (chronic endometritis), and infertility, resulting in declined reproductive efficiency (Amin *et al*., 2021). The results of the study of Sharma *et al* (2009) showed that there were *E. coli* and *S. aureus* bacteria from the cervical mucus of repeat breeder cows.

The bacteria found in the reproductive tract were most likely contaminants from the environment (*Bacillus spp*.), cowhide (*Staphylococcus spp*.) or fecal contamination (*E. coli*), which did not replace the normal flora in the reproductive tract (Pohl *et al*., 2015). Recent follow-up studies stated that, bacteria could also move from the intestines to the uterus (endogenous pathways), which were also associated with reproductive tract disorders (Appiah *et al*., 2020). The use of antibiotics was a choice step in carrying out the treatment (drug of choice) in cases of infections caused by bacteria (Ribeiro *et al*., 2016). The occurrence of antibiotic resistance was caused by the use of antibiotics that were not at the right dosage for treatment in animals (Agga *et al*., 2015). When antibiotics were used appropriately, they could provide unquestionable benefits, but when used or prescribed improperly (irrational prescribing) it could cause widespread losses in terms of health, economy and even for future generations (Utami, 2011).

Antibiotics used in this study were tetracycline, gentamicin, amoxicilin, and ciprofloxacin. These antibiotics were broad-spectrum antibiotics and were often used in farms (Kaur *et al*., 2011). Ciprofloxacin and gentamicin were considered effective in the treatment of infections in the reproductive tract of cattle (Öztürkler and Ucar, 2006). Amoxicilin and tetracycline were antibiotics that have been used quite frequently in Danamulya Agribusiness Cooperative. Based on this information, this study was conducted to determine the sensitivity of *S. aureus* and *E. coli* isolated from cows reproductive tract to tetracycline, gentamicin, amoxicillin, and ciprofloxacin.

MATERIALS AND METHODS

Sensitivity test was carried out using mucus samples of dairy cows in Danamulya Agribusiness Cooperative area, Pacet District, Mojokerto, East Java, Indonesia. A total of twenty-four cervical mucus sample was collected during estrus. Samples were collected using an insemination gun with a plastic sheath and a protective sleeve. The plastic sheath that has cervical mucus was cut at the end of the approximately 2cm and put into a tube prefilled with PBS and stored in a cool box.

Isolation and identification

Bacterial samples obtained was isolated in selective media Eosin Methylene Blue Agar (EMBA) and Mannitol Salt Agar (MSA) using streak plate method. EMBA encourage growth of coliforms and inhibit growth of Gram-positive bacteria, while MSA was selective for staphylococci. After incubation at 37^{o}C for 24 hours, bacteria from colonies grew in each media were Gram stained (Leininger *et al*., 2001) to distinguish between Gram-positive and Gram-negative cells and also to determine cell morphology, size, and arrangement.

For further identification of *E. coli*, identification was performed by means of indole, Methyl Red-Voges-Proskauer (MR-VP), citrate, and Triple Sugar Iron agar (TSIA) tests; while for *S. aureus*, catalase and coagulase tests were performed.

The biochemical tests of indole, MR-VP, and citrate was a test series referred to as the IMViC, and together with TSIA were microbiological tests used to distinguishes between members of the family *Enterobacteriaceae* and differentiates them from other Gram-negative rods based on their metabolic by-products.

The indole test served as an identification of the ability of bacteria to produce indole using the enzyme tryptophanase. This test was carried out by inoculating metallic green colonies selectively grown on EMBA media, that were Gram-negative with short rod-shaped (Coccobacilli) into Sulfide Indole Motility (SIM) media. An inoculating needle was stabbed

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down the center of the medium to 2/3 of the media. Tube was incubated at 37ºC for 24 hours. Two to three drops of chloroform solution and Kovacs reagent were added. The indole test showed positive results when a red ring was formed on the upper surface of the media. For motility, motile organisms diffused from the stab line and produced cloudiness throughout the medium. The growth of non-motile bacteria was restricted along the stab line and left the surrounding medium clear (Shakya *et al*., 2013).

The MR test aimed to detect bacteria with mixed acids and the VP test to identify bacteria passing through carbohydrate fermentation. For bacteria that have the ability to utilise glucose with production of a stable acid**,** the colour of the methyl red changes from yellow to red**.** *E. coli* was MR positive and VP negative. The MR-VP test was performed by inoculating the colony from the EMBA media into 5 mL MR-VP broth. The inoculum was incubated at 37ºC for 48 hours. Then the medium was divided into two tubes, each for MR, and VP test. For the MR test, 5 drops of 0.05% methyl-red indicator were added. MR-positive result shows a red coloration as a result of high acid production and a decrease in the pH of the culture medium. For the VP test, the culture was added with 0.6 mL of 5% α-naphthol solution followed by 0.2 mL of 40% KOH. Tube was shaken gently and left undisturbed for 15 minutes. VP negative result showed yellow to brown colour with no red coloration on top of the culture (Aditi *et al*., 2017).

The citrate test was used to distinguish between *E. coli* (citrate negative) from other coliforms. Colony from EMBA media were inoculated into Simmons Citrate Agar (SCA) media by stabbing inoculating needle followed by streaking up the surface of the slant. The inoculum was incubated aerobically at 37ºC for ±24 hours. Development of blue colour was observed along the slant.

TSIA test was used to distinguishes between members of the family *Enterobacteriaceae* and differentiates them from other Gram-negative rods based on the ability of a bacteria to ferment sugars and to produce hydrogen sulfide. Colonies were inoculated into TSIA media by stabbing an inoculating needle through the center of the medium to the bottom of the tube and then the surface of the agar slant was streaked. Tube was then incubated at 37ºC for 24 hours with the cap on loosely. *E. coli* in TSIA showed acid (yellow in colour) production in the slant and butt areas, negative H_2S, and positive gas (Saimin *et al*., 2020).

Catalase test was used to identify organisms that produce the enzyme catalase, in this study to determine whether the Gram-positive cocci was a staphylococcus or a streptococcus. Yellow colonies of bacteria that grew on MSA media, and were Gram-positive with round-shaped (Coccus) clustered on Gram staining were taken, then placed on an object glass and added with 3% H_2O_2, then oxygen bubbles were observed.

Coagulase test was used to differentiate *S. aureus* from other *Staphylococci* species based on the ability to produce the coagulase enzyme. *S. aureus* was a Gram-positive, catalase and coagulase positive coccus. Yellow isolated colony from the MSA media was taken using an inoculation loop, and placed in the nutrient broth media. After incubation at 37ºC for 24 hours, 1 ml rabbit plasma was added and mixed gently. Plasma clotting was observed within 4-24 hours incubation.

Antibiotic sensitivity test

Antibiotic sensitivity test was performed using the Kirby-Bauer (disc diffusion) method. Bacterial isolates were made into suspension and adjusted to 0.5 Mc Farland standard which was equivalent to 1.5 x 10^8 CFU/mL. Media were incubated at 37ºC for 24 hours and the inhibition zone was measured using a caliper, and interpreted using the CLSI (2017) guidelines (Qomariyah and Indrayudha, 2021).

RESULTS

Inoculation of samples on EMBA showed 15 samples that grew metallic green colonies with clear boundaries (Figure 1A), while inoculation of samples on MSA showed 8 samples that grew yellow colonies with halo (Figure 1B). Gram staining results showed that bacteria from the metallic green colonies from all 15 samples were Gram-negative (Figure 2A), and further identification confirmed that they were *E. coli*. Gram staining of the yellow colonies showed that all 8 samples were all Gram-positive (Figure 2B). Catalase test showed that all 8 samples were catalase positive.

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However, out of the 8 samples only 1 sample was positive for coagulase test, confirming there was only 1 sample contained *S. aureus.*

The sensitivity of *E. coli* and *S. aureus* to gentamicin was very good, indicated by successive results reaching 93.33% and 100%, while 6.7% of *E. coli* were resistant to gentamicin (Table 1, Table 2).

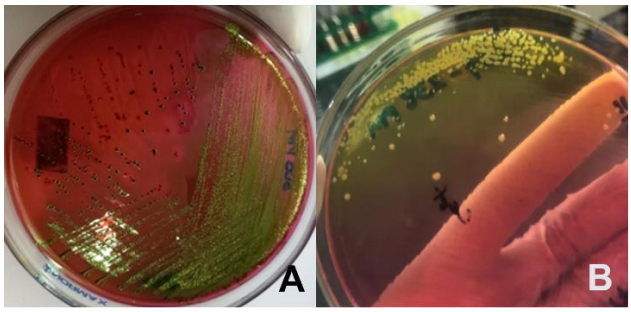

Figure 1 Streak plate isolation; EMB agar streaked for isolation of *E. coli* (A) and MS agar streaked for isolation of *S. aureus* (B) show a portion of an isolated colony.

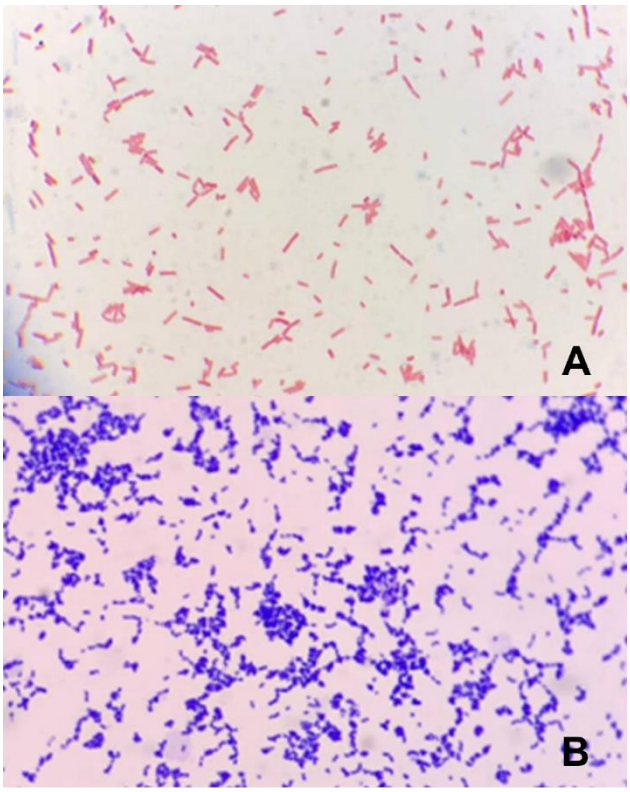

Figure 2 Gram staining; negative Gram staining show pink short rods (coccobacilli) of *E. coli* (A); positive Gram staining show clustered bluish purple cocci of *S. aureus* (B); Nikon E-200 light microscope,1000x magnification.

Table 1 Results of *E. coli* sensitivity test to antibiotics

sample code	antibiotic disc inhibition zone			
	ciprofloxacin	tetracycline	gentamicin	amoxicillin
2	29 (S)	15 (S)	17 (S)	22 (S)
3	29 (S)	16 (S)	19 (S)	17 (I)
4	32 (S)	18 (S)	18 (S)	25 (S)
5	29 (S)	15 (S)	18 (S)	18 (S)
6	29 (S)	15 (S)	18 (S)	26 (S)
7	40 (S)	23 (S)	23 (S)	25 (S)
9	40 (S)	22 (S)	25 (S)	23 (S)
2	29 (S)	15 (S)	17 (S)	22 (S)
3	29 (S)	16 (S)	19 (S)	17 (I)
4	32 (S)	18 (S)	18 (S)	25 (S)
5	29 (S)	15 (S)	18 (S)	18 (S)
6	29 (S)	15 (S)	18 (S)	26 (S)
21	33 (S)	22 (S)	22 (S)	23 (S)
23	28 (S)	20 (S)	19 (S)	21 (S)
24	30 (S)	20 (S)	20 (S)	23 (S)
R	0%	0%	6,67%	0%
I	0%	0%	0%	6,67%
S	100%	100%	93,33%	93,33%

R= resistant; I= intermediate; S= sensitive

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Table 2 Results of *S. aureus* sensitivity test to antibiotics

sample code	antibiotic disc inhibition zone			
	ciprofloxacin	tetracycline	gentamicin	amoxicillin
1	38 (S)	9 (R)	29 (S)	20 (S)
R	0%	100%	0%	0%
I	0%	0%	0%	0%
S	100%	0%	100%	100%

R= resistant; I= intermediate; S= sensitive

DISCUSSION

Isolation and identification on 24 samples of mucus of the reproductive tract of dairy cows during estrus found *E. coli* in 15 samples, and *S. aureus* in 1 sample, of which both types of bacteria were non-specific bacteria in the female reproductive tract. This was supported by research conducted by Nur (2020) where *Staphylococcus* and *Escherichia* bacteria were found from dairy cows in the follicular and luteal phases.

Metallic green colonies grew on EMBA media was characteristic for *E. coli* because *E. coli* could ferment lactose resulting in increased levels of acid in the medium. High acid levels precipitated methylene blue in EMBA. These colonies that were suspected to be *E. coli* were Gram stained. Gram staining of the 15 samples showed all colony samples in the form of short rods (coccobacilli) and were classified as Gram-negative bacteria indicated by pink cell colour. *E. coli* bacteria could not maintain violet crystal dyes during the Gram staining process due to the structure of the cell wall (Froböse *et al*., 2020). Gram-negative bacterial cell walls did not contain teichoic acid and because they contained only a small amount of peptidoglycans, therefore, the Gram-negative bacteria cell walls were more susceptible to mechanical damage (Sudrajad *et al*., 2018).

Inoculation of 24 samples on MSA media resulted in 8 samples growing yellow colonies with halo, while the other 16 samples did not. The yellow colour indicated the presence of mannitol fermentation. Mannitol, which was the acid produced, caused the phenol red colour of the agar to change from red to yellow (Mathew *et al*., 2019). Yellow colonies that grew on MSA media were then subjected to Gram staining. Gram staining of colonies from 8 samples showed clustered coccus (round in shape) and classified as Gram-positive bacteria indicated by bluish-purple cells. The purple colour was caused by the retention of crystal violet colour in bacteria. The difference in Gram's properties was influenced by the content of the cell wall, where Gram-positive bacteria contained thicker peptidoglycans than Gram-negative bacteria (Dewi, 2013).

Colonies suspected to be *E. coli* bacteria were identified using IMViC and TSIA tests. Indole test of all metallic green, Gram-negative colonies (from 15 samples) suspected of *E. coli* on SIM media showed the formation of a red ring on the surface of the media after the addition of chloroform solution and Kovac's reagent. The red ring was caused by indole reacting with the aldehydes when added with Kovach reagent. This was the result of the breakdown of tryptophan by *E. coli* (Ullah *et al*., 2021). Indole test showed that all colonies were motile positive, indicated by the growth of bacteria on the puncture marks that resembled cloud that reflected the motile *E. coli* (Hananto *et al*., 2015).

In the MR test, a positive result was obtained because there was a change in colour to red after the addition of methyl-red. The formation of mixed acids in the medium will lower the pH to 5.0 or lower, therefore when a methyl indicator was added to the culture with a pH as low as that, the indicator becomes red (Bambang *et al*., 2014). The VP test was negative for *E. coli* because *E. coli* ferments carbohydrates into acidic products and did not produce neutral products such as acetoin (Rahayu and Gumilar, 2017). This was indicated from the results of the VP test which showed a yellow-brown colour after being added with a solution of α-naphthol and KOH 40%. The citrate test was used to see the ability of bacteria

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to use citrate as the only carbon source. If bacteria were able to use citrate as a carbon source, it would raise the pH and change the colour of the culture medium from green to blue. In this study the results were negative, because *E*. coli could not use citrate as a carbon source (Bambang, *et al*., 2014).

TSIA test of all fifteen samples resulted acid/acid, H_2S negative and gas positive, which meant acid was produced both in the slant and butt areas, marked by changes in media colour which indicated that the bacteria ferment lactose (Isnan *et al*., 2017). H_2S negative was indicated by the absence of blackening in the butt area of the tube. Gas was produced due to the fermentation process of carbohydrates that appear as gaps in the medium and lifted agar at the bottom of the tube (Leboffe and Pierce, 2011).

Catalase tests conducted on the yellow colonies grew on MSA media (from 8 samples) showed all of them were catalase positive indicated by the presence of bubbles or foam. If bubbles appeared, it meant the bacteria was catalase positive, which confirmed the bacteria was *Staphylococcus*; if there were no bubbles, the bacteria were catalase negative which confirmed the bacteria was Streptococcus spp. (Mustafa, 2014). Gas bubbles (O_2) were produced by bacteria of the genus *Staphylococcus*, because *Staphylococcus* produced a catalase enzyme capable of hydrolyzing hydrogen peroxide (H_2O_2) into water (H_2O) and gas bubbles (O_2) (Toelle and Lenda, 2014). Coagulase test performed on 8 catalase positive samples showed 1 positive sample indicated by the occurrence of plasma clotting (Leboffe and Pierce, 2011). The coagulase test was a test used to determine the presence or absence of the coagulase enzyme produced by *Staphylococcus* sp.

Antibiotic sensitivity test

The sensitivity of *E. coli* and *S. aureus* to ciprofloxacin was very good, reaching 100% (Table 1, Table 2). These results were in accordance with the research of Kumar, *et al*. (2014) which showed that ciprofloxacin had a recovery rate of 87.5% in repeat breeder cows. The mechanism of action of ciprofloxacin was through inhibition of nucleic acid synthesis where this class of antibiotics can enter cells by passive diffusion through water-filled protein canal (porins) on the outer membrane of bacteria intracellularly. Uniquely, these drugs inhibited bacterial DNA replication by interfering with the work of DNA gyrase (topoisomerase II) during growth and reproduction of bacteria (Hangas *et al*., 2018).

The results of the sensitivity test of *E. coli* to tetracycline were very good, reaching 100% (Table 1), but *S. aureus* was declared to be 100% resistant (Table 2). This was in accordance with the study of Grossman (2016) which reported that 64 % of *S. aureus* isolates were resistant to tetracycline. Tetracycline was an antibiotic that can interfere with the protein synthesis process and was the antibiotic of choice that was able to inhibit Gram-positive and Gram-negative bacteria. Bacteria from the *Staphylococcus* group have beta-lactamase enzyme that can break down the beta-lactam ring on antibiotic and make it inactive (Effendi *et al*., 2013). The mechanism of bacterial resistance to gentamicin was through the process of inactivating antibiotics by aminoglycoside converting enzymes, in the form of attachment of aminoglycosides to specific protein receptors of the 30S subunit on the bacterial ribosome, then aminoglycosides would inhibit the activity of the initiation complex of peptide formation (Garneau-Tsodikova and Labby, 2016).

The sensitivity of *E. coli* and *S. aureus* to amoxicillin was very good, indicated by the results reaching 93.33% and 100%, respectively. Meanwhile, 6.67% of *E. coli* were intermediate to amoxicillin. These results indicated a higher level of sensitivity compared to the study of Sharma *et al*. (2009) where the sensitivity of amoxicillin was 70.7%. Todar (2002) stated that amoxicillin along with ampicillin belongs to a semisynthetic β-lactam chemical class and was effective against Gram-positive and -negative bacteria by inhibiting the steps of peptidoglycan cell wall synthesis (Bush and Bradford, 2016).

The results of the above study indicated the presence of non-specific bacteria (*S. aureus* and *E. coli*) in the reproductive tract of female dairy cows in the Danamulya Agribusiness Cooperative. This non-specific bacterial infection can cause repeat breeding. This is in line with data from practitioners at the Danamulya Agribusiness Cooperative which stated that the level of services per conception

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was still quite high. The sensitivity of bacteria isolates of *E. coli* from the reproductive tract of female dairy cows in the Danamulya Agribusiness Cooperative to ciprofloxacin, tetracycline, gentamicin and amoxicillin was very good. Meanwhile, *S. aureus* was sensitive to ciprofloxacin, gentamicin, and amoxicillin, but resistant to tetracycline. The widespread use of tetracycline in various countries with a relatively cheap price and as the second choice after penicillin caused the potential for resistance to increase (Trzcinski *et al*., 2000). Based on the information obtained, tetracycline was the antibiotic that have been used in the Danamulya Agribusiness Cooperative. Therefore, the use of tetracycline antibiotics for the treatment of *S. aureus* bacterial infections needs to be considered.

CONCLUSION

Based on the results of the research that has been done, it can be concluded that the antibiotics which *E. coli* bacteria isolated from the reproductive tract of dairy cows at the Danamulya Agribusiness Cooperative, Pacet District, Mojokerto Regency during estrus were most sensitive to were ciprofloxacin and tetracycline with a sensitivity level of 100%. then followed by gentamicin and amoxicillin with a sensitivity level of 93.33%. Antibiotics that *S. aureus* bacteria were sensitive to were ciprofloxacin, gentamicin, and amoxicillin.

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