Antimicrobial-resistant bacteria isolated from clinical mastitis cases

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Abstract. Bovine clinical mastitis is a global problem because it decreases milk quality and quantity. Treatment of bovine clinical mastitis becomes more complicated due to the emergence of antimicrobial resistance of the causative pathogen. The purposes of this study were to identify bacteria from clinical mastitis cases and investigate their antimicrobial resistance. Using standard bacterial culture techniques, bacteria were isolated from milk samples of cattle with clinical mastitis. Identification was performed through colony morphology, Gram-staining, and biochemical assays. Antimicrobial susceptibility was tested against seven antimicrobials using disk diffusion method. Identified bacteria included *Escherichia coli, Enterobacter aerogenes*, *Pseudomonas* sp., and *Staphylococcus aureus*, among others. Multridrug-resistance bacteria were identified. *E. coli* was resistant to all tested antimicrobials, *E. aerogenes* was resistant to multiple classes of antimicrobials, particularly beta-lactams and macrolides. *Pseudomonas* sp. was sensitive only to gentamicin. The *S. aureus* isolate demonstrated resistance to beta-lactam antimicrobials. The identification of multidrug-resistant bacteria in milk samples from clinical mastitis cases highlights the importance of antimicrobial stewardship.

1 Introduction

Bovine mastitis is a widespread contagious disease worldwide [1]. It poses a major challenge globally in the dairy industry, impacting public health and causing considerable economic burden [1]. In Indonesia, dairy farming plays a crucial role in supporting rural livelihoods and national food security [2]. A meta-analysis study estimated that 59.44% of dairy animals in Indonesia are affected by mastitis [3]. Bovine mastitis significantly affects milk production, quality, and animal welfare, leading to considerable economic losses.

Mastitis is commonly treated with antimicrobials [1]. However, concerns over antibiotic residues, antimicrobial resistance, and public health risks have led to increased restrictions on their uncontrolled use globally [1]. The rise of multidrug resistance (MDR) bacteria inhibits the effectiveness of mastitis treatment [1, 3]. Furthermore, MDR transfer from non-

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pathogens to mastitis pathogens could be a threat not only for the dairy industry but also for public health [1, 3].

In regions like Bogor, Indonesia, where small- and medium-scale dairy farms are predominant, the misuse of antimicrobials could increase the risk of antimicrobial resistance development. Studies investigating the resistance of bacterial pathogens causing clinical mastitis are essential to provide valuable insights into the dominant bacterial species contributing to mastitis cases and to highlight the current state of antimicrobial resistance. This study aimed to isolate and identify bacteria causing clinical mastitis in dairy cattle in Bogor, Indonesia, and to evaluate their antimicrobial resistance patterns. The findings contribute to understanding the epidemiology of clinical mastitis and support the formulation of evidence-based antimicrobial stewardship programs.

2 Method

2.1 Materials

The materials that were used were milk sample from bovine clinical mastitis cases, KOH 3%, H_2O_2 3%, Alcohol 70%, Alcohol 96%, distilled water, Blood Agar (BA), MacConkey Agar (MCA), Tryptic Soy Agar (TSA), Triple Sugar Iron Agar (TSIA), Mannitol Salt Agar (MSA), Mueller Hinton Agar (MHA), Gram staining reagents, indole, xylol, immersion oil, oxidase reagent, methyl red (MR) reagent, Voges-Proskauer (VP) reagent, Ehrlich reagent, Simon's citrate, sulphur indole motility (SIM), Triple Sugar Iron Agar (TSIA), peptone water, urease test media, Mannitol Salt Agar (MSA), sugars for fermentation tests (including glucose, lactose, sucrose, maltose, mannitol), antibiotic discs, and disinfectants.

2.2 Sample collection, bacterial isolation and identification

Milk samples used in the study were collected aseptically from two areas in Bogor. Samples were collected from four cattle suffering from clinical mastitis. Samples were delivered immediately to the Laboratory of Bacteriology, the School of Veterinary Medicine and Biomedical Sciences (SVMBS) IPB University for further analysis.

Clinical mastitis milk was inoculated onto BA and MCA and then incubated (37°C, 24 hours, aerobically). After incubation, single colonies were sub-cultured on TSA, with the same incubation condition.

Bacteria were identified by observing colonies macroscopically, including their morphology, color, size, edges, and elevation on selective media. Gram staining was performed for microscopic identification. For Gram-negative bacteria, the urease test, Simmon’s Citrate test, TSIA test, indole motility test, MR, VP, and carbohydrate fermentation tests were performed according to standard bacterial identification methods [4]. Gram-positive bacteria were tested for catalase test, glucose fermentation test in microaerophilic conditions, coagulase test, and growth on MSA [4].

2.3 Antimicrobial susceptibility test

Antimicrobial susceptibility was tested using the Kirby-Bauer disk diffusion method [5]. The bacteria from TSA were diluted with sterile physiological NaCl to make a suspension with a concentration equal to 0.5 McFarland standard. After that, 100 µl of the suspension was spread using a sterile cotton swab onto MHA. Once the surface had settled, seven antibiotic disks were placed on the MHA plates then incubated (37°C, 24 hours, aerobically). Antibiotics used in this study included ampicillin, carbenicillin, cephalothin, erythromycin,

gentamicin, tetracycline, and trimethoprim (OXOID). The test was performed in triplicates. The inhibition zones of each antibiotic disk were measured with a caliper. Interpretation (susceptible, intermediate, resistant) was performed according to the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI) to determine the bacteria's resistance levels [5].

3 Results and discussion

The clinical symptoms of the cows in which the samples were taken were inflammation of the udder, characterized by exudate, swelling, redness, and a quarter had changes in its function. This study identified various bacteria from clinical mastitis milk (Table 1**)**. The identified bacteria included *Staphylococcus aureus* (1 sample), *Staphylococcus epidermidis* (1 sample), and *Bacillus* sp. (2 samples), *Yersinia* sp. (3 samples), *Escherichia coli* (1 sample), *Pseudomonas* sp. (4 samples), *Pseudomonas diminuta* (1 sample), *Enterobacter aerogenes* (1 sample), *Alcaligenes* sp. (1 sample), and *Serratia* sp. (1 sample).

Table 1. Bacteria isolated from milk of clinical mastitis cases

Sample ID	Gram Negative Bacteria	Gram-Positive Bacteria
MKM1	*Yersinia* sp. *Pseudomonas* sp. *Pseudomonas diminuta*	*Bacillus* sp.
MKM2	*E. coli* *Pseudomonas* sp.	*Bacillus* sp. *Staphylococcus aureus*
MKM3	*Serratia sp.* *Pseudomonas* sp. *Yersinia* sp.	*Staphylococcus epidermidis*
MKM4	*E. aerogenes* *Alcaligenes* sp. *Yersinia* sp. *Serratia* sp.	

The identified Gram-positive bacteria in this study were *S. aureus*, *S. epidermidis*, and *Bacillus* sp. *Staphylococcus aureus* is a primary pathogen causing mastitis worldwide and has zoonotic potential, being transmissible between animals and humans [1, 6]. A study conducted on Dangke products from dairy farms in Indonesia reported a close genetic relationship between *S. aureus* isolates from animals and humans [6]. This commensal bacterium can be found on the skin and mucosal surfaces, as well as in the environment [1, 6, 7]. It can infect cows through open teat sphincter muscles post-milking or through teat injuries. *Staphylococcus epidermidis* is a coagulase-negative bacterium that can cause mastitis [1]. *Bacillus* spp. have been reported to cause mastitis [8]. *Bacillus* sp. is commonly found in soil, water, dust, air, and feces [8].

Bacteria isolates identified in this study were predominantly Gram-negative bacteria, including *E. coli*, *E. aerogenes*, *Pseudomonas* sp., *P. diminuta*, *Yersinia* sp., *Serratia* sp., and *Alcaligenes* sp. Gram-negative bacteria caused 97.5% of mastitis cases in another study [9]. The prevalence of these pathogens is linked to environmental contamination, as Gram-negative bacteria are usually present in the environment, increasing the risk of infection under poor sanitation conditions [1].

Escherichia coli is the most common Gram-negative bacterium causing bovine mastitis cases [1]. *Escherichia coli* is significant from a public health standpoint, as they serve as potential reservoirs for antimicrobial resistance genes [1]. In Bogor, MDR *E. coli* was identified from cow fecal samples [10]. This bacterium is commonly found in organic

materials, bedding, and feces. Cows can be infected through direct contact with environmental reservoirs or during milking.

Environmental bacteria such as *Pseudomonas* sp. were also identified in this study. *Pseudomonas* sp. was found in 6.5% of mastitis milk samples in another study [10]. Contaminated water used for udder washing and improperly cleaned milking machines are primary sources of *Pseudomonas* infection. Additionally, cows lying on soiled, wet bedding immediately after milking are at higher risk of infection [11]. *Enterobacter aerogenes*, a coliform bacterium, was also found in this study. *Yersinia* sp. was also identified from the samples. Previous studies reported a prevalence rate of 5% for mastitis caused by *Yersinia* sp., with infection sources including rodents, vegetables, waste, and water [12]. Similarly, *Serratia* sp. was detected in this study and has been previously identified in 2% of mastitis milk samples [13]. Contaminated milking machines are a common source of *Serratia* sp. infection [13]. *Alcaligenes* sp., though identified, is not typically associated with mastitis. It is primarily found in water and soil.

Pathogenic microorganisms can be transmitted during milking through various routes, including the milker's hands, contaminated water, cleaning cloths, or milking equipment [1, 3]. These findings underscore the need to enhance hygiene, sanitation, and management practices to prevent mastitis in dairy cattle.

3.1 Antimicrobial susceptibility test

Selected bacterial isolates, including *S. aureus, Pseudomonas* sp., *E. aerogenes*, and *E. coli*, were tested for their antimicrobial susceptibility. Seven antibiotics representing five classes were evaluated (Figure 1). The disk diffusion method by Kirby-Bauer was performed in triplicates, and results were interpreted following CLSI guidelines [5]. The detailed susceptibility patterns are presented in Table 1.

The results revealed the resistance of *E. coli* to all antibiotics tested, categorizing it as a multi-drug-resistant (MDR) bacterium. *E. aerogenes* displayed resistance to two antibiotic classes: beta-lactams (ampicillin, carbenicillin, cephalothin) and macrolides (erythromycin). The *Pseudomonas sp.* isolate was also classified as MDR, demonstrating resistance to all antibiotics tested except gentamicin. The *S. aureus* isolate showed resistance to beta-lactams, intermediate susceptibility to erythromycin, and susceptibility to gentamicin, tetracycline, and trimethoprim.

Table 2. Inhibition zone of the tested antimicrobials against the respective bacteria

Antimicrobial	**Antimicrobial class**	**Average size of inhibition zone (mm)***							
		E. coli		***E. aerogenes***		***Pseudomonas* sp.**		***S. aureus***	
Ampicillin	Beta-lactam	6±0	R	6.3±0.58	R	6±0	R	6±0	R
Carbenicillin	Beta-lactam	6±0	R	6.3±0.58	R	6.3±0.58	R	6.2±0.29	R
Cephalothin	Beta-lactam	6±0	R	6.2±0.29	R	6.3±0.58	R	6±0	R
Erythromycin	Macrolides	6±0	R	6.2±0.29	R	6.2±0.29	R	16.3±0.58	I
Gentamicin	Aminoglycoside	6±0	R	18.7±1.15	S	19.3±1.15	S	20±0	S
Tetracycline	Tetracycline	6±0	R	15.7±0.58	S	14.7±1.15	R	21.3±0.58	S
Trimethoprim	Folate pathways antagonists	6±0	R	27±1	S	6±0	R	17.7±0.58	S

* S: susceptible, I: intermediate susceptibility, R: resistant

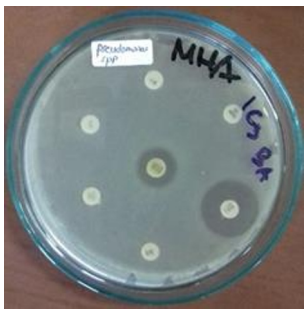

Fig. 1. Antimicrobial susceptibility test using the disc diffusion method.

The *E. coli* isolate exhibited resistance to all tested antibiotics. Similar findings have been reported in previous studies of pathogen causing mastitis, indicating that *E. coli* exhibited resistance to cephalothin (100%) and gentamicin (77%). Previous studies have reported similar findings [1]. *E. coli* resistance to beta-lactams is attributed to beta-lactamase enzymes encoded by plasmid genes, which hydrolyse and inactivate these antibiotics [1]. Tetracycline resistance is due to intrinsic genes, while gentamicin resistance is due to enzymatic modifications that inhibit binding to the 30S ribosomal subunit [1]. Trimethoprim resistance occurs via dihydrofolate reductase modification, encoded by the *dfr* gene [1].

The *E. aerogenes* isolate was resistant to beta-lactams and erythromycin, consistent with prior studies [8]. Beta-lactam resistance in *E. aerogenes* arises from AmpC beta-lactamase enzymes, which hydrolyze cephalosporins [1]. Erythromycin resistance is mediated by efflux pump systems that actively pump antibiotics from bacterial cells [1].

The *Pseudomonas sp.* isolate exhibited resistance to ampicillin, carbenicillin, cephalothin, tetracycline, erythromycin, and trimethoprim but remained susceptible to gentamicin. This aligns with prior reports showing *Pseudomonas* resistance to beta-lactams and other antibiotics. Resistance mechanisms include AmpC beta-lactamase production, efflux pumps targeting multiple antibiotic classes, and additional adaptations such as hypermutation and altered membrane permeability [1].

The *S. aureus* isolate was resistant to beta-lactams, displayed intermediate susceptibility to erythromycin, and remained sensitive to gentamicin, tetracycline, and trimethoprim. These findings align with previous studies reporting methicillin-resistant *S. aureus* (MRSA) with MDR patterns [1, 14, 15]. The emergence of MRSA poses a significant global threat. MRSA resistance to beta-lactams is driven by the mutation of the penicillin-binding protein 2a (PBP2a) encoded by the *mecA* gene [1, 15]. MRSA has been reported in mastitis cases, underscoring the need for control measures. A study in Indonesia by detecting the *mecA* gene reported that 83.3% *S. aureus* in that study were MRSA [7]. MRSA was most prevalent in isolates from human, cattle, and goats with a prevalence of 100%, 55.5%, and 40%, respectively [7].

The findings of this study underscore the identification of MDR bacteria in clinical mastitis cases. These results emphasize the importance of antimicrobial stewardship in dairy farms and regular antimicrobial resistance surveillance. Actions to address antimicrobial resistance is crucial to ensuring the effective management of clinical mastitis and safeguarding both animal and public health.

4 Conclusion

Multidrug-resistant bacteria were identified among isolates from clinical mastitis cases. *E. coli* was resistant to all tested antimicrobials, while *Pseudomonas* sp. was resistant to most of the tested antimicrobials. Similarly, *E. aerogenes* was resistant to multiple classes of antimicrobials, particularly beta-lactams and macrolides. The *S. aureus* isolate demonstrated resistance to beta-lactam antimicrobials. This finding emphasizes the need for effective antimicrobial stewardship to prevent further resistance development.

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