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Isolation and Identification of Gram-Negative Bacteria on Cattle Farms Indicated by Mastitis

Siti Rani Ayuti [1*], Zahwa Amelia Pratiwi[2], Masda Admi[3], Darniati[4], M. Isa[1], Herrialfian[1]

[1]*Laboratory of Biochemistry, Faculty of Veterinary Medicine, Universitas Syiah Kuala, Banda Aceh, Indonesia*
[2]*Bachelor Program of Veterinary Medicine, Faculty of Veterinary Medicine, Universitas Syiah Kuala, Banda Aceh, Indonesia*
[3]*Laboratory of Microbiology, Faculty of Veterinary Medicine, Universitas Syiah Kuala, Banda Aceh, Indonesia*
**Corresponding author: sitirani_ayuti@usk.ac.id*

Abstract

Mastitis is one of the causes of diseases that can reduce livestock production. Mastitis can be caused by various types of bacteria that cause a decrease in livestock production that is difficult to cure with antibiotics has been reported resistance. This study aims to determine Gram-negative bacteria that can cause of mastitis in cattle. The study used samples taken from folk farms through livestock udder swabs. Bacterial isolation is carried out by inoculating in Mannitol Salt Agar (MSA) media and identifying by Gram staining, then biochemical tests (maltose and lactose). The data analysis used is descriptive analysis, namely the types of bacteria as mastitis causative agents. The study results obtained bacteria that cause mastitis of Gram-negative species *Klebsiella* sp. and *Enterobacter* sp. which were classified as Gram-negative bacteria. It can be concluded that in cows indicated mastitis there are Gram-negative bacteria, that are morphologically identified, namely *Klebsiella* sp and *Enterobacter* sp.

Keywords: Mastitis, Isolation, Identification, Gram negative, cow

Background

Mastitis is an inflammation of the udder caused by microorganisms and easily transmitted in healthy animal livestock (Contreras *et al.,* 2007; Amri *et al.,* 2020). Mastitis can occur due to injuries to the nipples or udder tissue, resulting in contamination of microorganisms through the injured nipples (Grohn *et al.,* 2004; Contreras *et al.,* 2007). Mastitis can be caused by pathogenic microorganisms including *Staphylococcus aureus*, *Streptococcus agalactiae*, *Klebsiella* spp, *Escherichia coli*, and *Enterobacter* spp (Zadoks *et al.*, 2011; Artdita *et al*., 2018; Suarnata *et al.,* 2018). The bacteria attack locally or systemically in the ruminant glands during the initial phase of breastfeeding and can also be caused by hygiene conditions of the cage or equipment (Azis *et al*., 2013; Zhao *et al.,* 2015; Viennaa *et al.,* 2018).

The bacterial infection process that causes mastitis can occur due to several factors including the cleanliness of cages, equipment, workers, and the livestock environment (Windaria *et al.,* 2018; Nisa *et al.,* 2019). According to Aziz *et al.* (2013) Cage buildings that have concave floors can cause stagnant water and dirt, so they have the potential to be a source of disease. One of the diseases caused by maintenance management in newly given cows is mastitis, the losses caused by mastitis are a decrease in milk production, a decrease in milk quality, medical costs and low selling value of livestock (Utami, 2012; Riyanto *et al*., 2016).

In addition to reducing mil quality, livestock indocated by mastitis can also cause farms to experience losses due to a decrease in livestock productivity (Nisa *et al.,* 2019). Leitner *et al.* (2004) and Luengo *et al.* (2004) state that intramamari infection due to mastitis greatly affects the quality and quantity of milk produced, compared to other factors such as dry period, lactation period and the amount of lactation period. The bacteria that cause mastitis are classified as Gram-positive and Gram-negative in ruminants. Some Gram-positive bacteria such as *Staphylococcus aureus,*

Staphylococcus epidermidis, Streptococcus agalactiae, Streptococcus dysagalactiae, and Streptococcus uberis (Zadoks *et al*., 2011) and Gram-negative bacteria are grouped into *coliforms*, such as *Escherichia coli*, *Klebsiella pneumonia* (Artdita *et al.*, 2018), *Klebsiella varicola, Klebsiella oxytoca, and Enterobacter aerogenes* (Hall and Rycroft, 2007; Zadoks *et al.,* 2011).

This disease can cause is economic losses, significantly the decrease in milk production in dairy animals (Riyanto *et al.,* 2016; Nisa *et al.,* 2019). Other disadvantages include the cost of treating infected cows, falling selling points in cattle, and the cost of medicines and livestock health services and can sometimes lead to death (Windaria *et al.,* 2018; Nisa *et al.,* 2019). Rapid treatment and treatment must be done in mastitis after the initial symptoms (Amri *et al*., 2020). Handling mastitis in livestock using antibiotics requires enormous costs due to cases of antibiotic resistance (Nisa *et al.,* 2019; Maida and Lestari, 2019). Resistance of Gram-negative bacteria to gentamicin is often caused by the spread of plasmids that regulate enzymes that inactivate gentamicin (Surjowardojo, 2011; Nurhayati and Martindah, 2015). The antibacterial activity of gentamicin is mainly focused on aerobic Gram-negative bacillus bacteria while the activity against anaerobic bacteria is very low (Wahyuningsih and Zulaika, 2018).

The determination of the positive status of mastitis in ruminantlivestock can be done by the California Mastitis Test (CMT) examination. The status of mastitis can be ascertained using Gram staining examination, catalase test, mannitol sugar test, coagulase test, Voges-Proskauer test (Aziz *et al.,* 2013; Wasita & Hendrayana, 2016; Bulele *et al.,* 2019). Gram-negative mastitis can be identified using catalase, coagulase, simmon citrate, and biochemical assays (maltose, lactose) to identify gram-positive bacteria in milk associated with subclinical mastitis (Zadoks *et al.,* 2011; Leboffe and Pierce, 2011). Based on the background above, it is necessary to research to see the type of Gram-negative bacteria on cattle farms indicated by mastitis.

Methods and Materials

This research was carried out at the Laboratory of Microbiology, Faculty of Veterinary Medicine, Universitas Syiah Kuala in April 2022. The samples used in this study came from dairy cattle breeding farms. Determination of subclinical mastitis using the California Mastitis Test (CMT). The bacteria were isolated by cultivation on *Muller Hinton* Agar (MHA), and *Sorbitol MacConkey Agar* (SMAC) media and identified by Gram staining, mannitol sugar test, coagulase test, and Voges-Proskauer test. Samples are taken directly from cows that show characteristic symptoms of mastitis. Samples are collected and accommodated in sterile tubes and then tightly closed and stored in a freezer with an ice pack to maintain a stable temperature of 5-10°C to prevent bacterial growth until they arrive in the laboratory. Bacterial isolation on MSA media was carried out by taking one ose inoculum from goat milk identified as mastitis, then inoculated on mannitol media and incubated at 37 °C for 24 hours. If the positive result shows a change in color in the medium from red to yellow while the negative result shows no color change at all.

Bacterial Isolation

Isolation is carried out based on *Bergey's Manual Determination* method to grow bacteria on NB media. Samples were taken using ose and etched on *Blood Agar Plate* (BPA) media for 24 hours at 37 °C. Then bacterial planting was carried out using *Nutrient agar* (NA) obliquely incubated for 24 hours at a temperature of 37 ^{O}C as a stock of bacterial isolates.

Gram Staining

Gram staining serves to see gram properties and bacterial morphology. Then the preparations were made on the object glass, and then the preparations were fixed on the Bunsen, then the crystal violet solution was dripped onto the object glass and allowed to stand for 1-2 minutes. The remaining dyes are cleaned and washed with running water. All preparations were dripped with Lugol's solution and left for 30 seconds. Lugol's solution was discarded and

rinsed with running water. The preparations were bleached with 96% alcohol until all the colors faded, then immediately washed with running water. Furthermore, safranin dye was added, and the preparation was left for 2 minutes, rinsed with running water, and allowed to dry. The final step is that the preparations are observed under a microscope with an objective magnification of 100 x using emersion.

Bacterial Identification using Biochemical Test

The biochemical test conducted in this study was a maltose and lactose test with the carbohydrate broth labeled, then the bacterial culture inoculated into the carbohydrate broth and incubated at 37 °C for 24 hours. The sugar test tests positive if there is a change in color to yellowish while negative if the color remains red or there is no change in color (Lay, 1994).

Data Analysis

The data analysis used in this study is descriptive analysis. The data analyzed are the type of bacteria as the causative agent of mastitis.

Results and Discussion

Morphology of Bacteria

The results of sample inoculation in NB media show turbidity in NB media indicating bacterial growth in utilizing nutrients for their survival (Nuraeni and Sebayang, 2018; Wahyuningsih & Zulaika, 2018). If the environmental conditions and nutrients are suitable, bacteria can continue to develop and multiply (Rahmi *et al*., 2015). According to Dwita *et al*. (20 18), a positive result will be shown if the media turns cloudy and a negative result if there is no change in the media (remains clear). Bacterial inoculation in *MacConkey* media indicates the growth of separate bacterial colonies and visible discoloration of the media to orange. The results of the study are presented in Figure 1 and Table 1.

Table 1. Morphology of bacterial colonies on *MacConkey* media

Sample	Colony Morphology					
	Size	**Shape**	**Pigmentation**	**Surface**	**Edge**	**Elevation**
MM	1 mm	Round	Pink	Shiny	Flat	Convex
M	1 mm	Round	Red	Shiny	Flat	Convex

Description: MM (Pink), M (Red)

Based on Figure 1, it can be seen that the growth of separate colonies on *MacConkey* media with two different morphologies, namely pink pigmentation (MM) and red pigmentation (M). Tabulation of the results of morphological observations is presented in Table 1. visible bacterial colonies in the MM and M samples show the same size, namely 1 mm, round shape, shiny surface, flat edges, and convex elevation. This is following Dwita *et al.* (2018) that bacteria grown on *MacConkey* media have almost the same colony characteristics but have different sizes. The growth of colored colonies indicates that bacteria can ferment lactose and the growth of colorless colonies indicates that bacteria cannot ferment the lactose (Wasita and Hendrayana, 2016; Wahyuningsih and Zulaika, 2018).

Identification of differences in colonies growing on *MacConkey* media was done gram staining to see the structure of bacteria. Based on the results of observations, it shows two different bacterial colonies, including the MM colony with a long *bacilli-shaped* bacterial structure and an M colony with a cocco-*bacill* bacterial structure. This Gram staining also in addition to proves the type of Gram in a bacterium can be used to see the shape of the bacteria (Hemraj *et al.,* 2013; Maida and Lestari, 2019). Differences in bacterial structure are suspected to be 2 types of bacteria isolated from cattle with mastitis samples. According to Zadoks *et al.*

(2011), the form of bacill is a bacterium with a shape like a small stem and cylindrical. Some *bacilli*-shaped bacteria exist that go hand in hand, holding two or detached from each other with differences in bacterial species.

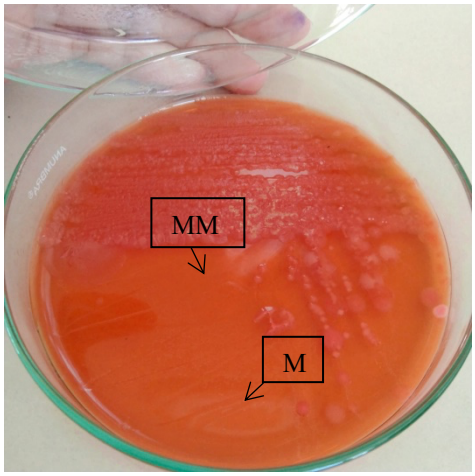


Figure 1. Bacterial growth on *MacConkey* media

According to Surjowardojo (2011), mastitis is the disease that affects cows the most in Indonesia, reaching 85%. Some research results in state that the causes of mastitis are inadequate, such as milking management and poor cage hygiene. Surjowardojo (2014) supported this, which stated that factors of poor cage hygiene and hygiene when milking can cause mastitis in ruminants. Nurhayati and Martindah (2015) added that the cause of very high mastitis in Indonesia occurs due to poor livestock management and almost all milking does not use antiseptic solutions after milking is completed. Added Mahardhika *et al*., (2012) cow hygiene before and after milking can also reduce the likelihood of mastitis events. Another thing that causes mastitis is a dirty environment such as a fecal dump close to the cage. Azis *et al.* (2013) states that feces are a source of microbial contamination that can also cause mastitis in cows.

Biochemical Test Results

Biochemical tests are carried out to identify bacterial species through the physiological properties of bacteria related to the metabolic activity of cells in generating or using energy for the synthesis of cell components (Hemraj *et al*., 2013; Zhao *et al.,* 2015). Steps to determine the type or species of bacteria based on microscopic morphology. Bacterial colonies that grow on solid media (plates) and can be identified in biochemical tests to ascertain the type o f bacteria that causes mastitis (Zadoks *et al.,* 2011). The results of the study are presented in Figure 2 and Table 2.

Table 2. Biochemical test results

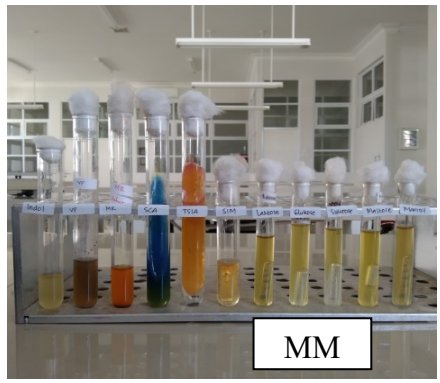
Sample	Biochemical Test										
	Indol	MR	VP	TSIA	Driver's license	SCA	Man nitol	Malt ose	Sucr ose	Gluc ose	Lact ose
MM	-	-	+	K/K	-	+	+	+	+	+	+
M	-	-	+	M/K	+	+	+	+	+	+	+

Description: K (Yellow), M (Red)

Based on the results of biochemical tests in Figure 3 and Table 2 that MM and M bacteria are bacteria that cannot degrade tryptophan, so they do not produce indole and are called negative results (-), in the MR test MM and M bacteria also cannot produce acid for glucose fermentation so that they show negative results (-), in the VP test MM and M bacteria can be seen the reaction of changing the media to red color is called a positive reaction (+). The SCA test showed positive results (+) because bacteria utilize citrate as a carbon source. The confectionery test of MM and M bacteria showed all positive results (+) where there was a change in the media from purple to yellow. The biochemical tests that differ between MM and M bacteria are in the TSIA test and the SIM test, namely, MM bacteria have positive results (+) which are characterized by changes in media color, namely yellow on the *butt* (base) and yellow on the *slant* (inclined surface). This is following the statement of Artdita *et al.* (2018) that

Klebsiella sp bacteria are specific to TSIA with yellow color at the base of the media and the surface of the media is tilted, this indicates that the bacteria ferments 3 types of carbohydrates, namely lactose, maltose and sucrose. The MM bacterial SIM test shows that bacterial growth results do not spread on the media as a sign that bacteria cannot move (non-motile). The M bacterial TSIA test obtained a positive result (+) where there was a gas marked by a change in media color, namely yellow on the *butt* (base) and red on the *slant* (inclined surface). This is under the statement of Luengo *et al.* (2004) that in TSIA media shows red on the surface and yellow on the bottom of the tube, as a sign of glucose fermentation but no fermentation of lactose and sucrose occurs. The SIM test on M bacteria shows growths that spread on the media as a characteristic of movable bacteria (motiles). After a series of laboratory analyses on swab samples of labour cow mastitis udders, with conventional microbiological research starting from inoculation in NB, *MacConkey* media, Gram staining, and biochemical tests showed the cause of mastitis in aceh cattle was Gram-negative bacteria species *Klebsiella* sp. and *Enterobacter* sp.

Examining of the bacterial phenotype is conducted to determine whether the bacteria can be classified as Gram-positive or Gram-negative bacteria. Both bacteria can cause cases of mastitis on ruminant farms in both cattle and goat farms (Artdita *et al.,* 2018). To find out the species of bacteria can use selective media because only Gram-negative bacteria grow and this media contains toxic dyes to Gram-positive (Quinn *et al.,* 2006). The shape of the Klebsiella colony on MCA media is circular, pink and mucoid, giving positive results on the simmon's citrate test which is characterized by the color of the media becoming blue, and in the MIO test a negative result of the motility test (absence of fog on the puncture marks) but the bacteria are able to decarboxylate ornithin (characterized by the presence of purple on the top of the media) (Leboffe & Pierce, 2011; Artdita *et al.,* 2018).



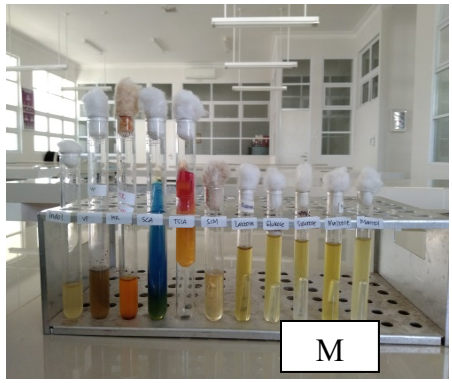


Figure 2. Biochemical test results (MM) pink, (M) red

Grohn *et al.* (2009) also reported that Klebsiella is a species of bacteria that is pathogenic and often causes mastitis. Mastitis infection can occur when the nipple muscles are open, it can cause an immune reaction in livestock characterized by a buildup of white blood cells. If the reaction does not occur, microorganisms will multiply and goats can show other reactions such as fever, if the endurance of cattle is weak, mastitis will occur (Guha *et al.,* 2012). The appearance of mastitis as a great threat to the survival of livestock breeds, since the toxin produced by bacteria on the mammary glands, in addition to weakening milk production can also cause death in calves and broods. Mastitis can not only cause death in offspring and mothers, but also cause considerable economic losses due to reduced milk production (Surjowardojo, 2011; Mahardika *et al.,* 2012). Maintenance management is one of the preventive measures that must be taken to control mastitis. Regular examination with California Mastitis Test (CMT) is required to monitor mastitis. Washing the udder with clean, warm water before milking can also

help prevent mastitis (Milk, 2014; Nurhayati & Martindah, 2015).

Conclusion

Based on the results of this research can be concluded that in cows indicated mastitis there are Gram-negative bacteria. Based on the morphologically characteristics, the bacteria were succesfully identified, namely the species *Klebsiella* sp and *Enterobacter* sp.

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