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Klebsiella pneumoniae Isolated from Subclinical Mastitis Milk of Etawah Crossbreed Goat

Isolasi *Klebsiella pneumoniae* dari Susu Kambing Peranakan Etawah yang menderita Mastitis Subklinis

Clara Ajeng Artdita*, Fajar Budi Lestari, Achmad Fauzi, Erian Pemila Ayu Tanzila

Veterinary Paramedic Study Program, Department of Bioresources Technology and Veterinary, Vocational College Universitas Gadjah Mada, Yogyakarta
*Email: clara.ajeng@ugm.ac.id

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Abstrak

Klebsiella spp. merupakan salah satu bakteri penyebab mastitis. *Klebsiella pneumoniae* dan *Klebsiella varicola* adalah jenis *Klebsiella spp* yang paling menginfeksi dalam suatu peternakan sapi perah. Sebanyak 16 sampel susu dari 8 ekor kambing masa laktasi di Peternakan Sahabat Ternak, Sleman, Yogyakarta digunakan pada penelitian ini. Penentuan status mastitis dilakukan dengan reagen *California Mastitis Test (CMT)*, selanjutnya dilakukan inokulasi pada plat agar darah (PAD) dan dilanjutkan dengan inokulasi pada *Mac Conkey Agar (MCA)* yang berfungsi sebagai media selektif terhadap bakteri Gram negatif. Koloni yang tumbuh pada PAD dan MCA dilakukan pewarnaan Gram. Fungsi pewarnaan Gram selain untuk menentukan bakteri Gram positif atau Gram negatif, juga dapat digunakan untuk membedakan morfologi sel bakteri. Koloni yang tumbuh pada MCA selanjutnya dilakukan identifikasi dengan uji biokimia meliputi uji fermentasi gula (glukosa, laktosa, maltose dan sukrosa), arginin, *ornithine decarboxylase*, indol, sitrat, hidrolisis urea (*Christensen's method*), lisin dan malonat. Hasil uji status mastitis menunjukkan bahwa dari 8 ekor kambing masa laktasi, sebanyak 5 kambing positif mastitis dan 3 kambing negatif. Hasil pewarnaan Gram terhadap sampel bakteri adalah Gram negatif dan batang pendek (*rods*), selanjutnya dilakukan dengan uji biokimia. Hasil identifikasi berdasarkan uji biokimia adalah *Klebsiella pneumoniae*. Tahapan identifikasi dilakukan di Laboratorium Preklinis Program Studi Kesehatan Hewan, Sekolah Vokasi Universitas Gadjah Mada dan konfirmasi hasil uji dengan kontrol positif *Klebsiella pneumoniae* dilakukan di Balai Laboratorium Kesehatan Yogyakarta. Hasil ini menunjukkan bahwa sampel susu kambing Peranakan Etawah terkontaminasi *Klebsiella pneumoniae*.

Kata kunci : Kambing Peranakan Etawah; *Klebsiella pneumoniae*; mastitis subklinis

Abstract

Klebsiella spp. is a common bacteria causing mastitis. *Klebsiella pneumoniae* and *Klebsiella varicola* is the most infected *Klebsiella spp* in the dairy farm. This study used 16 milk samples from 8 lactation goats in Sahabat Ternak farm, Sleman, Yogyakarta. Samples were tested by California Mastitis Test (CMT) reagent to determine the mastitis status, inoculated on blood agar (BA) then on Mac Conkey Agar (MCA) as a selective media for Gram negative bacteria. The colonies from BA and MCA were stained by Gram staining to determine Gram-positive or Gram-negative bacteria and its cell morphology. The colonies from MCA were identified by biochemical tests such as sugar fermentation tests (glucose, lactose, maltose, and saccharose), arginine, ornithine decarboxylase, indole, citrate, urea hydrolysis (Christensen's method), lysin and malonate. The result of mastitis test showed 5 goats were positive result and 3 samples were negative. Bacterial staining showed 2 samples were Gram-negative, rods and the others were Gram-positive, coccus. The samples with rods shapes were continued by biochemical tests. The characterization result of biochemical test indicated that the rods shapes bacteria were *Klebsiella pneumoniae.* These bacteria identification conducted in Laboratorium Preklinis Program Studi Kesehatan Hewan, Sekolah Vokasi Universitas Gadjah Mada and the result confirmation using positive control of Klebsiella pneumoniae conducted in Balai Laboratorium Kesehatan Yogyakarta. These results showed that milk from Etawah crossbreed goat was infected by *Klebsiella pneumoniae*.

Key words : Etawah Crossbreed Goat; *Klebsiella pneumoniae*; Subclinical Mastitis;

Introduction

Mastitis is one of big problem in dairy cattle, including Etawah Crossbreed goat, which produce milk beside cows. One of the cost factors of subclinical mastitis (without any symptom sign) is milk production loss. Mastitis is the condition of the mammary gland inflammation which caused by either Gram-positive or Gram-negative. Another microbial agents causing mastitis is algae and fungi. Many Gram-positive microbial species that commonly causing dairy mastitis are *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysagalactiae,* and *Streptococcus uberis* (Zadoks et al., 2011). Besides, many Gram-negative microbial species, group of coliforms, are *Escherichia coli, Klebsiella pneumoniae, Klebsiella varicola, Klebsiella oxytoca,* and *Enterobacter aerogenes* (Bannerman et al., 2003; Munoz et al., 2008; Zadoks et al., 2011). Coliform pathogens are essentially opportunistic. Their primary reservoir in transmission is represented by feces, water, soil, sawdust, and shavings that contaminate the canal of the teats. One of pathogen *Klebsiella* which causing mastitis is *Klebsiella pneumoniae* (Grohn et al., 2009)

Klebsiella pneumoniae is a Gram-negative bacteria, non-motile, encapsulated, sugar (glucose, lactose, maltose, sucrose) fermented, facultative anaerobic, rod shape. Unlike another coliforms, *Klebsiella* invade deeply into the udder tissue and damage the secretory capacity of the gland. This means that some *Klebsiella* infections become chronic, and affected animals suffer long-term decreased milk production. Mastitis adversely affects milk production and generally the herd do not regain full production levels post recovery (Grohn et al., 2004), this case is leading to considerable economic losses. It has also been reported that the amount of decrease in milk production depends on the specific pathogen causing the infection and that Gram negative bacteria are responsible for greater reduction than Gram positive bacteria and other non-bacterial organisms (Grohn et al., 2004; Schukken et al., 2009; Habrun and Kompes, 2014). New infections can occur at any time during lactation and may also occur during the dry period. However, goats in early lactation are at an increased risk for new infections due to the increased stress and immune suppression associated with the postpartum period. Additionally, goats are at an increased risk for mastitis immediately after drying-off. Following milk cessation, goats do not experience the daily flushing of the mammary gland and are at an increased risk for mastitis in the early dry period (Christina et al., 2011). These Gram–negative microorganisms possess lipopolysaccharides (LPS), so called endotoxins, in the outer layer of the cell wall, which in contact with the immune system lead to liberation of potent pro–inflammatory mediators (cytokines). Mammary glands of the domestic animals are extremely sensible to LPS (Munoz et al., 2006). The endotoxins induce to severe changes in vascular permeability, and increase of somatic cells in mammary gland and milk, resulting in edema, depression, toxemia, and severe per-acute or acute clinical signs of mastitis (Radostits et al., 2009).

When *Klebsiella* bacteria die, a toxin is released. This toxin is the primary cause of the clinical signs observed in a local mastitis infection. Antibiotics act to kill bacteria; consequently, in the case of these infections, the use of an antibiotic results in the toxin release. Thus, intra-mammary antibiotic treatment is not a generally recommended practice for local infections (Christina et al., 2011). Occasionally, complication of coliform mastitis occurs when pathogens disseminate from the mammary gland to systemic circulation, leading the animal to severe clinical signs of bacteremia and/or septicemia (Radostits et al., 2009). However, in cases in which

Klebsiella infections can become systemic, therefore antibiotic treatment and supportive therapy are required. Although there has been discussion in recent years regarding the presence of chronic infections caused by *Klebsiella spp.*, it is not yent known how these infections become chronic. Veterinary consultation is recommended prior to the start of any treatment protocol. Due to the nature of these bacteria, emphasis needs to be placed on prevention of these infections, rather than on treatment (Christina et al., 2011).

Although, not routinely performed for diagnostic purposes, further characterization of bacterial solates from infected animals helps to identify the pathogen which responsible of the infection (Munoz et al., 2007; Zadoks et al., 2011; Ohnishi et al., 2013). These data allow veterinarian and the farmer to understand the nature of transmission within herds and to implement targeted prevention strategies. The aim of this study was to identify the causative agent of subclinical mastitis in Etawah Crossbreed goat.

Materials and methods

Collecting samples

Sixteen (16) milk samples were collected from 8 goats (16 udders) in the morning. The goats were in standing position, the teats were cleaned up using 70% alcohol. The first milk threw away, to ensure the teats were free from debris. Approximately 10 cc of milk was collected into the sterile conical, a part of that milk was tested with CMT reagent (1:1) and the rest of it was taken into the cooler box immediately then the samples took to the laboratory for bacterial identification (Hall and Rycroft, 2007).

Klebsiella pneumoniae phenotypic identification

Conventional methods were used to detect *Klebsiella pneumoniae* based on the phenotypic system include microscopic examination and biochemical characterization. There were sixteen (16) udder samples from eight (8) lactation goats screened directly by *Californian Mastitis Test* (CMT, Dairy Research Products Co., Ontario, Canada) with 1:1 ratio between milk and reagent to determine the mastitis status. Milk samples for somatic cell counts had been taken immediately before milking, after removing three squirts of milk. It was measured by forming a stringy mass from the reagent (3% sodium lauryl sulfate) and the somatic cells with visual observation. The positive result was shown by the observation of somatic cells (forming gel). There were 4 categories which shown by this test. Negative result with no evidence of formation gel; trace was a slight slime with no tendency towards gel formation; positive 1 (weak) was a distinct slime with no tendency towards gel formation; positive 2 (distinct positive) was a gel formation mixture when thickens immediately and on continued swirling, mass moves around the periphery the bottom of the cup exposed; positive 3 (strong positive) was a distinct gel forms which tends to adhere to the bottom of the paddle and during swirling a distinct central peak was formed (Guha et al., 2012). The 2+ and 3+ result was isolated and phenotypic identified with standard phenotypic and biochemical testing for pathogen identification. Milk samples were streaked or inoculated on blood agar (BA, Oxoid) with 5% sheep blood then on Mac-Conkey Agar (MCA, BD), and then incubated in incubator at 37^0C for 24 hours. The colonies from BA and Mac Conkey Agar were stained by Gram staining to determine Gram positive or negative bacteria and its cell morphology. The colonies from Mac Conkey Agar were characterized by biochemical tests such as sugar fermentation tests (glucose, lactose, maltose, and saccharose), arginine agar, motility indole ornithin (MIO), Simmon's citrate, urea hydrolysis

(Christensen's method), lysin iron agar and malonate broth all were incubated at 37^{0}C for 24 hours as recommended (Andrews and Hammack, 2001; Forbes et al., 2002; Sirois, 2015). The report is focused on the microbiological analysis following the isolation of bacteria from the milk of Etawah Crossbreed goat samples. These results were analyzed descriptively.

Results and Discussion

The etiology of infectious mastitis in goats and cows is similar but goat affected less frequently. In the current study of CMT observation, showed that from 8 goats (16 udder), there were 5 goats (62.5%) positive result and 3 goats (37.5%) negative result. Five goats with positive result, there were 2 left udder and 2 right udder with 3+ (25%) also 2 left udder and 2 right udder with 2+ (25%). Three goats with negative result, there were 4 left udder and 4 right udder (50%) (Table 1).

Table 1 California Mastitis Test Result

Lactation goat number	Udder	
	Left	Right
1	-	-
2	-	-
3	-	-
4	+++	+++
5	++	-
6	+++	+++
7	-	++
8	++	++

Note: + is for positive result
-is for negative result

The positive result was shown by the observation of somatic cells (forming gel). The somatic cells are the white blood cells in milk, together with a relatively small number of epithelial cells from milk secreting tissues. These cells are an important part of the goat's natural defense mechanism. When udder tissue is injured or becomes infected, significant numbers of white blood cells accumulate in the milk. Normal goat milk has a higher cell count than normal milk from cows. The CMT reagent reacts with genetic material of somatic cells present in milk to form a gel (Paape and Capuco, 1997; Guha et al., 2012).

Somatic cell counts in milk from goats are higher than somatic cell counts in milk from cows. Although it is recognized that increased cell counts in cow milk result in decreased milk yield, there is no evidence to indicate that this situation exists in goat milk production. Several factors contribute to this elevated cell count. Milk secretion in the cow is merocrine, and secretion in the goat is apocrine (Wooding et al, 1970), and the apocrine results in the shedding of cytoplasmic particles into milk. Cytoplasmic particles in the size range of milk somatic cells commonly found in goat milk can be mistakenly counted as somatic cells. Further, neutrophils make up 50 to 70% of the somatic cell count in milk from goats free of intra-mammary infection, whereas neutrophils only make up 5 to 20% of the total cell count in bovine milk. Unlike in milk from cows, cell counts in goat milk increase with stage of lactation and parity (Paape and Capuco, 1997). In general, milk from non-infected glands will yield a negative (-), trace, or <1+ (< 1.000.000 SCC/ml) reaction. Scores of >2+ (500.000 – 2.000.000 SCC/ml) or >3+ (>1.500.000 SCC/ml) are indicated of mastitis. Somatic cell counts in excess of 1,500,000/ml are suggestive of intra-mammary infection, then diagnose confirmation by identification the pathogenic bacteria (Wooding et al, 1970; Paape and Capuco, 1997; McDougall et al., 2001; Persson and Olofsson, 2011; Guha et al., 2012)

In this study, one of bacteria causing goat mastitis was *Klebsiella pneumoniae* by phenotypic identification from milk which positif result CMT, there were 2 samples. A study found the *Klebsiella pneumoniae* (5.7%) from clinical mastitis goats in Ibadan, Nigeria (Ajuwa pe et al., 2005). Another study

also found the *Klebsiella spp.* (9.52%) from clinical mastitis goats (Jeph et al., 2013). Milk samples were streaked on Blood Agar with 5% sheep blood showed circular, medium-large colony size, sticky, mucoid, whitish color and non-hemolytic colonies. Mac-Conkey Agar is a selective media which relatively to grow up the Gram-negative bacteria as well as *Klebsiella* (Quinn et al., 2004). *The Klebsiella* colony in Mac-Conkey Agar showed circular, pink color and mucoid colony (Sirois, 2015) (Fig. 1).

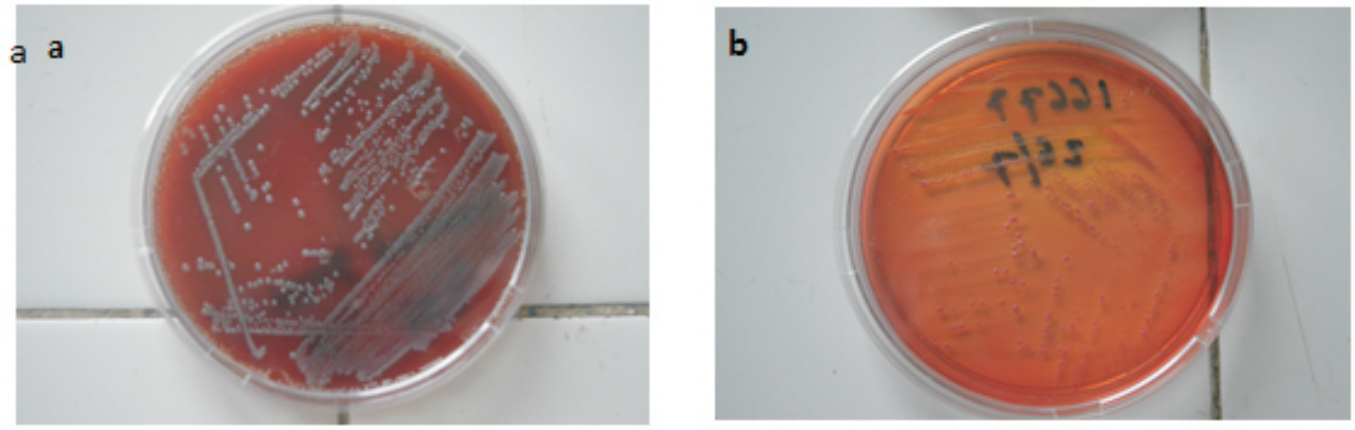

Fig. 1 *Klebsiella pneumoniae* culture 1; a Blood Agar, b Mac-Conkey Agar

In the Mac-Conkey Agar, these bacteria could ferment the lactose, acidic metabolic product were produced and the colonies were pink because of the pH indicator (neutral red) (Quinn et al., 2004). The Gram Staining of these colonies showed Gram-negative bacteria, facultative anaerobic rods (Carter, 1979; Quinn et al., 2004) (Fig. 2).

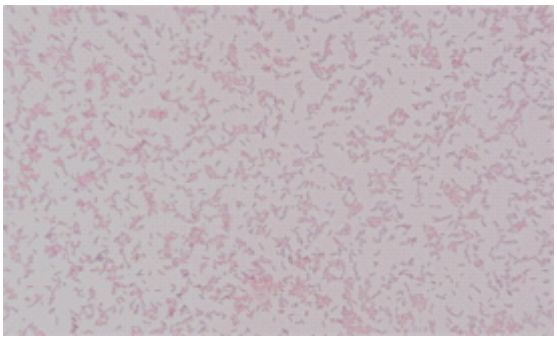

Fig. 2. Gram Staining : rods, Gram-negative

The colonies from MCA were characterized by biochemical tests such as sugar fermentation tests (glucose, lactose, maltose, and sucrose), arginine, ornithine decarboxylase, indole, citrate, urease, lysine and malonate all were incubated at 370C for 24 hours. The result of these biochemical test is shown in Table 2. The indicator in fermentation media changed to yellow, indicating *Klebsiella pneumoniae* was fermented in all glucose (produced gas), lactose, maltose, and sucrose. These bacteria fermented glucose with the production of gas (visible as bubble in the Durham tube) (Forbes et al., 2002). Arginine dehydrolase, ornithine decarboxylase and indole were negative result (Carter, 1979; Carter and Wise, 2004). To ensure the absence of motile structures such as flagella, a motility test can be performed by inoculation in semisolid agar (MIO agar) (Forbes et al., 2002). Arginine and ornithine are amino acids.

Klebsiella pneumoniae was inoculated in decarboxylase broth with arginine and ornithine (Moeller's method), after the incubation, no color changed (acid), indicating the fermentation of the dextrose in the medium causes the acid color. Indole test is used to determine the ability of an organism to split tryptophan to form the compound of indole and in this study, the color didn't change after the addition of appropriate reagent. Test result showed that the bacteria remain at the site of inoculation and the flagella show absence of the motile (Forbes et al., 2002).

Table 2. Biochemical characterization

Biochemical test	**Result**	**Another result**
Sugar fermentation:		
Glucose	+	Gas
Lactose	+	
Maltose	+	
Saccharose	+	
Arginin	-	
Ornithin decarboxylase	-	Non-motile
Indole	-	Non-motile
Simmon's Citrate	+	
Urea hydrolisis	+	
Lysin Iron Agar	+	Alkaline slant/alkaline butt
Malonate	+	

Note: + is for positive result
- is for negative result

The biochemical test of citrate, urea hydrolysis, lysine decarboxylase and malonate were positive result (Carter, 1979; Carter and Wise, 2004). The positive result of Klebsiella pneumoniae on the Simmon's Citrate agar was showed by the bacteria growth in the agar and changing the bromothymol blue indicator from green to blue, indicating the metabolic feature of citrate utilization (Forbes et al., 2002; Collin et al., 2004). The positive result in this biochemical test is showed by the bacteria growth in the medium, with or without a change in the color of the indicator. The positive result in urea hydrolysis (Christensen's method) was shown color changing from light orange to magenta. This biochemical test indicated that these bacteria produce urease enzyme which hydrolyze urea. The product of hydrolyze urea was ammonia and carbon dioxide (CO2). The ammonia made the medium alkaline and the pH shift was detected by the color changed of phenol red from light orange (pH 6.8) to magenta (pH 8.1). Another biochemical test is lysine decarboxylase (Forbes et al., 2002). These bacteria inoculated in lysine iron agar (LIA), showed positive result, it meant that cadaverine is formed because the bacteria produce lysine decarboxylase. The function of cadaverine is for neutralizing the organic acids formed by glucose fermentation and the butt of the medium

reverts to purple (alkaline) (Forbes et al., 2002). Malonate broth was used to differentiated Enterobacter from Escherichia, based on their ability to utilize malonate. Klebsiella pneumoniae showed positive reaction with dark-blue color, indicating these bacteria utilize sodium malonate as its carbon source and ammonium sulfate as its nitrogen source produced alkalinity due to the formation of sodium hydroxide. This condition changed the color of bromothymol blue indicator in the medium to light blue and finally Prussian blue (dark-blue color) (MacFaddin, 1985).

Conclusions

In conclusion, subclinical mastitis was detected by the monitoring of somatic cell counts using CMT. These results showed that milk from Etawah crossbreed goat was contaminated by *Klebsiella pneumoniae.*

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