Isolation and identification of bacterial pathogen from mastitis milk in Central Java Indonesia

by Dian Wahyu Harjanti

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Abstract. Mastitis is a multi-etiologic disease of the mammary gland characterized mainly by reduction in milk production and milk quality due to intramammary infection by pathogenic bacteria. Nearly 83% of lactating dairy cows in Indonesia are infected with mastitis in various inflammation degrees. This study was conducted to isolate and identify the pathogen in milk collected from mastitis-infected dairy cows. The study was carried out in ten smallholder dairy farms in Central Java Indonesia based on animal examination, California mastitis test, isolation bacterial pathogens, Gram staining, Catalase and Coagulase test, and identification of bacteria species using Vitek. Bacteriological examination of milk samples revealed 15 isolates where *Streptococcus* was predominant species (73.3%) and the coagulase negative *Staphylococcus* species was identified at the least bacteria (26.7%). The *Streptococcus* bacteria found were *Streptococcus uberis* (2 isolates), *Streptococcus sanguinis*(6 isolates), *Streptococcus dysgalactiae*ssp*dysgalactiae*(1 isolate), *Streptococcus mitis* (1 isolate) and *Streptococcus agalactiae* (1 isolate). The *Staphylococcus* isolates comprising of *Staphylococcus simulans* (1 isolate) *and Staphylococcus chromogens* (3 isolates). Contamination of raw milkwith pathogenic bacteria can cause outbreaks of human disease (milk borne disease). Thus, proper milk processing method that couldinhibit the growth or kill these pathogenic bacteria is important to ensure the safety of milk and milk products.
Keywords *: milk, mastitis, pathogen, Central Java, Streptococcus*

1. Introduction

Indonesian dairy industry is based on smallholder farms grouped into cooperatives. Most of the farms have three to five head lactating cows. The dairy farms are based on the cut and carry system, in which the forage grasses are being gathered from the outside of the farms. Nearly 97% dairy farms are concentrated in West, Central and East Java, whereas around 3% are located in Sumatra. According to the data from the Indonesian Government of Statistic Agency, Indonesia is estimated to have more than 260 millions inhabitants in 2017, with the population growth nearly 1.67% annually. Therefore, milk and dairy product consumptions continue to increase rapidly in Indonesia. Nonetheless, the domestic milk production fails to meet demands of the population in the country, also the needs of the processing industry in terms of both quantity and quality. The Indonesian dairy farmers are facing many challenges to improve milk production and quality. One of the main challenges that impact both milk quantity and quality is a disease named Mastitis.

Mastitis is a multi-etiologic disease of the mammary gland characterized mainly by reduction in milk production and milk quality due to intramammary infection by pathogenic bacteria.The mastitis

prevalence in tropical country like Indonesia is very high as 75-83% [1]. Mastitis treatment with intramammary antibiotic has been done for many years in Indonesia. Antibiotic treatment of clinical and subclinical mastitis is a key component of mastitis control program. However, in fact, single injection may not enough to cure the inflammation. Many factors could influence to this problems. The bacteria may be resistant to the antibiotic or the commercial antibiotic used may notsusceptible to the specific bacteria species in udder. Previously, research had been conducted to identify the bacteria-causing mastitis in Indonesia. In 2002, Estuningsih et al. [2] found *Streptococcus agalactiae*from 7 farms in Java area by PCR. Furthermore, in 2005, Salasia et al. [3] did the comparative study on phenotypic and genotypic of *Staphylococcus aureus*isolates from bovine subclinical mastitis in Java and comparing them to the *Staphylococcus aureus*from Germany. Nonetheless, the bacteria causing mastitis is not only*Streptococcus agalactiae*and *Staphylococcus aureus* and the pathogenicity of the same bacteria species might be change due to environment and the specific antibiotic used. Increasing antibiotic resistance has become a serious concern worldwide. The resistance patterns of coagulate negative bacteria species were highly correlated with the antibiotic used in herd [4]. Since the species bacteria will be killed or inactivated with the specific antibiotic, hence, the antibiotic must be chosen based on the bacteria species in the infected animals. Therefore, determination of bacteria species from mastitis-infectedcow is extremely important to choose the appropriate antibiotic for treatment, also the proper milk processing method for specific dairy products. Nowadays, highly automated identification system the new VITEK gram positive and negative identification cards provide stable and decisive result of bacteria identification [5,6,7]. The result from this study will provide a data of specific mastitis-causing bacteria for further rapid detection, proper mastitis treatment and control, also precise method for milk processing to ensure food safety.

2. Material and Method

2.1.*Sample collection and bacteria isolation*

A total of twelve individual milk samples were collected from subclinical mastitis cows aseptically,after performing California Mastitis Test (CMT). The cows were obtained from 10 smallholder dairy farms in three different geographic regions in Central Java during June – July 2016. All milk samples were cultured in Microbiology Laboratory Faculty of Medicine, Diponegoro University.Refrigerated milk samples were warm at room temperature (25°C) for half an hour and then homogenized by gently shaked it in order to disperse bacteria from milk fat. One standard loop (10 µL) of milk sample was streaked on 5% sheep blood agar (Oxoid™ Blood Agar Base, CM0055) and streaked using the quadrant streaking method for each sample. The inoculated plates were then incubated at aerobic condition at 37 °C and checked after 24 and 48 hto eliminate slow growing bacteria.The plates were examined for growth, morphologic features such as colony size, shape, color and hemolytic characteristics.Growth more than one type of colonies was determined as mixed growth.Presumptive colonies were selected and sub cultured on 5% sheep blood agar (Oxoid™ Blod Agar Base CM0055) and incubated at aerobic condition at 37 °C for 24 h to get a pure culture. After incubation, pure colonies were stained using Gram stain reagent (BD Difco BBL™ ref. 212525 (Gram Crystal Violet), 212542 (Gram Iodine), 212527 (Gram Decolorizer), 212531 (Gram Safranin) and bacteria were differentiated based onGram reaction (Gram-positive or Gram-negative), cellular morphology and arrangements of the bacteria.Additional catalase test (Hydrogen Peroxide 3%) was done for Gram positive cocci, followed by coagulase test for catalase positive.

2.2.Bacteria Identification

Identification has been done by automatic method (Vitek® 2 Compact, Biomérieux, France). Gram positive and negative bacteria were identify using GP ID card and GN ID card respectively (Biomérieux, France)

3. Result and Discussion

Relatively up to date data on the etiology of bovine mastitis are available in other subtropical countries, mainly Europe America, Australia and Japan [8,9,10], but not in tropical country like Indonesia. Management conditions in Indonesia greatly differ from management systems in developed countries, in particular the feeding and milking management also the high temperature and humiditycontributing risk factorfor developing mastitis.Hence, the species and pathogenicity of pathogens in Indonesia may be differed from those in other countries.

A total of 12 mastitis milk samples were collected and streaked on the sheep blood agar. The examination was conducted on the growth, morphologic features such as colony size, shape, colour and haemolytic characteristics as presented in Table 1. There were 3 samples showed the mixed growth of two bacteria species, whereas the others only showed single bacteria species. Four isolates were positive in the catalase test, indicating that the bacteria were respire using the oxygen as aerobic or facultative anaerobic bacteria. The catalase negative bacteria may be anaerobe or they may be facultative anaerobes that only ferment and do not respire using oxygen as terminal electron acceptor. Four catalase positive bacteria that have been further tested were negative in the coagulase test. Coagulase negative bacteria have become common bovine mastitis causing pathogen in many countries and could therefore be described as emerging mastitis pathogens [11].

Identification of bacteria species from all samples were conducted by Vitek as shown in Table 2. The identified mastitis-causing bacteria were all gram positive Streptococci and Staphylococci bacteria. The *Streptococcus* bacteria found were *Streptococcus uberis* (2 isolates), *Streptococcus sanguinis* (6 isolates), *Streptococcus dysgalactiae* ssp *dysgalactiae*(1 isolate), *Streptococcus mitis* (1 isolate) and *Streptococcus agalactiae* (1 isolate), whereas *Staphylococcus* isolates comprising of *Staphylococcus simulans* (1 isolate) *and Staphylococcus chromogens* (3 isolates). Evaluation on the occurrence of mastitis and identification of causing pathogen had been conducted in many countries. In Indonesia, previous studies in 2010 atthe dairy farms in West and Central Javareported that*Staphylococcus aureus* and *Streptococcus agalactiae*were the major pathogen in mastitis milk with the prevalence rate 8.5% and 37.5% [12]. However in the present study, the *Staphylococcus aureus* is not present from all isolates. Previous study of Sugiri and Anri [12] was using a biochemical method to identify the mastitis causing bacteria, whereas we use Vitek in this study. Wallet et al [6] and Crowley et al [7] stated that Vitek system provided rapid and accurate bacteria identification. *Staphylococci* comprises 45 species and 21 subspecies which are known to be the main etiologic agents of mastitis in dairy cows in worldwide [13]. Nonetheless, the clinical relevance of *Staphylococcus aureus* when isolated and cultured from mastitis milk are remained debatable. Some consider the *Staphylococcus aureus* as the major mastitis pathogens [14] and the others regard these bacteria as minor pathogen in bovine mastitis [15,16]. Therefore, we conducted an identification of coagulase negative Staphylococcus (CoNS) at the species level using Vitekto develop the effective control strategies for CoNS in bovine mastitis. Currently, our data showed that the *Staphylococcus chromogenes* was the most frequent isolated CoNS, indicating that this bacteria probably the most predominant CoNS causing mastitis in dairy cattle in Central Java. Previously in Iran, Hosseinzadeh and Saei [13] reported that *Staphylococcus haemolyticus* (40.7%) and *Staphylococcus chromogenes* (15.7%) were the predominant CoNS in bovine mastitis cases. The catalase negative bacteria *Streptococcus uberis, Streptococcus sanguinis, Streptococcus dysgalactiae*ssp*dysgalactiae, Streptococcus mitis, Streptococcus uberis,* and *Streptococcus agalactiae* can cause persistent infections, which result in increased somatic cell count and decreased milk quality. The CMT results for those catalase negative bacteria found were varied from +++ to ++++, which can be estimated containing 1.200.000 to 5.000.000 somatic cell/ ml milk [17]. Based on current knowledge, it is difficult to know whether the coagulase negative bacteria behave as environmental or as contagious pathogens. Physiologically, lactoferrin secreted from secretory cells of mammary gland may act as antibacterial against the infection, thus, the lactoferrin concentration in milk will be higher during mastitis infection [18].Dried manure, bedding and the decreased teat sphincter patency due to the high humidity or late lactation can be the main factors of intramammary bacteria infection. Biosecurity against contagious mastitis pathogens such as post milking teat dip disinfection reduce coagulase negative species in the herd and will ultimately reduce the number of somatic cells in milk [19]. The continuous

monitoring of the bacteria found in current study in herds is needed. Moreover, special attention on the prevention and control of mastitis pathogens need to be placed in the top priority.

Table 1. California Mastitis Test Result, Morphologic features and characteristics of Isolates.

Sample number	CMT result	Colony types	Morphologic features	Haemolytic characteristics	Catalase test	Coagulase test
1	+++	Mixed growth	Gram positive, coccus, translucent white	Weak hemolyis	Positive	Negative
			Gram positive, coccus and twisted (like a chain), translucent white	No hemolysis	Negative	-
2	+++	Mixed growth	Gram positive, coccus, translucent white	Weak hemolysis	Negative	-
			Gram positive, coccus, yellow	β- hemolytic	Positive	Negative
3	+++	Single species	Gram positive, coccus, white	β- hemolytic	Negative	-
4	+++	Single species	Gram positive, coccus	α-hemolytic	Negative	-
5	+++	Mixed growth	Gram positive, coccus and twisted (like a chain), translucent white	No hemolysis	Negative	-
			Gram positive, coccus, yellow	β- hemolytic	Positive	Negative
6	+++	Single species	Gram positive, coccus and chained, white	α-hemolytic	Negative	-
7	++++	Single species	Gram positive, coccus and chained, white	α-hemolytic	Negative	-
8	++++	Single species	Gram positive, coccus and chained, white	α-hemolytic	Negative	-
9	++	Single species	Gram positive, coccus, yellow	β- hemolytic	Positive	Negative
10	++++	Single species	Gram positive, coccus and chained, white	α-hemolytic	Negative	-
11	++++	Single species	Gram positive, coccus and chained, white	β- hemolytic	Negative	-
12	++++	Single species	Gram positive, coccus and chained, white	α-hemolytic	Negative	-

Table 2. Identification of mastitis causing pathogens.

Identification Result	Number of isolate (s)	Prevalence
Staphylococcus simulans	1	6.7 %
Staphylococcus chromogens	3	20%
Streptococcus uberis	2	13.3%
Streptococcus sanguinis	6	40%
Streptococcus dysgalactiae ssp *dysgalactiae*	1	6.7 %
Streptococcus mitis	1	6.7 %
Streptococcus agalactiae	1	6.7 %

Conclusion

In conclusion, *Streptococcus* is predominant and the coagulase negative *Staphylococcus* species is identified at the least mastitis causing pathogens. The mastitis causing pathogens are *Streptococcus uberis*, *Streptococcus sanguinis*, *Streptococcus dysgalactiae* ssp *dysgalactiae, Streptococcus mitis* and *Streptococcus agalactiae.*

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