Metagenomic analysis of pathogen mastitis in cow's milk from Cicurug, Sukabumi, West Java, Indonesia

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Abstract. Mastitis is a complex disease in cattle that involves interactions between management practices and infectious agents. The common microorganisms causing mastitis are bacteria, besides this disease can be caused by mycoplasma, algae, and yeast. Pathogen microorganisms in milk can be obtained from cattle, human hands, equipment, and the environment. This study aims to analyze the metagenomic of pathogen mastitis in cow's milk from Cicurug, Sukabumi, West Java. ZymoBIOMICS™ DNA Miniprep Kit was used for genome isolation to metagenomic analysis. The 16S rRNA PCR amplification was used for analysis the results of miniprep. Metagenomic analysis from subclinical mastitis milk showed that bacteria in cow's milk were the genera of Corynebacterium (20.53%), Corynebacterium (11.67%), Solibacillus (8.78%), Romboutsia (5.45%), Micrococcus (4.18%), Acinetobacter (3.64%), Aerospheara (1.94%), Ignavigranum (1.90%), Lysinibacillus (1.49%), and Staphylococcus (1.38%).

1. Introduction
Mastitis is a complex disease in cattle that it involves interactions between infectious agents and management practices. Mastitis can cause changes in milk quality, milk quantity, and milk's physicochemical characteristics [1]. Pathogen microorganisms in milk come from the cow itself, equipment, human hands, and the environment. Pathogen microorganisms enter through the nipple ducts and attack the udder tissue causing an inflammatory response so that causes changes in the milk. Transmission of mastitis mostly occurs during the milking process through contaminated milking machines, clothes, and hands of machine operators [2]. Some factors contributing to mastitis incidence in cows are pathological, physiological, environmental, and genetic [3].

The main microorganisms causing mastitis are bacteria, other else are algae, mycoplasma, and yeast. The pathogens causing mastitis are classified as environmental and contagious pathogens which depend on the main reservoir and the transmission mode. Contagious pathogens are commonly found in the udder and nipples of infected cows so they are the main infection source during the milking process.
Environmental pathogens are found in the environment around livestock, such as pens, bedding, manure, soil, feed, and contaminated water [4, 5]. Common contagious pathogens causing mastitis are *Streptococcus agalactiae*, *Staphylococcus aureus*, *Mycoplasma* spp., and environmental pathogens include *Streptococcus dysgalactiae*, *Streptococcus uberis*, and Coliform bacteria (*Klebsiella pneumoniae*, *Escherichia coli*, and *Serratia*) [4].

The proportion of pathogens varies between farms, regions, and countries due to differences in animal cage systems and livestock management [3]. Isolation of pathogenic mastitis is a basic aspect of the control program of udder health and milk quality monitoring. This is related to food safety and public health issues connected to foodborne pathogens found in milk and milk products [6]. This study aims to analyze the metagenomic of pathogen mastitis in cow's milk. Through this metagenomic analysis, a diversity of pathogenic bacteria would be found in subclinical mastitis cow's milk.

2. Materials and Method

2.1. Study area and sample preparation
Milk samples were obtained from Cicurug Subdistrict, Sukabumi District, West Java, Indonesia. Milk was stored at iceboxes until observation in the laboratory. The volume of fresh milk was about 30 mL, collected in sterilized tubes. The milk was examined using the IPB-1 mastitis test to determine positive and negative mastitis. The research was conducted at the Laboratory of Applied Genetic Engineering and Protein Design, Research Center for Biotechnology, Indonesian Institute of Sciences, Bogor, West Java, Indonesia.

2.2. Genome isolation for metagenomic analysis
ZymoBIOMICSTM DNA Miniprep Kit (Zymo Research, USA) was used for genome isolation. For about 5 mL of milk was centrifuged at 1000 xg for 10 minutes. The supernatant was transferred into a lysis tube and centrifuged at 6000 xg for 10 minutes. Pellet was added by 750 μL of lysis solution and mixed using vortex for 20 minutes. Centrifugation at 10000 xg; for 1 minute, was used for solution separation. Supernatant for about 400 μL was moved into a zymo filter in a microtube then centrifuged at 8000 xg for 1 minute. The filtrate was mixed with DNA binding buffer for the amount 1200 μL. The mixture was moved to the IICR column then centrifuged at 1000 xg. 1 minute. DNA wash buffer was added as much 3 times (400, 700, and 200 μL) followed by 1-minute centrifugation at 10000 xg for each addition. The IICR column was transferred to a clean microtube. DNA wash buffer was added as much 3 times (400, 700, and 200 μL) followed by 1-minute centrifugation at 10000 xg for each addition. The IICR column was transferred to a clean microtube. The ICR column was added 50 μL of DNase/RNase Free Water and incubated for 1 minute. Centrifugation at 10,000 xg for 1 minute was used to elute the DNA. The concentration of the result was measured using a nanophotometer.

2.3. 16S rRNA PCR analysis
A pair of primers for 16S rRNA PCR analysis was primary sequences primer 8F (5'AGAGTTGTGATCATGGCTCAG-3') and 16R (5'AAGGAGGTGATCCACACGGCA-3'). The PCR mix was ddH2O 6.4 μL, MyTaq DNA Polymerase 0.2 μL, 5 x MyTaq buffer 2 μL, primers 8F 0.2 μL, primers 16R 0.2 μL, DNA template 1 μL with total volume was 10 μL. The PCR conditions were 95°C 5 min; 35 cycles consisting of 95°C 1 min, 55°C 3 min, and 72°C 1 min; and 72°C 7 min. Agarose gel electrophoresis on 1% (w/v) was used to analyze of PCR products with marker 100 bp DNA Ladder as a standard DNA size. The agarose gel was stained by ethidium bromide staining and visualized under a UV transilluminator.

2.4. Sequencing analysis of metagenomic
Sequencing analysis of metagenomic was conducted at Novogene CO., Ltd Japan using Illumina (MiSeq) platform (paired-end reads). Paired-end reads were merged by FLASH [7]. Sequencing analysis used primer 341F (5'-CCTAYGGGBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAAT-3'). Filtering of quality on the raw tags was done in specific filtering conditions so it can acquire high-quality clean tags based on the quantitative insights into microbial ecology (QIIME) (V1.7.0) [8, 9]
quality-controlled process. The algorithm of UCHIME was performed to detect chimera sequences [10]. Uparse software was for the analysis of sequences [11]. References from the SILVA database were for the OTU similarity alignment analysis [12].

3. Results and discussion
Mastitis is udder inflammation that emerges because of various infectious agents. Mastitis is grouped into clinical mastitis and subclinical mastitis. Clinical mastitis is easily detected due to the appearance of clinical symptoms; otherwise, subclinical mastitis is laborious to detect for the absence of lesions. Clinical mastitis is characterized by udder inflammation and physical changes in milk whereas subclinical mastitis is looked like milk normally and no signs of udder inflammation, but inflammatory reactions can be detected [13]. The mastitis test results on cow's milk samples showed that 4 samples of subclinical mastitis were positive (+/++) and 3 samples were normal (Figure 1). Positive mastitis is characterized by a change in the milk viscosity.

![Figure 1. Mastitis test of cow's milk samples. (a) Milk with positive mastitis +/++ (Number 1, 2, 3, 4). (b) Normal milk (Number 5, 6, 7).](image)

Diagnosis is the key in the mastitis case. The golden standard in the diagnosis is the identification of bacterial pathogens in mastitis milk [14]. The best approach to mastitis treatment is identifying the causative agent due to the risk of antibiotic resistance and its multifactorial etiology [15]. Methods to identify bacteria in mastitis milk were by molecular analysis. In this study, the results of genome isolation from 2 samples of subclinical mastitis milk (numbers 1 and 4) were analyzed using 16S rRNA PCR. 16S rRNA PCR analysis using primer 8F and 16R primers can be seen in Figure 2. The concentration of genomic isolation was measured using a nanophotometer. The result concentration was 28.4 ng/µl (number 1) and 8.6 ng/µl (number 4). Based on PCR amplification results, a band of 1500 bp was obtained from 2 samples of subclinical mastitis milk. The genome isolated from 2 samples of subclinical mastitis milk was successfully carried out.

![Figure 2. 16S rRNA profile of subclinical mastitis milk. 1. Milk number 4; 2. Milk number 1.](image)

Metagenomic analysis can be used to describe components in milk microbiome. Metagenomic mapping of bacteria contained on sample subclinical mastitis milk (number 1) was analyzed through
this research. Figure 3 and Figure 4 are results of analysis metagenomic data from bacteria contained on subclinical mastitis milk. In the taxa-phylum, bacteria are dominated by Actinobacteria (36.38 %), Firmicutes (20.94%), and Proteobacteria (3.63%). In the taxa-class, bacteria are dominated by unidentified_Acinobacteria (36.38%), Bacilli (15.49%), Clostridia (5.45%), and Gammaproteobacteria (3.63%). In the taxa-order, bacteria are dominated by Corynebacteriales (32.19%), Bacillales (11.65%), Clostridiales (5.45%), Micrococcales (4.18%), and Lactobacillales (3.84%). In the taxa-family, bacteria are composed of Corynebacteriaceae (32.19%), Planococcaceae (10.27%), Peptostreptococcaceae (5.45%), Micrococcaceae (4.18%), Aerococcaceae (3.84%), Moraxellaceae (3.63%), Ruminococcaceae (3.42%), Carnobacteriaceae (2.63%), Actinomycetaceae (2.36%), and Staphylococcaceae (1.38%). In the taxa-genus, bacteria are composed of Corynebacterium_1 (20.53%), Corynebacterium (11.67%), Solibacillus (8.78%), Romboutsia (5.45%), Micrococcus (4.18%), Acinetobacter (3.64%), Aeronaera (1.94%), Ignavigranum (1.90%), Lysinibacillus (1.49%), and Staphylococcus (1.38%). The bacterial species detected on the metagenomic analysis were Corynebacterium variabile, Micrococcus lylae, Acinetobacter harbinensis, Acinetobacter soli, and Acinetobacter ursingii.

Figure 3. The relative abundance of bacteria in subclinical mastitis milk. A. Relative abundance of taxa-phylum; B. Relative abundance of classes; C. Metagenomic of the family; D. Metagenomic of the genus.
Figure 4. Phylogenetic of bacteria in subclinical mastitis milk. Phylogenetic of bacteria found in subclinical mastitis milk based on the metagenomic analysis. The larger circle/sign indicates the greater number of bacteria at each taxon level which is also indicated by a percentage.

Microbiota in milk has complex microbial communities because of the contamination source multiplicity [16]. The pathophysiology of bovine mastitis was influenced by milk microbiomes [17]. The metagenomics method used in this study successfully identified several bacterial from subclinical mastitis milk. This method allows a more complex view of the composition of the microbiota of subclinical mastitis milk. The metagenomics results in the taxa-phylum bacteria were showed dominated by Actinobacteria, Firmicutes, and Proteobacteria. This result is similar to previous research [18] that showed Firmicutes and Proteobacteria as the predominant phyla observed in the milk metagenome of subclinical mastitis milk.

Based on the metagenomic analysis, the genus of *Corynebacterium* and *Staphylococcus* are the agents causing mastitis disease. *Corynebacterium* spp. are a pathogen related to subclinical mastitis in dairy cows [19] and often being mentioned as a contagious pathogen. To identify specific *Corynebacterium* spp. species in bacterial culture are occasionally difficult because of their nature of slow-growing [20]. *Corynebacterium* spp. was checked by a DNA sequencer [21]. The genus of *Staphylococcus* is a potential agent of mastitis. *Staphylococcus aureus* is one of the contagious pathogens as a cause of mastitis. It has pathogenic characteristics (exotoxins) and is transmitted to the teat easily. This species is especially important because of the generation of resistance to antibiotics.
[22]. S. aureus is one of the main pathogens of subclinical and clinical mastitis in dairy cows of various countries [3]. Mastitis due to S. aureus is clinical and also become subclinical if the absence of clinical changes in mammary glands [23].

These study findings may help formulate strategies of prevention and treatment for mastitis and reduce economic losses. With the identification of bacteria types in subclinical mastitis milk through metagenomics, the treatment and use of antibiotics can be appropriate for pathogenic agents. Identifying the pathogens causing mastitis is important to know the route of spread, evaluate the transmission of mastitis cases, determine the prognosis and number of cows affected. This is needed for decision making, determination of preventive actions and appropriate vaccination programs to be implemented in dairy farms.

4. Conclusion
Mastitis is an important disease of cattle that multi-etiological in nature. Identification of bacterial pathogen provides important information to prevent and control mastitis disease. Metagenomics of cow milk samples with mastitis subclinical showed microbiome composition. Metagenomic of the genus in bacteria isolated from subclinical mastitis milk from Cicurug, Sukabumi is composed of Corynebacterium _1 (20.53%), Corynebacterium (11.67%), Solbacillus (8.78%), Romboutsia (5.45%), Micrococcus (4.18%), Acinetobacter (3.64%), Aerosphaera (1.94%), Ignavigranum (1.90%), Lysinibacillus (1.49%), and Staphylococcus (1.38%).

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