Mammary Gland Health and Identification of β-defensin Gene of Holstein-Friesian Cows

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Abstract. β-Defensin in bovine had known as an antimicrobial peptide group with a broad-spectrum activity. The defensin peptide found abundant in neutrophils and epithelial cells including in the mammary gland. The objectives of this research are to identify the β-defensin gene and mammary gland health status. Blood and milk samples were obtained from various lactated cows located from several farms in Bogor. The research consisted of two main activities including collecting blood and milk then laboratory examinations which carried out through amplifying genomic DNA fragments of β-defensin using polymerase chain reaction (PCR) method and milk analysis through somatic cell count. Therefore, based on these results, the β-defensin are found and identified in all samples with a low or high number of somatic cell. Hence, the genetic diversity of β-defensin gene need to be calculated to be used as genetic marker prior to select dairy cattle with increased resistance to infection of the mammary gland method. Other defensin genes need to be hold to assisted breeding programmes in accordance with mammary gland health.

1. Introduction
Nowadays, milk consumption has rapidly increases due to public awareness of nutritional need obtained from milk. High protein content of milk can be used as functional drink for improving health status. Unfortunately, there is only 30% of local need fulfilled with national production. Low number of cow population, milk production and milk yield due to udder health infection worsen the condition. Mastitis or udder infection considered as one of the most prevalent diseases in dairy cattle. It is reported causes great impact of economic losses to the dairy industry worldwide [1]. Mastitis is classified as clinical mastitis and subclinical mastitis. The incidence rate of subclinical mastitis was greater than clinical mastitis. Mastitis is mainly caused by pathogens, however, unprincipled management practices, health status of dairy cattle and genetics factor also took a part.

A considerable amount of association and quantitative trait loci (QTL) related to udder health have been studied in large farm animals due to its economic impact to milk production. However, the implementation progress has been relatively slow. Mastitis is the most frequent and important disease which infects the udder in dairy cattle. Therefore, it is considered important in improving the resistance of mastitis as breeding objective. Resistance to mastitis classified as a prime candidate for...
marker-assisted selection (MAS) in breeding programmes. Milk yield and somatic cell count (SSC) are known as indicator traits for this selection [2].

According to the database, there are 943 genes and genetic markers involved in mammary gland development and function [3]. One of them, defensin genes, is considered to be an outcome for selection against natural pathogen. Defensin is a group of peptide that plays an important role in innate immunity in livestock. β-defensins have been found in cells and various tissue such as neutrophils, leucocytes, epithelial cells, urine and blood plasma [4]. When inflammation occurred, neutrophils and monocytes secrete defensin as microbicidal agents. Defensins also contribute in adaptive immunity during microbial infection by disrupting the cytoplasmic membrane function and integrity [5]. Defensins well known for the ability as broad-spectrum antibiotic and also viruses and fungi [6]. Defensins main role in inflammation and immunity are known as host-defense peptide which function as mediators between innate and acquired immune mechanism [7].

Bovine β-defensins (tracheal antimicrobial peptide, lingual antimicrobial peptide and bovine neutrophils β-defensins) showed antimicrobial activity against bacteria such as Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, and Candida spp. which is known as common pathogens causing mastitis [8]. Besides, bovine β-defensins (TAP, LAP, BNBD1–13) also found in reproductive tracts in cattle and play an important role against infection during parturition and might be helpful in increasing resistance of reproductive system in Holstein Friesian dairy cows [9, 10]. Defensin has 29-49 amino acids and 3.5-6 kDa of molecular weight. It is small but rich in cysteine peptides whose structure is stabilized by three disulphide bounds, formed by six strongly conserved cysteines [11]. In mammals, defensins are divided into three classes, the α-, β- and θ-defensins, but the θ-defensins were found only in plants and some primates [12]. It is based on different spacings of the six cysteine residues and arrangement of the disulfide bonds [13]. The gene encoding defensin (all α- and most β-defensin) occur in a cluster at the chromosome 8p23.1 of human genome, while in bovine was mapped to bovine chromosome 27. The genomic structure of bovine b-defensins consists of two exons and one intron of approximately 1.5 kb [14] and they are located in the same cluster in synteny group U25 on chromosome 27 [15].

Based on previous studies, innate immunity and genetic variety of β defensin could be used as tool selection for breeding programs. Many genes associated with mastitis including β-defensin has been researched, yet, there is no related report in Indonesia till date. So, the purpose of this study was to identify β defensin gene and udder health status of Holstein Friesian cattle.

2. Materials and methods

2.1. Animals and Data Samples

A total of 44 Holstein-Friesian dairy cows between first and six lactation from several farms in Bogor (Kunak, Tegalwaru and Cibinong) were used randomly as DNA resources for genotyping of defensin gene. Three milliliters of blood were collected from coccygeal vein of each individual cow into vacutainer tube containing K3EDTA. Genomic DNA isolation was performed using Genomic DNA mini kit (Geneaid Biotech Ltd., Taiwan) by following to the manufacturer’s instructions. The collected DNA was used for genotyping by Polymerase Chain Reaction and then stored at -20°C for further use.

2.2. Genotyping

Amplification of the defensin gene along 429 BP uses primary forward 5-TGCTCCCATTGCTCCATAGAT-3; reverse 5-CCCCCTCTCCTCCCTTTTGT-3. A fair amount of 10 μl reaction mixture containing 5 μl PCR Master Mix (My Taq HS Red mix Bioline, USA), 1 μl each of primer (10 pmol/μl), 2 μl water free nucleases and 1 μl DNA template were used to perform PCR. Thermal cycling (Eppendorf, Germany) was carried out by following program pre-denaturation 94°C for 5 minutes, 34 cycles of denaturation at 94°C for 45 seconds, annealing 63.5°C for 45 seconds, extension 72°C for 1 minutes and final extension 72°C for 10 minutes. The amplified DNA fragments
were separated using 1% agarose gel (100 V, 60 minutes), stained with GelRed and visualized under UV light by UV Transilluminator (Major Science, USA).

2.3. Milk Analysis
Milk samples from each cow were collected to determine milk yield and SCC data. Milkoscan (FOSS A/S) was used to determine milk fat, protein, and lactose concentration. Somatic cell count were counted by means of a cell counter apparatus Fossomatic (FOSS A/S).

3. Result

3.1. Genotyping
The result of amplification of β-defensin gene by PCR was successful. Amplification of 50 DNA samples from all of HF dairy cattle resulted fragment lengths of 429 bp. The success of amplification of the defensin gene was determined by some factors, such as annealing temperature, primer concentrations, and targeted DNA concentration. Determination of defensins gene base pair in Holstein-Friesian cow was done by predicting through complete sequence data from the Bos taurus enteric beta-defensor (EBD) gene stored in GenBank (NCBI) with access number AF016539. Complete sequence data from the Bos taurus gene enteric beta-defensin (EBD) has 2701 base pairs. Complete sequence data from the Bos taurus enteric beta-defensor (EBD) gene, matched with the primary defensin gene owned {Primary 7 Forward (5'-TGC TCC CAT TGC TCC ATA GAT-3')} and {Primary 7 Reverse (5'-CCC CCT CCT TCC TTT GT -3')}. Based on primary matching with complete sequence data from the Bos taurus enteric beta-defensor (EBD) gene No.AF016539, the estimated product length is 429 bp (Figure 1).

3.2. Milk Yield and Somatic Cell Count
The interaction between milk yield including protein, fat, total solid, solid non-fat, lactose, density and acidity with somatic cell count from three farms in Bogor presented on Table 1.

4. Discussion
Tropical climate with hot weather and high humidity like in Indonesia is very favorable to disease causing microorganisms and parasite proliferation. Innate immunity is considered to be an outcome of natural selection and plays an important role against natural pathogens in livestock. Udder health and resistance to mastitis can be established by several antimicrobial peptides including defensin. Although mammary gland health not only as the result of some genes such as defensin, but also other factors such as environmental condition, management of rearing, age and lactation period, it is necessary to carried out this research along with the limitation. Previous studies showed that
polymorphisms in β-defensins genes could be used for breeding programs as selection for milk production trait and to detect cows with high resistance to mastitis [16,17]. Association on β-defensin polymorphisms to production traits and diseases in human, sheep and cattle have been published worldwide [17,18]. The aim of this study was to identify between β defensin gene and mastitis resistance with the result of milk yield and somatic cell count as indicators of udder health.

### Table 1. Number of observations on milk yield and somatic cell count

| Farm | Protein (%) | Fat (%) | Total Solid (%) | Solid Non Fat (%) | Lactose (%) | Density (g/mL) | FDP (°C) | Acidity (°SH) | SSC (thousand cell/mL) |
|------|-------------|---------|-----------------|------------------|-------------|---------------|----------|-------------|-----------------------|
| 1    | 3.04±0.99   | 1.71±1.31| 9.73±3.08       | 7.32±3.08        | 3.70±1.34   | 1029.71±9.95 | 0.45±0.16 | 6.69±2.36   | 381.12                |
| 2    | 3.15±0.83   | 0.95±0.69 | 9.12±2.15       | 8.00±1.99        | 3.73±1.16   | 1029.09±8.24 | 0.48±0.10 | 6.68±1.96   | 334.04                |
| 3    | 3.24±0.81   | 1.59±0.83 | 9.55±2.37       | 7.74±2.15        | 3.41±1.37   | 1026.25±8.72 | 0.43±0.17 | 6.74±2.19   | 415.02                |
| SNI  | 2.8         | 3.0     | 10.8            | 7.8              | -           | 1027         | -0.520    | 6.0-7.5     | 400                   |

The PCR product of β defensin gene fragments with primers DEF1A and DEF1B resulted in a 429 bp. It was identical with the fragment of the β1-defensin (enteric) gene (position 1441-1869; GenBank AF016539) except for a G/A transition at position 1615, the nucleotide substitution indicating the new defensin gene polymorphism [16]. PCR-RFLP analysis with TaqI restrictase enzyme characterized by bands arranged specifically in pairs, arbitrarily marked A1 A2 , B1 B2 , and C1 C2. Based on these results it is hard to specify whether it was polymorphism at one or several gene loci of various defensins, or which particular combined defensin genotypes (CDGs) [16]. In this research, a 429 bp gene fragments was successfully amplified with PCR. The mutation should be analyzed and identified by adding restriction enzyme to the PCR product. The finding of defensin PCR products yield was different with Ryniewicz et al (2003) finding which describe defensin in 1300-1650 bp including 1638 bp fragment, exons 1 and 2 and intron 1, of β1DEF gene [16]. Other sequence of the bovine β4-defensin gene (GenBank no. AF008307) amplified the fragments in the 924-bp or 393-bp. The PCR RFLP with NlaIII (Hin1II) from 212 samples showed CC genotype was the most common (72%), the heterozygous CT genotype was found in 26% of the genotyped cows and four cows (2%) were TT homozygotes. The statistical analysis found there are significant associations between the RFLP-NlaIII and milk fat, protein and lactose contents. This genotype also have correlation on the somatic cell count in the milk [17].

In sheep, defensin analysis (β-Defensin 1 gene and β-Defensin 2 gene) showed two SNPs identified in β-Defensin 2 gene coding region, at position 1659 and position 1667, were nonsynonymous, leading to amino acid changes in the protein product. This substitutions may not have effect on β-defensin 2 protein function [18]. Furthermore, SBD2 mutant sequence shows changes in mRNA secondary structure. According to RNA structure software, SBD1 SNPs did not affect the mRNA shape whereas both SBD2 SNPs (at position 1750 and 1761). Therefore, the structural difference in SBD2messenger RNA may be related to a possible role in translation efficiency with a modulation in the protein produced amount [20]. The results suggest that identified SNPs could play a role in the modulation of the immune response.

In addition to genetic variation, there are several factors related with milk production yield such as diseases occurrence, age, period of lactation, climate/season, or feed intake [21]. In particular, somatic cells such as leukocytes and epithelial mammary cells occurs in milk in quite large number. This somatic cells actively protect mammary gland against infectious disease caused by bacterial, viruses or fungi due to high susceptibility to inflammation and infection [22]. This number of somatic cells will increased rapidly at the time bacterial infection arise. Furthermore, milk yield, milk composition and milk quality affected by bacterial infection is well documented [23].
In this study, milk yield from three farms in Bogor showed number of protein appeared higher than SNI while fat is lower. Low measurable fat content can be in consequence of inadequate feed intake, age, period of lactation, season and milking time. The mean daily milk yield and fat, protein and lactose content of milk in investigated group of cows was around the SNI number while SCC was below the SNI except for farm 3. However, high lactose content might indicated that the cows suffered neither from sub-, nor clinical mastitis. In this study, there are no clinical mastitis samples but some cows showed higher number of SCC than SNI. Indeed it has been shown in sheep that animals bred for low somatic cell count possess a better ability to limit and eliminate mastitis infections than their high SCS counterparts [18].

The circumstances of defensin gene as markers related to mastitis and udder infection has been reported widely [16, 17]. This conclusion is due to expression of defensin in the cow’s udder. However, defensins that are recognized as one of the innate defensive response systems against the pathogenic microorganisms can affect the quality of cow milk. As well as the milk production in correlation with the defense system especially the innate immune system against the bacteria which infect the mammary glands [24]. Other genes reported to be responsible in increasing of somatic cell count is TLR4 gene. The effect of polymorphism of TLR4 gene on somatic cell score (SCS) was analyzed, the results indicated that the cattle with allele A in T4CRBR1 showed lower somatic cell score than that of allele B (P < 0.05). In short, the allele A might play an important role in mastitis resistance in bovine [25]. This result is against the statement that genetic correlations between milkability and clinically recorded mastitis were negative or almost zero (-0.50 - 0.02). The genetic correlation among udder health traits was moderately (0.31 - 0.49). The milkability and leakage were not genetically correlated with milk production [26]. The significant effect of the polymorphism of defensin genes on milk productivity performance trait found in Friesian (high milk production) and Egyptian (low milk production) cows may lead to the use of defensin genes as genetic marker(s) in the breeding programmes aiming at selecting highly productive dairy cattle with increasing resistance to udder infections [27].

The β defensin gene on udder health performance trait found in this study. The polymorphism of this gene need to be conducted and the result might lead to the use of defensin as genetic marker for breeding programmes in Indonesia.

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