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Toll-Like Receptor 4 Gene Diversity, Its Potential as a Molecular Marker in Indonesian Dairy Cattle

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Abstract: Toll-like receptor 4 (TLR4) gene plays a role in pathogen recognition and regulation of the immune system. The identification of TLR4 gene diversity has been studied in several livestock commodities. However, there is a lack of information on the polymorphism of this gene in Friesian Holstein (FH) dairy cows that have adapted to the Indonesian environment, and its relationship with several breeding parameters is not yet known. This research was conducted to determine the genotype diversity of the TLR4 gene in Indonesian FH cattle and its potential as a genetic marker for several breeding parameters. Polymorphisms were detected using the polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) technique in 100 deoxyribonucleic acid (DNA) samples, and mutation locations were confirmed by sequencing. The records from cows were used to obtain information on milk quantity, milk quality, and reproductive parameters. Estrogen levels during estrous phase were measured from serum using the ELISA method. The relationship of genotype on breeding parameters was tested using the procedure of the general linear model. Genotyping results with the AluI restriction enzyme produced three types of genotypes (CC, CT, and TT) of the TLR4 gene. The population was in Hardy-Weinberg equilibrium, with the dominant genotype being CT. TLR4|AluI gene diversity was correlated ($P<0.05$) with milk quantity but not with milk quality, estrogen level, or reproductive parameters. In conclusion, the TLR4|AluI gene in the Indonesian Friesian Holstein cattle population was polymorphic. This research reveals the potential of genes to be considered genetic marker candidates for superior milk quantity. The findings of this study can serve as preliminary information for a molecular technology-based dairy cattle selection program.

Keywords: dairy cattle, genotype, sequencing, toll-like receptor.

收费-与受体 4 基因多样性一样，其作为印度尼西亚奶牛分子标记的潜力

摘要：收费样受体 4 (TLR4) 基因在病原体识别和免疫系统调节中发挥作用。TLR4 基因多样性的鉴定已在多种牲畜商品中进行了研究。然而，目前缺乏关于适应印尼环境的弗里斯兰荷斯坦（跳频）奶牛中该基因多态性的信息，其与几个育种参数的关系尚不清楚。本研究旨在确定印度尼西亚跳频牛 TLR4 基因的基因型多样性及其作为多个育种参数的遗传标记的潜力。采用聚合酶链式反应-限制性片段长度多态性（聚合酶链式反应-限制性片段长度多态性）技术对 100 份脱氧核糖核酸（脱氧核糖核酸）样本进行多态性检测，并通过测序确认突变位置。奶牛的记录用于获取有关牛奶数量、牛奶质量和繁殖参数的信息。使用酶联免疫吸

附试验方法从血清中测量动情期的雌激素水平。采用一般线性模型的程序检验了基因型与育种参数的关系。铝限制性内切酶的基因分型结果产生了 TLR4 基因的三种类型的基因型（抄送、CT 和 TT）。群体处于哈迪-温伯格平衡，优势基因型是 CT。TLR4|铝基因多样性与乳量相关（磷<0.05），但与乳质、雌激素水平或生殖参数无关。总之，印度尼西亚弗里斯兰荷斯坦牛群体中的 TLR4|铝基因具有多态性。这项研究揭示了基因有可能被视为优质产奶量的候选遗传标记。 这项研究的结果可以作为基于分子技术的奶牛选择计划的初步信息。

关键词：奶牛，基因型，测序，收费样受体。

1. Introduction

Toll-like receptor 4 (TLR4) was the first discovered member of the family of toll-like receptors (TLRs), which are type 1 transmembrane receptors and are present in a variety of organisms [1]-[3]. In cattle, ten distinct functional TLRs (TLR1 to TLR10) have been found and mapped, with each TLR identifying different pathogen-related molecular patterns. TLR4 is one of these genes that has been linked to mastitis resistance and innate immunity [3], [4].

The TLR4 gene is a receptor that recognizes gram-negative bacterial endotoxins and is involved in pathogen detection [5], [6] as well as the activation of inflammatory and immunological responses [1]. The bovine TLR4 gene is found on chromosome 8 [7], it consists of a 5'-untranslated region (UTR), complete coding sequence, 3'-UTR, encodes 841 amino acids [8], has 3 exons and two introns, with nucleotide length of approximately 3,739 bp [9], [10].

Polymorphism has been identified in the bovine TLR4 gene at various SNPs [4, 9, 11-14]. Research on the TLR4 gene polymorphism in several breeds of livestock has revealed influences on milk traits [11, 14], mastitis resistance and somatic cells [8, 12], endometritis resistance [5], and reproductive traits [9, 13]. A meta-analysis study on the TLR4 gene in humans showed the importance of the correlation of SNP rs4986790 and mainly gram-negative infectious agents, mediated by lipopolysaccharides, although there was no association in the results of this investigation [2]. Research on the TLR4 gene in water buffalo has detected 13 SNPs in exons 1 and 3, which are associated with somatic cell score [6], and two SNPs correlated with endometritis resistance [5].

Genetic mapping plays an essential role in the livestock genomic selection process, becoming an alternative to conventional selection due to limited data and pedigree. Information on genetic markers related to essential economic traits can be obtained by detecting gene diversity [15]. Molecular technology has been applied to livestock development strategies in various countries, including one that uses marker genes. Using candidate genes as genetic markers speeds up selection and decreases the time required to accomplish breeding targets [16], [17]. Milk trait is one of the characteristics related to economic value, and because milk is widely developed by industry into processed products, it is necessary to investigate the function of genes that can enhance this trait [18]. In addition to milk production, reproductive characteristics are currently significant in designing dairy cattle breeding programs [16]. Several genes are associated with milk traits, including the prolactin gene [19], lactoferrin [9], LOC514211 gene [18], and leptin gene [16].

Several previous studies have reported TLR4 gene polymorphisms, among others, in cattle [1], [11], [20], buffalo [5]-[6], and goats [17], [21], but these polymorphisms have not been studied in Friesian Holstein (FH) dairy cows in Indonesia. This study was designed to investigate genotype diversity in SNPs at partial exon 3 of the TLR4 gene. The detection of this polymorphism has never been reported in Indonesian FH dairy cows, and the relationship between the TLR4 gene polymorphism and breeding parameters remains unknown. The results of this study may provide scientific information that could assist dairy cows in developing better genetics.

2. Methodology

2.1. Samples and DNA Isolation

A total of 100 isolated bovine DNA samples were used in this study. The DNA genome was obtained through an isolation process from blood cows using the protocol extraction kit of Genomic DNA Mini (Geneaid, Taiwan). The female Indonesian Friesian Holstein dairy cows that provided the source of the DNA sample were selected based on the criteria of being multiparous, having milk production and reproduction records at parity I, generally healthy, and not suffering from reproductive disorders. All cows were maintained under the same management at a dairy breeding center in Baturraden, Central Java. An illustration of the research design is presented in Figure 1. The quantity and quality of the DNA produced were measured using a nanodrop spectrophotometer. The

purity and concentration of DNA were calculated on the basis of the ratio of absorbance at 260 and 280 nm wavelengths.

2.2. Primer

A pair of specific primers were used to amplify a portion of the TLR4 gene that is the location of the expected single-nucleotide polymorphism (SNP), according to [8], with the forward primer (F): 5'-AGACAGCATTTCACTCCCTC-3' and the reverse primer (R): 5'-ACCACCGACACACTGATGAT-3'. The TLR4 gene amplification target sequence is located at partial exon 3 (382 bp) according to sequencing data from the National Center of Biotechnology Information (NCBI) with gene access code DQ839567.1.

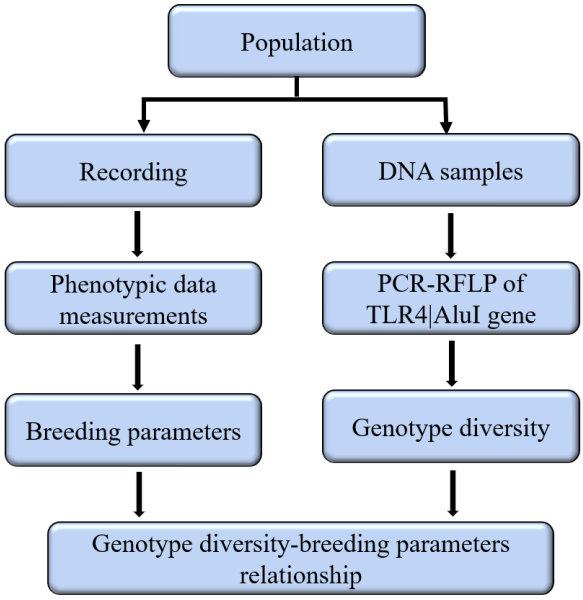


Fig. 1 Research design

2.3. PCR-RFLP Assay and Data on Breeding Parameters

Amplification of fragments of the TLR4 gene was performed using the PCR method. A PCR working solution of 50 µL volume consisting of 4 µL of DNA samples, 19 µL of water nuclease-free, 1 µL of forward primer and 1 µL of reverse primer, and 25 µL of PCR master mix. The reaction PCR mixture was inserted into a 0.2 mL PCR tube, and the complete amplification of TLR4 gene fragments was performed using a thermocycler PCR machine (Biobase). The amplification step procedures included 1 cycle of initial denaturation at 98°C 3 min; 30 cycles of denaturation at 98°C 20 s, annealing at 58°C 30 s, extension at 72°C 25 s, and termination by 1 cycle of final extension at 72°C 10 min. The electrophoresis technique using 2% agarose gel with ethidium bromide was used to evaluate the PCR results. A 50-bp marker ladder was employed to determine the amplicon length using a UV transilluminator.

Genotype detection of the TLR4 gene was performed by analysis of restriction mapping of PCR products using the AluI enzyme. As much as 10 µL PCR product of the TLR4 gene was made as a reaction mixture by adding 1µL AluI enzyme (10U/µL), 2 µL buffer, and 17 µL water nuclease-free. The solution that had been thoroughly mixed was set for 4 hours at a temperature of 37°C in a dry bath (Cleaver, UK). The fragments displayed in the electrophoresis results of 2% agarose gel were used to determine the type of genotype. Following this, forward and reverse primers were used to sequence 25 µL of PCR samples from each genotype. Sequencing was conducted at 1stBase, Selangor, Malaysia. SNPs were identified from sequenced results displayed and aligned with nucleotides from GenBank using the Molecular Evolutionary Genetic Analysis (Mega XI) program. The nucleotide sequence was then translated into amino acids to analyze the type of mutation caused by nucleotide substitution.

Breeding parameter data were collected from a recording of quantitative milk performance, data on heifer reproduction, and data on first parity reproduction. Milk quantity data were considered, and the amount of milk yield of one lactation period (in the first lactation) was corrected using 305-day and mature equivalent correction factors. For determining the levels of estrogen in cows with different genotypes, serum samples collected during estrus were used. The Estradiol Kit (Calbiotech, USA) was used to calculate the levels of estrogen using the ELISA technique. The reproductive data were calculated as follows: length of pregnancy (date of calving - date of insemination resulting in pregnancy), number of inseminations per pregnancy at heifer (total insemination resulting in first pregnancy), age at first calving (date of first calving - date of birth), first mating after calving (date of first insemination after first calving - date of first calving), and number of inseminations per pregnancy at parity 1 (total insemination resulting in second pregnancy). To assess milk quality, dairy cows in the lactating phase were tested (fat percentage, protein percentage, lactose percentage, and solid nonfat percentage). Lactoscan analyzer (MCC, UK) measurements were taken monthly for a minimum of six measurements during the tests.

2.4. Statistical Analysis

According to [22], genotype frequency, allele frequency, and heterozygosity data were computed on the basis of the genotype results. The chi-square test was used to investigate Hardy-Weinberg equilibrium. SAS was run to evaluate the relationship between genotype and phenotype breeding characteristics using the following general linear model approach:

$$Y=\mu+G+\text{Ɛ}$$

where Y denotes the value of the breeding parameters observed (dependent variable), µ denotes the average, G denotes the fixed effect of genotype, and Ɛ denotes the residual random effect. At $P<0.05$, the difference

was significant. Significant differences between each different genotype were calculated using Tukey's test.

3. Results and Discussion

3.1. DNA Amplification and Genotyping

The results of DNA quantity and quality measurements revealed that the average DNA concentration was 32.28±11.75 µg/ml and the average purity was 2.08±0.18. According to the kit protocol instructions, the minimum DNA concentration for the PCR process is 20–30 µg/ml; hence, this concentration is adequate. The TLR4 gene primer, which is located in partial exon 3 at positions 9305 to 9686 in GenBank DQ839567.1 (*Bos taurus*), was successful in amplifying PCR results along 382 bp, as shown in the results of the agarose electrophoresis assay (Figure 2). This result is in line with those of earlier studies [8, 20].

Figure 3 illustrates the position of the target of the TLR4 gene and the site of the digestion point by restriction enzymes. PCR products cut using restriction enzyme AluI indicated three different types of genotypes were produced based on the pattern of fragments formed (Figure 4).

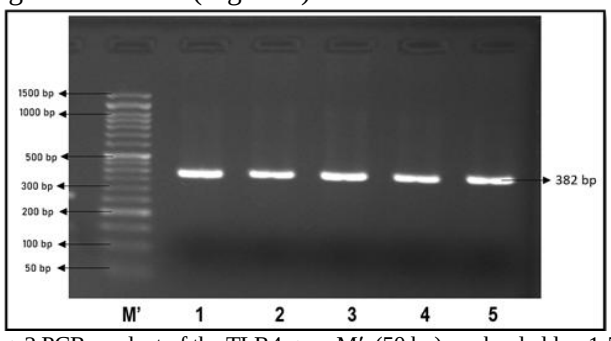


Fig. 2 PCR product of the TLR4 gene M′: (50 bp) marker ladder; 1-5: amplicon product (382 bp)

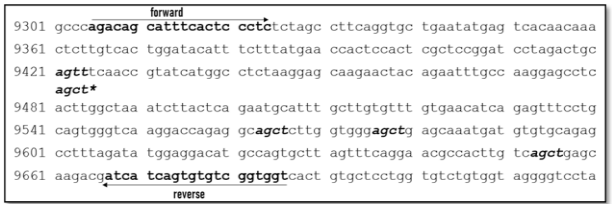


Fig. 3 Sequence target of TLR4 gene in *Bos taurus* GenBank (Code: DQ839567.1) with primer, SNP target (*), and AluI restriction site location

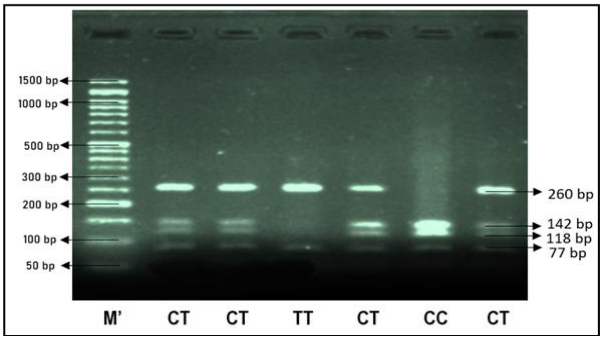
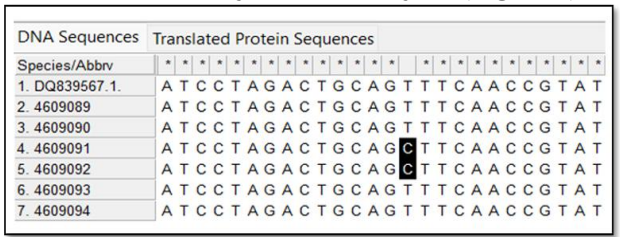


Fig. 4 Representative genotype pattern of TLR4|AluI from the PCR-RFLP product. M′: (50 bp) marker ladder

The AluI (*Arthrobacter luteus*) enzyme works by cutting fragments of the AG_CT base sequence. The formed T allele is characterized by an uncut fragment with a size of 260 bp. Mutation of thymine bases with cytosine produces the C allele, which is characterized by truncated fragments with sizes of 118 and 142 bp. Thus, the TT genotype has fragments of 260 bp, 77 bp, 32 bp, and 13 bp. The CC genotype has fragments of 142 bp, 118 bp, 77 bp, 32 bp, and 13 bp. The heterozygote genotype (CT) is formed by a combination of both alleles characterized by size fragments 260 bp (1-260), 142 bp (119-260), 118 bp (1-118), 77 bp (274-350), 32 bp (351-382), and 13 bp (261-273) bp. Small fragments (32 and 13 bp) cannot be observed in the illustration.

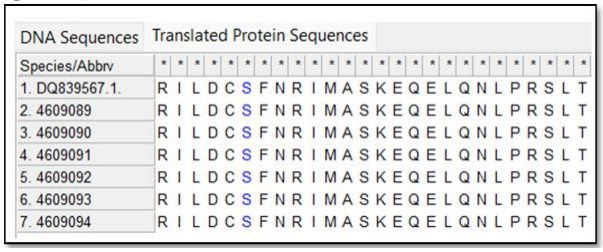
Single-nucleotide polymorphism (SNP) was determined from the results of alignment of nucleotides from TLR4 gene sequencing with nucleotide sequences from GenBank. Different nucleotides indicate the presence of an SNP. The SNP detected is located at nucleotide sequence 119th from the primary forward or at nucleotide sequence 9423 based on GenBank DQ839567.1. The results correspond to [23], calling this mutation SNP g.9423T>C. This variation in mutation occurs due to the transition substitution of the thymine (T) base with cytosine (C) in the TLR4 gene, which was detected by the AluI enzyme (Figure 5).



DNA Sequences	Translated Protein Sequences
Species/Abbrv	* *
1. DQ839567.1.	A T C C T A G A C T G C A G T T T C A A C C G T A T
2. 4609089	A T C C T A G A C T G C A G T T T C A A C C G T A T
3. 4609090	A T C C T A G A C T G C A G T T T C A A C C G T A T
4. 4609091	A T C C T A G A C T G C A G C T T C A A C C G T A T
5. 4609092	A T C C T A G A C T G C A G C T T C A A C C G T A T
6. 4609093	A T C C T A G A C T G C A G T T T C A A C C G T A T
7. 4609094	A T C C T A G A C T G C A G T T T C A A C C G T A T

Fig. 5 Variations in the nucleotide sequence of the TLR4 gene and reference, indicating a T>C base mutation

Mutation of T to C is in the exon 3 region, which is the coding area, so that it can be translated into the amino acid sequence. The type of mutation in this SNP is synonymous and does not cause changes in the amino acid, i.e, serine (Figure 6). The identification of polymorphisms in the PCR-RFLP results was confirmed by a chromatogram of sequencing results at the location of the mutation point, obtained homozygous genotypes TT and CC (indicated by single peaks) and heterozygous genotypes CT (double peaks) (Figure 7).



DNA Sequences	Translated Protein Sequences
Species/Abbrv	* *
1. DQ839567.1.	R I L D C S F N R I M A S K E Q E L Q N L P R S L T
2. 4609089	R I L D C S F N R I M A S K E Q E L Q N L P R S L T
3. 4609090	R I L D C S F N R I M A S K E Q E L Q N L P R S L T
4. 4609091	R I L D C S F N R I M A S K E Q E L Q N L P R S L T
5. 4609092	R I L D C S F N R I M A S K E Q E L Q N L P R S L T
6. 4609093	R I L D C S F N R I M A S K E Q E L Q N L P R S L T
7. 4609094	R I L D C S F N R I M A S K E Q E L Q N L P R S L T

Fig. 6 Sequence of amino acids that does not change due to the T>C nucleotide substitution

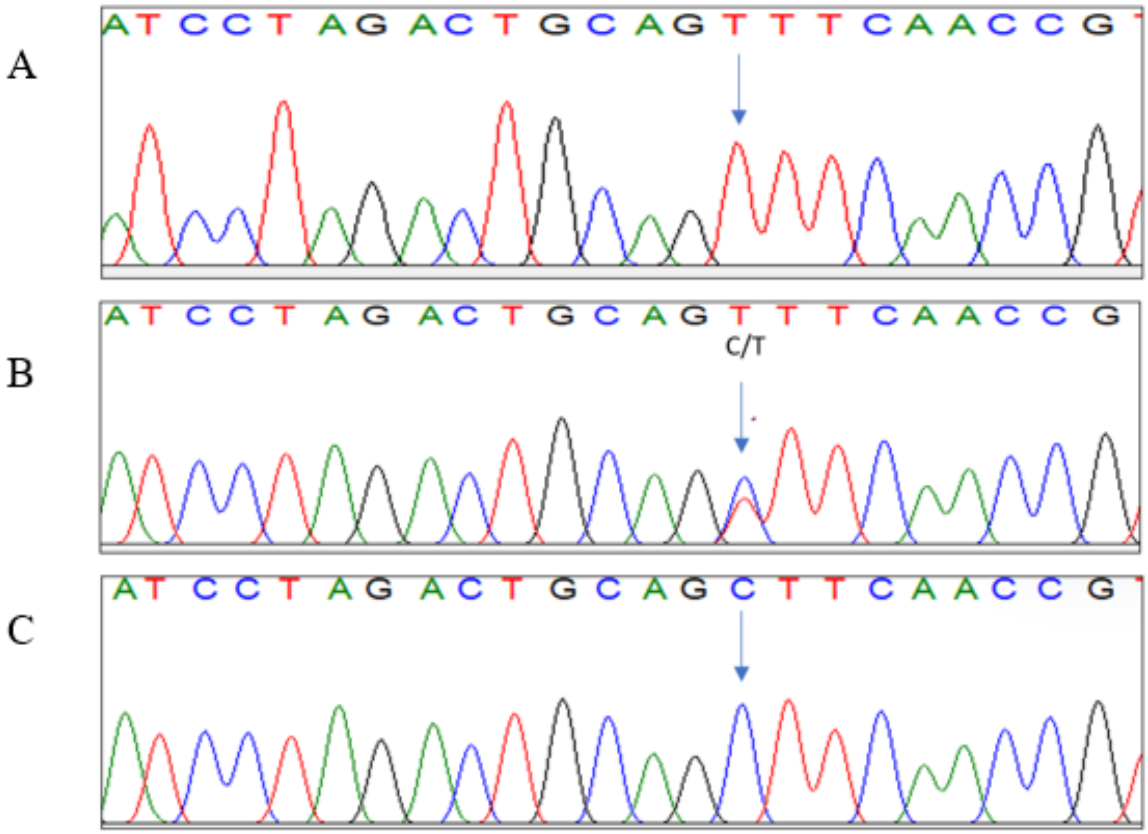


Fig. 7 Variants of genotype TLR4 gene based on partial sequencing results: A. genotype homozygote TT, B. genotype heterozygote CT, C. genotype homozygote CC

Locus SNP g.9423T>C in partial exon 3 of the TLR4 gene was polymorphic in the study population from PCR-RFLP results. The locus is termed polymorphic if one of the alleles has a frequency greater than 0.01 [22]. Data analysis of TLR4|AluI gene polymorphism is presented in Table 1.

Table 1 Data analysis of TLR4|AluI gene polymorphism in the population of Friesian Holstein cattle in Indonesia

Genotype	Genotype frequencies	Allele	Allele frequencies
TT	18 (0.180)	T	0.425
CT	49 (0.490)		
CC	33 (0.330)	C	0.575

The frequency of the C allele (0.575) is higher than T allele (0.425) with the dominant genotype frequency of the heterozygous CT (0.490). The diversity of TLR4 gene genotypes obtained in this study was nearly identical to other studies that found heterozygous CT to be the genotype most commonly found in Lithuanian Holstein cattle (0.507) [20], in SNP g.46372T>C in water buffalo (0.4059) [6], and in Chinese Simmental cattle (0.4167) [8]. However, the frequency of the dominant allele is in contrast to other studies using the same primer, such as in Kanrej and triple cross cattle in India [12] and in Holstein cows in Egypt [9]. The observed heterozygosity (0.490) in the study population was higher than the expected heterozygosity (0.489). The chi-square test results (0.00) indicate that Hardy-Weinberg equilibrium (HWE) occurs in the study population where the chi-square values were smaller than X^2 in the table value (5.99).

The lack of a specific breeding and selection program using the TLR4 gene is a potential cause of HWE in the study population. Other studies of TLR4 genes identified populations in HWE in Holstein cattle in Egypt [4], Kanrej and triple cross cattle in India [12], and dairy goats in Czech [17] but not in HWE in Holstein cattle in Iran [11].

3.2. Relationship between TLR4|AluI Gene Diversity and Breeding Parameters

The relationship analysis of TLR4 gene diversity with breeding parameters and milk quality is presented in Tables 2 and 3. As demonstrated in Table 2, there was a significant relationship ($P<0.05$) between TLR4 gene genotype variation and milk quantity, but it was not correlated with other breeding parameters or estrogen levels. Although SNPs in the TLR4 gene in this study did not result in amino acid changes (synonymous), genetic linkages with other loci in the TLR4 gene to this SNP can affect phenotype [11].

Table 2 Relationship analysis of TLR4|AluI gene diversity with breeding parameters

Breeding parameters	**Genotype**		
	TT	**CT**	**CC**
Milk quantity (kg)	5,759±1,228[a]	5,051±772[b]	4,710±1120[b]
Length of 1st pregnancy (days)	276.17±5.76	276.45±6.14	276.24±8.28
Number of inseminations per pregnancy in heifers (times)	1.11±0.32	1.12±0.33	1.12±0.32
Age at first calving (months)	27.03±3.50	27.60±3.44	27.49±2.96
First mating after calving(days)	77.94±28.92	99.06±48.94	92.58±54.72
Number of inseminations per pregnancy at parity 1 (times)	1.50±0.61	1.45±0.68	1.52±0.57
Length of 2nd pregnancy (days)	281,50±9,21	281,55±11,92	279,58±6,72
Estrogen level (pg/ml)	72.02±57.42	57.42±38.48	46.29±9.10

Note: Values in the same row with different letters indicate a significant difference at α 5%.

The TLR4 gene resides on chromosome 8 near the quantitative trait locus that affects somatic cells and mastitis [11, 23]. The TLR4 gene polymorphism may be used as a candidate gene for mastitis resistance because it is linked to the somatic cell score [6], [12]. The TT genotype is advantageous for milk quantity characteristics since it has much lower somatic cell score values than other genotypes [20]. Total milk production correlates negatively with mastitis. Somatic cell score and mastitis are correlated with the proliferation of pathogenic bacteria and inflammatory responses in glandular tissue. These conditions may damage and modify the udder tissue and have a negative impact on milk production and quality [12].

Table 3 Relationship analysis of TLR4|AluI gene diversity with milk quality performance

Genotypes	**Fat percentage**	**Protein percentage**	**Lactose percentage**	**Solid nonfat percentage**
TT	4.09±0.28	3.05±0.10	4.28±0.13	8.15±0.14
CT	4.17±0.17	3.06±0.11	4.34±0.17	8.25±0.17
CC	4.08±0.21	3.07±0.86	4.32±0.14	8.20±0.16

Note: Values in the same column with different letters indicate a significant difference at α 5%.

The TLR4 gene plays a role in protecting against microorganisms [2] that cause inflammation, particularly in the udder glands [6], by recognizing pathogens [1] and mediating signals to trigger the body's immune response, both innate and adaptive [8]. TLR4 is a surface cell receptor that is developed with accessory proteins to detect gram-negative bacterial lipopolysaccharides (LPS) [11], [14]. After attaching to a particular LPS of microorganisms, it triggers a cascade of immune regulatory mechanisms that allow microorganisms to be captured and phagocytosed [21]. Toll-like receptors (TLRs), including TLR4, function in the immune system by recognizing microorganisms, such as viruses, bacteria, fungi, parasites, and inflammation-causing substances, such as fibrinogen, heat shock protein, and hyaluronic acid [3], [10]. TLRs detect persistent pathogen microorganism structures known as pathogen-associated molecular patterns (PAMPs) and initiate the formation of a complete immune response [4], [14].

Polymorphism of SNP c.9421C>T in Lithuanian Holstein cows corresponds with milk somatic cells and mastitis resistance; however, SNP c.2021C>T polymorphism does not correlate with somatic cells in milk and resistance to mastitis [20]. T4CRBR2 expression in China Holstein and China Simmental cattle is also unrelated to somatic cell score [8]. The mechanism of the correlation between udder inflammation and milk production causes a relationship between the diversity of TLR4 genes and milk quality and quantity; however, genotype diversity in this study did not correspond with milk quality, as demonstrated in Table 3. The findings of this study contradict those of previous studies on Holstein cattle in Iran and New Zealand, which discovered that the TLR4 gene polymorphism affected milk fat and protein content [11], [14].

The potential influence of the TLR4 gene on bovine reproductive traits is based on the role of TLR in protecting the genital tract from infection and inflammation [7] and in affecting the functioning of ovaries independently of pathogenic stimulus during the follicular development period [9]. TLR4 is expressed in the endometrium and is involved in the immune system and uterine infections [5]. The T/C genotype in the TLR4 gene increased polymorphonuclear leukocyte migratory ability and apoptosis resistance while reducing the inflammatory

responses produced by bacterial infections [13]. This condition promotes uterine rehabilitation, implantation, and uterine environment conditioning for embryo development, resulting in improved reproductive performance.

Various patterns of TLR expression, particularly TLR4 in cattle, occur along with changes in the condition of the animal's ovaries during the estrous cycle and transition period. The findings revealed a lack of association between genotype diversity and reproductive parameters and estrogen hormone levels during estrous, in contrast to previous research that found a link between the TLR4 gene and calving interval, service per conception in Holstein cattle in Egypt [9], days open in dairy cattle in Japan [13], and calving interval in Czech Fleckvieh cattle [7].

Increasing the population of cows with the TT genotype could increase milk quantity, and this TLR4 gene could be used as a potential genetic marker for milk quantity. Gene diversity can be used in cattle selection programs for specific desirable traits. Studies related to the TLR4 gene in cattle are still rare; therefore, polymorphisms in other SNPs need to be further identified, especially in dairy cows living in the Indonesian environment. In addition, it is necessary to investigate the relationship between genes and disease resistance and other production traits.

4. Conclusion

The TLR4|AluI gene is polymorphic in the Indonesian Friesian Holstein cattle population used in this study. The population is at Hardy-Weinberg equilibrium, with the dominant genotype being CT. Polymorphisms in the TLR4|AluI gene are related to the quantity of milk but not related to reproductive parameters, estrogen levels, or milk quality. In conclusion, the TLR4|AluI gene could be considered as a genetic marker candidate for genomic selection in the Indonesian Friesian Holstein cattle population. Cows with a superior genotype are recommended for use as parents in the population. The findings of this study are preliminary information that can be applied in molecular technology-based dairy cattle breeding programs as a genetic improvement effort to obtain dairy cows with superior milk quantity.

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