REVIEW ARTICLE

ASSOCIATION STUDIES BETWEEN POLYMORPHIC VARIANTS OF TLR-ENCODING GENES AND *MASTITIS* IN DAIRY CATTLE. AN OVERVIEW OF THE CURRENT STATE OF KNOWLEDGE

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ABSTRACT

Mastitis is an inflammation of the mammary gland which occurs as a defensive reaction to microorganisms that have penetrated the body. The disease generates huge financial and economic losses as treatment of affected cows is expensive but not always effective, which translates into a decrease in the amount of milk that they produce. Due to the huge number of pathogens and their molecular diversity, the immune system has developed various mechanisms to combat them. What raises hopes for improved udder health is marker assisted selection (MAS) or genomic selection. The molecular diversity of microbes allows them to be considered genetic markers, and Toll-like receptors (TLRs) are suitable candidates. TLRs recognize infectious agents and trigger mechanisms to combat them, including by the production of pro-inflammatory cytokines, chemokines and interferons. Due to the role these receptors play in the body, TLR-encoding genes may be associated with bovine mastitis resistance.

Key words: *mastitis*, TLR, MAS, polymorphism

INTRODUCTION

Mastitis is caused by various types of pathogens. These may be bacteria (mainly streptococci and staphylococci) as well as fungi and viruses, but the former are the most common cause of inflammation. A diagnostic parameter indicative of the presence or absence of *mastisis* is somatic cell count (SCC) in milk or sometimes somatic cells score (SCS) in milk [Caraviello et al. 2005]. Increased SCC levels cause a loss of milk's nutritional value and difficulties in its processing. In milk of healthy cows, SCC is below 200 thousand/ml. The permissible counts of microorganisms (less than 100 thousand/ml) and somatic cells (less than 400 thousand/ml) laid down in the Polish standard for raw milk comply with the EU requirements [PN-A-86031:1965].

Another diagnostic criterion of great importance is the number of clinical *mastitis* (CM) cases [Abebe et al. 2016]. In the clinical form of the disease, there are palpable or visible changes in the udder or milk. Hyperacute/acute *mastitis* usually develops violently and

is accompanied by systemic symptoms such as fever, lack of appetite, dehydration and weakness. The affected udder quarter is reddened, swollen, hard and tender to touch, and produces less milk, whose parameters do not meet requirements [Sinha et al. 2022].

As for the etiology of *mastitis*, there are both genetic and environmental causes of the disease. A very important role in controlling *mastitis* is played by proper hygienic measures and milking conditions. Dairy farmers are obliged to comply with specific standards, and the equipment they use should be sterilized as it comes in direct contact with cows' teats and, if it is contaminated, microbes can be easily transferred to the udder. SCC is also heavily influenced by the stage of lactation, season, stress, diet, age of the animal or even milk yield [Harmon 1994, Beecher et al. 2010].

Due to the economic implications of *mastitis*, research teams around the world are making efforts to develop an easy-to-use and, above all, effective method of reducing the incidence of the disease. So far, this aim has not been achieved with traditional methods of treatment

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and improvement of the sanitary conditions of milk production. The measures taken to prevent the occurrence of udder inflammation in dairy cows are time-consuming and expensive, and do not produce the expected results [Wang et al. 2021].

Susceptibility or resistance to *mastitis* in dairy cows is determined genetically and depends on multiple genes. Research is ongoing on genes that may be associated with immunity/susceptibility to this disease. Researchers have discovered numerous *loci* in various genes, which are used in marker-assisted selection (MAS) or genomic selection to reduce the incidence of *mastitis* [Pawlik et al. 2009]. It is crucial to find markers that are linked to improved health of the udder and at the same time do not affect milk yield, which is a prerequisite for the profitability of dairy farming.

Elements of the immune system involved in antigen recognition and presentation in *mastitis*

The immune system has developed various mechanisms, specific (adaptive) and nonspecific (innate), for combating pathogens. Nonspecific immune response is less precise compared with specific response, but it occurs faster and constitutes the first line of defence of the host organism. Innate immunity is formed by anatomical and physiological barriers as well as cellular and humoral elements, whereas adaptive immunity only consists of cell-mediated and humoral responses [De Souza et al. 2012].

Nonspecific immunity involves a number of pathogen-recognition receptors (PRRs) which detect two different types of molecular structures: pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). The receptors also play a role in initiating a specific response to a particular antigen and in triggering the release of cytokines. PRRs have been localized in macrophages, monocytes, dendritic cells, neutrophils and epithelial cells. They are currently classified into many subgroups, including: Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), cytosolic DNA sensors (CDS), and C-type lectin receptors (CLRs) [Takeda et al. 2002].

While most TLRs are found in the cell membrane (TLR1, TLR2, TLR4, TLR5, TLR6, TLR10 and TLR11), some are located in the membrane of cytoplasmic vesicles (TLR3, TLR7, TLR8 and TLR9). Activated by molecular patterns, TLRs recognize the infectious agent and trigger a number of mechanisms leading to its eradication. The mechanisms include promoting the production of pro-inflammatory cytokines, chemokines and interferons, and increasing the expression of costimulatory molecules on antigen presenting cells (APCs)). The type of TLR involved in the recognition of a microbe as well as the ligand that binds to the TLR determine the type and course of the developing immune response. Due to the role that these receptors play in the body, the genes encoding TLRs may be associated with resistance to *mastitis* in cattle [Ashraf et al. 2018].

An overview of TLRs associated with resistance to *mastitis*

Ten different TLRs (from TLR1 to TLR10) have been identified in cattle. Among them, TLR1, TLR2, TLR4, TLR6 and TLR9 are associated with resistance to *mastitis*. Table 1. shows associations between selected polymorphisms of TLR-encoding genes and CM, SCM, SCC and SCS. Most studies focused on TLR2, TLR 4 and TLR 6, which is due to their involvement in the immune mechanism, and more specifically their ability to recognize bacterial cell components that are the main epidemiological factor in *mastitis*.

TLR1

TLR1 has been found to be expressed in the immune system cells (macrophages, dendritic cells, B lymphocytes, mast cells, eosinophils and neutrophils), epithelial cells, vascular endothelium, adipocytes, cardiomyocytes, fibroblasts and keratinocytes. TLR1 is highly homologous to TLR2 in structure and they are jointly involved in the recognition of specific lipoprotein/lipopeptide patterns [Bjelka and Novák 2020]. It participates in the innate immune response to microbial agents as it specifically recognizes diacylated and triacylated lipopeptides. Stimulation of TLR1 leads to the recruitment of MYD88 and TRAF6, resulting in the activation of NF-kappa-B, secretion of cytokines and an inflammatory response.

Russell et al. [2012] carried out a study in which they analysed the relationship between SNPs (-79 T>G, +1762 A>G and +2463 C>T) in the TLR1 gene and susceptibility to CM in Holstein-Friesian cows. They reported statistically significant associations between CM and two of the three analysed SNPs (-79 T>G and +2463 C>T), but no such associations were found in the case of the third SNP (+1762 A>G). What is more, no genetic antagonism was observed between resistance to CM and milk composition. This means that genetic improvement of resistance to CM through selection based on the favourable allele will not have an adverse effect on milk composition. In the same study, Russell et al. [2012] reported that the lowest resistance, expressed as the number of CM cases, was observed in individuals with genotypes TG (0.75 CM cases per cow per year) and GG (0.7 cases per cow per year). Moreover, an analysis of SNP -79 T>G revealed that animals with genotype GG were characterized by lower levels of mRNA in milk somatic cells compared with other cows. Animals with this genotype were additionally stimulated with a TLR1 ligand and were found to have significantly lower levels of mRNA and CXCL8 protein. An analysis of another SNP

Table 1. An overview of association studies between polymorphic variants of TLR-encoding genes and susceptibility to mastitis in dairy cattle

Gene	Polymorphism	Trait	Cattle breed	Author
TLR1	−79 T>G; +2463 C>T	SCC − SCS 0 CM +	Holstein	Russell et al. 2012
TLR2	T385G	SCC 0 SCS CM 0	Holstein, Sahne and Simmental	Zhang et al. 2009
	−79 T>G	SCC 0 SCS CM 0	Holstein	Huang et al. 2011
	E+945; E+978; E+1010; E+1688; E+1707; E+1779; E+1782; E+1995; E+2025; E+2055; E+2214	SCC 0 SCS + CM 0	Xinjiang Brown	Bai et al. 2012
	T385G (rs55617172)	SCC − SCS + CM 0	Holstein	Prebavathy et al. 2015
	C2010T (rs68343170)	SCC + SCS − CM 0		
	313 pb	SCC 0 SCS 0 CM +	Crosses of Holstein/Brown Swiss/Jersey with Hariana	Ranjan et al. 2018
	Taq1–TLR2	SCC 0 SCS − CM +	Holstein	Elmaghraby et al. 2018
TLR4	P–226	SCC + SCS 0 CM 0	Holstein	Wojdak-Maksymiec et al. 2015
	T4CRBR1	SCC 0 SCS + CM 0	Chinese Holstein, Sanhe and Chinese Simmental	Wang et al. 2007
	P–226 (ss49839679); E3 +1656; E3 +2021	SCC 0 SCS + CM 0	Holstein bulls	Sharma et al. 2006
	TLR32	SCC 0 SCS 0 CM +	Murrah buffaloes, Sahiwal cattle	Sentitula et al. 2011
	T4CRBR2	SCC 0 SCS + CM 0	Holstein	Noori et al. 2013
	Exon 1 (*Hae*III) Exon 2 (*Hinf*I)	SCC 0 SCS 0 CM +	Jersey	Razak et al. 2015
	g.9788C>T	SCC 0 SCS + CM 0	Simmental, Holstein, Sahne	Wang et al. 2008
	T4CRBR1	SCC 0 SCS + CM 0	Kankrej and Triple cross cattle	Gupta et al. 2015
TLR	T853A; G855A; A1980G	SCC 0 SCS + CM 0	Holstein	Chu et al. 2009
	C1859A; A1980G	SCC 0 SCS + CM 0	Holstein	Morales et al. 2017

+ the results for the trait were statistically significant
– the results for the trait were statistically non-significant
0 trait was not studied

(+2463 C>T) showed significant associations with CM. It was also found that both of the analysed SNPs have a pleiotropic effect and do not affect the quality of milk.

TLR2

As it has high affinity for several ligands present in pathogen structures, TLR2 is involved in the recogni-

tion of various pathogens and hence is of relatively high interest to researchers. In addition, TLR2 has the ability to form heterodimers with TLR1 and TLR6 and thus expands the range of recognition of PAMPs present in the pathogen structure [Takeda et al. 2002]. The ligands for TLR2 include bacterial lipoproteins, peptidoglycan, lipoteichoic acid, zymosan, glycolipids, bacterial porins and lipoarabinomannan [Feng et al. 2020]. TLR2 is present in the cytoplasm of resting CD4+ effector lymphocytes (Teffs) and regulatory T cells (Tregs), and upon their activation it is expressed on the surface of the cell membrane. This prevents excessive activation of lymphocytes by common microbial patterns that are TLR2 ligands [Liu et al. 2006].

Prebavathy et al. [2015] analysed the relationship between SNP T385G (rs55617172) in the TLR2 gene and SCS in cattle and revealed an association between the analysed polymorphism and *mastitis*. The same study also looked at other non-synonymous SNPs (rs111026127, rs68268256, rs68268260, rs68343170, rs68268268) and their possible associations with udder inflammation. Among them, only one (rs68343170) was shown to be significantly associated with *mastitis*. The authors emphasize that the C/T transition in the analysed SNP may lead to amino acid substitution of threonine to methionine. The replacement of a polar amino acid with a non-polar amino acid may have serious consequences for the protein structure and therefore the analysed SNP may predispose to a greater resistance or susceptibility to *mastitis*.

Elmaghraby et al. [2018] carried out a study in which they assessed the relationship between polymorphisms in the TLR2-encoding gene (2102 T>G, 2105 T>G, 2107 C>T) and CM, SCM (subclinical mastitis) and milk performance traits in Holstein cows. The analysis showed a statistically significant association between Taq1-TLR2 (2107 C>T) and CM. Furthermore, animals with GG genotype had the lowest SCC and were therefore more resistant to *mastitis*.

A study by Huang et al. [2011] showed that the c.+189 T>G polymorphism of the TLR2 gene is associated with SCS. The highest value of this trait was found in cows with TT genotype. Ranjan et al. [2018] searched for associations between a 313 bp fragment of the TLR2 gene and CM using the SSCP method. It was found that animals with AA genotype were characterized by lower susceptibility to CM. Thus, the studied polymorphism was shown to be significantly associated with CM.

Bovine TLR2 gene as a candidate gene for resistance to *mastitis* was also studied by Zhang et al. [2009]. Through molecular analysis, three missense mutations were detected in +385 T>G, +398 G>A, and +1884 G>A. Genotypes and allele frequencies were determined for a total of 240 cows of three dairy breeds (Holstein, Simmental and Sanhe). The effect of TLR2 polymor-

phisms on SCS was analysed and a significant association was revealed between T385 G and SCS. No significant associations were found between G398 A and G1884 A and SCC [Zhang et al. 2009].

TLR4

TLR4 is an important receptor that recognizes endotoxins associated with Gram-negative bacterial infections. It is capable of recognizing lipopolysaccharides (LPS) of bacteria such as *Escherichia coli* and *Klebsiella* spp., which are a common etiological factor in *mastitis* [De Mesquita et al. 2012, Abebe et al. 2016]. Moreover, TLR4 has been proven to recognize molecules released by damaged cells and tissues, leading to the induction of an inflammatory response. Most studies carried out to date have confirmed the existence of associations between genetic variants of the TLR4 gene and resistance to *mastitis* [Deb et al. 2013].

Gupta et al. [2015] investigated polymorphism in the bovine TLR4 gene for associations with *mastitis*. Using PCR technique, they amplified two fragments of the TLR4 gene in Kankrej and triple cross cattle, namely T4CRBR1 and T4CRBR2 of 316 bp and 382 bp, respectively. The results showed that two alleles (A and B) were present in each of the studied *loci* in both populations, and the genotype distribution followed Hardy-Weinberg equilibrium (P ≤ 0.05). An analysis of associations between the TLR4 gene polymorphism and SCS showed that cattle with allele A in T4CRBR1 had a lower SCS value than cows with allele B (P ≤ 0.05). Allele A may therefore play an important role in cows' resistance to *mastitis*.

The same polymorphic sites as those studied by Gupta et al. [2015] were also analysed by Wang et al. [2008] in Holstein, Sanhe and Simmental cows bred in China. The results showed that both alleles (A and B) in the two *loci* were found in all the three populations. The effect of TLR4 gene polymorphism on SCS was analysed and the results indicated that cattle with allele A in T4CRBR1 had a lower SCS than those with allele B (P ≤ 0.05).

Sharma et al. [2006] searched for associations between three SNPs of the TLR4 gene and SCS. One of the SNPs was located in the putative promoter region (P-226) and the other two in the third exon (E3+1656 and E+2021). All the analysed SNPs were found to be significantly associated with SCS. Similar results were reported by Wojdak-Maksymiec et al. [2015], who analysed the SNP in the putative promoter region P-226 of the TLR4 gene. The analysed polymorphic site showed a significant association with SCC.

TLR4 gene variants in dairy cattle and buffalo were also studied by Sentitula et al. [2013]. The study included 218 animals (110 Sahiwal and 108 Murrah). The cows were analysed for *mastitis* case records and subjected to microbiological screening. It was shown that the anal-

ysed polymorphism was associated with CM. BB genotype was found to be significantly associated with *mastitis*, while AB genotype was associated with healthy animals.

Similarly, a research by Razak et al. [2015] indicated the TLR4 gene as a candidate gene for resistance to *mastitis* in cattle. The authors analysed exons 1 and 2 of the TLR4 gene for CM and SCM in Jersey and Holstein-Friesian crossbred cattle. The first exon was digested with *Hae*III and *Dpn*I enzymes, while the second exon was digested with *Hinf*I and *Tru*I. A significant association between CM and polymorphisms identified by *Hae*III and *Hif*I was only observed in Holstein-Friesian cross bred cattle.

Noori et al. [2013] showed in their research the existence of a statistically significant association between T4CRBR2 and SCS. The result contradicts the results of similar studies conducted by Wang et al. [2007] and Gupta et al. [2015], which did not show any association between T4CRBR2 and SCS.

The rs8193069 in bovine TLR4 is significantly associated with both production and blood parameters in Holstein cows, with the rs8193069 C allele being the major allele. The mutation of rs8193069 from T to C contributes to mastitis resistance with reduced SCC in milk, possibly by impairing the ability of the TIR domain to bind to TLR4 adapters and inflammation mediated by LPS/TLR4-NF-κB signaling [Chen et al. 2022].

TLR6

The structure of TLR6, like that of TLR1, is highly homologous to TLR2, and the two receptors work in conjunction to recognize specific lipoprotein/lipopetid patterns. The specific property of TLR6 is its ability to recognize diacyl lipopeptides and lipoteichoic acids in Gram-positive bacteria and zimosan in fungi.

To date, few studies have been carried out on possible correlations between TLR6 gene polymorphisms and *mastitis*. Chu et al. [2009] looked for associations between six SNPs (T853A, G855A, G1793A, C1859A, G1934A and A1980G) and *mastitis* expressed as SCS. Three out of the six polymorphisms (T853A, G855A and A1980G) were found to be significantly associated with the trait under analysis. All three types of genotypes were identified for T853A and G855A (AA, AB and BB) and A1980G (II, IJ, JJ). In addition, the results of the study indicated alleles B and J of the TLR6 gene as potential genetic markers for improved resistance to *mastitis* [Chu et al. 2009].

Possible associations between C1859A and A1980G polymorphisms in the TLR6 gene and *mastitis* resistance expressed as SCS were analysed by Morales et al. [2017]. The study proved the existence of associations between the studied SNPs and SCS. What is more, the results for

A1980G polymorphism were similar to the results reported by Chu et al. [2009].

CONCLUSION

Mastitis is a disease that has a tremendous impact on the condition and functioning of the dairy herd, which is why researchers are constantly working on a deeper understanding of its etiology. To date, they have discovered many links between polymorphisms of various genes and the development of the disease.

Most studies have confirmed associations between genetic variants of the genes encoding different TLRs and mastitis resistance. The TLR genes listed in this review, due to their role in the immune response against bacteria, can be postulated as molecular markers in the selection against bacteria of infectious diseases that pose serious problems in animal production.

Research on TLR2 and TLR4 should be continued in larger dairy herds, in other cow breeds and under different environmental conditions. Due to their function, TLR1 and TLR6 also have great research potential, but so far there has been little research on their relationship to mastitis and more research needs to be undertaken.

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BADANIA ASOCJACYJNE POMIĘDZY POLIMORFICZNYMI WARIANTAMI GENÓW KODUJĄCYCH TLR A *MASTITIS* U BYDŁA MLECZNEGO. PODSUMOWANIE STANU WIEDZY

STRESZCZENIE

Mastitis jest zapaleniem gruczołu mlekowego powstałym jako reakcja obronna na drobnoustroje, które wniknęły do organizmu. Generuje ogromne straty gospodarcze i ekonomiczne, ponieważ leczenie chorych zwierząt jest kosztowne, a nie zawsze efektywne, co przekłada się na spadek ilości wyprodukowanego przez nie mleka. Ze względu na ogromną liczbę i molekularną różnorodność patogenów układ odpornościowy wykształcił różne mechanizmy ich zwalczania. Nadzieję na uzyskanie poprawy stanu zdrowia wymienia daje selekcja wspomagana markerami (MAS) lub selekcja genomowa.

Słowa kluczowe: *mastitis*, TLR, MAS, polimorfizm

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