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Toll-like Receptor Gene Polymorphism and Its Relationship with Somatic Cell Concentration and Natural Bacterial Infections of the Mammary Gland in Sheep

W.P. ŚWIDEREK[a], M.R. BHIDE[b], J. GRUSZCZYŃSKA[a], K. SOLTIS[c], D. WITKOWSKA[a], I. MIKULA[b,c]

[a]*Department of Genetics and Animal Breeding, Warsaw Agricultural University – SGGW, 02-786 Warsaw, Poland*
e-mail swiderek@alpha.sggw.waw.pl

[b]*Laboratory of Biomedical Microbiology and Immunology, University of Veterinary Medicine, 040 13 Košice, Slovakia*
[c]*Institute of Neuroimmunology, Slovak Academy of Sciences, 842 45 Bratislava, Slovakia*

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ABSTRACT. Possible correlation between Toll-like receptor (TLR)-gene mutations and the susceptibility of the mammary gland to bacterial infections and also the associate breed-dependent aspects of somatic cell concentration (SCC), bacterial infection and TLR-gene mutations in sheep are described. In Polish Lowland Sheep (PLS), milk samples exceeding the level of 500/μL (*i.e.* 5×10^5 per mL) of SCC were recorded almost twice more frequently than in Polish Heath Sheep (PHS) (40 and 22.3 %, respectively). The frequency of bacterial infections was also found in a similar ratio (20 and 12.7 %, respectively). During detection of the TLR-gene mutation we recorded 2 alleles of *TLR1*, 6 alleles of *TLR2* and 10 alleles of *TLR4* genes in PHS sheep, while PLS sheep possessed 2, 4 and 6 alleles, respectively. Statistical analyses revealed a relationship between the specified TLR alleles, SCC and the frequency of incidence of bacterial inflammations of mammary gland. The data may serve as a benchmark for further study of TLR-gene mutation-dependent predisposition of mammary gland defensive cells to recognize the pathogen properly and initiate the immunological response, and may help in identifying one of the markers of natural resistance against sheep mastitis.

Abbreviations

CI	confidence interval	PHS	Polish Heath Sheep (ewes)
OR	odds ratio	PLS	Polish Lowland Sheep (ewes)
PAMP	pathogen associated molecular patterns	SCC	somatic cell concentration
PCR-SSCP	polymerase-chain reaction-single strand conformational polymorphism analysis	SSC	sub-clinical mastitis occurrence
		TLR	Toll-like receptor

The health state of the mammary gland is one of the most important factors decisive for the effectiveness in sheep production. Mastitis may lead to a disturbance in general health status, lowering of production and quality of milk, and in acute cases it may even cause death. Many factors favorable for the development of mastitis invade mammary gland; the most important of them include pathogenic microorganisms, mainly staphylococci and streptococci (Bergonier *et al*. 2003; Godány *et al*. 2004; Tkáčiková *et al.* 2004). The enormous diversity in the makes of mastitis causes its recognition and treatment very difficult and often insufficiently effective.

Recent research in the field of natural resistance of animals and humans has increased the chances of controlling the pathogenic, metabolic and hereditary disease. In particular, the discovery of TLR receptors created not only a chance for better elucidation of defensive mechanisms of the organism against pathogens but also facilitated identification of genes which decide on the resistance of animals to mastitis (Connor *et al*. 2006; Franchini *et al*. 2006; Werling *et al.* 2006). TLR receptors recognize permanent structures of microorganism PAMP; as a result, they initiate the development of full immunological response and influence the course of the process, affecting the specialized regulatory cells (Gelman *et al*. 2004; Drennan *et al*. 2004). Several types of TLR receptors which recognize PAMP elements in different microorganisms have been identified (Abreu *et al.* 2005; Anders *et al.* 2005; Cario 2005). Genes responsible for the expression of TLR receptors show considerable variability (Menzies and Ingram 2006). The polymorphism of TLR genes may affect the differentiation of properties of individual alleles and thus change their function.

Here we evaluate the correlation between *TLR1*, *TLR2* and *TLR4* genes and the health state of the sheep udder.

MATERIAL AND METHODS

Animals. The study was conducted with 130 Polish Heath Sheep ewes and 130 Polish Lowland Sheep ewes in the *Experimental Station Żelazna* (belonging to *Warsaw Agricultural University*).

Cytological tests of milk. Ten-mL milk sample from each half of udder was collected separately from ewes during the 4th week of lactation, 2 h after weaning of lambs. SCC in 1 mL of milk was determined by using in-flow cytometer Somacount 150 (*Bentley*); the level of 300/μL (*i.e.* 300×10^3 cells per mL) was considered as a physiological standard of SCC for healthy ewes.

Bacteriological tests of milk for mastitis were conducted in accordance with the standard methods. Aseptically collected milk samples were cooled down to 4 °C and delivered to the laboratory. Inoculation was done within a few hours after sampling; 100 and 10 μL of each sample was inoculated on nutrient agar (containing 5 % defibrillated sheep blood) and on MacConkey medium. The plates were incubated in aerobic conditions at 37 °C. After a 1-d incubation, colonies were counted and total bacterial concentration in each sample was determined. Biochemical tests for identification bacterial species were done using API microtests (*bioMérieux*, France). The coagulase test using rabbit plasma was used for characterizing staphylococci, the presence of *Streptococcus agalactiae* was demonstrated by the CAMP test.

Detection of TLR-gene mutations. DNA extraction. Genomic DNA was isolated from the whole blood samples by using the phenol–chloroform extraction. The concentration and purity of the DNA were determined spectrophotometrically.

Construction of primers. Primers were constructed (*DNAStar*) for *TLR4* in such a way that they covered both previously described major polymorphism sites: Asp299Gly and Thr399Ile. The nucleotide sequence of primers was *TLR4*/F – 5′-GGG ACT GTG CAA CCT GAC CA-3′ and *TLR4*/R – 5′-GCT CTA AGC CCA TGA AGT TTG AA-3′; the primer set produced an amplicon of 434 bp. Conditions for PCR were: initial denaturation (4 min 95 °C), followed by 35 cycles (1 min 95 °C, 45 s 53 °C, 1.5 min 72 °C) and final extension (10 min 72 °C). Primers for *TLR2* were constructed (*DNAStar*) to the amplified segment containing previously described Pro681His and Arg677Trp mutations (*TLR2*/F – 5′-CAG GAG CTG GAG CAC TTG TAC C-3′ and *TLR2*/R – 5′-GTC TCA TCC ACG GGC CAG ACC A-3′); these primers amplified 362 bp amplicons. Cycling conditions for *TLR2* were similar as *above* except for annealing temperature (45 s at 56 °C).

Mutations or polymorphisms in *TLR1* gene in sheep or other production animals are known. Because of that, nucleotide sequences and amino acid sequences of *TLR1* of *Bos taurus*, *Ovis aries* and humans were retrieved from *GenBank* and PDB (**www.ncbi.nlm.nih.gov**), aligned (*Bio-Edit* software), putative pattern recognizing site was determined and primers were constructed for sheep *TLR1* gene. The nucleotide sequences of both primers were: *TLR1*/F – 5′-GGA GAT ACT TAT GGG GAA AGA GAA-3′ and *TLR1*/R – 5′-GTG TAT AGA CAA GGC CTT CAG TGA-3′. Annealing temperature was 52 °C (for 45 s).

After amplification all products were checked for the absence of nonspecific amplification by 1.2 % agarose-gel electrophoresis and then subjected to mutation detection.

SSCP analysis. Briefly, 5 μL of amplified product was mixed with an equal amount of loading dye (98 % formamide, 10 mmol/L EDTA, 250 ppm bromophenol blue, 250 ppm xylene–cyanol), subjected to denaturation (10 min at 95 °C) and cooled rapidly on ice. Denaturated single-stranded amplimers were loaded on 14 % acrylamide–bisacrylamide (37.5 : 1, *V*/*V*) gels (*Bio-Rad*) containing 10 % glycerol. Electrophoresis was performed in 0.5 % TBE buffer in an electrophoresis chamber (*Ingeny*, The Netherlands) using 200 V for 20 h at 8 °C. Gels were silver-stained.

Statistical analysis. Statistical package SPSS 11.5 PL for Windows was employed for statistical calculations. Relationship between TLR alleles and SCC, and the presence of bacteria in the ewe milk was evaluated by a logistic regression method, estimating OR and 95 % CI.

RESULTS

Cytological and bacteriological tests of the sheep milk (Table I) showed that in the range of <100/μL SCC, there were >39 % of PHS ewes and ≈28 % of PLS ewes. A considerably higher inter-breed differentiation of the milk samples was recorded in the range >500/μL SCC (PLS 40.0 %, PHS 22.3 %). In the remaining ranges, the distribution of SCC in milk samples in the two breeds was similar. In PLS ewes, the presence of bacteria in milk was found more frequently (PLS 20 %; PHS 12.7 %). The highest proportion (>90 %) of isolated bacteria in both sheep breeds was represented by staphylococci and streptococci.

The PCR-SSCP tests showed the incidence of considerable polymorphism of TLR genes (Table II). In the case of *TLR1* gene, only two alleles were recorded. Allele *1* occurred more frequently than allele *2*. Analysis of *TLR2* gene revealed the incidence of 6 alleles in PHS ewes and 4 in PLS ewes. The greatest

polymorphism was observed within the *TLR4* gene. Among 10 alleles found in PHS ewes, three (*TLR4* – alleles *6*, *7* and *9*) were not recorded in PLS ewes.

Table I. Somatic cell concentration (SCC, 1/μL)[a] and incidence of bacterial infections of mammary gland in the PHS and PLS sheep breeds

SCC	Number of samples		Bacteria in milk			% of total bacteria
	n	%	total	streptococci	staphylococci[b]	
PHS						
≤100	102	39.2	9[c]	–	7 (2)	8.8
101–200	50	19.2	5	–	5	10.0
201–300	20	7.7	3	–	3	15.0
301–500	30	11.5	1	–	1 (1)	3.3
>500	58	22.3	15[c]	4	10 (1)	25.9
Total	260	100	33[c]	4	26 (5)	12.7
PLS						
≤100	72	27.7	8[c]	–	6	11.1
101–200	40	15.4	3	–	3 (3)	7.5
201–300	24	9.2	6	2	4	25.0
301–500	20	7.7	4	–	4	20.0
>500	104	40.0	31[c]	9	20 (4)	29.8
Total	260	100	52[c]	11	37 (7)	20.0

[a]To make an analysis easier, the data concerning SCC in milk are distinguished into 5 levels.
[b]In *parentheses* – number of findings of *S. aureus*. [c]Including other bacteria.

The relationship between TLR alleles and the presence of bacteria is illustrated in Table III. In the case of *TLR1* alleles, OR and CI indices were not calculated because all milk samples from which the bacteria had been isolated arrived from ewes carrying *TLR1* allele no. *1* (*TLR1_1*), whereas no bacteria were found in ewes carrying *TLR1_2*. This indicates possible natural resistance against bacterial infection in the ewes carrying the *TLR1_2* allele. The correlation between bacterial concentration, SSC and *TLR1* genotypes was also evident. The incidence of subclinical mastitis showing SSC > 300/μL was most common (OR 2.253 in PHS and 2.033 in PLS) in the sheep carrying *TLR1_1* genotype (Table IV), whereas the presence of high SSC in *TLR1_2* genotype decreased by 45 %.

In *TLR2*, allele *TLR2_2* showed the lowest OR values in both sheep breeds, which probably indicate a negative correlation between susceptibility to bacterial infection and the presence of this allele in sheep. In the other words, it may also be considered as a preventive factor for bacterial udder infections. On the other hand, PHS carrying *TLR2_4* allele (OR 1.33; CI 0.149–11.929) and PLS carrying *TLR2_1* allele (OR 3.275; CI 1.033–10.380) were more prone to bacterial infection of udder, indicating these alleles as risk factors for mammary gland infections. While correlating SSC

Table II. Frequency of alleles of the *TLR1*, *TLR2* and *TLR4* genes

Allele	PHS		PLS	
	n	%	*n*	%
TLR1				
1	114	87.7	126	97.7
2	16	12.3	3	2.3
TLR2				
1	90	69.2	108	83.7
2	14	10.8	18	13.9
3	3	2.3	–[a]	–[a]
4	7	5.4	–[a]	–[a]
5	10	7.7	2	1.6
6	6	4.6	1	0.8
TLR4				
1	37	28.5	31	23.9
2	16	12.3	40	30.8
3	20	15.4	30	23.1
4	5	3.8	22	16.9
5	15	11.5	5	3.8
6	16	12.3	–[a]	–[a]
7	11	8.5	–[a]	–[a]
9	3	2.3	–[a]	–[a]
10	7	5.4	2	1.5

[a]Allele not found.

and the risk of *TLR2* genotypes we found higher OR value in PHS carrying *TLR2_1* allele (OR 2.481) and OR 1.787 in PLS, while lower OR values were found between *TLR2_2* genotype and SSC. Similarly, a lower OR value was also recorded between *TLR2_2* genotype and bacterial infection (*see above*). This confirms the inter-correlation between SSC, bacterial infection and *TLR2* genotypes. The highest value of OR, however, recorded for allele *TLR2_4* (OR 3.111) in PHS ewes may be due to the small frequency of the samples. Similarly, in the case of alleles *TLR1_1* and *TLR2_1* a wide range of confidence intervals was found that may be caused by high variability of the analyzed trait.

Table III. Relationship between alleles of the TLR genes (*TLR1*, *TLR2* and *TLR4*) and the presence of bacteria in milk[a]

Allele[b]	PHS				PLS			
	TLR alleles[c]	bacteria[d]	OR	CI[f]	TLR alleles[c]	bacteria[d]	OR	CI[f]
TLR1								
1	114[e]	33	–	–	126[e]	51	–	–
2	16[e]	0	–	–				
TLR2								
1	90	21	0.304	0.057– 1.622	108	47	3.275	1.033–10.380
2	14	2	0.167	0.019– 1.491	18	4	0.389	0.120– 1.258
4	7	4	1.333	0.149–11.929				
5	10	2	0.250	0.027– 2.319				
TLR4								
1	37	9	0.482	0.069– 3.357	31	12	1.322	0.585– 2.987
2	16	6	0.900	0.115– 7.031	40	12	0.536	0.242– 1.185
3	20	4	0.375	0.046– 3.056	30	16	2.032	0.890– 4.638
4					22	10	1.310	0.520– 3.299
5	15	4	0.545	0.065– 4.562				
6	16	2	0.231	0.023– 2.366				
7	11	3	0.563	0.061– 5.218				

[a]Estimated by the logistic regression method.
[b]Calculations were made for alleles with frequency ≥5 %.
[c]Number of samples with TLR alleles.
[d]Number of samples showing significant bacterial concentration.
[e]OR value was not calculated.
[f]95 % CI for OR.

Association between *TLR4* alleles and bacterial infection varied significantly depending on the sheep breeds. *TLR4_3* allele was calculated as a preventive factor (OR – 0.375) against bacterial infection in PHS, whereas an increased risk of bacterial infection was displayed in PLS (OR – 2.023: CI – 0.890–4.638). Other alleles of *TLR4* showed similar breed-dependent association with bacterial infection. On correlating *TLR4* alleles with SSC, we found no significant difference either among *TLR4* alleles or between sheep breeds. These results suggest that *TLR4* cannot be recognized to be a good gene marker for decision concerning the relationship of natural resistance in sheep against bacterial infection and SSC.

DISCUSSION

The observed inter-breed differentiation of health states of the ewe udder (Table I) in correlation with SCC levels and the incidence of bacterial infections was also described by Hueston *et al*. (1986), Nizamlioglu *et al*. (1989) and Watkins *et al*. (1991); these authors recorded 10–40 % of mastitis cases in ewes caused by bacterial infections. Romeo *et al*. (1998) found that the mean SCC in the milk from healthy ewes varied around 140/μL whereas in the milk samples obtained from the ewes with bacterial infection of mammary gland the mean SCC exceeded 340/μL. Pengov (2001) observed that 98.9 % of milk samples from which bacteria were isolated had SCC levels exceeding 250/μL. In milk samples from ewes with subclinical mastitis, coagulase-negative staphylococci and streptococci were mainly found, indicating their major role in mammary gland infections (Fruganti *et al*. 1985; Bor *et al*. 1989; Lafi *et al*. 1998; Bergonier and Berthelot 2003). Similar results were also obtained in our study. In ewes with the clinical form of mastitis, *S. aureus*

was the most frequently isolated bacterium (17–57 %), being generally considered as the main pathogen of udder diseases (Krzyżanowski *et al*. 1983; Bor *et al*. 1989).

Table IV. Relationship between alleles of the TLR genes (*TLR1*, *TLR2* and *TLR4*) and somatic cell concentration (SCC, 1/μL) in milk[a]

Allele	PHS				PLS			
	SCC[b]		OR	CI	SCC[b]		OR	CI
	≤300	≥301			≤300	≥301		
TLR1								
1	75	39	2.253	0.606– 8.382	61	62	2.033	0.180–23.006
2	13	3	0.444	0.119– 1.651				
TLR2								
1	57	33	2.481	0.981– 6.274	50	55	1.787	0.684– 4.670
2	13	1	0.146	0.018– 1.157	11	7	0.591	0.213– 1.639
4	3	4	3.111	0.662–14.614				
5	9	1	0.222	0.027– 1.817				
TLR4								
1	27	10	0.693	0.297– 1.616	19	12	0.534	0.234– 1.222
2	10	16	1.286	0.433– 3.819	17	21	1.321	0.616– 2.833
3	13	7	1.140	0.417– 3.116	15	15	0.980	0.432– 2.222
4					11	10	0.875	0.343– 2.235
5	10	5	1.042	0.332– 3.272				
6	10	6	1.448	0.478– 4.383				
7	8	3	0.697	0.191– 3.028				

[a]*See* footnotes *a*, *b* and *f* in Table III.
[b]Number of samples with SCC ≤300/μL or ≥301/μL.

On the above data, TLR receptors, which have an immense importance for appropriate recognition of pathogens and regulation of immunological response (Werling and Jungi 2003; Werling *et al*. 2006), were studied for polymorphism and/or mutations present in pattern recognition receptor encoding domains. Changes in the structure of TLR genes due to mutation may lead to changes in the properties of the receptors and their functions. The PCR-SSCP method is one of the methods employed in detection of mutations. Molecular studies (using PCR-SSCP) assessed the occurrence of polymorphism of the TLR genes in humans and animals (Schroder *et al*. 2005; Mockenhaupt *et al*. 2006; Menzies and Ingham 2006). In our study, the greatest polymorphism was observed within *TLR4* gene (*see* Table II). By contrast, only 2 alleles were identified in the *TLR1* gene. Since the analyzed receptors (*TLR1*, *TLR2* and *TLR4*) recognize characteristic structures (PAMP), mainly those of bacteria, the results of the SSCP analysis of TLR genes could be compared with the results of bacteriological tests (Table III). This revealed differentiation in the incidence of mastitis pathogens in milk samples, depending on the presence of the specified TLR allele in the genotype of the ewes. A special attention should be paid to allele *TLR1_2* because no mastitis pathogens were found in the milk of the ewes carrying this allele. Similar relationship was also observed if allele *TLR2_2* was present. The risk of occurrence of bacterial infection of mammary gland in ewes with the *TLR2_2* allele was estimated within the limits of 17 % (PHS) to 39 % (PLS). Slightly more frequent was the presence of bacteria in the milk in ewes carrying alleles *TLR1_1*, *TLR2_1* and *TLR2_4*.

The observed correlation was confirmed when evaluating the relationship of the TLR alleles and SCC in the milk (*see* Table IV). In ewes carrying alleles *TLR1_1* and *TLR2_1*, in which the cases of bacterial infections were recorded more often, the probability of occurrence of subclinical forms of mastitis was ≈2× higher. The above results demonstrate the difference in the predispositions as being due to the specified alleles that can recognize the pathogen properly and initiate an effective immunological response. It creates the possibility of identifying the markers of natural resistance of the ewes toward mastitis. Similar situation was observed in studies concerning certain diseases in humans. A relationship was found between certain alleles and a predisposition to tuberculosis (Arg677Trp *TLR2*) (Ben-Ali *et al*. 2004), resistance to borreliose

development (Arg753Gln *TLR2*) (Schröder *et al.* 2005), and the risk of falling sick to malaria (Thr399Ile *TLR4*) (Mockenhaupt *et al*. 2006).

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