

ASSOCIATIONS BETWEEN MICROSATELLITE POLYMORPHISM WITHIN THE MACROPHAGE EXPRESSED LYSOZYME (MLYS) GENE AND MILK INDICES PROPERTIES IN POLISH BLACK-AND-WHITE COWS

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Received: September 30, 2004

Abstract

WALCZAK-WÓJCIAK, M., KLUPCZYŃSKI, J., MICIŃSKI, J., HOŠEK, M.: *Associations between microsatellite polymorphism within the macrophage expressed lysozyme (mLys) gene and milk Indices Properties in Polish Black-and-White Cows*. Acta univ. agric. et silvic. Mendel. Brun., 2005, LIII, No. 2, pp. 99-106

The experiment was performed in the years 1998–2000 on two farms located near to Vistula Lowlands. The experimental materials were comprised of 52 daughters of bull Paran, among which 21 possessed the mLys-mic 7 allele and 31 – the mLys-mic 3 allele. The serum and whey bacteriolytic activity of the lysozyme, concentrations of selected mineral elements, technological properties of milk at successive the first lactation stages (30, 100, 150 and 200 days), somatic cell count and bacterial count were investigated in this study. The results obtained indicate that Lys-mic polymorphism has low suitability as a marker for milk production capacity in cows. The lysozyme gene, treated as a mastitis resistance factor, showed no effects on somatic cell count and bacterial count. Further investigations, conducted not only during the first lactation, but also during the next lactation in a herd threatened by a variety of pathogenic factors, would be carried to validate this Lys-mic gene polymorphism on mastitis resistance.

alleles, lysozyme, mastitis, somatic cells, bacterial cells, lactation

The genetic aspects of differentiation of the bacteriolytic activity of the bovine lysozyme have been already studied in Norway and Poland. At the beginning statistical detection methods were employed to confirm the presence of a hypothetical gene for high activity, denoted by the symbol LZM⁺. Three breeding bulls (one Red-and-White in Norway, two Black-and-White in Poland), independently segregating with high and normal capability to lyse bacterial cells, have been identified. The structure and organization and the bovine lysozyme gene have been de-

scribed. A microsatellite fragment has been detected recently within the immuno-relevant lysozyme gene (mLys-mic) exhibiting expression in both macrophages and mammary gland tissue (Weikard et al., 1996). One of the polymorphic microsatellite variants, denoted by the symbol mLys-mic 7, showed an analogy to the phenotypically identified high-activity allele LZM⁺. Moreover, three mutations located in introns 2 and 3 have been identified. One of them, found at the position 8603 bp, also shows full compatibility with the hypothetical LZM⁺ allele. There-

fore, DNA molecular markers enable the identification of genotypes determining differentiated phenotypic effect and, in consequence, directional mating in cattle herds, resulting in a threefold higher capability of bacterial pathogen destruction in the progeny (Steinhoff et al., 1994; Pareek et al., 2003; Prusinowska et al., 2003).

The objective of the present study was to determine the associations between the occurrence of the mLys-mic 7 allele, and concentrations of mineral elements and physicochemical properties of milk at successive lactation stages in primiparous cows. The activity of the serum and whey lysozymes, and the somatic cell and bacteria counts in milk, as dependent upon the activity of the Lys-mic 7 and Lys-mic 3 alleles, were also determined.

MATERIAL AND METHODS

The experiment was performed in the years 1998–2000, on Black-and-White cows kept under similar conditions on two farms in the Vistula Lowlands (Zuławy Wislane). The cows were free from tuberculosis, brucellosis and bovine leukemia. Both herds were characterized by comparable productivity. The cows were kept indoor and fed farm-made roughage, i.e. green forage (grass-alfalfa mixtures) in summer, and maize, grass and pulp silage supplemented with hay in winter. The cows were also given feed concentrate composed of ground grain, soybean meal and cake, supplemented with premixes.

The experimental materials were comprised of daughters of bull Paran (51681 - 1 – 8). This bull was characterized by genetically differentiated bacteriolytic activity of the serum lysozyme, and was identified (Walawski et al., 1997) as a carrier of the allele for high lysozyme activity (LZM⁺/LZM^o genotype) and a mLys-mic 7/ mLys-mic 3 heterozygote (Pareek et al., 1998). His semen (100 doses) was used for cow insemination on both farms. Both the number of heifers and bulls produced, and the presence of the sire allele, were purely accidental. Among 52 Paran's daughters, one group (21 cows) possessed the sire mLys-mic 7 allele for high bacteriolytic activity, and the other (31 cows) – the mLys-mic 3 allele for normal activity, were identified according to Weikard et al. (1996). The heifers were successively introduced into the production herd and kept under the same environmental conditions.

Tab. I presents the indices analyzed and methods used for their determination. Samples for analyses were taken at successive lactation stages, i.e. 30 days (stage I), 100 days (stage II), 150 days (stage III) and 200 days (stage IV). The serum and whey lysozyme activity was expressed as a percentage of lysis of *Micrococcus lysodeicticus*, according to the formula:

$$\% \text{ lysis} = (E_z - E_c) \times 100\% / E_z,$$

where:

E_z = extinction before incubation;

E_c = extinction after 10 min. incubation.

I: Indices analyzed and methods used for their determination

Index	Unit of measure	Determination/analysis	Authors
Calcium	g/l	Spectrophotometry	Whiteside and Miner (1984)
Phosphorus	g/l	Colorimetry	Rutkowska (1981)
Sodium	g/l	Photometry	Rutkowska (1981)
Potassium	g/l	Photometry	Rutkowska (1981)
Chlorides	g/l	Mohr method	Budslawski (1973)
Density	°Ld	lacto-densitometry	Manual
Active acidity	pH	“PICCOLO –PLUS” pH-meter	PN-68/A-86122
Clotting	sec.	Rennet method	Budslawski (1973)
Thermostability	ml ethanol per 10 ml milk	Ethanol method	Polish Standard PN-68/A-86122
Somatic cell count	10 ³ /ml	“FOSSOMATIC” apparatus	Manual
Bacterial count	10 ³ /ml	“BACTOSCAN” apparatus	Manual
Lysozyme activity	% lysis Micrococcus lysodeicticus	Turbidimetric method	Smolelis and Hartsell acc. to Metzger (Ślopek 1970)

The results were analyzed statistically (STATISTICA 6.0), by calculating arithmetic means (\bar{x}) and standard deviations (Sd). The effects of mLys-mic polymorphism and lactation stage on productivity parameters, milk composition and properties, were verified by a two-factor analysis of variance in a non-orthogonal design and the Duncan test. In the case of somatic cell count and bacterial count the analysis was also performed for logarithmized values.

RESULTS

The effects on the Lys-mic gene on calcium content (1.13 g/l in both Lys-mic 7 and Lys-mic 3 groups – Tab. II) was statistically found non-significant. The lysozyme gene had no influence on the phosphorus content of milk (0.90 g/l in the Lys-mic 3 group and

0.87 g/l in the Lys-mic 7 group). The highest phosphorus concentration was recorded at stages III (0.93 g/l) and IV (0.91 g/l). Phosphorus content was the lowest at the time of the highest milk yield, to increase from 0.84 g/l to 0.93 g/l along with a decrease in milk yield. It remained at this level, fluctuating a little, until the end of the lactation period (statistically significant differences at $P = 0.01$ between stages II and III, and at $P = 0.05$ between stages I and III, and II and IV). An important indicator of functional disorders of the udder and reduced technological suitability of milk was noticed in case of potassium to sodium ratio. It should be the ratio of 2:1 and its lower threshold value indicates mastitis. In the present experiment the potassium to sodium ratio was observed as 4.11:1, which can confirm as good condition of the udder.

II: Mineral element concentrations in milk

Mineral element	Sire allele	Determinations during the first lactation								Mean	
		stage I 30 days		stage II 100 days		stage III 150 days		stage IV 200 days			
		\bar{x}	Sd	\bar{x}	Sd	\bar{x}	Sd	\bar{x}	Sd	\bar{x}	Sd
Calcium g/l	Lys-mic 7	1.17	0.17	1.03	0.13	1.10	0.12	1.23	0.12	1.13	0.15
	Lys-mic 3	1.09	0.14	1.04	0.14	1.10	0.10	1.28	0.18	1.13	0.17
	\bar{x}	1.13 ^B	0.15	1.04 ^B	0.13	1.10 ^B	0.12	1.26 ^A	0.16	1.13	0.16
Phosphorus g/l	Lys-mic 7	0.84	0.10	0.82	0.10	0.92	0.10	0.89	0.11	0.87	0.10
	Lys-mic 3	0.87	0.12	0.86	0.15	0.93	0.11	0.92	0.08	0.90	0.12
	\bar{x}	0.86 ^{bc}	0.11	0.84 ^{Bc}	0.13	0.93 ^{Aa}	0.11	0.91 ^{ab}	0.09	0.88	0.12
Potassium g/l	Lys-mic 7	1.46	0.10	1.38	0.13	1.47	0.11	1.41	0.11	1.44	0.12
	Lys-mic 3	1.45	0.12	1.44	0.15	1.47	0.13	1.43	0.15	1.45	0.13
	\bar{x}	1.46	0.11	1.41	0.14	1.47	0.12	1.42	0.13	1.44	0.13
Sodium g/l	Lys-mic 7	0.36	0.03	0.31	0.04	0.34	0.05	0.39	0.07	0.35	0.06
	Lys-mic 3	0.36	0.06	0.33	0.05	0.33	0.05	0.37	0.06	0.35	0.06
	\bar{x}	0.36 ^{ABa}	0.05	0.32 ^C	0.04	0.33 ^{BCb}	0.05	0.38 ^A	0.06	0.35	0.06
Chlorides g/l	Lys-mic 7	1.14	0.15	1.15	0.13	1.24	0.20	1.34	0.16	1.22	0.18
	Lys-mic 3	1.18	0.14	1.18	0.15	1.25	0.15	1.32	0.17	1.23	0.16
	\bar{x}	1.16 ^B	0.14	1.17 ^B	0.14	1.25	0.17	1.33 ^A	0.16	1.23	0.17

Means followed by different letters (lactation stages-dates of determination) or x (genotype) differ significantly;

Capital letters or xx - $P = 0.01$; small letters or x - $P = 0.05$

The average chloride content of milk during the first lactation was 1.23 g/l (Tab. II). The lysozyme gene had no effects on its level, which was 1.22 g/l in the Lys-mic 7 group, and 1.23 g/l in the Lys-mic 3 group. The observed chloride content was much below the threshold value, indicating that cows of both genotypic groups were mastitis-free. A regular increase in chloride concentration was noted over the lactation period. The date of its determination had a

significant effect on this parameter, whose value was the lowest at stage I (1.16 g/l) and the highest at stage IV (1.33 g/l).

The average milk density recorded in the experiment (Tab. III), i.e. 29.9 °Ld, can be considered normal. The analysis performed showed that genotype was not a statistically significant factor as regards milk density differentiation, which was affected by date of determination (lactation stage) only. The low-

est milk density was noted at stage I (28.9 °Ld), and differed significantly from its level recorded at stage II (29.8 °Ld), and highly significantly from the levels observed at stages III (30.4 °Ld) and IV (30.7 °Ld).

III: Physicochemical properties of milk

Parameter	Sire allele	Determinations during the first lactation								Mean	
		stage I 30 days		stage II 100 days		stage III 150 days		Stage IV 200 days			
		\bar{x}	Sd	\bar{x}	Sd	\bar{x}	Sd	\bar{x}	Sd	\bar{x}	Sd
Density °Ld	Lys-mic 7	28.8	1.7	29.9	1.7	30.2	1.1	30.5	1.3	29.8	1.6
	Lys-mic 3	28.9	1.3	29.6	1.6	30.5	1.3	30.8	1.1	30.0	1.5
	\bar{x}	28.9 ^{Cb}	1.4	29.8 ^{BCa}	1.6	30.4 ^{AB}	1.2	30.7 ^A	1.1	29.9	1.5
Active acidity pH	Lys-mic 7	6.70	0.12	6.68	0.10	6.78	0.14	6.72	0.14	6.72	0.13
	Lys-mic 3	6.67	0.07	6.71	0.08	6.72	0.21	6.65	0.25	6.68	0.17
	\bar{x}	6.69	0.10	6.70	0.09	6.75	0.18	6.69	0.21	6.71	0.16
Clotting /sec./	Lys-mic 7	724	726	591	387	776	592	933	627	756	598
	Lys-mic 3	662	626	589	297	671	326	754	473	669	448
	\bar{x}	693	662	590	333	724	451	844	542	713	515
Thermostability (ml etanol/10 ml)	Lys-mic 7	5.03	1.41	5.35	1.46	5.46	2.46	4.22	1.72	5.02	1.84
	Lys-mic 3	5.70	1.24	5.23	1.19	4.77	1.54	3.71	1.95	4.85	1.67
	\bar{x}	5.37 ^A	1.34	5.29 ^A	1.29	5.12 ^A	1.97	3.97 ^B	1.86	4.94	1.74

Means followed by different letters (lactation stages-dates of determination) or x (genotype) differ significantly;

Capital letters or xx - P = 0.01; small letters or x - P = 0.05

Active acidity of milk from the experimental cows was normal in average, i.e. pH 6.71. The mean time of curd formation amounted to 713 seconds (11.7 min.) and was slightly above the norm. The process of milk clotting showed fluctuations during lactation, and clotting time varied from 590 to 844 seconds (9.8–14.1 min.), but these differences were not confirmed statistically.

In the present study the mean of thermostability was characterized by alcohol amount of 4.94. In the group of cows with the Lys-mic 7 allele the thermostability index was slightly higher than in the group with the Lys-mic 3 allele (5.02 ml vs. 4.85 ml). However, the differences between these values were not statistically significant. Milk thermostability was decreasing gradually over the lactation period, to reach the lowest level at stage IV (3.97 ml), highly significantly different from the levels recorded at stages I (5.37 ml), II (5.29 ml) and III (5.12 ml). The reason for reduced milk thermostability at stage IV was the absence of the acid-base equilibrium,

which could be caused by a slight increase in calcium content.

The average somatic cell count was $280 \times 10^3/\text{ml}$ milk (Tab. IV). The statistical analysis performed after logarithmic transformations did not show significant differences in this parameter, so both arithmetic means and standard deviations are presented as the absolute value of somatic cell count, in accordance with the generally accepted veterinary and technological norms. The above value corresponds with the norms for extra grade milk (to 400 000/ml) (Polish Standard PN-A-86002 1999). The variations noted in its level indicated the possibility of periodic subclinical inflammation, especially at stages III and IV. This is confirmed by a distinct decrease in milk thermostability on the 200th day of lactation (stage IV) in both groups. It seems that these parameters could be affected by season. In one third of the experimental cows stage III occurred in summer. High variation of this index (Sd > 100%) made it difficult to verify the results obtained within genotypes and dates of determination (lactation stages).

IV: Somatic cell count and bacterial count in milk

Parameter	Sire allele	Determinations during the first lactation								Mean	
		stage I 30 days		stage II 100 days		Stage III 150 days		stage IV 200 days			
		\bar{x}	Sd	\bar{x}	Sd	\bar{x}	Sd	\bar{x}	Sd	\bar{x}	Sd
Somatic cell count (10 ³ /ml)	Lys-mic 7	231	451	88	103	556	1132	451	967	332	787
	Lys-mic 3	256	543	112	134	292	558	248	329	227	428
	\bar{x}	244	504	100	122	426	838	350	664	280	560
Bacterial count (10 ³ /ml)	Lys-mic 7	219	519	225	394	173	289	180	264	199	400
	Lys-mic 3	165	289	181	320	100	85	105	90	138	205
	\bar{x}	192	394	203	349	137	196	143	190	169	302

The average bacterial count was 169 x 10³ per ml milk and did not fulfill the extra grade requirements. Worse values of this parameter were achieved in the group with the Lys-mic 7 allele, and much better in the Lys-mic 3 group. A statistical analysis of these differences was difficult due to very high variation of this index (Sd >100%). The results obtained also indicated periodic subclinical mastitis.

The group of cows with the sire mLys-mic 7 allele, compared with the mLys-mic 3 group, was characterized by a threefold higher capacity to lyse the tested *Micrococcus lysodeicticus* strain (35.2% vs. 12.9% lysis) (Tab. V). The effect of the mLys-mic genotype was highly significant at all lactation stages. The ly-

tic activity of the lysozyme was gradually decreasing over the lactation period, from on average 25.9% lysis at stage I to 21.4% lysis at stage IV. The average whey bacteriolytic activity of the lysozyme was 7.77% lysis. The date of determination (lactation stage) had a considerable influence on the activity of the whey lysozyme. Both groups of cows were characterized by an increasing activity of the whey lysozyme during lactation. The lowest lytic activity of the enzyme was recorded at stage I (5.56% lysis), and the highest – at stage IV (10.27% lysis), and this difference was statistically highly significant. However, the expected effect of the mLys-mic 7 allele on the lytic activity of the lysozyme in whey was not confirmed statistically.

V: Serum and whey lysozyme activity

Parameter	Sire allele	Determinations during the first lactation								Mean	
		stage I 30 days		stage II 100 days		stage III 150 days		stage IV 200 days			
		\bar{x}	Sd	\bar{x}	Sd	\bar{x}	Sd	\bar{x}	Sd	\bar{x}	Sd
Activity of the serum lysozyme % lysis	Lys-mic 7	38.2 ^{xx}	5.7	36.4 ^{xx}	5.4	35.0 ^{xx}	5.0	31.3 ^{xx}	4.8	35.2 ^{xx}	5.4
	Lys-mic 3	13.5	5.0	13.4	4.8	13.0	4.8	11.5	4.4	12.9	4.7
	\bar{x}	25.9	11.0	24.9	10.4	24.0	10.0	21.4	9.0	24.1	10.4
Activity of the whey lysozyme % lysis	Lys-mic 7	5.93	2.53	8.76	6.18	6.97	6.08	9.25	7.58	7.73	5.94
	Lys-mic 3	5.19	3.07	6.84	4.98	7.92	9.81	11.29	10.34	7.81	7.93
	\bar{x}	5.56 ^B	2.86	7.80	5.52	7.45	8.44	10.27 ^A	9.29	7.77	7.18

Means followed by different letters (lactation stages-dates of determination) differ significantly;

Capital letters P = 0.01; small letters P = 0.05

Means of genetic groups differ significantly at xx - P ≤ 0.01.

DISCUSSION

The assumed calcium content of milk is 1.10 to 1.20 g/l. The calcium content observed in the present experiment agrees with the reference values and does not indicate the occurrence of mastitis. The Ca:P ratio should oscillate around 1.2:1. In our study it mean value was 1.27:1, ranging from 1.19 (stage III) to 1.38:1 (stage IV).

Sodium chloride, similarly as lactose, affects considerably the osmotic pressure in milk. It compensates for the differences between soluble milk salts and lactose, stemming from a variety of reasons. A decrease in lactose content is accompanied by an increase in sodium chloride content. Its level exceeding 2.0 indicates disadvantageous functional changes in the mammary gland. However, such high values were not noted in this experiment.

A growing tendency observed in milk density during lactation was also reported by Sowiński (1993). A relatively higher milk density (29.0 to 30.2 °Ld) was noted by Walawski et al. (1997).

In the studies conducted by Walawski et al. (1997), the average milk clotting time was 339 to 380 seconds (5.6–6.4 min.). Kiszka et al. (1988) recorded a much longer clotting time in Jersey and Belgian Red cows and their crossbreeds (526 to 718 seconds). Sowiński (1993) found that coagulation time was increasing during lactation, and the differences observed were statistically significant.

Curd forming ability depends on the contents of milk constituents and their ratios, as well as the health condition of the mammary gland. According to reference data, there is a significant positive correlation between coagulation time and somatic cell count. It is generally assumed that the clotting of milk produced by mastitic cows is lower, resulting in a longer coagulation time. Milk obtained from mastitic cows does not clot even after 20 minutes, in some cases does not clot at all. The basis for evaluation should be ethanol stability of at least 75, or even 80 and higher, which roughly corresponds to the amount of alcohol of 6.5 and 7.0 respectively (Jurczak, 1999).

The total number of cells referred to as somatic cells (Max Paape, as cited in Malinowski, 2001) is

generally slightly higher at the beginning and end of lactation, but in a normal and healthy mammary gland does not exceed $100 \times 10^3/\text{ml}$ milk (Deluyker et al., 1993; Laevens et al., 1997). The composition of somatic cells is differentiated, including mammary gland epithelial tissues, as well as granulocytes, lymphocytes, monocytes and macrophages (Koralewska 2002), whose presence is connected with the immunological responses of the udder. Somatic cell count undergoes natural fluctuations, associated with lactation stage and cow's age (Pytlewski and Dorynek, 2000). According to Scheppers et al. (1997), the somatic cell count in the uninfected quarter is lower than $200 \times 10^3/\text{ml}$ milk, which constitutes a threshold value between infection-free and infected udder. In the opinion of Hortet and Seegers (1998), the threshold value is 50×10^3 somatic cells per ml milk. According to their estimates, the doubling of this number may result in daily losses of 0.4 kg milk in primiparas and 0.6 kg milk in multiparas.

Malinowski (2001) reported that an increase in somatic cell count in summer was a consequence of temperature stress which, combined with high air humidity, is conducive to pathogen proliferation. Also Rupp et al. (2000) observed a higher somatic cell count in summer and autumn. In the investigations carried out by Pytlewski and Dorynek (2000), somatic cell count was the lowest during the first 100 days of lactation, higher during the next 100 days, and the highest – at the end of lactation. Ng-Kwai-Hang et al. (1984) and Sowiński (1993) recorded the highest somatic cell count at the beginning and end of lactation.

CONCLUSIONS

The results obtained indicate that Lys-mic is of low suitability as a marker for milk production capacity in cows. The lysozyme gene, treated as a mastitis resistance factor, showed no effects on somatic cell count and bacterial count. Further investigations, conducted not only during the first lactation, but also during the next lactations, in a herd threatened by a variety of pathogenic factors, would enable a more detailed analysis of the influence of the Lys-mic gene on mastitis incidence.

SOUHRN

Vztah mezi mikrosatelitním polymorfismem makrofágového projevu lysozymového (mLys) genu a vybranými vlastnostmi mléka polských černobílých krav

Sledování proběhlo v letech 1998–2000 na dvou farmách v oblasti Zulawy Wislane. Do sledování bylo zahrnuto 52 dcer – prvotetek býka Parana, z nichž 21 bylo nositelkami alely 7 mLys-mic genu a 31 nositelkami alely 3 mLys-mic genu. V 30, 100, 150 a 200 dnech laktace byla sledována sérová a syrovátková bakteriolytická aktivita lysozymu, koncentrace vybraných minerálních látek, technologické vlastnosti mléka, počet somatických buněk a bakterií. Zjištěné výsledky ukazují, že polymorfismu Lys-mic se

nedá využit jako markeru pro mléčnou produkci krav. U lysozymového genu, hodnoceného jako faktor rezistence mastitid, nebyl pozorován vliv na počet somatických buněk a počet bakterií. Přesto v dalších sledováních, včetně dalších laktací, ve stádech ohrožených různými patogenními vlivy, by mohlo být využito polymorfismu Lys-mic genu při resistenci mastitid.

alela, lysozym, mastitis, somatické buňky, bakterie, laktace

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