Breed-related differences in blood lysozyme concentration and complement activity in cows in Bulgaria

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SUMMARY

The aim of this study was to compare serum lysozyme concentrations and activities of the alternative pathway of complement activation (APCA) measured in 7 different cow breeds (Jersey, Hornless Hereford, Limousine, Bulgarian Black and White, Bulgarian Brown, Bulgarian Rhodope and Aberdeen Angus, with 30 cows for each breed) reared in 3 Bulgarian public farms. The Hornless Hereford breed presented a significantly higher average serum lysozyme concentration than the dairy breeds and the Aberdeen Angus breed. A high mean serum lysozyme concentration was also encountered in the Limousine breed but the difference with the other breeds was not significant because of the great value dispersion. Furthermore, exceptionally enhanced values for cows (above 1.104 μg/ml*)* were found in 4 cows belonging to the Hornless Hereford and Limousine breeds, confirming the existence of a particular gene responsible for the high enzyme synthesis. The higher blood APCA activities were evidenced in meat-type breeds (Hornless Hereford, Limousine and Aberdeen Angus) and the lower in 2 dairy breeds, i.e. the Bulgarian Black and White and the Bulgarian Brown, whereas intermediate values were obtained in the Jersey and Bulgarian Rhodope breeds. These results show that serum lysozyme concentrations and APCA activities were partially influenced by the cow breed and that some meat-types (Hornless Hereford and Limousine) exhibited a more efficient natural humoral immunity than dairy breeds.

Keywords : lysozyme, complement alternative pathway, breed, cow.

RÉSUMÉ

Concentrations en lysozyme et activité du complément selon la race bovine.

L'objectif de cette étude était de comparer chez la vache, les concentrations sériques en lysozyme et 1'activation du complément par la voie alterne mesurées dans 7 races différentes (Jersey, Hornless Hereford, Limousine, Noire et Blanche de Bulgarie, Brune de Bulgarie, Rhodope bulgare et Aberdeen Angus, avec pour chaque race un échantillon de 30 animaux) élevées dans 3 centres publiques Bulgares. La race Hornless Hereford a présenté une concentration sérique moyenne en lysozyme significativement plus élevée que les races laitières et que la race Aberdeen Angus. Une valeur moyenne élevée a également été obtenue dans la race Limousine mais la différence avec les autres races n'a pas été significative en raison d'une importante variabilité interindividuelle. De plus, des concentrations exceptionnellement fortes pour l'espèce bovine (au-dessus de 1.104 _g/ml*)* ont été mises en évidence chez 4 animaux de races Hornless Hereford et Limousine, ce qui confirmerait l'existence d'un gène particulier responsable d'une synthèse élevée de 1'enzyme. Les plus fortes activités de complément ont été mises en évidence dans les races à viande (Hornless Hereford, Limousine et Aberdeen Angus) et les plus faibles dans 2 races laitières, la Noire et Blanche de Bulgarie et la Brune de Bulgarie, tandis que des valeurs intermédiaires ont été obtenues dans les races Jersey et Rhodope bulgare. Ces résultats montrent que les concentrations sériques en lysozyme et le degré de l'activation du complément par la voie alterne sont partiellement déterminés par la race et que certaines races à viande (Hornless Hereford et Limousine) présentent au total une immunité humorale naturelle plus efficace que les races laitières.

Mots-clés : lysozyme, complément, voie alterne, race, vache.

Introduction

The activity of phagocytosis, complement, β-lysines and the concentrations of lysozyme, interferons and immunoglobulins determined the level of systemic natural and specific immune responses [1, 2, 4, 5, 7, 40, 43] and these markers could be used as biological tests for systemic immune status.

Lysozyme is one of the principal factors of the natural immunity in humans, mammals and birds [3, 6, 27]. Its bactericide effect against Gram-positive and some Gram-negative micro organisms is due to its lytic, cationic and hydrophobic properties [9, 13, 14, 22, 28, 29]. Complement performs various defence roles via activation of three different pathways: alternative, classical and lectin pathways [19, 20].

The studies of some authors proved that lysozyme and complement concentrations differ in the various animal species and that they are also breed-dependent. Considerable breed-related differences were evidenced in swine, sheep, horses [34-37] and cattle [17, 18]. The aim of the present study was to establish the breed-related differences in serum lysozyme and complement activities in cows, reared in different regions of the Bulgaria.

Material and methods

ANIMALS

The experiment was carried out in November-December 2005. The animals were 4– 6 years old and were reared in different farms. Seven cattle breeds were studied : cows of Jersey (n = 30), Aberdeen Angus (n = 30), Hornless Hereford (n = 30), and Limousine (n = 30) and breeds were reared in Institute of Mountain Animal Husbandry and Agriculture – Troyan whereas cows of Bulgarian Black and White cattle (BBW) (n = 30) and Bulgarian Rhodope cattle (BR) (n = 30) – in the Experimental Farm of the Trakia University – Stara Zagora and Bulgarian Brown cattle (BB) (n = 30) in the farm of the Professional High School of Veterinary Medicine "I. P. Pavlov" – Stara Zagora.

Blood for analysis was sampled in 10 ml tubes from *v. jugularis*. The blood was allowed to clot for one hour at room temperature (25°C) and the samples were centrifuged at 2000 g for 10 min at room temperature.

METHODS

Measurement of lysozyme concentrations

Blood serum lysozyme concentrations were determined according the method of LIE [23]. Twenty ml of 2% agarose (ICN, UK, Lot 2050) dissolved in phosphate buffer (0.07 M Na_2HPO_4 and NaH_2PO_4, pH = 6.2) were mixed with 20 ml suspension of 24 hours culture of *Micrococcus lysodeicticus* at 67°C. This mixture was poured out in Petri's dish (14 cm diameter). After solidifying at room temperature 32 wells were made (5 mm diameter). Fifty microliters of undiluted sera were poured out in each well. Eight standard dilutions (from 0.025 to 3.125 μg/ml) of lysozyme (Veterinary Research Institute, Veliko Tirnovo) were used in the same volume as well. The samples were incubated for 20 hours at 37°C and lytic diameters were measured. The final lysozyme concentrations were calculated using special computer program developed in Trakia University and expressed as μg/ml.

Determination of alternative pathway of complement activity

The alternative pathway of complement activation (APCA) was studied by the method of SOTIROV [34]. Each serum sample was first diluted by mixing 100 μl serum with 300 μl veronal-veronal Na buffer (in final concentrations: 146 mM NaCl; 1.8 mM 5,5-diethylbarbituric acid sodium salt; 3.2 mM 5,5-diethylbarbituric acid; 1 mM EGTA and 0.8 mM $MgCl_2$). In U bottomed microplates (Flow Laboratories, UK), 7 other dilutions from each diluted serum were again prepared in veronal-veronal Na buffer: 70 μl diluted serum + 30 μl buffer; 60 μl diluted serum + 40 μl buffer; 50 μl diluted serum + 50 μl buffer; 40 μl diluted serum + 60 μl buffer; 30 μl diluted serum + 70 μl buffer; 20 μl diluted serum + 80 μl buffer; 10 μl diluted serum + 90 μl buffer. Then, 50 μl buffer and 100 μl of 1% rabbit erythrocyte suspension were added to each well. After incubation for 1 hour at 37°C, samples were centrifuged at 150 g for 3 min at room temperature (25°C). Thereafter, 150 μl of each supernatant were removed and placed in flat-bottomed plates for measurement of optic density at 540 nm by "Sumal-PE2" ELISA reader (Karl Zeiss, Germany). The final APCA activity was calculated using a special computer program developed in the Trakia University and expressed as CH50 units (CH50 units correspond to 50% of complement-induced haemolysis of applied erythrocytes).

Statistical analysis

Data were analysed using the fixed effect MANOVA model (Program STATISTICA, StatSoft, Inc., USA) according to the following formula:

$$y_{ij} = \mu + \alpha_i + \varepsilon_{ij},$$

where y_{ij} is the observation value of the investigated trait, μ the population mean, α_i the breed effect and ε_{ij} the random errors. Differences were considered as significant when p values were less than 0.05.

Results

Serum lysozyme concentrations in the different cattle breeds are shown in Table I. The highest values were obtained for the Hornless Hereford cows (0.699 ± 0.136 μg/ml) and they were statistically significantly higher than values observed in dairy breeds ($p < 0.001$) and in the meat-type Aberdeen Angus cows ($p < 0.01$). The Limousine cows also presented high serum lysozyme concentrations (0.609 ± 0.330 μg/ml) but the differences with the other cow breeds were not significant because of the great value dispersion in this meat-type breed. Besides, some exceptional high values (6.245; 2.208; 1.156 and 1.104 μg/ml) were observed in 4 animals belonging to these 2 breeds whereas serum lysozyme concentrations ranged from 0.098 to 0.195 μg/ml and from 0.195 to 0.781 μg/ml in the other Limousine and Hornless Hereford cows respectively. By contrast, mean serum lysozyme concentrations varied from 0.167 ± 0.014 (Bulgarian Brown) to 0.241 ± 0.061 μg/ml (Bulgarian Rhodope) in the dairy breeds and in the Aberdeen Angus breed and the value dispersion within these breeds was lower than in Limousine and Hornless Hereford breeds except for the Bulgarian Rhodope breed.

The complement activities in the studied breeds are shown in Table II. The highest ACPA values were observed in the meat-type breeds, i.e. Aberdeen Angus, Limousine and Hornless Hereford, whereas the lowest values were obtained in Bulgarian Black and White and Bulgarian Brown breeds ($p < 0.01$) and the Jersey and Bulgarian Rhodope breeds presented intermediate values.In order to investigate the relationship between lysozyme concentrations and complement activity, we studied correlations in the different breeds and in all studied animals (Table III). .

Breed	n	X ± SE	Extreme values (min – max)	VC (%)
Hornless Hereford	30	0.699 ± 0.136^{b}	0.195 – 1.104	43.38
Limousine	30	0.609 ± 0.330^{ab}	0.098 – 1.561	121.39
Bulgarian Black and White	30	$0.184 \pm 0.020^{a***}$	0.116 – 0.232	24.51
Jersey	30	$0.201 \pm 0.024^{a***}$	0.138 – 0.276	26.56
Bulgarian Brown	30	$0.167 \pm 0.014^{a***}$	0.138 – 0.195	18.75
Bulgarian Rhodope	30	$0.241 \pm 0.061^{a**}$	0.098 – 0.464	56.72
Aberdeen Angus	30	$0.197 \pm 0.014^{a***}$	0.164 – 0.232	15.46

[a,b] Mean values with different superscripts within the same column differ significantly with $p < 0.001$ (***) or with $p < 0.01$ (**).

TABLE 1. Lysozyme concentrations (μg/ml) in the different cow breeds. Results are expressed as mean ± standard error (SE). VC (%) : Coefficients of variations.

Breed	n	X ± SE	Extreme values (min – max)	VC (%)
Aberdeen Angus	30	626.05 ± 10.91^{a}	590.39 – 662.33	3.90
Hornless Hereford	30	621.09 ± 10.09^{a}	599.57 – 628.25	3.63
Limousine	30	623.78 ± 10.84^{a}	583.86 – 655.36	3.89
Bulgarian Black and White	30	566.82 ± 9.41^{b}	547.38 – 604.41	3.71
Jersey	30	604.8 ± 10.84^{ab}	585.46 – 650.53	4.01
Bulgarian Brown	30	559.08 ± 7.70^{b}	530.88 – 583.76	3.08
Bulgarian Rhodope	30	593.13 ± 13.30^{ab}	557.20 – 643.60	5.02

[a,b] Mean values with different superscripts within the same column differ significantly ($p < 0.05$).

TABLE 2. APCA activity (CH50) in the different cow breeds. Results are expressed as mean ± standard error (SE). VC (%) : Coefficients of variations.

Breed	n	Correlation coefficient (r)	P
Aberdeen Angus	30	-0.59	< 0.01
Hornless Hereford	30	- 0.01	NS
Limousine	30	0.58	< 0.01
Bulgarian Black and White	30	0.24	NS
Jersey	30	0.41	< 0.05
Bulgarian Brown	30	- 0.67	< 0.01
Bulgarian Rhodope	30	0.17	NS
Overall population	**210**	**0.39**	**< 0.05**

TABLE 3. Correlations between lysozyme concentrations (μg/ml) and APCA activity (CH50) in the overall population of cows and in the different cow breeds. r = correlation coefficient.

Globally, these 2 parameters were positively but moderately associated ($r = 0.39$, $p < 0.05$). This positive association was also found in Jersey and Limousine breeds ($p < 0.05$), whereas lysozyme concentrations were negatively correlated with APCA activities in Bulgarian Brown and Aberdeen Angus cows ($p < 0.05$). Finally, in the other breeds (Hornless Hereford, Bulgarian Rhodope and Bulgarian Black and White) no significant correlation was obtained.

Discussion

In the present study, high mean serum lysozyme concentrations were observed in Limousine and in Hornless Hereford cows and very high values for this specie (1.104 to 6.245 μg/ml) were recorded in 4 animals (6.7%). However, dramatically elevated serum lysozyme concentrations are exceptional in cattle. WALASWKI *et al.* [41] reported that out of

10 000 studied animals, only two bulls and some cow dams with high lysozyme concentrations were discovered. Their progeny (regardless of its gender, age or other factors) has inherited a very high or a normal lysozyme concentration. On the basis of a rich experimental material (294 young bulls from 19 lines) LIE [22] assumes that the serum lysozyme concentration was influenced genetically:

1. With regard to the observed heredity coefficient ($h^2 = 0.27$) he supposed that lysozyme concentration was polygenically regulated.

2. The existence of a particular gene with a relatively low frequency, responsible for the exceptionally high enzyme concentrations in some individuals within the population, was also supposed.

Later, LIE and SOLBU [24] studied 329 bulls from the Red Norwegian cattle breed and they concluded that lysozyme concentration in cattle was probably inherited as a simple Mendelian sign and that the frequency of the dominant gene determining the high enzyme concentration in this breed was 6%. Besides the authors observed a statistically significantly high positive correlation ($r = 0.63$; $p < 0.01$) between serum and colostrum lysozyme concentrations in this breed. According to them, this genetic bond was highly influenced by the presence of the dominant gene, described earlier [24]. Thus, they hypothesized that the frequency of the dominant gene in cows should be increased via selection of bulls on this trait leading to an increased resistance to diseases, caused by Gram-positive micro organisms. Consequently, the 4 animals which presented exceptional high serum lysozyme concentrations in the present study could be probably carriers of the "main" gene (or allele) responsible for the high blood enzyme concentration. This finding may support the previous Dr. LIE's hypothesis.

During the last 15 years, several investigators [15, 16, 18, 39] have elucidated at a significant extent the genetic structure of the gene (or genes), coding for lysozyme in the various animal species and its importance in systemic defence against pathogens. On the basis of these works, we could assume that in breeds with a high phenotypic diversity of the trait, the primary gene determining high lysozyme concentrations was encountered in a homozygous state. The supposition is also supported by the studies of IRWIN and WILSON [15] and IRWIN *et al*. [16], which have reported the existence of at least 10 genes coding for the enzyme in ruminants. On the other hand, STEINHOFF *et al*. [39] have evidenced in cows 2 other genes expressed in both neutrophil granulocytes and the udder coding for lysozyme in blood and milk respectively. These 2 new genes are different from genes coding for gastric enzymes [32]. In the other organs and body fluids (lungs, liver, heart, spleen, lymph nodes, cartilage, amniotic fluid, sperm etc.) lysozyme is also detected, but at different concentrations and with additional functions [6]. This finding allows assuming that in the other body cells, specific genes for lysozyme synthesis could be found.

Other investigators [17, 27, 33] also communicate breed-related differences in lysozyme concentrations in cows. MEYER *et al*. [27] reported that milk lysozyme concentrations in Black-and-White or Jersey cows were significantly different and varied from 0.18 to 0.45 μg/ml. According to the authors, the hereditability coefficient of muramidase activity was $h^2 = 0.13$. They could not provide a direct response to the opportunity of using lysozyme as a selection trait in cows for improving resistance to mastitis. SIEFERT [33] has studied lysozyme activity in different cattle breeds and presented the following conclusions: i) local breeds (Criollo and Ndama) had higher concentrations of serum lysozyme than imported breeds (Holstein-Friesian, Grey Brown Alpine) and than their crosses (German Brown x Yellow cattle); ii) crossing with imported breeds negatively affected lysozyme concentrations; iii) meat cattle breeds had higher serum lysozyme concentrations compared to dairy breeds; iv) cows had higher muramidase activities than bulls and v) lysozyme concentrations decreased with age. The same conclusions were made by KADIMOV *et al*. [17] in a study on lysozyme in Zebu, Aberdeen Angus x Zebu, Aberdeen Angus x Zebu x Black-and-White cattle, and Black-and-White cattle. MEYER *et al*. [27] consider that the mechanism of inheritance of lysozyme levels could be elucidated by investigating the inter-group correlations in cattle [1, 27, 33].

Although lysozyme concentrations and APCA activities positively correlated in the overall studied population of cows, the correlation coefficients obtained in the 7 different breeds varied within broad ranges and negative correlations were even found in Bulgarian Brown and Aberdeen Angus cows. These findings suggested that the biosynthesis of lysozyme and globulins of complement were not or poorly genetically linked [15, 16, 19] and that different mechanisms of regulation dependent of the targets [6, 8, 9, 12, 21] or of other humoral factors [32] were involved. WAMBURA *et al*. [42] compared 3 zebu breeds (Meru, Mbullu, and Iringa Red) and their crosses with Friesian cattle according to their resistance against ticks. It was observed that purebred zebu were more resistant to parasites compared to crossbreds. Also, the activity of complement in purebred zebu was higher than that in crossbreds. It was assumed that the highest complement activity was important for the higher resistance of the three zebu breeds. This suggestion was supported by the statistically significant negative correlation between the degree of infection with parasites and the complement activity. The alternative pathway of complement activation plays an important role in systemic defence against parasites. MACHADO *et al*. [25] proved that *Schistosoma mansoni* could be inactivated and killed by the APCA *in vitro*. These results are also supported by SANTORO *et al*. [31]. RICKARD *et al*. [30] reported that APCA is actively involved in the lysis of *Echinococcus granulosus*. The same effect is observed against *Taenia taeniaeformis* by HAMMERBERG and WILLIAMS [11]. STANKIEWICZ [38] and MACKENZIE *et al*. [26] observed reactions of cellular binding in trichinellosis and proved that APCA also participates in t his process. FLEMMINGS and DIGGS [10] reported that APCA plays a primary role in systemic defence against *Trypanosoma rhodesiense*.

In the present study, it could be concluded that in cattle, breed-related differences in lysozyme concentrations and complement activities existed, similarly to other animal species.

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