ANALYSIS OF FACTORS INFLUENCING IMMUNOGLOBULIN G CONCENTRATION IN MILK OF DAIRY COWS

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Abstract. In this study, the purpose was to estimate immunoglobulins G (IgG) concentration in milk depending on cows' breed, number of lactations and somatic cell count (SCC). Twenty cows of different breeds (Lithuanian Black-and-White, Dutch Black-and-White, and German Black-and-White) and lactations (from 1st to 6th) were used in the study. The research material was split into four groups according to SCC detected in the milk samples: 1st group – SCC up to 100 x10^3/ml; 2nd group – 101– 400 x10^3/ml; 3rd group – 401 – 1,000 x10^3/ml and 4th group – 1,001 x10^3/ml. Samples of fresh milk were analysed for SCC performed by the heavy-duty counter-measurer *Somascope*. The contents of IgG were determined by the enzymatic method ELISA based on the competition between marked antigen-antibodies. The mean value for IgG concentration obtained from all the samples was 0.29±0.14 mg/ml. Milk IgG content increased with the increment of SCC. The relation between SCC and IgG content correlated significantly r_p=0.931, r_s=0.854 (P<0.0001). It was determined that IgG concentration varied from 0.26±0.15 mg/ml (1st-2nd lactations) to 0.41±0.11 mg/ml (5th–6th lactations). IgG concentration in milk was not significantly different among numbers of lactations and breeds of cows (P>0.05).

Keywords: immunoglobulin G, somatic cell count, cow, milk

Introduction

Bovine mastitis remains the disease causing the biggest economic losses to the dairy industry, despite the intensive research and prevention measures at herd level carried out for decades (Sobczuk-Szul et al., 2014). The immunological response to intramammary infection is a normal function of the cow's immune system (Harmon, 1994). Natural immunological protective reactions of the animal can successfully restrict and eliminate the udder infection (Sandholm and Pyörälä, 1995; Korcine et al., 2012). Investigations show evidence that humoral immunity components, such as immunoglobulins A and G, play an important role in immunological defence response of the cow udder (Korhonen and Kaartinen, 1995; Marnila and Korhonen, 2002; Korcine et al., 2012). Immunoglobulins form specific immunity components and they mainly penetrate into the udder during inflammation but are also formed locally (Burvenich et al., 2000; Kehrli and Harp, 2001; Pyörälä, 2002). Immunoglobulins are able to prevent the adhesion of microbes, inhibit bacterial metabolism, agglutinate bacteria, augment phagocytosis of bacteria, kill bacteria through activation of complement-mediated bacteriolytic reactions, and neutralize toxins and viruses (Pyörälä, 2002; Park, 2009).

The amount of immunoglobulins in milk varies and all factors influencing their concentration are not discovered yet (Korhonen et al., 2000; Krol et al., 2010). Some publications reported that during the middle stage of lactation, the concentration of immunoglobulins in healthy cow milk is low but it increases during the udder inflammation (Korhonen and Kaartinen, 1995; Korhonen et al., 2000; Korcina et al., 2012). As somatic cell count (SCC) also forms a part of the defence system of the udder against mastitis, it has been suggested that with very low SCC the risk to environmental mastitis may increase (Schukken et al., 1990; Pyörälä, 2002). Pyörälä (2003) and Smith (2002) reported that a bovine quarter producing milk with an SCC >200 x10^3/ml shows the symptoms of subclinical mastitis. According Burvenich et al. (2000), the quarters with elevated SCC are more resistant to mastitis than the quarters with low SCC. However, it is not likely that SCC levels of dairy cows will, through breeding, reach 'too low' levels in the near future. Dairy cows have been bred for high milk production, and there is a positive correlation between high milk yield and mastitis (Fleischer et al., 2001; Pyörälä, 2002).

It is generally accepted that changes in the content of milk components are caused in more than 50 % by genetic factors and in about 40 % by environmental ones (Brodziak et al., 2012). Nowadays, thirteen breeds of cattle are in use in Lithuania, while the milk production is dependent mainly on the Lithuanian Black-and-White breed. The productivity of cows and physico-chemical parameters of milk are influenced not only by breed of cows and feeding system but also by physiological factors such as age and stage of lactation (Krol et al., 2013; Krol et al., 2014).

Work should be concentrated on ways of minimising the negative influence on immune functions and ways of stimulating these functions, especially during periods of immune suppression, to increase the natural ability of the cow to resist, or defend herself, against udder infections. Detailed knowledge about the immune responses and important host defence factors is essential in order to find

better ways for the prevention and treatment of udder infections and mastitis (Mol and Clegg, 2002).

The aim of the present study was to evaluate the concentration of IgG in milk depending on cow breed, number of lactations and somatic cell count.

Material and methods

Selection of animals and collection of milk samples

The experimental part of the study was carried out at the Lithuanian University of Health Sciences, Veterinary Academy (LUHS VA), Practical training and testing centre. Overall, 20 cows were selected. Cows enrolled in the study were from the 1st to 6th lactations of the best production (22–30 kg per day). Milk samples collected from three different breeds (Lithuanian Black-and-White, Dutch Black-and-White, and German Black-and-White) of dairy cows maintained in Lithuania. Milk samples were collected during control evening milking time as total quarter milk from each cow in pairs. After cleaning and disinfection of the teats, 50ml of milk were aseptically collected in sterile plastic tubes according to LST EN ISO 707:1999+P: 2003 standard. Samples were kept under refrigeration until arrival to laboratory facilities and were tested within 6h from collection.

Methods/technique

For determination of somatic cell count milk samples were preserved and analysed with the flow cytometric analysis method using a Somascope cell counter (Foss, 3400 Hillerød, Denmark) according to LST EN ISO 13366-1:2008+AC:2009 microscopic method standard.

The enzyme-linked immunosorbent assay (ELISA) used to determine IgG concentration in bovine biological fluids is distributed as Biopanda Reagents (United Kingdom). This assay has been designed for the quantification of IgG by means of specific antibodies and it has been validated in an interlaboratory study with satisfactory results of repeatability. The procedure of the assay was carried out as follows. The kit provided the 96-well plate coated with specific antibodies against bovine IgG. Standards or samples (100µl) in duplicate were added into each well and the plate was incubated at room temperature (RT) for 30 min. Afterwards, the wells were emptied by inverting the plate and tapping firmly onto absorbent tissue. The plate was washed in Labsystems Multiwash microtiter plate automatic washer (Helsinki, Finland) with 300 µl of diluted wash buffer per well, making five cycles of washing. After removing the excess liquid as described above, 100 µl of the diluted horseradish peroxidase (HRP) - antibody conjugate solution was added into each well and incubated at RT) for 30 min. After this second incubation, the plate was washed as previously described and 100µl of the substrate 3.3‘, 5.5‘- tetramethybenzidine (TMB) solution was added into each well. In order to allow the colour development, the plate was incubated at RT for 10 min. Finally, 100µl of the stop solution were added into each well. The concentration of antibodies in a sample was indicated by the blue colour appearing during the reaction and turning yellow after suppression of reaction with acid. The test results were estimated by measuring the optical density (OD) of samples at wave length λ=450 nm using spectrophotometer Thermo Scientific Multiskan EX (Thermo electron corporation, China, 2005). In order to calculate the IgG concentration of each sample, a graphic representation was made by plotting the concentrations of the standards (y axis) versus the mean values of the corresponding absorbances (x axis) for each plate. The IgG concentration of the samples was determined by interpolating the corresponding absorbances in the standard curve, which was adjusted to a second-order polynomial equation.

Statistical analysis

GraphPad Prism version 4.0 statistical package was used for biometric data analysis. Arithmetic averages ($\bar{x}$), medium standard deviations (SD), minimum and maximum values were calculated for cow's milk IgG parameters and somatic cell count. Pearson's (r_p) and Spearman's (r_s) correlation coefficients and linear regression were used to investigate the relationship between SCC levels and milk IgG. Results were statistically significant when R^2 was not less than 0.25.

The following factors were taken into consideration:

- The research material was split into four groups according to somatic cell count detected in the milk samples: 1st group – SCC up to 100 x10^3/ml (n=3); 2nd group – 101– 400 x10^3/ml (n=6); 3rd group – 401 – 1,000 x10^3/ml (n=8) and 4th group – 1,001 x10^3/ml (n=3).
- Age classes mostly noticed as subsequent lactation: 1, 2, 3, 4, 5, 6.
- Three breeds of cows (Lithuanian Black-and-White, Dutch Black-and-White, German Black-and-White) were involved in this study.

Results. The mean value for IgG concentration obtained from all the samples was 0.29±0.140 mg/ml. Immunoglobulin values in milk obtained in our research and the standards given in literature are summarized in Table 1.

Table 1. **Immunoglobulin G values mg/ml (mean ± SD) in cow's milk**

Immunoglobulin class	Data obtained in our studies		IgG values given in literature
IgG	$\bar{x}$ ± SD	Range of values	0.30-0.60 (Pakkanen et al.,1997; Krol et al., 2010) 0.30-0.50 (Collin et al., 2002) 0.25±0.12 the range of values 0.008-1.457 (Conesa et al., 2005) 0.72 (Grapper et al., 2007) 2.05±0.83 the range of values 0.16-4.30 (Korcina et al., 2012)
	0.29±0.14	0.11-0.58	

With the growth of SCC, the concentration of IgG significantly increased. A substantial effect of SCC on IgG content was confirmed by relatively high positive values of calculated correlation coefficients. The relation between SCC and IgG content correlated significantly r_p=0.931, r_s=0.854, P<0.0001, regression analysis was significant R^2=0.867. The results are shown on Figs 1 and 2.

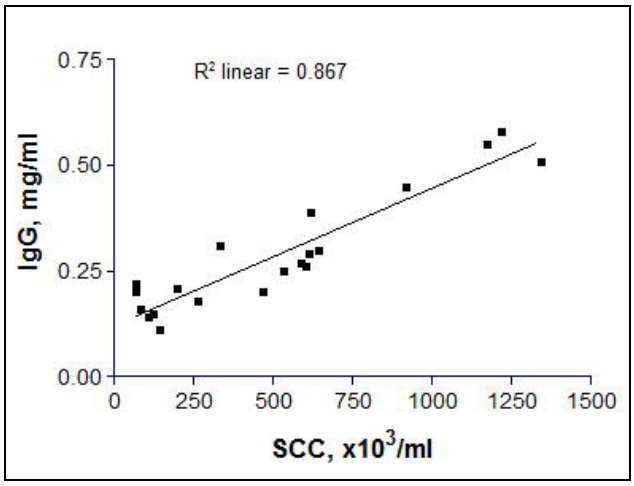

Fig. 1. **Linear regression model of the effect of SCC** (x10^3/ml) **on milk IgG mg/ml**

It was shown that among the many breeds of cows involved in milk production in Lithuania, the cows of the German Black-and-White breed produce a higher content of immunoglobulins G (the range from 0.14 to 0.58). A slightly lower level of IgG was established in milk of Dutch Black-and-White cows (the range from 0.15 to 0.39) as well as Lithuanian Black-and-White cows (the range from 0.11 to 0.51). The cows breed influence on IgG concentration was not significant (P>0.05). The results are shown in Table 2.

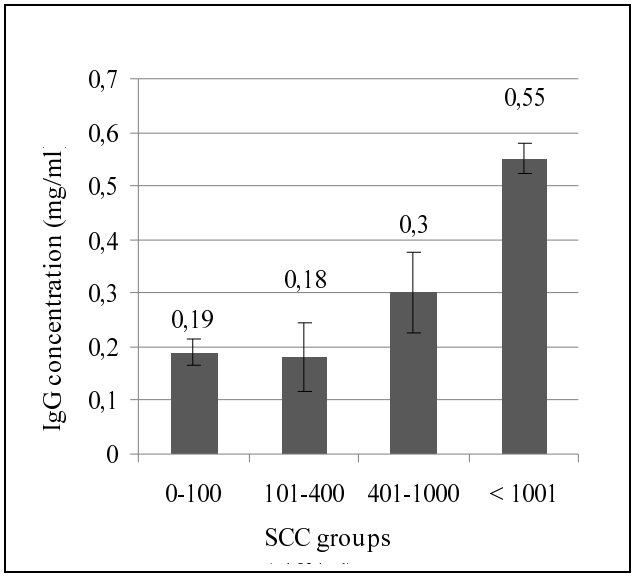

Fig. 2. **Distribution of immunoglobulin G content by SCC groups**

The obtained average results of IgG at different lactations ranged from 0.26±0.15 mg/ml (1st and 2nd lactations cows) to 0.41±0.11 mg/ml (5th and 6th lactations cows). Calculated congruency between cows lactations and IgG concentration in milk was not significant (r=0.02). We observed that the concentration of immunoglobulins in milk increases proportionally with increasing number of lactations as shown in Table 3.

Table 2. **Effect of breed on IgG** (mean±standard deviation) **content in cow's milk**

Breed	Number of cows, n	Immunoglobulins G, mg/ml
Lithuanian Black-and-White	7	0.25±0.12
Dutch Black-and-White	4	0.27±0.08
German Black-and-White	9	0.33±0.15

Table 3. **Effect of lactation on IgG** (mean±standard deviation) **content in cow's milk**

Lactation	Number of cows, n	Immunoglobulins G, mg/ml
1-2	11	0.26±0.15
3-4	6	0.28±0.08
5-6	3	0.41±0.11

Discussion. The amount of immunoglobulins in milk varies and all factors influencing their concentration are not yet discovered (Korhonen et al., 2000; Krol et al., 2010). Different authors have indicated that concentration of immunoglobulins A, G, and M in the cow milk varies not only depending on the degree of udder infection but it is also considerably affected by the cow age, lactation period, keeping conditions, and feeding (McFadden et al., 1997; Korcina et al., 2012).

The mean levels obtained for IgG in the whole population studied are 0.29±0.14 mg/ml, a value close to the range referred to as normal for mature milk, which is 0.3-0.5 mg/ml (Collin et al., 2002). Similar IgG values were obtained by other researchers as well: 0.30–0.60 (Pakkanen, 1997; Krol et al., 2010). In the analysis of bovine IgG in milk, Grapper et al. (2007) ordered a higher IgG value 0.72 mg/ml. The highest concentration of the immunoglobulin G in milk 2.05±0.83 mg/ml was

indicated by Latvian researchers (Korcina et al., 2012). As mentioned above, the amount of immunoglobulins in milk varies depending of many factors; therefore, all indicated immunoglobulin G values in milk are different.

Somatic cell count is a commonly recognized indicator of bovine udder health and milk quality. SCC has been shown to influence the immunoactive protein content (Krol et al., 2012). Milk with the highest number of the somatic cells (group 4) contained the highest IgG concentration 0.55 mg/ml. The same results were obtained from another research carried out in Poland (Krol et al., 2012). A substantial effect of SCC on IgG content was confirmed by high positive values of calculated correlation coefficients r_p=0.931, r_s=0.854, P<0.0001, where they were higher than those indicated by Krol et al., (2012 and 2014) in their research r=0.79 and r=0.507 respectively. Also the effect of SCC growth on the content of IgG was reported by Liu et al. (2009).

Cow's breed is one of the factors influencing the amount of immunoglobulin in milk. Krukowski et al. (2006) determined IgG content in milk from Black-and-White variety cows. IgG concentration in these cows' milk was 0.628 mg/ml. The studies of Levieux and Ollier (1999) revealed that milk gained from Holstein-Friesian cows contained 0.47 mg/ml of IgG at average. A significant amount of IgG was reported in milk of Polish Black-and-White cows 0.42±0.18mg/ml (Krol et al., 2010) and 0.53±0.29 mg/ml (Krol et al., 2012). In the present research, a lower IgG content was found. Following our test data, the richest content of IgG in the milk of the breed German Black-and-White was 0.33±0.15 mg/ml. The poorest source of IgG content in milk was the breed Lithuanian Black-and-White (0.26±0.15 mg/ml). Some authors have reported that in healthy cow milk during the middle stage of lactation the concentration of immunoglobulin is low (Korhonen et al., 2000; Korcina et al., 2012). Other authors indicated that there is a positive correlation between high milk yield and mastitis (Fleischer et al., 2001; Pyörälä, 2002). Our results possibly indicate that the breeds with a low amount of IgG were in the middle stage of lactation and the milk yield of these cows was low too.

The age of the cow and the number of lactations are considered as two relevant factors, which determine IgG concentration in milk. The poorest source of IgG proved to be the milk obtained from the 1st and 2nd lactations cows (0.26±0.15 mg/ml). Older cows, in the 5th and 6th lactations, produced milk with higher concentration of IgG compared to younger ones (0.41±0.11 mg/ml). These findings are similar with the Krol et al. (2010; 2012) research where primiparous cows showed to produce significantly less IgG as compared to cows at 2 to 4 lactations (P≤0.05) and older (P≤0.01). The lowest level of IgG was found in the 1st lactation (0.454±0.16 mg/ml) and in subsequent lactations IgG compounds increased gradually (Krol et al., 2012).

In conclusion, it should be stated that somatic cell count had a significant effect on content of the IgG in cows' milk evaluated. The increase in SCC caused a significant increase (P<0.0001) in IgG concentrations in milk. Their source proves to be milk obtained from multiparous cows of German Black-and-White breed.

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