OCCURRENCE OF MASTITIS PATHOGENS IN RELATION TO SOMATIC CELLS

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The incidence of mastitis pathogens is a persistent problem not only in dairy cows but also for other dairy animals. For breeders it always presents the economic losses. Rajala–Schultz *et al*. (1999) studied the effect of mastitis dissease on milk yields and found that the daily loss during the first 2 wk after the occurrence of mastitis varied from 1.0 to 2.5 kg, and the total loss over the entire lactation varied from 110 to 552 kg. The milking hygiene control, which is directly associated with the development disease caused by environmental microorganisms, often sticking mainly to time and financial cost of laboratory tests. The higher incidence of environmental microorganisms, which include *S. aureus* and *S. uberis*, can be explained by the development and change of milking technology, when the pipeline milking equipment in stall occurred more frequent contamination of the udder by hands of nursing staff or by handling with milking equipment. Therefore, the milking equipment influences whole row of indicators by its function and use, for instance by overmilking (Pařilová *et al.*, 2010, 2011), and can be factor and vector of mastitis origination. Aggravated function grows up mastitis occurrence and subsequently also somatic cell count (Hanuš and Ticháček, 1997). The current types of milking parlours provide better comfort and hygiene of milking (Vyletělová *et al*., 2009; Janštová *et al*., 2011). Beside good quality of milking equipment function also other ways of reduction of mastitis occurrence are looking for as cow vaccination for instance (Toušová *et al.*, 2011).

One of the respected indicators of health status of the mammary gland (Sava and Piwczynski, 2002; Berry *et al.*, 2006; Heck *et al.*, 2009) is the somatic cells count (SCC) similarly as mammary associated isotype of serum amyloid A (Kováč *et al.*, 2011). Golebiewski *et al.* (2011) found the higher average SCC value in long–term observation for Polish Holstein–Friesian as compared to Montbéliarde breed (642 and 455 ths.ml^{-1}). Similar conclusion published Frelich and Šlachta (2011) for Holstein and Czech Fleckvieh without interaction to farm and seasonal effect. The permissible SCC limit value in a pool sample is < 400 thousands in 1 ml of raw cow's milk. As a healthy mammary gland is generally taken to mean that, where the SCC is around 100 ths.ml^{-1} in one quarter. Sheldrake *et al*. (1983) determined the number of somatic cells in uninfected mammary gland from 83 ths.ml^{-1} (from the 35th day after calving) to 160 ths.ml^{-1} (285th day). The higher values indicate a possible infection of the mammary gland. Reneau (1986) in his work provides the value of SCC 283 ths.ml^{-1} as a limit value suitable for the determination of suspicion from subclinical mastitis occurrence. The less numbers of SCC (250 and 228 ths.ml^{-1}) indicate also Andrews *et al.* (1983) and Dohoo *et al*. (1981) as the threshold for selection in the case of treatment of mastitis in cows. The similar relationship (but in the case of the bulk milk samples) describe in their work Benda *et al*. (1997). They estimated 1.7% mastitis diseases incidence caused by *Staphylococcus aureus* at the somatic cells count 160 ths in 1 ml, and 43.5% incidence at the SCC 410 ths.ml^{-1}. In the case of streptococcal mastitis caused by *Streptococcus agalactiae* estimated 1% incidence of mastitis at SCC 160 ths.ml^{-1}, and 24.6% at SCC 400 ths.ml^{-1}.

In time of areal application of antibiotic mastitis therapy in dairy cows and increase of pathogen strain resistance against these medicaments the topicality of return to selective cure and also importance of methods which support this kind of therapy is growing up. At present, much attention is given to methicillin–resistant staphylococci strains, especially *S. aureus* (MRSA), which represent a serious problem in human and veterinary medicine (Holmes and Zadoks, 2011). These strains, which are associated with livestock, are called as LA–MRSA.

The spread of resistant strains in cattle can be problematic in the case of farms with breeding of dairy and meat animals. Because the transfer of resistance going horizontally, the spread of resistant strains is also possible through working staff (Holmes and Zadoks, 2011). An improvement of methods for support of selective cure could be effective in terms of cost saving and mentioned risk reduction as well.

Aim of this work was to assess the relationship between mammary gland pathogen occurrence and somatic cell count (SCC) and possibility to find SCC discrimination limit for estimation of start for subclinical mastitis treatment according to SCC under current conditions.

MATERIAL AND METHODS

Animals and milk sample collection

The suspect animals were selected by responsible worker of dairy production and according to NK test (viscosity) results, clinical symptoms and SCC. There were mainly the higher lactation cows (> 1st lactation) with subclinical mastitis. Milk samples were collected from all four teats into sterile sample containers, kept in a cooler at 4 °C, transported to the laboratory and then immediately processed. There were examined 161 cows from four farms in total. Holstein and Czech Fleckvieh dairy cows were included in the experiment. The mean herd milk yield varied from 5 600 to 8 900 kg per standard lactation. The binding stabling with pipeline milking was used in two stables and free stabling with milking parlour in other two stables. All animals were milked twice a day.

Microbiological laboratory analyses

For microbiological analysis of samples there were used Blood agar, Edwards and Endo agar (Oxoid, Basingstoke, UK). The cultivation of the samples proceeded at 36 °C for 24 hours. Suspicious colonies were further isolate on Blood agar and cultivated at 36 °C for 24 hours. For species identification there were used biochemical tests STREPTOtest, STAPHYtest and ENTEROtest and identification program TNWpro7.0 (Erba Lachema, CZ). In addition, in strains confirmed as *S. aureus* was detected *mec*A gene, encoding resistance to methicillin, using PCR method (Boşgelmez–Tinaz *et al.*, 2006). The determination of SCC in individual milk samples was performed according to CSN EN ISO 13366–2 (2007) using the Somacount instrument (Bentley Chasca Minnesota, USA).

Statistical methods

The SCC classification into classes according to the positive and negative findings of microbiological pathogens was used for statistical evaluation of the results. The classes were created by respectable, literature–based professional practice. Somatic cell counts were evaluated in their original values (thousands ml^{-1}), but also in logarithmically transformed form. This was done for a legitimate presumption absence of normal frequency distribution of SCC results in sets of individual milk samples (Ali and Shook, 1980; Raubertas and Shook, 1982; Reneau *et al*., 1983, 1988; Reneau, 1986; Wiggans and Shook, 1987). For the same reason, for statistical hypothesis testing of differences between groups of samples (t–test on log SCC), there were used geometric means and medians (in the position of the mean values) because of the given purpose. These are more representative characteristics than a simple arithmetic indicator.

RESULTS AND DISCUSSION

Pathogen species identification

Table I shows the results of species identification of the mastitis pathogens. We have confirmed 52 positive dairy cows from all of 161 tested animals and identified 55 mastitis pathogens. The most frequently isolated species was *Enterococcus faecalis* (n = 20), followed by *Staphylococcus aureus* (n = 6) and *Streptococcus uberis* (n = 5). In *S. aureus* strains, the presence of the *mec*A gene, or genes for enterotoxins production was not found. A similar incidence of mastitis pathogens and negative incidence of MRSA was confirmed also in previous work (Vyletělová *et al.*, 2011).

The interesting thing is the finding of only one case of pathogen *Streptococcus agalactiae* from all of positive samples. Previously this pathogen was the main cause of contagious mastitis with high SCC together with *S. aureus* (Erskine *et al*., 1987; Benda *et al*., 1997; Keefe, 1997). Today is *S. agalactiae* after changing technologies, housing and milking hygiene regimes, essentially replaced by other environmental pathogen *S. uberis*. Next to *S. aureus* and *S. uberis* are the most important other species of pathogenic microorganisms such as *E. coli*, coagulase–negative staphylococci CNS, *Corynebacterium bovis* (Bradley, 2002; Pitkälä *et al*., 2004; Vyletělová, 2006; Vasiľ, 2009; Kalmus *et al.*, 2011).

Occurrence of mastitis pathogens in relation to somatic cells

Somatic cell count ranged from 9 to 24 204 thousands (ths.) in 1 ml. In Table I, there is divided incidence of mastitis pathogens in relation to somatic cells into three groups: I) positive / negative mastitis incidence of disease in the case of the SCC $\leq$ 100 ths.ml^{-1}; II) incidence of mastitis disease if SCC > 100 and $\leq$ 283 ths.ml^{-1}; III) incidence of mastitis disease in case of SCC > 283 ths.ml^{-1}. As regards, the positive or negative mastitis disease incidence in the case of I. and II. classes, significant differences are apparent. This also confirms the mentioned limit value for the determination of suspicion of subclinical mastitis (Reneau, 1986). However, differences are between species proportion of isolated bacterial strains.

From the results, it is interesting that at lower SCC (about 50 ths.ml^{-1}) were present bacteria of the genus *Staphylococcus*, followed by bacteria of genus *Enterococcus* (75 ths.ml^{-1}), and only at higher numbers of SCC (346, 554 and 594 ths.ml^{-1} respectively) bacteria genus *Streptococcus*, *Enterobacter* and *Escherichia coli*.

From all results it was found 23 negative results (14%) in samples with higher SCC than 400 ths.ml^{-1} (from 430 to 9 006 ths.ml^{-1}). The absence of pathogens in this case may be caused by another load of mammary glands (stress, nutrition, milking equipment). Similar experiences, but with quarter (individual) samples describe Emanuelson and Wever (1989), when the SCC correctly classified 82.9% of all cases of suspected bacterial infection.

Results of SCC statistical analyses

Statistical analysis results of SCC sets are shown in tables (Table II and III) and in graph (Figure 1). In Table II, there are shown mean SCC values for individual classes of milk samples from health and infected mammary glands and in Table III significance of differences between them. It is remarkable at typically higher arithmetic means of SCC in individual infected classes (from 1 923 to 3 728 ths.ml^{-1}) and in contrast to non infected samples (295 ths.ml^{-1}) that their relative variability is quite balanced (vx from 163.9 to 234.8 versus 173.5%). Also values of geometrical means (Table II) and medians (Figure 1) of SCC individual classes are characteristically very well–balanced compared to values of arithmetical means. Therefore, these values are more representative, more reliable and hence preferable for interpretation. This conclusion is in accordance with previous results and estimations by Ali and Shook, (1980), Raubertas and Shook (1982), Reneau *et al*. (1983, 1988), Reneau (1986) and Wiggans and Shook (1987).

Differences in SCC (arithmetic and geometric means) according to pathogen classes (Table II) were statistically significant ($P < 0.001$) first of all as for about differences non infection group against infected groups which are represented by pathogens (Table III; xg 131

SCC versus 491 for positive, 611 for staphylococci and 464 ths.ml^{-1} for other positive). These significant differences in SCC are as group median with negative findings against group medians with positive findings shown in Figure 1. Insignificant difference ($P > 0.05$; Table II, III and Figure 1) in SCC was noted only between infected groups with positive pathogen findings (staphylococci versus other pathogens, xg 611 and 464 ths.ml^{-1}).

In the file, there was minority representation of *S. agalactiae* infection, wich marked again highest SCC loading (15 199 ths.ml^{-1}; Table I). Under mentioned conditions which are again in good accordance with current general proportions of mastitis ethiology (Vyletělová, 2006; Vasiľ, 2009) the staphylococci infection means higher milk loading by somatic cells (xg 611 ths.ml^{-1}; Table II) and its quality aggravation than in the case of other infection (xg 464 ths.ml^{-1}; Table II). Of course, this fact increases economical relevancy of *S. aureus* infection.

It is possible to calculate a SCC discrimination limit (on 95% conventional level of probability) for practical likelihood of pathogen occurrence estimation in infectious sample groups under presumption of normal frequency distribution of logarithmic transformed SCC values (comparable obliqueness and acuteness of data files). This SCC discrimination limit for suspicion of infection is 159 ths.ml^{-1} for positive group (Table II) according to procedure: negative $2.1157 + 0.5354 \times 1.96 = 3.165084$; positive $2.6911 - 0.7407 \times 1.96 = 1.239328$; $3.165084 - 1.239328 = 1.925756 / 2 = 0.962878 + 1.239328 = 2.202206 = 159$ ths.ml^{-1} of SCC. Along the same procedure for sample groups staphylococci and other positive these limits are 113 and 174 ths.ml^{-1}. It is evident that these limits are mutually relatively similar. Perhaps this is possible to recommend the value 174 ths.ml^{-1} as limit for suspicion of infection and for practical use with target to apply selective mastitis antibiotic treatment or other preventive or curative measures. This could be used for instance as indication for selective antibiotic dry cow therapy in case of its exceeding as compared to SCC lactation geometric mean. This limit is lower as compare to limit 283 ths.ml^{-1} which was defined previously in dependence on milk yield losses by Reneau *et al.* (1983 and 1988), Reneau (1986) and Wiggans and Shook (1987) for similar purposes. However, this estimation is more similar to limits which were previously mentioned by Dohoo *et al*. (1981), Sheldrake *et al*. (1983), Andrews *et al*. (1983) and also Benda *et al*. (1997). One of the reasons for this could be also the development (changes) in pathogen and mastitis situation in cow herds (Sava and Piwczynski, 2002; Berry *et al.*, 2006; Vyletělová, 2006; Heck *et al.*, 2009; Vasiľ, 2009; Frelich and Šlachta, 2011; Golebiewski *et al.*, 2011) during included years in comparison.

CONCLUSIONS

From these obtained results and in consequence of related results by Vyletělová *et al.* (2009 and 2011) there is possible to conclude as follows:

- *S. aureus* is permanent risk in veterinary medicine and its systematic checking should be important part of HACCP and in milk primary production;
- during last years it is clear from results of development of mastitis pathogens that *S. aureus* is henceforth one of main causes of mastitis and *S. uberis, E. faecalis* and CNS happen important microorganisms in mastitis pathogenesis;
- the highest frequency of mammary gland disorder was in subclinical mastitis and *S. aureus* was the most frequently identified pathogen and *S. uberis, S. haemolyticus* and *S. agalactiae* were following pathogens in order according to their frequency of occurrence;
- the results of milking equipment impact (pipeline milking (PM) and milking parlour (MP)) showed significantly higher occurrence of clinical mastitis in PM as compared to MP and also lower ratio of health mammary gland occurrence.

In general the results pointed out by good correspondence of their main characteristics the interpretation importance of milk sample microbiological investigation on pathogen occurrence in indicated cases for possibility how to put into effect the preventive and curative measures in mastitis elimination including the investigation of pathogen antibiotic sensitivity. This is underevalued relatively and improperly by farmer practice today. In the fact it means change of present routine approach.

SUMMARY

The aim of this work was to assess the relationship between mammary gland pathogen occurrence and somatic cell count (SCC) and possibility to find SCC discrimination limit for estimation of start for subclinical mastitis treatment according to SCC under current conditions. The suspect animals were selected according to NK test (viscosity) results, clinical symptoms and SCC. There were mainly the higher lactation cows (> 1st lactation) with subclinical mastitis. Milk samples were collected from all four teats and there were examined 161 cows from four farms in total. Holstein and Czech Fleckvieh dairy cows were included in the experiment. For microbiological analysis there were used standard cultivation agars and the isolated species were identified using biochemical tests STREPTOtest, STAPHYtest and ENTEROtest and identification program TNWpro7.0. In addition, in strains confirmed as *S. aureus* was detected *mec*A gene, encoding resistance to methicillin, using PCR method. The determination of SCC in individual milk samples was performed using the Somacount instrument. The SCC classification into classes according to the positive and negative findings of microbial pathogens was used for statistical evaluation of the results. For the same reason, for statistical hypothesis testing of differences between groups of samples (t–test on log SCC), there were used geometric means and medians (in the position of the mean values) because of the given purpose. We have confirmed 52 positive dairy cows and identified 55 mastitis pathogens from our results. The most frequently isolated species was *Enterococcus faecalis* (n = 20), followed by *Staphylococcus aureus* (n = 6) and *Streptococcus uberis* (n = 5). In *S. aureus* strains, the presence of the *mec*A gene, or genes for enterotoxins production was not found. Somatic cell count ranged from 9 to 24 204 ths. in 1 ml. We have divided incidence of mastitis pathogens in relation to somatic cells into three groups: I) positive / negative mastitis incidence of disease in the case of the SCC $\leq$ 100 ths.ml^{-1}; II) incidence of mastitis disease if SCC > 100 and $\leq$ 283 ths.ml^{-1}; III) incidence of mastitis disease in case of SCC > 283 ths.ml^{-1}. As regards, the positive or negative mastitis disease incidence in the case of I. and II. classes, significant differences are apparent. From the results, it is interesting that at lower SCC (50 ths.ml^{-1}) were present bacteria of the genus *Staphylococcus*, followed by bacteria of genus *Enterococcus* (75 ths.ml^{-1}), and only at higher numbers of SCC (346, 554 and 594 ths.ml^{-1} respectively) bacteria genus *Streptococcus*, *Enterobacter* and *Escherichia coli*. From all results it was found 23 negative results (14%) in samples with higher SCC than 400 ths.ml^{-1} (from 430 to 9 006 ths.ml^{-1}). The absence of pathogens in this case may be caused by another load of mammary glands (stress, nutrition, milking equipment). Differences in SCC according to pathogen classes were significant ($P < 0.001$; negative xg 131 SCC versus 491 for positive, 611 for staphylococci and 464 ths.ml^{-1} for other positive). Insignificant difference in SCC was noted only between infected groups with positive pathogen findings (staphylococci versus other pathogens, xg 611 and 464 ths.ml^{-1}). It increases economical relevancy of *S. aureus* infection. It is possible to calculate a SCC discrimination limit for practical likelihood of pathogen occurrence estimation in infectious sample groups under presumption of normal frequency distribution of logarithmic transformed SCC values. This limit for suspicion of infection is 159 for positive group, 113 for staphylococci and 174 ths.ml^{-1} for other positive. This could be possible to recommend the value 174 ths.ml^{-1} as limit

for suspicion of infection and for practical use with target to apply selective mastitis antibiotic treatment (for instance at dairy cow drying) or other preventive or curative measures.

I: Results of genus and species identification of mastitis pathogens, the lowest somatic cell count (SCC in ths.ml^{-1}) at pathogen confirmation

finding/SCC	I. ≤ 100	II. > 100 ≤ 283	III. > 283	SCC ths.ml^{-1}
negative (interval)	47	36	26	9 – 3 379
positive (interval)	6	11	38	52 – 24 204
Staphylococcus aureus	-	2	4	216
Staphylococcus haemolyticus	1	-	1	56
Staphylococcus simulans	1	-	-	64
Staphylococcus xylosus	2	-	-	52
Staphylococcus intermedius	-	-	1	711
Enterococcus faecalis	-	7	13	104
Enterococcus faecium	-	1	2	187
Enterococcus gallinarum	1	-	1	87
Enterobacter amnigenus bv. 1	-	1	-	123
Escherichia coli	-	-	1	594
Streptococcus uberis	-	-	5	594
Streptococcus agalactiae	-	-	1	15 199
Staphylococcus sp.	-	-	1	4 165
Streptococcus sp.	-	-	7	346
Enterobacter sp.	-	-	1	554
Enterococcus sp.	1	-	-	75

II: Statistical characteristics of case groups of somatic cell counts (SCC in ths.ml^{-1}) in milk with various mastitis state of mammary gland of animals with different pathogen occurrence respectively

Parameter	total	negative	positive	staphylococci	other positive
n	161	97	64	13	51
x	1 088	295	2 290	3 728	1 923
sd	3 241	511	4 882	6 109	4 517
vx	297.8	173.5	213.2	163.9	234.8
x log SCC	2.3444	2.1157	2.6911	2.7862	2.6669
sd	0.6839	0.5354	0.7407	0.9419	0.6895
xg	221	131	491	611	464

n = number of cases of individual milk samples; x = arithmetic mean; sd = standard deviation; vx = coefficient of variation in %; xg = geometric mean

III: Test of differences between groups of somatic cell counts (SCC) in milk with different mastitis state with different pathogen occurrence respectively (from Table II)

Difference	n	t	significance level
negative versus positive in total	161	5.68	$P < 0.001$
negative versus staphylococci	110	3.76	$P < 0.001$
negative versus other positive	148	5.34	$P < 0.001$
staphylococci versus other positive	64	0.51	$P > 0.05$

t = t–test criterion value

1: Medians of case groups of somatic cell counts (SCC in ths.ml^{-1}) in milk with various mastitis state of mammary gland of animals with different pathogen occurrence respectively