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ENTEROTOXIGENIC POTENTIAL THE SAME BACTERIA OF *STAPHYLOCOCCUS SPP.* IN MASTITIS OF DAIRY COWS AND SHEEP

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Abstract

The aim of this work was to evaluate the occurrence of *Staphylococcus spp.* on the overall prevalence of mastitis in cows and sheep farms in eastern Slovakia, and determine incidence of enterotoxigenicity and other characteristics of bacterial isolates. Were tested presence of genes encoding production of enterotoxins (*sea*, *seb*, *sec*, *sed*, *see*) and the production individual types of staphylococcal enterotoxins (SEA, SEB, SEC, SED, SEE) in milk samples of cows and sheep. In a two-year experiment of testing 2916 individual samples of cow's and sheep's milk were recorded bacteria *Staphylococcus spp.* 18.41% (537). Using PCR methods was confirmed the presence of the gene (*seg*) encoding staphylococcal enterotoxins (SE) in 5.21% (28 cases). By using the Ridascreen® Set, (B), (C), (D), (E) was recorded *in vitro* production of SE in 4.28% (23 cases). Four species isolated from samples of cow's milk produced the following types of SE: *S. aureus* - SEC (4) and SEE (1), *S. haemolyticus* - SEA, *S. chromogenes* - SEC (2) and SED (1), *S. simulans* - SEA (1). The staphylococci isolated from samples of sheep's milk produce SE: *S. aureus* - SEA, SEB (1) and (2) SEC (3), *S. intermedius*, *S. chromogenes* - SEC-SEC (3), *S. xylosus* - SED (1). A common feature of the enterotoxigenic bacteria was the creation of a biofilm. Formation of hyaluronidase and lysins α , β and δ have been confirmed as positive for *S. aureus* and *S. intermedius*. Creation of lysine α has been confirmed for strains of *S. chromogenes*. The production of hyaluronidase in CPS was significant on significant level $\alpha = 0.001$. Formation of hemolysines only on significant level $\alpha = 0.01$. Other following properties were non significant.

In spite legislatively obligatory pasteurization of milk before further processing, a relatively high percentage of enterotoxigenic strains of staphylococci isolated from the milk of sheep and cows can pose a significant risk especially in the marketing of milk and milk products directly from the farm as organic products.

Keywords: mastitis; dairy cow; sheep; staphylococcal enterotoxins; genes

Introduction

Bacteria of *Staphylococcus spp.* as the cause of a wide range of diseases in humans and animals are well known, but in recent decades, especially coagulase-negative staphylococci (CNS) were subsequently diagnosed as the aetiological agent of a wide range of infectious processes and healthy complications (Bansal et al. 2004, Favre et al. 2005, Huang et al. 2006, Lalani et al. 2006). Common are also reported increases frequency of CNS as ruminant mastitis agents in many countries, with significant impact on the economy of milk production.

From samples of cows and sheep milk were isolated strains of bacteria, in which by ELISA and PCR methods were determined the production some of the types of staphylococcal enterotoxins (SE), and was confirmed the presence of the gene (*seg*) responsible for the production of SE (Fagundes et al. 2010).

Enterotoxigenic staphylococci activity is an important virulence factor and was mentioned in relation to their specific contribution to the pathogenesis of mastitis (da Silva et al. 2005) with very frequent

property is the ability to naturally acquire resistance to antibiotics (Orwin et al. 2001). Among equally important virulence factors of pathogens mammary gland include biofilm formation (Melchior et al. 2006).

Treatment of dairy milk for consumption ensures a standard of consumer protection to the public prior to the occurrence of staphylococcal enterotoxigenesis. The free market, however, allows the consumption of milk in homes and other alternative sources without pasteurization. The solution for reducing the risk of enterotoxigenesis in these cases is treatment by boiling milk.

The aim of this work was to evaluate the occurrence of *Staphylococcus spp.* on the overall prevalence of mastitis in cows and sheep farms in eastern Slovakia, and determine incidence of enterotoxigenicity and other characteristics of bacterial isolates.

Materials and methods

In the course of two years in the framework of the periodic complex examination were in herd of 120 dairy cows taken 968 samples, and in herd of 350 sheep examined 1948 individual milk samples. Clinical examination of udder, classification of mastitis and bacteriology examination were carried out according to Vasil (2004).

Taxonomy of staphylococci was used by set STAPHYtest 24 and evaluated by program TNW, version 7.0 ProAuto (Erba Lachema, Brno, ČR). Identified bacteria *Staphylococcus spp.* were examined on presence of genes coding their enterotoxigenic activity (*sea, seb, sec, sed, see*) by PCR method according to (Becker et al. 1998). On the separation of DNA was used QiAMP tissue kit (OIAGEM, Hilden, Germany). Within the PCR method were used reference strains for SEA, SAB, SEC, SED and SEE types of enterotoxins – (Bergdoll; CNCTC, Brno) and commercial oligonucleotide primers. Every strain with confirmed gene was examined for production of enterotoxins by Ridascreen® Set A, B, C, D, E (R-Biopharm AG, Darmstadt, Germany).

In relation to forms of mastitis were in enterotoxigenic strains of staphylococci followed production of hemolysins α , β , δ , hyaluronidase and production of biofilm by methods according to Türkyilmaz and Kaya (2006), Boynukara et al. (2008), Krukowski et al. (2008).

On statistical evaluation was used Chi-square test, was tested individual properties of enterotoxigenic CNS compare to enterotoxigenic CPS at significant level $\alpha = 0.001$ (0.1%), and significant level $\alpha = 0.01$, testing criteria $G = 45.387$; critical value $df = 32.91$.

Results and discussion

From 2916 examined milk samples were (24.24%) bacteriological positive while *Staphylococcus spp.* was found in 537 cases (18.41%). Summary of isolated staphylococci are described in Table 1.

Table 1

| Total result of bacteriology examinations, prevalence of mastitis, percentage ration of the <i>Staphylococcus spp.</i> isolated from individual milk samples of dairy cows and sheep | | | | | | | | | |
|--|---------------------------|----------|-------------------|----------------------------|------|----------|-------|----------|-------|
| Sample | Individually milk samples | | | <i>Staphylococcus spp.</i> | | | | Σ | |
| | examined | positive | find ¹ | CPS | | CNS | | | |
| | <i>n</i> | <i>n</i> | % | <i>n</i> | % | <i>n</i> | % | <i>n</i> | % |
| dairy cow | 968 | 280 | 28.92 | 21 | 2.17 | 170 | 17.56 | 191 | 19.73 |
| sheep | 1948 | 427 | 19.73 | 38 | 1.95 | 308 | 15.81 | 346 | 17.76 |
| summary | 2916 | 707 | 24.24 | 59 | 2.02 | 478 | 16.39 | 537 | 18.41 |

1 prevalence of mastitis (percentage of positive cases from total number of examined cows and sheep); CPS – coagulase-positive staphylococci (*S.aureus*; *S.intermedius*); CNS – coagulase-negative staphylococci

By PCR and ELISA analyses of all strains of *Staphylococcus spp.* was determined enterotoxigenic potential in 28 cases (5.21%). Four species isolated from samples of cow's milk produced the following types of SE: *S. aureus* - SEC (4) and SEE (1), *S. haemolyticus* - SEA, *S. chromogenes* - SEC (2) and SED (1), *S. simulans* - SEA (1).

Presence only gene coding relevant enterotoxigenic activity was certified in *S. aureus* (*sea*, *sec*), and *S. simulans* (*sec*). General 13 (6.80%) strains of *Staphylococcus spp.* had an enterotoxigenic potential, but only in 10 cases (5.23%) were determined *in vitro* production of SE.

The staphylococci isolated from samples of sheep's milk produce SE: *S. aureus* - SEA, SEB (1) and (2) SEC (3), *S. intermedius*, *S. chromogenes* - SEC-SEC (3), *S. xylosus* - SED (1). In two cases was certified presence of gene *sec* in strains of *S. intermedius*, *S. xylosus*. General 15 (4.33%) strains of *Staphylococcus spp.* had an enterotoxigenic potential, but only in 13 cases (3.75%) were determined *in vitro* production of SE.

Enterotoxigenic staphylococci isolated from milk of ruminants were in generally presented by strains of *S. aureus* (7/6), and *S. chromogenes* (7/6). While cow's milk accounted for generic representation of *S. haemolyticus* (1) *S. simulans* (2), originating from sheep's milk has been linked to the species *S. intermedius* (2), *S. epidermidis* (2) and *S. xylosus* (2). Up to half of the strains produced SEC, in particular, it was the *S. aureus* and *S. chromogenes*.

Table 2

Occurrence of enterotoxigenic staphylococci (n = 28) according to number of samples and species of isolated bacteria (n = 537)

| Enterotoxigenic bacteria <i>Staphylococcus spp.</i> (n / %) | Production of SE, presence of genes | | | | | <i>sec</i> | | |
|--|-------------------------------------|------|-----|-----|-----|-------------------------|---|------------|
| | SEA | SEB | SEC | SED | SEE | | | |
| Isolated from mastitis in cow (n = 191) | | | | | | | | |
| <i>S. aureus</i> | 7 | 3.66 | 4 | | 1 | <i>sea</i> , <i>sec</i> | | |
| <i>S. haemolyticus</i> | 1 | 0.52 | 1 | | | | | |
| <i>S. chromogenes</i> | 3 | 1.57 | 2 | 1 | | | | |
| <i>S. simulans</i> | 2 | 1.04 | 1 | | | <i>sec</i> | | |
| summary | 13 | 6.80 | 2 | 0 | 6 | 1 | 1 | 3 |
| Isolated from mastitis in sheep (n = 346) | | | | | | | | |
| <i>S. aureus</i> | 6 | 1.73 | 2 | 1 | 3 | | | |
| <i>S. intermedius</i> | 2 | 0.57 | | | 1 | | | <i>sec</i> |
| <i>S. chromogenes</i> | 3 | 0.86 | | | 3 | | | |
| <i>S. epidermidis</i> | 2 | 0.57 | 1 | | 1 | | | |
| <i>S. xylosus</i> | 2 | 0.57 | | | | 1 | | <i>sec</i> |
| summary | 15 | 4.33 | 3 | 1 | 8 | 1 | 0 | 2 |

Accompanying the origin and some properties of the tested species of *Staphylococcus spp.* are shown in Table 3. Staphylococci isolated from cow's milk are equally involved in acute, subacute and subclinical mastitis. In contrast enterotoxigenic staphylococci from sheep's milk came primarily from acute and subacute mastitis. The common feature of enterotoxigenic strains, were the biofilm formation. The production of hyaluronidase and lysine α , β and δ were certified as properties characteristic for coagulase positive *S. aureus* and *S. intermedius*. Formation of lysine α was determined also in *S. chromogenes*.

Increased incidence of coagulase-negative staphylococci (CNS) at intramammary infections in cows is closely associated with an increased number of somatic cells (SCC) in milk, which is an important indicator of the quality of milk in its economic recovery (Lam et al. 1997, White et al. 2001).

Correlation between the prevalence of mastitis caused by CNS and increased SCC in cow's milk confirmed Borm et al. (2006), when antibiotic therapy reduced the proportion both variables to a minimum. Taponen et al. (2006) report the occurrence of CNS in the context of severe inflammatory reactions of udder in primiparous before calving, which threaten favourable onset of lactation.

Table 3

Forms of mastitis and selected factors of pathogenicity in enterotoxigenic species of staphylococci (n = 28)

| Enterotoxigenic <i>Staphylococcus spp.</i> (n) | Forms of mastitis | Lysine | Hyaluronidase | Biofilm | |
|---|-------------------|--------------------|---------------------------------|---------|----|
| Isolated from mastitis in cows | | | | | |
| <i>S. aureus</i> | 7 | AK 3; SBA 2; SBK 2 | α 4; β 2 | 3 | 7 |
| <i>S. haemolyticus</i> | 1 | SBK | - | - | 1 |
| <i>S. chromogenes</i> | 3 | AK 1, SBA 2 | α 2 | - | 2 |
| <i>S. simulans</i> | 2 | SBK 2 | - | - | - |
| summary | 13 | AK 4; SBA 4; SBK 5 | α 6; β 2 | 3 | 10 |
| Isolated from mastitis in sheep | | | | | |
| <i>S. aureus</i> | 6 | AK 4; SBA 2; SBK 1 | α 1; β 1; δ | 5 | 6 |
| <i>S. intermedius</i> | 2 | AK 1; SBA 1 | α 1 | 1 | 2 |
| <i>S. chromogenes</i> | 3 | AK 2; SBA 1 | α 1 | - | 3 |
| <i>S. epidermidis</i> | 2 | SBA 1; SBK 1 | - | - | 2 |
| <i>S. xylosus</i> | 2 | SBA 1; SBK 1 | - | - | 2 |
| summary | 15 | AK 7; SBA 6; SBK 2 | α 3; β 1; δ | 6 | 15 |

AK - acute mastitis; SBA – subacute mastitis; SBK - subclinical mastitis

Among the many literary sources follows that CNS frequently isolated from mastitis cows and sheep are: *S. chromogenes*, *S. haemolyticus*, *S. capitis*, *S. capri*, *S. cohnii*, *S. epidermidis*, *S. hominis*, *S. saprophyticus*, *S. sciuri*, *S. simulans*, *S. warneri* and *S. xylosus*, while in many of whom were recorded production of any type of staphylococcal enterotoxins *in vitro*, and has been validated gene (*seg*) responsible for the production of SE (Cenci-Goga et al. 2003; Loncarevic et al. 2005).

From the mutual comparison of selected properties in individual lines in Table 4 resulted, that the production of hyaluronidase in CPS was significant on significant level $\alpha = 0.001$. Formation of hemolysines only on significant level $\alpha = 0.01$. Other following properties were non significant.

Table 4

Evaluation of relation of coagulase-positive and coagulase-negative enterotoxigenic staphylococci causing different forms of mastitis to selected properties

| Selected properties of enterotoxigenic staphylococci | | CPS (n = 15) | | CNS (n = 13) | | Test* ($\alpha = 0.001$) |
|--|------------------------------------|----------------|----|----------------|----|-------------------------------|
| | | + | - | + | - | |
| caused | clinical mastitis | 12 | 3 | 8 | 5 | 1.171 ^N |
| | subclinical mastitis | 3 | 12 | 6 | 7 | 2.181 ^N |
| produced | hemolysine α, β, δ | 12 | 3 | 3 | 10 | 9.053 ^X |
| | hyaluronidase | 9 | 6 | 0 | 13 | 11.502 ^Z |
| | biofilm | 15 | 0 | 10 | 3 | 3.890 ^N |

CPS (coagulase-positive staphylococci – *S. aureus*; *S. intermedius*); CNS (coagulase-negative staphylococci – *S. haemolyticus*; *S. chromogenes*; *S. epidermidis*; *S. simulans*; *S. xylosus*);

*- Chi-square test (in significant level $\alpha = 0.001$ (0.1%) critical value $df = 10.828$); ^N – independence of individual signs is on significant level $\alpha = 0.001$; ^Z – dependence of individual signs is on significant level $\alpha = 0.001$; ^X – dependence of individual signs is only on significant level $\alpha = 0.01n$ (1.0%), valid critical value $df = 6.635$

Conclusion

The work presents results from testing of 968 individual milk samples originating from herd of 120 dairy cows for a period of two years. In the total bacteriological finding 280 positive samples (28.92%), was recorded incidence of *Staphylococcus spp.* in 191 cases (19.73%). Was confirmed the presence of the gene (*seg*) encoding staphylococcal enterotoxins (SE) in 6.80% (13) cases, *in vitro* production of SE was observed in 5.23% (10) cases.

From the examination of 1948 individual milk samples originating from herd of 350 sheep for the same period were 427 samples positive (19.73%), and was recorded 17.76% incidence of *Staphylococcus spp.* (346 bacteria). The presence of the gene responsible for production of SE was reported in 4.33% (15) cases. *In vitro* production of SE was determined in 3.75% (13) cases. The production of hyaluronidase in enterotoxigenic CPS was significant on significant level $\alpha = 0.001$. Formation of hemolysines only on significant level $\alpha = 0.01$. Other following properties were non significant.

The observed results highlight the need for increased attention to the species of coagulase-negative staphylococci groups who was demonstrated enterotoxigenic potential, for example in evaluating the effectiveness of hygienic programs in facilities processing the milk into dairy products.

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