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Original article

Haemolytic and proteolytic activity of coagulase-negative staphylococci isolated from *mastitis* cows

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Abstract

The aim of the present study was to assess the haemolytic and proteolytic activity of coagulase-negative staphylococci (CNS) isolated from cows with *mastitis*. The study was conducted on 100 CNS strains: *S. xylosus* (n=28), *S. chromogenes* (n=26), *S. haemolyticus* (n=25), *S. sciuri* (n=14), *S. warneri* (n=4), *S. hominis* (n=2), *S. saprophyticus* (n=1); 22 CNS were isolated from cows with clinical mastitis and 78 from those with subclinical *mastitis*. The CNS studied showed the ability to produce only α -haemolysin and belonged to one strain – *S. haemolyticus* (21.0% of isolated CNS strains). Haemolysin-positive CNS were responsible for both clinical and subclinical *mastitis* (22.7% and 20.5%, respectively). The ability to produce protease was found in 31.0% of CNS belonging to two strains: *S. chromogenes* and *S. sciuri*. Protease-positive CNS were the etiological factor of both clinical and subclinical *mastitis* (31.8% and 30.8%, respectively). All *S. xylosus*, *S. warneri*, *S. hominis*, and *S. saprophyticus* strains were found protease-negative and haemolysin-negative, irrespective of whether they caused clinical or subclinical *mastitis* in cows.

Key words: cows, *mastitis*, coagulase-negative staphylococci, haemolytic activity, proteolytic activity

Introduction

Coagulase-negative staphylococci (CNS), for years considered non-pathogenic, have become the primary etiological factor of cow mastitis in many countries (Macovec and Ruegg 2003, Pitkälä et al. 2004, Rajala-Schultz et al. 2004, Malinowski et al. 2006, Taponen et al. 2007, Pyörälä and Taponen 2009).

Pathogenicity of microorganisms is expressed by two parameters: invasiveness (capability to permeate the protective barriers and to spread) and toxicity

(ability to produce enzymes and toxins) (Archer 1998, Kłossowska and Malinowski 2001, Małachowa 2003).

Coagulase-negative staphylococci are capable of producing enzymes other than coagulase, which enable the conversion of host tissues and spread of the inflammatory process (e.g. lipase, fibrinolysin, urease) (Szymańska and Buczek 1999). Moreover, they were found capable of producing proteolytic enzymes and haemolysins, which facilitate the uptake of iron (Lisiecki and Mikucki 1996).

The essential proteins binding iron in various secretions, including milk, are lactoferrin from phagocytes or milk-secreting cells (Weinberg 1986, Lisiecki and Mikucki 1996, Chaneton et al. 2008) and transferrin produced by the liver (Weinberg 1986, Lisiecki and Mikucki 1996). Insufficient availability of this element in tissues and body fluids may be the factor limiting the multiplication of staphylococci in the host organism (Weinberg 1986, Krajewska-Pietrasik and Różalska 1995, Zimecki and Artym 2005).

Haemolysins belong to cytolytic exotoxins affecting the cell membrane (Bedidi-Madani 1998, Stachowiak and Bielecki 2000). Staphylococci produce several types of haemolysins: α , β , γ , δ , which degrade the cell membrane of erythrocytes, thus facilitating for the staphylococci the access to iron contained in haemoglobin (Krajewska-Pietrasik and Różalska 1995, Różalska et al. 1995, Bedidi-Madani 1998). Moreover, each of them also attacks some other cells: α -haemolysin – platelets, monocytes and keranocytes, β -haemolysin destroys macrophages and leucocytes whereas γ -haemolysin monocytes and macrophages (Hryniewicz and Roszkowski 1980, Bedidi-Madani 1998, Szymańska and Buczek 1999). β -haemolysin degrades sphingomyelin in the cell membrane and therefore the susceptibility of various tissues depends on its content in the membrane (Jonsson and Wadström 1993, Bedidi-Madani et al. 1998). However, its toxicity is lower than that of α and γ -haemolysins (Szymańska and Buczek 1999). Furthermore, coagulase-negative staphylococci were demonstrated to produce δ -like haemolysin (δ -like Hf), whose structure and action resemble the δ -haemolysin of coagulase-positive staphylococci (Różalska et al. 1995).

The aim of the study was to assess the haemolytic and proteolytic activity of coagulase-negative staphylococci isolated from clinical and subclinical *mastitis*.

Material and Methods

The study was conducted on 100 CNS strains: *S. xylosus* (n=28), *S. chromogenes* (n=26), *S. haemolyticus* (n=25), *S. sciuri* (n=14), *S. warneri* (n=4), *S. hominis* (n=2), *S. saprophyticus* (n=1), with 22 strains isolated from clinical, and 78 strains from subclinical, *mastitis*. Milk samples were taken from 86 cows from Lublin farms.

Prior to collection of samples for bacteriological tests, the clinical state of the cows was assessed, i.e. presence of general symptoms and mammary gland lesions, and macroscopic evaluation of milk. The cows sampled did not receive any drugs during the ongoing

lactation. Milk samples were collected according to the accepted procedure. After cleansing and drying of the udder skin and disinfection of teats with 70% alcohol, milk was collected to sterile test-tubes without preservatives, chilled to 4°C and delivered to the laboratory of the Department of Animal Reproduction in Lublin.

Bacteriological testing of milk was performed according to standard procedures: milk culture on agar medium with 5% sheep blood added, 24-hour incubation under oxygen conditions at 37°C; morphology of bacterial colonies and Gram-stained specimens, catalase testing (3% hydrogen peroxide solution, Polfa), lisostaphin susceptibility (Sigma, USA), and free coagulase testing using rabbit plasma (Biomed, Kraków). The isolated CNS strains were identified using the API STAPH system (Biomerieux, France).

Classification of cow mastitis. Based on the results of milk bacteriological cultures, the somatic cell count determined using Fossomatic apparatus, and clinical examinations of cows, the type of mastitis was defined. A somatic cell count exceeding 200 000 in 1 ml of milk and positive bacteriological cultures of milk without general symptoms were considered to be indicators of subclinical *mastitis*. Macroscopic changes in milk and/or local changes within the mammary gland as well as general symptoms with increased somatic cell count > 200 000/ml milk indicated clinical *mastitis* (De Vliegher et al. 2003, Gentilini et al. 2002, Moon et al. 2007).

Evaluation of ability to produce haemolysins. A prepared solution of Columbia Agar Base (Oxoid, England), 39 g in 1 l of distilled water, was sterilized in an autoclave at 121°C for 15 minutes. After cooling to 50°C, defibrinated sterile sheep blood was added (5% of the medium volume) and placed on Petri plates, 9-10 cm in diameter. A Columbia Agar Base with 5% defibrinated sterile rabbit blood added, was prepared similarly. Once the medium set, cultures of strains were performed. The plates were incubated at 37°C for 24 h. The reference *Staphylococcus aureus* from the ATCC 25923 collection was used as a positive control.

Evaluation of ability to produce protease. A solution of Nutrient Gelatin medium (Oxoid, England), 128 g in 1 l of distilled water, was sterilized in an autoclave at 121°C for 15 minutes and placed on Petri plates, 9-10 cm in diameter. Once the medium set, thin-line cultures were performed. The base-coated plates were incubated at 37°C for 24 h. The results were interpreted as follows: positive – when the zone was 4 times wider than the growth line of a particular strain. The reference *Staphylococcus aureus* from the ATCC 25923 collection was used as a positive control.

Statistical analysis

The significance of differences between the percentage of CNS capable of producing protease and haemolysins isolated from clinical and subclinical *mastitis* cows was assessed using the Statistica 6.0 program based on normal distribution for comparisons of fractions ($p \leq 0.01$, $p \leq 0.001$ were considered statistically significant).

Results

The results regarding the ability of CNS to produce haemolysins and protease are presented in tables 1, 2 and 3.

The coagulate-negative staphylococci strains studied showed the ability to produce only α -haemolysin; all of them belonged to one strain – *Staphylococcus haemolyticus* (21.0% of isolated CNS strains). Haemolysin-positive coagulase-negative staphylococci were responsible for clinical *mastitis* (22.7%). Two haemolytic CNS strains were from milk of two different udder quarters of the cow diagnosed with acute *mastitis*. The remaining 16 *S. haemolyticus* strains demonstrating haemolysis around the colonies were isolated from milk of cows with subclinical *mastitis* (20.5%).

The ability to produce protease was found in 31.0% of coagulase-negative staphylococci belonging to two strains: *S. chromogenes* (20 strains) and *S. sciuri* (11 strains). Protease-positive CNS were isolated from

Table 1. Ability to produce haemolysins and protease by CNS isolated from milk of *mastitis* cows.

Phenotypic features	CNS strain														Total (100)	
	<i>S. xylosum</i> (28)		<i>S. chromogenes</i> (26)		<i>S. haemolyticus</i> (25)		<i>S. sciuri</i> (14)		<i>S. warneri</i> (4)		<i>S. hominis</i> (2)		<i>S. saprophyticus</i> (1)		n	%
	n	%	n	%	n	%	n	%	n	%	n	%	n	%		
Production of haemolysin	0	0.0	0	0.0	21	84.0	0	0.0	0	0.0	0	0.0	0	0.0	21	21.0
Production of protease	0	0.0	20	76.9	0	0.0	11	78.6	0	0.0	0	0.0	0	0.0	31	31.0

Table 2. Ability to produce haemolysin and protease by CNS isolated from milk of clinical *mastitis* cows.

Phenotypic feature	CNS strain										Total (22)	
	<i>S. xylosum</i> (8)		<i>S. haemolyticus</i> (7)		<i>S. chromogenes</i> (4)		<i>S. sciuri</i> (3)		n	%	n	%
	n	%	n	%	n	%	n	%				
Production of haemolysin	0	0.0	5	71.4	0	0.0	0	0.0	0	0.0	5	22.7 ⁽¹⁾
Production of protease	0	0.0	0	0.0	4	100.0	3	100.0	7	31.8 ⁽²⁾		

Table 3. Ability to produce haemolysin and protease by CNS isolated from milk of subclinical *mastitis* cows

Phenotypic feature	CNS strain														Total (78)	
	<i>S. chromogenes</i> (22)		<i>S. xylosum</i> (20)		<i>S. haemolyticus</i> (18)		<i>S. sciuri</i> (11)		<i>S. warneri</i> (4)		<i>S. hominis</i> (2)		<i>S. saprophyticus</i> (1)		n	%
	n	%	n	%	n	%	n	%	n	%	n	%	n	%		
Production of haemolysin	0	0.0	0	0.0	16	88.9	0	0.0	0	0.0	0	0.0	0	0.0	16	20.5 ⁽¹⁾
Production of protease	16	72.7	0	0.0	0	0.0	8	72.7	0	0.0	0	0.0	0	0.0	24	30.8 ⁽²⁾

Explanations to tables 2 and 3:

- (1) statistically insignificant difference at $p \leq 0.001$ between the percentage of CNS strains producing haemolysin isolated from milk of clinical and subclinical *mastitis* cows.
- (2) statistically insignificant difference at $p \leq 0.001$ between the percentage of CNS strains producing protease isolated from milk of clinical and subclinical *mastitis* cows.

milk of both clinical and subclinical *mastitis* cows (31.8% and 30.8%, respectively). Acute inflammation of two udder quarters was caused by *S. sciuri* (2 strains) capable of producing this enzyme. Moreover, protease-positive CNS were also the etiological factor of less strongly manifested clinical *mastitis*: *S. chromogenes* – 4 strains and *S. sciuri* – 1 strain. The remaining CNS strains capable of producing protease were isolated from the milk of cows with subclinical *mastitis*: *S. chromogenes* – 16 strains and *S. sciuri* – 8 strains.

Discussion

The requirement of staphylococci for free, easier assimilated iron (Fe^{+3}) is substantial compared to other microorganisms (Różalska et al. 1995, Lisiecki and Mikucki 1996). In inflammations, including cow *mastitis*, the level of proteins (lactoferrin and transferrin) binding this element increases significantly, thus reducing its availability (Weinberg 1986, Komine et al. 2005, Chaneton et al. 2008). In response to environmentally limited access to iron, staphylococci have developed mechanisms enabling them to uptake this relevant constituent, e.g. the capacity to produce proteolytic enzymes and haemolysins (Krajewska-Pietrasik and Różalska 1995, Szymańska and Buczek 1999).

The thesis that production of haemolysin may be important for the pathogenesis of *mastitis* was put forward by Jarp (1991). In his studies, *S. haemolyticus* producing haemolysin was found to be most commonly associated with acute forms of cow *mastitis*, with elevated body temperature. Likewise, our findings indicate that haemolysin-positive *S. haemolyticus* was responsible for *mastitis* with acute symptoms and slightly increased body temperature in cows. However, no statistically significant differences were observed in the ability to produce haemolysins by CNS isolated from various forms of cow *mastitis*. The percentages of h- haemolytic strains of CNS were almost identical in clinical and subclinical *mastitis* (22.7% and 20.5%, respectively).

In contrast to Jarp (1991) Bedidi-Madani et al. (1998), who studied the haemolytic activity of CNS isolated from milk of healthy goats, found that each of 165 strains was capable of producing at least one haemolysin, despite the lack of inflammatory reaction in the udder. Over 50% of CNS strains produced α -haemolysin, 75.1 % – β -haemolysin and 76.3% – δ -haemolysin. On the other hand, the author indicates that CNS present in milk, despite the lack of clinical symptoms of *mastitis*, is not indifferent to the mammary gland, which is evidenced by a slight increase in somatic cell count.

Furthermore, according to Birgersson et al. (1992) who studied 25 CNS strains isolated from *mastitis* cows, none of the strains showed the ability to produce α - or β -haemolysin, whereas δ -haemolysins were produced by 3 *S. simulans* strains and 1 *S. haemolyticus* strain; 2 *S. epidermidis* resulted in a slight reaction. Moreover, the author studied the ability of these strains to produce proteolytic enzymes. Two out of three *S. chromogenes* and 8 *S. hyicus* strains were protease positive. Slight reactions were caused by 3 *S. epidermidis* and one *S. sciuri* strain. The highest number of extracellular enzymes was produced by *S. hyicus*.

In our study, the ability to produce protease was found only in 31.0% of all CNS strains responsible for *mastitis*, all of which belonged to two strains (*S. chromogenes* and *S. sciuri*). Moreover, similar percentages of protease-positive strains were observed in clinical and subclinical *mastitis* (31.8% and 30.8%, respectively).

A similar low number of CNS strains producing protease was determined by Devriese et al. (1994). The authors studied 65 CNS strains isolated from milk of *mastitis* cows: *S. hyicus* – 3, *S. chromogenes* – 8, *S. simulans* – 22, *S. warneri* – 16, *S. epidermidis* – 6, *S. hominis* – 2, *S. xylosus* – 8 strains. Only *S. hyicus* and *S. chromogenes* reacted strongly in the protease assay.

According to the results presented above (Birgersson et al. 1992, Devriese et al. 1994, Bedidi-Madani 1998), the pathogenicity of a microorganism is determined by a combination of various virulence factors, which are responsible for its toxicity and invasiveness.

It should be stressed that in our study none of the CNS strains had the ability to produce both haemolysin and protease. In one cow, haemolysin-positive yet protease-negative *S. haemolyticus* was responsible for *mastitis* with pronounced acute symptoms and slightly elevated body temperature. In another, acute clinical *mastitis* was caused by non-haemolytic, yet protease-producing *S. sciuri*. However, due to too low numbers of CNS strains isolated from milk of cows with acute *mastitis*, the significance of the features studied for the development of clinical *mastitis* cannot be conclusively determined. Moreover, there were no statistically significant differences in the capacity to produce haemolysins and protease between CNS isolated from milk of clinical and subclinical *mastitis* cows. Furthermore, all *S. xylosus*, *S. warneri*, *S. hominis*, and *S. saprophyticus* strains were found protease-negative and haemolysin-negative, irrespective of whether they caused clinical or subclinical *mastitis* in cows.

To conclude, our findings indicate the lack of correlation between the ability to produce haemolysins or protease and *mastitis* of a particular severity of symptoms.

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