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INFLUENCE OF SOME FACTORS ON ADHERENCE OF *S. AUREUS* AND *S. AGALACTIAE* TO A BOVINE MAMMARY SECRETORY EPITHELIAL CELLS (BME-UV1)

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The bovine mammary epithelial cell line (BME-UV1) was incubated with a suspension of S. aureus (10^8/ml) for 30min at 37 ^{0}C in a 5% CO_2 atmosphere. Observation under the light microscope (10x100) in 20 visible fields showed visible fields with 100 epithelial cells on average were selected for counting. The results were presented as the number of visible fields out of 20 in which adherence was observed. The results showed that adherence of isolated strains of S. aureus occurred in 2-20 visible fields, and 0-16 for S. agalactiae, which indicates a high degree of variation in adherence among the isolated strains tested. The difference in adherence between tested strains of a single species of bacteria was statistically significant, but there was no difference between the examined strains of S. aureus and S. agalactiae. Strains of S. aureus which formed capsules did not show significantly less adherence for the bovine mammary gland secretory epithelial cells (BME-UV1) than strains of S. aureus which did not form capsules. The strains of S. aureus which produced slime did not show significantly higher adherence for the BME-UV1 cells than the strains which did not produce slime. The adherence of isolated strains of S. aureus to the BME-UV1 cells did not depend on their capability to produce toxins, or on their plasmid profile.

Key words: adherence, bovine mammary epithelial cells, capsule, mastitis, plasmids, S. agalactiae, S. aureus, slime

INTRODUCTION

Adhesion of bacteria to epithelial cells is recognized as a critical stage in the colonization of mucous membranes (Beachey, 1981). Bacteria which cause bovine mastitis adhere to mammary epithelial cells and adhesion may have a role in pathogenesis (Frost, 1975, 77). Mechanisms of adhesion among causes of mastitis are known. Bacteria have different mechanisms of adhesion on an immunological, physico-chemical and genetic basis. Success of adhesion depends on the balance between different synergistic and antagonistic reactions

in the microenvironment in which adherence is occurring (Freeter and Jones, 1983). The main adhesins of *S. aureus* to epithelial cells are proteins and in *S. agalactiae* LTA and M protein (Beachey, 1981). Milk is a suitable medium for growth and multiplication of *S. aureus* and *S. agalactiae*, which is very significant at the beginning of infection (Sutra, 1994). Inflammation processes in the mammary gland lead to changes of the physical and chemical characteristics of milk, and on increase in the number of somatic cells. About 89,7% of *S. aureus* strains produce a capsule, that appears important as a factor for virulence of these microorganisms in the challenge of mastitis. Slime is a product of certain bacteria that is different from the capsule. Slime may influence the process of adherence (Baselga *et al.* 1993). In consideration of the complexity of the adherence process, and the large number of factors participating in its beginning, we decided to examine the adherence of one of the most frequent causes of mastitis to bovine mammary epithelial cells in culture and the influence of some bacterial factors (capsule, slime, toxins, plasmids) on the adherence of *S. aureus* and *S. agalactiae*.

MATERIAL AND METHODS

Epithelial cells: In the test of adherence bovine mammary gland secretory epithelial cell line (BME-UV1, Zavizion *et al.* 1996) was used.

Strains: A total of 22 strains of *S. aureus* from the collection of the Istituto Malattie Infettive (Universita di Milano), 2 strains of *S. agalactiae* from the collection of the Faculty of Veterinary Medicine (University in Belgrade), the Smith diffuse strain of *S. aureus* (2286) and Smith compact strain of *S. aureus* (2287) from the Czech collection of microorganisms, Brno, a strain of *S. agalactiae* from the collection of microorganisms of the Institute for Hygiene and Epidemiology, Prague, Czech Republic was examined.

Preparation of the bacterial suspension: The strains of *S. aureus* and *S. agalactiae* were grown in brain heart infusion broth for 18h at 37 °C. The bacteria were then centrifuged at 3500 r.p.m. for 10 min., washed two times in HBSS (Hanks balanced saline solution) and resuspended in DMEF12 to a content of 10^8CFU/ml.

Adherence of bacteria to epithelial cells: Suspensions of the bacteria were incubated with the monolayer of BME-UV1 for 30 min. at 37 ^{0}C in a CO_2 incubator. After 4 washes with HBSS, the slides were fixed in methanol and stained with Giemsa reagent. Slides were read by light microscopy at 1000x magnitude, 60 fields were a counted for each slide and the number of fields with adhering *S. aureus* or *S. agalactiae* was recorded.

The presence of slime: The presence of slime in the examined strains of *S. aureus* was determined by the method of Freeman *et al.* (1989).

Toxins: For examining toxins, strains of *S. aureus* were grown in agar with 5% bovine blood.

Plasmid profile: The plasmid profile of the *S. aureus* strains was determined by electrophoresis using a modified Quiagen protocol (Brinboim *et al.*, 1979; Vicenzoni *et al.*, 1996).

RESULTS

The results showed that the bovine mammary gland secretory epithelial cell line (BME-UV1) can be used to examine adherence of *S. aureus* and *S. agalactiae.* In suspension the (BME-UV1) cells have a clear, uniform, circular shape. When they adher to plastics they take a cuboidal or polygonal form, becoming flat. A confluent monolayer has the characteristic shape of a paved road. In relation to myoepithelial cells, the nucleus of these cells has a regular form and the cell membrane is not involved.

The level of adherence of *S. aureus* strains to the bovine mammary secretory epithelial cells (BME-UV1) is shown in Table 1.

Table 1. Adherence of *S. aureus* strains to bovine mammary gland secretory epithelial cell line (BME-UV1).

Code of *S. aureus* strain	Mean values for number* of visible fields with adhering *S. aureus*	SE	SD	CV	x min	x max
1615	11,67	3,18	5,51	47,21	8	18
1616	6,33	1,33	2,31	36,46	5	9
1625	10,67	1,45	2,52	23,59	8	13
1673	18,00	1,00	1,73	9,62	17	20
1674	6,67	1,20	2,08	31,22	5	9
1676	6,00	1,15	2,00	33,33	4	8
1694	5,67	1,20	2,08	36,74	4	8
1709	9,00	1,53	2,65	29,40	6	11
1632	10,00	2,52	4,36	43,59	7	15
1641	12,67	2,85	4,93	38,94	7	16
1667	15,67	1,20	2,08	13,29	14	18
1692	12,00	0,58	1,00	8,33	11	13
1702	14,67	1,45	2,52	17,16	12	17
1703	10,67	0,88	1,53	14,32	9	12
1706	10,33	2,67	4,62	44,70	5	13
1711	10,33	1,76	3,06	29,57	7	13
1697	7,67	1,33	2,31	30,12	5	9
1698	12,33	0,33	0,58	4,68	12	13
1757	11,33	1,86	3,21	28,36	9	15
1758	15,67	0,33	0,58	3,69	15	16
1195	15,33	2,91	5,03	32,83	10	20
1583	17,33	0,88	1,53	8,81	16	19
2286	3,33	0,67	1,15	34,64	2	4
2287	8,33	1,86	3,21	38,57	6	12

*Mean values of three determinations

The average values for the adherence straines of *S. aureus* ranged from 3,33 to 18 visible fields with bacteria adhering to epithelial cells out of twenty observed.

The results for adherence of *S. agalactiae* to the bovine mammary gland secretory epithelial cell line (BME-UV1) are shown in Table 2.

Table 2. Adherence of *S. agalactiae* to bovine mammary gland secretory epithelial cell line (BME-UV1)

Code of *S. agalactiae* strain	Mean values for number* of visible fields with adhering bacteria	SE	SD	CV	x min	x max
Str 1.	0,33	0,33	0,58	173,21	0	1
Str 3.	13,67	1,20	2,08	15,23	12	16
Str 4.	12,67	1,67	2,89	22,79	11	16

*Mean value of three determinations

The average values for the adherence of *S. agalactiae* to epithelial cells (BME-UV1) ranged from 0,33 to 13,67. Significant differences were faund in adherence between tested strains of each species of bacteria but no difference was found when the adherence was compared for *S. aureus* and *S. agalactiae.*

The most pathogen is strains of *S. aureus* produce a slime which is considered to have a role in adherence of these microorganisms, because it comes in to contact with cells of the host first. The results for adherence of slime producing, slime non-producing and slime variable strains of *S. aureus* to the bovine mammary gland secretory epithelial cell line are shown in Table 3.

Table 3. Adherence of slime producing, slime non-producing and slime variable *S. aureus* strains to bovine mammary gland secretory epithelial cell line (BME-UV1)

Code of slime producing *S. aureus*	Nnumber* of visible fields with adhering *S. aureus.*	Code of slime non producing *S. aureus*	Number* of visible fields with adhering *S. aureus*	Code of slime variable *S. aureus*	Number* of visible fields with adhering *S. aureus.*
1616	6,34	1615	11,67	1673	18,00
1625	10,67	1709	9,00	1667	15,67
1674	6,67	1632	10,00	1692	12,00
1676	6,00	1697	7,67	1703	10,67
1694	5,67	1698	12,34	1195	15,34
1641	12,67	1757	11,34	2286	3,33
1702	14,67	1758	15,67		
1706	10,34	1711	10,34		
1583	17,34	2287	8,33		

* Mean values of three determinations

Average values for adherence of slime producing of *S. aureus* ranged from 5,67 to 17,34, slime non-producing strains from 7,67 to 15,67 and slime variable strains from 3,34 to 18 visible fields with adhering bacteria to epithelial cells out of twenty observed. No significant differences were found when the adherence was compared between slime producing, slime non-producing and slime variable strains of *S. aureus*.

Most strains of *S. aureus,* isolated from bovine mastitis milk, produce alpha-toxin and beta-toxin which damage epithelial cells and help adherence and invasion of these microorganisms in to epithelial cells. Because of that the adherence of alpha-toxin producing, beta-toxin producing and delta-toxin producing strains of *S. aureus* was assessed. Values for adherence of *S. aureus* producing different toxins are shown in Table 4.

Table 4. Adherence of *S. aureus* strains producing different toxins to bovine mammary gland secretory epithelial cells (BME-UV1)

S. aureus (alpha toxin)		*S. aureus* (beta toxin)		*S. aureus* (delta toxin)		*S. aureus* (alpha and beta toxin)		*S. aureus* (beta and delta toxin)		*S. aureus* without toxins	
Code of strain	X*	Code of strain	X*	Code of strain	X*	Code of strain	X*	Code of strain	X*	Code of strain	X*
1692	12,00	1615	11,67	1673	18,00	1616	6,34	1625	10,67	1709	9,00
1702	14,67	1674	6,67	1667	15,67	1195	15,34	1676	6,00	1697	7,67
1706	10,34	1632	10,00			15,83	1734	1703	10,67	1698	12,34
		1711	10,34					1758	15,67		
		1694	5,67					1757	11,34		
		1641	12,67								

X*- Mean value for number of visible fields with adhering *S. aureus*

The average values for adherence of alpha- toxin producing of *S. aureus* ranged from 10,34 to 14,67 visible fields with adhering bacteria out of twenty observed, for beta-toxin producing strains it ranged from 5,67 to 12,67, for delta-toxsin producing strains it ranged from 15,67 to 18,00, for the alpha-toxin and beta-toxin producing ranged from 6,34 to 17,34, for beta-toxin and delta-toxin producing strains it ranged from 6 to 15,67 and for the toxin non-producing strains it ranged from 7,67 to 12,34 visible fields with adhering bacteria out of twenty observed. No significant differences were found in adherence between strains wich produce different toxins.

Since, specific wall proteins the synthesis of which is genetically contrlold are responsible for adherence of *S. aureus*, the dependence od adherence of *S. aureus* on the plasmid profile was examined. The results for adherence of *S.*

aureus strains with different plasmids to the bovine mammary gland secretory epithelial cell line (BME-UV1) are shown in Table 5.

Table 5. Adherence of *S. aureus* strains with different plasmid profiles to a bovine mammary gland secretory epithelial cell line (BME-UV1)

S aureus with plasmid 23KD		*S aureus* with plasmid 23KD and other plasmids		*S. aureus* without plasmid 23K but with other plasmids		*S. aureus* without plasmids	
Code of strain	X*	Code of strain	X*	Code of strain	X*	Code of strain	X*
1615	11,67	1641	12,67	1709	9,00	1676	6,00
1616	6,34	1667	15,67	1195	15,34	1697	7,67
1625	10,67	1703	10,67	1583	17,4		
1673	18,00	1711	10,34				
1674	6,67	1698	12,34				
1694	5,67	1758	15,67				
1632	10,00	1757	11,34				
1702	14,67	1692	12,00				
1706	10,34						

* Mean value for number of visible fields with adhering *S. aureus*

The average values for adherence of *S. aureus* with plasmid 23KD ranged from 6,34 to 18, for strains with plasmid 23KD and other plasmids from 10,34 to 15,67, for strains without plasmid 23 KD and without other plasmids it ranged from 9 to 17,34 and for strains without plasmids it ranged from 6 to 7,67 visible fields with adherence of *S. aureus* to epithelial cells out of twenty observed. Significant differences were not found in adherence between strains of *S. aureus* which possess different plasmids.

DISCUSSION

Adherence of bacteria to host cells is an interaction between bacteria and the surface of the host cells and is an important factor in the virulence of bacteria. Adherence is a complex process involving specific as well nonspecific factors of the host and bacteria and also factors of the micro-environment in which adherence is occurring are involved (Mamo *et al.* 1986).

According to the results obtained in our study significant differences in adherence occurred between the tested strains of a single species of bacteria, but were not found between *S. aureus* and *S. agalactiae* species (Table 1). The results our examination are in agreement with those of Opdebeeck et al (1988)

who observed that the adherence 15 strains of *S. aureus* to a bovine mammary gland ductular epithelial cell line varied from strain to strain.

The cell wall of Gram positive bacteria is composed of proteins, teichoic acid, peptidoglycan, LTA and polysaccharide which participate in the adherence to host cells (Verceloti *et al.* 1985). Opdebeeck and Frost (1987) indicated many mechanisms of adherence of staphylococci, whose expression and degree of activity generally depend on composition and environmental characteristics. Our findings concerning the influence of slime producing on the adherence of *S. aureus* to the bovine mammary gland epithelial cell line are in agreement with the results of Haagen *et al.* (1990) and confirm that production slime is not a significant factor in adherence of staphylococci. Our data are also in agreement with the results of Cifrian *et al.* (1994) who established that noncapsular strains of *S. aureus* had a tendency of increased adherence in relation to capsular strains of *S. aureus*, showing that the bacterial cell wall has great affinity for extracellular protein matrix, nondamaged and damaged epithelial cells.

Comparison of the adherence of different toxin producing strains of *S. aureus* indicated no significant differences in adherence and it could be concluded that adhesins are not controlled by same genetic material as toxins. However, toxins have a role in the process of adherence because damaged cell membranes and cells, exposed basal membranes and extracellular matrix are more attractive to bacteria (Cifrian *et al.* 1994).

Comparison of adherence of strains of *S. aureus* in relation on plasmid content indicated no significant differences between the strains of *S. aureus* which possessed different plasmids.

Investigation of the factors influencing adherence whether of bacterial or host origin are in the focus of interest of current science and contribute towards getting more precise answers to many questions relating to prevention and pathogenesis of mastitis and bacterial diseases, generally.

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UTICAJ NEKIH FAKTORA NA ADHERENCIJU *S. AUREUS I S. AGALACTIAE* ZA LINIJU SEKRETORNIH EPITELNIH ĆELIJA MLEČNE ŽLEZDE KRAVA (BME-UV1)

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SADRŽAJ

U testu adherencije, linija epitelnih ćelija mliječne žlijezde krava (BME-UV1) je inkubirana sa suspenzijom sojeva *S. aureus* (10^8/ml) ili *S. agalactiae* (10^8/ml) 30min. pri 37^0C u atmosferi sa 5%$C0_2$. Preparati su posmatrani svjetlosnim mikroskopom, uvećanjem 10 x 100. Na preparatima je posmatrano vezivanje bakterija za epitelne ćelije na 20 vidnih polja, a za brojanje su odabirana vidna polja na kojima je bilo prosječno 100 epitelnih ćelija. Rezultati su prikazani kao broj vidnih polja na kojima je utvrđena adherencija u okviru 20 posmatranih.

Rezultati su pokazali da se adherencija izolovanih sojeva *S. aureus* kretala od 2-20, a sojeva *S. agalactiae* od 0 -16 vidnih polja, što pokazuje visok stepen variranja adherencije između ispitivanih sojeva. Razlika u adherenciji između ispitivanih sojeva iste vrste bakterija bila je statistički značajna, što nije utvrđeno između sojeva *S. aureus* i *S. agalactiae*. Ispitivanjem uticaja faktora mikroorganizama na adherenciju utvrđeno je da soj *S. aureus*, koji stvara kapsulu, nije pokazao značajno manju adherenciju od soja *S. aureus* koji ne stavra kapsulu. Sojevi *S. aureus*, koji stvaraju sluz, nisu pokazali značajno veću adherenciju od sojeva koji ne stvaraju sluz. Adherencija izolovanih sojeva *S. aureus* nije zavisila od njihove sposobnosti da stvaraju toksine, kao ni od plazmidskog profila.