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ADHERENCE AS A PREREQUISITE FOR INFECTION OF THE BOVINE MAMMARY GLAND BY BACTERIA

By

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WANASINGHE, DON D.: *Adherence as a prerequisite for infection of the bovine mammary gland by bacteria.* Acta vet. scand. 1981, 22, 109—117. — Using a simple in vitro test it was demonstrated that staphylococci, Streptococcus agalactiae, and micrococci, the species of bacteria which are commonly isolated from udder infections, adhered to mammary gland epithelial cells readily and in large numbers. Some strains of organisms which are associated with sporadic outbreaks or occur less commonly, like Str. dysgalactiae and Str. uberis, adhered moderately. Escherichia coli, Klebsiella spp., Corynebacterium pyogenes, C. bovis, Str. bovis, and Str. faecalis, species which are isolated occasionally, adhered poorly. From these studies, it appears that selective adherence of bacteria to the epithelial cells is a factor contributing to the ability of organisms to infect the mammary gland and may, therefore, be considered an important stage in the pathogenesis of bovine mastitis.

bovine mammary gland; mastitis; adherence;
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In most bacterial diseases, the infecting organism enters the host via a mucosal surface. Hence, pathogenic bacteria must be able to colonize such surfaces and this is now recognized as an important step in the infectious process (Smith 1972, Gibbons 1973). To colonize mucosal surfaces, the organisms must first adhere to the epithelial cells of these tissues or they will be removed by the host's physiological-anatomical defence mechanisms (Ofek et al. 1977). Thus, it seems likely that the ability of a pathogen to attach to a surface would influence the extent to which it may colonize, and, thus, may be related to its virulence (Gibbons 1974).

It is commonly accepted that most bacterial udder infections take place through the teat orifice via the streak canal. The bovine udder is exposed to a wide variety of species of bacteria, yet the majority of udder infections are caused by a limited number of bacterial species. It has been proposed that selective adherence of bacteria to mammary gland epithelial cells may explain why only a limited range of bacteria cause udder infections (Frost 1975).

An *in vitro* test system to evaluate the ability of a number of species and strains of bacteria to adhere to bovine mammary gland epithelial cells (Wanasinghe 1977, 1981) has been used herein to test the above hypothesis.

MATERIALS AND METHODS

Cells

The epithelial cells were collected principally from the lactiferous sinuses of clinically healthy lactating cows immediately after slaughter. The cells were washed and suspended in phosphate buffered saline (PBS) to contain approximately 10^4 cells per ml (Wanasinghe 1981). The cell suspensions were used within 3 h of collection.

Bacteria

Strains of *Staphylococcus aureus*, *S. epidermidis*, *Micrococcus* spp., *Streptococcus agalactiae*, *Str. uberis*, *Str. bovis*, *Str. faecalis*, *Str. dysgalactiae*, *Escherichia coli*, *Klebsiella* spp., *Pseudomonas aeruginosa* (PS1), *Corynebacterium pyogenes* and *C. bovis* were isolated from the milk of cows affected with mastitis while conducting a survey of mastitis in Queensland, Australia. *Ps. aeruginosa* (PS2) was isolated from milk of a goat with chronic mastitis. For use in the adherence test, all strains were routinely grown in Todd-Hewitt broth (Oxoid Ltd.) for 12–14 h, except *C. pyogenes* and *C. bovis* which were grown on blood agar for 48 h and harvested into PBS. The bacteria were washed twice and resuspended in PBS to obtain suspensions with approximately 10^6 colony forming units (c.f.u.)/ml.

Adherence of bacteria to mammary gland epithelial cells

Aliquots of 2 ml of washed epithelial cell suspensions and of bacterial suspensions were mixed in test tubes. These mixtures

were incubated at 37°C for 1 h. The unattached bacteria were removed by 3 cycles of centrifugation (1500 r.p.m., 15 min) and washing of the cells with PBS. Smears were prepared by spreading 0.2 ml of the resulting suspensions on glass slides over approximately 1 cm². The smears were air-dried, stained with Gram crystal violet for 10–15 s and examined under the oil immersion lens of a microscope.

Counting of numbers of bacteria adhering to epithelial cells

The numbers of bacteria adhering to 100 epithelial cells and the numbers of epithelial cells which had bacteria attached were recorded.

Adherence index

It was found inconvenient to handle more than 6 species or strains of bacteria in any one experiment. Accordingly, strains were grouped into 5 experiments. To enable ready comparison of results across a range of experiments, a common relative adherence index was used for evaluation. The adherence of each strain was expressed as a percentage of the adherence of a standard strain (usually *S. aureus* Sa1, which was known to adhere well) included with each test series. On the basis of the adherence index, i.e., the percentage adherence value, the relative adherence of the test bacteria was graded arbitrarily as high (over 70), medium (50–70), low (50–30) and very low (below 30).

RESULTS

Applying the classification of relative adherence, the results of experiment 1 (Table 1) show that the adherences of 3 strains of *Str. agalactiae* were medium while those of 2 strains were very low, compared to the standard strain of *Str. agalactiae*.

In experiment 2, the adherence of 1 strain of *S. aureus* was high, those of 3 strains were medium and that of 1 strain was low, compared to the standard strain of *S. aureus*. In experiment 3, the adherences of the strains of *Str. faecalis*, *Str. bovis*, *Str. uberis*, *Str. dysgalactiae*, and *E. coli* were very low compared to that of the standard *S. aureus* strain. However, the adherence of one of the strains of *Str. uberis* (Ub 4) was higher than for the other strains of the same species (low).

Table 1. Adherence of different strains of bacteria isolated from cases of mastitis to bovine mammary gland epithelial cells.

Experiment	Species	Strain	No of cells ^a with adher- ing bacteria per 100 cells	No of adher- ^a ing bacteria per 100 cells	Adher- ence index	
1	Str. agalactiae (control)	Ag1	63	461	100	
		Ag2	18	62	13	
		Ag3	12	43	9	
		Ag4	45	242	52	
		Ag5	55	312	64	
		Ag6	49	353	68	
2	S. aureus (control)	Sa1	63	144	100	
		Sa2	42	80	55	
		Sa3	37	65	45	
		Sa4	41	86	59	
		Sa5	64	152	105	
		Sa6	44	77	53	
3	S. aureus (control)	Sa1	66	219	100	
		Str. dysgalactiae	DS1	19	32	14
			DS2	22	42	19
			DS3	6	11	5
	DS4		5	18	8	
	Str. uberis	Ub1	11	6	2	
		Ub2	9	14	6	
		Ub3	8	15	6	
		Ub4	34	89	40	
	E. coli	EC1	19	26	11	
		EC2	9	11	5	
		EC3	17	18	8	
	Str. bovis	Sb1	2	3	1	
	Str. faecalis	Sf1	6	7	3	
	4	S. aureus (control)	Sa1	83	355	100
Sa2			77	357	100	
Micrococcus spp.		M2	66	200	56	
S. epidermidis		Se1	41	132	37	
5	S. aureus (control)	Sa1	84	371	100	
		Sa8	67	281	75	
	Micrococcus spp.	M4	74	400	107	
	S. epidermidis	Se2	69	288	77	

Table 1 (continued).

Experiment	Species	Strain	No of cells ^a with adher- ing bacteria per 100 cells	No of adher- ^a ing bacteria per 100 cells	Adher- ence index
6	<i>S. aureus</i> (control)	Sa1	84	444	100
	<i>C. pyogenes</i>	CP1	5	7	1
	<i>C. bovis</i>	Cb1	21	45	10
		Cb2	2	9	2
7	<i>S. aureus</i> (control)	Sa1	50	141	100
	<i>Ps. aeruginosa</i>	PS1	24	35	24
		PS2	63	191	135
	<i>E. coli</i>	EC2	3	4	2
	<i>Klebsiella</i> spp.	K2	11	12	8

^a These data represent the mean values from counts in triplicate.

In experiments 4 and 5, the adherences of *S. aureus* were high, and those of strains of *Micrococcus* spp. and *S. epidermidis* were medium or high, compared to the standard *S. aureus* strain.

In experiment 6, the adherences of *C. bovis* and *C. pyogenes* were very low compared to the standard strain of *S. aureus*.

In experiment 7, the adherences of *Klebsiella* spp., *E. coli*, and of one of the strains of *Ps. aeruginosa* were very low, while the adherence of the other strain of *Ps. aeruginosa* (PS2 from goat) was high.

When the adherence indices of bacterial species were low, the number of cells which had bacteria attached were also low (see Table 1).

DISCUSSION

In a survey of the incidence of mastitis (Wanasinghe 1977) among the herds from which the bacterial strains for this study were isolated, the infection rates due to various species of bacteria in 1207 cows tested were as follows: *S. aureus*, 19 %; *Str. agalactiae*, 15 %; *Str. uberis*, 4 %; *Str. dysgalactiae*, 4 %; *C. pyogenes*, 0.2 %; *Micrococcus* spp., 40 %. *Micrococcus* spp. is regarded as a low grade pathogen (Dodd *et al.* 1977). When the results of all adherence experiments are viewed as a whole, it is generally seen that those species of bacteria which were responsible for the higher incidences of udder infections, i.e., *S. aureus*, *Str. agalactiae* and micrococci, adhered more readily to the mam-

mary gland epithelial cells (high or medium relative adherence). The strains of *E. coli*, *Klebsiella* spp. and *Corynebacterium* spp. adhered very poorly (relative adherence was very low) and the incidences of udder infections due to these species was also very low.

Although the incidences of udder infections due to *Str. dysgalactiae* and *Str. uberis* were very low, 3 herds had a high incidence of *Str. dysgalactiae* infections (11—13 %) and 5 had a high incidence of *Str. uberis* infections (9—13 %). These data show that *Str. dysgalactiae* and *Str. uberis* can occasionally occur as major problems in herds. It was found that the relative adherence of *Str. dysgalactiae* and *Str. uberis* was generally very low, but some strains of both of these species adhered better than others to the mammary gland epithelial cells. The high incidence of udder infections due to these 2 streptococcal species in some of the herds surveyed may have been due to the prevalence of more adhesive strains. However, it is not known whether these strains which adhered better (Table 1, experiment 3) were representative of those causing infections in these herds.

Recently, significant differences in intramammary infectivity between strains of *Str. dysgalactiae* have been demonstrated (Higgs *et al.* 1980). As few as 12 cells of one strain, CE 127, were shown to be capable of causing an infection upon intramammary instillation. Interestingly, this strain of *Str. dysgalactiae* gave the highest adherence index to ductular epithelial cells of any of the strains of *Str. dysgalactiae* tested in a previous study (cf. strain 127 in Table 4, Frost *et al.* 1977). It would seem worthwhile to examine the ability of strains of *Str. dysgalactiae* and *Str. uberis* showing differences in relative adherence to ductular epithelium to cause *in vivo* infections, as such information would provide a means of cross-checking the hypothesis concerning the relationship of adherence to prevalence.

The strain of *Ps. aeruginosa* which adhered well was from a goat with chronic mastitis and from a herd in which mastitis due to *Pseudomonas* was prevalent. Perhaps bovine and caprine mammary gland epithelial cells might be similar in relation to bacterial adherence. This is not unlikely as goats have been used as a model for studies of bovine mastitis (Pattison 1958). Again, *in vivo* infections with strains showing differences in relative adherence to ductular epithelium may provide insight into factors determining adherence and/or infectivity.

Coliform mastitis has been described as different from other major forms of bovine mastitis. The organisms grow in secretions and usually there is little tissue invasion. Thus, very low adherence might be expected. *C. bovis* has been shown to be an inhabitant of the streak canal (*Black et al.* 1972), which might explain its relatively low adherence to ductular cells. The pathogenesis of *C. pyogenes* mastitis is poorly understood. The association of *Peptococcus indolicus* with *C. pyogenes* in mastitic infections suggests that the pathogenicity of this form of mastitis could be different from that of other udder infections (*Sørensen* 1974, *Schwan & Holmberg* 1978/79). The role of adherence of these bacteria in this mixed infection remains to be elucidated.

The epithelial cells used in the present study were collected mainly from the lactiferous sinuses. However, since no attempt was made to strictly confine collection to the lactiferous sinuses, some of the cells almost certainly came from the large ducts. It has been shown that there is a significant difference in the adherence of *S. aureus* to cells from different areas of the same udder quarter and from different cows (*Frost et al.*). Nevertheless, this factor should not have influenced the present studies in which a number of strains included in a particular experiment were compared with one another, unless the ability of different species or strains to adhere to cells of the lactiferous ducts and sinuses varies. In the author's experience, this seems unlikely.

However, one may have to consider this factor when comparing the results from a series of experiments, particularly when the differences in bacterial adherence are marginal. In the present study, the grouping of species and of strains of bacteria had been arranged so that organisms of direct comparative interest were placed within an experimental group. When the adherence of a bacterial species was low, the number of cells which had bacteria adhering was also low. Thus, it appears that bacterial adherence to mammary gland epithelial cells involves surface components of both cells and bacteria (*Ofek et al.* 1975).

From the present study, it thus appears that there is a relationship between adherence of organisms to bovine mammary gland epithelial cells and their general prevalence in udder infections. These findings further strengthen the argument that selective adherence of bacteria to bovine mammary gland epithelium is a prerequisite for the organism to be a successful parasite of the mammary gland. Selective adherence may, there-

fore, be considered an important stage in the pathogenesis of mastitis.

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SAMMANFATTNING

Adhesion (vidhäftning) som en förutsättning för bakteriell bovin mastit.

Genom att använda en enkel in vitro test kan man demonstrera hur stafylokker, *Str. agalactiae*, mikrokker och andra bakterier som ofta påträffas vid bovin mastit „fastnar“ på juverepitelceller. Antalet bakterier som fastnar på epitelcellerna varierar emellertid. Bakterier av de förutnämnda arterna fastnar ofta i stort antal. Bakterier som ses vid mer sporadiskt inträffade mastiter (t ex *Str. dysgalactiae* och *Str. uberis*) fastnar däremot i mindre antal. *E. coli*, *Klebsiella*, *C. pyogenes*, *C. bovis*, *Str. bovis* och *Str. faecalis* slutligen fastnar endast i ringa antal. Det tycks som om den selektiva adhesionen är en betydelsefull faktor för bakteriens förmåga att framkalla mastit. Den kan därför tänkas bidra till en ökad förståelse av den bovina mastitens patogener.

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