PROTECTIVE EFFECT IN THE LACTATING BOVINE MAMMARY GLAND INDUCED BY COAGULASE-NEGATIVE STAPHYLOCOCCI AGAINST EXPERIMENTAL STAPHYLOCOCCUS AUREUS INFECTIONS

B. Poutrel, Catherine Lerondelle

Ann. Rech. Vét., 1980, **11** *(4)*, 327-332.

PROTECTIVE EFFECT IN THE LACTATING BOVINE MAMMARY GLAND INDUCED BY COAGULASE-NEGATIVE STAPHYLOCOCCI AGAINST EXPERIMENTAL *STAPHYLOCOCCUS AUREUS* INFECTIONS

B. POUTREL and Catherine LERONDELLE

Institut National de la Recherche Agronomique, Station de Pathologie de la Reproduction, 37380 Nouzilly, France

Résumé

PROTECTION CONFÉRÉE DANS LA MAMELLE BOVINE EN LACTATION PAR DES STAPHYLOCOQUES COAGULASE-NÉGATIVE CONTRE DES INFECTIONS EXPÉRIMENTALES A *STAPHYLOCOCCUS AUREUS*. — La résistance de quartiers préalablement infectés soit expérimentalement par *Staphylococcus epidermidis* ou *Staphylococcus xylosus*, soit naturellement par d'autres staphylocoques coagulase-négative, a été mesurée après inoculation d'épreuve par deux souches différentes de *Staphylococcus aureus*. Le taux de quartiers contrôles infectés (95 %) a été significativement plus élevé que celui des quartiers préinfectés par des staphylocoques coagulase-négative (24,5 %). Des différences ont été observées dans la fréquence de surinfection des quartiers selon la souche de staphylocoque coagulase-négative préinfectante, la durée de cette préinfection, le niveau cellulaire au moment de l'inoculation d'épreuve.
Le mécanisme de protection conférée par la préinfection par les staphylocoques coagulase-négative vis-à-vis de *Staph. aureus* a été discuté.

Among bacteria involved in mammary infection in cattle, coagulase-negative micrococci are minor pathogens (Griffin *et al.*, 1977), rarely associated with clinical symptoms. They are frequently isolated from aseptically collected samples of milk and their influence on the production of the quarter, composition and somatic cell count of the milk have been reported (Bramley, 1975 ; Holmberg, 1973 ; Linzell and Peaker, 1972 ; Natzke *et al.*, 1972).

Systematic treatment with antibiotics may be discussed following observations and experiments which suggest that quarters infected by coagulase-negative micrococci are less susceptible to superinfection by major pathogens. Bramley's work (1978) seems to show that this protective effect varies according to the species of bacteria used for the challenge. He found that the effect observed for *Streptococcus agalactiae* was superior to that for *Escherichia coli*. In the experiment reported by Linde *et al.* (1975), the reduced number of quarters in which *Staphylococcus epidermidis* was reisolated at the time of the challenge with *Staphylococcus aureus* does not permit a valid evaluation of the defence induced against this species of bacteria. According to Edwards and Jones (1966), an antibiotic substance secreted by certain coagulase-negative staphylococci could be capable of inhibiting the growth of coagulase-positive staphylococci. According to Bramley

Table 1. — Major characteristics of the coagulase-negative staphylococci strains

Characteristics	Strains		
	Staph. epidermidis 128.59	*Staph. xylosus* 137.11	Natural
Acid from glucose (anaerobically)	+	+	+
Susceptibility to lysostaphin	+	+	+
Clumping factor	–	–	–
Protein A	–	–	–
Arginine dihydrolase	+	–	ND[1]
Hemolysis	+	–	V[2]
Acid from			
xylose	–	+	ND
trehalose	–	+	ND
mannose	+	+	ND
maltose	+	+	ND
mannitol	–	+	ND
saccharose	+	+	ND

1 : ND = Not determined
2 : V = Variable

(1978), the increase in the number of polymorphonuclear leucocytes (PMN) in quarters preinfected by *Staph. epidermidis* could be solely responsible for the lower susceptibility of these quarters to the challenge.

The purpose of the work reported here was to measure the protective effect induced in lactating quarters preinfected by coagulase-negative staphylococci against a challenge with *Staph. aureus* and to define the mechanism in question.

Materials and Methods

Cows and management

Forty-four Friesian cows of our experimental herd were used. All lactating cows were in the second to seventh month of lactation. Before milking, udders were washed with individual cloths, sterilized by boiling after each milking. Post-milking teat dipping was carried out systematically with a solution containing 5 $^0/_{00}$ available iodine.

Strains of bacteria

Staphylococcus epidermidis 128.59 and *Staphylococcus xylosus* 137.11 (Devriese, 1979) (table 1) were isolated in pure culture from milk samples from bovine mammary glands. Organisms isolated from spontaneous mammary infections were identified as « coagulase-negative staphylococci » by colonial morphology, Gram staining, clumping factor, protein A and coagulase tests, ability to ferment glucose anaerobically and lysostaphin sensitivity.

Staph. aureus strains 106.6 and 107.59 have been described and used extensively in previous experiments (Postle *et al.*, 1978 ; Poutrel and Lerondelle, 1978).

Experimental design

1. Collection and examination of quarter foremilk samples

All quarters of all lactating cows were sampled once every fortnight. Quarter foremilk samples from cows on test were collected daily from the fifth day prior to infusion up to day 14 post-infusion of coagulase-negative staphylococci strains (128.59 and 137.11). Quarters samples were taken 14 days afterwards in each case to establish the result of the challenge with *Staph. aureus* strains (106.6 and 107.59).

About 20 ml of quarter foremilk samples were aseptically collected at evening milking.

Table 2. — Effect of coagulase-negative staphylococci infection on susceptibility of quarters to experimental superimposed infections with *Staph. aureus*.

	Coagulase-negative staphylococci strains				
	128.59	137.11	Spontaneous infections	Total	Control quarters
Challenge strains					
106.6	2/7[a]	3/9	1/4	6/20	16/17
107.59	0/3	4/12	0/6	4/21	18/19
Total infected (%)	2/10 (20)	7/20 (35)	1/10 (10)	10/41 (24.5)	34/36 (94.5)

a : No. quarters infected/No. quarters challenged.

Milk samples were plated (0.025 ml) on sheep blood agar for isolation of bacteria present. Typical colonies were identified after 24 or 48 hours of incubation at 37°C. Somatic cell count was determined using a Coulter Counter (model F) according to the method recommended by the International Dairy Federation. As a control, California Mastitis Tests were also carried out.

2. Preparation of infusion dose and procedure for infusion

Methods used for the growth of bacteria and preparation of the inoculum were similar to the procedure described by Postle *et al.* (1978). Appropriate dilutions were made in buffered saline solution to obtain an infusion dose (0.2 ml) of between 1000 and 2000 colony forming units (CFU) for coagulase-negative staphylococci strains (128.59 and 137.11) and about 100 CFU for *Staph. aureus* strains (106.6 and 107.59).

Quarters free of intramammary infection after repeated sample examination and with a cell count of less than 500 000/ml were selected for infusion with coagulase-negative staphylococci strains. Quarters which had been diagnosed as infected by these strains were superinfected by *Staph. aureus* strains, 15 days or between 30 and 60 days after this first infusion. Quarters in which spontaneous coagulase-negative staphylococci infections occurred were superinfected by *Staph. aureus* strains at least three months after the first positive bacteriological examination.

When all four quarters of the cows were eligible for injection, two of them, chosen at random, were infused with the two coagulase-negative staphylococci strains and challenged with either 106.6 or 107.59 *Staph. aureus* strains. The two remaining quarters were considered as control quarters and infused at random with one of the two *Staph. aureus* strains. Cows having only two or three eligible quarters were infused in one quarter with either 128.59 or 137.11 strains. In this case the same *Staph. aureus* strain was used for the challenge and for control quarters.

The number of infusions with each strain was balanced as far as possible.

Quarters selected for the test were infused after evening milking and infusions for the challenge and for control quarters were performed the same day. The infusion technique used was that described by Poutrel and Lerondelle (1978).

Results

The experimental infection with coagulase-negative staphylococci became established in 11 of 45 quarters infused with *Staph. epidermidis* (20.8 %) and in 20 quarters of 43 infused with *Staph. xylosus* (46.5 %). No clinical infection developed in these quarters or in control quarters after experimental infusions with the *Staph. aureus* strains.

Of 41 quarters preinfected by coagulase-negative staphylococci, 10 (24.5 %) became infected by *Staph. aureus* after the challenge (table 2). The proportion of quarters which became infected by *Staph. aureus* differs very significantly from that of control quarters (95 %) ($\chi^2 = 38$, df = 1, $P < 0.001$). Protection observed thereby was independent of the challenge strain (106.6 or 107.59) of *Staph. aureus* used ($\chi^2 = 0.7$, df = 1, $P \simeq 0.4$).

Table 3. — Effect of the foremilk cell counts of the coagulase-negative staphylococci infected quarters prior to challenge on susceptibility of quarters to infection with *Staph. aureus*.

	Quarters				
	Infected with				
	128.59	137.11	Natural strains	Total	Uninfected (control)
No. quarters	10	20	10	40	36
Geometric mean total cell count (X 10^3/ml)	230	306	398	...	224
No. quarters infected with Staph. aureus/*No. quarters challenged (%)*					
Range of total cell counts					
⩽200	2/3	5/9	0/2	7/14 (50)	26/26 (100)
201 to 500	0/7	2/8	1/6	3/21 (14)	8/10 (80)
501 to 1000	...	0/2	0/1	0/3 (0)	...
>1001	...	0/1	0/1	0/2 (0)	...

Natural infections by coagulase-negative staphylococci gave best protection (10 % of quarters infected), *Staph. xylosus* infections giving least protection (35 % of quarters infected). However, there was no significant difference according to preinfective strain (χ^2 = 2.4, df = 2, P ≃ 0.3).

Overall, frequency of infection appears to be linked to the number of somatic cells at the time of challenge (table 3). No infection developed in quarters with a somatic cell count of more than 500 000 cells/ml milk which were preinfected by coagulase-negative staphylococci. The highest frequency of infection was found with a cell count of less than 200 000 cells/ml, both for control quarters and all quarters preinfected. The latter, however, were very significantly less susceptible to the challenge than control quarters (χ^2 = 15.8, df = 1, P < 0.001).

With a duration of preinfection with coagulase-negative staphylococci strains of 15 days, 6 of 17 quarters (35 %) having received the challenge with *Staph. aureus* became infected with this species, compared to 3 of 13 quarters (23 %) for a preinfection duration of between 30 and 60 days. The difference observed thereby was not significant (χ^2 = 0.29, df = 1, P ≃ 0.59).

Discussion

The results show that in the given experimental conditions, the natural or induced coagulase-negative staphylococci preinfections induce a considerable protective effect against a challenge using *Staph. aureus*. This observation confirms results reported by Linde *et al.* (1975). The method used for the challenge, with *Staph. aureus* placed directly into the cistern without precolonisation of the teat, makes this challenge particularly severe and suggests that this protective effect could at least be as important in natural conditions.

A number of hypotheses may be advanced to explain the resistance of preinfected quarters. The inhibiting effect *in vitro* of substances secreted by *Staph. epidermidis* upon the growth of *Staph. aureus* described by Edwards and Jones (1966) and by Linde *et al.* (1975) was not found by Bramley (1978) for *Streptococcus agalactiae* and *E. coli* and was not investigated in the present experiment. According to Bramley, the increase in the number of polymorphonuclear leucocytes in quarters preinfected with *Staph. epidermidis* induces a greater resistance to superinfection. This idea of a « cell barrier » was put forward by Schalm *et al.* (1964) and recently developed by Paape *et al.* (1979) who showed that it is possible to induce a slight leukocytosis after inserting a polythene loop into the cistern of the udder.

The present results favour this hypothesis, as none of the quarters with a somatic cell count of more than 500 000/ml developed an infection of *Staph. aureus* and as the proportion of infection was 14 % and 50 % for those

with a count of between 200 000 and 500 000 and lower than 200 000, respectively.

However, comparison of control quarters and preinfected quarters shows that at a somatic cell count as equal as can be determined, frequency of infection in preinfected quarters is half as high (50 % as compared to 100 %). It is probable that the coagulase-negative staphylococci strains stimulate another defence mechanism in the udder. The fact that this protective effect is more effective against a gram-positive species of bacteria *(Streptococcus agalactiae)* than against a gram-negative species *(E. coli)* (Bramley, 1978) could favour a certain specificity of the phenomenon. Variability of the susceptibility of quarters according to the preinfection strain used and the duration of preinfection before the challenge, although not significant in the conditions of this experiment, may equally constitute an argument in favour of a specific mechanism.

Other studies, in particular on the opsonizing ability of milk samples from preinfected quarters using different species of bacteria, would make it possible to determine to what extent the protection observed is imputable to a specific mechanism.

Accepted for publication, October 10th, 1980.

Summary

The susceptibility of quarters preinfected either experimentally with *Staphylococcus epidermidis* or *Staphylococcus xylosus* or naturally by other coagulase-negative staphylococci was measured after challenge with two different strains of *Staphylococcus aureus.* The proportion of infected control quarters (95 %) was raised significantly higher than that of those preinfected by the coagulase-negative staphylococci (24.5 %). Differences were observed in the frequency of superinfection of the quarters according to the strain of the preinfective coagulase-negative staphylococcus, duration of preinfection and somatic cell count at the time of challenge.
The protective mechanism against *Staph. aureus* induced by preinfection with coagulase-negative staphylococci is discussed.

References

BRAMLEY A.J., 1975. Infection of the udder with coagulase negative micrococci and *Corynebacterium bovis. In Proceeding of Seminar on Mastitis Control,* International Dairy Federation, Bruxelles, 377-381.

BRAMLEY A.J., 1978. The effect of subclinical *Staphylococcus epidermidis* infection of the lactating bovine udder on its susceptibility to infection with *Streptococcus agalactiae* or *Escherichia coli. Br. Vet. J.,* **134**, 146-151.

DEVRIESE L.A., 1979. Identification of clumping-factor-negative staphylococci isolated from cows' udders. *Res. Vet. Sci.,* **27**, 313-320.

EDWARDS S.J., JONES G.W., 1966. The distribution and characters of coagulase-negative staphylococci of the bovine udder. *J. Dairy Res.,* **33**, 261-269.

GRIFFIN T.K., DODD F.H., NEAVE F.K., WESTGARTH D.R., KINGWILL R.G., 1977. A method of diagnosing intramammary infection in dairy cows for large experiments. *J. Dairy Res.,* **44**, 25-45.

HOLMBERG O., 1973. *Staphylococcus epidermidis* isolated from bovine milk. Biochemical properties, phage sensitivity and pathogenicity for the udder. *Acta. Vet. Scand.* Suppl. **45**, 1-144.

LINDE C., HOLMBERG O., ASTRÖM G., 1975. An attempt to superimpose *Staphylococcus aureus, Streptococcus agalactiae* and *Streptococcus dysgalactiae* upon *Staphylococcus epidermidis* infections in the cow's udder. *In Proceeding of Seminar on Mastitis Control,* International Dairy Federation, Bruxelles, 391-394.

LINZELL J.L., PEAKER M., 1972. Day-to-day variations in milk composition in the goat and cow as a guide to the detection of subclinical mastitis. *Br. Vet. J.,* **128**, 284-296.

NATZKE R.P., EVERETT R.W., GUTHRIE R.S., KEOWN J.F., MEEK A.M., MERILL W.G., ROBERTS S.J., SCHMIDT G.H., 1972. Mastitis control program : effect on milk production. *J. Dairy Sci.,* **55**, 1256- 1260.

PAAPE M.J., SCHULTZE W.D., KORTUM W.T., 1979. Leukocyte response in mammary quarters fitted with an intramammary device. *J. Anim. Sci,* **49**, suppl. 1, 320.

POSTLE D.S., ROGUINSKY M., POUTREL B., 1978. Induced staphylococcal infections in the bovine mammary gland. *Am. J. Vet. Res.,* **39**, 29-35.

POUTREL B., LERONDELLE C., 1978. Induced staphylococcal infections in the bovine mammary gland. Influence of the month of lactation and other factors related to the cow. *Ann. Rech. Vét.,* **9**, 119-128.

SCHALM O.W., LASMANIS J., CARROLL E.J., 1964. Effects of pre-existing leukocytosis on experimental coliform *(Aerobacter aerogenes)*. Mastitis in cattle. *Am. J. Vet. Res.,* **25**, 83-89.