DIFFERENCES IN INTRAMAMMARY PATHOGENICITY OF FOUR STRAINS OF *STREPTOCOCCUS DYSGALACTIAE*

T. M. HIGGS, F. K. NEAVE AND A. J. BRAMLEY

National Institute for Research in Dairying, Shinfield, Reading RG2 9AT

STREPTOCOCCUS DYSGALACTIAE has long been recognised as an udder pathogen. It was first described by Diernhofer (1932) and subsequently classified as a group-C streptococcus by Lancefield (1933). It can be found in various extramammary sites, including the vagina (Francis, 1941) and tonsils (Daleel and Frost, 1967) of adult cattle, but the significance of these sites as reservoirs of infection in a herd is not known. Udder infection with *S. dysgalactiae* is generally less frequent than with either *S. agalactiae* or *S. uberis* (Slanetz and Naghski, 1940). Results from 32 herds in the United Kingdom showed that 2.9% of cows were infected with *S. dysgalactiae* compared with 4.0% and 4.4% for *S. agalactiae* and *S. uberis* respectively (Wilson and Kingwill, 1975). McDonald and McDonald (1976) found that 41 of 455 (9%) streptococcal isolates from infected udders were *S. dysgalactiae* compared with 7 (1.5%) for *S. agalactiae* and 257 (56%) for *S. uberis*.

Experimental infections of goats and cattle with *S. dysgalactiae* have been successful (Edwards, 1932; Holman, Pattison and Gordon, 1952; Pattison and Smith, 1953) but we are not aware of published results comparing the infectivity of strains. It was partly for this reason and partly to identify an infective strain for milking-machine studies that this work was done.

The ability of streptococci to form chains means that the number of colony-forming units per unit volume could be a misleading indication of the number of viable organisms in a challenge volume, particularly if one of the strains tended to produce long chains whilst others produced short chains in the growth conditions used. To investigate the possible effects of this factor, strains for infection studies were compared before and after treatment that would disrupt chains.

MATERIALS AND METHODS

Strains of S. dysgalactiae. Strains were isolated as follows: strain CE 127 in 1969 from a clinical case of mastitis on Farm CE, strain BE63 in 1968 from a clinical case of mastitis on Farm BE, strain BC93 in 1970 from a clinical case of mastitis on Farm BC, strain 401/10 from mastitic milk in 1970 by the Veterinary Investigation Centre, Coley Park, Reading. Strains CE127 and BC93 are deposited in the National Collection of Dairy Organisms and numbered 2043 and 2023 respectively. Cultures were grown on nutrient-agar slopes containing 10% (v/v) calf blood at 37°C for 18 h. The slopes were stored at 4°C and subcultured at 2–3-month

intervals. All four strains showed the typical biochemical reactions of *S. dysgalactiae* (Cowan and Steel, 1974) but differed in their abilities to produce acid from sorbitol; strains BC93 and 401/10 were sorbitol positive whilst CE127 and BE63 were sorbitol negative. From all four strains, an HCl (0.2N) extract gave a precipitin reaction with Lancefield Group-C Streptococcal Antiserum (Burroughs Wellcome, Beckenham, Kent).

Cultures of S. dysgalactiae for udder challenge. The organisms were subcultured on nutrient-agar plates containing 7-8% (v/v) calf blood and 0.1% aesculin (ABA). The plates were incubated at 37°C for c. 40 h and several typical colonies were transferred to 10 ml of litmus milk which was incubated at 37°C for 17 h. After incubation the litmus-milk cultures were cooled to 4°C and divided into two equal parts; one part was ultrasonically treated for 1 min. (Soniprobe, Dawes Ltd, London) and the other part left untreated. Earlier tests had shown that this treatment increased the percentage of single or diplococcal units in a culture of strain CE127 from 66% to 100%. Samples of sonicated and untreated culture were diluted in half-strength nutrient broth ($\frac{1}{2}$ NB) (Oxoid Broth No. 2) and five 0.1-ml volumes of a 10^6 dilution were plated on ABA to determine the number of colony-forming units/ml (c.f.u./ml). The increases in c.f.u./ml resulting from ultrasonic treatment are shown in table I. The undiluted cultures were

TABLE I
Effect of ultrasonic treatment for 1 min. on the number of colony-forming units of *Streptococcus dysgalactiae*

<i>S. dysgalactiae</i> strain no.	Colony-forming units (10^6)/ml of culture		(b) ÷ (a)
	before ultrasonic treatment (a)	after ultrasonic treatment (b)	
BC93	620	1100	1.8
CE127	1080	1700	1.6
BE63	947	1370	1.4
401/10	260	633	2.4

stored overnight at 4°C and then diluted to contain about 40 c.f.u./ml; the diluted suspensions were introduced into the udder sinus.

Experimental animals. Sixteen lactating Friesian cows were used. They were housed in cubicles on sawdust bedding and milked twice daily, at 06.00 and 14.30 hours, in a six-unit tandem milking parlour. Before milking, udders were washed and dried; after milking, teats were dipped in a hypochlorite solution containing 4% available chlorine. The milking clusters were pasteurised by the circulation of water at c. 80°C for about 10 s between milking of each cow. Quarter foremilk samples were collected on two occasions during the 7 days before challenge to establish the level of infection at the start of the experiment. Of the 64 quarters, 61 were free from bacterial infection during this period. Of the remaining three quarters, two were excreting coagulase-negative staphylococci while the third was infected with *Corynebacterium ulcerans*. The distribution of these infected quarters is shown in the footnotes to tables II and III. Of the 16 animals, 12 were in their first lactation and the oldest cow was in its sixth lactation. Ten cows had calved not more than 6 weeks earlier but one animal had been in milk for 72 weeks.

Experimental design. Cows nos. 1-8 were challenged with suspension (U) prepared by ultrasonic treatment whilst nos. 9-16 were challenged with the untreated control suspension (C). Each cow was infused in all four quarters, each quarter being challenged with a different strain according to a latin-square design.

Collection and examination of quarter foremilk samples. The methods used for the aseptic collection and examination of quarter foremilk samples have been described by Bramley (1976). Samples were taken on the day of infusion, 1, 1½, 2 and 5 days after infusion and then at frequent intervals up to a maximum of 20 days. All samples were examined for bacteria, clots and

discoloration, and by the modified Whiteside test as an indirect measure of the somatic-cell content (Murphy and Hanson, 1941).

Infusion of the quarters. Infusions were done after an afternoon milking. When the cow entered the parlour, samples were taken from each quarter and the cow was milked. A sample of strippings milk was then collected and the teat orifice scrubbed for 15–20 s with 70% ethyl alcohol. After cleaning of the teat, 0.25 ml of the bacterial suspension was infused through the teat duct into the teat sinus as described by Bramley (1976). The sample of stripping milk was examined for somatic cells by the microscopic method of Prescott and Breed (1910). Within 20 min. of infusion, 0.25 ml of each infusion suspension was plated in triplicate on ABA for calculation of the number of organisms infused.

Detection of clinical signs. A careful examination of the foremilk was made with a strip cup before each milking and clots were recorded on a scale from 1 to 4 (grade 1 indicating one or two small clots and grade 4 severe clotting). Milk samples were also examined for clots after standing overnight at 4°C. After milking, the udder was palpated to detect hardness, swelling or tenderness of the quarters.

*Growth of *S. dysgalactiae* in raw milk.* Subcultures of *S. dysgalactiae* were made from nutrient blood agar in a medium containing 30 parts of yeast dextrose litmus milk and 70 parts of dextrose Lemco (Oxoid) broth; these were incubated at 37°C for 5 h, cooled rapidly in iced water, stored at 4°C and then diluted in $\frac{1}{2}$ NB to give c. 40 c.f.u./ml. Whole milk was obtained by aseptic cannulation of three cows free from intramammary infection. The milk was pooled and dispensed into sterile plugged test tubes in 10-ml portions. After warming to 37°C the tubes were inoculated with 1 ml (40 c.f.u.) of the diluted culture and incubated in a water bath at 37°C. Samples were taken after 5- and 18-h incubation for colony counting.

RESULTS

*Comparison of the pathogenicity of four strains of *S. dysgalactiae*.* The results of challenge with each strain are shown in table II. Of the 36 infections, 35 were detected within 5 days of challenge; and one quarter challenged with strain BC93 became infected after 14 days. "Infection" in this context has been defined as the presence of 20 or more of the challenge organisms/ml of milk—the minimum number detectable by the technique used—with an increase in Whiteside score of two grades or distinct clinical signs of mastitis on at least one occasion. Although strain CE127 infected all 16 challenged quarters, strain 401/10 was reisolated only once after infusion and did not produce definite clinical disturbance or increase in Whiteside score. Clots were found once in one quarter after challenge with strain 401/10 but this quarter had a pre-infusion strippings cell count of $> 14 \times 10^6$ /ml that may have been related to the subsequent clinical disturbance. Pathogens were not isolated in the period immediately before or after challenge. Strains BE63 and BC93 yielded similar results, infecting 10 quarters.

Analysis of variance of mean infection scores showed strain CE127 to be significantly more infective than strains BC93 and BE63 ($p=0.01$), and these three strains to be more infective than strain 401/10 ($p=0.005$). Differences between the infectivity of control and ultrasonicated suspensions were not significant. The small differences between the viable counts of the treated and the untreated suspensions (table I) indicated that little chaining had occurred in the untreated suspension.

Relationship between pre-challenge milk cell count and infection. Table III shows the proportion of challenged quarters that became infected in relation to

TABLE II
Infection of four strains of Streptococcus dysgalactiae into the teat sinus

Strain no.	Ultrasonic treatment before challenge	Number of colony-forming units in challenge suspension	Number of quarters					<i>S. dysgalactiae</i> c.f.u./ml of milk from infected quarters
			challenged	showing clinical signs	showing increase in Whiteside grade of 2 or more	yielding <i>S. dysgalactiae</i> > 20 c.f.u./ml of milk	infected*	
BC93	+	10	8	2(0)†	6(1)	4(3)	4	220 > 10 000
CE127	-	10	8‡	5(0)	5(0)	6(3)	6	20 > 10 000
	+	12	8	8(6)	8(5)	8(8)	8	1200 > 10 000
BE63	-	16	8	8(6)	8(4)	8(8)	8	5000 > 10 000
	+	9	8§	4(1)	4(0)	4(4)	4	500 > 10 000
401/10	-	9	8	5(3)	6(3)	6(6)	6	60 > 10 000
	+	9	8	1(1)	0(0)	1(1)	0	1200
	-	14	8‡	0(0)	0(0)	0(0)	0	0

* For definition of "infection", see text.

† Figures in parentheses indicate number of quarters showing reaction within 24 h.

‡ One quarter yielded coagulase-negative staphylococci before challenge.

§ One quarter yielded *Corynebacterium ulcerans* before challenge.

TABLE III

Relationship between pre-challenge milk cell count of strippings milk and outcome of challenge with *Streptococcus dysgalactiae*

Strain no.	Number of quarters infected/number of quarters challenged that had pre-challenge cell counts (10^3 /ml) of		
	< 250	250-500	> 500
CE127	7/7	6/6	3/3
BC93	4/4	3/6	3/6*
BE63	6/6	2/6	2/4†
401/10	0/5	0/4	0/7*
Totals	17/22	11/22	8/20

* One quarter yielded coagulase-negative staphylococci before challenge.

† One quarter yielded *Corynebacterium ulcerans* before challenge.

the pre-challenge strippings cell count. Strain CE127 infected all the challenged quarters, and strain 401/10 none, irrespective of initial cell count. But for the other two strains, quarters with cell counts of less than 250×10^3 /ml were significantly more susceptible to these strains than quarters with counts greater than this number ($p=0.01$). The three quarters that were already infected at the time of challenge had cell counts ranging from 600×10^3 to 8000×10^3 /ml and none became infected with the test strains.

Growth of S. dysgalactiae in milk. All four strains grew in raw milk and the small differences detected between them (table IV) did not correlate with their infectivity.

TABLE IV

Growth of four strains of *Streptococcus dysgalactiae* in raw milk

Strain no.	Colony forming units/ml of milk* after incubation at 37°C for		
	0 h	5 h	18 h
CE127	23	45×10^3	35×10^6
401/10	68	9.5×10^3	16.5×10^6
BC93	24	5.3×10^3	6.7×10^6
BE63	68	18.4×10^3	34×10^6

* Mean of three counts

DISCUSSION

This study shows clearly that there are differences in intramammary infectivity between strains of *S. dysgalactiae*. It also emphasises the susceptibility of the udder to disease because infection always followed the introduction of 12 or more cells of *S. dysgalactiae* strain CE127 into the gland after milking.

The extent to which differences in infectivity between strains of a pathogen influence rates of infection in commercial dairy herds is unclear. Dodd and Neave (1970) reported that herds with high rates of streptococcal udder disease also tended to have high rates due to other pathogens such as *Staphylococcus aureus*. This observation suggests that, in field conditions, the strain of micro-organism is less important than some other factor(s) influencing the incidence of udder disease.

A successful pathogen, having gained entrance to the udder, must be able to multiply in milk and withstand the flushing-out effect of milking. All four of the strains of *S. dysgalactiae* that we studied multiplied in raw milk but the small differences detected between strains in their rates of growth did not correlate well with the large differences in infectivity. We were able to show a significant relationship between the milk cell count at the time of infusion and susceptibility to infection, supporting other data which indicate that increased leucocyte numbers enhance the resistance of quarters to infection. (Schalm, Lasmanis and Carroll, 1964; Bramley, 1976). Frost (1975) suggested that attachment of bacteria to the epithelial surfaces of the mammary gland was an important initial stage in mastitis. Further study led Frost, Wanasinghe and Woolcock (1977) to propose that differences in infectivity might be related to the ability of strains to adhere to ductular epithelium. They included strains 401/10 and CE127 (termed 137 and 138, respectively) in their study of adhesion and found that strain CE127 gave the highest adherence index of any of the strains of *S. dysgalactiae* they tested. However, the adherence index was low when compared with that of *Staph. aureus* and most strains of *S. agalactiae*.

This work adds to the accumulating evidence that even when some strains penetrate the teat duct they rarely cause disease. Carroll *et al.* (1973) showed that strains of coliform bacteria that were sensitive to the bactericidal action of bovine serum were avirulent for the lactating udder. Garvie and Bramley (1979) found differences in infectivity between strains of *S. bovis*. In the present study the differences between the strains of *S. dysgalactiae* in the severity of the infection that they caused suggest that, after infusion, some strains were rapidly eliminated without causing a major inflammatory response. The elimination of serum-sensitive strains of *Escherichia coli* can also occur without measurable inflammation (Anderson, Burrows and Bramley, 1977). Although many antibacterial mechanisms of milk have been described (Reiter and Bramley, 1975) our knowledge of their importance in influencing rates of udder disease is limited.

SUMMARY

The intramammary pathogenicity of four strains of *Streptococcus dysgalactiae* was measured by infusion of small numbers of bacteria (8–16 colony-forming units) into the teat sinus after milking. Significant differences in the infectivity of strains were detected.

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