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ISOLATION AND SIGNIFICANCE OF ANAEROBIC BACTERIA ISOLATED FROM CASES OF BOVINE MASTITIS

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ABSTRACT

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The role of obligate anaerobic bacteria in the aetiology of mastitis of lactating dairy cows was investigated. Anaerobes were isolated from 12% of lactating mastitic cows, which were representative of 50% of the 10 dairy herds examined. *Bacteroides fragilis* was the most frequently isolated organism (50%), followed by *Peptococcus indolicus* (33%), *Eubacterium lentum* (33%), *E. aerofaciens* (17%), *Propionibacterium granulosum* (17%) and an anaerobic *Streptococcus* sp. (17%). These obligate anaerobes were always isolated together with organisms classically involved in mastitis. It was possible to induce overt clinical mastitis in healthy lactating udders within 24 hours by infection with single pure cultures of anaerobes via the teat canal. All *B. fragilis* strains were resistant to penicillin G and tetracycline. In addition, one strain was also resistant to ampicillin, cephalothin and amoxicillin. Anaerobic gram positive cocci and bacilli were sensitive to most antibiotics. These findings imply an important role for anaerobes in the aetiology of mastitis.

Résumé

ISOLEMENT ET SIGNIFICATION DE BACTÉRIES ANAEROBIES ISOLÉES A PARTIR DE CAS DE MAMMITE BOVINE

Le rôle des bactéries anaérobies dans l'étiologie de la mammite de vaches laitières en lactation a été investigué. Des anaérobes furent isolés de 12% *de vaches atteintes de mammite en lactation qui représentaient* 50% *de* 10 *troupeaux laitiers examinés.* Bacteroides fragilis *fut l'organisme le plus fréquemment isolé* (50%) *suivi par* Peptococcus indolicus (33%), Eubacterium lentum (33%), E. aerofaciens (17%), Propionibacterium granulosum (17%) *et un streptocoque anaérobie* (17%). *Ces anaérobes furent toujours isolés ensemble avec des microbes classiquement associés à la mammite. Il fut possible de provoquer une mammite clinique ouverte dans des pis sains en lactation endéans les* 24 *heures par infection avec des cultures pures uniques d'anaérobes par le canal du trayon. Toutes les souches de* B. fragilis *furent resistantes à l'penicilline G et la tetracycline. En plus, une seule souche de* B. fragilis *fut résistante à l'ampicilline, la cephalothine et l'amoxicilline. Les coques et les bacilles anaérobies gram positifs furent sensibles à la plupart des antibiotiques. Ces observations impliquent un rôle important pour les anaérobies dans l'étiologie de la mammite bovine.*

INTRODUCTION

Routine bacteriological dianosis of mastitis does not provide an index to the obligate anaerobic flora involved. Recent developments in techniques for the isolation of even the most fastidious genera of obligate anaerobic bacteria have provided new perspectives on their significance as infectious agents (Finegold, Rosenblatt, Sutter & Attebery, 1972; Anonymous, 1974; Greeff, Du Preez & Eksteen, 1980). Isolation frequencies of these organisms in pure culture or in association with others from a wide range of purulent human infections often exceed 60% (Nichols, Schumer Nyhus, Bartlett & Gorbach, 1976).

The role of anaerobes in bovine mastitis is obscure. *Peptococcus indolicus*, a gram positive anaerobic micrococcus, has been consistently isolated together with *Corynebacterium pyogenes* from cases of summer mastitis (Stuart, Buntain & Langridge, 1951; Sorensen 1974; Weber, Schliesser & Steiner, 1977) and from healthy cattle (Sorensen, 1976). Stuart *et al.* (1951) and Sorensen (1972) showed experimentally that *P. indolicus* in mixed culture with *C. pyogenes* could induce mastitis in healthy non-lactating heifers. Recently, Shinjo, Shimizu, Nagatomo, Nosaka, Hamana, Otsuka, Hataya, Sakanoshita & Shindo (1976) reported on the isolation of obligate anaerobic Peptococcaceae, *Bacteroides* spp. and *Fusobacterium necrophorum* from outbreaks of mastitis and from the healthy udders of non-lactating heifers.

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We report here our findings on the isolation of various anaerobic species from sporadic cases of mastitis in lactating cows and the experimental induction of mastitis by pure cultures of anaerobes.

MATERIALS AND METHODS

Collection of samples

Milk samples were obtained from 180 quarters representative of 75 lactating cows from 10 dairy herds. After disinfection of the teats, all milk samples were taken anaerobically via the teat canal from the gland cistern of the udder by means of a 150 mm × 1,0 mm catheter attached to a 10 mℓ disposable syringe. The syringe and catheter were preflushed with oxygen-free CO_2 to remove atmospheric oxygen from the system. Pus from an udder abscess was aspirated under similar conditions by means of a needle and, syringe. Samples were immediately injected into 100 × 30 mm vaccine type bottles, equipped with crimped butyl rubber sealers and containing only an atmosphere of oxygen-free CO_2. Samples were transported on ice, and analysis was initiated within 6 hours. Clinical, subclinical and healthy udders were classified according to Kastli (1967). In all positive cases of mastitis (Kastli, 1967) somatic cell counts exceeded 10^7 cells/mℓ as measured by Coulter counter.

Isolation procedures

Facultative aerobic and microaerophilic organisms were isolated by streaking a loopful of the sample on each of 2 blood agar plates. Both plates were incubated for 48 h at 37 °C, one aerobically and the other under microaerophilic conditions. Characterization of species was done according to Cowan & Steel (1974).

Anaerobes were isolated on supplemented brain heart infusion (BHI) agar* by streaking onto rotating rolltubes under an atmosphere of oxygen-free CO_2 (Holdeman, Cato & Moore, 1977). All media used for the propagation of obligate anaerobes were pre-reduced with oxygen-free CO_2 and anaerobically sterilized (PRAS) according to the methods of Holdeman *et al.* (1977). The rolltubes were sealed anaerobically and incubated for up to 7 days at 37 °C. Subculturing and isolation of single colonies were accomplished on rolltubes or PRAS blood agar plates, using standard anaerobic jar methods. The analysis of acids and alcohol products for generic identification was accomplished by gas chromatography. Ether extracts and methyl derivatives from culture products grown in both chopped meat carbohydrates (CMC)* medium and peptone yeast extract glucose (PYG)* broth were prepared according to Holdeman, *et al.* (1977). Analysis was performed on a Pye Unicam model 242 gaschromatograph equipped with 200 cm × 0,6 cm glass columns. The columns were packed with 5% free fatty acid phase Carbowax 20M on 80/100 mesh chromosorb-G and operated for thermal condictivity at 150 °C. Helium was used as carrier gas at a flow speed of 120 mℓ/min. Speciation of anaerobes was done according to Holdeman *et al.* (1977) and Sutter, Varno & Finegold (1975).

Antibiograms

The susceptibility of anaerobic organisms to attainable blood level concentrations of penicillin-G (10 units/mℓ), ampicillin (4 μg/mℓ) cephalothin (6 μg/mℓ), clindamycin (3,2 μg/mℓ), chlorampenicol (12 μg/mℓ) and erythromycin (3 μg/mℓ) was determined by a broth disc method according to Wilkins & Thiel (1973).

Induction of mastitis

Pure cultures of 11 strains of anaerobic bacteria isolated during the course of this study (Table 5) were used in an attempt to induce mastitis by udder infection in 5 lactating cows which had previously been found to be clinically and bacteriologically free from mastitis. The organisms were grown to a density of approximately 2–6 × 10^6 colony-forming units/mℓ in BHI broth. One millilitre of culture suspension was introduced anaerobically into the udder via the teat canal by means of a catheter and syringe. With some strains the dose was repeated once 24 h later in a different cow. Control quarters were injected with the same volume of BHI only. Quarters were monitored daily for 3 days for evidence of mastitis. Clinical and cyto-bacteriological criteria were used to establish the existence of mastitis according to the International Dairy Federation (Kastli, 1967).

RESULTS

Isolation of anaerobic bacteria from the udders of mastitic lactating cows

Fifty per cent of the herds examined harboured anaerobic bacteria (Table 1). Their incidence in lactating cows with subclinical mastitis was more than twice as high (16,6%) as in those with clinical mastitis (7,4%). In contrast to a 12% isolation rate of anaerobes from mastitic quarters no bacteria were isolated from healthy control quarters (Kastli, 1967). Anaerobic bacteria isolated from 6 lactating cows were consistently found in combination with organisms classically involved in bovine mastitis (Table 2). In the case of Cow 1 the bacteria isolated from the milk were identical with those from an abscess in the udder. The isolation frequency of the various anaerobic isolates is recorded in Table 3. *B. fragilis* was most frequently found in herds (60%) and in individual cows (50%). It was also consistently resistant to penicillin G and tetracycline (Table 4).

TABLE 1 Incidence of anaerobic bacteria in lactating cows

Experimental group	a	b	Anaerobes present	Incidence of anaerobes %
Herds...........	10	180	5	50
Healthy animals..	24	64	0	0
SC-mastitis......	24	82	4	16,6
C-mastitis........	27	34	2	7,4
Total............	75	180	6	8 (12)*

* % of anaerobes in subclinical (SC) and clinical (C) mastitis
a = Number of animals per group
b = Number of quarters examined

TABLE 2 Concurrence of anaerobic bacteria and recognized mastitogenic bacteria isolated from lactating mastitic udders

Animal	Sample	Anaerobic bacteria isolated	Aerobic bacteria isolated
Cow 1......	Milk	*E. aerofaciens*	*Staphylococcus aureus*
		E. lentum	*C. pyogenes*
		B. fragilis	*Streptococcus agalactiae*
		P. indolicus	
Cow 1......	Udder abscess	*E. aerofaciens*	*S. aureus*
		E. lentum	*S. agalactiae*
		B. fragilis	*C. pyogenes*
		P. indolicus	
Cow 2......	Milk	*P. indolicus*	*S. aureus*
Cow 3......	Milk	*B. fragilis*	*S. aureus*
Cow 4......	Milk	*E. lentum*	*S. aureus*
Cow 5......	Milk	*B. fragilis*	*S. agalactiae*
		P. granulosum	*S. aureus*
Cow 6......	Milk	*Streptococcus* spp.	*S. aureus*

TABLE 3 Isolation frequency of anaerobic bacteria

Organism	Frequency of isolation from herds	Frequency of isolation from mastitic cows
B. fragilis..............	3/5 (60%)	3/6 (50%)
P. indolicus............	2/5 (40%)	2/6 (33%)
E. lentum..............	2/5 (40%)	2/6 (33%)
E. aerofaciens..........	1/5 (20%)	1/6 (17%)
P. granulosum.........	1/5 (20%)	1/6 (17%)
Streptococcus spp.......	1/5 (20%)	1/6 (17%)

Induction of mastitis

From the data shown in Table 5 it is evident that most anaerobic strains are capable of inducing mastitis within 24 h in lactating udders and, in most cases, clinical symptoms were apparent within 24 h after infection. The cows were slaughtered 48 h after induction of mastitis. With the exception of *P. granulosum*, the relevant organism was subsequently isolated in pure culture from quarter milk.

* Difco Laboratories, Detroit, USA

TABLE 4 Susceptibility of some anaerobic bacterial isolates to antimicrobial agents

Anaerobes	Strain	Antimicrobial agents								
		PEN	TET	CHL	CLI	ERY	MET	AMP	CEP	AMX
B. fragilis....................	(133/3)	−	−	+	+	+	+	+	+	+
B. fragilis....................	(MN3/1)	−	−	+	+	+	±	+	+	+
B. fragilis....................	(M28/1)	−	−	+	+	+	+	−	−	−
P. indolicus..................	(133/4)	+	+	+	+	+	+	+	+	+
P. indolicus..................	(116/1)	+	+	+	+	+	+	+	+	+
E. lentum.....................	(133/2)	+	+	+	+	+	+	+	+	+
E. lentum.....................	(150/1)	+	+	+	+	+	+	+	+	+
E. aerofaciens................	(133/a1)	+	+	+	+	+	+	+	+	+
P. granulosum................	(M28/2)	+	+	+	+	−	±	+	+	+
Streptococcus spp............	(MN6/1)	+	+	+	+	+	±	+	+	+

PEN=Penicillin G, TET=Tetracycline, CHL=Chloramphenicol CLI=Clindamycin, ERI=Erythromycin, MET=Metronidazole, AMP=Ampicillin, CEP=Cephalot n, AMX=Amoxicillin, +=susceptible, −=resistant, ±=partial resistance

TABLE 5 Induction of mastitis in lactating cows, with various anaerobic bacteria

Anaerobes	Strain	Experiment 1	Experiment 2	Anaerobes isolated
B. fragilis........	(133/3)	+	+	+
B. fragilis........	(133/a3)	+	ND	+
B. fragilis........	(MN3/1)	+	ND	+
B. fragilis........	(M28/1)	+	ND	+
E. aerofaciens....	(133/1)	+	+	+
E. lentum........	(133/a2)	+	ND	+
E. lentum........	(150/1)	+	+	+
P. indolicus.......	(133/a4)	−	ND	+
P. indolicus.......	(116/1)	+	ND	+
P. granulosum....	(M28/2)	+	+	−
Streptococcus spp.	(MN6/1)	+	ND	+

In all positive cases, mastitis was evident within 24 h

Experiment 1=Initial experimental infection

Experiment 2=Repeat experimental infection in a different cow after 24 h

ND=Not done

Discussion

The pathogenicity of several pure cultures of anaerobic bacteria has been demonstrated by their ability to induce clinical mastitis in healthy lactating udders. In this respect *B. fragilis* may be of particular importance. *B. fragilis* is commonly isolated from purulent infections of man (Finegold *et al.*, 1972) and besides being the organism most frequently encountered in this experiment (Table 3), it showed the widest spectrum of resistance to various antimicrobial agents (Table 4). Its virulence has been studied in various animal models (Onderdonk, Weinstein, Sullivan, Bartlett & Gorbach, 1974; Weinstein, Onderdonk, Bartlett, Louie & Gorbach, 1975). The propensity of some strains to form abscesses is related to the presence of polisaccharide capsular material (Onderdonk, Kasper, Cisneros & Bartlett, 1977). Furthermore, Tally, Goldin, Jacobus & Gorbach (1977) found that pathogenic strains possess significantly higher amounts of superoxide dismutase which enable them to survive in highly oxygenated tissues of the lungs and blood until proper reduced conditions are established for their growth.

We consistently isolated anaerobes concurrently with aerobic bacteria which are known to be associated with bovine mastitis (Table 2). Mixed infections are often found in situations where anaerobes are isolated from human infections (Bartlett & Finegold 1972; Sabbaj, Sutter & Finegold, 1972). Secondary infection involving non-sporulating obligate anaerobic organisms often arise in the wake of predisposing factors related to a lowering of the oxydation-reduction (redox) potential of tissue to values favourable for growth and multiplication. Normal healthy tissue has a redox potential around +120 millivolt (mV). Most anaerobes, however, grow best at redox values below −150 mV (Holdeman *et al.*, 1977). Primary infection by aerobic, microaerophilic or facultative organisms may cause reduced blood supply due to tissue necrosis, abscess and gas formation, all creating low redox conditions (Finegold *et al.* 1972). Isolating an identical flora from the milk and an abscess from the same cow (Table 2) suggests at least a secondary role for the anaerobic isolates. Furthermore, in contrast to a significant isolation rate of anaerobes from mastitic cows, we were unable to demonstrate the presence of these bacteria in healthy udders. Although it is evident from this study that various pathogenic anaerobic bacteria may induce clinical mastitis under experimental conditions, their propensity to act as primary pathogens in nature is still unclear. Very few reports exist that deal with the isolation of obligate anaerobic bacteria from mastitic cows. This could possibly be explained by a lack, at least until recently, of suitable techniques for the growth of these exacting organisms. Also, recent developments in anaerobic technology is yet to be introduced in routine veterinary bacteriology. Gram positive anaerobic bacteria were generally sensitive to various antimicrobials.

On the other hand a number of reports have listed *B. fragilis* as the most resistant anaerobic isolate from clinical material (Sutter & Finegold, 1976; Willis, 1979). More than 40% of the clinical isolates of *B. fragilis* were found by Kislak (1972) and by Martin, Gardner & Washington (1972) to be resistant to tetracycline. Most *B. fragilis* isolates contain at least small amounts of β-lactamase, and the degree of resistance to benzylpenicillin was found to be proportional to the amount of β-lactamase produced (Percival & Cumberland, 1978). However, very few strains resistant to clindamycin, chloraphenicol, metronidazole or erythromycin have been reported (Martin *et al.* 1972; Dornbusch, Nord & Olsson, 1975; Jones & Fuchs, 1977). It thus seems prudent for antibiotic therapy of mastitis, where indicated, to be specifically directed at the anaerobic component.

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