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STUDIES ON MICRO ORGANISMS CAUSING BOVINE MASTITIS WITH PARTICULAR REFERENCE TO ANAEROBES

BY

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INTRODUCTION

Mastitis is considered to be one of the most important destructive disease producing monetary losses at extremely high figure for nations and also causes losses to dairy industry.

Every year, there was an appreciably higher incidence of obligatory anaerobic organisms being found in various countries (Hakogi et al., 1980; Tadkod et al., 1984 ad Sole et al. x1986). The aetiology of obligatory anaerobic mastitis is still very pooly understood and the discovery of various new techniques for identification and typing of anaerobic organisms invitably led to their isolation as the causative agents of mastitis (Vihan, 1990).

Investigation and identification of the specificity of anaerobic organisms isolated from bovine mastitis might help to answer completely the causative agents of bovine mastitis. *Clostridium perfringens* has been known to cause bovine mastitis either alone or in combination with other facultative anaerobic organisms and present in many cases of bovine mastitis (Shimizu et al., 1981 and Tadkod et al., 1984). On the other hand. Bacteroides species, Eubacterium, *Fusobacterium necrophorum* and Peptococcus species were investigated from mastitic cases (Shinjo et al., 1980 and Hillerton and Bramley, 1989).

This work was aimed to identify the prevalence rate of obligatory anaerobic as well as facultative anaerobic organisms causing bovine mastitis and to study in vitro the response of the prevalent obligatory anaerobic isolates to different chemotherapeutic agents.

MATERIAL AND METHODS

A total of 369 samples were collected from both cows and buffaloes showing clinical mastitis. milk samples was drawn by hand x aseptically as possible, the first four streams being discarded and transferred to the laboratory with minimum delay.

Milk sample was divided into two portions, the first portion was inoculated into cooked meat broth and incubated anaerobically for 24 hrs, then streaked onto each of reinforced clostridial blood agar plates containing 70/ug/ml neomycin sulphate and incubated anaerobically at 37°C for 48 x hrs and examined.

The second portion was inoculated directly onto the following media in duplicate: Nutrient agar, blood agar, MacConkey lactose bile salt agar, mannitol salt agar, Staphylococcus medium No. 110, crystal violet blood agar and Sabouraud's maltose agar plates and incubated aerobically at 37°C for 24-48 hrs except the last plates which were incubated at room temperature for 5-7 days.

Suspected colonies were examined for their appearance, haemolytic activity, morphological characters. Anaerobic micro-organisms were propagated into reinforced clostridial broth and then onto Lombard Dowell media for clostridia and neomycin sulphate sheep blood agar for other anaerobic organisms. Biochemical identification was done according to **Konemann et al.** (1983).

Determination of the type of *C. perfringens* toxins by dermonecrotic test was done using diagnostic *C. perfringens* antisera type A, B, C, D and F (Burroughs, Wellcome, Beckenham, London, England). The test was applied into albino guinea- pigs intradermally, the degree of the dermonecrotic reaction and its neutralization were done according to **Stern and Batty** (1975).

Isolates belonging to facultative anaerobic and aerobic microorganisms were identified x biochemically according to **Finegold and Martin** (1982) and **Konemann et al.** (1983).

Antibiotic sensitivity test on the prevalent obligatory anaerobic isolates were done using the disc diffusion method described by **Cruickshank et al.** (1975).

RESULTS AND DISCUSSION

Out of 269 mastitic cows, 94 cases (34.9%) revealed either single pure obligatory anaerobic organisms (19.0%) or concurrently mixed with facultative anaerobic bacteria (16.0%). These findings are nearly inagreement with that described by **Seno et al.** (1983) who showed that 42.0% of the cow's milk smaples has anaerobic organisms.

From Table (1), it was of interest to note that the most predominant strict anaerobic organisms found in mastitic cows were: *C. perfringens* (4.1%); *Bacteroides fragilis* (3.7%); *Eubacterium lentum* and *Fusobacterium necrophorum* (3.3%) and *Peptostreptococcus anaerobius* (3.0%). On the other hand, *Propinobacterium acnes* and *Peptococcus asaccharolyticus* were isolated in an incidence of 0.8% each. These

Table (1): Prevelance of obligatory anaerobes either as the sole causative agent of cow's mastitis or combined with other organisms.

Microorganisms.	No.	%
I. Anaerobic single organism:		
C.perfringens	11	4.1
Bacteriodes fragilis	10	3.7
Eubateriodes fragilis	9	3.3
Eusobacterium necrophorum	9	3.3
Peptostreptococcus anaerobius	8	3.0
Propinobacterium acnes	2	0.8
Peptococcus asaccharolyticus	2	0.8
Total	51	19.0
II. Mixed infection		
C. perfringens+ A. pyogenes	8	3.0
C. perfringens+ S.aureus	7	2.6
C. perfringens+ Str. pyogenes	7	2.6
Peptostreptococcus anaerobius+S.aureus	5	1.8
Fusobacterium necrophrum+ S.aureus	5	1.8
Bacteriodes fragilis+ Str. pyogenes	5	1.8
Propinobacterium acnes + E. coli	2	0.8
Eubacterium lentum +S. epidermidis	2	0.8
F. necrophorum + E. agglomerans	2	0.8
Total	43	16.0
Overall total	94	34.9%

findings agree with Shinjo et al. (1980) who isolated *F. necrophorum* and peptococcaceae from 50.0% and Bacteroides species from 33.0% of mastitic cows. Moreover, Madsen et al. (1980) examined milk samples from heifers suffering from mastitis and they found that the majority of samples revealed *Actinomyces pyogenes, Peptindolicus, F. necrophorum* and *Pept. asaccharolyticus.*

As shown in Table (1), the most prevalent mixed infection was : C. perfringens and A. pyogenes and *A. pyogenes* (3.0%); *C. perfringens* and *S. aureus* (2.6%); *C. perfringens* and *Str. pyogenes* (2.6%); *Peptostreptococcus anaerobius* and *S. aureus* (1.8%); *F. necrophorum* and *S. aureus* (1.8%) and *Bacteroides fragilis* and *Str. pyogenes* (1.8%). Nearly similar observations have been reported by Shinjo et al. (1980) and Hillerton and Bramley (1989).

In the present work, a total of 175 cows suffering from mastitis secured a single or mixed infection

Table (2) : Prevelance of facultative anaerobic organisms either alone or combined obtained from cows mastitis.

Organisms	No.	%
S. aureus	41	15.2
str. agalactiae	23	8.5
A. pyogenes	20	7.4
Str. pyogenes	19	3.7
E. coli	10	7.0
Str. bovis	9	3.3
Str. uberis	8	3.0
Entr. agglomerans	6	2.2
Klebsiella species	6	2.2
Cory. bovis	5	1.9
Proteus vulgaris	4	1.4
Proteus mirabilis	3	1.1
Cory. renale	3	1.1
S. haemolyticus	3	1.1
Str. faecalis	3	1.1
Str. pyogenes +E.coli	3	1.1
S. aureus +Citr. freundii	3	1.1
Citr. diversus +yeasts	2	0.9
A. pyogenes +S. aureus	2	0.9
A. pyogenes +Str. pyogenes	1	0.4
S. aureus +Enter. aerogenes	1	0.4
Total	175	65.1

Table (3) : Relative incidence of species of strict anaerobes alone or mixed with other microorganisms recovered from mastitc milk from buffaloes

Organisms	No.	%
I. As a pure infection:		
C. perfringens	4	4
Bacteriodes fragilis	3	3
F. necrophorum	2	2
Peptostreptococcus anaerobius	1	1
II. As mixed infection:		
C. perfringens + A. pyogenes	3	3
Actinomyces viscosus + S. aureus	1	1
C. perfringens Str. pyogenes	1	1
Eubacterium lentum + S. aureus	1	1
Peptostreptococcus anaerobius +yeast	1	1
C. perfringens +E. coli	2	2
	19	19%

of facultatiove anaerobic microorganisms as shown in Table (2). The

Table (4) : Relative incidence of species of facultative anaerobes either alone or mixed togther obtained from mastitic milk from buffaloes.

Organisms	No.	%
S. aureus	20	20
A. pyogenes	14	14
Str. agalactiae	10	10
E. coli	10	10
Kl. pneumoniae	5	5
Str. lactis	4	4
Enterobacter aerogenes	3	3
Morganella morganii	3	3
Proteus vulgaris	1	1
Yeast	1	1
Cory. bovis	1	1
Mixed infection		
S.aureus + Enter. aerogene	2	2
S. aureus+E.coli	2	2
Str. agalactiae+S. aureus	2	2
E.coli+S. haemolyticus	1	1
A. pyogenes+S. haemolyticus	1	1
A. pyogenes+Ps. aeruginosa	1	1
Total	81	81%

Table (5) : Typing of *C.perfringenns* strain recovered from mastitis cows and buffaloes.

Animal species	Total No of tested isolates	Non toxigenic isolates		Toxigenic isolates		Types of the toxigenic isolates			
						Type A		Type D	
		No.	%	No.	%	No.	%	No.	%
Cows Buffaloes	33 10	6 2	18.2 20.0	27 8	81.8 80.0	26 8	96.3 100.0	1 0	3.7 0.0
Total	43	8	18.6	35	81.4	34	97.1	1	2.9

Fig.(6): Antibiotic sensetivity of the prevalentes of obligatory anaerobic organisms.

Antimicrobial disc	C. perfringens (43)			Bacteroides fragilis (18)			F. necrophorus (18)			Peptostreptococcus anaerobuis		
	R	I	S	R	I	S	R	I	S	R	I	S
Ampicillin	33 (76.7)	2 (4.7)	8 (18.6)	3 (16.7)	7 (38.9)	8 (44.4)	18 (100.0)	0 (0.0)	0 (0.0)	10 (66.7)	3 (20.0)	2 (13.3)
Erythromycin	40 (93.0)	3 (7.0)	0 (0.0)	2 (11.1)	4 (22.2)	12 (66.7)	18 (100.0)	0 (0.0)	0 (0.0)	1 (6.7)	6 (40.0)	8 (53.3)
Chloramphinicol	0 (0.0)	0 (0.0)	43 (100.0)	18 (100.0)	0 (0.0)	0 (0.0)	18 (100.0)	0 (0.0)	0 (0.0)	15 (100.0)	0 (0.0)	0 (0.0)
Gentamycin	43 (100.0)	0 (0.0)	0 (0.0)	6 (33.3)	2 (11.1)	10 (55.6)	13 (72.2)	1 (5.6)	4 (22.2)	7 (46.7)	1 (6.7)	7 (46.7)
Tetracycline	0 (0.0)	0 (0.0)	43 (100.0)	0 (0.0)	0 (0.0)	18 (100.0)	16 (88.9)	2 (11.1)	0 (0.0)	10 (66.7)	0 (0.0)	5 (33.3)
Neomycin	0 (0.0)	10 (23.3)	33 (76.7)	0 (0.0)	0 (0.0)	18 (100.0)	0 (0.0)	0 (0.0)	18 (100.0)	0 (0.0)	0 (0.0)	15 (100.0)
Nitrofurantoin	35 (81.4)	8 (18.6)	0 (0.0)	18 (100.0)	0 (0.0)	0 (0.0)	18 (100.0)	0 (0.0)	0 (0.0)	7 (46.7)	0 (0.0)	8 (53.3)
Sulphaquinoxallin	39 (81.4)	4 (9.3)	0 (0.0)	18 (100.0)	0 (0.0)	0 (0.0)	18 (100.0)	0 (0.0)	0 (0.0)	9 (60.0)	1 (6.7)	5 (33.3)
Oxytetracycline	22 (51.2)	11 (25.5)	10 (23.3)	0 (0.0)	4 (22.2)	14 (77.8)	18 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	15 (100.0)
Pencillin-G	0. (0.0)	0. (0.0)	43 (100.0)	15 (83.3)	0 (0.0)	3 (16.7)	0 (0.0)	0 (0.0)	18 (100.0)	0 (0.0)	0 (0.0)	15 (100.0)
Trimethoprim sulfamethoxazole	38 (88.4)	4 (9.3)	1 (2.3)	12 (66.7)	0 (0.0)	6 (33.3)	18 (100.0)	0 (0.0)	0 (0.0)	7 (46.7)	1 (6.7)	7 (46.7)
Colistine sulphate	43 (100.0)	0 (0.0)	0 (0.0)	8 (44.4)	0 (0.0)	10 (55.6)	18 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (33.3)	10 (66.7)

R = Resistant I = Intermediate S = Sensetive * Figures between brackets indicates the percentages calculated.

most predominant pathogenic species were: *S. aureus* (15.2%), *Str. agralactiae* (8.5), *A. pyogenes* (7.4%); *Str. pyogenes*, (3.7%), *E.*

Coli (7.0%), *Str. bovis* (3.3% each), *Str. uberis* (3.0%), *E. agglomerans* and *Kl. pneumoniae* (2.2% each). Similar findings were also reported by **Sol et al. (1986)** and **Rhsn (1987).**

As shown in Table (3), a total of 19 buffaloes showing mastitis revealed obligatory anaerobic organisms either in pure form or concurrently mixed with other pathogens in an incidence of 19.0%. Single infection included *C. perfringens* (4.0%), *Bacteroides fragilis* (3.0%), *F. necrophorum* (2.0%) and *Peptostreptococcus anaerobius* (1.0%). On the other hand, 9 buffaloes revealed mastitis due to concurrently mixed infection. Three buffaloes revealed *C. perfringens* mixed with *A. pyogenes*, 2 cases *C. perfringens* in combination with *E. coli*. In addition to one case due to each of *Actinomyces viscosus + S.aureus, C. perfringens and Str. pyogenes, E. lentum + S. aureus* and *Pepto streptococcus anaerobius* and yeast in order of their frequencies.

Facultative pathogens together work with obligatory factors. The antagonistic bacteria include infectious agents that interfer with each other to form pathological changes in the udder tissues. The opportunistic and ubiquitous nature of obligatory anaerobic organisms has been well documented in the present work and their role in producing mastitis in buffaloes must be considered.

As shown in Table (4), the isolation rate of facultative anaerobic organisms from buffaloes with mastitis was 80.1%. This finding support the statement of **Jaffery and Rizvi (1975)** found that the incidence of mastitis in buffaloes was 83.9%.

In the present work, the most prevalent organisms obtained from mastitic buffaloes were *S. aureus* (20.0%), *A. pyogenes* (14.0%), *Str. agalactiae* and *E. coli* (10.0% each). Meanwhile, the least micro-organisms were *Kl. pneumoniae* (5.0%), *Str. lactis* (4.0%), *Enter. aerogenes, Morganella morganii* (3.0% each), *Pr. vulgaris, A. bovis* and yeast (1.0% each). Nearly similar findings have been described by **Jaffery and Rizvi (1975)** who reported the strains obtained from 740 milk sample from 194 buffaloes were as follows: 44.0% yielded *S. aureus*, 30.0% *Str. agalactiae*, 8.7% *Str. dysgalactiae*, 3.2% *Str. uberis*, 12.4% coliforms, 5.1% , *Coryn. pyogenes*, 3.2% *Ps. aeruginosa* and 0.4% yeasts. Also, **Narendra et al. (1982)** observed that staphylococci were the only incriminated agents in 78.0% of clinical cases of mastitis in cows and 61.0% in buffaloes, while streptococci were from 20.0% and 23.0% of cases, respectively.

As shown in Table (5), out of 33 strains of *C. perfringens* obtained from mastitic cows, 6 were non-toxigenic and the remaining 27 strains were toxigenic (81.8%). The organism was subsequently typed and sero-typed, identifying it as *C. perfringens* typed A in high incidence rate (96.3%) but only one strain (3.7%) was sero-typed as type D only. On examination of 10 strains obtained from mastitic buffaloes, 10.0% were non-toxigenic and the remaining 80.0% were toxigenic and all of them were belonging to serotype A only. These findings nealry coincide with the results obtained by **Nesbakken and Helmen (1975)** found an acute case of mastitis due to *C. perfringens* type A only. **Furthermore, Shimizu et al. (1981)** recorded that all strains *C. perfringens* isolated from mastitic Friesian cows were identified as type A by toxin test.

As shown in Table (6), all tested strains of *C. perfringens* were highly sensitive to chloramphenicol, tetracycline and penicillin-G (100.0% each). In contrast, they were completely resistant to gentamicin and colistin sulphate (100.0% each). Most of *C. perfringens* strains were sensitive to neomycin (76.7%) but highly resistant to trimethoprim-sulfamethoxazole (88.4%), oxytetracycline (51.2%), sulphaquinoxallin (90.7%). nitrofurantoin (81.4%) and erythromycin (93.0%). Nearly similar observations have also been reported by **Shimizu et al. (1981)** who found that all *C. perfringens* isoaltes were identified as type A by toxin tests and all were very sensitive to tetracycline and chloramphenicol but not to kanamycin or gentamicin.

As shown in Table (6) ***Bacteroides fragilis, F. necrophorum*** and ***Peptostreptococcus anaerobius*** were highly sensitive to neomycin sulphate (100.0% each). All of them were resistant to chloramphenicol (100.0% each). ***F. necrophorum*** and ***Peptostreptococcus anaerobius*** were highly resistant to ampicillin, tetracycline, sulphaquinoxallin and trimethoprim - sulfamethoxazole. ***Baceroides fragilis*** strains were highly resistant to nitrofurantoin, sulphaquinoxallin, penicillin-G, trimethoprim-sulfamethoxazole and colistin sulphate. Nearly similar findings were reported by **Larouche (1979)** who recorded the specific antibiotic for use in anaerobic mastitis therapy were; Neomycin, oxytetracycline and to a lesser extent penicillin-G; nitrofurantoin and colistin sulphate.

SUMMARY:

The incidence of strict anaerobes present alone or combined with other facultative anaerobes causing mastitis among cows was 34.9% *C. perfringens* (4.1%), ***B. fragilis*** (3.7%), ***E. lentum*** (3.3%), ***F. necrophorum*** (3.3%) and ***Pept. anaerobius*** (3.3%) secured the more frequently obligatory anaerobic organisms causing mastitis in cows either in pur-

form or combined with facultative anaerobes.

The prevalent facultative anaerobic organisms recovered from mastitic cows were: ***S. aureus*** (15.2%), ***Str. agalctiae*** (8.6%), ***Coryn. pyogenes*** (7.4%) ***Str. pyogenes*** (3.7%), ***E. coli*** (7.0%), and ***Str. bovis*** (3.3%).

C. perfringens, B. fragilis, F.necrophorum, Pept. anaerobius, E. lentum and ***A. viscosus*** were x present in mastitic buffaloes either in pure culture or mixed with other facultative anaerobes with an overall incidence reached 19.0%.

The most prevalent facultative anaerobic isolates obtained from mastitic buffaloes: ***S. aureus, A. pyogenes, Str. agalactiae, E. coli, Kl. pneumoniae*** and ***Str. lactis*** in order of their frequencies.

A total of 43 strains of ***C. perfringens*** were recovered from mastitic cows and buffaloes. Out of these, 81.4% were toxigenic and the remaining 16.8% were non-toxigenic. Among cows, strains, 96.3% were belonging to type A and 3.7% were of type D. Meanwhile, all toxigenic ***C. perfringens*** isolated from buffaloes were belonging to type A only.

In vitro sensitivity of the prevalent obligatory anaerobic isolates to various chemotherapeutic agents was discussed in details.

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