BACTERIAL ISOLATIONS FROM "CARA INCHADA"-LESIONS OF CATTLE[1]

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Foi conduzido um estudo para investigar o possível envolvimento de agentes bacterianos em lesões de "cara inchada" em bovinos (CI). *Corynebacterium pyogenes* foi isolado das lesões peridentárias de todos os 23 bezerros com CI necropsiados para fins de estudo bacteriológico, e somente 1 das 22 biópsias da gengiva feitas em bezerros negativos para a doença. *Bacteroides melaninogenicus* ocorreu, junto com *C. pyogenes,* em todas as lesões peridentárias, mas somente em 1 das biópsias dos animais sadios de controle. *Bacteroides bivius* foi isolado de 13 e *Fusobacterium nucleatum* de 9 dos 23 bezerros com CI, mas de nenhum dos animais de controle. *Actinomyces israelii* ocorreu mais freqüentemente nas biópsias da gengiva dos bezerros de controle do que nas lesões peridentárias dos animais com CI. As bactérias isoladas mostraram-se, *in vitro,* sempre sensíveis à penicilina G, à tetraciclina e à eritromicina. Repetidas inoculações intra-gengivais de *C. pyogenes,* realizadas em 5 bezerros e 2 cordeiros, e de *C. pyogenes* junto com *B. melaninogenicus,* não causaram lesões peridentárias progressivas; mas em 2 bezerros observaram-se, na região das inoculações, retração moderada e, em 2 outros, retração leve da gengiva. Estes resultados não indicam que as bactérias isoladas tenham papel primário na etiologia da "cara inchada" dos bovinos, mas parecem sugerir algum envolvimento destas bactérias na patogênese das lesões peridentárias.

TERMOS DE INDEXAÇÃO: Doença peridentária, "cara inchada", bovinos, isolamentos de bactérias, *Corynebacterium pyogenes, Bacteroides melaninogenicus, Bacteroides bivius, Fusobacterium nucleatum, Actinomyces israelii,* susceptibilidade antimicrobiana.

ABSTRACT.- Attempts were made to study a possible involvement of bacteria in the periodontal lesions of "Cara inchada" in cattle (CI). *Corynebacterium pyogenes* could be isolated from all of 23 CI-positive bovines and from the tissue samples of 1 of 22 CI-negative controls. *Bacteroides melaninogenicus* occurred together with *C. pyogenes* in all CI-lesions and only in 1 of the CI-negative tissue samples. *Bacteroides bivius* was cultivated from 13 and *Fusobacterium nucleatum* from 9 of the 23 CI-positive, but from none of the CI-negative animals. *Actinomyces israelii* occurred more frequently in the samples of the CI-negative than those of the CI-positive animals. The isolated bacteria were consistently susceptible *in vitro* to penicillin G, tetracycline and erythromycin. Repeated intragingival injections of *C. pyogenes* alone and together with *B. melaninogenicus* into 5 calves and 2 young sheep did not produce progressive periodontal lesions. However, 2 of the calves developed distinct and 2 slight retractions of the gingiva near the sites of injections. This did not indicate a primary role of the isolated bacteria in "Cara inchada" of cattle, but could suggest some bacterial involvement in the development of the periodontal lesions.

INDEX TERMS: Periodontal disease, Cara inchada, cattle, bacterial isolations, *Corynebacterium pyogenes, Bacteroides melaninogenicus, Bacteroides bivius, Fusobacterium nucleatum, Actinomyces israelii,* antimicrobial susceptibility.

INTRODUCTION

"Cara inchada" of cattle (CI) is a disease affecting young animals, causing loss of teeth, leading to malnutrition and sometimes to death (Fig. 1-3). It occurs mainly in central and northern Brazil and constitutes in some regions a serious economic problem. The incidence of CI varies greatly and may exceed 50% in some herds. The etiology of the disease is still unknown. A possible alimentary cause was suggested by Döbereiner et al. (1974). In subsequent studies 34 heifers, severely affected with CI, were transferred from their original grazing areas to native "cerrado" rangeland. Of these 18 exhibited a definite improvement of the disease and a better

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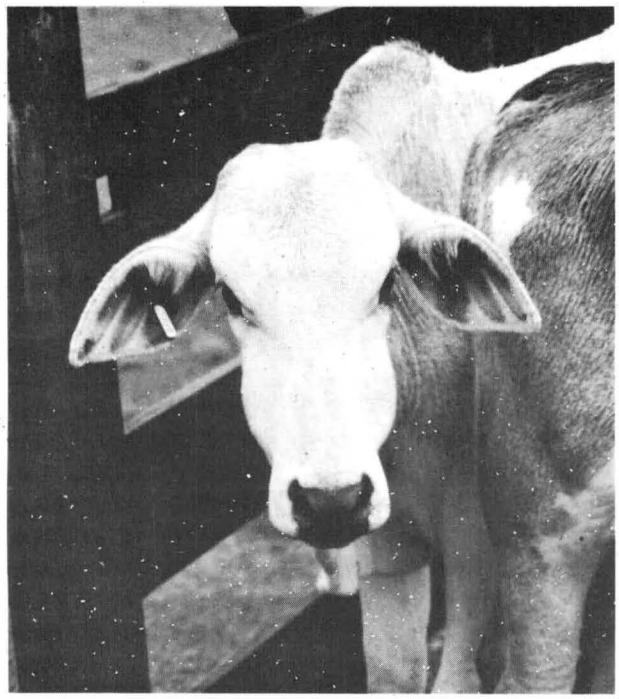

Fig. 1. *Cross-bred Zebu-calf, 5 months of age, with a pronounced "bulging" of the face ("Cara inchada").*

animals appeared to play a role in the etiology of CI (Döbereiner et al. 1976, Döbereiner 1980). Nunes et al. (1979) studied 7 CI-positive bovines, 7 to 13 months of age, and diagnosed "a generalized osteodystrophia fibrosa caused by a secondary nutritional hyperparathyroidism in association with osteoporosis and hypothyroidism".

In a comparative review on periodontitis in man and other animals Page and Schroeder (1980) suggested a possible bacterial role in the etiology of the disease. Cutress and Schroeder (1982) concluded from histopathological studies of periodontitis ("broken-mouth") in sheep that these lesions "result from an active progression of plaque-forming oral microorganisms (of unknown species) advancing along the root surface between the cementum and periodontal tissues". These considerations stimulated the present studies on the bacteriology of the periodontal lesions of CI.

MATERIALS AND METHODS

Selection of animals. In August 1982 the bacterological studies were started on 3 beef cattle herds with a high incidence (25-50%) of CI in Mato Grosso, Brazil. The herds consisted of approximately 4800, 850 a 2300 animals. After extensive clinical examinations (Döbereiner et al. 1974, 1975) 10 diseased bovines (3-14 months old) with typical periodontal lesions were sacrificed for these studies. In February and August 1983 this research continued on 5 additional beef cattle herds with an estimated incidence of CI ranging from 30 to 50%. The herds consisted of about 2000, 8000, 6000, 2200 and 2000 head. Again, on the basis of clinical findings 13 typically affected CI-animals were selected for post mortem examinations and bacteriological samplings. In addition 22 CI-free (3-5 months of age) control animals from

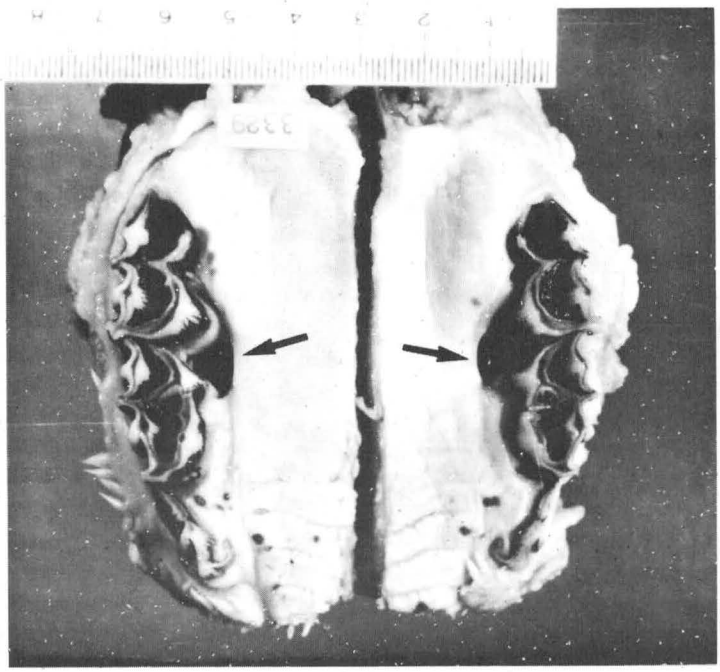

Fig. 2. *Bilateral periodontal lesions (Pd_{3-4} max.) of a 2 months-old calf (Döbereiner et al. 1974).*

Fig. 3. *Bilateral periodontal lesions with loss of teeth (Pd_3 max.) of a 5 months-old calf.*

nutritional condition within 6 months, the remaining 16 died (Döbereiner et al. 1975). Furthermore, 2 groups of 6 and 10 calves with periodontal lesions of CI were confined on CI-positive farms, and given a balanced ration for 5 to 6 months. The CI-lesions improved and all animals gained weight (Rosa et al. 1976). None of the plant species available to the grazing

2 experimental cattle herds without CI were subjected to gingival tissue biopsies for bacteriological studies. At the end of these studies another 2 heifers, 8 and 10 weeks of age, with very early periodontal lesions, near the second and third maxillary premolars (Pd_3 and Pd_4 respectively), were sacrificed for sampling.

Tissue samples. Following post mortem examinations of the 23 CI-positive animals and superficial cleaning of the lesions, the affected maxillary premolar and molar teeth were removed. From the depth of the CI-lesions respectively 2 tissue-samples were taken uni- or bilaterally, depending on wether 1 or both sides were involved. In addition, samples were collected aseptically from the retropharyngeal lymph nodes. The 22 CI-free bovines were subjected to gingival biopsies using a 23 cm-forceps ("Hohlmeisselzange nach Stille-Ruskin", Fa. Kretschmer, Giessen, West Germany). All tissue-samples were immediately deposited in 2 ml-plastic "Kryo"-ampules (Fa. Nunc, Wiesbaden, West Germany) each containing approximately 1 ml thioglycolate broth (Fa. Oxoid, Wesel, West Germany) enriched with hemin and vitamin K_1 (Vera & Power 1980) and submersed in fluid nitrogen. For transport and storage in the fluid nitrogen container (Fa. Union Carbide, Düsseldorf, West Germany) the "Kryo"-ampules had been arranged in rows of 5 and packs of 15 aluminium frames (Fa. Kretschmer, Giessen, West Germany).

Cultivation of bacteria. In the first series of isolation attempts sheep blood agar (Blobel & Schliesser 1980) was used aerobically, chocolate agar from defibrinated sheep blood (Vera & Power 1980) microaerobically (Blobel & Schliesser 1980) and anaerobically CDC anaerobe sheep blood agar, kanamycin-vancomycin blood agar and Bacteroides bile esculin agar (Vera & Power 1980). All isolation experiments were repeated 3 times with a reduced set of media, using sheep blood agar for both aerobic and microaerobic cultivations and CDC anaerobe sheep blood agar in a self-contained anaerobic system (Gas Pak, Fa. Becton and Dickinson, Cockeysville, Maryland, USA). The agar plates were examined after 1-2 days of aerobic, after 2-3 days of microaerobic and after 5-6 days of anaerobic incubation at 37°C.

Identification procedures. Generally, the appearance of isolated colonies, microscopic observations of the Gram-stained bacteria and their biochemical reactions (Blobel & Schliesser 1980, Lennette et al. 1980) served to arrive at the bacteriological diagnosis (Buchanan & Gibbons 1974). For recognition of *Corynebacterium pyogenes* the recommendations of Hartwigk (1980) were followed. The biochemical reactions of the anaerobic bacteria were determined with a commercially available microsystem (API 20 A, Fa. API-System S.A., Montalien, Vercieu, France) (Hansen & Stewart 1976). In addition, fluorescent antibodies (Fluorotec, Fa. General Diagnostics, Division of Warner-Lambert Company, Morris Plains, N.J., USA) served for microscopic detection of *Bacteroides fragilis* and *B. melaninogenicus.*

Antibiotic sensitivity tests. Susceptibility (Blobel & Schliesser 1980) of the isolated bacteria to antimicrobial substances was determined with "Sensi-discs" (Fa. Becton and Dickinson).

Experimental infections. For attempts to reproduce the periodontal lesions of CI 5 crossbred Holstein-Zebu-calves, 3 to 4 weeks of age, were injected into the interdental papilla of the lingual gingiva between the second and third maxillary premolars (Pd_3 and Pd_4 respectively), left and right. Each animal received a total of 10 to 12 injections over a period of 3 to 4 weeks. Each inoculum given on the left side contained approximately 10^4 colony-forming units (CU) *Corynebacterium pyogenes,* that on the right side 10^4 CU *C. pyogenes* and $3x10^4$ CU *Bacteroides melaninogenicus.* All inocula were prepared from lyophilized cultures. In addition, 2 sheep, about 3 months of age, received identical inocula at the same time-schedule into the lingual marginal gingiva of the anterior teeth, left and right. The oral cavities of all animals were examined clinically at times of injections and at weekly intervals for 1 month after the last injection.

RESULTS

The first series of cultivation revealed the frequent occurrence of *Corynebacterium pyogenes* in the samples from the CI-lesions. Their detection on chocolate agar, however, proved to be more difficult than on unheated sheep blood agar, where the hemolytic reactions of the bacteria could be easily recognized. Furthermore, chocolate agar did not yield any additional pathogenic bacteria from the CI-samples which could not be isolated on unheated blood agar. Therefore, chocolate agar was replaced by unheated sheep blood agar for microaerobic cultivation.

In the 3 subsequent isolation series, conducted respectively under aerobic, microaerobic and anaerobic conditions, highly reproducible results were obtained, since the samples were kept in liquid nitrogen and only briefly withdraw for culturing. From all CI-lesions *C. pyogenes* was isolated repeatedly and in great numbers (Fig. 1). Corynebacterial growth and hemolysin production were significantly enhanced under microaerobic incubation. The hemolytic reactions were more pronounced on rabbit and bovine than on sheep blood agar. From the gingival biopsy samples of the 22 CI-negative bovines the corynebacteria could be isolated only in 1 case and then in relatively small numbers. *Bacteroides melaninogenicus* occurred also in all 23 CI-positive and only in 1 of the CI-negative tissue samples together with *C. pyogenes.* Its colonies were easily recognizable by their black pigment formation after incubation for 5-7 days on the CDC anaerobe sheep blood agar. For detection of *B. melaninogenicus* the fluorescent-antibody test also proved to be useful. *Bacteroides bivius* could be isolated from 13 and *Fusobacterium nucleatum* from 9 of 23 CI-positive bovines. Neither of these 2 bacterial species were demonstrable in the samples of the 22 CI-negative animals. On the other hand, *Actinomyces israelii* occurred more frequently in samples from the CI-negative control animals than in those from the CI-positive bovines (Fig. 4). Samples from lymph nodes of the CI-positive bovines yielded no bacterial growth.

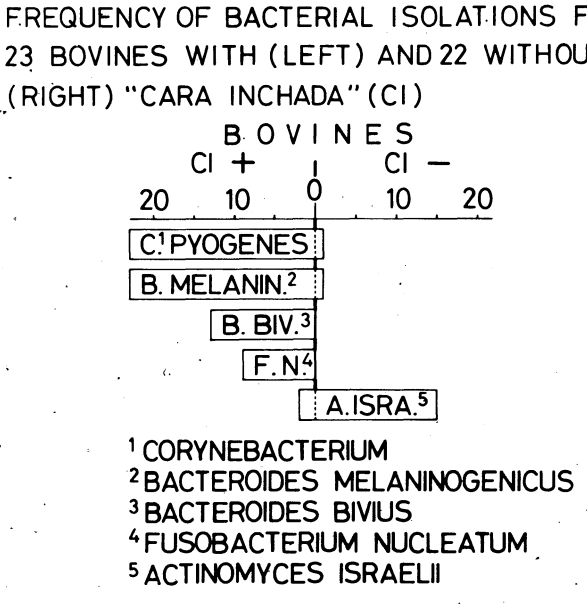

Fig. 4. *Occurrence of bacteria in the periodontal lesions of 23 "Cara inchada"(CI)-positive bovines and in the gingival biopsy samples of 22 CI-negative animals.*

Table 1. *Antimicrobial susceptibility of bacteria isolated from "Cara inchada"-lesions of cattle*

Bacterial species	Penicilin G	Erythro-mycin	Ampicillin/ Cloxacillin	Tetra-cyclin	Strepto-mycin	Genta-mycin	Vanco-mycin	Kana-mycin	Neomycin	Chlor-amphenicol
Corynebacterium pyogenes	+++[a]	+++	+++	+++	++	++	++	++	R	+++
Bacteroides melaninogenicus	+++	+++	+++	+++	R	R	+++	R	R	+++
Bacteroides bivius	+++	+++	+++	+++	R	R	+	R	R	+++
Fusobacterium nucleatum	+++	+++	++	+++	+	+	++	R	R	++
Actinomyces israelii	+++	+++	++	++	R	+	+++	++	R	++

[a] +++ Highly susceptible, ++ moderately susceptible, + weakly susceptible, R resistant.

From the 2 calves with very early periodontal lesions α-hemolytic streptococci were isolated from the superficial scrapings and numerous *B. melaninogenicus* and few *B. bivius* from the depth of the lesions.

Antimicrobial susceptibility tests revealed that penicillin G, tetracycline and erythromycin exhibited consistently high antibiotic activities against the bacteria isolated from CI-lesions. On the other hand, streptomycin, gentamycin, vancomycin, kanamycin and neomycin had little or no antibiotic effects (Table 1).

Attempts to reproduce the periodontal lessions in 5 calves and 2 young sheep were made by repeated (10-12) intragingival injections of *C. pyogenes* alone and *C. pyogenes* together with *B. melaninogenicus.* None of the 5 calves and 2 sheep developed the progressive disease. However, 2 of the calves showed distinct and 2 slight retractions of the gingiva near the sites of injections about 3 weeks after the begin of the trial. No significant difference was observed in the severity of the lesions caused by *C. pyogenes* alone or in combination with *B. melaninogenicus*. The 2 sheep developed neither retractions nor any other lesions.

DISCUSSION

In the present bacteriological studies *Corynebacterium pyogenes* and *Bacteroides melaninogenicus* could be isolated from the periodontal lesions of all 23 CI-positive bovines and only from 1 of 22 CI-negative controls. In addition, *Bacteroides bivius* and *Fusobacterium nucleatum* were demonstrated in some (respectively 13 and 9) of the 23 CI-positive, but in none of the 22 CI-negative animals. These findings proved to be highly reproducible in 3 subsequent isolation series, since the tissue samples were kept in fluid nitrogen throughout and only briefly withdrawn for cultivation.

The frequent occurrence of *C. pyogenes* and *B. melaninogenicus,* some times together with *B. bivius* and *F. nucleatum*, suggested a role of these bacteria in the development of the periodontal lesions in CI. This led to attempts to produce these lesions by repeated intragingival injections of *C. pyogenes* alone and together with *B. melaninogenicus* into 5 calves and 2 sheep. Although none of the exposed animals developed the progressive periodontal disease, 2 of the 5 calves showed distinct and 2 slight gingival retractions near the sites of injections. This might indicate the importance of predisposing factors. Such factors could include a weakness in the supporting structures of the teeth, an impaired antibacterial body defense and nutritional imbalances, still to be elucidated. In this connection studies are in progress to evaluate the relatively high incidence of mastitis in cows nursing calves affected with CI.

The findings of the present investigations did not indicate a primary bacterial etiology of CI, but could suggest an involvement of the isolated bacteria in the development of the periodontal lesions.

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