Faculty of Graduate Studies and Research

M.Sc.

DEVELOPMENT OF A SUCCESSFUL METHOD FOR QUANTIFYING VIABLE ORAL ANAEROBIC SPIROCHETES FROM PURE CULTURE AND PERIODONTAL POCKETS

by

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ABSTRACT

Spirochetes are markedly prevalent in periodontal disease but are not included as predominant cultivable organisms because of the inability to quantify them by viable count. A successful method was developed for enumerating viable oral spirochetes in pure culture as colony-forming units (CFU) in new oral spirochete (NOS) medium with 0.7% agarose and using small tissue culture flasks. Three species of oral spirochetes in log-phase growth in NOS broth were used for evaluation of the method. Reliable, consistent and reproducible viable counts of pure spirochete cultures were obtained.

This method was extended to enumerate viable oral spirochetes from periodontal pockets. The antibiotic rifampin (20 μg/ml) was found to be an excellent selective agent for such a count when added to NOS-agarose medium. Counts of cultivable oral spirochetes from 10 subgingival plaque samples ranged from 12.5% to 28.2% of the total cultivable anaerobic bacteria. In addition, by the use of this method thirteen new oral spirochetes were isolated.

The viable count technique was modified and employed to study the locomotion of spirochetes. Migration of oral spirochetes out of NOS-Bacto agar medium into NOS-agarose medium was observed and two locomotory phenotypes of oral spirochetes were detected.

RÉSUMÉ

Il est bien établi que les bactéries des spirochètes sont associées dans la periodontite. Bien que ces bactéries soient prédominantes au site d'infection, les méthodes de culture courantes qui permettent le décompte des cellules viables, ne sont pas representatives.

Une nouvelle méthode a été mise au point afin d'évaluer la viabilité des spirochètes en culture pure. Dans cette méthode la viabilité est évaluée par le décompte des colonies en milieu NOS (new oral spirochete) content 0.7% d'agarose. La culture est faite dans de petits flacons conçus pour la culture de tissus.

Trois espéces de spirochètes ont été cultivées en milieu NOS liquide, jusqu'en phase logarythmique, puis utilisées afin d'evaluer la méthode. Des décomptes fiables, consistants et reproductibles ont été obtenus avec ces cultures pures.

Subséquemment, la méthode fut adaptée afin de permettre le dénombrement des spirochètes viables provenant des cavités périodontales. La rifampin (20 μg/ml), ajoutée au milieu NOS-agarose, s'est révélée un excellent agent de selection pour le dénombrement. Un dénombrement des spirochètes viables, réalisé à partir de prélévements effectués au niveau de 10 plaques sous-gingivales, a démontré que les spirochètes représentaient de 12.5% à 28.2% des bactèries anaérobiques

totales pouvant être cultivées. De plus, l'utilisation de cette méthode a permis l'isdement de treize nouvelles espèces de spirochètes oraux

La technique d'évaluation de la viabilité a été modifiée et utilisée afin d'étudier la mobilité des spirochètes. La migration des spirochètes oraux à partir du milieu NOS-Bacto agar jusqu'au milieu NOS-agarose a ainsi pu être observée, et deux types de mouvements des spirochètes oraux ont pu être identifiés.

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TABLE OF CONTENTS

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

INTRODUCTION

Periodontal disease is the loss of tooth-supporting tissue that results from either the presence or the numerical overgrowth of certain bacterial types in dental plaque (Loesche et al, 1985; Moore, W. E. C., 1987). It is widely prevalent and is the major cause of tooth loss in adults.

Oral anaerobic spirochetes have been implicated as etiologic agents in periodontal disease because they are often the predominant bacterial types observed in subgingival plaque samples. Their increased number in diseased periodontal pockets is associated with the presence of inflammatory periodontal disease .

However, so far, oral spirochetes are still one of the least-studied and -understood microorganisms in the plaque flora because of the difficulty encountered in their isolation and quantification from dental plaque. Various efforts have been made to isolate and to characterize a number of spirochetes from subgingival plaque. The percentage isolated, however, has been relatively low compared with the numbers observed by phase-contrast and darkfield microscopy count (Cheng and Chan, 1983; Loesche, 1988; Simber and Burmeister, 1983). For this reason, most investigations of subgingival mixed microbiota never included oral anaerobic spirochetes as part of the " predominant cultivable organisms" whenever cultural methods were used (Loesche et al., 1992). At the

present time, we do not know how many different species of spirochete can be cultivated. Neither do we know whether certain types, such as the intermediate- sized and large spirochetes, can be cultivated. Nor do we know what proportion of the total spirochetes is composed of the known cultivable species.

This study was initiated to develop a reliable, consistent and reproducible method for quantifying viable oral anaerobic spirochetes from pure culture and subgingival plaques. In addition, better methods of cultivation of spirochetes will be valuable for the understanding of their taxonomy, physiology and pathogenicity in periodontal disease. It will also be valuable for the clinician to select appropriate antimicrobial treatment and monitor patients undergoing antimicrobial therapy when the biology of these etiologic agents of periodontal disease is better understood.

LITERATURE REVIEW

I. SPIROCHETES

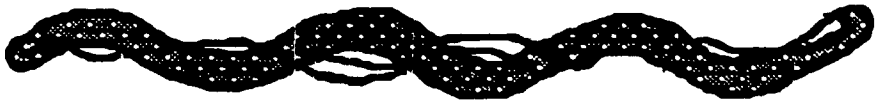
i. GENERAL CHARACTERISTICS OF SPIROCHETES

Spirochetes have a unique morphology. They are flexuous, thin, Gram-negative, chemoheterotrophic, helical-shaped organisms. Spirochetes are found widespread in nature and occur in a variety of habitats. Many are present as free-living forms in aquatic environments, such as the water and mud of ponds, lakes, rivers, and oceans. Other spirochetes are members of the normal microflora indigenous to eukaryotic hosts. Thus, various spirochetes inhabit the colon of mammals, the gingival crevice of humans, the body surface of protozoa, etc. A relative few spirochetes are pathogenic, cause disease such as syphilis, relapsing fever, Lyme disease, leptospirosis, and others. They also differ morphologically from other procaryotes by the presence of an axial fibril, known also as an endoflagellum, or axial filament, or periplasmic fibril. Spirochetes thus far examined invariably possess an outer sheath, which surrounds the cell and axial fibrils, a protoplasmic cylinder consisting of the cell wall, cell membrane, and the enclosed cytoplasm (Fig.1). Spirochetes divide by transverse binary fission (Hovind-Hougen, K. 1974). The structures and their specific functions are briefly reviewed.

Fig. 1. (A). Schematic diagram of a spirochete cell in longitudinal profile with one axial fibril from each pole.

(B). Cross-section of a spirochete with mucoid layer, protoplasmic cylinder, outer sheath and six axial fibrils.

A



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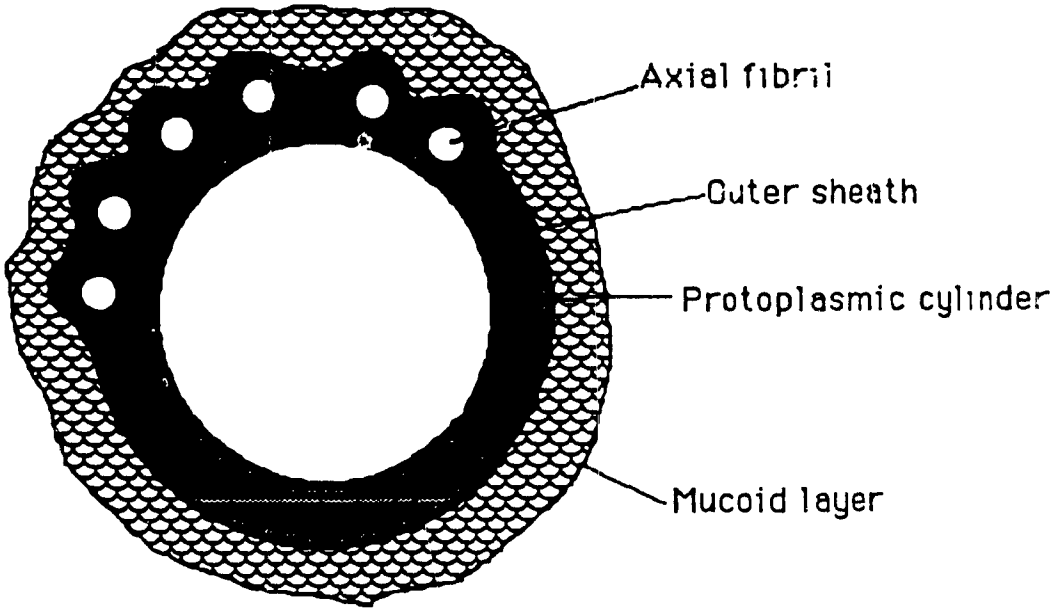

Axial fibrii
Outer sheath
Protoplasmic cylinder
Mucoid layer

(a). Outer Sheath (OS)

The outer sheath is seen as the most external layer of the cell. Electron microscopy has shown that the outer sheath of spirochetes is a three-layer membrane that completely surrounds both protoplasmic cylinder and periplasmic fibrils.

Since the OS is the most external layer of spirochetes, it may interact with the host defense mechanism. The OS of the leptospire is the primary target for the antibody-complement bactericidal reaction. The same is probably true for the pathogenic Borrelia and Treponema since they are also immobilized and/or killed by the antibody-complement system . The viability of spirochetes is dependent on an intact outer sheath. Damage of the OS mediated by the action of antibody and complement results in the loss of intracellular components and cell death (Anderson and Johnson, 1968).

Outer sheath preparations from a treponema and from a leptospire, were found to contain the following: protein, lipid, and carbohydrate. Several reports in the literature indicated the presence of a lipopoly-saccharide (LPS) layer in spirochetes, but the precise physical location of spirochete LPS was not known. A recent study by Gopalsami et al. (1993) suggested that the addition of OS of T.denticola increased the release of ^{45}Ca from bones. They showed that a LPS-like material present in the outer membrane of T. denticola may be responsible for bone

resorption in the *in vitro* system. The OS of spirochetes is known to contain LPS different in compositions from that of other bacteria. Thus it is possible that the LPS of spirochetes have biological activity quite different from other Gram-negative bacteria (Zeigler and Van Eseltine, 1975).

Some investigators studied the protein content of the OS of spirochetes. Haapasalo et al.(1992) reported that the 53-kDa OS protein of T. denticola bound to fibronectin, fibrinogen, and laminin Such bindings may help the association of T. denticola with periodontal tissue. Recently, Egli et al.(1993) purified this 53-kDa protein and found it is a porin with a very large channel diameter. This 53-kDa protein serves as a crude filtration device for spirochetes.

(b). Axial fibril

The filamentous structures wound around the helical protoplasmic cylinder of spirochetes have been designated by a variety of names, such as axial filaments (AF), periplasmic fibrils, endoflagella, and flagella. The number of AF per spirochetal cell ranges from two to hundreds (Canale-Parola, 1978).

Electron microscopy has shown that individual AF is similar to eubacterial flagella in fine structure. Major difference between AF and eubacterial flagella is that the latter are external structures and propel the cell by acting against the external environment; they cannot actively

bend inasmuch as they lack mechanisms for interconverting chemical and mechanical energy. In contrast, filamentous structures of spirochetes are able to bend actively, and serve to flex the cells around which they are wound, thus originating the bending motions, wave propagations, and other movement typical of spirochetes.

Purified AFs from various spirochetes were found to consist almost entirely of protein. Their amino acid composition resembled that of bacterial flagella. Acrylamide gel electrophoresis of dissociated AFs purified from diverse spirochetes indicated the presence of several protein species, whereas the filamentous portion of flagella usually consists of a single protein component, flagellin. The results of these studies suggest that the AFs of spirochetes are chemically more complex than the flagella of most bacteria (Holt, S. C. 1978).

It is interesting to notice that AFs could also be external on motile cells on two species of spirochetes: T. denticola ATCC 33520 and Treponema phagedenis Kazan 5. Ruby, J. D. et al.(1990) purified and characterized the AFs from these two species. Dark-field microscopy revealed that swimming cells of both species had structures extending out from the cell ends which resembled the structure of purified AFs. These external AFs were observed more frequently in old cultures. The results suggested that the AFs of spirochetes could become external and still display motion. Studies strongly suggest that AFs play a role in spirochete motility.

(c). Protoplasmic cylinder

The protoplasmic cylinder is directly beneath the outer sheath. It encloses the cytoplasmic and nuclear components and has been referred to as the peptidoglycan-cytoplasmic membrane complex (Johnson, R. C., 1977) because the presence of a peptidoglycan layer has been suggested and muramic acid has been detected in Leptospira and Borrelia cells (Ginger, C. D., 1963). The precise location of the peptidoglycan layer has been demonstrated by lysozyme treatment.

Peptidoglycan layer was found to maintain the helical shape of spirochetes. This has been demonstrated by investigators who found that the purified peptidoglycan layer of Spirochaeta species retained its coiled configuration (Joseph and Canale-parola, 1972), and the treatment of spirochete with agents that affect the integrity or synthesis of the peptidoglycan layer resulted in loss of the helical cell shape (Joseph et al, 1973).

ii. TAXONOMY

The classification of spirochetes is based on their extensive physiological diversity, great variation in etiology and morphology, and difference in DNA homologies.

i. Classical Taxonomy

In classical taxonomy, spirochetes belong to the order Spirochaetales which is divided into two families, Spirochaetaceae and Leptospiraceae . The family Spirochaetaceae comprises the genera Treponema, Spirochaeta, Borrelia, and Cristispira, and the family Leptospiraceae comprises the genera Leptospira and Leptonema. However, the information currently available is insufficient to define satisfactorily the genus Leptonema and it has been retained in the genus Leptospira (Johnson and Faine, 1984). Five genera are briefly reviewed below.

(a). Spirochaeta

Cells are free living in sewage and polluted water and in H_2S-containing mud. Five species of Spirochaeta have been described, four of them have been grown in pure culture and they are obligate and facultative anaerobes. Spirochaeta are 0.2-0.75 µm in diameter and 5-250 µm in length. G+C content of DNA ranges 51-65 mol%. They are not pathogenic (Canale-Parola, 1984).

(b). Cristispira

Cristispirae are the large spirochetes present in the crystalline style and in the digestive tract fluid of marine and fresh water mollusks

(Breznak, 1973). They are 0.5-3.0 µm in diameter and 30-150µm in length. Since no strains of Cristispira have been grown in pure culture, G + C content of DNA is unknown. More than 100 axial fibrils are intertwined with its periplasmic cylinder When removed from or disturbed in its habitat, the axial fibrils may become detached from the protoplasmic cylinder within a distended spirochetal envelope and form the so-called **crista;** spirochetal spheres may develop, motility becomes irregular and cells may lyse (Breznak, 1984). Cristispira is usually found in healthy, palatable univalve and bivalve molluscs obtained from well aerated beds. It is not pathogenic to its host (Berkeley, 1959)

(c). Treponema

Treponemes are found in the oral cavity, intestinal tract and genital regions of man and animals Most species stain poorly if at all with Gram's or Giemsa's stain. They are best observed with darkfield or phase contrast microscopy. Those grown in pure culture *in vitro* are obligate anaerobes or microaerophilic. Cells are 0 2-0.5µm in diameter and 5-20µm in length. G+C content of DNA ranges 25-54 mol%. Some species are pathogenic, which occur in the oral cavity, rumen, intestinal tract and genitals of man and animals. The pathogenic treponemes causing syphilis, yaws, pinta and nonvenereal endemic syphilis in man have not been cultivated (Smibert, 1984).

(d). Borrelia

Borreliae are anaerobic or microaerophilic. They are 0.2-0.5 μm in diameter and 3-20 μm in length. Their growth occurs at 20-37 C, optimum at 28-30 C. G+C content of DNA ranges 27-32 mol%.

Borrelia is zoonotic in nature; it has an animal reservoir and is able to infect humans via direct or indirect contact with the animal reservoir. Members of Borrelia cause two dissimilar diseases, relapsing fever and Lyme disease. .

Since Lyme disease became an important public health problem in the late 1970's, the etiologic agent, Borrelia burgdoferi, has been isolated from patients. This multisystemic and tick-borne spirochete is of world-wide distribution. Lyme disease is the most common arthropod-borne disease in the United States and Europe (Steere, 1989). This disease may be acute and self-limited or may progress to a chronic state with skin, joint, cardiac, and neurological involvement. The inflammatory response is the result of amplification by potent host-derived mediators released as a result of contact with the spirochete or its products, i.e., interleukin-1 (IL-1). Spirochete dissemination to distant locations involves adherence to and penetration across the endothelium and may be facilitated by host responses that increase vessel permeability. Many antibiotics have been used successfully in the treatment of the disease, including penicillin, amoxicillin, ceftriaxone, tetracycline, erythromycin,

and clarithromycin (Agger, et al., 1992, Dever, et al., 1992, Johnson, et al., 1990).

(e). Leptospira

Leptospires are slender spirochetes and may have one or both ends bent or hooked. They are 0.1-0.2 μm in diameter and 6-12μm in length. G+C content of DNA ranges 36-53 mol%. They are obligate aerobes and free living in surface of waters and in soil, or present in association with animals and humans. Some strains are parasitic and may be pathogenic for man and animals. For example, L. interrogans strains are the cause of leptospirosis. The primary mode of transmission of leptospirosis is by contact with the urine of animals directly or through contact with urine-contaminated soil and water. The reservoirs of L. interrogans are rodents and other domestic animals. In vitro, leptospires are sensitive to many antibiotics. Penicillin, streptomycin, and tetracycline are commonly used for the treatment of leptospirosis(Johnson and Faine, 1984).

ii. Attempts in Phylogenetic Classification

Since spirochetes are one of the few major bacterial groups whose natural phylogenetic relationships are evident at the level of gross phenotypic characteristics (Woese, 1987), attempts to produce a phylogenetic systematic for the spirochetes were carried out, e.g., using the rRNA oligonucleotide cataloging method (Paster, et al., 1984).

In 1991, Paster et al. showed that analysis of nearly complete 16S rRNA sequences (>95%) is a more powerful and accurate method for determining phylogenetic relationship among the spirochetes. The 16S rRNA sequences of Spirochaeta, Treponema, Borrelia, Leptospira, Lepotonema, and Serpula (includes the former species Treponema hyodysenteriae and Treponema innocens) were determined and the results indicated that the spirochetes formed a coherent taxon composed of six major clusters or groups. The first group, termed the treponemes, was divided into two subgroups. The first treponema subgroup consisted of T. pallidum, T. phagedenis, T. denticola, a thermophilic spirochete strain, and two species of Spirochaeta, S. zuelzerar and S. stenostrepta, with an average interspecies similarity of 89.9%. The second treponema subgroup contained T. bryantii, T. pectinovorum, T. saccharophilum, T. succinifaciens, and rumen strain CA, with an average interspecies similarity of 86.2%. The second spirochete group contained S. aurantia, S. bajacaliforniensis, S. litorelis, S. halophila, and S. isovalerica, with an average similarity of 87.4%. The spirochaeta group was related to the treponema group, with an average similarity of 81.9%. The third spirochete group contained borrelias, including B. burgdorferi, B. anserina, B. hermsii, and a rabbit tick strain. The borrelias formed a tight phylogenetic cluster, with average similarity of 97%. The borrelia group shared a common branch with the spirochaeta group and was closer to this group than to the treponemas. A single spirochete strain isolated from the shrew constituted the fourth

group (Shrew spirochete). The fifth group was composed of strains of Serpula (Treponema) hyohysenteriae and Serpula (Treponema) innocens. The two species of this group were closely related, with a similarity of greater than 99% and these strains do not branch with other Treponema species and were renamed as species of Serpula(Stanton, et al., 1991). Leptonema illini, Leptospira biflexa,and Leptospira interrogans formed the sixth and most deeply branching group. The average similarity within this group was 83.2%. The phylogenetic position of Cristispira was not determined because spirochetes of this genus could not be cultured in vitro. The phylogenetic structure determined from 16S rRNA cataloging is in approximate agreement with the classical taxonomy for spirochetes.

iii. CLASSIFICATION OF ORAL ANAEROBIC SPIROCHETES (OAS)

Oral anaerobic spirochetes are ubiquitous in those dental plaques associated with gingivitis and periodontitis. Listgarten and Socransky (1965) differentiated the OAS on the basis of their cellular diameter and number of axial filaments. The small OAS are 0.1 to 0.25 μm in diameter, possess 1 to 2 flagella at each end of the cell, and comprise the cultivable species T. denticola, T. socranskii, T. macrodentium, T. pectinovorum, and T. orale. The intermediate spirochetes are 0.2 to 0.5 μm in diameter, possess from 3 to 20 flagella at each end, and are represented by T. vincentii. The large spirochetes are 0.5 μm or more in diameter, contain

12 to more than 20 flagella originating at each end (Loesche and Laughon, 1981). There is presently no cultivable representative of large oral spirochetes. The four species of oral spirochetes, T. denticola, T. socranskii, T. vincentii, and T. pectinovorum, are well characterized. Table 1 is the summary of characteristics of these four species of spirochetes. OAS strains of T. denticola have been divided into two serovarieties (Cheng, et al., 1985).

iv. MOTILITY OF SPIROCHETES

Noguchi in 1912 observed the ability of spirochetes to migrate through agar away from the site of inoculation, forming hazes of growth. Spirochetes have a unique motility system that is characterized by flagella filaments contained within the outer membrane sheath. Direct evidence using video microscopy has indicated that the spirochete swims due to the rotation of periplasmic flagella between the outer membrane sheath and cell cylinder (Berg, 1976). A proton motive force drives the axial filament to rotate (Goulbourne and Greenberg,1983).

AFs of spirochetes consist of a filamentous portion or shaft, a hooked region near the insertion into the cell body, and an insertion apparatus that includes a number of disks. The AFs are wound around the helical protoplasmic cylinder of spirochetes and are attached subterminally at each cell end and extend toward the opposite pole of the cell. The association of the disks with the cytoplasmic membrane could

Table 1. Taxonomic characteristics of cultivable oral spirochetes*

Characteristics	T. denticola	T. vincentii	T. socranskii	T. pectinovorum
Cell length (μm)	6-16	5-16	6-15	7-15
Cell diameter (μm)	0.20±0.02 0.22±0.03	0.2-0.25	0.16-0.18	0.28-0.30
Carbohydrate fermentation	-	-	glucose	pectin
Amino acids fermentation	+	+	ND	ND
H_2S production	+	+	+	-
Final pH of glucose broth	6.7	6.9-7.2	5.1-5.9	5.3-5.9
Indole	+	+	-	-
No. of axial fibrils	2-4-2, 5-10-5	5-10-5	1-2-1	2-4-2
G+C content of DNA (mol %)	37-38	44	51	39

*Summary of information from Krieg and Holt, 1984; Smibert and Burmeister, 1983 and Smibert, et al., 1984.

have functional significance for the axial fibril just as in the case of the eubacteria flagellum. The disks may insert into the membrane and be able to rotate freely, permitting the shaft to rotate as well, and thus function in cell movement. Energy required for movement could be generated in the cytoplasmic membrane portion of the protoplasmic cylinder and could then be transmitted to the shaft through the insertion disks.

Spirochete chemotaxis has been most thoroughly studied in S. aurantia which has served as one of the model organisms for studying and understanding spirochete motility and chemotaxis. S. aurantia exhibits three distinct behaviours, runs of smooth swimming, reversals and flexing (Fosnaugh and Greenberg, 1988). These organisms show adaptation to chemical stimuli in a manner similar to that of other bacteria (Macnab, 1987). The difference of chemotaxis between the prototypical bacterium, E. coli, and S. aurantia is that chemotaxis in S. aurantia involves transmembrane ion gradients or potential fluctuations in signalling to the AF. One interesting finding by Charon et al.(1991) was that wild-type cells had bent-shaped ends, but the ends of mutant cells which lacked AF were straight. There is a complex interaction between the AF and the cell cylinder that results in bending of the cell ends. Their results indicated that the AF cause the end of the cell to bend and also to gyrate.

The motility of spirochetes enables them to translocate in certain environments, for example, highly viscous gel-like environments, that

immobilize most other bacteria including E. coli and S. typhimurium (Kaiser and Doetsch, 1975; Canale-Parola, 1978).It has been reported that OAS can migrate (locomote) through a viscous medium (Klitorinos et al., 1993).

However, so far, spirochete motility in general is still poorly understood.

II. PERIODONTAL DISEASE

i. General Characteristics and Prevalence of Periodontal disease

Periodontal diseases, more commonly known as gum diseases, are an inflammatory response of gingiva and connective tissue to the bacteria accumulations on the teeth and characterized by destruction of the supporting tissues of the teeth.

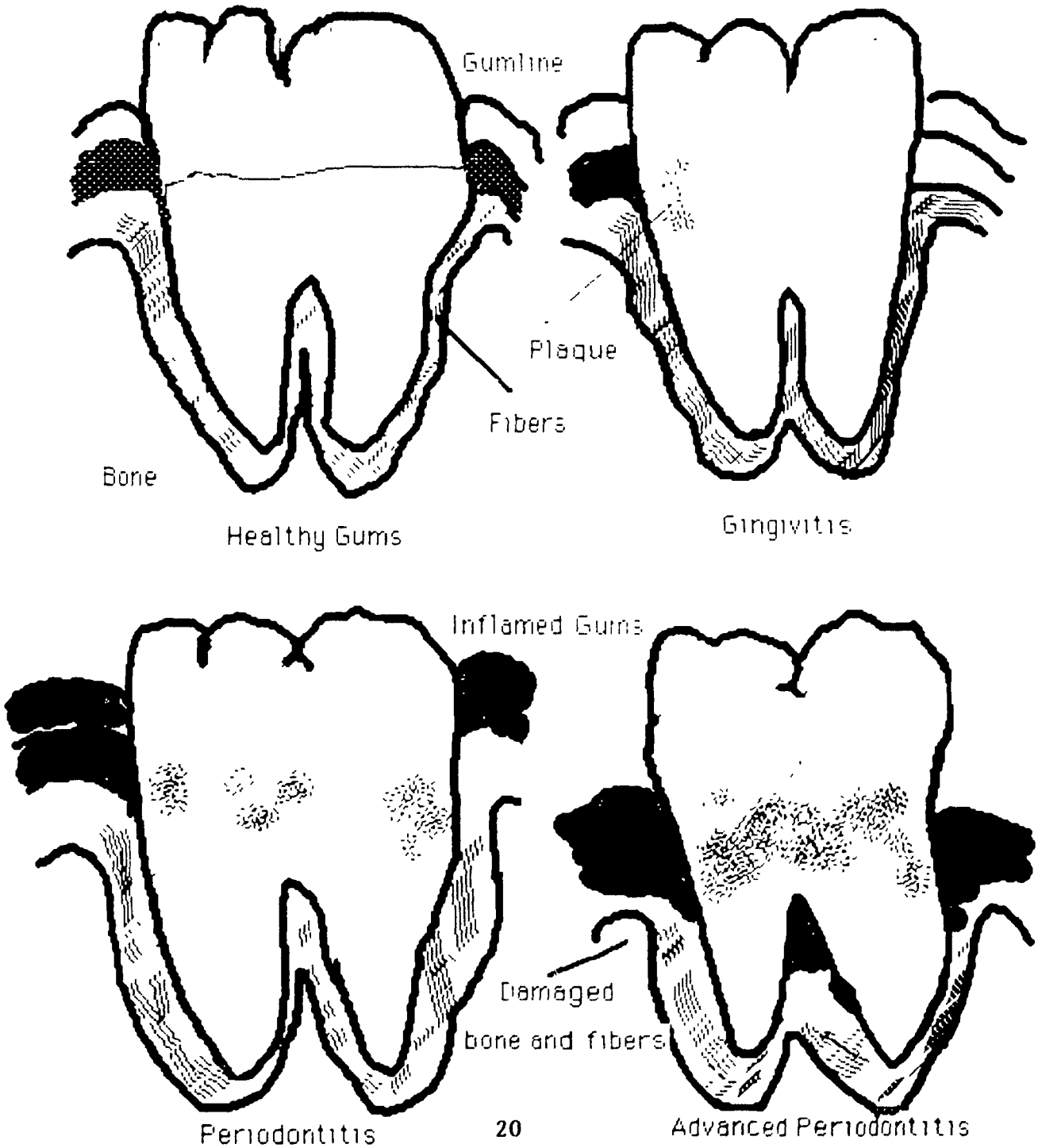
Inflammatory periodontal diseases are divided into two general clinical groupings: gingivitis and periodontitis.

The early stage of gum disease is called gingivitis, a minor inflammation of the gums. Gums become red and may bleed during brushing or flossing. There is no clinical bone loss or mobility. This type of disease includes acute necrotizing gingivitis, HIV-associated gingivitis, etc.

At the stage of periodontitis, bacterial plaque moves down the teeth to the roots, damaging the bone and fibers that support the teeth in place. Periodontitis is typified by the loss of attachment and bone. The loss of attachment creates a space or pocket between the tooth and gingival epithelium which can reach a depth of > 10mm. Many types of bacteria have been found in the periodontal pocket. Gums generally feel painful and the tooth may shift or loosen. There are different types of periodontitis, including adult periodontitis, early-onset periodontitis, necrotizing ulcerative periodontitis, etc.. Present activity may be indicated by bleeding on gentle probing from the base of the pocket, pus formation or other signs of active inflammation (Fig.2) (Loesche,1993; Clerehugh, 1991).

Periodontal disease can begin in childhood and progress through adolescence into adult life, eventually resulting in tooth loss. Severe periodontal disease is highly prevalent in older populations. For example, the Piedmont 65+ Dental Study found that 16% of white subjects and 46% of black subjects had severe periodontal disease (Beck et al., 1990). Locker and Leake(1992) reported that in Ontario, Canada, 22% of older adult population were classified as having severe disease. This was defined as four or more sites with 5mm or more of attachment loss, with at least one of those sites having a pocket of 4 mm or more. Risk markers of periodontal disease include aging, smoking, dental hygiene, etc..

Fig 2 THE STAGES OF PERIODONTAL DISEASE



ii. Microbes as Etiological Agents in Periodontal Diseases

It has been known since the 19th century that periodontal disease is due to clinical responses to the overgrowth of bacteria on the dento-gingival surfaces (Miller, 1890). This concept was reintroduced in the 1950s when it was suggested that plaque control was essential in the treatment of periodontal patients (Loesche, 1976). The so-called nonspecific plaque hypothesis was introduced to describe the belief that all bacterial types are equally capable of initiaing periodontal disease. Later studies suggested that all organisms in plaque may not be equally capable of causing destructive periodontal disease. The relative and absolute increase in the numbers of only certain members of the subgingival plaque was held to be associated with periodontitis. The inflammatory response in the gingival tissue to these specific organisms results in tissue loss. This belief was embodied in the specific plaque hypothesis in destructive periodontal disease (Loesche, 1992).

The healthy gingival crevice consists of relatively few microbes and is predominanted by Gram-positive microorganisms. The proportion of Gram-negative, anaerobic bacteria has been shown to increase in human subgingival plaque as the severity of periodontal disease increases. These bacteria include Porphyromonas gingivalis, Bacteroides intermedius, Actinobacillus actinomycetemcomitans, Eikenella corrodens, Fusobacterium nucleatum, Peptostreptococcus mictos, etc. (Loesche and Syed, 1978). Periodontal pocket between the tooth and gingival

epithelium serves as a protected environment for the growth of different types of bacteria. Over 300 bacterial species have been cultured from the periodontal pockets of different individuals, and as many as 30 to 50 bacterial species have been recovered from a single diseased site (Moore, et al.,1983; Wolff, et al., 1988). Although we do not know which bacterium is responsible for which type of periodontal disease, mutiple investigations have indicated that A. actinomycetemcomitans may be an etiologic pathogen in localized juvenile periodontitis (Slots, 1979; Zambon, 1985). P. gingivalis was suggested to be important in adult periodontitis (Tanner et al, 1979). Socransky and Haffajee (1992) reported that the level of P. gingivalis was elevated in lesions of periodontitis, but not in healthy or gingivitis subjects. They also suggested that" black-pigmented Bacteroides" species produced ß-hemolysis on blood agar plates and the mean counts of this species at sites where ß-hemolysis species were not detected were much lower compared to the sites where ß-hemolytic species were detected. They presumed that maybe ß-hemolysis species cause damage to red blood cells or other host tissue cells and foster the colonization of the suspected periodontal pathogen leading to tissue damage.

The approach to determine whether an organism is virulent necessitates the demonstration that it must invade the tissue or its products or components must penetrate the tissue and be able to cause a response in the host. The periodontopathic organisms A. actinomycetemcomitans, P. gingivalis, and spirochete species T.

denticola have in common the ability to penetrate the gingival epithelium and thus to present their virulence factors. Mikx, et al. (1990) were able to induce necrotizing ulcerative gingititis (NUG) in beagle dogs by supplying plaque debris from a NUG carrier to experimental dogs. Coccoidal, rod-shaped bacteria and spirochetes (intermediate-size) were observed intercellularly and intracellularly in sulcular gingival epithelium. The important finding was that the detection of these microorganisms in sulcular epithelium preceded clinical evidence of NUG. Their results suggested that invasion by intermediate spirochetes and other bacteria plays a role in initiating ulcerative gingivitis. In the literature, periodontal diseases have also been induced in the monkey (Dreizen and levy, 1977; Smith, et al., 1993) , the gnotobiotic rat (Socransky and Haffajee, 1990), mouse , rat, and hamster (Burckhardt, et al., 1981; Chen, et al., 1987). Experimental periodontitis has proven to be a useful model for studying and understanding naturally occurring periodontal diseases. In the monkey model, Smith, et al.(1993) were able to provide evidence that changes in inflammatory mediators regulate the magnitude of the tissue-destructive response in progressive periodontitis. Now we know that the periodotopathic organisms can produce components or products, including endotoxin, immunogenic antigens, enzymes that degrade tissue components, and a variety of noxious low-molecular-weight by-products such as H_2S, NH_4^+, volatile fatty acids, and peptides, that provoke an inflammatory response (Meikle, et al., 1986; Socransky and Haffajee, 1992). Fine et al.(1992) compared the endotoxin levels and percentage of Gram-negative bacteria in periodontally healthy and diseased sites.

They found that at a sample dilution of 1x 10^{-4}, the majority of healthy sites yielded a negative endotoxin test while the majority of periodontitis sites yielded a positive test. They suggested that determination of site-specific endotoxin levels may have potential application in monitoring the results of periodontal therapies. A. actinomycetemcomitans was found to produce leukotoxin (LT) that may be responsible for inducing localized juvenile periodontitis because there are significantly higher proportions of LT^+ isolates in diseased patients than in healthy individuals (Zambon, J. J., 1985). LT could recruit and activate neutrophils and monocytes and could cause the release of the neutrophils' array of tissue-destroying proteolytic enzymes.

At present, there is no direct evidence that any plaque microorganism is the etiologic agent of gingivitis or periodontitis. Since the numbers of bacteria inhabiting a single pocket site are extremely large, it is possible that human adult periodontal disease may involve a progression of certain synergistic bacterial species. Simonson, et al. (1992) showed that occurrence of T. denticola requires the presence of P. gingivalis and P. gingivalis secretes a substance that stimulates the growth of T. denticola.

The foregoing discussion indicates that microbes in the periodontal pocket are putative periodontal pathogens . However, many other factors may also be involved, e.g., susceptibility of the host. For most people, periodontal health and teeth are well maintained by the body defenses

despite the high bacteria load on the teeth. Based on current research, if one asks " What causes periodontal disease?", a likely answer might be "specific bacteria of the right clonal type with the essential genetic elements in sufficient numbers for that host with appropriate additional species in the right enviroment" (Socransky and Haffajee, 1992).

iii. ROLE OF SPIROCHETES IN PERIODONTAL DISEASE

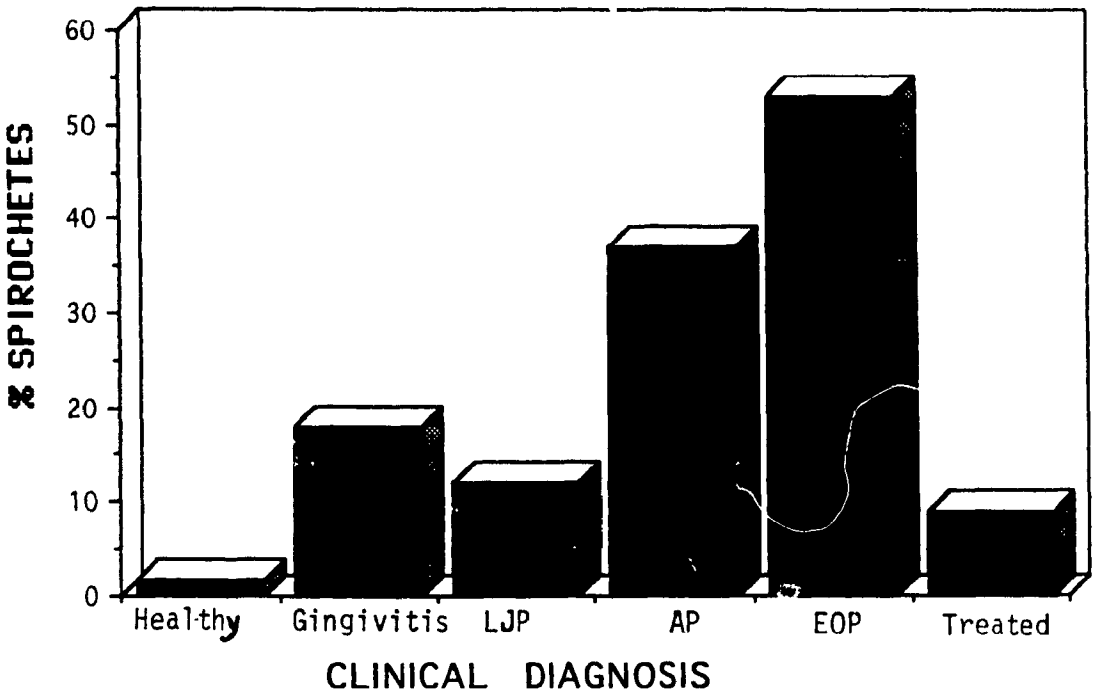
Oral anaerobic spirochetes are often the dominant bacterial type observed in subgingival plaque removed from diseased periodontal sites. In the mid-1960s, it was suggested that a specific spirochete might be the cause of acute necrotizing ulcerative gingivitis on the basis of observing large numbers of this organism in lesions of this disease (Listgarten, 1965), but the investigators have been handicapped by difficulty to grow this spirochete. With the development of darkfield microscopy and other techniques, further studies made an impressive argument that spirochetes are pathogenic in periodontal disease (Listgarten, 1976).

Oral anaerobic spirochetes are usually not detected, or are present in low numbers and proportions in plaque removed from healthy gingivae (Moore et al., 1984). Their number increased greatly within the diseased periodontal pocket and reached their highest values in plaque removed from sites with periodontitis (Fig. 3) (Loesche, 1988). Their increased number in diseased pockets(range from19 to 57%) has been found to be associated with the severity and/or activity of inflammatory

periodontal diseases. Moore et al. (1985) reported that certain specific spirochetes were more closely associated with severe periodontitis than they were with healthy sites or gingivitis. They identified Treponema denticola as one of the spirochete species more frequently isolated from severely diseased sites in young adults. By using an enzyme-linked immunosorbent assay, Simonson et al. (1988) were able to show a positive correlation between an increase in the OAS species T. denticola and increasing severity of periodontal disease. This quantitative study showed that the ratio of T. denticola content per milligram of plaque in the deep pocket groups to that of the healthy control or moderate disease was about 2:1.

Many investigators have tried to elucidate the virulence mechanisms of OAS. Spirochetes were found to have the ability to invade the sulcular epithelium and initiate ulcerative gingivitis (Mikx, et al., 1990). The invading spirochetes can directly present their endotoxins, immunologically active compounds, and cytotoxic enzymes and molecules to the host inflammatory cells. Another finding is that invading spirochetes in the connective tissue can inhibit polymorphonuclear leukocyte (PMNL) function. Thus the PMNLs were able to phagocytize these spirochetes, but were unable to degrade them. This was associated with a failure of the lysosomal granules to degranulate and suggested that the spirochetes may limit the fusion of the lysosomes to the phagosomes (Boehringer, et al., 1986; Isogai, et al., 1993) . The immunological studies also showed that antispirochetal antibodies did not help in associating spirochetes with disease. Steinberg (1970) reported high

Fig. 3. Association between spirochetes and various periodontal diseases. LJP is localized juvenile periodontitis; AP is adult periodontitis; EOP is early-onset periodontitis; treated represents the spirochete levels found in subgingival plaque of patients who have received either mechchanical debridement and/or chemical antimicrobial treatment. Height of bar reflects the average percentage of spirochetes found in plaque taken from individuals with the cited clinical diagnosis.


% SPIROCHETES
60
50
40
30
20
10
0
Healthy
Gingivitis
LJP
AP
EOP
Treated
CLINICAL DIAGNOSIS

titers of circulating antibodies to spirochetes in individuals with moderate periodontitis but not in individuals with advanced disease. Tew et al. (1985) reported tnat those individuals with the most severe periodontal involvement exhibited a lower serological response to T. denticola and T. socranskii compared with healthy controls and with individuals with less periodontal morbidity. These findings demonstrated that spirochetes may cause some type of immune suppression which enables them to escape the normal immunologic surveillance of the host.

The small T. denticola is one of the few cultivable spirochetes and is more frequently isolated. T. denticola possesses a wide variety of proteolytic enzymes and produces many of the cytotoxic products which can elicit an inflammatiry response (Loesche, 1993). It can produce phospholipase C(Siboo, et al., 1989) and a trypsin-like enzyme (Ohta, et al., 1986). T. denticola also can inhibit fibroblast proliferation by containing a cell-bound factor of approximately 50,000 molecular weight (Isogai, et al., 1993).

Recently, pathogen-related oral spirochetes (PROS) were found in greater numbers in both supragingival and subgingival plaque of disease sites. PROS are unknown spirochetes that are similar in size and shape to other oral spirochetes and they are uncultivable at present. Riviere, et al.(1991) found that PROS in dental plaque from patients with ulcerative gingivitis or chronic periodontitis have antigens that are thought to be unique to patnogenic treponemas. Monoclonal antibodies against pathogen-specific determinants on T. pallidum subspecies pallidum

were used. They found that spirochetes with a pathogen-specific epitope were found in plaque samples from ulcerative gingivitis and periodontitis, but not in any of the healthy controls. This close antigenic relation suggests that T. pallidum or a closely related organism may be involved in the pathogenesis of periodontal diseases. This organism has not been observed before probably because it is one of the uncultivable spirochetes that exist in dental plaque. .

OAS can be used as a diagnostic and prognostic marker in periodontal disease. Loesche et al.(1990) indicated that T. denticola and P. gingivalis possess a trypsin-like enzyme which can be detected by the hydrolysis of benzoyl-DL-arginine-2-naphthylamide (BANA) in subgingival plaque. This BANA hydrolytic enzyme was statistically related to the plaque levels and proportions of spirochetes and to probing depth. By using monoclonal antibodies, Simonson, et al.(1992) reported that T. denticola is a specific spirochete marker that quantitatively indicates periodontal disease recurrence. Their results suggest that T. denticola levels of successfully treated sites decreased, while non-responding sites have levels of these microbial markers which are equal to or greater than the pre-treatment levels.

Overall, because of the difficulty encountered in the isolation and cultivation of oral spirochetes, they are still one of the least-studied and -understood members of the plaque flora. Many species that heve been observed directly have not been isolated, cultivated, or characterized.

v. HOST RESPONSES IN PERIODONTAL DISEASE

The patient's responses to periodontal infections are complex. For most people, periodontal health is protected by the body defenses despite the high bacterial load on the teeth. Some of the factors which have been suggested to increase susceptibility of periodontal subjects include impaired neutrophils, inadequate or unregulated host immunologic response, AIDS, and diabetes.

Host responses in periodontal disease involve both cellular and humoral immunity as it is for most inflammatory diseases. Stouffi et al.(1987) and Johannessen et al. (1990)found that T-cells and B-cells may predominate in lesions, but there was a variation in the number of plasma cells. Polyclonal B-cell activation is increased in some patients with periodontitis. B-cells undergo blastogenesis and produce large quantities of antibodies and lymphokines
(Tew, et al., 1989).

Various studies suggest that the neutrophil/ antibody/ complement axis is critical for protection against periodontal disease. Abnormalities in this axis often lead to increased susceptibility to periodontal disease. Patients with neutrophil disorders, such as diabetes and Down's syndrome, all have been found to have severe periodontal disease (Genco and Slots, 1984; Van Dyke and Hoop; 1991).

The polymorphonuclear leucocyte (PMNL) is the principal cell of

the gingival crevicular fluid and pocket exudate. PMNLs come into direct contact with dental plaque in the gingival crevice and actively phagocytose plaque micro-organisms (Barnett and Baker, 1982). There is a significant association of localized juvenile periodontitis (LJP) with PMNL abnormalities (Genco et al., 1980). In a recent study, Kimura et al. (1992) showed that about 50% of their patients with LJP had depressed PMNL phagocytic functions, while only a minimal number of patients with adult periodontitis and no healthy subjects showed a depressed phagocytic response. They also suggested that the phagocytosis was mainly complement-dependent. The depressed phagocytic responses in the patients could be due to PMNL cell-associated defect(s), perhaps complement receptor-related defect(s) on the PMNL. The major protective function of the PMNL , phagocytosis and killing of bacteria , is often dependent on serum opsonization. Serum antibody and complement both mediate opsonization but in sightly different functions. Complement C3b and iC3b mediate opsonization by binding to CR1 and CR 3 receptors on PMNL to enhance adherence and killing by the PMNL(Genco, 1992). This theory helps us to understand that abnormalities in neutrophil/antibody/complement axis often lead to increased susceptibility to periodontal disease. Activation of complement by T. denticola has been reported. Schenkein and Berry (1991) showed that T. denticola was capable of activating both the classical and alternative complement pathways in human serum and was opsonized with the major C3-derived opsonins C3b and iC3b. Although proteolytic enzymes are likely to be virulence factors for T. denticola (Loesche, 1988), they did not appear to influence the activation of complement or deposition of

complement-derived opsonins on the cell surface. So complement activation and deposition of complement-derived opsonins may be important defense mechanisms in the control of infection with T. denticola.

In a study of humoral immune response, Whitney et al.(1992) reported that the humoral immune response against a whole-cell homogenate of P. gingivalis in rapidly progressive periodontitis, as well as in matched control subjects that mount a response, is dominated by IgG2 followed by IgG3. The IgG subclasses differ greatly with regard to their functional activities. IgG2 is considered less effective than IgG1 (and IgG3) as a bacterial opsonin because of its lower binding efficiency to the Fc receptors of phagocytic cells. The low avidity of patient anti-P. gingivalis IgG antibodies suggest that humoral responsiveness to infection with P. gingivalis may be ineffective in clearing this organism.

The destructive periodontal diseases can cause tooth loss. The role of periodontal bacteria may well be to stimulate chronic inflammatory cells of the gingiva and fibroblasts to produce cytokine including IL-1, TNF, IL-6, and others which act to stimulate osteoclastic bone resorption (Genco, 1992). The role of cytokines in human disease is a rapidly developing area. The role of IL-1 is pleotrophic. IL-1 stimulates bone resorption (Gowan et al.,1983, Dewhirst et al., 1985). IL-1ß was localized in gingival tissue (Jandinski et al., 1991). They also reported that IL-1 levels decreased after periodontal therapy. Kono et al. (1991) suggested

that IL-6 was important in regulation of the plasma cell infiltrate in periodontal disease. These cytokines mediate their activity through the arachidonic acid metabolites, mainly the prostaglandins, but also possibly the prostacyclines and lipoxygenase products to increase osteoclastic activity which in turn results in bone resorption (Genco, 1992).

Overall, development of oral infections depends on the outcome of the initial oral pathogenic interaction with the host defence system. If this reaction leads to a rapid immune response, the microorganisms should be eliminated or contained without disease. Alternatively, the initial immune response may fail and the host may therefore be left immunologically compromised, leading to oral infections by the pathogenic microorganisms. The associated immnuosuppression may be temporary. Many patients eventually develop detectable humoral and/or cellular responses to plaque-derived infections.

vi. CLINICAL TREATMENT

Generally speaking, the treatment of periodontal disease involves mechanical debridement and the administration of antibiotics.

Loesche (1992) suggested that antimicrobial therapy should only be used when there is an appropriate diagnosis of an infection and when the

clinical situation is serious enough to warrant this treatment. He also suggested that if the spirochetes accounted for more than 20% of the flora in three or four of the plaques , then diagnosis was an anaerobic infection. The most common antibiotics used in the clinic are tetracyclines, penicillins, erythromycin and metronidazole. Metronidazole is the treatment of choice of anaerobic infections in many countries, as it has excellent pharmacodynamics and efficacy (Baines and McFadzean, 1981). Loesche suggested that metronidazole should only be chosen after diagnosing an anaerobic infection that was serious enough to warrant the use of systemic medication. Debridement is an essential preceding component of metronidazole treatment. Experiments also showed that when a week of medication preceded the debridement, the need for surgery was reduced by an average of 40% in the metronidazole group and 14% in the positive-control group. When the medication was given after the debridement was completed, the metronidazole reduced the surgical needs by about 60%, compared to 27% in the positive-control group (Loesche, et al., 1991). It has been demonstrated that debridement should precede systemic metronidazole therapy.

Since inflammatory periodontal diseases are initiated by dental plaque, effective removal of plaque deposits on a regular basis is required to return inflamed periodontal tissues to health. Oral hygiene is sufficient to reverse an early stage of gingivitis.

III. ISOLATION AND QUANTIFICATION OF OAS FROM SUBGINGIVAL PLAQUES

Oral spirochetes are usually isolated by the following two general methods: (i) membrane filters placed on the surface of agar media and (ii) incorporation of antibiotics, i.e. rifampin (RFP) into media as a selective agent (Stanton and Canale-Parola, 1979; Leschine and Canale-Parola, 1980).

In the first method, the sample is placed on the filter and the Petri dish containing agar medium quickly placed in an anaerobic jar or chamber and incubated. After incubation the filter is removed and a white haze can be seen in the agar. Spirochetes migrate through the filter and grow into the agar. A plug of agar is removed and inoculated into a prereduced culture medium. In the second method, filter-sterilized RFP is added to either prereduced broth or molten agar medium in tubes to a final concentration of 1.0-10.0 μg/ml. Serial dilutions of samples can be made in the selective medium. The medium was then poured into Petri dishes or solidified in the tubes. By using the first method, Loesche and Socransky (1961) were able to isolate small spirochetes which can penetrate membrane filters. This procedure is quite useful for small spirochetes with a cellular diameter of approximately 0.3μm or less, but the intermediate and large spirochetes require filter pore sizes which permit the penetration of motile bacteria such as Vibrio, Selenomonas, and Capnocytophaga. Smibert et al.(1984) isolated many strains of T. socranskii. By the combined use of membrane filters and the antibiotic rifampin , intermediate-size anaerobic spirochetes could be selectively

isolated (Cheng and Chan, 1983). Stanton and Canale-Parola (1979) isolated seven morphological types of spirochetes differing in cell size, cell coiling pattern, and number of periplasmic fibrilla per cell from bovine rumen.

Although certain types of spirochetes were isolated, it was still not possible to quantitatively isolate spirochetes from the plaque at present. Spirochetes averaged about 55% of the microscopic count but were less than 1% of the viable count(Salvador, et al., 1987; Loesche, 1988). The membrane procedure did not lend itself to the quantitative isolation of spirochetes. Difficulties encountered in the isolation and quantification of oral spirochetes also involve the specific nutritional requirement of spirochetes. Most of the information available on the growth requirements of oral spirochetes concerns the small-sized species (Breznak, J. A., 1973, Canale-Parola, E., 1977, Johnson, R. C., 1977). Socransky et al.(1964) reported that oral treponemes grew best in the medium with an Eh between -185 to -220 mv. The optimum Eh was reported to be -190 mv. Loesche (1969) reported that oral treponemes would grow in an anaerobic chamber with 0.1% or less oxygen in the atmosphere, but would not grow with oxygen concentrations of 0.3% or greater. Special nutritional requirements have been noted for different types of spirochetes. For example, T. denticola, T. macrodentium, and T. vincentii require thiamine pyrophosphate for growth, and extracts of T. denticola require this cofactor for acetyl-phosphate formation from pyruvate (Hespell and Canale-Parola, 1971).

Quantification of spirochetes in periodontal plaque has been hampered by their extreme sensitivity to sonication. Salvador et al.(1987) compared three dispersion procedures, including sonication, mechanical mixing, and homogenization for their ability to permit the isolation of T. denticola, T. vincentii, T. socranskii, and T. pectinovorum from plaque samples on media that support the growth of these species They found that the highest viable recoveries of spirochetes were observed when the plaques were dispersed with a homogenizer, but the highest recoveries averaged only about 1% of the total cultivable counts.

Loesche et al.(1992) compared the ability of several detection methods currently used in research, including the serial dilution anaerobic culture and/or microscopic procedure, the DNA probe procedure, and the immunologic reagents using both an enzyme-linked immunosorbent assay and indirect immunofluorescence assay to detect T. denticola, P. gingivalis, B. forsythus, and A. actinomycetemcomitans in subgingival plaque samples taken from 204 periodontally diseased tooth sites. The prevalence of the four monitored species varied as a function of both the species and the detection method. Spirochetes were present in 99% of the plaques. The culture method yielded the lowest prevalence values for the cultivable species. Their results showed that the DNA probes and immunological reagents to be significantly superior to the culture approach for the detection of A. actinomycetemcomitans, P. gingivalis and B. forsythus and to be comparable to the microscopic

approach in the detection of T. denticola.

Since difficulty was encountered in the isolation and quantification of oral spirochetes, the quantification of spirochetes in subgingival plaque has always been microscopy-based using cytoimmunochemistry, darkfield or phase-contrast optics. By using enzyme-linked immunosorbent assay, Simonson et al.(1988) showed a positive correlation between an increase in the T. denticola serovar c and increasing severity of periodontal disease.

In 1990, Umeda et al. in Japan developed a plate-in-bottle anaerobic culture method. They found that high recovery of spirochetes was obtained when M 10 medium supplemented with 10% rumen fluid was employed in the plate-in-bottle method(culture median:18.5%, phase contrast microscope median:19.2%). Their set of culture vessels consisted of a glass bottle, a small stainless steel pan, and a butyl rubber stopper. All of the culture vessels were oven-sterilized (except for the stopper, which was autoclaved) and gassed with oxygen-free CO^2 for a minimum of 2 min. It is obvious that this was a very complicated procedure and the culture vessels are not commercially available.

Many investigators attempted to obtain quantitative recovery of spirochete cells by culture but none of their efforts produced methods that yielded quantitative viable counts conveniently, reliably and reproducibly. For this reason, most investigations of subgingival mixed microbiota never included OAS as part of the " predominant cultivable

organisms" whenever cultural methods were used(Loesche, et al., 1992). Since culturing with accompanying biochemical analysis constitutes the so-called gold standard in detection and enumeration of subgingival microflora, a successful method is needed to be developed.

IV. SUMMARY OF MICROBIOLOGICAL TESTS USED IN PERIODONTAL RESEACH

Microscopic assays. One of the simplest and oldest assay is the microscopic monitoring of bacterial morphotypes. Its main advantage includes the ability to account for all bacteria in the sample; i.e., estimates of total counts of bacteria morphotypes in the sample can be reliably established. The main drawback of the technique is the inability to determine microorganisms' susceptibility to antimicrobial agents and whether the the microorganisms are alive or dead.

Culture assays. The use of culture technique enabled investigators to isolate microorganisms and do futher identification, but not all species grow equally well in routine culture conditions, and in some cases some may not grow at all (the majority of spirochetes).

Immunologic assay. The immunologic techniques, such as immuno-fluorescence or the enzyme-linked immunosorbent assay (ELISA), have also proved useful in detecting the presence and relative proportions of selected microbial species. These techniques depend on the availability

of specific antibodies that will bind to selected bacterial antigens and can then be detected by labelling the primary antibody directly with a fluorescent marker or with a fluorescent secondary antibody. The main drawback of both techniques is that only those species will be detected for which the specific antibody is available .

DNA probe assays. DNA probes are able to detect the presence of as few as 10^3 cells in the sample and provide information on the presence of selected species that is as reliable as the information obtained with culture methods. However, DNA probe technology has not been developed to the point where it will routinely provide accurate quantitative data. Like immunofluorescence assays and the ELISA, DNA probe technology may be subject to problems of specificity and may be limited by the number of available probes.

Enzyme-based asssays. Another approach to the detection of selected species is to look for the presence of an enzyme that is unique to one or more of the clinically relevant species. For example, BANA (benzoyl arginine naphthylamide) is hydrolyzed by a trypsin-like enzyme produced primarily by T. denticola, Bacteroides forsythus and P. gingivalis. However, this method only can be used in detection but not quantification of periodontal bacteria.

MATERIALS AND METHODS

I. MEDIUM

The spirochete medium used throughout this study was the"New Oral Spirochete"(NOS) medium devised by Leschine and Canale-Parola (1980).

The composition of complete NOS medium was (in grams per 100ml of distilled water): brain heart infusion broth, 1.25; trypticase, 1.0; and yeast extract, 0.25, all purchased from BBL (Becton Dickinson and Company, Cockeysville, M.D.); sodium thioglycollate, 0.05; L-asparagine, 0.025 and glucose, 0.2, all purchased from Fisher Scientific (Fair Lawn, NJ); L-cysteine hydrochloride (BDH, The British Drug House, Toronto), 0.1; Noble agar (Difco laboratories, Detroit, MI), 0.3 for maintenance and 0.7 for isolation of spirochetes; SeaPlaque agarose (FMC BioProducts, Rockland, ME), 0.7 for viable counts in place of Noble agar. The medium was autoclaved at 121 °C for 20 minutes before the addition of the following filter-sterilized supplements (ml/100ml medium): 0.2% (wt./vol.) thiamine pyrophosphate (Nutritional Biochemical Corporation, Cleveland, Ohio), 0.3; 10% sodium bicarbonate (Fisher Scientific), 2.0 ; normal rabbit serum (Flow Laboratories, Mclean, Virginia), 2.0; and a mixture of volatile fatty acids, 0.2, made from 0.5 ml of each of the following volatile fatty acids dissolved in 100 ml 0.1 N KOH: isobutyric acid (Fisher Scientific); D, L-2-methylbutyric acid; isovaleric and valeric acids (Eastman Kodak Company, Rochester, N Y). The medium was placed in

the anaerobic glove box for a period of 24 hours for reduction as well as a purity check before inoculation.

II. ANAEROBIC CULTIVATION CONDITION

The oral spirochetes employed throughout the study were maintained in the 0.3 % NOS Noble agar medium by subculturing monthly. They were grown anaerobically at 35 °C in an atmosphere of 85% N_2, 10% H_2 and 5% CO_2 in an anaerobic glove box (Coy Laboratory Products, Ann Arbor, MI).

III. BACTERIA

The four oral treponemes used were: Treponema denticola ATCC 35404, 35405 isolated and characterized in our laboratory (Cheng and Chan, 1983); Treponema vincentii ATCC 35580 and Treponema socranskii subspecies socranskii ATCC 35536 purchased from the American Type Culture Collection (Rockville, MD.) and maintained in our laboratory as well.

IV. STERILIZATION

Media and glassware were sterilized at 121°C, 15 lb per square inch for 20 min in a pressure-steam sterilizer. Heat-unstable solutions were sterilized by passing through Millipore membrane filters with average pore size of 0.45 μm (Millipore Corporation, Bedford, MA).

V. DEMINERALIZATION AND DISTILLATION OF WATER

Single-distilled water was demineralized by passing it through a demineralizer (Corning, Fair Lawn, NJ. Model Ld-2) and double-distilled with a water distillation apparatus (Corning Model AG-2).

VI. METHODS OF STUDY FOR QUANTIFYING VIABLE ORAL SPIROCHETES

i. Various Modifications of Pour Plate Method

The pour plate method has been used by many investigators(Moore, et al., 1982; Leschine and Canale-Parola, 1980) for the isolation of oral spirochetes so it was chosen as the primary method for evaluation of colony-counting of spirochetes.

(a). General Procedures of the Pour Plate Method

Two ml of the spirochete suspension to be counted were placed into 18 ml 0.7% molten NOS agar medium in a screw-capped test-tube held at 45-50°C water bath and thoroughly mixed by vortexing. Serial ten-fold dilutions were made by removing 2 ml of medium from one test-tube and adding into the next one containing 18 ml medium. The molten NOS agar medium containing the diluted spirochete cells was then poured into a 100 x15mm Petri plate (Fisher Scientific) and allowed to solidify.

All plates were either kept in an anaerobic jar with a cup of distilled water at the bottom or sealed with adhesive tape after gaseous exchange at the air-lock chamber of the anaerobic glove box to prevent any moisture loss and then incubated anaerobically at 35 °C for about two weeks.

Various modifications of the pour plate method were used based on the above general procedures.

(b). Two-layer pour plate method I

10 ml (or 20 ml) 0.7% sterile NOS-Noble agar medium were used to overlay the spirochete-inoculated layer after it gelled in each plate (after the above procedures in (a)) in order to displace the air volume present at the top of each plate.

(c). Two-layer pour plate method II

10 ml (or 20 ml) 0.7 % sterile NOS-Noble agar medium with supplements were poured into each Petri plate and allowed to solidify before the pouring of spirochete-inoculated medium .

(d). Three-layer pour plate method

The three-layer (sandwich) experiment was the combination of two

two-layer techniques. 10 ml sterile NOS-Noble agar medium with supplements were poured into each Petri plate and allowed to solidify as the first layer. The spirochete-inoculated medium was poured into each of the above plates and allowed to solidify before the addition of 10ml 0.7% NOS-Noble agar medium used as top layer.

ii. Test-tube method

The test-tube method was described by Stanton and Canale-Parola (1979) for isolation of rumen spirochetes.

0.7% NOS-Noble agar medium was prepared and dispensed into each screw-capped test-tube. The medium within the test-tube was then autoclaved and cooled to 45 °C before the adding of NOS supplements. The total volume of medium within each test-tube was 9 ml. 1 ml log-phase growth spirochetes was removed from one test tube to the next in a series and thoroughly mixed by vortexing. All test-tubes were allowed to stand for 20 min before incubation in an anaerobic glove box at 35 °C. Duplicate plates and test tubes were employed.

iii. Tissue Culture Flask Method

25-cm^2 polystyrene tissue culture flasks (ICN Biomedicals Canada, Mississauga, Ontario, Canada) were chosen since they have optically clear flat surfaces to facilitate counting of colony-forming units (CFU). They

are also permeable to gases so that anaerobiosis can be attained easily upon incubation within an anaerobic chamber.

NOS medium with 0.7% (w/v) Noble agar was prepared , autoclaved and cooled to 45-50 °C in a water bath. The medium was supplemented then transferred into a 500 ml dispensing burette and 27 ml of the medium was dispensed into each 40-ml polystyrene tissue-culture flask and held at 45-50°C in a water-bath. Three ml of the suspension of spirochetes to be counted were placed into the first flask and thoroughly mixed by gentle shaking and inversion. Ten-fold dilutions were made by removing 3 ml of medium from one flask to the next in a series. (Alternatively, a suspension of spirochetes to be counted was serially diluted out in fluid NOS medium in 10-fold dilutions, and 1 ml of each dilution was placed into 29-ml medium volumes of NOS-Noble agar medium in tissue culture flasks).

SeaPlaque agarose (FMC) was subsequently used in place of Noble agar at the same concentration of 0.7% in NOS medium. NOS-agarose medium (autoclavable at 121 °C for 20 min) could be kept molten at 37 °C in a water-bath throughout the dilution steps of a suspension of spirochetes to obtain widely dispersed cells, whereas 0.7% NOS-Noble agar medium must be held at 45 °C or above to keep molten. All flasks containing diluted spirochete cells were placed at 4 °C for 20 min to allow gelling of the NOS-agarose medium.

In order to displace air volume present at the top of the culture

flasks and to obtain highest recovery of spirochetal colony-forming units, various top layers were tested and compared, including distilled water gels of Noble agar and Bacto agar (without NOS medium ingredients) at different concentrations(0.7%, 1.0% and 1.5%); such gels with the incorporation of sodium thioglycolate reducing agent at 1.1g/L and methylene blue (0.002g/L) or resazurin (0.0001%) anaerobic indicators to test the anaerobiosis at the top of the flasks; 0.7% NOS-Noble agar medium and 0.7% NOS-Bacto agar medium without supplements.

Upon solidification of NOS-agarose medium with diluted spirochete cells in the culture flasks, it was topped off with 5-7 ml of one of the tested top layers described above. All culture flasks were incubated in an anaerobic glove box at 35 °C.

iv. Wide-Mouth Jar Method

A 30-ml straight-side wide-mouth jar (Naglene Company, Rochester, NY) was chosen as culture vessel because it has a wide opening and thus is easy to open to remove the incubated agar medium containing CFUs (in order to do subsequent tests or identifications).

5ml,10ml or 15 ml of 0.7% molten NOS-agarose medium were pipetted into each jar held at 37 °C in a water bath. A suspension of logarithmic-phase growth spirochetes to be counted was serially diluted out in fluid NOS medium in 10-fold dilutions, and 1ml of each dilution was

placed into each jar containing molten NOS-agarose medium. The jars were gently shaken to obtain a thorough distribution of cells and kept at 4 °C for 20 min to allow the gelling of NOS-agarose medium before adding 10 ml 0.7% NOS-Noble agar medium (without supplement) as topping layer.

When the jars were placed into an anaerobic chamber, the caps of the jars were loosened for about 1 hour to facilitate gaseous exchanging within the jar. All of the jars were incubated anaerobically at 35 °C.

II. TEMPERATURE SENSITIVITY EXPERIMENTS

Two holding temperatures, 37 °C (to keep molten 0.7% NOS-agarose medium) and 50 °C (to keep molten 0.7% NOS-Noble agar medium) were compared by the rate of spirochete CFU formation and the numerical recovery of CFU since it was the purpose to determine whether the holding temperature throughout the dilution steps could injure the spirochetes.

The tissue culture flask method described above was used. A suspension of logarithmic-phase T. denticola ATCC 35404 was diluted out at 50 °C in molten NOS-Noble agar medium and at 37 °C in NOS-agarose medium contained in tissue culture flasks. An alternative method used was prior serial dilution of spirochete cells in fluid NOS medium and then planting 1 ml of each dilution into molten NOS-Noble agar medium and

NOS-agarose medium. All flasks were incubated anaerobically in the glove box at 35 °C.

III. GROWTH EXPERIMENTS

Three techniques were used to follow the growth of oral spirochetes in fluid NOS medium on a daily basis : optical density reading, darkfield cell counting and viable counting. The spirochete cultures used were I. denticola ATCC 35404 and T. vincentii ATCC 35580 grown in fluid NOS medium in erlenmeyer flasks. The flasks containing spirochete cultures were gently and continuously agitated with a magnetic spinner in the anaerobic glove box.

a. Optical Density Reading

5 ml of each cell culture were pipetted, under anaerobic condition, to spectrophotometer tubes. The cell cultures in the spectrophotometer tubes were vortexed and the turbidity measurements were then recorded with a Gilford Starsar II Spectrophotometer (Gilford Instruments Laboratory Inc., Oberlin, Ohio, U. S. A) at 620 nm.

b. Darkfield Cell Count

The number of spirochete cells per ml of growth medium was counted using a Petroff-Hausser counting chamber (C. A Hausser and Son,

Philadelphia, U.S.A) under darkfield illumination with a final magnification of 500X. One drop of the suspension was placed onto the ruled area of a clean Petroff-Hausser counting chamber and carefully covered with a cover-slip to avoid air bubbles. The counting chamber was then allowed to stand for 10 min to permit the bacteria to settle into the same focal plane as much as possible.

The counting chamber is ruled into squares that are 1/400 mm^2 in area and 1/50 mm in depth, so that the volume over a square is 1/20,000 mm^3 or 1/20,000,000 ml. Spirochete cells in at least 20 squares were counted and the average number was taken as the average number per square in calculating.

c. Viable Count

The viable count of spirochetes in fluid NOS medium was followed by using the tissue culture flask method described above. Ten-fold dilutions were made by pipetting 1 ml of cell suspension under anaerobic condition from one test-tube to the next containing 9 ml fluid NOS medium, and 1 ml of each high dilution (from 10^{-4} to 10^{-8} or 10^{-5} to 10^{-9} depending on the stage of growth) was pipetted into 29 ml 0.7% NOS-agarose medium in each tissue flask held at 37 °C to obtain countable colonies. The suspensions of spirochetes in the flasks were thoroughly mixed by gently shaking and inverting the flasks. All flasks were kept at 4 °C for 20 min to allow gelling of NOS-agarose medium with dispersed spirochete cells

and then topped with 5-7 ml NOS-Noble agar medium. The viable count of spirochetes was made after 7 to 10 days of anaerobic incubation at 35 °C.

IV. ANTIBIOTIC SUSCEPTIBILITY EVALUATIONS

i. Antibiotics

Polymyxin B was purchased from ICN Pharmaceuticals Inc. (Cleveland, OH). Vancomycin was obtained from Eli Lilly Canada Inc. (Toronto, Ontario). Spectinomycin, rifampin, nalidixic acid and neomycin were the products of Sigma Chemical Company (St. Louis, MO).

ii. Evaluations of Antibiotic Susceptibility

The viable count technique using NOS-agarose medium in tissue culture flasks described above provides a unique opportunity to test the effect of selected antibiotics on the oral spirochetes. Logarithmic-phase cultures of T. denticola, T. vincentii and T. socranskii were used in this study. Rifampin (4 µg/ml, final concentration) has been used by Leschine & Canale-Parola (1980) as a selective agent for isolation of oral spirochetes and so was a prime candidate for testing. Rifampin at 20 µg/ml final concentration was incorporated into NOS-agarose medium to observe the growth of oral spirochetes by viable counts. The minimal inhibitory concentrations of rifampin for different species of oral spirochetes are from 25 µg/ml to 50 µg/ml as determined by turbidity of growth of spirochete cells in fluid NOS medium (S.-L. Cheng, 1984). Other

antibiotics tested were the combination of nalidixic acid (20 μg/ml) and neomycin (30 μg/ml), since these were used successfully for the selective growth of Borrelia burgdorferi (Steere, et al., 1983); polymyxin B (854 units/ml or 100 μg/ml); the combination of rifampin (2μg/ml) and polymyxin B (800U/ml) which was described to be the best antibiotics for the suppression of other oral bacteria by Fiehn and Frandsen (1984) and the combination of rifampin 20 μg/ml and polymyxin 100μg/ml.

The antibiotics vancomycin (6.25 μg/ml) and spectinomycin (200μg/ml) were used by Kunkle and Kinyon (1988) in the isolation of T. hyodysenteriae. The susceptibilities of oral spirochetes to vancomycin and spectinomycin were evaluated. Different concentrations of those antibiotics were tested by incorporating them into fluid NOS medium and the results were read by O. D. reading. The highest concentrations of these two antibiotics which did not inhibit the growth of spirochetes were incorporated into NOS-agarose medium. The growth of oral spirochetes in such medium containing antibiotics was observed by CFU.

V. PLAQUE SAMPLE COLLECTIONS

Subgingival plaque samples were obtained by inserting sterile paper points into periodontal pockets 6 to 7 mm in depth for about 15 sec. Patients from both sexes and all ages were selected. All patients used in this study received no medications including antibiotics during the course

of study. The paper points were placed into 2 ml reduced transport medium in a screw-cap vial at chairside. Samples were then taken immediately to the laboratory for processing.

Transport medium contained the following constituents: resazurin solution (Allied Chemical Corporation, Rochester, NY), 0.4 ml; salts solution (see below; Fisher Scientific), 4.0 ml; distilled water, 100.00 ml; 0.05% hemin solution (Sigma), 1.0 ml; Vitamin K1 (1 µg/ml; ICN), 0.02g and cysteine HCl (BDH), 0.05g. The salts solution consisted of (grams per liter distilled water): $(NH_4)_2SO_4$, 0.5; K_2HPO_4, 1.13; KH_2PO_4, 0.9; NaCl, 1.0. The transport medium was sterilized by autoclaving and was placed in the anaerobic glove box for a period of 24 hours for reduction before sampling.

VI. PROCESSING OF SAMPLES

Reduced transport medium containing a subgingival plaque sample on paper point(s) was vortexed vigorously. All samples were examined under darkfield microscopy and the number of spirochetes was counted by using the darkfield cell count method described before.

(a). Viable Spirochete Count

After evaluating several antibiotics, rifampin at 20 µg/ml was chosen as selective antibiotic and incorporated into NOS-agarose medium

to inhibit the growth of virtually all other oral eubacteria in subgingival plaques. Two-tenths (0.2) ml of the sample was pipetted into a 40-ml polystyrene tissue-culture flask containing 19.8 ml molten NOS-agarose-rifampin (NAR) medium kept at 37 °C. Such an initial flask represented 1:100 dilution of the plaque sample. Serial 1:10 dilutions were made by pipetting 3 ml mixed molten NAR medium containing suspended bacteria into successive flasks each containing 27 ml molten NAR medium. Each flask was thoroughly but gently mixed by inversion before a 3-ml aliquot was removed for dilution. After the gelling of NAR medium containing diluted sample bacteria, 5-7 ml NOS-Noble agar medium was placed into each flask as topping layer.

(b). Total Viable Cell Count

Brain-heart infusion (BHI) agar medium was used for total viable cell counts from subgingival plaques. BHI agar medium had the following composion: brain heart infusion broth (dehydrated), 3.7g; and yeast extract, 0.5g, all purchased from BBL; distilled water, 100 ml; and Bacto agar, 1,5g. The medium was boiled and cooled to room temperature before the addition of the following constituents: cysteine-HCl· H_2O(BDH), 0.05g; 0.05% hemin solution, 1.0 ml; and Vitamin K_1 (1 µg/ml), 0.02 ml. The medium was dispensed into each screw-capped test-tube and autoclaved at 121 °C for 20 min and then cooled to 45 °C in the water bath. Subgingival plaque samples were similarly diluted out in the test tubes as described above. The molten BHI agar medium, kept at 45 °C, containing

dispersed bacterial cells was vortexed and poured into Petri dishes.

Both tissue culture flasks and Petri dishes were incubated in the anaerobic chamber at 35 °C. The results were obtained by counting CFU.

VII. VERIFICATION OF SPIROCHETAL CFUs

Spirochete colonies in NAR medium were readily recognizable because they exhibited typical morphology, i.e., spherical, cottony with a denser center. The colony increased in size by diffusing characteristically through the agar medium. After incubation in the anaerobic glove box, each CFU exhibiting the typical colonial morphology of oral spirochete in flasks containing 20 to 30 CFU was picked by stabbing a sterile Pasteur pipette through each selected colony. An alternative method involved cutting off the top of the flask by melting the polystyrene with a hot wire and the slab of NOS semi-solid medium with spirochete colonies was removed into a sterile dish. Selected CFUs were verified to be spirochetes by darkfield examination of wet mounts. If the spirochete colony was verified, it was then transferred into 0.3% NOS-Noble agar medium for maintenance and further identification.

VIII PURIFICATION OF NEWLY ISOLATED OAS

The colonies with the typical morphologies described did not always consist of pure spirochetes; some of them contained a few of other kinds of eubacterial cells, which were slow growers. Subsequent purifications

those colonies were required.

i. Repetitive High Dilutions

A portion of the spirochete colony mixed with other bacteria examined by darkfield microscopy was transferred into fluid NOS medium in a screw-capped test tube. The test tube was mixed thoroughly using a Vortex mixer, serial 1:10 dilutions were made again to 10^{-8} in 0.7% molten NAR medium held at 37 °C in tissue culture flasks. (Per cent values preceding medium refers to concentration of gelling agent here and hereafter.) The tissue culture flask method was subsequently performed again to get viable spirochete counts.

ii. Membrane Filter Method

This was a modification of the wide-mouth jar method. 15 ml molten NAR medium were pipetted into each jar and allowed to solidify at 4 °C for 20 min. A sterile membrane filter with an average pore size of 0.45 µm (Millipore Corporation, Bedford, Mass.) was placed on the surface of the agar medium. The suspension containing spirochete colony was vortexed and one drop of the suspension was placed on the surface of the filter. The alternative method was that a portion of the colony was directly placed on the surface of the membrane filter sitting on the surface of NAR medium in the wide-mouth jar. Both methods were compared. After incubation at 37 °C for a period of 2 weeks, the

membrane filter was removed with a pair of sterile forceps. If the spirochetes had migrated through the filter, an area of hazy growth beneath the filter and below the agar surface could be observed.

The purity of isolated colonies from both methods was examined microscopically. Pure isolated colonies of spirochetes were maintained in soft NOS agar medium (containing 0.3% Noble agar) with no rifampin in screw-capped test tubes.

IX. CHEMOTAXIS ASSAYS

These experiments were designed based on the observations when distilled water gels of Bacto agar or Noble agar used as topping layer inhibited the growth of oral spirochetes.

i. Test Agents

The viable count method using tissue culture flasks as culture vessels also provided a unique opportunity to study the chemotaxis of oral spirochetes. Different gelling agents , 0.7% agarose (FMC), 0.5% Noble agar (Difco), and 0.5% Bacto agar were tested in this study by incorporating them into NOS medium. They all were kept molten at 37 ^{o}C in a water bath throughout the dilution steps of a suspension of spirochetes so that temperature effects were eliminated.

The growth requirement of oral spirochetes for NOS supplements has been determined by S.-L. Cheng (1984) in our laboratory by using optical density reading. In this study, the nutritional requirement of spirochetes for the complete NOS supplement was determined by viable count in the tissue culture flasks.

ii. Two-layer method

A four-day old culture of T. denticola ATCC 35405 grown in fluid NOS medium was serially diluted out in fluid NOS medium in screw-capped test tubes.

1 ml of 10^{-3} or 10^{-4} dilution spirochete cells(spirochete cells usually form individual colonies at these dilutions) was pipetted into each of 20 ml screw-capped test tube containing 20-ml molten 0.7% NOS-agarose medium, 0.5% NOS-Noble agar medium , or 0.5% NOS-Bacto agar medium with NOS supplement kept at 37 °C in a water-bath. The spirochete cells in the NOS agar media were vortexed and then pipetted carefully by using a 10 ml glass pipette into 40-ml polystyrene tissue-culture flasks as bottom layer. The flasks containing diluted spirochete cells and NOS agar medium were gently placed at 4 °C for 20 min to allow the gelling of the agar medium. Any shaking of the flasks was avoided in order to prevent the spirochete cells in the flasks from contaminating the top part of the flasks.

Upon solidification of the bottom layer, the cell-free top layer , with and without supplement, was introduced into each of the flasks in the following manner: 20 ml 0.7 % molten NOS-agarose medium was introduced into each flask containing gelled 0.5% NOS-Noble agar medium or 0.5% NOS-Bacto agar medium with spirochete cells; and 20 ml 0.5 % molten NOS-Noble agar medium was topped into each flask containing NOS-agarose medium with spirochete cells. The flasks were then kept at 4 °C for 20 min to allow the solidification of the top layer media before they were incubated anaerobically in the glove box. The chemotaxis of spirochete cells at the different agar media was detected by colony-forming units after 7 to 10 days of anaerobic incubation.

iii. Three-layer Method

The three-layer method was used to place different NOS agar media and spirochete cells in the middle layer (sandwich) to observe the migration of spirochete cells to the top and/or bottom layer by the presence of CFUs.

(a). Original Spirochete Cell Assays

Four-day old culture of T. denticola ATCC 35405 was used in this experiment.

10 ml supplemented 0.7 % molten NOS-agarose medium was dispensed into each 40-ml polystyrene tissue culture flask and allowed to

solidify at 4 °C for 20 min as first layer. Upon solidification of the first layer, 10 ml 0.5 % molten NOS-bacto agar medium (with and without NOS supplement) in a test tube containing 1 ml diluted spirochete cells (10^{-3} to 10^{-4} dilutions) were carefully pipetted into each of the above flasks and allowed to solidify as a second layer. The third layer which has the same constituents as the first layer was placed into each flask containing the two different layers and allowed to gel at 4 °C for 20 min before 5 ml 0.7 % NOS-Noble agar medium (without supplements) was added in to overlay the third layer in order to displace the air volume present at the top of each flask thus creating equal conditions for both the first and third layers. Only 0.5 % NOS-Bacto agar medium was used in the meddle layer in this study since Bacto agar was found to have more inhibitory effects for the growth of oral spirochetes than that of Noble agar when used as the top layer in viable spirochete count experiments.

The control flasks had the second layer consisting of 0.7% NOS-agarose medium (with and without supplements) and diluted spirochete cells.

Duplicate flasks were performed for each different middle layer and all of the flasks were kept flat in an anaerobic chamber at 35 °C for about 7 to 10 days' for the formation of spirochete colonies.

(b). Different Locomotory Phenotype Assays

The spirochete cells employed in this experiment were: four-day old culture of origin T. denticola ATCC 35404 in fluid NOS medium used as control; spirochete cells taken from the third layer (top layer) and the first layer (bottom layer) from the above experiments.

The first and third layer NOS agar medium slabs with spirochete colonies in the tissue culture flasks were separately removed into sterile dishes by cutting off the polystyrene flasks with a hot wire. The colonies picked up from both layers by using a sterile bacteriological loop were separately transferred into fluid NOS medium in the test tubes, which were then mixed vigorously using a Vortex mixer. These suspensions of spirochetes were used to make serial dilutions in fluid NOS medium as described above and inoculated into10 ml 0.5% NOS-Bacto agar medium with and without supplements in tissue culture flasks and served as the middle layer.

This method was designed to determine whether oral spirochete cells can move in different directions. This work was repeated three times from the original T. denticola ATCC 35405 cells to check the reproducibility of the movements of spirochete cells.

RESULTS

I. Development of Method for Quantifying Viable Oral Spirochetes

i. Various Modifications of the Pour Plate Method

Spirochete colonies of T. denticola ATCC 35404 and ATCC 35405 were sometimes present in the Petri plates containing complete NOS medium with 0.7% Noble agar after 10 days' anaerobic incubation in the glove box. About 10-fold more colonies were observed in the two-layer I and three-layer techniques than in the two-layer II method, but no isolated colonies of T. vincentii ATCC 35580 and T. socranskii ATCC 35536 were observed even when prolonged incubation was provided. The colonies were subsurface and exhibited the appearance of being white and cottony (Fig. 4). They were examined by darkfield examination of wet mounts and helically-shaped spirochetes were observed. However, these methods were not reproducible even though the exact same procedures were performed.

ii. Test-tube Method

Spirochete colonies with the typical colonial morphology of spirochetes. were consistently formed in test tubes containing complete NOS medium with 0.7% Noble agar However, spirochete colonies formed

within the deeps of the tubes would not be sampled easily for subsequent testing and identification. Furthermore, the round glass culture vessels did not facilitate counting of CFU (Fig. 5).

iii. Tissue Culture Flask Method

Widely dispersed spirochete cells of T. denticola ATCC 35404, ATCC 35405, T. vincentii ATCC 35580 and T. socranskii ATCC 35536 in high dilutions consistently formed individual colonies in tissue culture flasks containing complete NOS medium with 0.7% Noble agar or 0.7% agarose When such colonies were examined by darkfield examination of wet mounts, helical-shaped spirochetes in pure culture were observed. In a comparison study, a higher number of spirochete colonies (8.0 $X10^7$ CFU/ml) were obtained using the tissue culture flask method than the number (1.8 X 10^5 CFU/ml) obtained using the pour plate technique. The darkfield cell count was 4.9 $X10^8$ cells/ ml.

Distinctive colonial morphologies of different species of oral spirochetes were detected by this method. T. denticola ATCC 35404 and ATCC 35405 formed small to medium size compact colonies (Fig. 6a) after 10 days' incubation, whereas T. vincentii ATCC 35580 and T. socranskii ATCC 35536 formed large diffuse colonies(Fig. 6b) and prolonged incubation (about 3 weeks) was required for their colony formation.

An inhibitory zone was observed below the topping-layer consisting of distilled water gels of Noble agar or Bacto agar at different

concentrations (0.7%, 1.0% and 1.5%). The zone was not eliminated even when the reducing agent sodium thioglycolate was incorporated. At the same concentrations, Bacto agar exhibited more inhibitory effect than Noble agar for the growth of oral spirochetes. However, such inhibition was not observed when Noble agar was incorporated with NOS medium constituents (without supplements). But the NOS medium constituents failed to neutralize completely the inhibitory activity of the cruder Bacto agar; the inhibition was reduced but still existed (Fig. 7).

The anaerobic indicator methylene blue or resazurin became colorless on the second day of incubation in the anaerobic glove box when incorporated into the medium. This indicated that anaerobiosis was achieved within the flasks.

iv. Wide-Mouth Jar Method

Spirochete colonies of T. denticola ATCC 35404 , ATCC 35405, T. vincentii ATCC 35580 and T. socranskii ATCC 35536 were obtained in the jars containing 10 ml or 15 ml NOS-agarose medium (Fig.8), and no colonies were observed in the jars containing 5 ml NOS-agarose medium (which may due to the limited volume of the medium). But such round culture vessels did not facilitate the counting of CFU and the small volume within the jar could not provide sufficient space for numerous bigger spirochete colonies, such as the colonies of T. vincentii ATCC 35580 or T. socranskii ATCC 35536. When the jar contained about 20

colonies, some of the colonies touched each other which might affect the accuracy of the counting of CFU by coincidence loss.

II. Comparative Temperature Sensitivity Experiment

Table 2 exhibits the viable counts of T. denticola ATCC 35404 obtained from two agar media whcih were held at two differernt temperatures throughout the dilution steps. As can be seen the spirochete colonies appeared earlier (in 6 days) and were more numerous ($3.0X10^8$ CFU/ml) in NOS-agarose medium (spirochete cells were diluted out at 37^oC), but countable CFU did not appear in NOS-Noble agar medium (spirochete cells were diluted out at 50^oC) till at least the 8th day of incubation and a lower viable count ($1.3 X 10^7$ CFU/ml) was seen. Similar results were obtained when the alternate method used. i e., prior serial dilution of spirochete cells in fluid NOS medium and then planting 1 ml of each dilution into each molten NOS-Noble agar medium and NOS-agarose medium.

The results indicate that the holding temperature of 50 ^oC of molten NOS medium containing 0.7% Noble agar injured the fragile spirochete cells during their dilution and dispersion in the warm medium. The results also suggest that complete NOS-agarose medium is optimal for obtaining the highest recovery of CFU when compared with the NOS-Noble agar medium.

III. Growth Experiments (Quantifying Oral Spirochetes from Pure Cultures)

The growth of two treponemes in fluid NOS medium was followed by optical density readings, darkfield cell counting and viable spirochete counting in the culture flasks. The viable flask count technique described previously made it possible to follow the growth of oral spirochetes by viable count and it provided reliable and reproducible results. The growth curves of two treponemes are shown in Figures 9a and 9b. The average growth yield at stationary growth for: T. denticola ATCC 35404 was 1.87 $X10^9$ cells/ml by darkfield cell count and 1.41 X 10^9 CFU/ml by viable count; T. vincentii ATCC 35580 was 6.47 $X10^8$ cells/ml by darkfield cell count and 1.9 $X10^8$ CFU/ml by viable count. The curve of log CFUs of both T. denticola ATCC 35404 and T. vincentii ATCC 35580 declined after anaerobic incubation of cultures. The start and progression of decline stages of the growth of oral spirochetes could not be detected by darkfield cell counting because both viable and dead cells were counted.

IV. Evaluation of Antibiotics for Use as Selective Agents of Oral Spirochetes

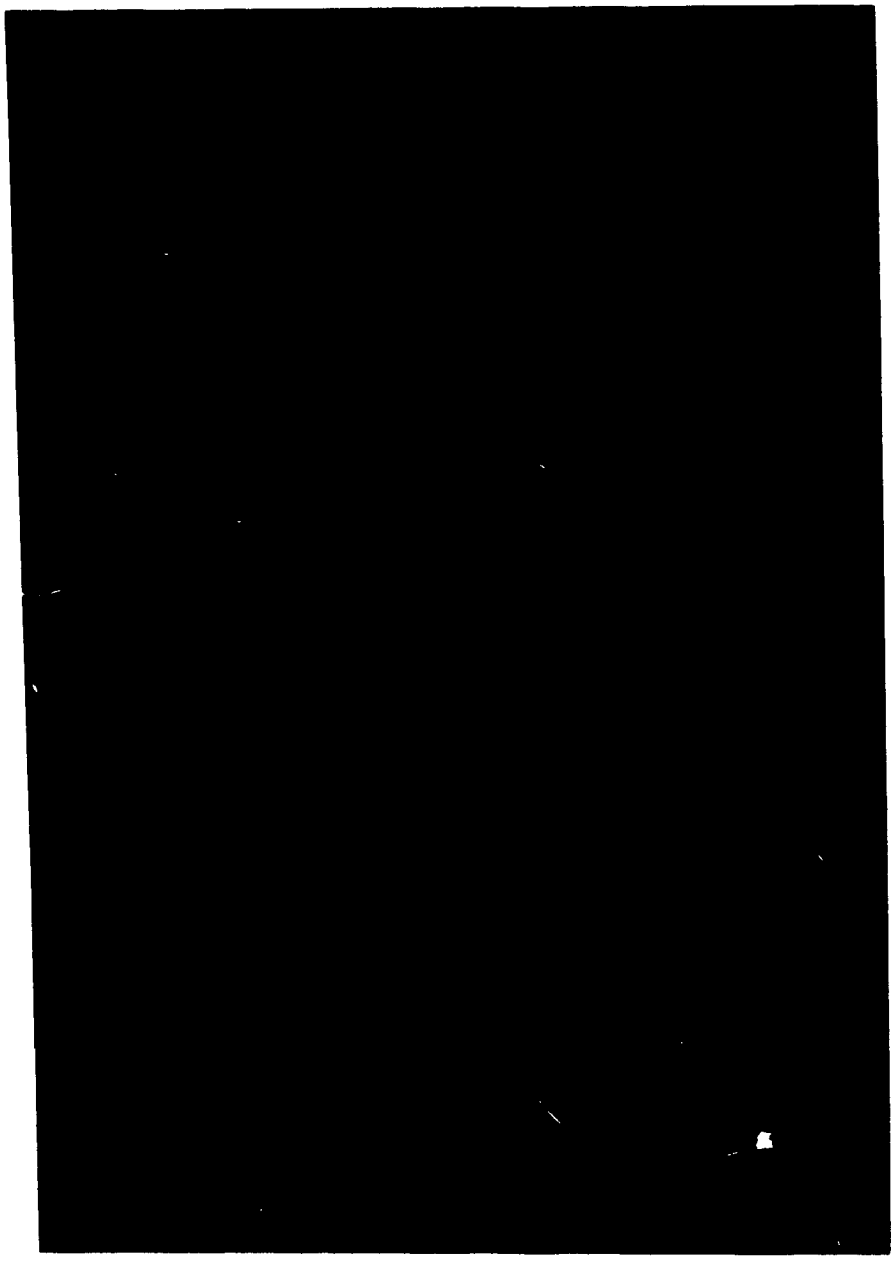
After evaluating the antibiotics rifampin, polymyxin,nalidixic, neomycin, vancomycin and spectinomycin for their efficacy as selective agents for the recovery of oral spirochetes by viable count technique,

cultivable species of oral spirochetes were found to be highly resistant to two antibiotics: rifampin and polymyxin. There was no decrease in CFU of T. denticola ATCC 35404 when rifampin at either 4 μg/ml or 20 μg/ml final concentration was incorporated into NOS-agarose medium. Table 3 shows the viable count of T. denticola ATCC 35404 in NOS-agarose medium without and with rifampin at 20 μg/ml final concentration .

Table 4 shows the viable count of T. denticola ATCC 35404 in NOS-agarose medium with and without polymyxin B at 854 U/ml or 100 μg/ml final concentration. As can be seen there was no decrease in CFU of T. denticola ATCC 35405 in the medium containing polymyxin B, but prolonged incubation was required for their colony formation (about one more week) compared to the control culture to which no polymyxin had been added. However, the CFU of T. denticola ATCC 35404 were decreased in NOS-agarose medium with the combined antibiotics rifampin 20 μg/ml and polymyxin B 40 μg/ml (2.5 $x10^6$ CFU/ml), i.e., much less than the number obtained in the NOS-agarose medium without antibiotics (2.5 $x10^6$ vs.1.17 $x10^9$ CFU/ml), The results are shown in Table 5. It appears that the synergistic effect of the two antibiotics together was inhibitory to the formation of oral spirochete colonies.

In order to determine whether rifampin or polymyxin B could serve individually as a selective agent for the recovery of oral spirochetes by viable count, a subgingival plaque sample was collected from a periodontal patient and equal aliquots of it were inoculated into NOS-agarose medium incorporated with rifampin at 20 μg/ml, polymyxin B at

Fig.4. Serially diluted suspension of Treponema denticola ATCC 35404 grown in Petri plates containing 0.7% NOS-Noble agar medium.



854 U/ml and the combination of rifampin (20 μg/ml) and polymyxin B (40 μg/ml), respectively. The results showed that oral spirochetes were highly resistant to polymyxin B but so were many other eubacterial species in dental plaque. The growth of spirochetes also might be inhibited by the growth of other eubacteria in the dental plaque in the same medium in the presence of polymyxin. In the NOS-agarose medium containing both rifampin (20 μg/ml) and polymyxin (40 μg/ml) , no spirochete colonies were observed.

The results suggest that rifampin alone (at 20 μg/ml final concentration) selects for a higher count of spirochetes than polymyxin alone or in conjunction with polymyxin and inhibited virtually all other bacterial species. The typical spirochete colonies were obtained from high dilutions and spirochete colonies were confirmed by darkfield examination of wet mounts. The viable count of spirochetes in rifampin-containing NOS-agarose medium was 1.5 $x10^6$ CFU/ml and the darkfield spirochete count was 1.6 $x10^7$ cells/ml. The antibiotic rifampin serves as a good selective agent for the recovery of oral spirochetes

When a combination of nalidixic acid (20 μg/ml) and neomycin (30 μg/ml) was added to the NOS-agarose medium, the growth of T. denticola ATCC 35404 was completely inhibited. Oral treponemes were also found to be very susceptible to vancomycin and spectinomycin; growth of the spirochetes was inhibited by low concentrations of vancomycin (3 μg/ml) and spectinomycin (20 μg/ml). Since these concentrations cannot

Fig. 5. Serially diluted suspension of Treponema denticola ATCC 35404 grown in test tubes containing 0.7% NOS-Noble agar medium.

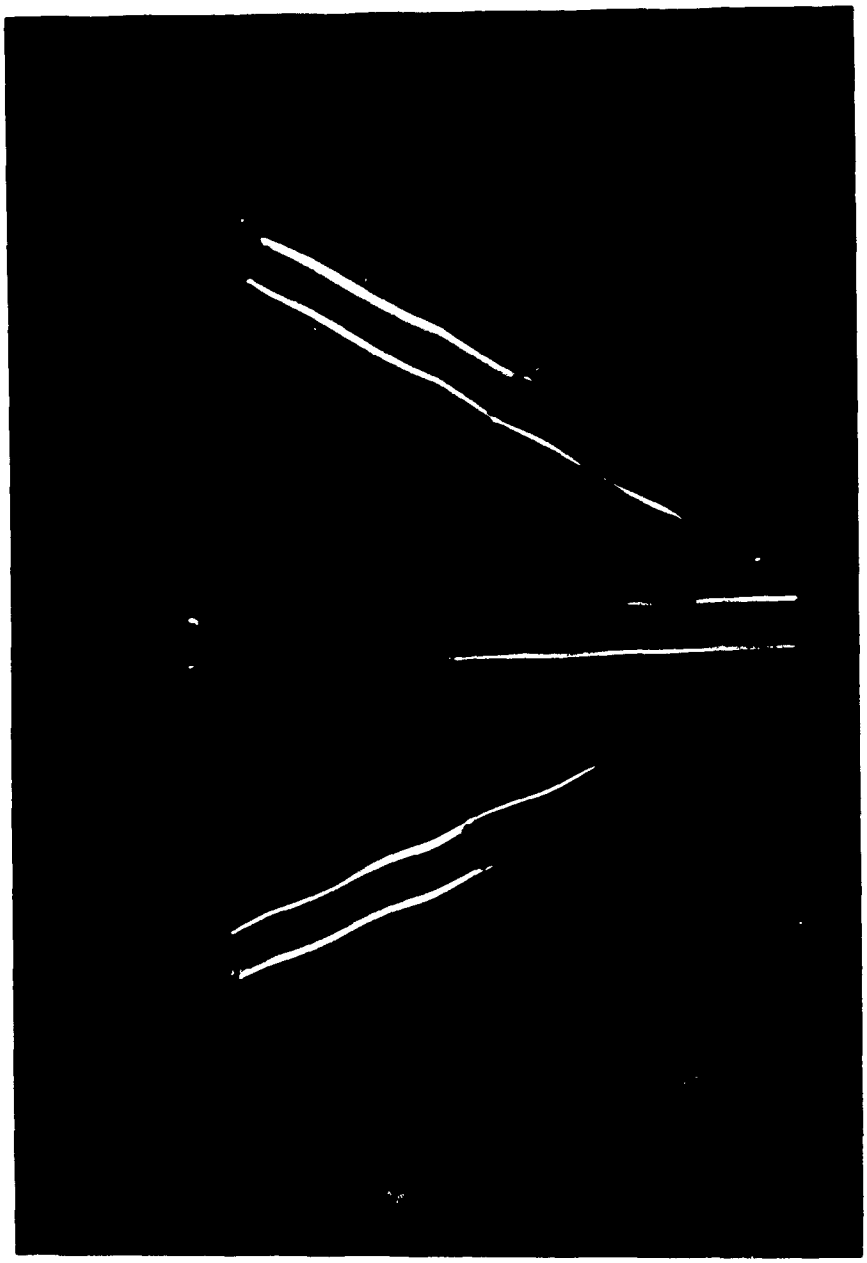
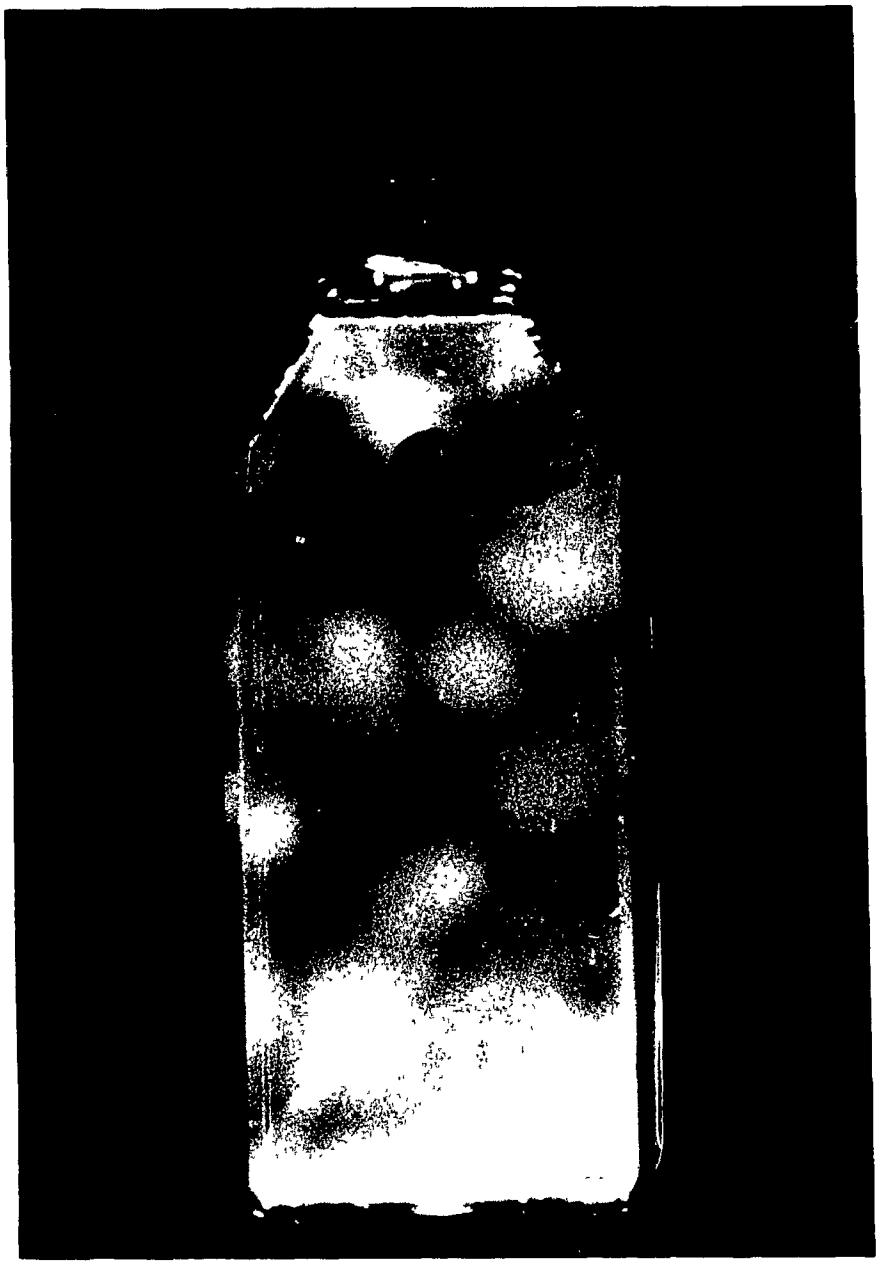


Fig. 6a. Serially diluted suspension of Treponema denticola ATCC 35404 grown in tissue culture flasks containing 0.7% NOS-agarose medium.

Fig. 6b. Colonial morphology of Treponema socranskii ATCC 35536 grown in tissue culture flask containing 0.7% NOS-agarose medium.



efficiently inhibit the growth of other bacterial species in the dental plaque, vancomycin and spectinomycin are not suitable to be used as selective agents for oral spirochetes.

V. Enumeration of Viable Spirochetes From Periodontal Pockets

(a). Enumeration of viabie oral spirochetes by use of NOS-Agarose-Rifampin (NAR) medium

Since results obtained showed that rifampin at 20 µg/ml can serve as a selective agent for oral spirochetes, this antibiotic was added to NOS-agarose medium in the enumeration of viable spirochetes from subgingival plaque samples (Fig. 10). Table 6 shows the specific counts of oral spirochetes obtained by means of darkfield microscopy and CFU in NOS-agarose rifampin (NAR) medium from 16 subgingival plaque samples of periodontal patients. The mean of spirochete colonies in NAR medium was only 10-fold lower that that of the darkfield cell count and the recovery of viable spirochetes ranged from 4.1 to 100% (the mean was 39.3%$\pm$ 7.4). Figure 11 shows the percentage recoveries of the viable spirochete counts over the total anaerobic cultivable flora(CFU on BHI medium plus the specific spirochete counts) from 10 subgingival plaque samples and the percentage range of cultivable oral spirochetes from 12.5% to 28.2% of the total anaerobic cultivable flora.

Fig.7. Tissue culture flasks containing spirochete CFU in NOS-agarose medium topped by NOS-Noble agar medium and distilled water gel of Bacto agar.

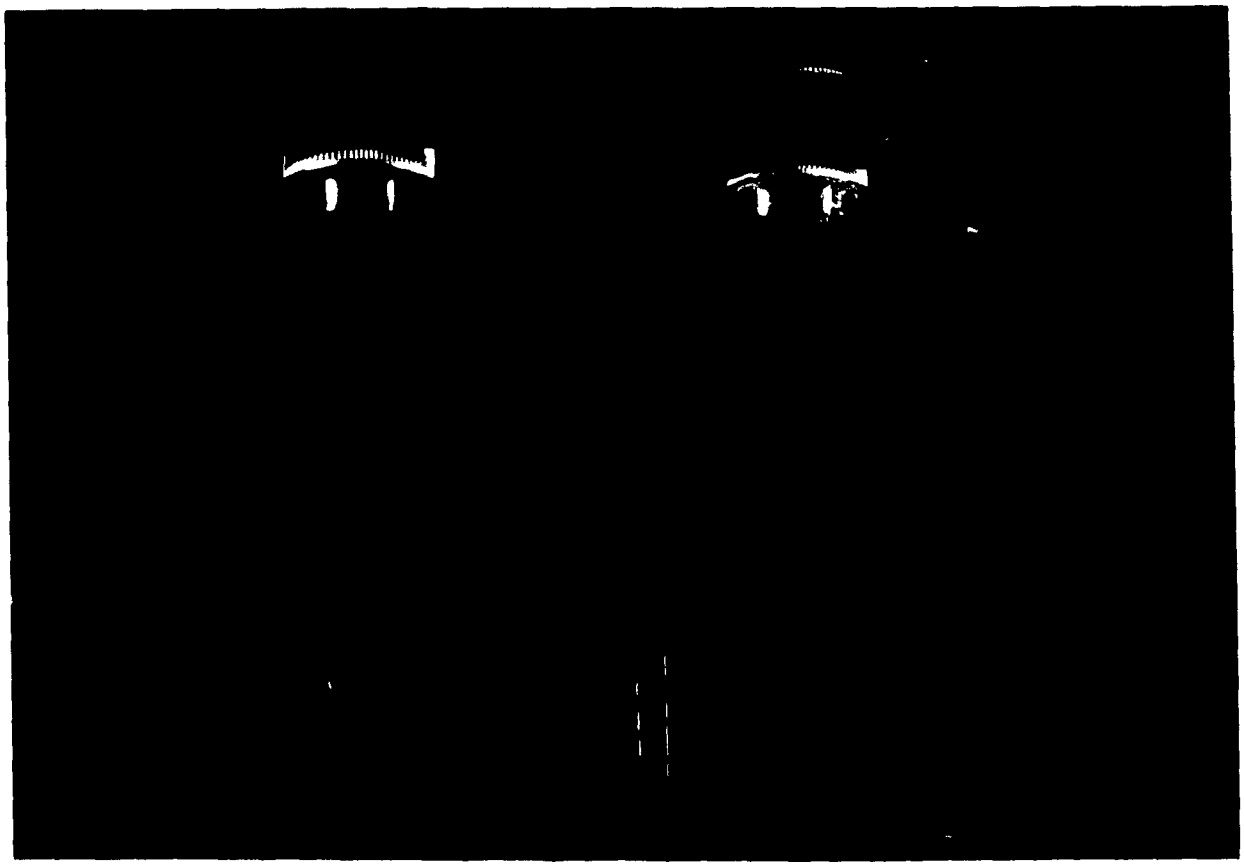
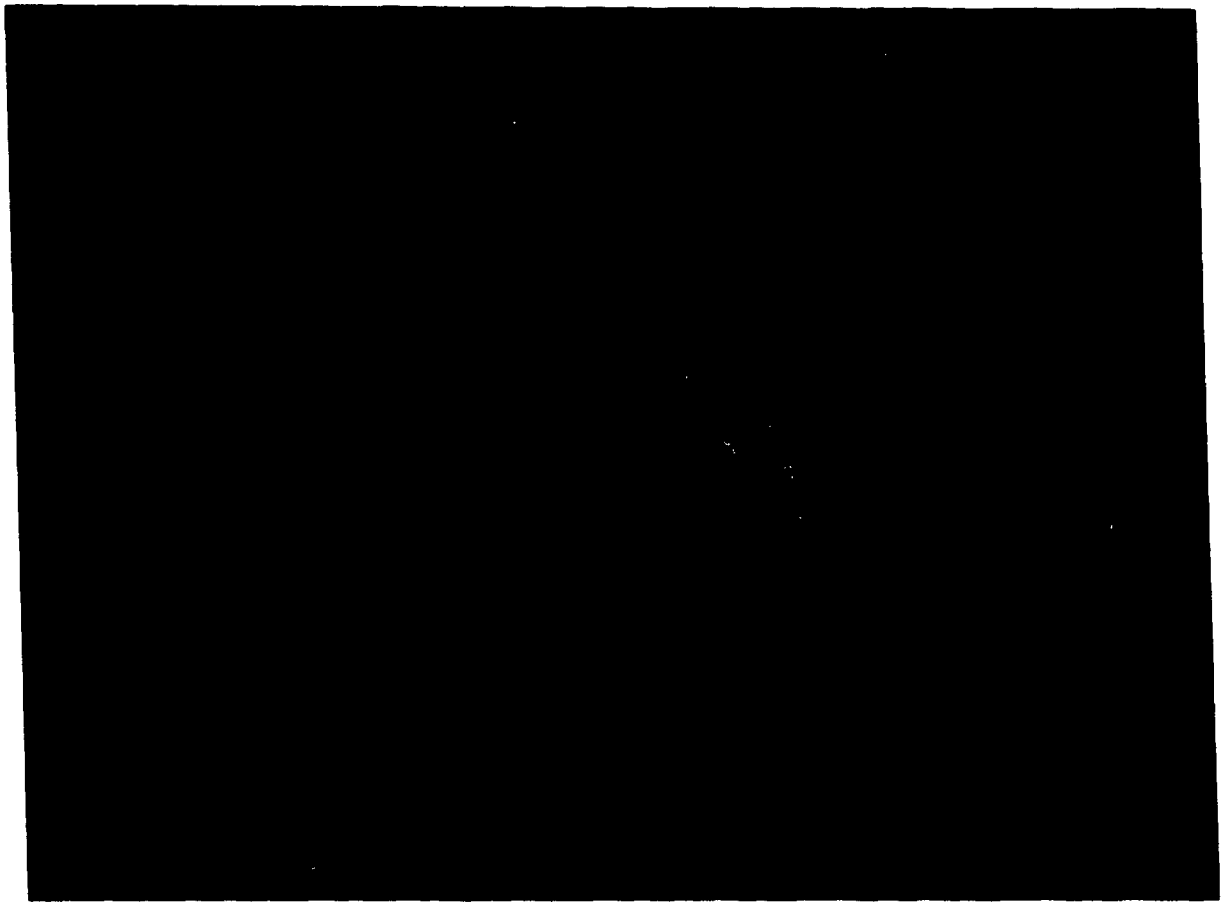


Fig. 8. Treponema denticola ATCC 35404 grown in wide-mouth jar containing 15 ml 0.7% NOS-agarose medium.



(b). Verification of spirochetal CFU

CFU that exhibited typical morphology of spirochete colonies described were verified to be spirochetes by darkfield examination of wet mounts. Attempts were made to render spirochete colonies differential (chromogenic) in situ (within the medium) by means of their enzymatic activity on substrates incorporated into NAR medium. For example, p-nitrophenylphosphoryl-choline was incorporated for the detection of phospholipase C activity and BANA was incorporated for the detection of a trypsin-like enzyme activity. None of these attempts were successful probably because the reaction products diffused away from the colonies. But examination of the bacteria in each colony by darkfield microscopy proved to be quite expedient for verification of spirochete colonies.

(c). Isolation of New Spirochetes

Thirteen new and uncharacterized anaerobic oral spirochetes were isolated from sixteen subgingival plaque samples by using viable count technique and NAR medium. The strains isolated differ in cell morphology, colonial morphology and growth rate. A large spirochete isolated is shown in Fig. 12. This spirochete appears larger than any known cultivable spirochetes observed by darkfield microscopy. Very tiny spirochetes and very motile spirochetes were also isolated which could not be isolated before. Some species of new spirochetes formed spherical, almost transparent colonies and increased greatly in size

during incubation whereas others formed cottony, dense, ball-like with an even edge colonies, etc.. Identifications of these new species are being undertaken by other workers in the laboratory.

However, not all discrete colonies of spirochetes isolated by the viable count technique proved to be entirely pure; some of them contained a few cells of other kinds of eubacteria, which were slow growers. This may be due to coaggregation.

(d). Purification of Newly Isolated Oral Spirochetes

Repetitive high dilution of isolated colonies in NAR medium was the best method to render them axenic. At high dilutions, isolated colonies consisting only of spirochetes were obtained consistently in flasks containing NAR medium. However, the membrane filter used in the Wide-Mouth Jar method did not provide consistent results and only one type of intermediate spirochete colony was obtained beneath the filter in the jar containing NAR medium, which was achieved when a portion of a mixed colony was placed directly on the surface of the membrane filter. This may be because the filter pore size did not permit the penetration of other newly isolated large oral spirochetes.

VI. Negative Chemotaxis Experiments

Negative chemotaxis of oral spirochetes was observed from both

two-layer and three-layer assays. The migration of spirochete cells was detected by spirochete colonies in NOS-agarose medium.

i. Two-layer Method

After 7 days' incubation of a culture in the anaerobic glove box, spirochete colonies were found in both top and bottom layers, that is, many spirochete cells of T. denticola ATCC 35405 migrated out of the NOS-Bacto agar or NOS-Noble agar medium into NOS-agarose medium and formed colonies. The results showed again that Bacto agar contained more inhibitory substances than Noble agar for the growth of spirochetes since more spirochete colonies were observed in NOS-agarose medium (examined visually since the colonies were too numerous to count) in flasks containing Bacto agar medium as the bottom layer when both NOS-Bacto agar medium and NOS-Noble agar medium were inoculated with the same concentration of T. denticola ATCC 35405 (results not shown). As expected, NOS-agarose medium with supplements used as topping layer in the flask attracted more spirochete cells from the bottom layer than that without supplements since more spirochete colonies were observed (Fig. 13). [NOTE: "top" and "bottom" refer to the geometry of the flask; all flasks were incubated horizontally.]

No spirochete migration was detected when the opposite way was performed, that is, NOS-Noble agar medium used as top layer (with and without supplements) and NOS-agarose medium used as bottom layer and inoculated with spirochete cells. Spirochete colonies were found only in

Fig. 9a. Growth curves of Treponema vincentii ATCC 35580 in fluid NOS medium followed by viable count and darkfield count.

Fig. 9a. Treponema. vincentii ATCC 35580

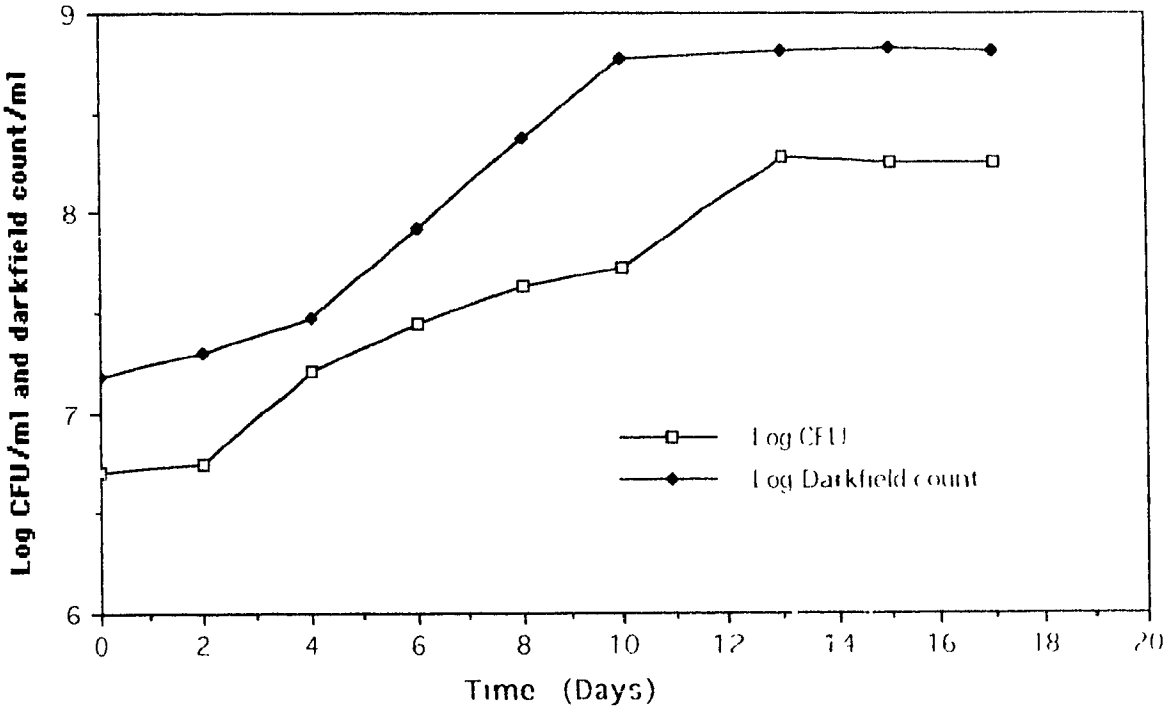
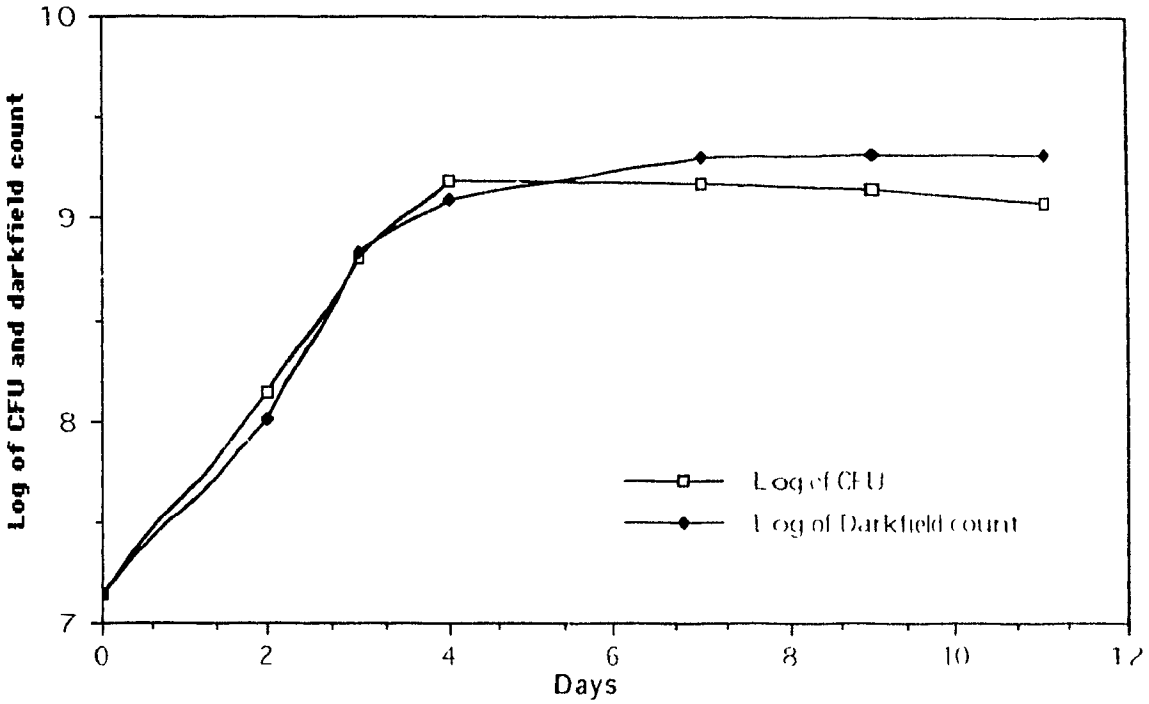


Fig. 9b. Growth curves of Treponema denticola ATCC 35404 in fluid NOS medium followed by viable count and darkfield count.

Fig.9b. Treponema denticola ATCC 35404



inoculated with spirochete cells. Spirochete colonies were found only in the bottom layer. The two-layer experiment was repeated several times and the results were reproducible.

ii. Three-layer Method

When NOS-Bacto agar medium with T. denticola ATCC 35405 was placed in the middle layer in the flasks, two locomotory phenotypes of oral spirochetes were observed. However, more spirochete cells migrated to the top layer and less spirochete cells migrated to the bottom layer (both top and bottom layers contained NOS-agarose medium with supplements) and formed colonies (Fig. 14). When the two different locomotory phenotypes of spirochetes were removed from top or bottom layer and placed in the middle layer to repeat the same experiment, the remarkable observation was made that top layer cells all migrated to the top layer and bottom layer cells all migrated to the bottom layer (Fig. 14), whereas original spirochete cells employed still migrated in both directions. The experiment was repeated by using spirochete cells from the original stock culture of T. denticola ATCC 35405; top layer spirochete cells and bottom layer spirochete cells obtained from previous experiments. Migrations of original spirochete cells to two different directions was consistently observed from stock culture cells. However, the migration of isolated spirochete cells of the two locomotory phenotypes varied somewhat. Sometimes isolated top layer cells or bottom layer cells still migrated in two directions and spirochete

Table 2. Effect of medium-holding temperature on the viable count of Treponema denticola ATCC 35404

Days	NOS-agarose medium at 37°C	NOS-Noble agar medium at 50 °C
6	4.7×10^6 *	No CFUs
8	8.4×10^7	8.8×10^5
9	2.4×10^8	3.6×10^6
12	2.7×10^8	5.1×10^6
14	2.9×10^8	5.3×10^6
16	3.0×10^8	7.3×10^6
19	3.0×10^8	1.3×10^7
21	3.0×10^8	1.3×10^7
24	3.0×10^8	1.3×10^7

* CFUs are means of duplicate flask cultures

Table 3. Colony-forming units in NOS-agarose medium with and without rifampin (20 μg/ml)

Dilution	Without rifampin	With rifampin
10^{-3}	Too numerous to count	Too numerous to count
10^{-4}	Too numerous to count	Too numerous to count
10^{-5}	78, 72 (avg. 75)	80, 75 (avg. 78)
10^{-6}	21, 18 (avg. 20)	23, 18 (avg. 21)
10^{-7}	5, 2 (avg. 4)	6, 4 (avg. 5)
10^{-8}	None	None

Table 4. Colony-forming units of T. denticola ATCC 35404 in NOS-agarose medium with and without polymyxin B (854 U/ml)

Dilutions	Without polymyxin B	With polymyxin B
10^{-4}	Too numerous to count	Too numerous to count
10^{-5}	Too numerous to count	Too numerous to count
10^{-6}	60, 50 (avg. 55)	59, 56 (avg. 58)
10^{-7}	10, 8 (avg. 9)	11. 13 (avg. 12)
10^{-8}	2, 0 (avg. 1)	1, 2 (avg. 2)

Table 5. Colony-forming units of Treponema. denticola ATCC 35404 in NOS-agarose medium with and without rifampin (20 μg/ml)+polymyxin B (40 μg/ml)

Dilutions	Without rifampin and polymyxin B	With rifampin and polymyxin B
10^{-3}	Turbid, no isolated colonies	Too numerous to count
10^{-4}	Turbid, no isolated colonies	Too numerous to count
10^{-5}	Too numerous to count	30, 20 (avg. 25)
10^{-6}	Too numerous to count	2, 1 (avg. 2)
10^{-7}	115, 120 (avg. 117)	None
CFU:	1.17×10^{9}	2.5×10^{6}

Table 6. Specific spirochete counts in plaque samples and per cent viable spirochete cells

Sample	Darkfield count	Viable count (CFU)	% viable spirochetes*
1	1.6×10^7	7.5×10^6	46.9
2	2.5×10^7	5.5×10^6	22.0
3	7.5×10^6	7.0×10^6	93.3
4	1.6×10^7	4.5×10^6	28.1
5	6.0×10^6	6.0×10^6	100
6	8.0×10^6	8.0×10^5	10
7	1.8×10^7	1.4×10^7	77.8
8	1.8×10^7	7.5×10^6	41.7
9	4.2×10^7	2.0×10^7	47.6
10	1.7×10^7	7.0×10^5	4.1
11	8.5×10^6	4.0×10^6	47.1
12	1.4×10^7	6.0×10^6	42.8
13	1.1×10^7	2.4×10^6	21.8
14	1.5×10^7	1.2×10^6	8.0
15	1.4×10^7	4.0×10^6	28.6
16	1.5×10^7	1.4×10^6	9.3

* CFU/Darkfield count x 100

Fig. 10. Serial dilution of a subgingival plaque sample exhibiting isolated spirochete colonies in high dilutions (10^{-4}, 10^{-5}, 10^{-6}).

Fig. 11. The per cent viable counts of oral spirochetes from subgingival plaque samples calculated from the relationship CFU spirochetes/ (CFU anaerobic bacteria + CFU spirochetes) x 100.

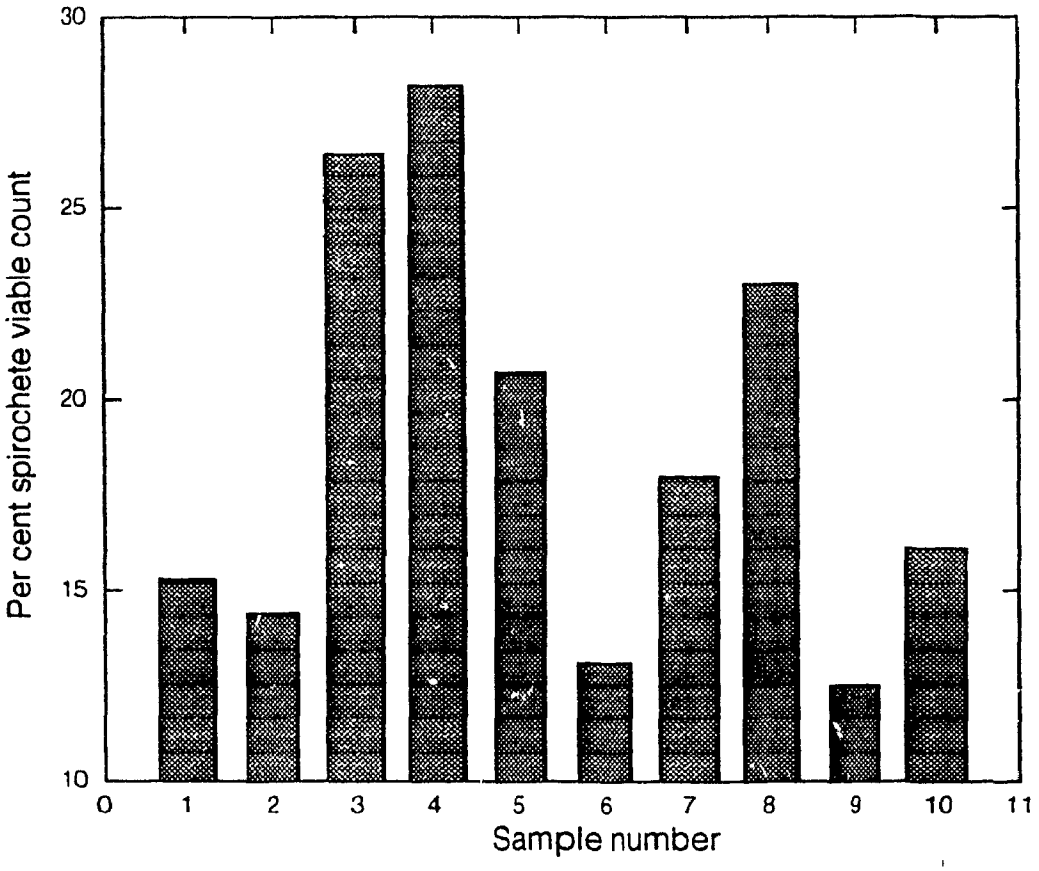

Per cent spirochete viable count
30
25
20
15
10
0
1
2
3
4
5
6
7
8
9
10
11
Sample number

Fig. 12. Large spirochetes isolated by viable count. Note the large whorl of intertwined spirochete cells. Such whorls are common in large spirochete cultures.

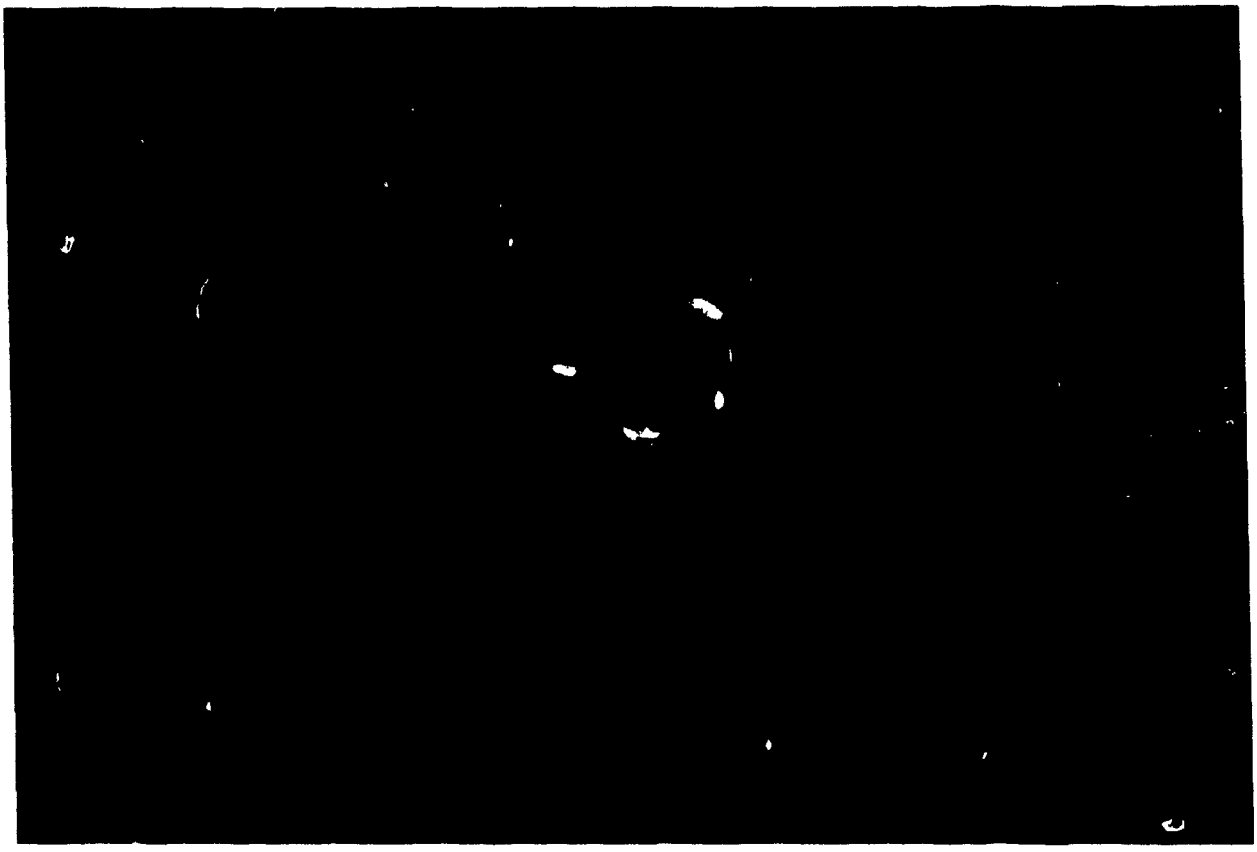


Fig. 13. Two-layer chemotaxis experiments. In A and B, the flasks contained NOS-Noble agar medium with supplements inoculated with *T. denticola* ATCC 35405 as bottom layer, NOS-agarose medium without supplements (A) or with supplements(B) as top layer. In C and D, the flasks contained NOS-agarose medium with supplements inoculated with same spirochete cells as bottom layer, NOS-Noble agar medium without supplements (C) or NOS-agarose medium with supplements (D) as top layer.

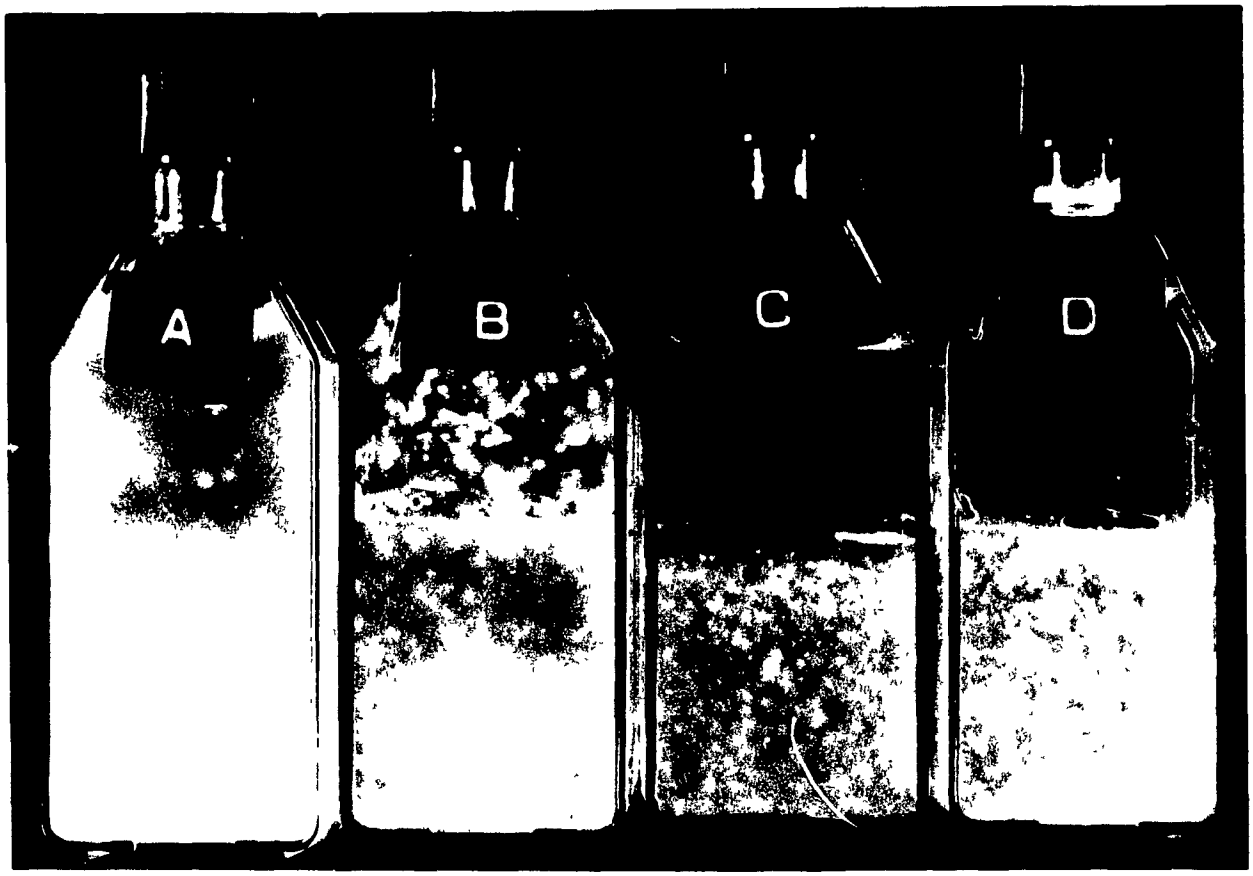


Fig.14. Two locomotory phenotypes of oral spirochetes. The top and bottom layers contained NOS-agarose medium with supplements and the middle layer contained NOS-Bacto agar medium inoculated with T. denticola ATCC 35405(middle flask) and top layer spirochete cells (right) and bottom layer spirochete cells (left) obtained from previous experiments.

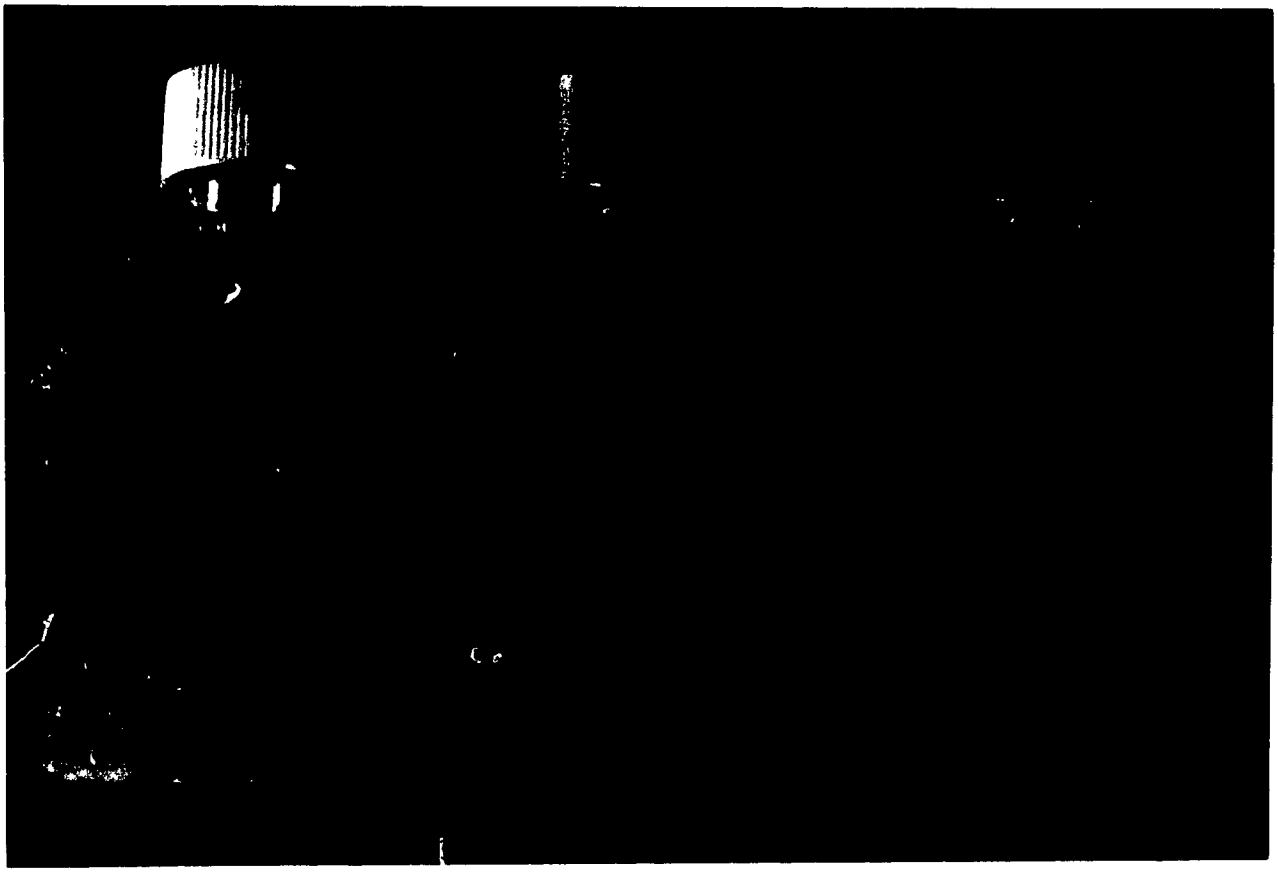
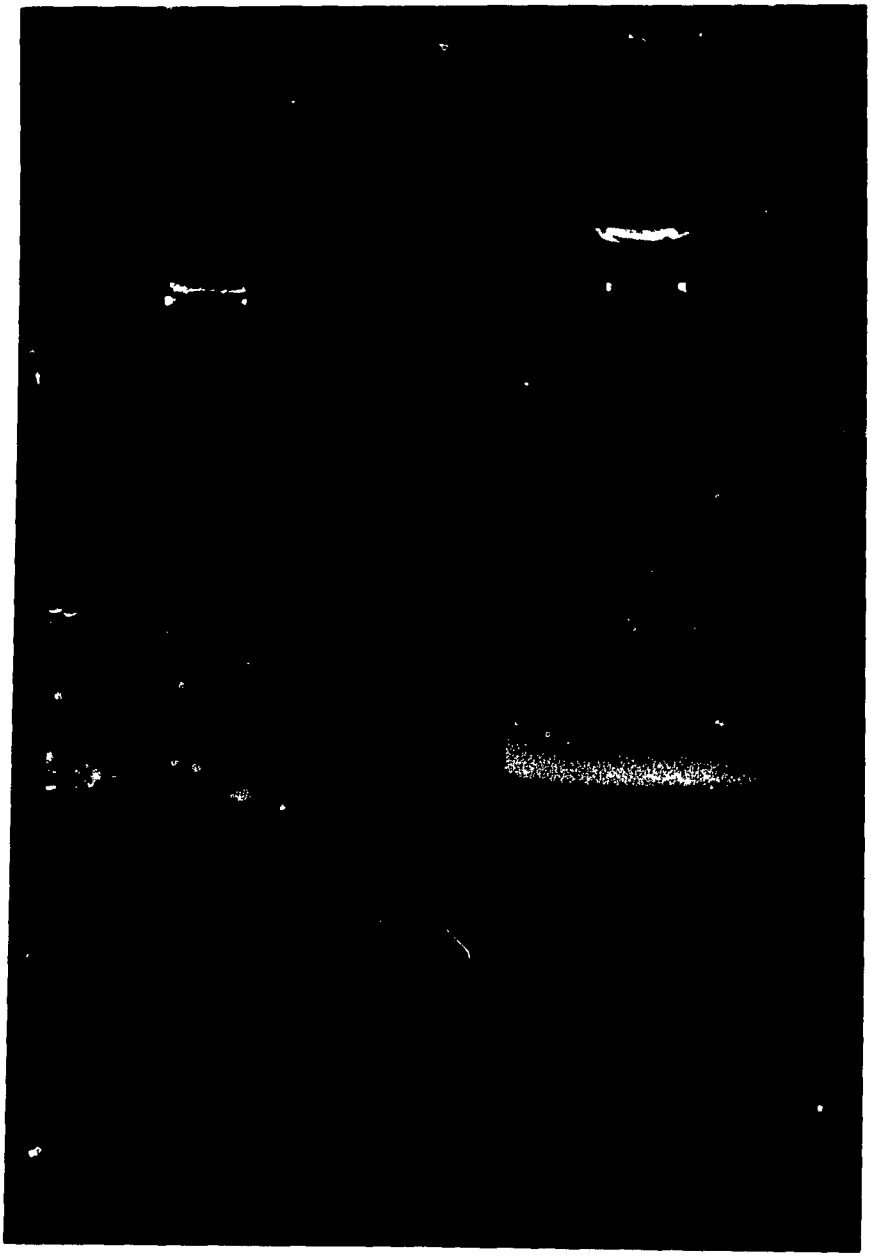


Fig.15. NOS-agarose medium used in all of three layers and spirochete cells were inoculated in the middle layer with (right) and without (left) supplements.



When NOS-Bacto agar medium without supplements was used as the middle layer and inoculated with spirochete cells, the middle layer was clear (no growth). The majority of the spirochete cells migrated to the top layer and the minority of cells migrated to the bottom layer and formed colonies.

In control flasks in which NOS-agarose medium was used in all of the three layers and spirochete cells of T. denticola ATCC 35405 were inoculated in the middle layer, spirochete colonies were only observed in the middle layer. Many more spirochete colonies were obtained in the middle layer when supplements were used than that without supplements (Fig.15).

DISCUSSION

Previous studies of Salvador, et al.(1987) showed that the overwhelming majority of the spirochetes observed microscopically in subgingival plaques were not cultivable by the methods used. Serial dilution of oral spirochetes, when plated in the conventional manner in Petri plates containing appropriate growth medium, would not result in a reliable viable count of CFU and the highest recoveries of oral spirochetes averaged only about 1 % of the total cultivable counts. Therefore, the issue has been raised as to what is the "gold standard" in detection and enumeration of subgingival microflora in periodontal infection (Loesche, et al., 1992).

Various modifications of the pour plate method were made in this study and were used to enumerate viable oral spirochetes from pure culture in fluid NOS medium. None of these modifications was successful in giving a consistent viable count of CFU. The recoveries of oral spirochetes from pure cultures of Treponema denticola by these modified culturing techniques were only about 0.1 to 1 % of the darkfield count, which are in general agreement with previous findings (Salvador, et al.1987). No isolated colonies of T. vincentii ATCC 35580 and T. socranskii ATCC 35536 were obtained even after prolonged incubation (about 3 to 5 weeks). These two species of oral spirochetes grew consistently well in fluid NOS medium in our laboratory and cell densities reached 10^8 to 10^9 cells/ml by darkfield counting. These results suggest

that plate methods cannot be used in quantitative recovery of spirochetes even from pure cultures because the viable count of oral spirochetes obtained is too low even when the spirochetes' nutritional needs are met in the agar medium. The results provided by these methods are not reliable and reproducible. The inability of the viable count to coincide with the darkfield count of spirochetes from pure culture by plate methods may be explained by several factors. For example, the deficiency of the culture vessel- Petri plate in providing a favorable environment for the growth of oral spirochetes, such as in the attainment of anaerobiosis since the medium depth is shallow. Medium depth is important because spirochetes all form subsurface colonies within the medium (Fig.5, Fig 6a and 6b). Also, Petri plates may not be good in retaining moisture and volatile fatty acid(VFA) which are important components in the growth of spirochetes(Canale-Parola, 1984).

Although an increased isolation frequency of T. denticola from diseased sites has been reported previously by using the tube method (Moore, et al. 1985) and oral spirochete colonies were consistently formed in test tubes containing NOS medium with 0.7% Noble agar (from our results), the test tube method was found to be inconvenient to use for the quantification of oral spirochetes. The round culture tube does not facilitate the accurate counting of CFU, especially when numerous spirochete colonies are present. Another disadvantage in using this method is the difficulty in picking cells from a CFU formed in the depth of the medium to carry out identifications (Fig.5). As can be seen from Fig. 8, the small volume contained within the wide-mouth jar makes it

impractical to use such jars as culture vessels to quantify numerous spirochetes in the dental plaques.

The results indicate that cultural methods tested by us as decribed above are not suitable for use in the isolation of spirochetes from dental plaques or in biochemical testing of isolated oral spirochetes.

A successful viable count of oral spirochetes was finally obtained with the utilization of an alternate culture vessel: the polystyrene tissue culture flask. The use of polystyrene tissue culture flasks is convenient because of their commerical availability, sterility on receipt, clear flat optical surfaces (facilitate counting of CFU), capacity for gaseous exchange (to attain anaerobiosis in the anaerobic chamber), prevention of medium dehydration and VFA escape during incubation, and ease of removal of the colonies formed in the medium to do subsequent tests.

Spirochetes of T. denticola ATCC 35404, ATCC 35405, T. vincentii ATCC 35580 and T. socranskii ATCC 35536 in log-phase growth in fluid NOS medium were used for evaluation of this viable count method. Reliable, consistent and reproducible viable counts of these pure spirochete cultures were obtained. As can be seen in Figures 6a and 6b, colonial morphologies of different species of oral spirochetes are distinctive. The colonies of T. denticola are dense and compact, whereas those of T. vincentii and T. socranskii are diffuse and large. In addition, T. vincentii colonies form earlier than those of T. socranskii. The

distinctive colonial morphologies of oral spirochetes may be used to identify some species as is done for other bacteria, such as red and large colonies of Serratia marcescens, yellow and small colonies of Micrococcus luteus, etc. This viable count technique is currently used in our laboratory to assess the purity of oral spirochete cultures by means of colonial morphology. For instance, two different colonial morphotypes have been found in a "pure" spirochete culture (T. denticola ATCC 35405) which is widely used in oral studies.

Another critical component to obtain successful viable count of oral spirochetes was the use of low temperature-gelling agarose at 0.7% in complete NOS medium. Noble agar and Bacto agar, commonly used as gelling agents in many media, have been proven to contain inhibitory substances which inhibit the growth of oral spirochetes (Fig. 7) The inappropriate agar incorporated in spirochete media may be one of the reasons for the low recovery of spirochetes from subgingival plaques by culturing by other workers (Salvador, et al., 1987)

It is apparent that oral spirochetes are highly sensitive to heat exposure. As can be seen in Table 2, oral spirochetes are injured or killed at 50°C, although most other procaryotes may survive at exposure in molten agar medium. The heat sensitivity of oral spirochetes is evidenced by their late colonial development and decreased numbers of CFU at this temperature. The delay in colony formation may due to heat injury necessitating time for cell recovery or physiological adaptation. It might

be noted that, historically, fever therapy was used to treat syphilis (Youmans, et al., 1985). However, the information on heat sensitivity of oral spirochetes is extremely limited, which may be because of the inability to quantitatively recover them from plaques.

The NOS-agarose medium method in tissue culture flasks has been successfully used to follow the growth curves of two species of oral spirochetes in fluid NOS medium. As can been seen in Figures 9a and 9b, T. vincentii showed a slower growth rate than T. denticola. More importantly, this method allowed us to quantitatively enumerate the growth of oral spirochetes by a viable count, thus overcoming the disadvantage of a darkfield count in which both viable and dead cells are counted.

One of the difficulties that investigators have in the quantitative recovery of spirochetes from dental plaque is the choice of an effective selective agent. Selective agents are important. As shown by Stanton and Canale-Parola (1979), the ratio of the number of rumen spirochete colonies to the total number of colonies was markedly greater in the medium containing rifampin than that in the absence of rifampin; therefore, rifampin had been proven to be a selective agent. However, as noted, the concentrations of rifampin when employed as a selective agent in agar medium were very low, e.g., 1μg/ml (Stanton and Canale-Parola, 1979), 2 μg/ml (Leschins and Canale-Parola, 1980), etc., although oral treponemes are found resistant to rifampin at 50 μg/ml (Cheng, et al.,

1985). The evaluation of antibiotics for use as selective agents of oral spirochetes has been hampered by lack of a reliable method to quantify them by viable count.

Rifampin at 4 μg/ml was shown to have no inhibitory effect on the growth of T. denticola ; furthermore, we found that rifampin is a suitable selective agent to use in NOS-agarose medium since its concentration at 20 μg/ml had no effect on spirochete counts (Table 3) and inhibited virtually all other bacterial species (Fig.12).

By the method described above, it is now possible to obtain a viable count of oral anaerobic spirochetes from subgingival plaque samples. Paper point sampling was chosen since significantly higher numbers of spirochetes have been found for paper point sampling than for curette sampling (Renvert, et al., 1992). To be expected, the specific count of spirochetes in most samples was approximately 10-fold lower by viable count than by microscopic count (Table 6). This may be explained by the obvious reasons for discrepancies between microscopic counts and viable counts: dead spirochete cells could not be enumerated by a viable count; the NOS-agarose rifampin medium used might not have met the nutritional requirements of some oral spirochetes; some oral spirochetes could have been inhibited by the high concentration of rifampin used (although T. denticola, T. vincentii, and T. socranskii are not inhibited by rifampin at 20 μg/ml); spirochetes forming atypical colonies would not have been detected by our viable count procedures. Such limitations are inherent in all viable count methods and the one we have described above for recovery

of oral spirochetes by culture is no exception. The per cent viable spirochetes, calculated from the ratio of the colony-forming units to the darkfield count expressed as a percentage, is also shown for the different samples in Table 6. The mean was calculated to be 39.3 with a standard error of 7.4. However, an inspection of these values shows the range to be very large, from 4.1 to 100%, and that the variation between samples was considerable. In the very high value samples, the assumption may be made that the recovery of spirochetes by culture approached that of the microscopic counts. This implies that all, or nearly all, the spirochetes in the sample were viable, the medium employed satisfied their nutritional needs and the tissue culture flasks used as culture vessels provided a favourable environment for their growth.

The per cent recoveries of viable spirochetes based on the total anaerobic cultivable bacterial flora, i.e., the ratio of spirochete colony-forming units to total anaerobic bacteria colony-forming units expressed as a percentage, ranged from 12.5 to 28.2% as shown in Fig. 11; the average value was about 20%. Loesche (1988) summarized the per cent recoveries, obtained by microscopic examination of spirochetes in subgingival plaque removed from sites classified as adult periodontitis, to range from 19 to 57% (from 16 citations) with an average of 40%. It is apparent that our method for enumerating viable counts of spirochetes from subgingival plaque samples provides per cent recoveries approaching that of values obtained by microscopic counts. Therefore, the viable count values obtainable by the method advocated in this investigation are within

the range of acceptability for studies on the association of spirochetes with periodontal disease. It may also be noted that previous work on viable counts of oral spirochetes (Salvador, et al., 1987) yielding the highest recoveries that averaged only about 1% of the total cultivable counts. Inappropriate procedure probably accounted for their results, i.e., curette sampling, a pour plate method which we have evaluated, and incorporating a low concentration of rifampin (5 μg/ml) as selective agent which may not be efficient enough to inhibit the growth of other bacterial species.

The value of the viable count technique we have developed for enumeration of viable spirochetes in periodontal pockets will only be proven when investigators in other laboratories employ it in their work, for example, using patients with different clinical conditions For the present, this method as described allows for the culturing and concomitant biochemical analyses of subgingival spirochetes, thus meeting the "gold standard" by which detection and enumeration of subgingival microflora is carried out. Just as important, this method permits the simultaneous isolation of tiny, intermediate-sized, as well as large spirochetes in the course of counting viable spirochetes from periodontal pockets. In the case of the large spirochetes, probably membrane filters used conventionally in the isolation of spirochetes from subgingival plaque might have prevented their migration through the filters. However, with the viable counting method in NAR medium, the plaque sample is placed directly into the medium without the limiting

porosity of a filter, and spirochetes of all sizes, large and small, can grow and form colonies provided the nutrition of these spirochetes is satisfied by the medium used. Thirteen new oral spirochetes have been isolated. Characterization of such oral anaerobic spirochetes, isolated and maintained in pure cultures, is underway in our laboratory.

The negative chemotaxis of T. denticola ATCC 35405 was detected when spirochete cells were inoculated into NOS-Bacto agar and NOS-Noble agar media. (Bacto agar and Noble agar, especially Bacto-agar, have been demonstrated to contain inhibitory substances for the growth of spirochetes when used as topping layers in the flasks.). The oral spirochetes migrate out of NOS-Bacto agar and NOS-Noble agar media into NOS-agarose medium and form colonies since agarose does not contain growth-inhibiting substances for these microorganisms. Viscosity-dependent locomotion of oral spirochetes determined by using darkfield optics coupled to time-lapse video technique (Klitorinos, et al., 1993) has been suggested to be a virulence factor to initiate periodontal disease. Chemotaxis may be one of the several methods governing the interaction of spirochetes with gingival tissues. However, chemotaxis of spirochetes in general is poorly understood (Goldstein and Charon, 1990). The viable count technique now allows us to detect the migration of spirochete cells, either positive or negative chemotaxis, by observing colony-forming units. In this way, the method we developed is also valuable in spirochete locomotory studies.

The finding that NOS-agarose medium with supplements attracted more spirochete cells was not surprising Supplements provide nutrients such as protein (rabbit serum) and various VFA which are required for the growth of spirochetes.

The phenomenon of two locomotory phenotypes of oral spirchetes is of great interest to us. What causes this differing direction of migration or locomotion may form the subject of further investigation The different directions of gyration of Leptospira illini have been observed by Goldstein and Charon (1990). They showed that the rotation of the internal periplasmic flagella directed the shape and the gyration of cell ends, either clockwise or counterclockwise. The further investigations on the chemotactic behaviour of oral spirochetes will lead to better understanding of the pathogensis of oral spirochetes in periodontal disease.

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