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Penicillin Resistance in the Subgingival Microbiota Associated with Adult Periodontitis

Susan Ann Kinder

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PENICILLIN RESISTANCE IN THE SUBGINGIVAL MICROBIOTA ASSOCIATED WITH
ADULT PERIODONTITIS

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Master of Dental Science Thesis

PENICILLIN RESISTANCE IN THE SUBGINGIVAL MICROBIOTA ASSOCIATED WITH ADULT PERIODONTITIS

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Susan A. Kinder
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SECTION I. INTRODUCTION AND REVIEW OF THE LITERATURE
INTRODUCTION

The selection of an antimicrobial agent for the treatment of an infectious disease involves the consideration of a wide variety of factors. A primary concern, however, is the identification and antimicrobial susceptibility of the infecting organism. This knowledge, combined with pharmacological and host factors, provides the data base from which appropriate therapy may be determined (Sande and Mandell, 1980). Empiric therapy involves the choice of an antimicrobial agent on the basis of the microorganisms expected to be involved in a particular infection, and a general knowledge of their susceptibility patterns (Neu, 1983a). This process of antibiotic selection is of particular importance in the treatment of anaerobic infections. The relatively long period of time required for the growth and isolation of these species makes antibiotic selection on the basis of susceptibility testing impractical (Sutter and Finegold, 1976). An underlying assumption in the use of empiric therapy is the predictability of the microorganisms involved in a specific infection, and their predictable susceptibility to the agents of choice. However, the development of antimicrobial resistance among pathogenic organisms is a well documented phenomenon, and one which has clearly altered empiric therapy for a number of infectious diseases (for reviews see Neu, 1983a, 1984).

Since penicillin became available in the 1940's, it has been considered the drug of choice in the treatment of odontogenic infections (Peterson, 1981; Crawford, 1984). In recent years,
however, documentation of odontogenic infections which failed to respond to penicillin therapy (Heimdahl et al., 1980; Bahn et al., 1981; Whitcher et al., 1983; von Konow and Nord, 1983; Brook 1984a) and reports of increasing resistance to penicillin among bacterial species known to be associated with odontogenic infections (Murray and Rosenblatt, 1977; Edson et al., 1982) have initiated a reevaluation of the empiric use of this antibiotic.

The investigation presented here was undertaken to evaluate antibiotic susceptibility in the penicillin-resistance component of the subgingival microbiota associated with adult periodontitis. Bacterial species which have been frequently associated with odontogenic infections are also routinely isolated from the periodontal pocket (e.g. Bacteroides spp.: Chow et al., 1978; Williams et al., 1983), in addition to being found in increased numbers in adult periodontitis (Slots, 1977). The subgingival microbiota undoubtedly serves as a reservoir for species involved in odontogenic infections. Thus, this microbial niche represents part of the "normal" microbiota from which antibiotic susceptibility patterns may be monitored and applied to the evaluation of antibiotic therapy for odontogenic infections. Subjects either with or without a history of recent penicillin therapy were evaluated to further assess patterns of antibiotic resistance, since the detection of resistant species has been correlated to the previous use of the antimicrobial agent (Heimdahl et al., 1981; Neu, 1984).
REVIEW OF THE LITERATURE

The term "odontogenic infection" refers to an infection arising from the microbiota surrounding the teeth. Although technically this term encompasses both periodontal disease and dental caries, conventional usage of the term does not. Thus, odontogenic infections, as discussed in this work and others, refer to various infectious states related to the dentition; including pulpal, periapical, dentoalveolar, fascial space, and pericoronal infections, as well as periodontal abscesses (Chow et al., 1978).

The Microbial Etiology of Odontogenic Infections

Over the past 20 years, profound advances have been made in anaerobic microbiological techniques. These have included the recognition of, and supplementation of growth media to accommodate, the complex nutritional requirements of anaerobic bacteria (Loesche, 1968); as well as improvements in the maintenance of a continuous anaerobic environment achieved through the development and implementation of pre-reduced transport and growth media (Syed and Loesche, 1972), and anaerobic techniques of sample manipulation (Gordon et al., 1971). The results of these technological developments have resulted in 4 to 8 fold increases in the cultivable recovery of bacteria from anaerobic environments (Gordon et al., 1971).

Recognition of these improvements provides an important perspective when evaluating the literature available on odontogenic infections. Investigations into the microbiological basis of these
infections employing inadequate anaerobic techniques have typically reported streptococci and Staphylococcus spp. to be predominant isolates, often in pure culture; and the prevalence, or rate of detection, of anaerobic species in the range of 0 to 30% (Sims, 1974; Turner et al., 1975; Matusow, 1981).

In contrast, in investigations using improved anaerobic techniques, the prevalence of anaerobic species from odontogenic infections has been reported to range from 90 to 100% of the specimens, with more than one bacterial species isolated from 88 to 100% of the specimens (Chow et al., 1978; Bartlett and O'Keefe, 1979; Aderhold et al., 1981; Brook et al., 1981; von Konow et al., 1981; Williams et al., 1983). Typically, an average of from 3 to 6 different organisms have been recovered from individual infections (Goodman, 1977; Bartlett and O'Keefe, 1979). The combination of microorganisms reported indicates a predominance of the anaerobic component (e.g. average of 3 anaerobic to 1 aerobic species per specimen in the report of Chow et al. (1978)), but often reflects a mixture of both anaerobic and facultative or aerobic species (e.g. 52% of specimens yielded both aerobes and anaerobes in the report of Chow et al. (1978)). Thus, the microbiological profile of odontogenic infections is now recognized to be primarily anaerobic and polymicrobial.

The microflora associated with odontogenic infections (Sabastian et al., 1976; Sundqvist 1976; Chow et al., 1978; Bartlett and O'Keefe, 1979; Newman et al., 1979; Aderhold et al., 1981; Brook et al., 1981; von Konow et al., 1981; Williams et al., 1983), with rare exceptions, consists of organisms indigenous
to the oral cavity (Tanner et al., 1979; Moore et al., 1985). Therefore, these infections are considered endogenous in nature. The microbiological recovery reported from odontogenic infections has encompassed a wide range of bacterial species, representing many different genera. Typically, these data have been reported in two forms: as the prevalence of microorganisms recovered, or as the number of different species isolated expressed as a percentage of the species isolated from all of the infections combined. Predominant genera consistently associated with odontogenic infections on the basis of these data include Bacteroides, Fusobacterium, Peptococcus, and Peptostreptococcus. Bacteroides spp. have been detected in as many as 80% of the odontogenic infections examined in one investigation (Williams et al., 1983), and as a percentage of the total number of isolates recovered from odontogenic infections have ranged from 10 to 14 percent (Goodman, 1977; Brook et al., 1981). Fusobacterium spp. have been reported with a prevalence as high as 60% (Williams et al., 1983), and generally account for from 2 to 20% of the isolates in these investigations (Goodman, 1977; von Konow et al., 1981). The involvement of Peptococcus and Peptostreptococcus spp. is indicated by detection rates as high as 23% and 52%, respectively (Chow et al., 1978). Although Streptococcus spp. have been reported in microbiological studies of odontogenic infections, they appear to be overrepresented in these data when quantitative considerations, and their possible role as sampling contaminants, were evaluated (Sabastian et al., 1976). It is of note that the genera which appear to be predominant in odontogenic infections include species
which are routinely isolated specifically from the periodontal microbiota (Slots, 1977; Moore et al., 1985).

The role of pigmented *Bacteroides* spp. in odontogenic infections has been of particular interest. Numerous investigators have examined the "infectivity" of plaque microorganisms, based on the production of transmissible infections in a guinea pig model (MacDonald et al., 1963; Socransky and Gibbons, 1965; Sundqvist et al., 1979; Mayrand and McBride, 1980). These studies demonstrated that when transmissible infections were produced after inoculation with subgingival plaque only a few species were recovered from the abscess produced, and pigmented *Bacteroides* spp. were uniformly present. Reinoculation with different combinations of the strains recovered from abscesses have indicated a key role of pigmented *Bacteroides* spp.; transmissible infections were produced only when a pigmented *Bacteroides* strain was included in the combination of organisms inoculated. With few exceptions, the pigmented *Bacteroides* spp. were not infective alone, but required the presence of at least one other organism. Mayrand and McBride (1980) have found that the minimum infective combinations consisted of pigmented *Bacteroides* spp. in addition to a second "helper" organism, and any succinate-producing organism was able to function as the "helper" strain. The succinate produced was shown to replace hemin in supporting the growth of the *Bacteroides* spp. This type of interbacterial growth dependency reflects the polymicrobial nature of anaerobic infections, and in particular illustrates the importance of pigmented *Bacteroides* in infections arising from the microbiota of the gingival crevice.
Many other bacterial genera, either anaerobic or facultative and including species with significant pathological potential, have been reported to be associated with odontogenic infections (e.g. *Eikenella*), but less frequently than the genera discussed above (Bartlett and O'Keefe, 1979). In addition, recently described genera such as *Capnocytophaga* and *Wolinella* (Leadbetter et al., 1979; Tanner et al., 1981) include species that have been associated with odontogenic infections (Newman and Sims, 1979; Tanner et al., 1982), and these species may therefore be underrepresented in the data currently available on the microbiology of odontogenic infections.

In summary, with the advent of improved anaerobic cultivation techniques, the role of anaerobic species in odontogenic infections has become apparent. These infections have been demonstrated to be endogenous, and associated with a primarily polymicrobial and anaerobic microbiota. Predominant genera involved appear to include *Bacteroides*, *Fusobacterium*, *Peptococcus* and *Peptostreptococcus*. Importantly, the bacterial species frequently recovered from odontogenic infections correspond to organisms which are indigenous in the periodontal microbiota. A key role of pigmented *Bacteroides*, in particular, in these infections is supported by investigations of the "infectivity" of subgingival species in experimental animal models.

**Antimicrobial Treatment of Odontogenic Infections**

Penicillin G became available for use clinically in the 1940's, and since that time has been considered the drug of choice in the
treatment of odontogenic infections (Peterson, 1981; Goodman and Newman, 1984). Penicillin and ampicillin are beta-lactam antibiotics, and function by binding to proteins located in the inner cytoplasmic membrane (penicillin binding proteins; PBP's). The PBP's are enzymes involved in the synthesis of peptidoglycan, and are inactivated when bound by penicillin. The result is a blocking of cell wall synthesis, and cell lysis in susceptible microorganisms (Neu, 1983b). Both penicillin G (oral form: penicillin V) and ampicillin are considered broad spectrum antibiotics, but ampicillin has an expanded range of activity including more Gram-negative species than penicillin G. The beta-lactams offer the advantage of low toxicity. Allergic reactions are the primary side effects, and have been reported to occur in 0.7 to 10% of patients administered the antibiotic (Mandell and Sande, 1980).

For penicillin allergic patients, alternative therapy with tetracycline or metronidazole has been recommended (Goodman and Newman, 1984). Tetracycline is a bacteriostatic agent which functions by binding to the 30S ribosome of the target organism, thus preventing the binding of transfer RNA and inhibiting protein synthesis (Chopra and Howe, 1978). The broad spectrum of activity of tetracycline includes many Gram-negative anaerobic microorganisms. Tetracycline is contraindicated for use in pregnant women or children due to it's incorporation into calcified tissues which will result in discoloration of developing teeth (Sande and Mandell, 1980).
Metronidazole is a synthetic compound with a spectrum of activity specific for anaerobic organisms. This compound is reduced by electron transport proteins in susceptible bacterial cells, and the reduced form is thought to disrupt DNA synthesis resulting in cell death. Ingestion of alcoholic beverages during metronidazole therapy results in an antibiotic effect, and the use of metronidazole is contraindicated in women in the first trimester of pregnancy due to reports of carcinogenesis in rodents and mutagenesis in bacteria (Rollo, 1980).

Clindamycin is a bacteriostatic antibiotic which binds to the 50S ribosome in susceptible microorganisms, resulting in an inhibition of protein synthesis and cell death. The spectrum of activity of clindamycin includes aerobic Gram-positive organisms, as well as Gram-positive and Gram-negative anaerobic species. Clindamycin is reported to cause diarrhea in 2 to 20% of the patients prescribed this antibiotic, with the potentially lethal side effect of pseudomembranous colitis to occur in as many as 20% of these patients. The latter condition has been associated with an intestinal overgrowth of Clostridium difficile. Due to the incidence of pseudomembranous colitis, the use of clindamycin has been recommended to be limited to severe infections, and consultation with a physician is recommended (Sande and Mandell, 1980; Goodman and Newman, 1984; Hammill, 1984).

Penicillin Resistance

Neu (1984) in a recent review summarized the mechanisms of antibiotic resistance in five categories: 1) altered receptors for
a drug, 2) decreased entry of a drug into the target cell, 3)
development of alternative resistant metabolic pathways, 4)
destruction of a drug, and 5) combinations of the first four
mechanisms. The best known mechanism of penicillin resistance,
and the most pertinent to this discussion, is drug inactivation by
the enzyme beta-lactamase. Both penicillins and cephalosporins are
characterized by a beta-lactam ring in their molecular structure,
and are thus termed beta-lactam antibiotics. These compounds are
degraded by a number of hydrolytic enzymes produced by Gram-negative
bacteria (Sykes, 1979). The most common and important degradation
of the beta-lactam antibiotics results from the action of beta-
lactamases. These enzymes hydrolyze the cyclic amide bond of beta-
lactams, producing cephalosporates or penicilloates, which are
antibacterially inactive. All beta-lactamases act on penicillins
and cephalosporins, but vary in the relative rate of hydrolysis of
the different antibiotics. As the number of beta-lactamases
described has continued to increase, a variety of classification
schemes have been proposed. The simplest classification relies on
the relative rate of hydrolysis of beta-lactam antibiotics and the
enzymes are classified as penicillinases or cephalosporinases on
this basis (Richmond and Sykes, 1973; Sykes and Matthew, 1976;
Ambler et al., 1980).

In Gram-positive microorganisms, beta-lactamase production is
the major factor in determining resistance. Gram-positive
microorganisms generally produce inducible beta-lactamases, which
are synthesized in large quantities in the presence of an inducer,
e.g., a beta-lactam antibiotic. In addition, the beta-lactamases of
Gram-positive organisms are almost all excreted extracellularly (Sykes and Matthew, 1976; Del Bene, 1979). This is of significance in that Gram-positive beta-lactamases may exert a "population effect"; with enough of the enzyme excreted into the environment it may inactivate the antibiotic to the extent that other, beta-lactam susceptible microorganisms, are protected (Brook et al., 1984).

In contrast, with few exceptions, the beta-lactamases of Gram-negative bacteria are cell bound, they may be either constitutive or inducible, and they are produced in smaller quantities than that produced by Gram-positive species (Sykes and Matthew, 1976; Del Bene, 1979). Beta-lactamase production in Gram-negative species accounts in part for resistance to beta-lactam antibiotics. A permeability barrier also contributes resistance. The term "crypticity" has been used to describe the relationship between the permeability barrier and the beta-lactamase activity of Gram-negative species. Crypticity is defined as the specific enzyme activity of disrupted cells divided by the specific enzyme activity of whole cells. If a permeability barrier exists, the enzyme activity of disrupted cells will be greater than that of whole cells, and the crypticity value will be greater than 1 (Sykes and Matthew, 1976). Whereas Gram-negative facultative microorganisms have crypticity values in the range of 10 to 20, obligate anaerobes typically have values less than 2 indicating a minimal contribution by permeability barriers in determining resistance among anaerobes (Del Bene, 1979).

The genetic basis of beta-lactamase production may be chromosomal or plasmid mediated. In general, the cephalosporinases
tend to be chromosomally mediated, and the penicillinases tend to be plasmid mediated (Richmond and Sykes, 1973). Plasmids are small pieces of DNA which exist separate from the chromosome, and plasmids may be transferred not only between bacteria of the same species, but between species of different bacterial genera (Del Bene, 1979). R-plasmids, or plasmids carrying the genetic information coding for antibiotic resistance, were first described in the 1950's and within eight years, the transmissibility of R-factors was recognized as a world-wide problem (for a review see Falkow, 1975). Bacterial species currently known to harbor R-plasmids include 18 Gram-negative and 4 Gram-positive genera. The significance of this information was extended by the discovery of transposons, pieces of DNA that appear to be freely mobile between plasmid and chromosomal DNA, as well as "promiscuously" mobile between bacterial species (Neu, 1984). The importance of R-factors and transposons to the acquisition and dissemination of resistance among bacteria cannot be overemphasized. An example of the potential for the dissemination of plasmid mediated resistance is provided by considering *Neisseria gonorrhoeae*. Penicillin resistance in this species was first reported in 1976, and the prevalence of resistant strains in this country has been seen to increase since that time. Resistant *N. gonorrhoeae* infections went from a level of 300 cases per year in the late 1970's to over 3,400 cases in the first 9 months of 1980 (Genco et al., 1984).

Beta-lactamase production in *Bacteroides* spp. other than *B. fragilis* was first reported by Pinkus et al. (1968) in *B. oralis*. Although a number of investigations on the beta-lactamases of these
species have been reported, the data is contradictory and comprehensive characterization lacking (Murray and Rosenblatt, 1977; Salyers et al., 1977; Timwell et al., 1981; Sherrill and McCarthy, 1981; Sherrill and McCarthy, 1984; Walker et al., 1984). Some reports have indicated that beta-lactamases produced by Bacteroides spp. are primarily penicillinases, whereas other report them to be primarily cephalosporinases. In addition, investigations on the genetic basis of beta-lactamase production in these species are not available in the literature. It is of note, however, that there have been numerous reports of penicillin-resistant infections documenting the presence of penicillin-resistant Bacteroides spp. (Heimdahl et al., 1980; Whitcher et al., 1983; Brook et al., 1984a).

**Penicillin Resistance and Odontogenic Infections**

In recent years, the empiric use of penicillin has been questioned. Reports of resistance* among microorganisms frequently associated with odontogenic infections, in addition to reports of clinical cases that have failed to respond to penicillin therapy have appeared in the literature with an apparent increasing frequency (data reviewed below).

*The determination of resistance versus susceptibility as discussed in this thesis is based on the classification of Finegold (1977). Because the focus of the discussion here relates primarily to outpatient therapy, resistance refers to the combination of Finegold's catagories of "intermediate" and "resistant". The minimum inhibitory concentration defining resistance therefore corresponds to 4 mcg/ml of penicillin G, 4 mcg/ml of ampicillin, 4 mcg/ml of clindamycin, 4 mcg/ml of tetracycline and 16 mcg/ml of metronidazole.
Bacteroides spp., in particular pigmented Bacteroides spp. have been frequently reported to be predominant among the microorganisms associated with odontogenic infections (data reviewed previously). Although the non-fragilis Bacteroides spp. were once considered routinely susceptible to penicillin, data reported since 1972 from the Mayo Clinic has indicated the development of resistance to penicillin in these species (Martin et al., 1972; Stanech and Washington, 1974; Murray and Rosenblatt, 1977; Edson, 1982). The minimum inhibitory concentration (MIC) required to inhibit 70% of the non-fragilis Bacteroides spp. tested was reported to be 1.6 mcg/ml of penicillin in 1972, 3.1 mcg/ml in 1974, 6.2 mcg/ml in 1977, and 50 mcg/ml in 1982. The Bacteroides spp. examined in these data are the pigmented Bacteroides and the B. oralis group. A clear trend of increasing resistance is evident in this these data, with a dramatic increase seen in the resistance to penicillin in the 1982 report. In interpreting the Mayo data, one should recognize that the isolates examined are of unspecified origin ("clinical isolates"), and from individuals of whom the history of antibiotic usage is not reported. However, the documentation of resistance, regardless of the source of the isolates, is significant in demonstrating the potential for the development of resistance in a particular organism in any environment.

Investigations of isolates from the periodontal microbiota have reported the concentration of penicillin G required to inhibit 90% of the strains (MIC$_{90}$) to be 0.5 U/ml for pigmented Bacteroides from patients with no history of recent antibiotic therapy (Sutter et al., 1983); and to range from 0.072 mcg/ml for B. melaninogenicus...
ss melaninogenicus to 4.8 mcg/ml for B. melaninogenicus ss intermedius from patients of whom the previous use of antibiotics was unspecified (Baker et al., 1983).

Penicillin resistance in Bacteroides species has been correlated with the production of the enzyme beta-lactamase (Murray and Rosenblatt, 1977; Salyers et al., 1977; Laatsch et al., 1982). In the periodontal microbiota, beta-lactamase-producing Bacteroides in periodontal sites were reported from 7 of 26 subjects examined by Valdés et al. (1982).

Reports have appeared in the literature on patients with odontogenic infections that have failed to respond to therapy with penicillin. Documented cases of infections clinically resistant to therapy with penicillin have included submandibular, perimandibular, periapical and dentoalveolar abscesses of odontogenic origin, and have been reported with an apparent increased frequency in recent years (Heimdahl et al., 1980; Bahn et al., 1981; Brook et al., 1981; von Konow and Nord, 1983; Whitcher et al., 1983; Brook, 1984a). Most of these infections have been associated with beta-lactamase-producing Bacteroides spp., underscoring the importance of penicillin resistance in these organisms.

The development of resistance among bacterial species is known to be enhanced through the use of certain antimicrobial agents, and this phenomenon has been demonstrated in the oral microbiota with penicillin therapy. Heimdahl et al. (1981) found beta-lactamase-producing Bacteroides spp. in the salivary microbiota to be significantly more prevalent in subjects with a recent history of penicillin administration in contrast to subjects with no recent
history of antibiotic use. The emergence of beta-lactamase-producing microorganisms in the oropharyngeal microbiota of children has been documented longitudinally; 3 of 21 (14.3%) children harbored beta-lactamase-producing species initially, and 10 of the same 21 subjects (47.6%) demonstrated beta-lactamase-producing species one week after a course of either penicillin or ampicillin (Brook, 1984a)

In summary, the efficacy of penicillin therapy in the treatment of odontogenic infections has been questioned subsequent to reports of penicillin resistance among *Bacteroides* spp. associated with these infections and reports of odontogenic infections which have failed to respond to penicillin therapy. Surveys of the "normal" microbiota from which these infections arise, have indicated significant levels of resistance among some species of concern. Furthermore the development, or emergence, of penicillin-resistant oral microorganisms has been associated with the recent use of the antibiotic.
SECTION II.

MANUSCRIPT SUBMITTED TO JOURNAL OF PERIODONTOLOGY

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PENICILLIN RESISTANCE IN THE SubGINGIVAL MICROBIOTA
ASSOCIATED WITH ADULT PERIODONTITIS

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The penicillin-resistant and beta-lactamase-producing subgingival microbiota associated with adult periodontitis was characterized, and the impact of a recent exposure to penicillin on the recovery of resistant organisms assessed in this investigation. Subjects with adult periodontitis were examined clinically and microbiologically: 21 had a documented history of penicillin therapy within the previous 6 months; 21 had no history of antibiotic use within 1 year. Subgingival plaque samples were cultured anaerobically on non-selective and penicillin-containing selective media. MIC's and beta-lactamase production were determined for isolates from the selective medium. The penicillin-resistant microbiota consisted primarily of Gram-negative organisms, including Bacteroides, Veillonella, Haemophilus, Actinobacillus, Eikenella, and Capnocytophaga spp. Significantly greater proportions of penicillin-resistant pigmented Bacteroides and Veillonella species were recovered from subjects with penicillin exposure. Beta-lactamase production was detected in pigmented Bacteroides, Capnocytophaga and Streptococcus species. The prevalence of beta-lactamase-producing Bacteroides was increased 2-fold in subjects with recent penicillin exposure. The susceptibility of penicillin-resistant strains to other antibiotics indicated that metronidazole may be useful for penicillin-resistant infections arising from the subgingival microbiota.
INTRODUCTION

Although penicillin has long been considered the drug of choice in the treatment of odontogenic infections (Peterson, 1981; Goodman and Newman, 1984), reports of infections resistant clinically to penicillin therapy (Heimdahl et al., 1980; Bahn et al., 1981; Brook et al., 1981; von Konow and Nord, 1983; Whitcher et al., 1983; Brook, 1984a), as well as reports of penicillin resistance among species associated with these infections (Murray and Rosenblatt, 1977; Edson et al., 1982), have initiated a reexamination of the empiric use of this antibiotic. A variety of bacterial genera have been associated with odontogenic infections, but predominant genera recovered include Bacteroides, Fusobacterium, Peptococcus and Peptostreptococcus spp. (Chow et al., 1978; Bartlett and O'Keefe, 1979; von Konow et al., 1983; Williams et al., 1983).

The involvement of Bacteroides species in odontogenic infections is of particular interest, as investigations of abscess formation in animal models indicate a key role of these species in the production and transmissibility of experimental infections (MacDonald et al., 1963; Sundqvist et al., 1979; Mayrand and McBride, 1980). Increasing patterns of penicillin resistance have been reported among non-fragilis Bacteroides species in surveys from hospital clinical laboratories (Murray et al., 1977; Edson et al., 1982). In addition, microbiological examinations of infections resistant to penicillin therapy have frequently yielded beta-lactamase-producing Bacteroides spp. (Heimdahl et al., 1980; von Konow et al., 1983; Brook, 1984a). The production of beta-lactamase has been shown to be mediated in some bacterial species by plasmids,
and associated with the widespread development of resistance to beta-lactam antibiotics (Neu, 1984). The use of beta-lactam antibiotics, in particular, has been associated with the emergence of beta-lactamase-producing Bacteroides species in the oral microbiota (Heimdahl et al., 1981; Brook, 1984b). Many of the bacterial species implicated in odontogenic infections are routinely isolated from the subgingival microbiota and, as in the case of Bacteroides spp., are found in increased numbers in the subgingival microbiota of individuals with adult periodontitis (Slots, 1977; Tanner et al., 1979; White and Mayrand, 1981). The present investigation was undertaken to characterize the penicillin-resistant and beta-lactamase-producing subgingival microbiota from individuals with adult periodontitis, and to examine the impact of a recent exposure to penicillin on the recovery of these species.

MATERIALS AND METHODS

Subjects.

Forty-two subjects, 26 males and 16 females with a mean age of 49 years, were selected from the outpatient dental clinics at the University of Connecticut School of Dental Medicine, Farmington, CT. The subjects selected were in generally good health; individuals with diabetes, autoimmune disorders, or other conditions potentially influencing their periodontal condition were not included. All subjects had adult periodontitis, based on the criteria of a minimum age of 30 years, and the presence of periodontal lesions with
probing depth >5 millimeters (mm) in addition to crestal alveolar bone loss >4 mm as measured on recent bitewing radiographs. The majority of subjects were untreated periodontally, and none had received scaling and root planning within two months prior to participation in the study. One half of the subjects had not received any antibiotics within the previous year, and were designated as the Pen (-) group. The remaining subjects, the Pen (+) group, had been prescribed a course of a non-penicillinase resistant penicillin (i.e., penicillin V, ampicillin) within the previous 1 to 6 months. The course of penicillin consisted of a minimum dosage of 1 gram per day and an average duration of 8 days in these subjects. The antibiotic prescription was confirmed through consultation with the subject's physician or dentist. Subjects with a history of chronic antibiotic use were excluded, and only one subject had received an additional separate course of penicillin within one year prior to participating in the study. Three subjects had received additional antibiotics within the previous 6 months; one subject having received tetracycline and bactrim, one received erythromycin, and one received metronidazole.

Clinical Procedures.

Clinical and microbiological examinations (see below) were carried out on two sites in the premolar/molar region per subject, with the exception of one Pen (+) subject who had only one site considered appropriate based on the selection criteria and one Pen (-) subject in which one of the microbiological samples was lost. Clinical examinations consisted of the Plaque Index (Silness and
Löe, 1964), a modified Gingival Index (Löe and Silness, 1963), probing depth and clinical attachment level measurements. The scoring protocol for the Gingival Index was modified to accommodate the sampling procedure; a score of 2 was used to indicate bleeding after gentle probing, with the remaining scores unchanged.

**Microbiological Procedures.**

Microbiological samples were obtained after careful debridement of the supragingival plaque, but prior to probing. Subgingival plaque samples were obtained and processed according to the method of Kornman et al. (1981). Briefly, three sterile paper points were placed to the depth of the periodontal pocket for a period of 10 seconds. The paper points were removed and immediately placed in a vial of sterile reduced transport fluid (RTF: Syed and Loesche, 1972), and transported to a Coy anaerobic chamber for processing (Coy Manufacturers, Inc., Ann Arbor, MI). The plaque samples were dispersed, diluted in RTF, and plated onto non-selective and selective media with an automatic diluting and plating devise (Spiral Systems, Bethesda, MD). Enriched trypticase soy agar (ETSA: Syed et al., 1980) was used as the non-selective medium, while ETSA supplemented with pencillin G was used as a selective medium. The pencillin concentration used in the selective medium was titrated to achieve a maximum level of 2 mcg/ml of pencillin G activity (data not shown). After 5 to 7 days of incubation in the anaerobic chamber at 37°C, the total number of colony forming units (CFU) was determined on both media. Colonies presumed to be pigmented
Bacteroides, on the basis of pigmentation and fluorescence under UV illumination, were subcultured from the non-selective medium. From the selective medium, representative colonies of the different morphological types found were subcultured.

Pure cultures of the strains isolated from the penicillin containing selective medium were characterized by their minimum inhibitory concentration of selected antibiotics and the presence or absence of beta-lactamase production (see below). For the purposes of this investigation, penicillin resistance was defined by an MIC > 4 mcg/ml of penicillin G or the detection of beta-lactamase production. All isolates from the non-selective medium, as well as the penicillin-resistant isolates from the selective medium were identified to the genus and species level according to current taxonomic schemes; based on colonial and cellular morphology, biochemical reactions, fermentation patterns, utilization of chromogenic substrates (An-Ident™: API, Plainville, NY), and gas-liquid chromatographic analysis of metabolic end-products (Holdeman et al., 1977; Krieg, 1984; Lennette, 1984). Selected strains were additionally characterized by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of precipitated and soluble proteins (see below).

Minimum Inhibitory Concentration Determinations.

The MIC's of penicillin G, ampicillin, metronidazole, tetracycline (Sigma Chemical, Inc., St. Louis, MO), and clindamycin (Upjohn Co., Kalamazoo, Michigan) were determined for all isolates from the selective medium. Antibiotic dilutions were prepared in sterile distilled water, and stored at -70°C until used. MIC
determinations were carried out according to the agar dilution technique recommended for anaerobic species (National Committee for Clinical Laboratory Standards [NCCLS], 1982), with the following modifications: the Wilkins-Chalgren test medium was supplemented with 3% (v/v) sheep blood (Colorado Serum, Colorado Springs, Colorado); 0.05% (w/v) sodium formate, and 0.02% (w/v) sodium fumarate. Bacterial strains to be tested were suspended in thioglycollate broth (prepared according to the NCCLS technique) to a McFarland standard of 0.5 immediately prior to inoculation. All MIC plates were inoculated in the Coy anaerobic chamber using a replicating device (Repliplate™, Cathra International, Inc.). Control cultures included in each test run were Bacteroides fragilis (ATCC 25285) and B. thetaiotaomicron (ATCC 29741), and the results for these strains were within the previously defined range of acceptable MIC values (NCCLS, 1982).

Beta-Lactamase Detection.

Beta-lactamase production was determined on all pigmented Bacteroides spp., as well as on all strains with an MIC $\geq 2$ mcg/ml of penicillin G, isolated from the selective medium. A chromogenic cephalosporin technique was used (Cefinase™ disks, BBL, Cockeysville, MD), as this method was shown to be the most reliable for anaerobic species (Burkholder et al., 1983). Positive and negative beta-lactamase controls consisted of pigmented Bacteroides strains VPI 9331 and VPI 8944, respectively (Salyers et al., 1977).
SDS-Polyacrylamide Gel Electrophoresis.

Trichloroacetic acid (TCA) precipitated and soluble proteins from selected bacterial strains were analyzed by the method of Pearlman et al. (1985). Bacterial cells were harvested in phosphate buffered saline (PBS: pH 7.2) from the surface of 3 to 6 ETSA plates which had been incubated 3 to 5 days. The cells were washed and centrifuged twice at 12,000 x g for 20 min., suspended in 2.0 ml of PBS to an approximate OD550 of 1, and centrifuged (as above). For TCA precipitation, the cells were resuspended in 0.2 ml of 10% (w/v) TCA, and incubated at 4°C for approximately 12 hours. The precipitated material was resuspended in distilled water after centrifugation (5 min.: Fisher Microcentrifuge, Model 235A) and the protein content of the preparations were estimated using the Bio-rad protein assay (Bio-Rad Laboratories, Richmond, CA). For preparation of soluble protein, the cells were resuspended in a detergent solution consisting of 1% (v/v) Triton X-100, 0.2% (w/v) SDS, and 10 mM EDTA in 10 mM Tris-hydrochloride (pH 7.8). Lysozyme and phenylmethylsulfonyl fluoride were added to a final concentration of 0.1 mg/ml and 1 mM respectively. The preparation was incubated at 37°C for approximately 12 hrs, and the insoluble material removed by centrifugation (5 min.: Fisher Microcentrifuge, Model 235A). Both preparations were stored at -70°C until used.

SDS-PAGE was carried out using the discontinuous system of Laemmli (1970), employing a 4% stacking gel and 12% resolving gel. Samples of approximately equal protein content were used, and the samples were run at a continuous current of 25 mA per gel. Gels were fixed in 1% TCA, stained for 12 hours with a Coomassie blue
solution, and destained in a solution of 10% acetic acid and 10% isopropanol for 48 hrs. Low molecular weight standards (Bio-Rad Laboratories, Richmond, CA) were included in each run. Banding patterns of the selected strains were compared to those of ATCC strains as well as strains of *Wolinella recta* 3222, and *Eikenella corrodens* S1075 and S3217 (kindly provided by Dr. G. McKinley, API, Plainville, NY).

**Statistical Analysis.**

Data analysis was carried out employing the Statistical Package for the Social Sciences (SPSS Inc, Chicago, Ill., version 9.1). Differences between the Pen (-) and Pen (+) groups were examined using the Student's t-test for parametric data, and the Mann-Whitney U-test or chi square test with Yate's correction for the non-parametric data. The focus of the statistical analysis was to examine the impact of a history of recent penicillin administration, which is a subject variable. The analysis, therefore, was based on subject means (N= 42), with the exception of the analyses of plaque and gingival indices which were based on the sample sites (N= 82).

**RESULTS**

The Pen (-) and Pen (+) groups were very similar in the clinical parameters examined (Table 1). With few exceptions, the periodontal sites examined were associated with supragingival plaque and demonstrated bleeding on probing. The mean probing depth and attachment loss for both groups was 6.1 and 6.8 mm, respectively.
In addition, the microbiological recovery from the non-selective medium was similar for the two study groups. All species of the pigmented Bacteroides group were recovered from these subjects, with no significant differences found in the total number of CFU's, in the detection of pigmented Bacteroides spp., or in the percentage of pigmented Bacteroides spp. between the two groups (Table 2). In the Pen (-) group, the penicillin-resistant strains recovered represented 1.7% of the total cultivable subgingival microbiota (Table 3), with anaerobic and facultative Gram negative species accounting for 71% of the resistant organisms recovered. The bacterial genera constituting the majority of these strains were Bacteroides, Veillonella, Eikenella, Capnocytophaga, and the Actinobacillus/Hemophilus group (Table 4 and 5). Of these, only Bacteroides spp. were detected in more than 50% of the Pen (-) subjects (Fig. 2). The most prevalent penicillin-resistant organism found was B. gracilis, which was recovered from 62% of these subjects (Fig. 2). Penicillin-resistant pigmented Bacteroides and Eikenella corrodens strains were detected in 33 and 29% of the Pen (-) subjects, respectively (Fig. 2 and 3).

In the Pen (+) group, penicillin-resistant species represented 3.8% of the total cultivable subgingival microbiota (Table 3). Comparing the two study groups, a significantly greater recovery of penicillin-resistant species was found in the Pen (+) subjects (p<0.05). Although the distribution of penicillin-resistant species in the Pen (+) group were the same as those found in the Pen (-) group, the increased recovery of penicillin-resistant species from the Pen (+) subjects was seen primarily in the anaerobic Gram-
negative component of the resistant microbiota. Significantly greater percentages of the pigmented Bacteroides spp., B. intermedius, the B. melaninogenicus/denticola group, Veillonella spp., and V. dispar were isolated from the Pen (+) subjects (Table 4). In addition, the prevalence of most resistant species was greater in the Pen (+) group compared to the Pen (-) group, with a significantly greater rate of detection of pigmented Bacteroides spp., B. intermedius, and Veillonella spp. (Fig. 2).

Beta-lactamase production was detected in all pigmented Bacteroides spp. with an MIC of penicillin G > 1 mcg/ml, and in the resistant Capnocytophaga and Streptococcus species. The prevalence of beta-lactamase-producing species was found to be 48% in the Pen (-) group compared to 76% in the Pen (+) group (Figure 1). The proportion of the total cultivable microbiota represented by beta-lactamase-producing pigmented Bacteroides spp. was significantly increased in the Pen (+) group (Table 4; p<0.005).

Antibiotic susceptibility data are reported in Table 6. No differences were observed in the susceptibility patterns of the isolates recovered from the Pen (-) subjects versus the Pen (+) subjects, so they were combined in these tables. The strains reported here were initially selected on the basis of their resistance to penicillin, and this is reflected in the MIC50 and MIC90 values of penicillin G reported for all isolates. Exceptionally high levels of penicillin resistance were noted among Eikenella corrodens, the Actinobacillus/Haemophilus group, and Streptococcus species (Table 6). In these groups of organisms, the MIC90 values were ≥ 64 mcg/ml of penicillin G. Ampicillin
demonstrated comparable or slightly greater activity for these strains, as compared to penicillin G. For example, for *B. intermedius* strains, the MIC<sub>50</sub> of ampicillin was 8 mcg/ml in comparison to the MIC<sub>50</sub> of penicillin G which was 16 mcg/ml.

Of the remaining antibiotics examined in this investigation, no single antibiotic was uniformly effective in inhibiting the growth of the different organisms examined. Tetracycline resistance was evident in some strains of each of the pigmented *Bacteroides* spp., with MIC<sub>90</sub> values ranging from 8 to 64 mcg/ml. In contrast, the pigmented *Bacteroides* strains were uniformly susceptible to both metronidazole (MIC<sub>90</sub> from values 0.5 to 4 mcg/ml) and clindamycin (MIC<sub>90</sub> < 0.25 mcg/ml). All of the facultative organisms, the *Actinobacillus/Haemophilus* group, *Eikenella corroden*, *Capnocytophaga* spp. and *Streptococcus* spp. were resistant to metronidazole (MIC<sub>90</sub> values from 16 to > 128 mcg/ml). Finally, while clindamycin inhibited the growth of most isolates, resistance to this antibiotic was seen in *B. gracilis* with an MIC<sub>90</sub> of 8 mcg/ml, *Eikenella corroden* with an MIC<sub>90</sub> > 128 mcg/ml, and in the *Actinobacillus/Haemophilus* group with an MIC<sub>90</sub> of 64 mcg/ml.

The penicillin-resistant subgingival species recovered in this investigation included numerous strains of the Gram-negative assacharolytic organisms, *B. gracilis* and *Eikenella corroden*. Characterization of these strains was complicated by their poor growth in liquid media, the use of SDS-PAGE proved to be a valuable tool in the differentiation of these organisms from other phenotypically similar species, such as *Wolinella*. Examples of the SDS-PAGE results are shown in Figures 4 to 6.
DISCUSSION

Penicillin resistance among organisms found in the subgingival microbiota is of significance in light of the routine use of penicillin in the treatment of infections involving these species (Peterson, 1981; Goodman and Newman, 1984). Subgingival microorganisms that have been associated with odontogenic infections and previously reported to be resistant to penicillin include Bacteroides, Fusobacterium and Streptococcus spp., as well as Eikenella corrodens (Chow et al., 1978; Jones and Romig, 1979; Walker et al., 1984; Brook et al., 1981; Baker et al., 1983; Sutter et al., 1983; Williams et al., 1983). Beta-lactamase-producing Bacteroides spp., in particular, have been recovered from odontogenic infections that have failed to respond clinically to penicillin therapy (Heimdahl et al., 1980; von Konow and Nord, 1983). In addition, oral Streptococcus spp. and Actinobacillus actinomycetemcomitans have been implicated in infective endocarditis (Geraci et al., 1980; Anolik et al., 1981; Facklam and Carey, 1985) and penicillin resistance among these species may present a significant problem in the treatment, as well as the prevention, of these infections (Mitchell and Gillespie, 1964; Slots et al., 1983).

Penicillin resistance in the subgingival microbiota associated with adult periodontitis, based on in vitro susceptibility testing has not been previously reported. Penicillin has been reported to be active against most microorganisms found in the subgingival region (Sutter et al., 1983), and in this investigation the penicillin-resistant microorganisms were found to constitute less
than 4% and 2% of the total cultivable microbiota from subjects with and without a history of recent penicillin therapy, respectively. The organisms identified as resistant to penicillin are generally consistent with previous investigations (Sutter and Finegold, 1976; Walker et al., 1980; Tanner et al., 1981; Laatsch et al., 1982).

Although penicillin has often been reported to be effective against Veillonella spp. (Baker et al., 1983; Sutter et al., 1983), resistance has been reported in at least one investigation (Hanson and Martin, 1980).

Beta-lactamase production is an important mechanism in penicillin-resistant microorganisms, and has been associated in some cases with plasmid-mediated resistance that can be transferred between microorganisms (for a review see Neu, 1984). Ampicillin resistance mediated by beta-lactamases in Haemophilus influenzae provides an example of the rapid dissemination of resistance that may occur. Beta-lactamase was first detected in this microorganism in 1972, and whereas prior to this time H. influenzae was routinely susceptible to beta-lactam antibiotics, the rates of resistant organisms recovered by 1982 ranged from 7 to 48% in different reports (Thornsberry and McDougal, 1982). Little information is available regarding the beta-lactamases produced by oral microorganisms, or the genetic basis of their production (Murray and Rosenblatt, 1977; Sherrill and McCarthy, 1984).

Beta-lactamase-producing subgingival isolates have been reported to consist primarily of pigmented Bacteroides species (Valdés et al., 1982). The beta-lactamase-producing species recovered in the present investigation accounted for 1.3% and 0.7%
of the total cultivable microbiota from subjects with and without a history of recent penicillin therapy, respectively. In contrast to the Valdés report in which no beta-lactamase-producing B. intermedius were reported, this species represented a significant proportion of the beta-lactamase-producing strains recovered in the present study. This difference may relate to the use of different media and different techniques of beta-lactamase detection in the previous study. Beta-lactamase-producing strains of B. intermedius isolated from the subgingival microbiota have been reported by others (Walker et al., 1984). It was of interest in this investigation, that although B. gingivalis accounted for 15% of the pigmented Bacteroides recovered on the non-selective medium, no beta-lactamase or penicillin-resistant strains were detected.

Other beta-lactamase-producing microorganisms recovered in the present study included Capnocytophaga and Streptococcus spp. Beta-lactamase production has not been previously reported in the genus Capnocytophaga. Although oral Veillonella spp. have been reported to produce beta-lactamase (Valdés et al., 1982), the enzyme was not detected in the penicillin-resistant Veillonella species recovered in this investigation.

Penicillin administration has been associated with an increased detection of beta-lactamase-producing Bacteroides spp. in the salivary flora (Heimdahl et al., 1981), and the emergence of beta-lactamase-producing species in the pharyngeal microbiota (Brook, 1984b). In the data reported here, beta-lactamase-producing species were recovered from the subgingival plaque of periodontitis sites from 76% of the subjects with a history of a recent course of
penicillin, in contrast to 48% of the subjects with no recent antibiotic exposure. In particular, the detection of beta-lactamase-producing *Bacteroides* was more than doubled in those subjects with a history of recent penicillin use (71% versus 33%). These data indicate that in a population with adult periodontitis, the chances of encountering a patient harboring beta-lactamase-producing pigmented *Bacteroides* spp. are 1 in 3 if the patient has no recent history of antibiotic use, but 1 in 1.4 if the patient has a history of recent penicillin administration. Although the actual numbers of penicillin-resistant and beta-lactamase-producing species in the subgingival microbiota associated with periodontitis are small, these species would be expected to proliferate under the selective pressure of penicillin administration. Thus in patients with adult periodontitis and odontogenic infections related to the subgingival microbiota, those with a history of recent penicillin administration should be considered to be at greater risk for infections that will not respond to therapy with penicillin.

The susceptibility of the penicillin-resistant subgingival microorganisms was of interest due to reports of odontogenic infections which have not responded clinically to penicillin therapy, and the pigmented *Bacteroides* spp. were of particular concern because of their relatively frequent association with penicillin-resistant odontogenic infections (Heimdahl et al., 1980; von Konow and Nord, 1983; Brook, 1984a). Of the antibiotics examined here, no single agent was uniformly effective in inhibiting all of the penicillin-resistant strains. Tetracycline was
effective against the majority of these strains. However, because of significant levels of resistance in the pigmented *Bacteroides* spp. tetracycline would not be appropriate for the treatment of penicillin-resistant odontogenic infections. Metronidazole and clindamycin were effective in inhibiting many of these strains, including the pigmented *Bacteroides* spp. The use of clindamycin has been associated with a significant incidence of pseudomembranous colitis and for this reason has generally not been considered appropriate in the treatment of odontogenic infections (Sande and Mandell, 1980; Hammill, 1984). The spectrum of activity of metronidazole is specific for anaerobic organisms, and this agent may be of use in the treatment of penicillin-resistant infections. Metronidazole is contraindicated in women in their first trimester of pregnancy, and concurrent alcohol ingestion is associated with an antibuse effect (nausea). The use of combined therapy has been recommended both as a strategy for dealing with antibiotic resistance, as well as for the treatment of mixed anaerobic infections (Sande and Mandell, 1980). A combination of metronidazole with ampicillin would potentially provide the advantage of better inhibition of *Eikenella corrodens*, as opposed to the use of metronidazole alone. Data on the pharmacokinetics and the *in vitro* susceptibility of potential pathogens for this combined regime would be of interest. Antimicrobial agents not examined in this investigation include newly developed combinations of a beta-lactam compound with a beta-lactamase inhibitor (Neu, 1983a). These may also be of benefit in the treatment of penicillin-
resistant infections, and investigation of the susceptibility of common pathogens to these agents are warranted.

In summary, in this investigation the penicillin-resistant and beta-lactamase-producing subgingival microbiota associated with adult periodontitis was characterized, and significant increases in the detection and proportion of penicillin-resistant pigmented Bacteroides and Veillonella spp. were demonstrated in those subjects with a history of recent penicillin administration. Beta-lactamase production was detected in pigmented Bacteroides, Capnocytophaga and Streptococcus spp. In vitro susceptibility data for antibiotics investigated here indicated that metronidazole would be a reasonable alternative in the treatment of penicillin-resistant infections related to the subgingival microbiota. Further investigation is warranted to characterize and determine the genetic basis of beta-lactamase production in oral microorganisms, and to examine the possibility of combined therapy with either metronidazole and ampicillin, or a beta-lactam and beta-lactamase inhibitor in cases of penicillin-resistant odontogenic infections.

ACKNOWLEDGMENTS:
The authors would like to acknowledge the excellent technical support provided by Ms. June Ellis.
TABLE I

CLINICAL PROFILE OF SUBJECT POPULATIONS*

<table>
<thead>
<tr>
<th></th>
<th>Mean ± S.D. by Subject**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pen (-) Group</td>
</tr>
<tr>
<td></td>
<td>N=21</td>
</tr>
<tr>
<td>Age (years)</td>
<td>52.6 ± 12.0</td>
</tr>
<tr>
<td>Plaque Index</td>
<td>1.5 ± 1.0</td>
</tr>
<tr>
<td>Gingival Index</td>
<td>1.9 ± 0.4</td>
</tr>
<tr>
<td>Probing Depth (mm)</td>
<td>6.0 ± 1.0</td>
</tr>
<tr>
<td>Attachment Loss (mm)</td>
<td>6.7 ± 1.3</td>
</tr>
</tbody>
</table>

*Both experimental populations consisted of adult periodontitis subjects. Pen (-) subjects had no history of antibiotic use within 1 year. Pen (+) subjects had a history of recent penicillin administration. See Materials and Methods for further description of these subjects.

**No significant differences based on Student's t-test (2-tailed) for age, probing depth and attachment loss; and Mann-Whitney U test for gingival and plaque indices (2-tailed).
<table>
<thead>
<tr>
<th></th>
<th>Mean ± S.D. by Subject* (%) of Pigmented Bacteroides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pen (-) Group</td>
</tr>
<tr>
<td>Total CFU X 10^6 + S.D.</td>
<td>32.7 ± 31.4</td>
</tr>
<tr>
<td>Total % CFU ± S.D.:</td>
<td></td>
</tr>
<tr>
<td>Pigmented Bacteroides</td>
<td>12.8 ± 6.5</td>
</tr>
<tr>
<td>B. intermedius</td>
<td>9.5 ± 0.9</td>
</tr>
<tr>
<td>(74.3)</td>
<td>(56.3)</td>
</tr>
<tr>
<td>B. melaninogenicus/denticola</td>
<td>0.5 ± 4.2</td>
</tr>
<tr>
<td>(4.2)</td>
<td>(5.8)</td>
</tr>
<tr>
<td>B. loescheii</td>
<td>&lt;0.1 ± 0.2</td>
</tr>
<tr>
<td>(0.3)</td>
<td>(2.5)</td>
</tr>
<tr>
<td>B. gingivalis</td>
<td>1.0 ± 8.1</td>
</tr>
<tr>
<td>(8.1)</td>
<td>(23.9)</td>
</tr>
<tr>
<td>Unspeciated Bacteroides</td>
<td>1.7 ± 3.0</td>
</tr>
<tr>
<td>(13.2)</td>
<td>(11.3)</td>
</tr>
</tbody>
</table>

CFU: Colony forming units.

*Unspeciated Bacteroides: strains not recovered on subculture.

*No significant differences, Mann-Whitney U test (2-tailed).
TABLE 3

PENICILLIN-RESISTANT SUBGINGIVAL MICROBIOTA

FROM SUBJECTS WITH ADULT PERIODONTITIS AND WITH OR WITHOUT A
HISTORY OF RECENT PENICILLIN ADMINISTRATION

I. GENERAL DESCRIPTION

Mean % of Total CFU ± S.D. by Subject
(% of Total Penicillin Resistant species)

<table>
<thead>
<tr>
<th></th>
<th>Pen (-) Group</th>
<th>Pen (+) Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Penicillin</td>
<td>1.7 ± 2.3</td>
<td>3.8 ± 4.9*</td>
</tr>
<tr>
<td>Resistant species</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anaerobic</td>
<td>0.7 ± 1.1</td>
<td>2.6 ± 3.1**</td>
</tr>
<tr>
<td></td>
<td>(35.3)</td>
<td>(68.4)</td>
</tr>
<tr>
<td>Facultative</td>
<td>1.0 ± 2.2</td>
<td>1.2 ± 3.6</td>
</tr>
<tr>
<td></td>
<td>(64.7)</td>
<td>(31.6)</td>
</tr>
<tr>
<td>Gram-negative</td>
<td>1.2 ± 1.5</td>
<td>3.7 ± 4.9**</td>
</tr>
<tr>
<td></td>
<td>(70.6)</td>
<td>(97.4)</td>
</tr>
<tr>
<td>Beta-lactamase-producing</td>
<td>0.7 ± 1.4</td>
<td>1.3 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>(41.1)</td>
<td>(34.2)</td>
</tr>
</tbody>
</table>
TABLE 4

PENICILLIN-RESISTANT SUBGINGIVAL MICROBIOTA

FROM SUBJECTS WITH ADULT PERIODONTITIS AND WITH OR WITHOUT A
RECENT HISTORY OF PENICILLIN ADMINISTRATION

II. ANAEROBIC MICROBIOTA

Mean % of Total CFU ± S.D. by Subject
(% of Total Penicillin Resistant species)

<table>
<thead>
<tr>
<th></th>
<th>Pen (-) Group</th>
<th>Pen (+) Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Bacteroides</td>
<td>0.5 ± 1.1</td>
<td>1.7 ± 2.2**</td>
</tr>
<tr>
<td>species</td>
<td>(29.4)</td>
<td>(44.7)</td>
</tr>
<tr>
<td>Total Pigmented</td>
<td>0.3 ± 1.1</td>
<td>1.3 ± 2.2**</td>
</tr>
<tr>
<td>Bacteroides species</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(17.6)</td>
<td>(34.2)</td>
<td></td>
</tr>
<tr>
<td>B. intermedius</td>
<td>0.2 ± 1.1</td>
<td>0.6 ± 1.7*</td>
</tr>
<tr>
<td></td>
<td>(11.8)</td>
<td>(15.8)</td>
</tr>
<tr>
<td>B. melaninogenicus/</td>
<td>0.1 ± 0.3</td>
<td>0.6 ± 1.6*</td>
</tr>
<tr>
<td>denticola</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(5.9)</td>
<td>(15.8)</td>
</tr>
<tr>
<td>B. loescheii</td>
<td>&lt;0.1 ± &lt;0.1</td>
<td>&lt;0.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>(0.5)</td>
<td>(2.4)</td>
</tr>
</tbody>
</table>

Table continued on next page.
TABLE 4 (CONT.)

PENICILLIN-RESISTANT SUBGINGIVAL MICROBIOTA®
FROM SUBJECTS WITH ADULT PERIODONTITIS AND WITH OR WITHOUT A
RECENT HISTORY OF PENICILLIN ADMINISTRATION

II. ANAEROBIC MICROBIOTA

Mean % of Total CFU + S.D. by Subject
(% of Total Penicillin Resistant species)

<table>
<thead>
<tr>
<th></th>
<th>Pen (-) Group</th>
<th>Pen (+) Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pen (-) Group</td>
<td>0.2 + 0.4</td>
<td>0.4 + 0.8</td>
</tr>
<tr>
<td></td>
<td>(11.8)</td>
<td>(10.5)</td>
</tr>
<tr>
<td>B. gracilis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Veillonella species</td>
<td>&lt;0.1 + 0.1</td>
<td>0.6 + 1.1**</td>
</tr>
<tr>
<td></td>
<td>(2.4)</td>
<td>(15.8)</td>
</tr>
<tr>
<td>V. parvula/</td>
<td>&lt;0.1 + 0.1</td>
<td>0.2 + 0.5</td>
</tr>
<tr>
<td>atypica</td>
<td>(1.8)</td>
<td>(5.3)</td>
</tr>
<tr>
<td>V. dispar</td>
<td>&lt;0.1 + &lt;0.1</td>
<td>0.4 + 0.8**</td>
</tr>
<tr>
<td></td>
<td>(0.6)</td>
<td>(10.5)</td>
</tr>
</tbody>
</table>

®Penicillin resistance defined by an MIC of penicillin G ≥ 4 mcg/ml or beta-lactamase production.

*p<0.05, Mann-Whitney U test (1-tailed).

**p<0.005, Mann-Whitney U test (1-tailed).
TABLE 5

PENICILLIN-RESISTANT SUBGINGIVAL MICROBIOTA§
FROM SUBJECTS WITH ADULT PERIODONTITIS AND WITH OR WITHOUT A
RECENT HISTORY OF PENICILLIN ADMINISTRATION

II. FACULTATIVE MICROBIOTA

Mean % of Total CFU ± S.D. by Subject*
(% of Total Penicillin Resistant species)

<table>
<thead>
<tr>
<th></th>
<th>Pen (-) Group</th>
<th>Pen (+) Group</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Eikenella corrodens</strong></td>
<td>0.2 ± 0.4</td>
<td>0.8 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>(11.8)</td>
<td>(21.1)</td>
</tr>
<tr>
<td><strong>Actinobacillus/ Haemophilus species</strong></td>
<td>0.2 ± 0.6</td>
<td>&lt;0.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>(11.8)</td>
<td>(1.2)</td>
</tr>
<tr>
<td><strong>Capnocytophaga species</strong></td>
<td>&lt;0.1 ± &lt;0.1</td>
<td>&lt;0.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>(&lt;0.1)</td>
<td>(&lt;0.1)</td>
</tr>
</tbody>
</table>

§Penicillin resistance defined by an MIC of penicillin G ≥ 4 mcg/ml or beta-lactamase production.

*No significant differences, Mann-Whitney U test (1-tailed).
### Table 6

**In Vitro Susceptibility of Penicillin-Resistant Subgingival Isolates to Selected Antibiotics**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Antibiotic</th>
<th>Range</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteroides intermedius</strong> (32)</td>
<td>Penicillin G</td>
<td>2-64</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Ampicillin</td>
<td>0.5-64</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Tetracycline</td>
<td>≤0.25-16</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Metronidazole</td>
<td>≤0.25-0.5</td>
<td>≤0.25</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Clindamycin</td>
<td>≤0.25-2</td>
<td>≤0.25</td>
<td>≤0.25</td>
</tr>
<tr>
<td><strong>Bacteroides melaninogenicus/ denticola</strong> (48)</td>
<td>Penicillin G</td>
<td>1-64</td>
<td>8</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Ampicillin</td>
<td>0.5-64</td>
<td>8</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Tetracycline</td>
<td>≤0.25-64</td>
<td>0.5</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Metronidazole</td>
<td>≤0.25-4</td>
<td>≤0.25</td>
<td>≤0.25</td>
</tr>
<tr>
<td></td>
<td>Clindamycin</td>
<td>≤0.25-1</td>
<td>≤0.25</td>
<td>≤0.25</td>
</tr>
<tr>
<td><strong>Bacteroides loescheii</strong> (24)</td>
<td>Penicillin G</td>
<td>2-64</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Ampicillin</td>
<td>1-64</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Tetracycline</td>
<td>≤0.25-64</td>
<td>0.5</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Metronidazole</td>
<td>≤0.25-2</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Clindamycin</td>
<td>≤0.25</td>
<td>≤0.25</td>
<td>≤0.25</td>
</tr>
</tbody>
</table>
### TABLE 6 (CONT.)

**IN VITRO SUSCEPTIBILITY OF PENICILLIN-RESISTANT SUBGINGIVAL ISOLATES TO SELECTED ANTIBIOTICS**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Antibiotic</th>
<th>Range</th>
<th>MIC₅₀</th>
<th>MIC₉₀</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteroides gracilis</strong></td>
<td>Penicillin G</td>
<td>4–&gt;128</td>
<td>4</td>
<td>32</td>
</tr>
<tr>
<td>(52)</td>
<td>Ampicillin</td>
<td>&lt;0.25–128</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Tetracycline</td>
<td>&lt;0.25–32</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Metronidazole</td>
<td>&lt;0.25–16</td>
<td>0.5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Clindamycin</td>
<td>&lt;0.25–64</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td><strong>Veillonella parvula/atyptica</strong></td>
<td>Penicillin G</td>
<td>4–32</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>(19)</td>
<td>Ampicillin</td>
<td>&lt;0.25–8</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Tetracycline</td>
<td>&lt;0.25–2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Metronidazole</td>
<td>&lt;0.25–4</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Clindamycin</td>
<td>&lt;0.25–2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><strong>Veillonella dispar</strong></td>
<td>Penicillin G</td>
<td>4–&gt;128</td>
<td>8</td>
<td>32</td>
</tr>
<tr>
<td>(19)</td>
<td>Ampicillin</td>
<td>&lt;0.25–128</td>
<td>8</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>Tetracycline</td>
<td>&lt;0.25–64</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Metronidazole</td>
<td>&lt;0.25–8</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Clindamycin</td>
<td>&lt;0.25–8</td>
<td>≤0.25</td>
<td>2</td>
</tr>
</tbody>
</table>
TABLE 6 (CONT.)

IN VITRO SUSCEPTIBILITY OF PENICILLIN-RESISTANT SUBGINGIVAL ISOLATES TO SELECTED ANTIBIOTICS

<table>
<thead>
<tr>
<th>Organism (# Isolates)</th>
<th>Antibiotic</th>
<th>Range</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinobacillus/</td>
<td>Penicillin G</td>
<td>4–&gt;128</td>
<td>8</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Haemophilus spp.</td>
<td>Ampicillin</td>
<td>≤0.25–&gt;128</td>
<td>8</td>
<td>32</td>
</tr>
<tr>
<td>(11)</td>
<td>Tetracycline</td>
<td>0.5–4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Metronidazole</td>
<td>2–&gt;128</td>
<td>4</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Clindamycin</td>
<td>4–128</td>
<td>8</td>
<td>64</td>
</tr>
<tr>
<td>Capnocytophaga spp.</td>
<td>Penicillin G</td>
<td>4–16</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>(7)</td>
<td>Ampicillin</td>
<td>≤0.25–8</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Tetracycline</td>
<td>≤0.25–2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Metronidazole</td>
<td>2–16</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Clindamycin</td>
<td>≤0.25</td>
<td>≤0.25</td>
<td>≤0.25</td>
</tr>
<tr>
<td>Eikenella</td>
<td>Penicillin G</td>
<td>4–&gt;128</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>corrodens (25)</td>
<td>Ampicillin</td>
<td>1–&gt;128</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Tetracycline</td>
<td>0.5–4</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Metronidazole</td>
<td>32–&gt;128</td>
<td>128</td>
<td>&gt;128</td>
</tr>
<tr>
<td></td>
<td>Clindamycin</td>
<td>8–&gt;128</td>
<td>128</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Organism ( # Isolates)</td>
<td>Antibiotic</td>
<td>Range</td>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>MIC&lt;sub&gt;90&lt;/sub&gt;</td>
</tr>
<tr>
<td>------------------------</td>
<td>------------</td>
<td>----------</td>
<td>-----------------</td>
<td>-----------------</td>
</tr>
<tr>
<td><em>Streptococcus</em> spp. (6)</td>
<td>Penicillin G</td>
<td>32–&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
</tr>
<tr>
<td></td>
<td>Ampicillin</td>
<td>16–&gt;128</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>Tetracycline</td>
<td>0.5–4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Metronidazole</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
</tr>
<tr>
<td></td>
<td>Clindamycin</td>
<td>&lt;0.25–1</td>
<td>0.5</td>
<td>1</td>
</tr>
</tbody>
</table>
Figure 1. Penicillin-Resistant Microbiota: Prevalence of Resistant Microorganisms.

Pen (-) subjects (N=21; □) had no history of antibiotic use within previous year; Pen (+) subjects (N=21; ■) had a history of penicillin administration within previous 6 months. No significant differences were found between the two groups.
Figure 2. Penicillin-Resistant Microbiota: Prevalence of Resistant Anaerobic Microorganisms.

Pen (-) subjects (N=21; □) had no history of antibiotic use within previous year; Pen (+) subjects (N=21; ▨) had a history of penicillin administration within previous 6 months. ★: p<0.05, Chi square test.
Figure 3. Penicillin-Resistant Microbiota: Prevalence of Resistant Facultative Microorganisms.

Pen (-) subjects (N=21; □) had no history of antibiotic use within previous year; Pen (+) subjects (N=21; ■) had a history of penicillin administration within previous 6 months. No significant differences were found between the two groups.
Figure 4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of representative Bacteroides and Wolinella strains after trichloroacetic acid (10% v/v) extraction of whole cell protein. Lanes 1,11: Bio-Rad low molecular weight standards, 1:20 and 1:10 dilution respectively; Lanes 2: W. recta strain 3222; 3: W. succogenes ATCC 29543; Lanes 4 to 9: B. gracilis strains, 4: P2398.04; 5: P2398.09; 6: P2415.06; 7: P2515.06; 8: P2256.01; 9: ATCC 33236; Lane 10: B. ureolyticus. Molecular weight standards: (►) = phosphorylase, MW 92,500; (►►) = bovine serum albumin, MW 66,200; (►►►) = carbonic anhydrase, MW 31,000. All strains beginning with 'P' (figures 4 to 6) were isolated the subgingival plaque of subjects from this investigation. See materials and methods for details of technique.
Figure 5. Representative *Eikenella corroden*s strains compared to *Eikenella corroden*s type strains with SDS-PAGE banding patterns of TCA precipitated (Lanes 2 to 7) and solubilized (Lanes 9 to 12) proteins. Lanes 1: Bio-Rad Low MW STD, 1:20 dilution; 2: S3217; 3: S1075; 4: ATCC 23834; 5: P2474.11; 6: P2472.12; 7: P2308.03; 8: Bio-Rad Low MW STD, 1:10 dilution; 9: ATCC 23834; 10: P2474.11; 11: P2472.12; 12: P2308.03.

Molecular weight standards: ( ▶ ) = phosphorylase, MW 92,500; ( ▶▶ ) = bovine serum albumin, MW 66,200; ( ▶▶▶ ) = carbonic anhydrase, MW 31,000.
Figure 6. Representative Veillonella and Actinobacillus/Haemophilus strains compared to type strains with SDS-PAGE banding patterns of TCA precipitated proteins. Lanes 1: Bio-Rad Low MW STD, 1:20 dilution; 2: V. parvula ATCC 10790; 3: V. dispar P2252.06; 4: V. parvula/atypica P2254.06; 5: V. dispar P2472.10; 6: V. dispar P2507.14; 7: H. aphrophilus ATCC 19415; Lanes 8 to 10 Actinobacillus/Haemophilus group, 8: P2595.01; 9: P2298.05; 10: P2595.03; 11: A. actinomycetemcomitans ATCC 29522; 12: Bio-Rad Low MW STD, 1:10 dilution.

Molecular weight standards: ( ➤ ) = phosphorylase, MW 92,500; ( ➤ ➤ ) = bovine serum albumin, MW 66,200; ( ➤ ➤ ➤ ) = carbonic anhydrase, MW 31,000.
SECTION III. CONCLUDING CHAPTER
THESIS DISCUSSION

Due to reports of penicillin resistance among microorganisms of significance in odontogenic infections (Edson et al., 1982) and the apparent increasing incidence of clinically penicillin-resistant infections in the region of the oral cavity (Heimdahl et al., 1980; Bahn et al., 1981; Brook et al., 1981; von Konow and Nord, 1983; Whitcher et al., 1983; Brook, 1984a), this investigation was undertaken to characterize, and to determine the antibiotic susceptibility patterns of the penicillin-resistant and beta-lactamase-producing subgingival microbiota associated with adult periodontitis. The administration of penicillin has been associated with increases in the prevalence of resistant organisms (Heimdahl et al., 1981; Brook, 1984b), indicating that patients with odontogenic infections and a history of penicillin use might be at greater risk for the presence of a clinically penicillin-resistant infection. For this reason, the present investigation sought additionally to examine the impact of a recent systemic exposure of penicillin on the penicillin-resistant subgingival microbiota.

Comparison of the Study Groups on the Basis of Clinical Parameters and Non-selective Microbiological Recovery

The sample population investigated consisted of 42 subjects with adult periodontitis: half of the subjects had no history of antibiotic use within the previous year, the Pen (-) group; and half had a history of recent penicillin exposure, the Pen (+) group. The two study groups were very similar based on the clinical parameters examined. The majority of sites investigated were
associated with supragingival plaque (35 of 41 in the Pen (-) group and 36 of 41 in the Pen (+) group), and demonstrated bleeding on probing (37 of 41 in the Pen (-) group and 36 of 41 in the Pen (+) group). Probing depths ranged from 5 to 9 mm, with a mean of 6.0 and 6.2 in the Pen (-) and Pen (+) groups, respectively. A similar correspondence was seen in the attachment loss measurements with a mean of 6.7 and 6.9 in the Pen (-) and Pen (+) groups, respectively. The criteria used for adult periodontitis included, in addition, that there be a minimum of 4 mm of alveolar bone loss as measured from the CEJ on recent bite wing radiographs. The demographic and clinical data on the subjects examined in this investigation support the diagnosis of adult periodontitis, as described for example by Page and Schroeder (1982).

The two study groups were also very similar in the microbiological recovery on the non-selective medium from the subgingival plaque samples obtained; with no significant differences found in the total number of CFU's, total cultivable pigmented Bacteroides, or in the various Bacteroides spp. The percentage of pigmented Bacteroides and B. gingivalis for all subjects was 11.7% and 1.8% of the total CFU's, respectively. These values are low relative to some investigations of adult periodontitis (Slots, 1977; White and Mayrand, 1981), but appear high relative to other investigations (Zambon et al., 1981; Moore et al., 1985). A significant proportion of pigmented Bacteroides strains remained unspeciated, as they failed to grow when subcultured from the primary isolation plate. During the latter part of this investigation, numerous strains of B. gingivalis were found that
grew on ETSA only in the proximity of a second, 'co-feeding' strain on the same plate. The required co-feeding strain was a *Micrococcus* strain, and probably provided some factor lacking in ETSA but required for the growth of the dependant *B. gingivalis* strains. Attempts to further supplement ETSA with compounds previously reported as growth factors for these strains were unsuccessful in eliminating the growth dependance. Laboratory stock strains, ATCC strains and other *B. gingivalis* strains did not show this growth dependance. It is possible that the percentage of pigmented *Bacteroides* which failed to grow when subcultured in this investigation was in part due to *B. gingivalis* strains which required previously undescribed factors for growth not provided in ETSA. Interestingly, pigmented *Bacteroides* strains which failed to grow when subcultured were found in 88% of the subjects from which *B. gingivalis* strains were recovered, but in only 24% of the remaining subjects who harbored pigmented *Bacteroides* but in whom no *B. gingivalis* strains were recovered. This association suggests that the strains which were not subcultured might have been *B. gingivalis* variants with a dependance on unmet growth factors.

In summary, the subjects employed in this investigation demonstrated a clinical and microbiological profile consistent with adult periodontitis. In addition, there were no significant differences between the two study groups in the clinical parameters examined or in the non-selective microbiological recovery from the subgingival microbiota of the sites sampled.
Composition of the Penicillin-Resistant Subgingival Microbiota Associated with Adult Periodontitis

The penicillin-resistant subgingival microbiota from all subjects was found to consist primarily of Gram-negative organisms. This is consistent with the reported spectrum of penicillin activity (Mandell and Sande, 1980). Anaerobic and facultative Gram-negative, and facultative Gram-positive organisms accounted for all organisms recovered that were found to be resistant to penicillin, and these findings agree with the observations of Valdés et al., (1982) in their description of the spectrum of organisms recovered on penicillin-containing selective media.

Of the strains isolated on the selective medium used in this investigation, 274 were determined to be resistant to penicillin based on the criteria of an MIC $\geq 4$ mcg/ml of penicillin G or the detection of beta-lactamase production. Ninety-two percent (253 of the 274 strains) were identified to the genus or species level. The remaining strains were inconsistent in their biochemical reactions and utilization of chromogenic substrates, and were therefore not interpreted to represent any single group of organisms. The penicillin-resistant strains identified were members of 7 different bacterial genera, and are briefly described below.

The genus *Bacteroides* was represented by *B. gracilis* and the pigmented *Bacteroides* group. The most prevalent penicillin-resistant organism recovered in this investigation was *B. gracilis*, detected in 13 (62%) of the Pen (-) subjects and 12 (57%) of the Pen (+) subjects. This species was described by Tanner et al. (1981) and has been reported to range in MIC values of penicillin from 1-32
mcg/ml. Additionally, Walker et al., (1980) reported 4 of 6 strains assayed to be resistant to 2 mcg/ml of penicillin. Thus the recovery of penicillin-resistant strains of B. gracilis in this investigation is consistent with the previous literature. The genus Wolinella, which is similar superficially to B. gracilis, has been reported to include penicillin-resistant species (Tanner et al., 1981). Penicillin-resistant Wolinella spp. were not recovered in this study, indicating either the lack of these species or penicillin-resistant strains in the population investigated or a failure of the medium employed to support the growth of resistant Wolinella spp. present.

As a group, the penicillin-resistant pigmented Bacteroides were detected in 7 (33%) of the Pen (-) subjects and 15 (71%) of the Pen (+) subjects. All saccharolytic species of pigmented Bacteroides were represented in the penicillin-resistant microbiota. B. melaninogenicus / denticola were the most frequently recovered group of the pigmented Bacteroides, detected in 6 of the Pen (-) subjects and 10 of the Pen (+) subjects. These two species are closely related biochemically (Holdeman et al., 1984), and were not differentiated in this investigation. Consistent with the data of Murray and Rosenblatt (1977), all strains of pigmented Bacteroides with a MIC \(\geq 1\) mcg/ml of penicillin G were found to produce detectable levels of beta-lactamase. The uniform detection of beta-lactamase production in pigmented Bacteroides spp. with MIC values greater than or equal to 1 suggests that the production of beta-lactamase accounts for the resistance to penicillin observed in these species. It was of interest that despite the recovery of B.
gingivalis on the non-selective medium from subjects in this study, penicillin-resistant (or beta-lactamase-producing) strains were not detected from the selective medium. Beta-lactamase-producing B. gingivalis have been reported from one investigation (Wasfy and Santos, 1984). This finding is somewhat unusual, as these strains were reported in relatively high proportions from the subgingival plaque of children, a subject population that would not be expected to harbor abundant levels of B. gingivalis (Delaney, 1984). The lack of beta-lactamase-producing or penicillin-resistant B. gingivalis in the present study is otherwise consistent with previous investigations (Valdés et al., 1982; Baker et al., 1983; Sutter et al., 1983).

Penicillin-resistant and beta-lactamase-producing oral B. oralis strains have been reported (Heimdahl et al., 1980; Valdés et al., 1982), but were not detected in the subgingival microbiota of the subjects in this investigation. B. oralis and B. loescheii are virtually identical biochemically and have typically been differentiated on the basis of pigmentation, a characteristic known to vary with the use of different media (Valdés et al., 1982; Holdeman et al., 1984). It is likely that there has been overlap in the identification of these two species. All strains designated as pigmented Bacteroides spp. in this investigation demonstrated pigmentation and fluorescence under UV illumination on primary isolation. Although these strains pigmented in pure culture, varying degrees of pigmentation and fluorescence were observed.
Penicillin-resistant *Veillonella* spp. were detected in 5 (24%) of the Pen (-) subjects and 13 (62%) of the Pen (+) subjects in this study, and accounted 2.4% of the penicillin-resistant microbiota of the Pen (-) subjects. On the basis of catalase activity, these strains were classified as *V. parvula* / atypica (catalase negative) or *V. dispar* (catalase positive: Rogosa, 1984). The MIC₉₀ values observed in these species were 16 to 32 mcg/ml of penicillin G for the catalase negative and positive strains, respectively, and beta-lactamase production was not detected in these species contrary to the report of Valdés et al. (1982). *Veillonella* spp. recovered from the subgingival microbiota have generally been reported to be susceptible to penicillin (Baker et al., 1983; Sutter et al., 1983), but penicillin resistance has been reported in at least one investigation (Hanson and Martin, 1980).

The most prevalent of the penicillin-resistant facultatively anaerobic organisms was *Eikenella corrodens*, recovered from 6 (29%) of the Pen (-) subjects and 10 (48%) of the Pen (+) subjects. This species accounted for 11.8% of the penicillin-resistant microbiota in the Pen (-) group. The recovery of resistant *E. corrodens* is in agreement with the data of Walker et al. (1980), who reported 47% of 17 strains resistant to 2 mcg/ml of penicillin.

Strains identified as representing either *Actinobacillus actinomyctetemcomitans* or *Haemophilus* spp. were isolated as a portion of the penicillin-resistant microbiota in 4 (19%) of both the Pen (-) and the Pen (+) subjects. The differentiation of these two closely related genera is based on the demonstration of factor V or X growth dependancy for *Haemophilus* spp., which was not determined
in this investigation, and they were thus treated as a single group. The combined group of strains accounted for 11.8% of the resistant microbiota of the Pen (-) group. Penicillin resistance has been previously reported for Actinobacillus actinomycetemcomitans (Slots et al., 1980, Walker et al., 1980; Baker et al., 1983). Little data is available on the susceptibility of periodontal Haemophilis isolates, and these species are generally considered susceptible to penicillin. Penicillin resistant beta-lactamase-producing H. paraphrophilus isolated from the laryngeal microbiota has been reported (Jones et al., 1976). In the investigation reported here, beta-lactamase production was not detected in any of the isolates comprising the Actinobacillus / Haemophilus group recovered.

Penicillin-resistant, beta-lactamase-producing Capnocytophaga spp. were recovered from one subject (5%) in both the Pen (-) and the Pen (+) groups. Previous investigations have not found Capnocytophaga spp. to be susceptible to penicillin, but beta-lactamase production has not been previously reported in this genus (Sutter et al., 1981, Walker et al., 1980; Baker et al., 1983; Sutter et al., 1983). The Gram-positive component of the penicillin-resistant microbiota was represented by beta-lactamase-producing Streptococcus spp., detected in 3 (14%) of the Pen (-) subjects and 1 (5%) of the Pen (+) subjects. These strains were facultative, non-hemolytic, catalase negative cocci, which fermented glucose, sucrose, and lactose, and did not ferment inulin or raffinose, or hydrolyze esculin. The biochemical patterns observed were not completely consistent with currently described species (Facklam and Carey,
Penicillin-resistant oral *Streptococcus* spp. have been reported (Baker et al., 1983), but beta-lactamase production has not been reported among the oral *Streptococcus* spp.

**Impact of a History of Recent Penicillin Therapy on the Recovery of Penicillin-Resistant Microorganisms from the Subgingival Microbiota of Subjects with Adult Periodontitis**

The effect of a history of recent penicillin therapy on the subgingival microbiota of subjects with adult periodontitis was assessed by comparison of the detection and percentage of penicillin-resistant microorganisms recovered in the Pen (-) and Pen (+) groups (Tables 3-5, Section 2; see comments on statistical management of the data below).

Penicillin at 4 mcg/ml was active against the majority of subgingival species, with the percentage of penicillin-resistant strains found to be less than 3% of the total cultivable microbiota of all subjects. The penicillin-resistant microbiota constituted 1.7% of the total CFU's in the Pen (-) group in contrast to 3.8% in the Pen (+) group, a difference significant at the p<0.05 level. The increased recovery of penicillin-resistant microorganisms in the Pen (+) group was evident in the Gram-negative anaerobic species, which represented 0.6% and 2.6% of the total CFU's, or 35.3% and 68.4% of the penicillin-resistant microbiota, in the Pen (-) and Pen (+) groups respectively (p<0.005). Of the penicillin-resistant microbial groups identified in this investigation, significantly greater detection rates and percentages of the total CFU's were seen in the total pigmented *Bacteroides* spp., *B. intermedius*, *Veillonella*
spp., and V. dispar. The percentage of the total Bacteroides spp.,
and the B. melaninogenicus / denticola group in the Pen (+) subjects
was significantly elevated relative to the Pen (-) group (p<0.05),
but no significant differences were found in detection.

The beta-lactamase-producing species recovered in this
investigation consisted of the pigmented Bacteroides, Capnocytophaga
and Streptococcus spp. No significant differences were seen in the
total number of beta-lactamase-producing species between the two
study groups, but the detection and percentage of the beta-
lactamase-producing pigmented Bacteroides group, as noted above, was
significantly increased in the Pen (+) group.

Previous investigations have examined the effect of systemic
penicillin therapy on the recovery of resistant pigmented
Bacteroides in the salivary (Heimdahl et al., 1980) and pharyngeal
(Brook, 1984b) microbiota, and have documented significant increases
in the detection of these species. The focus of these previous
investigations has been on the recovery of beta-lactamase-producing
species, thus the significant increases in detection of the non-
beta-lactamase-producing Veillonella spp. seen in this investigation
has not been documented elsewhere.

Comments on the Statistical Management of the Data

The data generated in this investigation was analyzed to
examine the effect of a recent history of systemic penicillin
therapy on the level of penicillin-resistant microorganisms in the
subgingival microbiota. Since the systemic administration of an
antibiotic is a subject variable the analyses were based on subject
means (N=42), with the exception of the analyses of plaques and gingival indices which were based on sample sites (N=82). In all cases the level of significance used was $\alpha = 0.05$. The clinical data was analyzed using the student's t-test for the parametric data and the Mann-Whitney U test for the nonparametric data.

Two separate microbiological data bases were generated; the quantitative data, and the prevalence data. The quantitative data consisted of the number of CFU's of specific microorganisms or groups of microorganisms recovered from each subgingival plaque sample. A high degree of variability in quantitative microbiological data is common (Socransky et al., 1983) and this has been attributed to the large range in actual numbers of microbial species encountered in plaque samples. The numbers of CFU's of microbial species recovered in this investigation were highly variable, and did not consistently conform to a normal distribution even with various transformations (square root, sine, cosine, arctangent, natural log and base 10 log transformations examined). Therefore, the approach taken was to convert these data to percentage counts. Specifically, the number of CFU's of each group of microorganisms was divided by the total number of CFU's for that sample and multiplied by 100 to obtain the percentage count per sample. The mean of these values from the two samples from each subject was calculated to obtain the percentage count per subject. The values used in the statistical analyses were the percentage counts per subject, except as previously noted. As the percentage data is ordinal in nature, it was analyzed using the Mann-Whitney U test. A potential problem in nonparametric analyses is the effect
of a large number of ties. This may be a particular problem in analyzing quantitative microbiological data, as a large number of zero values may result from the minimum detection level inherent in the microbiological techniques. However, an advantage of the Mann-Whitney U test is that a large proportion of ties in the data base (as great as 90%) in fact has a minimal effect (Siegel, 1956). A correction for ties is available, but results in an increase in the level of significance (i.e. decreases the p value). Thus the uncorrected Mann-Whitney U test, as employed, is a more conservative analysis. Of additional note, both 1-tailed and 2-tailed analyses were employed. Examination of the microbiological recovery from the nonselective medium was carried out to test the null hypothesis that no differences would exist between the two study populations, and was therefore based on 2-tailed tests. In contrast, a greater recovery of penicillin-resistant microorganisms has been documented in the salivary and oropharyngeal microbiota of subjects with a history of recent penicillin administration compared to subjects with no history of recent penicillin administration (Heimdahl et al., 1981; Brook, 1984a). On this basis, the examination of the resistant microbiota was carried out to test the null hypothesis that more resistant microorganisms would not be recovered from subjects with a recent history of penicillin therapy, and 1-tailed tests were used.

Prevalence data is nominal in nature and the statistical analyses were carried out using the chi square test corrected for continuity (Siegel, 1956). The chi square test requires that values in each cell of the contingency table not be too small. However,
when the sample size is greater than 40, Siegel (1956) recommends the chi square test corrected for continuity regardless of the cell values. An alternate approach, recommended by Scheffler (1980) is to use the Fisher Exact test in lieu of the chi square test when any cell of the contingency table is less than 5. When using the Fisher Exact test (BMPD Statistical Software, Inc., Los Angeles, CA) on the prevalence data generated in this investigation, the difference between the two study groups in the detection of penicillin-resistant *Bacteroides* spp. was found to be significant \((p<0.05)\), in addition to the significant differences seen with the chi square test. It is of note that the Fisher Exact test is more sensitive when extreme values of the expected frequencies occur. This phenomena explains why the detection of penicillin-resistant *Bacteroides* spp. with a difference between the two study groups of 5 subjects was significant, whereas the detection of beta-lactamase-producing species with a difference of 6 subjects was not significant (Table 1). The distribution of subjects relative to the detection of penicillin-resistant *Bacteroides* spp. yields more extreme expected cell frequencies (values closer to zero) than the detection of beta-lactamase-producing species, resulting in a more powerful analysis. As shown in Table 1, the differences in the \(p\) values of these two analyses are in fact quite small, but happen to straddle the predetermined level of significance.

Correlations between the microbiological parameters and the clinical parameters of probing depth, attachment loss, plaque and gingival indices were addressed using the Spearman rank correlation coefficient (2-tailed). A single positive correlation was found,
<table>
<thead>
<tr>
<th>Parameter</th>
<th># Subjects</th>
<th>Positive</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pen (-)</td>
<td>Pen (+)</td>
</tr>
<tr>
<td>Detection of</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Penicillin-Resistant</td>
<td>15</td>
<td>20</td>
<td>0.047</td>
</tr>
<tr>
<td><em>BMPD Statistical Software, Inc., Los Angeles, CA.</em></td>
<td></td>
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<td></td>
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</tbody>
</table>
between the percentage of penicillin-resistant strains and the plaque index ($\rho = 0.27$, $p < 0.05$). Within the Pen (+) group, there was no significant correlation between the percentage of resistant species and the number of months since the administration of penicillin therapy.

**Technical Considerations in the Recovery of Penicillin-Resistant Microorganisms**

In order to facilitate the recovery of penicillin-resistant microorganisms from the subgingival plaque samples, a selective medium consisting of ETSA with 1, 3 or 6 mcg/ml of penicillin G incorporated was used (designated as 'Pen 1', 'Pen 2' and 'Pen 3' plates, respectively). Because the Pen 1 plates were less selective than was desired for the purposes of this investigation, the concentration of penicillin was increased, as indicated. It was speculated initially that the lack of selectivity seen with this medium was due to binding of the penicillin molecule to serum proteins in the blood component of the medium. This may have played a role, but recently the inactivation of beta-lactam antibiotics by cysteine, one of the compounds used in ETSA, has been reported. This phenomena was illustrated by Markowitz and Williams (1985), and the inactivation of the antibiotic was evident in the increases in MIC values for of beta-lactam antibiotics when cysteine was incorporated in the media used for MIC determinations.

The actual antibiotic activity of the three different selective media used was assessed by standardized inoculations (NCCLS, 1982) of strains that had previously been characterized by their MIC of
<table>
<thead>
<tr>
<th>MIC of Penicillin G</th>
<th># of Inoculations</th>
<th>Amount of Penicillin G of incorporated (mcg/ml) into selective medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>≤0.25</td>
<td>190</td>
<td>V</td>
</tr>
<tr>
<td>0.5</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>1.0</td>
<td>25</td>
<td>+</td>
</tr>
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<td>2.0</td>
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</tr>
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<td>4.0</td>
<td>25</td>
<td>+</td>
</tr>
<tr>
<td>≥8.0</td>
<td>65</td>
<td>+</td>
</tr>
</tbody>
</table>

This data represents the growth of 67 strains with known MIC's of penicillin G on the different media used. Inocula were prepared according to the NCCLS technique, and five separate inoculations were carried out for each strain on each medium. The presence or absence of growth after 48 hours was determined, and this data is expressed as; +: >90% of inoculations yielded growth; V: 90-10% of inoculations yielded growth; -: ≤10% of inoculations yielded growth.
penicillin G (Table 2). These data indicated that the actual penicillin activity of the three different selective media ranged from \( \leq 0.25 \) to 2 mcg/ml. Previous investigations of penicillin resistance in the subgingival microbiota have used the recovery of microorganisms on penicillin containing selective media as a means of determining resistance (Valdes et al., 1982). Because of the range of activity of the selective media used in this investigation, the determination of resistance used was based entirely on MIC determinations and the detection of beta-lactamase production.

The use of the three different levels of penicillin containing selective media was not felt to significantly impact on the results of this study. The mean percentage of inhibition* on all plates was 82.0 and 83.0 for the Pen (-) and Pen (+) groups respectively. This issue was addressed statistically by examining the relationship between the level of penicillin in the selective medium and the percentage recovery of resistant strains using the Spearman rank correlation coefficient (1-tailed). A positive correlation was found for the percentage of resistant *Eikenella corrodens* (rho=0.34; \( p<0.05 \)), and the percentage of Gram-negative facultative species (rho=0.29, \( p<0.05 \)). However, these percentages were not significantly different between the two study groups examined.

**Significance**

The results of this investigation have demonstrated a significantly greater prevalence of penicillin-resistant pigmented

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**Number of CFU's on selective medium/ number of CFU's on ETSA.**
Bacteroides spp. in subjects with adult periodontitis and a history of recent penicillin administration, as compared to those subjects with a history of no recent antibiotic use. Pigmented Bacteroides spp. are recognized pathogens in odontogenic infections (Heimdahl et al., 1981; von Konow and Nord, 1983). These data indicate that patients with odontogenic infections, as well as adult periodontitis and a history of recent penicillin use, should be considered at greater risk for an infection that will not respond to penicillin therapy.

Beta-lactamase production by Capnocytophaga species and oral Streptococcus species described in this investigation has not been previously reported. Beta-lactamase production in pathogenic microorganisms is of particular importance, as many beta-lactamases are plasmid mediated. The dissemination of plasmid associated resistance may be rapid and of significant clinical impact in the management of infections involving beta-lactamase-producing species (Neu, 1984). Little is known of the genetic basis of beta-lactamase production in oral anaerobic microorganisms, and further investigation is clearly indicated.

The in vitro susceptibility data on the penicillin-resistant component of the subgingival microbiota is applicable to the assessment of odontogenic infections that fail to respond to penicillin therapy, as the resistant species involved would be likely to have been represented by the strains recovered in the present study. From the susceptibility data on the penicillin-resistant strains for the antibiotics tested, it appeared that metronidazole would be a good alternative choice. However, the lack
of uniform susceptibility of these species to the antibiotic agents examined underscores the need for microbiological and in vitro susceptibility data in the treatment of these infections. In light of the observation made in this investigation of an increase in the recovery of penicillin-resistant microorganisms from subjects with a recent history of penicillin use, further investigation to determine the prevalence of penicillin-resistant odontogenic infections and their clinical response to different antibiotic regimes is warranted.
CONCLUSIONS

1) In subjects with adult periodontitis and no history of recent antibiotic use:

A) The penicillin-resistant subgingival microbiota constituted less than 2% of the total cultivable microbiota.

B) The penicillin-resistant microbiota consisted primarily of anaerobic and facultative Gram-negative organisms, with the predominant genera represented by Bacteroides, Veillonella, Eikenella, Capnocytophaga, Actinobacillus/Haemophilus and Streptococcus spp.

C) The beta-lactamase-producing subgingival organisms constituted less than 1% of the subgingival microbiota, and consisted of pigmented Bacteroides, Capnocytophaga and Streptococcus spp.

2) In subjects with adult periodontitis and a history of recent penicillin administration:

A) The predominant microorganisms comprising the penicillin-resistant and beta-lactamase-producing subgingival microbiota were identical to those found in subjects with no history of recent antibiotic use.

B) A significantly greater prevalence of penicillin-resistant pigmented Bacteroides spp., B. intermedius, Veillonella spp.
and *V. dispar* was found as compared to the recovery from subjects with no history of recent antibiotic use.

C) Significantly greater percentages of total penicillin-resistant species, as well as the penicillin-resistant pigmented *Bacteroides*, *B. intermedius*, the *B. melaninogenicus/denticola* group, *Veillonella* spp. and *V. dispar* were found, as compared to subjects with no history of recent antibiotic use.

3) *In vitro* susceptibility testing of the penicillin resistant subgingival isolates revealed that:

A) Of the antibiotics examined, no single agent was found to be uniformly active against all of the penicillin-resistant subgingival isolates at concentrations achievable after standard oral doses.

B) Penicillin-resistant pigmented *Bacteroides* spp. were uniformly susceptible to metronidazole and clindamycin at levels readily achieved after standard oral doses.
SECTION IV. BIBLIOGRAPHY
BIBLIOGRAPHY


National Committee for Clinical Laboratory Standards. 2: 70, 1982.


