THE ANTIBIOTIC SENSITIVITY PATTERNS

AND

PLASMID DNA CONTENT

OF

GRAM-NEGATIVE ANAEROBIC BACTERIA

ISOLATED IN

PALMERSTON NORTH, NEW ZEALAND

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ABSTRACT

One hundred and seven Gram-negative bacteria, including 65 Bacteroides species, 28 fusobacteria and 14 veillonellae were isolated from 17 oral infections treated in two dental surgeries in Palmerston North. These bacteria, plus 37 isolates belonging to the B. fragilis group received from Palmerston North hospital, were surveyed for their antibiotic sensitivity levels, and their plasmid DNA content.

The hospital isolates of the B. fragilis group were found to have sensitivity levels comparable with those of B. fragilis group isolates reported in the literature recently. The oral isolates were more sensitive to penicillin, cefoxitin, and tetracycline than isolates of the same species reported in the literature.

Half the hospital isolates had plasmids, which were all between 8.5 and 2.7 kilobases (kb) in size except for one 60, and one 43 kb plasmid. Comparatively few of the oral anaerobes had plasmids. One Fusobacterium russii isolate had four plasmids, and five Bacteroides isolates had one plasmid each. These five Bacteroides isolates came from two specimens, R5 and R6.

Restriction enzyme analysis of all plasmids revealed that the three 5.6 kb plasmids from sample R5 may be related to a group of 5.8 kb plasmids harboured by four of the hospital isolates. Two different species of Bacteroides isolated from sample R5 harboured the 5.6 kb plasmid, and two species of the B. fragilis group bacteria harboured the 5.8 kb plasmid.

Plasmid DNA isolated from two tetracycline resistant hospital isolates was used to transform restriction negative E. coli to a low level of tetracycline resistance.

ACKNOWLEDGEMENTS

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INTRODUCTION

THE PATHOGENICITY OF GRAM-NEGATIVE ANAEROBES

The role of the body's commensal microflora as pathogens, is now firmly established (32). The majority of anaerobic infections are caused by bacteria from endogenous sources such as the oro'- and nasopharynx, gastrointestinal tract, genitourinary tract, and skin (32). The relative incidence of these endogenous anaerobic bacteria in various infections is given in Table 1.

The Gram-negative anaerobic bacilli of the genera Bacteroides and Fusobacterium are reported to be the most commonly encountered anaerobes in clinical infection (32). Various species belonging to this group have been associated with different types of infections as follows: the Bacteroides fragilis group, particularly B fragilis and B thetaiotamicron in intraabdominal infections mainly, and in many other infections throughout the body (32,70), the B melaninogenicus - B asaccharolyticus group, B ruminicola, B oralis and Fusobacterium nucleatum in oral and dental, head and neck, bite, pleuropulmonary and other infections (25,32,44,46,43,15,6), B bivius and B disiens particularly in female genital tract infections and in oral infections (32,26), and F necrophorum in widely disseminated infection commonly originating in a focus of membranous tonsillitis known as Vincent's angina (32).

Organisms from the commensal microflora which cause disease in their host do so generally as a result of the host being compromised in some way. Thus they are termed "opportunistic pathogens". Factors often associated with anaerobic infections generally create circumstances that allow these bacteria to gain access to tissues with poor blood supply and thus lowered oxygen tension (32,57). Examples are tissue damage due to wounds or infection by other microorganisms, surgery, the presence in tissue of inanimate objects such

TABLE 1

The relative incidence of endogenous
anaerobic bacteria in various infections (32)

Infection	Relative incidence
Brain abscess	89%
Chronic sinusitis	50%
Periodontal abscess	100%
Aspiration pneumonia	85-95%
Lung abscess	93%
Necrotizing pneumonia	85%
Empyema	76%
Peritonitis and abscess	90-95%
Tuboovarian abscess	92%
Vulvovaginal abscess	74%
Septic abortion and endometritis	73%

as splinters, sutures, implanted prosthetic devices or
dead teeth and underlying diseases such as malignant
neoplasm.

Anaerobic infections are often polymicrobial
(32,43). Bacterial assay of samples commonly reveals
the presence of several species of anaerobe, and
frequently facultative bacteria also. This can be
attributed to the organism involved in the infection
being derived from the polymicrobial microflora of
epithelial surfaces adjacent to the site of infection
(43). Their polymicrobial nature, and the absence of
any exogenously derived bacteria, made separation of
actively pathogenic bacteria from those passively present,
and those simply taking advantage of conditions created,
relatively difficult in infections involving commensal
anaerobes (43,58,40).

Classically, for a microorganism to be accepted as the aetiological agent of an infection, Koch's postulates had to be fulfilled. Thus, the organism had to be regularly found in lesions of the disease, grown in pure culture in artificial media, inoculation of this pure culture into experimental animals had to produce a similar disease, and the organism had to be recovered from lesions in these animals. The postulates served several generations of researchers well, but have proven inadequate for the situation presented by diseases involving members of the normal microflora such as dental abscess (55). Firstly, a large number of different species can often be isolated regularly from these lesions, and many of the fastidious anaerobes have only relatively recently been cultured and characterized on artificial media. Also, polymicrobial infections often involve several species or genera of bacteria behaving in a co-operative or symbiotic manner to produce sepsis (40).

To overcome the inadequacies of Koch's original postulates, certain modifications and additional criteria have been suggested (55). The original theme of the postulates is still adhered to, but not all of the new criteria must be fulfilled; each adds weight to the evidence that any one agent is an active pathogen (55).

The first criteria has been modified to association with disease. This implies enrichment of the organism at sites of pathology and the corollary that the organism is in lower numbers or proportions or absent at healthy sites or sites with different forms of disease (55). This criterion has been used to implicate Gram-negative anaerobic bacteria, particularly the black pigmented <u>Bacteroides</u> species in periodontal abscess (44).

Elimination of the organism has been added as a test of the role of an organism in active disease. The effectiveness of the anaerobe specific nitroimidazole antibiotics in treating anaerobe specific disease has

been suggested as evidence of their active role in these infections (30). Experiments involving abscess induction in guinea pigs with various combinations of bacteria commonly found in oral infections have demonstrated that when Bacteroides species were in the combination, induced abscesses failed to resolve, and there was a gradually increasing accumulation of polymorphnuclear leukocytes. When Bacteroides species were absent from the inoculum, the induced abscess did not progress but resolved (58). Bacteroides asaccharolyticus has been shown to be dependent on the presence of organisms which produce succinate to induce non-resolving, progressive abscesses in guinea pigs (40). The succinate replaced haemin as a growth factor, and was produced by facultative bacteria such as Klebsiella pneumoniae and Escherichia coli. While the growth of Bacteroides species has been shown to be enhanced by facultative bacteria they are often found associated with in mixed infection, it has been suggested that the facultative bacteria generally benefit to a greater extent than the anaerobes from the association (5). This has been speculated as being due to protection from phagocytosis and intracellular killing (5).

The demonstration of mechanisms of pathogenicity is considered fairly strong evidence of the role of an organism as an active pathogen (55). Gram-negative anaerobes are able to hydrolyse collagen, fibrin and other proteins (58,25,55,15,16,20,31,72), cause resorption of bone (54,41), produce destructive metabolites such as hydrogen sulphide, methyl mercaptan, indole and ammonia (32,55,34,47), and inhibit phagocytosis and killing by polymorphnuclear leukocytes, both by being encapsulated and by producing leukocidal toxins (58,23,32,55).

Periodontal and pericoronal abscesses (Figures 1 and 2) generally involve bacteria normally resident on adjacent mucosal membranes (43). These infections normally have a very heterogenous flora, with the actively

pathogenic Gram-negative anaerobes present in high numbers (44,15). The tooth root canal is normally sterile, with no adjacent mucosal membrane. Bacteria gain access to this region through channels created by carious lesions. Periapical abscesses (Figure 3) have a more specific flora with less species of bacteria present than other types of dental abscess (46,15,6).

ANTIBIOTIC SENSITIVITY OF GRAM-NEGATIVE ANAEROBES

Treatment of anaerobic infections generally involves surgical drainage of any pus, and the use of antibiotics to localise abscesses, arrest bacteremia, and clear tissues of bacteria after surgical drainage (43,9). It is generally accepted that due to the polymicrobial nature of many anaerobic infections and the relatively long periods of time required for growth and isolation of causal agents, rapid routine susceptibility testing of individual isolates of anaerobic bacteria is impractical (60). The initial choice of antimicrobial therapy to treat anaerobic infections must be made empirically (3).

The susceptibility of anaerobic bacteria to antibiotics has been reported since the mid-1950s, and for 20 years there was very little alteration of susceptibility patterns (3). Penicillin was used routinely for infections involving anaerobes above the diaphragm and tetracycline for those below, because the B fragilis group were not sensitive to penicillin. In 1972, two laboratories reported striking increases in resistance to tetracycline and erythromycin among isolates of Bacteroides, Clostridia, and anaerobic cocci. Penicillin resistance by black pigmented Bacteroides species was also reported in 1972 (3). Clinical failure with penicillin in treatment of orofacial infections caused by β-lactamase producing Bacteroides species have been reported in 1980, 1981 and 1982 (30).

PLASMIDS OF BACTEROIDES

The emergence of antibiotic resistance in bacteria
is hastened by expansion of the pool of genetic determinants
by the dissemination and amplification of plasmids (76).
The plasmid content of the B fragilis group of species
has been investigated by a number of researchers. Three
homology classes of small (< 5 M daltons) cryptic
plasmids have been identified in this group (8). Within
the classes there is close sequence similarity based on
restriction endonuclease digestion, and polypeptide
products. There is no evidence of species barriers for
these plasmids among the intestinal Bacteroides; all
three classes were found in seven of the 10 species
investigated, and the presence of all three classes in
one isolate demonstrated that they are not incompatible
(8).

Larger plasmids have also been isolated from
Bacteroides species, and a number of these have been
shown to carry antibiotic resistance markers.

In 1977 multiple resistance to ampicillin,
amoxacillin, cephalothin, tetracycline, minocycline and
chloramphenicol was transferred from B fragilis L010
to E coli K12 strain CSH1 (37). No plasmid could be
detected in either donor or recipient bacteria but it
was assumed that conjugal transfer of a plasmid carrying
resistance genes had occurred. A conjugative plasmid
was shown to be responsible for transfer of chloramphenicol,
tetracycline, and kanamycin resistance from B ochraceus
2228 to E coli K12 HB101 in 1978 (23). This was a
70 M dalton plasmid designated pGD10. A 27 M dalton
plasmid designated pBF4 isolated from B fragilis
was shown to conjugatively transfer resistance to
clindamycin, lincomycin, and erythromycin between
B fragilis and B uniformis and vice versa, in 1979 (68).
Also in 1979, resistance to clindamycin and erythromycin
conjugatively transferred between B fragilis and
B thetaiotamicron was associated with transfer of a pair

of plasmids; these were 2.0 and 10 M daltons in size (63).
In 1981, a much smaller plasmid of 1.95 M daltons,
designated pBY22 and isolated from B fragilis was found to
transform E coli to penicillin G, and tetracycline
resistance (51). This plasmid was found to be resistant
to 12 different restriction endonucleases, was stably
maintained in restrictive positive strains of E coli,
and mobilized by another plasmid, R1 drd-19.

        Evidence that antibiotic resistance in Bacteroides
species is carried on transposons began to accumulate in
1981. Resistance to tetracycline and clindamycin was
transferred from B fragilis to B uniformis without transfer
of a plasmid carried by the donor (36). The donor, B fragilis
V503, was shown to contain DNA sequences that shared homology
with the previously mentioned 27 M daltons plasmid
pBF4 (68). In 1984 two different species of clindamycin
resistance Bacteroides were isolated from the same
infection (24). A B thetaiotamicron strain contained a
15 kb plasmid (9.9 M daltons), designated pCP1 which encoded
transferable resistance to clindamycin. The other resistant
isolate, a strain of B distasonis, had a 10 kb plasmid (pCP2)
that shares extensive homology with pCP1 but doesn't
transfer resistance to clindamycin. Hybridization studies
revealed that pCP1 shares a 5 kb region of homology with
pBF4 which was shown in both plasmids to be bounded by
direct homologous repeats, and to contain the clindamycin
resistance determinant. This 5 kb region was missing from
the other plasmid pCP2, but was found in the whole cell
DNA of its clindamycin resistant host B distasonis strain.
Then in 1985 a compound transposon (Tn 4400), containing
active insertive elements as directly repeated sequences
at its ends was identified in a plasmid pBFTM10 carried by
B fragilis (52). This plasmid is described as being similar to
pCP1 (24). Transposon 4400 comprises a 5.6 kb region of
pBFTM10 and is capable of mediating replicon fusion and
transposition. As well as clindamycin resistance, this
transposon codes for tetracycline resistance that is
expressed in E coli but not in B fragilis.

Hybridization studies have demonstrated extensive homology between plasmid and chromosomal DNA segments from most clindamycin resistant Bacteroides strains and Tn 4400. This transposon also shares extensive homology with the 5 kb direct repeat bordered clindamycin resistance coding region of pBF4.

Since the development of antibiotic resistance in previously sensitive anaerobic bacteria, it has become the practice of large medical centres to carry out surveys of recent clinical isolates of these bacteria to monitor their changing sensitivity patterns (62,1,49).

Isolation of plasmid DNA from Bacteroides species of oral origin has been reported (32), but very little other work concerning the plasmid DNA of bile sensitive Gram-negative anaerobic bacteria has been published.

Surveys of the antibiotic sensitiveness of anaerobic bacteria generally concentrate on the B fragilis group (18), which are the most commonly encountered and antibiotic resistant anaerobic bacteria (32). When the sensitivities of the bile sensitive Bacteroides species and fusobacteria are reported, the source of these bacteria is frequently not indicated very specifically. Bacteria are generally described as being recent clinical isolates, and the diagnostic laboratory or hospital of origin is indicated. There is a suggestion that different clinical settings involving different patient populations and antibiotic usage affect the results of sensitivity surveys with anaerobic bacteria (17). Also, surveys that include anaerobes from various sites all over the body may not be applicable to predicting the susceptibility of anaerobes isolated from specific sites (26).

The first aim of this research was to isolate and identify Gram-negative anaerobic bacteria from oral infections treated in dental surgeries in Palmerston North, New Zealand and then to survey the susceptibility of the bacteria to antibiotics commonly used to treat oral and anaerobic infections.

The various types of dental abscess are described in Figures 1, 2 and 3.

Bacteria of the B _fragilis_ group of species were received in pure culture from Palmerston North hospital, and their sensitivities were surveyed to the same antibiotics as the oral isolates.

The agar dilution method was used to assess antibiotic susceptibilities because it has been recommended as the standard method for use with anaerobic bacteria (7).

The second aim of this research was to investigate the plasmid DNA content of all the isolates. Agarose gel electrophoresis, of cell lysates obtained by two methods, alkaline lysis (28), and the Eckhardt method (13), was used for this survey. Plasmids were then characterized by investigating the number and size of fragments generated from them by restriction endonucleases. This enabled comparison of plasmids isolated from different bacteria.

Finally, wherever there were apparent correlations between plasmid presence and antibiotic resistance, conjugation and transformation experiments using restriction negative E _coli_ K12 were carried out to try and confirm the association.
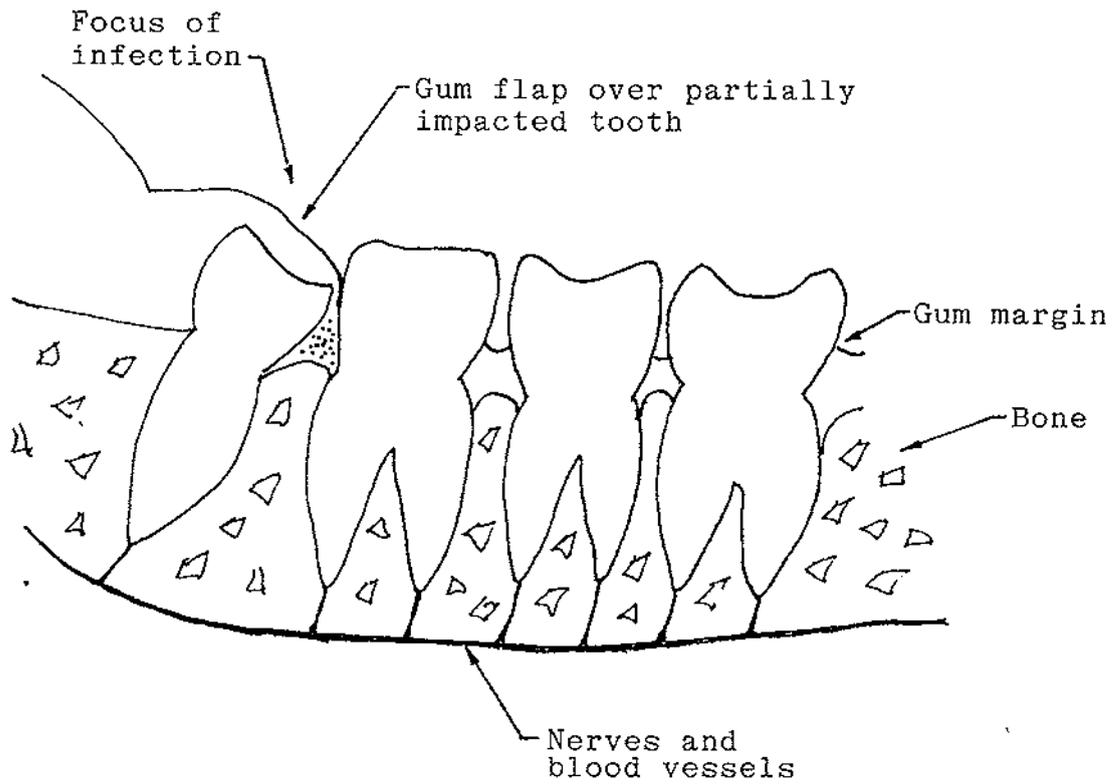
FIGURE 1: DIAGRAMATIC REPRESENTATION OF PERICORONITIS
Pericoronitis frequently involves wisdom teeth.
As teeth emerge, debri and bacteria accumulate
under the flap of gum tissue still covering the
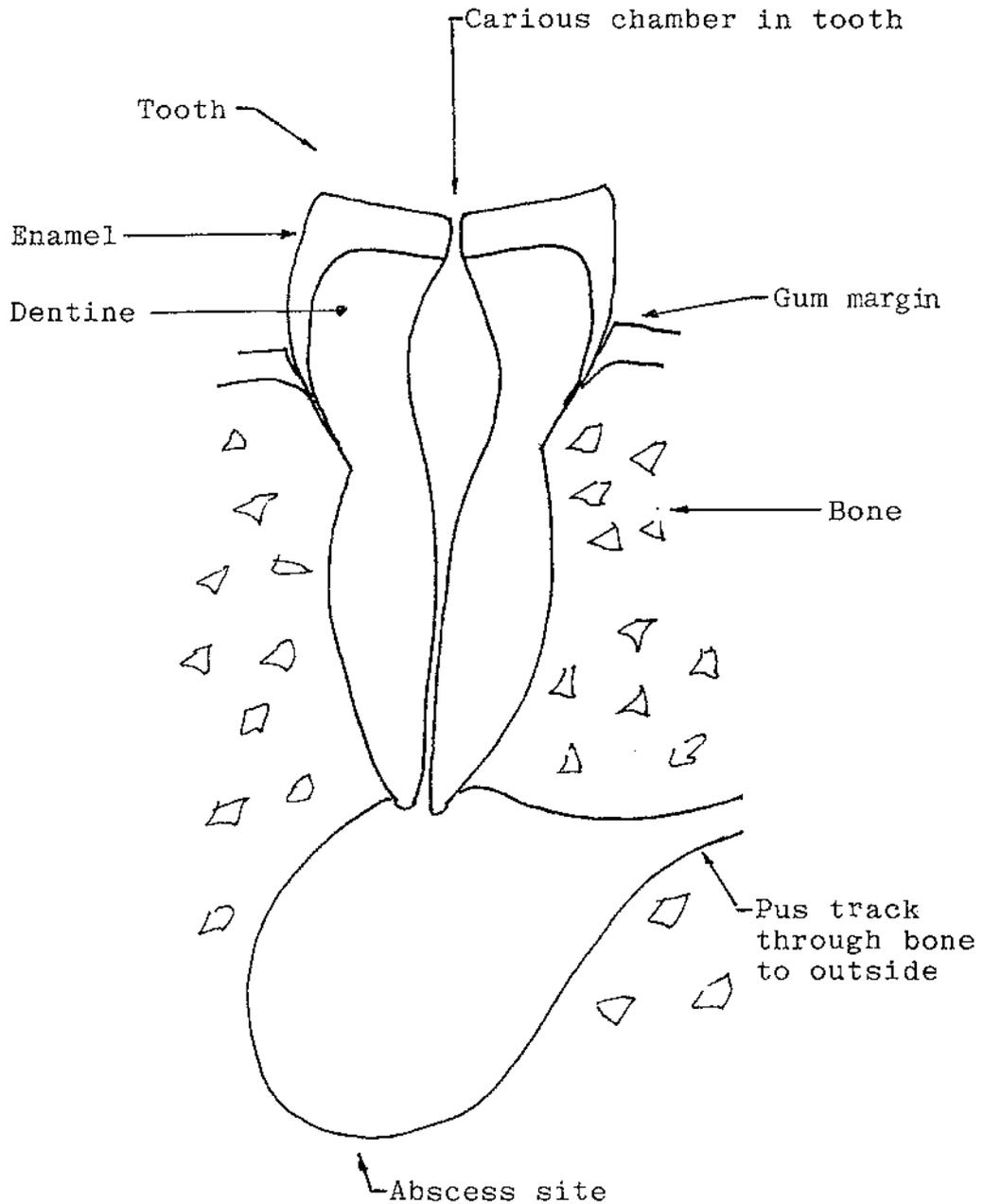tooth; infection of surrounding tissue often
results.

FIGURE 2:   DIAGRAMATIC REPRESENTATION OF A
            PERIAPICAL ABSCESS

Bacteria gain access to the interior of the tooth
through carious lesions, and infect the pulp and
root canal of the tooth. Destruction of
supporting bone results in an abscess which can
track to appear inside the oral cavity or
externally on the face.
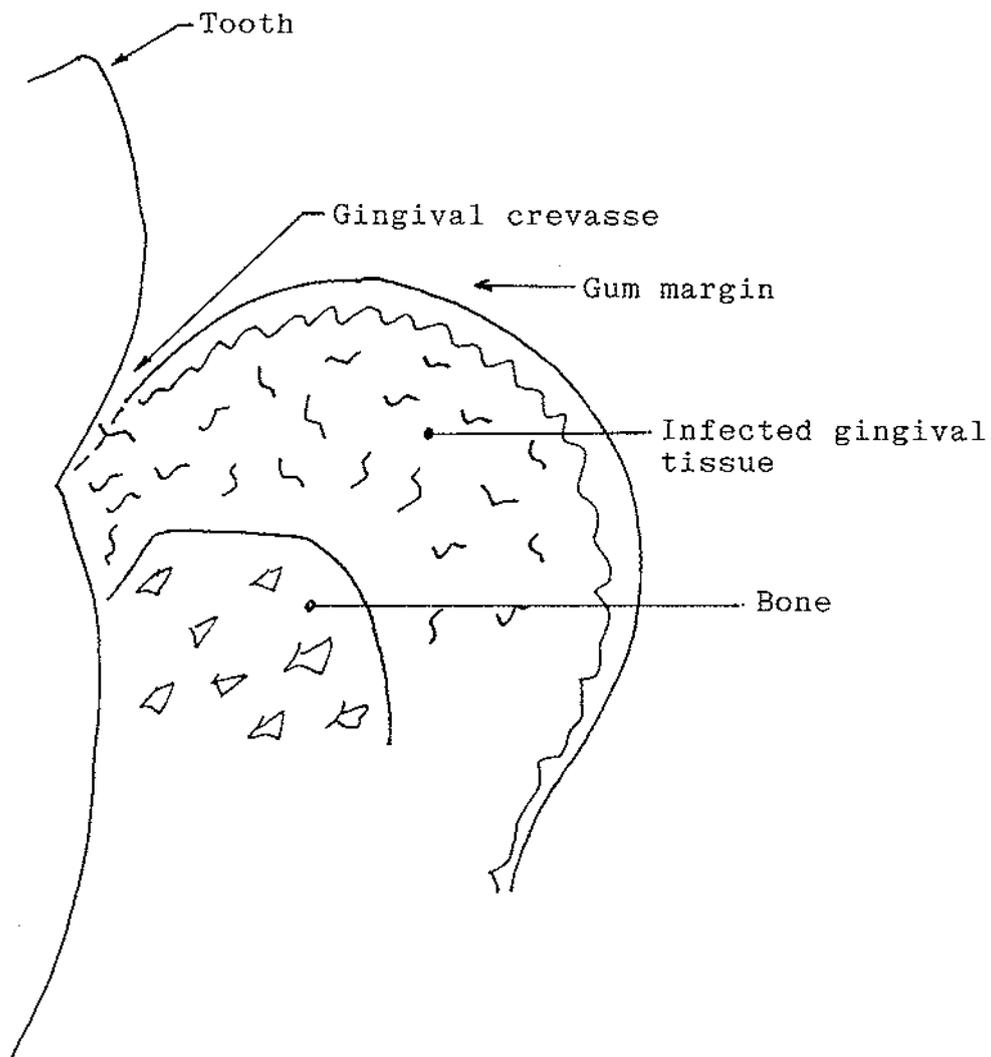
FIGURE 3:   DIAGRAMATIC REPRESENTATION OF A
            PERIODONTAL ABSCESS
            Bacteria originating from the sub-gingival
            plaque invade the soft gingival tissue;
            swelling and bone loss results.

MATERIALS AND METHODS

Media

All media were prepared as outlined in Appendix 1. All media were fully reduced when inoculated.

Anaerobic chamber

Kaltec medical design containing an atmosphere of 82.5% nitrogen, 7.5% carbon dioxide and 10% hydrogen.

Subjects

Specimens were collected from 30 patients; 20 males and 10 females. These people were seen at one of two dental clinics in Palmerston North, New Zealand between January 1985 and October 1986. Patients' ages ranged from 13 - 67 years.

Sampling

All samples were taken by the dental practitioners in their surgeries. Material was recovered by aspiration with a syringe through a sterile needle for unlanced abscesses or through a sterile cannula following lancing of infected sites. An aliquot was then transferred into anaerobic transport medium and the rest retained in the syringe which was sealed by inserting the needle into a rubber bung.

Samples were then stored at $4^{\circ}C$ until transport to the laboratory. Culture was initiated as soon as possible, usually within two hours of collection.