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THE ANTIBIOTIC SENSITIVITY PATTERNS
AND
PLASMID DNA CONTENT
OF
GRAM-NEGATIVE ANAEROBIC BACTERIA
ISOLATED IN
PALMERSTON NORTH, NEW ZEALAND

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Christopher Allan Mooney

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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.

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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 Bacteroides 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, amoxicillin, 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.

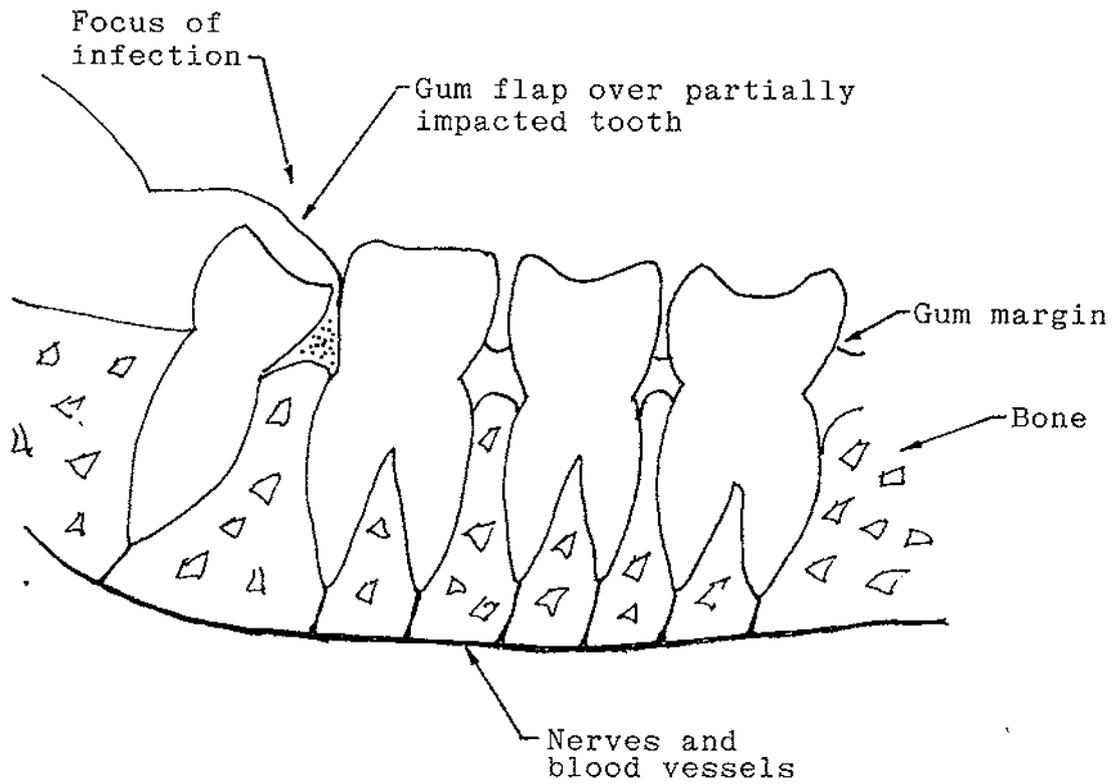


FIGURE 1: DIAGRAMATIC REPRESENTATION OF PERICORONITIS
Pericoronitis frequently involves wisdom teeth. As teeth emerge, debris and bacteria accumulate under the flap of gum tissue still covering the tooth; infection of surrounding tissue often results.

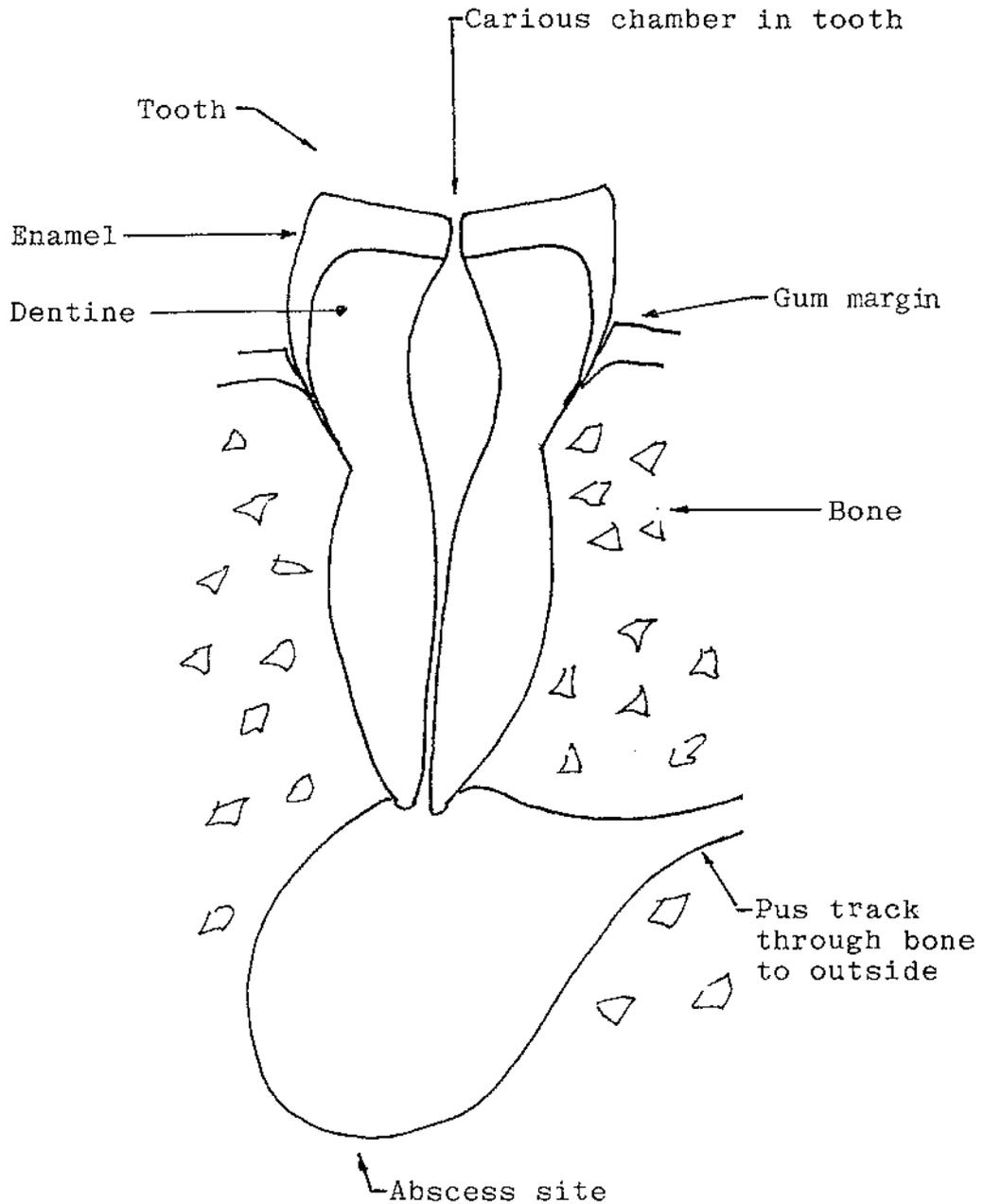


FIGURE 2: DIAGRAMATIC REPRESENTATION OF A PERIAPICAL ABSCESS

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.

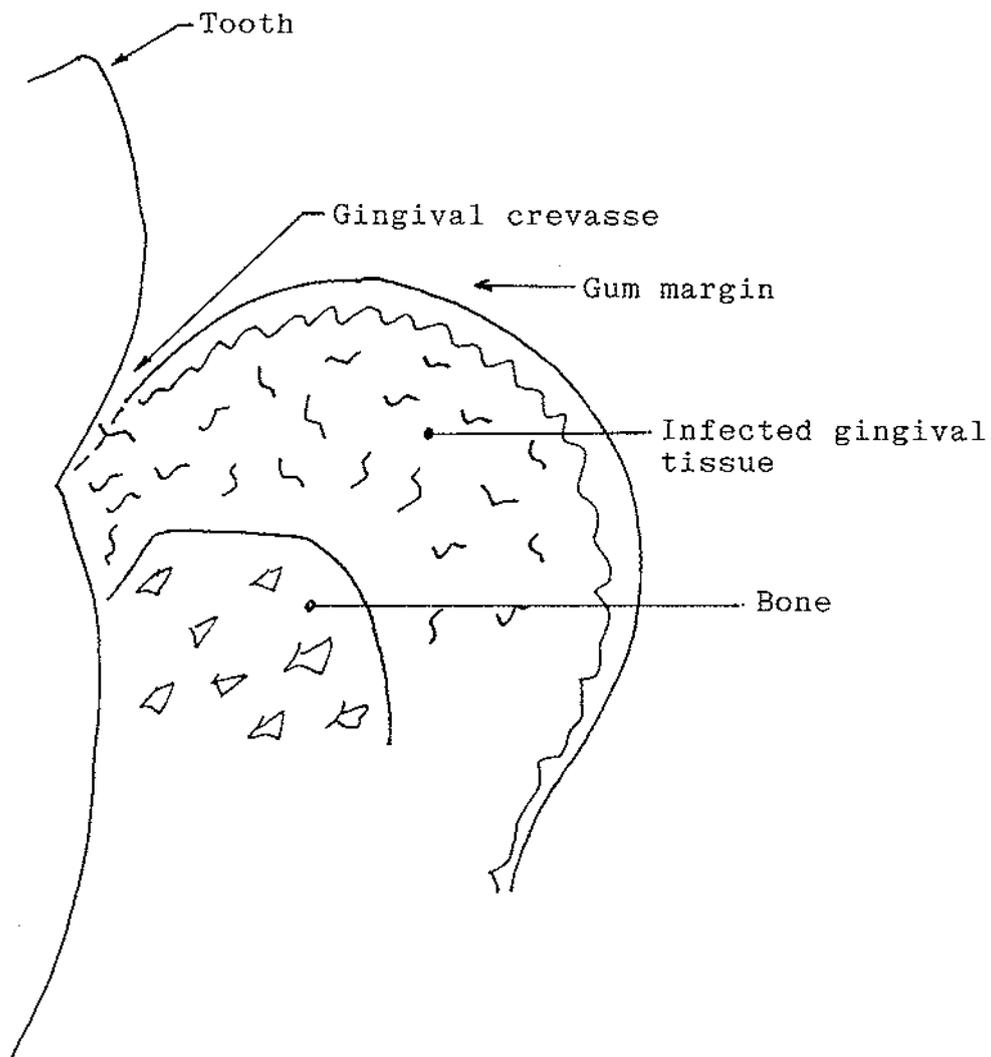


FIGURE 3: DIAGRAMATIC REPRESENTATION OF A PERIODONTAL ABSCESS

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 unlanded 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°C until transport to the laboratory. Culture was initiated as soon as possible, usually within two hours of collection.

SECTION 1: ISOLATION AND IDENTIFICATION OF BACTERIA

Isolation

Contents of syringes were transferred to 5.0 ml of 0.25 strength Ringers dispersion solution and vortexed. Serial ten fold dilutions were made in Ringers dilution solution. Three hundred microlitre aliquots from each dilution were spread on two supplemented blood agar (SBA), and one each of Fusobacterium selective agar (FSA) and Bacteroides selective agar (BSA). One set of SBA was incubated aerobically at 37°C, all others were incubated anaerobically at 37°C.

After four, and up to 10 days' incubation, colonies were picked from the selective plates mainly and subcultured on SBA. All colony types present on primary isolation plates were subcultured on SBA until obtained in pure culture. Only "strictly anaerobic" Gram-negative bacteria were retained for identification. Organisms that did not grow in 21% oxygen on SBA were deemed to be strict anaerobes.

Identification

Identification procedures included the following: macroscopic colony characteristics, Gram stain, oxygen tolerance, kanamycin and vancomycin sensitivity, nitrate reduction, gelatin liquification, catalase production, esculin hydrolysis, indole production, bile growth, and carbohydrate fermentations.

(see Appendix II).

Tests used to identify strictly anaerobic isolates

Gram-negative cocci which were non-fermentative and reduced nitrate were considered to be Viellonella parvula.

Gram-negative rods were subdivided on the basis of sensitivity to kanamycin. A zone of 10 mm or greater around a disc containing 10 mg base activity (10.13 mg powder) was considered sensitive.

Gram-negative rods were further subdivided into species on the basis of tests listed in Tables II and III.

TABLE II
Kanamycin resistant species

	Growth in 20% bile	Black Pigment	Indole	Esculin hydrolysis	Gelatin liquefaction	Catalase	Fermentations					
							Arabinose	Lactose	Sucrose	Trehalose	Glucose	Xylose
<i>B bivius</i>	-	-	-	-	+	-	-	+	-	+	-	
<i>B capillosis</i>	-	-	-	+	-	-	-	+	-	+	-	
<i>B disiens</i>	-	-	-	-	+	-	-	-	-	+	-	
<i>B oralis</i>	-	-	-	+	+	-	+	+	-	+	-	
<i>B praecutus</i>	-	-	-	-	+	-	-	-	-	-	+	
<i>B ruminicola</i>	-	-	-	+	+	+	+	+	-	+	+	
<i>B asaccharolyticus</i>	-	+	+	-	-	-	-	-	-	-	-	
<i>B melaninogenicus</i>	-	+	-	-	-	-	-	+	+	+	-	
<i>B denticola</i>	-	+	-	+	-	-	-	+	+	+	-	
<i>B loescheii</i>	-	+	+	-	-	-	-	-	+	+	-	
<i>B intermedius</i>	-	+	+	-	-	-	-	-	+	+	-	
<i>B corporis</i>	-	+	-	-	-	-	-	-	-	+	-	
<i>B levii</i>	-	+	-	-	-	-	-	+	-	+	-	
<i>B macacae</i>	-	+	+	-	-	-	-	+	-	+	-	
<i>B distasonis</i>	+	-	-	+	-	+	-	+	+	+	-	
<i>B fragilis</i>	+	-	-	+	-	+	-	+	-	+	-	
<i>B ovatus</i>	+	-	+	+	-	-	-	+	+	+	-	
<i>B thetaiotamicron</i>	+	-	+	+	-	+	-	+	+	+	-	
<i>B uniformis</i>	+	-	+	+	-	+	-	+	-	+	-	
<i>B vulgatus</i>	+	-	-	+	-	-	-	+	-	+	-	
<i>B eggerthii</i>	+	-	+	+	-	-	-	-	-	+	-	
<i>B splanchnicus</i>	+	-	+	+	-	-	-	-	-	+	-	

A slide agglutination test using rabbit anti *B gingivalis* sera kindly supplied by Dr K Frisken, Otago University, was carried out on all asaccharolytic black pigmented *Bacteroides* isolates. *Bacteroides gingivalis* strain JKG1 was used as a positive control.

TABLE III
Kanamycin sensitive species

	Growth in 20% bile	Indole	Esculin hydrolysis	Nitrate reduction	Fermentations		
					Glucose	Fructose	Mannose
F <u>gonidiaformans</u>	-	+	-	-	+	-	-
F <u>naviforme</u>	-	+	-	-	-	+	-
F <u>necrophorum</u>	-	+	-	-	-	-	-
F <u>nucleatum</u>	-	+	-	-	-	+	-
F <u>russii</u>	-	-	-	-	-	-	-
F <u>mortiferum</u>	+	-	+	-	+	+	+
F <u>varium</u>	+	+	-	-	+	+	+
B <u>ureolyticus</u>	-	-	-	+	-	-	-

SECTION 2: ANTIBIOTIC SENSITIVITY TESTING BY THE
AGAR DILUTION METHOD

The six antibiotics tested were:

1. Benzyl Penicillin (sodium) B P
(Crystapen, Glaxo Laboratories Ltd,
Greenford, England)
2. Cefoxitin sodium
(Mefoxin, Merck Sharp & Dome
New Zealand Ltd.)
3. Metronidazole
(Sigma, St. Louis M.O. USA)
4. Ornidazole
(Tiberol, Roche Products
New Zealand Ltd)

5. Tetracycline hydrochloride
(Sigma, St. Louis M.O. USA)

6. Clindamycin hydrochloride
(Dalacin C, Upjohn Kalamazoo
Michigan USA).

Stock solutions were prepared in appropriate solvents (Appendix III) and stored at -20°C for no longer than recommended by the manufacturer.

Agar plates containing dilutions of the antibiotics were poured after adding 1.0 ml of appropriate antibiotic in solution to 19 ml of Wilkens Chalgren (W/C) agar cooled to 50°C . Plates were poured and inoculated within 24 hours.

Cultures to be tested were removed from storage and grown on SBA or W/C agar, then inoculated into 4.0 ml PYG broth and grown overnight to reach a turbidity comparable to half that of a McFarland No. 1 standard (Appendix III).

Seven hundred microlitres of each broth culture were added to the wells in the replicator (Figure 4), which inoculated the antibiotic plates with approximately two microlitres of each culture.

Control plates containing no antibiotic were inoculated before and after each set of antibiotic plates.

Bacteroides thetaiotamicron (ATCC 29741) was included as a control.

Plates were incubated anaerobically at 37°C for 48 hours.

The minimum inhibitory concentration (MIC) was read as the lowest concentration of antibiotic which allowed three or fewer discrete colonies, or a barely visible haze of growth that was definitely less than the growth on the control agar plates.

All isolates were tested on at least two separate occasions.

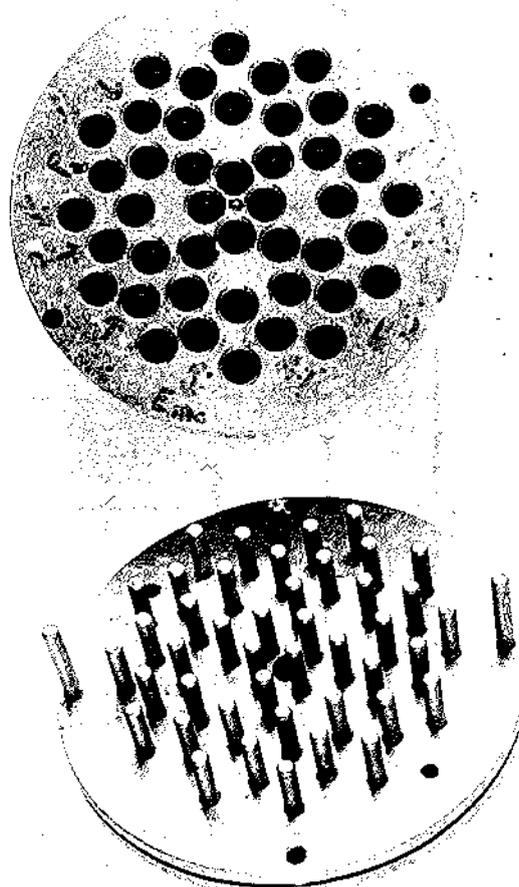


FIGURE 4: MULTIPLE INOCULATION REPLICATOR.
The replicator was made using aluminium rivets mounted in an aluminium plate. A 100mm diameter stub had 44 holes drilled in it to act as a reservoir for inoculation.

SECTION 3: PLASMID ANALYSIS

Details of buffers and reagents used in this section are listed in Appendix III.

Agarose gel slabs were made as follows:

0.42 g of agarose (type 1: low EEO, Sigma), was dissolved in 60 ml of tris borate buffer by boiling in a pressure cooker for five minutes. The gel was then cooled to 50°C and the slab poured directly in an electrophoresis box.

Plasmid analysis by the Eckhardt method:

The method of Thomas Eckhardt (13) was modified as follows:

Culture equivalent to a 1.0 mm diameter colony was removed from an agar plate with a toothpick and suspended in 20 ul of solution 1 in an Eppendorf tube. This was immediately transferred to a well in an agarose gel slab. After 10 minutes 20 ul of solution 2 was added and a flame sealed Pasteur pipette was passed across then back through the well once, the two phases were not mixed. The well was then flooded with tris borate buffer and subjected to electrophoresis at 20 volts for 17 hours.

Tris equilibration of phenol (38)

Clear (non-oxidized) phenol was melted at 60°C in a waterbath and 8-hydroxyquinoline added to give a final concentration of 0.1%. The phenol was then extracted twice with 1.0 M tris pH 8.0 in a separating funnel. Extractions were then made using 0.1 M tris with 0.2% B-mercaptoethanol (pH 8.0), until the pH of the aqueous phase was greater than 7.6.

Ethanol precipitation of DNA (38)

Aqueous solutions of DNA were either made 0.33 M with respect to sodium acetate, or 1.0 M with respect to sodium chloride.

Two volumes of cold (-20°C) absolute ethanol were then added and gently mixed.

The mixture was either left at -20°C overnight, or -70°C for 20 minutes.

Precipitated DNA was recovered by centrifugation.

Plasid analysis by the alkaline lysis method:

The method of Kado, C.I. and Liu, S.T. (28) was modified as follows:

Cells were grown overnight in PYG broth and 4.0 ml were harvested by centrifugation at 3000g for 15 minutes. These were washed once in tris acetate buffer containing 0.1% sarkosyl, then resuspended in 0.33 ml of tris acetate buffer. Then 0.67 ml of lysis mixture was added, and mixed gently. This mix was incubated at 55°C for one hour then cooled to room temperature. Eighty microlitres of 2.0 M tris (pH 7.0) was then added rapidly. Two millilitres of a 1:1 phenol : chloroform solution was then added to the lysate, and gently mixed until emulsified. The two phases were allowed to separate at 4°C, then the mix was centrifuged at 4000g for 15 minutes to bring precipitated cellular debris to the interface. The aqueous phase was removed, and extractions with phenol : chloroform, and chloroform on its own were repeated until the aqueous phase appeared clear, often only one extraction was required. The aqueous phase was then removed and ethanol precipitated using 3.0 M sodium acetate (see above). The DNA was recovered by centrifugation at 14000g or greater for 30 minutes, the liquid was gently poured off, and the DNA dried under vacuum. The DNA was resuspended in 20 ul of tris EDTA buffer, and loaded into a dry well in an agarose gel slab. The electrophoresis box was filled with tris

borate buffer until level with the surface of the gel slab, and 20 volts was applied to the gel for one hour. The gel was then flooded with tris borate buffer and electrophoresis at 20 volts continued for a further 16 hours.

DNA bands, stained with ethidium bromide, were visualized and photographed over a shortwave ultraviolet transilluminator using Kodac Tri-X Pan film and a number 7 filter.

The size of plasmids was estimated by comparing their mobility in agarose gel with that of the eight plasmids of known size isolated routinely from E coli V517 (kindly supplied by Dr F. Macrina, Department of Microbiology, Virginia Commonwealth University, Richmond, Virginia 23298 (35)) (Figure 5).

The percentage mobilities of the plasmids from V517 were plotted against Log base 10 (molecular size), and the line of best fit drawn (Figure 5a).

The molecular size of other plasmids in the same gel were read from the plot at their own percentage mobilities.

This method is less accurate for estimating the size of large plasmids, as the plot deviates from a straight line to a curve (Figure 5a).

The size of linear fragments of DNA generated from the plasmids by cleavage with restriction enzymes was estimated in the same way using linear fragments of known size generated from phage Lambda.

The error in the estimation of the size of plasmids in the range of 7.6 to 1.5 kb (5.0 - 1.0 Mda) by this method was about six percent. This was derived by assessing the variability of the sizes estimated for the plasmids from the B fragilis isolate PN9, which was used as a positive control during the plasmid content survey.



The photograph is of a 0.7% agarose gel. Lane B contains four plasmids of unknown size isolated from a *B fragilis* strain designated PN9. Lane A contains eight plasmids of known size isolated from *E coli* V517.

Plasmid	Distance travelled mm ^t	Percentage mobility	Plasmid size (M daltons)
V517A	3.50	6.4	35.84 ± 1.0
V517B	30.75	56.4	4.82 ± 0.11
V517C	36.50	67.0	3.67 ± 0.08
V517D	38.00	69.7	3.39 ± 0.09
V517E	42.75	78.4	2.63 ± 0.07
V517F	47.75	87.6	2.03 ± 0.06
V517G	49.75	91.3	1.79 ± 0.07
V517H	54.50	100*	1.36 ± 0.08
PN9A	6.00	11.0	28
PN9B	37.75	62.3	4.2
PN9C	42.25	77.5	2.65
PN9D	49.25	90.4	1.85

^tThe distance travelled by Plasmid bands was measured from the front of the well to the front of the band.

*The smallest V517 plasmid of 1.36 ± 0.08 M daltons (35) was designated as having 100% mobility.

FIGURE 5: Example of size estimation of plasmids.

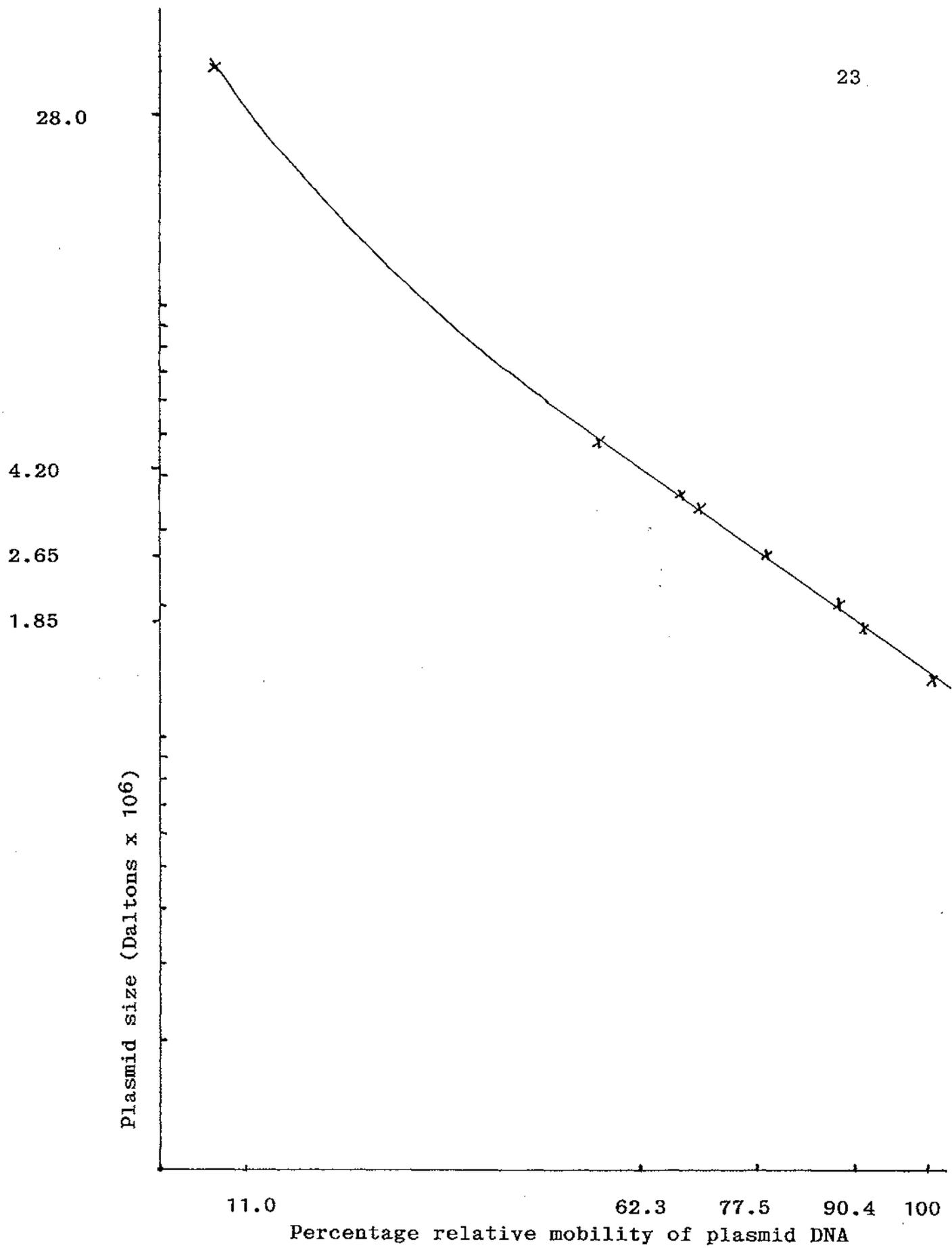


FIGURE 5a: PLOT OF RELATIVE MOBILITY OF PLASMID DNA AGAINST MOLECULAR SIZE.

Large scale isolation of plasmid DNA

Cells were grown as a lawn on one or two agar plates then harvested with a sterile wire loop into 4.0 ml PYG broth, and suspended by aspiration. The cell suspension was transferred to 250 ml of PYG broth which was incubated at 37°C anaerobically overnight.

Cells were harvested by centrifugation at 3000g for 15 minutes, washed once in 200 ml of tris acetate buffer with 0.1% sarkosyl, and resuspended in 10 ml tris acetate buffer. Thirty millilitres of lysis mix which had not been made alkaline by the addition of NaOH was then added, and the cells allowed to lyse. Heating at 55°C for one hour was sometimes needed to obtain a cleared lysate.

Ten millilitres of 5.0 M NaCl were then added, and the lysate left at 4°C overnight for a precipitate to form. The DNA was recovered in the supernatant after centrifugation at 4000g. Plasmid DNA was then precipitated by adding 100 ml of cold (-20°C) absolute ethanol, and placing at -70°C for 20 minutes. The plasmid DNA was recovered by centrifugation at 20,000g for 60 minutes, and resuspended in 5.0 ml of tris acetate buffer.

At this stage a sample of the DNA solution was run in an electrophoresis gel and visualized to ascertain the concentration of plasmid DNA, and whether it was clearly visible above background staining under longwave ultraviolet light.

If plasmid bands were clearly visible under longwave UV light then the sample was loaded into wide wells in agarose gel slabs made with ultra pure DNA grade agarose (Bio-Rad), electrophoresed, visualized with a hand held long wave UV lamp, and plasmid bands cut from the gel with a scalpel.

If background staining obscured plasmid bands then RNase was added to the sample at 10 ug/ml, and incubated at 37°C for one hour. Phenol : chloroform,

and chloroform extractions (as described for alkaline lysis plasmid screening) were also carried out until the DNA solution appeared clear. If plasmid DNA concentration was low, the DNA was ethanol precipitated and resuspended in a smaller volume of tris acetate buffer.

Extraction of DNA from agarose gel (64)

Plasmid bands were cut from ultra pure DNA grade (Bio-Rad) agarose under long wave ultraviolet light using a scalpel.

Excess buffer was blotted from the gel slice, then it was cut into small pieces and placed in a centrifuge tube.

The agarose was covered with tris equilibrated phenol, vortexed, then frozen, either at -20°C for four hours, or -70°C for one hour.

The still frozen mixture was then centrifuged until thawed. The aqueous phase (containing DNA) was removed and extracted once with phenol : chloroform, as previously described, then ethanol precipitated, and DNA recovered by centrifugation at 14000g or greater for 30 minutes.

Plasmid DNA was resuspended in sterile distilled water, and a portion electrophoresed through agarose gel and visualized to check plasmid DNA concentration, and that only one species of plasmid was present.

Purified plasmid DNA solutions were stored at -70°C .

Bacterial Restriction Endonuclease Analysis (BRENDA):

Restriction enzymes:

Alu I and *Hae III* were obtained from New England Biolabs, 32 Tozer Road, Beverly, MA. USA.

The method of Davis (10) was used as follows:

Twenty-five microlitres of purified plasmid DNA solution were placed in a DNase free Eppendorf tube (sterilised).

Three microlitres of medium salt restriction buffer were added.

Two microlitres of either *Alu I* or *Hae III*, diluted in their appropriate storage buffers to three units per microlitre, were added and mixed.

The digest was placed in a 37°C water bath for three hours.

Ten microlitres of stop buffer were added, and the tube transferred to a 70°C water bath for 10 minutes.

Digested plasmid DNA was stored at -70°C.

SECTION 4: CONJUGATION AND TRANSFORMATION EXPERIMENTS

The recipient organism for these experiments was Escherichia coli strain HB101.

The minimum inhibitory concentrations of benzyl penicillin and tetracycline for HB101 were determined to be 60 and 2.0 ug/ml respectively. These were determined by spotting 10 ul of overnight broth culture grown in L-Broth (Appendix I) onto Wilkens Chalgren agar containing appropriate dilutions of the antibiotics.

Conjugation:

Overnight broth cultures of strictly anaerobic donor, and facultative recipient bacteria were spotted

onto 0.45 um nitrocellulose filter paper on Wilkens Chalgren (W/C. agar in a ratio of 2:1 (Donor:recipient). The recipient HB101 was spotted onto filter paper on agar by itself as a control, and the plates incubated at 37°C for 24 hours in the anaerobic chamber. The filter paper was then transferred to PYG broth and vortexed to suspend the bacteria.

To select for penicillin resistant transconjugants, 0.3 ml aliquots of the suspended bacteria were plated directly onto W/C plates containing 80, 75, and 70 ug/ml penicillin.

To select for tetracycline resistant transconjugants, suspended bacteria were diluted to an optical density at 590 nm of 0.5, and 0.3 ml aliquots spread on W/C plates containing 6.0 ug/ml tetracycline.

Penicillin and tetracycline plates were then incubated anaerobically at 37°C for 24 hours.

Transformation

The method of Cohen et al (51) was used as follows:

E coli stain HB101 was grown in L broth to an optical density at 590 nm of 0.85 to 0.9. The cells were harvested by centrifugation and washed once with sterile 0.01 M NaCl, suspended in 0.3 M CaCl₂, and kept on ice for 20 minutes.

The suspension was centrifuged, and the cells suspended in one-tenth the volume of cold sterile 0.03 M CaCl₂. In another tube sterile distilled water replaced the DNA solution; these bacteria were used as a control. The tubes were left on ice for one hour, then warmed to 42°C and gently shaken for two minutes. After rechilling, the cells were diluted in 2.0 ml of fresh L broth and incubated at 37°C for six hours.

Recipient and control cells were plated undiluted in 0.3 ml aliquots onto W/C plates containing 80, 75 and 70 ug/ml of benzyl penicillin, and 6.0 ug/ml of tetracycline . The W/C agar plates were incubated at 37°C for 24 hours.

Experiment to quantify the extent to which tetracycline resistance in transformed E coli HB101 was inducible:

Transformed E coli were grown overnight on two sets of W/C agar plates, with and without tetracycline (induced and uninduced) at a concentration of 6.0 ug/ml. Induced and uninduced bacteria were then suspended in L broth (Appendix I) to an optical density equivalent to a half McFarland number one standard (Appendix III). Serial 10 fold dilutions of these suspensions were then made in more L broth, and 100 ul aliquots pipetted onto W/C plates with tetracycline at 6.0 ug/ml.

RESULTS

SECTION 1: ISOLATION OF ORAL BACTERIA

Thirty samples were examined and 17 of these yielded a total of 107 strictly anaerobic Gram-negative bacteria. Sixty five of these were Bacteroides species, 28 were fusobacteria, and 14 were the Gram-negative coccus Veillonella parvula.

Of the 65 Bacteroides species, 40 were black pigmented on blood agar. Of these 40, 28 were asaccharolytic. These were tested with rabbit anti-B gingivalis sera, and none of the 28 agglutinated, although a positive control did. Other black pigmented isolates were identified as B melaninogenicus and B intermedius. Non pigmented Bacteroides species identified were B bivius, B capillosis, B oralis, B praecutus, B ruminicola, and B ureolyticus.

Fusobacterium species isolated were F mortiferum, F nucleatum and F russii.

Details of samples and the bacteria they yielded are given in Table IV.

No sample yielded anaerobic bacteria only; eight samples described as post operative infections, and one described as an infected wisdom tooth yielded facultative bacteria but no Gram-negative anaerobes. Four samples yielded no bacterial growth at all. One of these was described as an infected cyst which was treated with ornidazole, and three were dental abscesses which had been treated with cefoxitin plus amoxicillin.

Of 11 samples described as coming from dental abscesses, eight yielded Gram-negative anaerobes, and three no growth at all. Three samples were described as coming from infected wisdom teeth, (pericoronitis). Two of these yielded Gram-negative anaerobes, and one had facultative, Gram-negative cocci but no Gram-negative anaerobes.

Fifteen samples were described as coming from post operative infections. Seven of these samples yielded Gram-negative anaerobic bacteria. All 15 of these samples contained facultatively anaerobic bacteria.

Thirty seven bacteria were received in pure culture from Palmerston North hospital. Twenty seven of these were Bacteroides fragilis, four were B thetaitomicron, three were B ovatus and one was described as a "Bacteroides species".

TABLE IV
DETAILS OF SAMPLES

Sample	Sample site and Patient description	Bacteria isolated	Ratio AnO ₂ /O ₂ *
B1	Periapical abscess 20 yr female	V <u>parvula</u> F <u>russii</u> x 2 F <u>mortiferum</u> B <u>asaccharolyticus</u>	4.0
B2	Post extraction infection 28 yr male	F <u>russii</u> F <u>nucleatum</u> x 2	79
B4	Dental abscess male age na	F <u>nucleatum</u>	na
B9	Dental abscess 26 yr male	V <u>parvula</u> B <u>ureolyticus</u> B <u>melaninogenicus</u> B <u>praecutus</u>	15
B13	Post surgical infection 23 yr female	V <u>parvula</u> B <u>asaccharolyticus</u> x 5 B <u>melaninogenicus</u>	na
B14	Dental abscess male, age na	V <u>parvula</u>	0.2
B16	Dental abscess 25 yr female	V <u>parvula</u> B <u>asaccharolyticus</u> x 3 B <u>capillosis</u> B <u>oralis</u>	25
B17	Post surgical infection male, age na	V <u>parvula</u> B <u>melaninogenicus</u> B <u>praecutus</u>	0.2
B19	Post surgical infection 24 yr female	V <u>parvula</u> B <u>melaninogenicus</u> <u>Fusobacterium</u> sp x 4	670
B20	Post surgical infection 44 yr male	B <u>asaccharolyticus</u> x 3 B <u>melaninogenicus</u> Non pigmented <u>Bacteroides</u> sp x 3 <u>Fusobacterium</u> sp x 5	1700

continued.../32

(TABLE IV continued)

Sample	Sample site and Patient description	Bacteria isolated	Ratio AN_2/O_2^*
B21	Post surgical infection 30 yr female	<u>B asaccharolyticus</u> x 7 <u>V parvula</u> x 2 Non pigmented <u>Bacteroides</u> sp x 2 <u>Fusobacterium</u> sp x 3	14000
B22	Post surgical infection 20 yr male	<u>V parvula</u> x 2 <u>F russii</u> <u>Fusobacterium</u> sp Non pigmented <u>Bacteroides</u> sp x 2	120
B23	Dental abscess 20 yr male	Non pigmented <u>Bacteroides</u> sp <u>Fusobacterium</u> sp x 6	2230
R3	Pericoronitis 24 yr male	<u>V parvula</u> <u>F russii</u> <u>B oralis</u> x 2 <u>B bivius</u> <u>B ruminicola</u> <u>B intermedius</u> <u>B asaccharolyticus</u>	5.0
R4	Pericoronitis 28 yr female	Non pigmented <u>Bacteroides</u> sp x 2 <u>Fusobacterium</u> sp	2250
R5	Dental abscess 42 yr male	<u>B asaccharolyticus</u> x 8 <u>B melaninogenicus</u> <u>B ruminicola</u> x 2 Non pigmented <u>Bacteroides</u> sp fusobacteria x 3	2100
R6	Dental abscess 13 yr male	<u>V parvula</u> x 2 <u>B intermedius</u> x 2 <u>B ureolyticus</u> x 2	145

(na = not available)

Ratio AN_2/O_2 - this indicates the proportion of anaerobic bacteria in the sample. This ratio is derived from the number of colonies on the two sets of non-selective SBA plates incubated at primary isolation. The total count of bacteria growing anaerobically is divided by the total count of bacteria growing aerobically. It should be noted that facultatively anaerobic bacteria will grow on both sets of plates, though many of these are oxygen sensitive on primary isolation. Thus this ratio gives an indication only of the proportion of strict anaerobes in the sample.

SECTION 2: ANTIBIOTIC SENSITIVITY TESTING

The results of antibiotic sensitivity testing by the agar dilution method are shown in Tables V to X. The break point of an antibiotic is defined as the serum or tissue concentration it attains during optimum therapy.

TABLE V
Minimum Inhibitory Concentrations of
benzyl penicillin for isolates

Concentration (ug/ml)	Break point = 2.0 ug/ml										
	Cumulative & susceptible to indicated concn.										
	0.005	0.05	0.5	1.0	2.0	4.0	8.0	16	32	80	160
<u>Bacteria</u> (number of isolates)											
Hospital isolates (37)						3	27	54	86	89	
Black pigmented <u>Bacteroides</u> sp (40)	20	75	82.5	87.5	92.5			NT	NT	95	NT*
Non pigmented <u>Bacteroides</u> sp (25)	17	67	96			100					
<u>Fusobacterium</u> sp (28)	7	89	100								
<u>Veillonella parvula</u> (14)	29	50	100								

*NT - These bacteria not tested at
this concentration.

TABLE VI

Minimum Inhibitory Concentrations of
cefoxitin for isolates

Concentration (ug/ml)	Break point = 20 ug/ml							
	Cumulative % susceptible to indicated concn.							
	0.05	0.5	5.0	10	20	40	80	240
<u>Bacteria</u>								
(numbers of isolates)								
Hospital isolates (37)			13.5	75.5	97		100	
Black pigmented <u>Bacteroides</u> sp (40)	22.5	87.5	100					
Non pigmented <u>Bacteroides</u> sp (25)	21	75	100					
<u>Fusobacterium</u> sp (28)	11	82	100					
<u>Veillonella parvula</u> (14)	50	86	100					

TABLE VII

Minimum Inhibitory concentrations of
metronidazole for isolates

Break point = 8.0 ug/ml
Cumulative % susceptible to indicated concn.

<u>Concentration (ug/ml)</u>	0.02	0.2	2.0	4.0	8.0	16	32
<u>Bacteria</u> (number of isolates)							
Hospital isolates (37)			100				
Black pigmented <u>Bacteroides sp</u> (40)	20	37.5	97.5	100			
Non pigmented <u>Bacteroides sp</u> (25)	8	30	100				
<u>Fusobacterium sp</u> (28)	11	57	100				
<u>Veillonella parvula</u> (14)		7	100				

TABLE VIII
Minimum Inhibitory Concentrations of
ornidazole for isolates

Concentration ug/ml	Break point = 8.0 ug/ml						
	Cumulative % susceptible to indicated concn.						
	0.02	0.2	2.0	4.0	8.0	16	32
<u>Bacteria</u> (number of isolates)							
Black pigmented <u>Bacteroides</u> sp (40)	20	37.5	97.5	100			
Non pigmented <u>Bacteroides</u> sp (25)	8	30	100				
<u>Fusobacterium</u> sp (28)	11	57	100				
<u>Veillonella parvula</u> (14)		7	100				

TABLE IX

Minimum Inhibitory concentrations of
tetracycline for isolates

Concentration ug/ml	Break point = 3.0 ug/ml									
	Cumulative % susceptible to indicated concn.									
	0.0075	0.075	0.75	1.5	3.0	6.0	12	24	48	120
<u>Bacteria</u>										
(number of isolates)										
Hospital isolates (37)			59.5	62		70		89	100	
Black pigmented <u>Bacteroides</u> sp (40)		2.5	95				100			
Non pigmented <u>Bacteroides</u> sp (25)			80	92		96	100			
<u>Fusobacterium</u> sp (28)	18	86	96		100					
<u>Veillonella parvula</u> (14)		79	100							

TABLE X
 Minimum inhibitory concentrations of
 clindamycin for isolates

Concentration ug/ml	Break point = 4.0 ug/ml							
	Cumulative % susceptible to indicated concn.							
	0.01	0.1	1.0	2.0	4.0	8.0	16	32
<u>Bacteria</u> (number of isolates)								
Hospital isolates (37)		5.5	81	89	100			
Black pigmented <u>Bacteroides</u> sp (40)	47.5	92.5	100					
Non pigmented <u>Bacteroides</u> sp (25)	4	83	100					
<u>Fusobacterium</u> sp (28)	7	68	96	100				
<u>Veillonella parvula</u> (14)	7	36	100					

All thirty seven hospital isolates were resistant to penicillin (Table V). One *B ovatus* isolate had an MIC of 4.0 ug/ml, and nine *B fragilis* isolates had MIC's between 4.0 and 8.0 ug/ml, the other 27 isolates of various species from the *B fragilis* group had MIC's from above 8.0 ug/ml to above the highest concentrations tested, 160 ug/ml.

Few oral *Bacteroides* isolates were resistant to 2.0 ug/ml of penicillin. Three of these were pigmented; they were designated R6B1, R6B2 and R3B6, and were isolated from samples R6 and R3 (Table IV). Neither of the infections these samples were taken from had been treated with antibiotics. R6B1 and R6B2 were almost certainly two isolates of the same bacterium. They were both isolated from sample R6, identified as *B intermedius*, and found to carry a 6.6 kb plamid (Figure 6). They have an MIC for penicillin somewhere in excess of 80 ug/ml. R3B6 was identified as *B asaccharolyticus*. Its MIC was somewhere between 8.0 and 80 ug/ml. The non pigmented resistant *Bacteroides* isolate, B23B4, from sample B23, had an MIC between 4.0 and 8.0 ug/ml. The infection this sample was taken from had previously been treated with amoxycillin. Six fusobacteria also isolated from this sample were all penicillin sensitive.

All the hospital isolates were sensitive to cefoxitin (Table VI) except for one, which was identified as *B distasonis*. Plasmid DNA was not detected in this isolate. Its MIC for cefoxitin was between 40 and 80 ug/ml. All oral isolates were sensitive to cefoxitin. R6B1 and R6B2, the most penicillin resistant oral bacteria, had an MIC of 5.0 for cefoxitin.

All isolates were sensitive to metronidazole and orindazole (Tables VII and VIII) and results were identical for the two.

Thirty percent of the hospital isolates were resistant to 6.0 ug/ml of tetracycline (Table IX). This included 22 percent or six of the *B fragilis* isolates, two of the four *B thetaiotamicron* isolates and all three *B distasonis* isolates. Seventy five percent of the

resistant hospital isolates harboured plasmids (see Section 3). Plasmid DNA was not detected in the three B distasonis isolates, but all nine of the other resistant isolates had plasmids. Forty four percent of the hospital isolates that were sensitive to tetracycline had plasmids. Tetracycline resistance could not be correlated with any particular plasmid size or group after plasmid analysis.

Most oral isolates were sensitive to tetracycline. Two black pigmented Bacteroides isolates designated B17B5 and R3B6, and two non pigmented isolates (B17B3 and R3B5) were resistant to 3.0 ug/ml. These resistant bacteria were isolated from samples B17 and R3 (Table IV); these samples also contained tetracycline sensitive bacteria. One Fusobacterium isolate identified as F russii was also resistant to 3.0 ug/ml of tetracycline.

All isolates were sensitive to clindamycin at 4.0 ug/ml (Table X).

In summary, all isolates in this survey were highly sensitive to the nitro-imidazoles and clindamycin, and only one isolate of B distasonis was resistant to cefoxitin. The B fragilis group isolates from hospitalized patients were resistant to penicillin and 30 percent of these isolates were also resistant to tetracycline. The oral isolates in contrast were generally highly sensitive to these antibiotics. A small proportion of the oral isolates were moderately resistant to tetracycline as were a low number to penicillin. Only one strain of B intermedius was highly resistant to penicillin.

SECTION 3: PLASMID ANALYSIS

Of the 110 oral bacteria, six (5.5 percent) were found to harbour plasmids (Figure 6), while 20 of the 37 Bacteroides isolates received from Palmerston North hospital had plasmids (54 percent).

Plasmids harboured by the hospital isolates were generally in the size range of 8.5 to 2.7 kilobases (kb)

(5.6 - 1.8 M daltons), with two large plasmids approximately 43 and 60 kb being the only exceptions (pPN9a and p136a, Figure 7). One of the isolates (PN9) had four plasmids, four had three, three had two and twelve had one plasmid.

The Fusobacterium russii isolate B22F3 had four plasmids (Figure 6), none of which had restriction sites for either of the restriction endonucleases *Alu I* and *Hae III*. The sizes of these plasmids were 15.3, 14.2, 11.4 and 10.2 kb. Two isolates of B. intermedius (R6B1 and R6B2) from the same sample carried a 6.6 kb plasmid (pR6, Figure 6), which had one restriction site for *Hae III* and no sites for *Alu I*. None of the plasmids isolated from hospital anaerobes resembled pR6.

Two isolates of B ruminicola (R5B10 and R5B11) and one of B melaninogenicus (R5B12), all isolated from the same sample, carried 5.6 kb plasmids (pR5B10, pR5B11 and pR5B12). These three plasmids were shown to have the same electrophoretic mobility and thus be of the same size (Figure 8). Two of these plasmids, pR5B10 and pR5B12, were cleaved by *Hae III* to give fragments of 4.3 and 1.3 kb. Digestion of the other plasmid pR5B11, from the other B ruminicola isolate, gave fragments of 5.0 and 5.4 kb (Figure 10). Digestion of these plasmids with *Alu I* gave a large number of small fragments which were not visible in the agarose gel.

A similar group of plasmids was found in four bacteria received from Palmerston North hospital. Three isolates of B fragilis, QLBfr, 1HBfr, and 9Bfr, and one of B thetaiotamicron, 61181, had plasmids of the same size of about 5.8 kb (pQLBfr, p1HBfr, p9Bfr and p61181; Figure 9). Digestion of pQLBfr, p9Bfr and p61181 with *Hae III* gave fragments of 4.3 and 1.5 kb (Figure 10). Digestion of p1HBfr with *Hae III* gave 5.6 and 5.2 kb fragments (Figure 10). Again digestion of these plasmids with *Alu I* gave numerous small fragments which were not discernible in agarose gels.

The size of the 5.6 kb oral plasmid pR5B10 is compared with that of the 5.8 kb plasmids from the four

hospital isolates in Figure 12.

Of the other plasmids harboured by hospital isolates, only two pairs showed any relatedness on the basis of their restriction endonuclease digestion patterns (Figure 11). Plasmid 0L9a and p7896 are 5.0 and 5.2 kb respectively, p0L9c and p0L7 are 2.7 and 4.0 kb respectively. None of these plasmids were cut by *Hae III*.

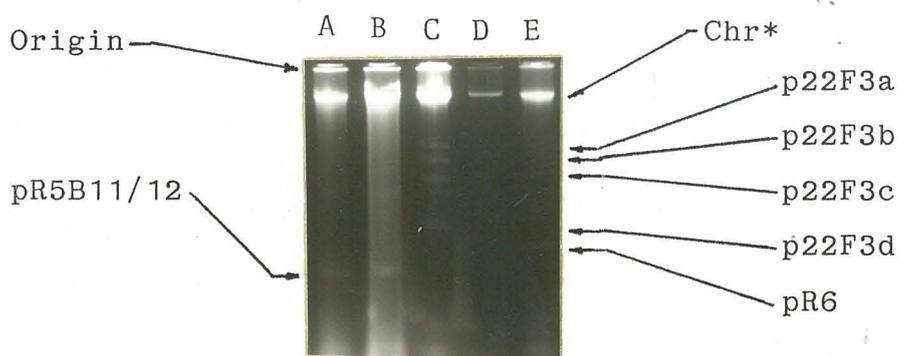


FIGURE 6: Plasmids harboured by oral bacteria.

Track A: 5.6 kb pR5B11, B: 5.6 kb pR5B12,
C: 15.3 kb p22F3a, 14.2 kb p22F3b, 11.4 kb
p22F3c, 10.2 kb p22F3d, E: 6.6 kb pR6.

* Chromosomal DNA.

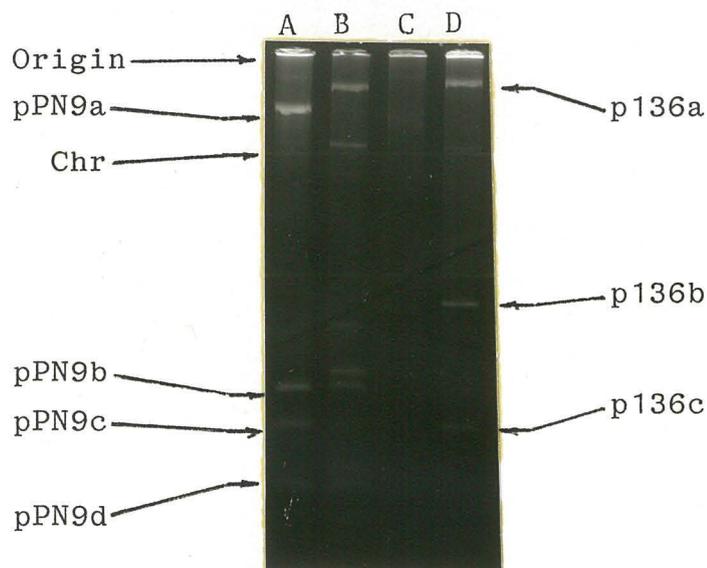


FIGURE 7: Large plasmids harboured by hospital isolates.

Track A: 43 kb pPN9a, 5.1 kb pPN9b, 4.1 kb pPN9c, 2.8 kb pPN9d, B: eight plasmids of *E. coli* V517, D: 60 kb p136a, 8.6 kb p136b, 3.4 kb p136c.

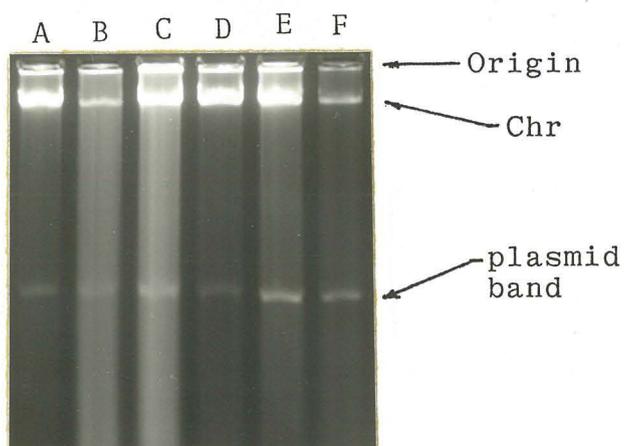


FIGURE 8: Size comparison of pR5B10, pR5B11 and pR5B12.

Track A: pR5B12, B: pR5B11 + pR5B12, C: pR5B11, D: pR5B11, E: pR5B10 + pR5B11, F: pR5B10.

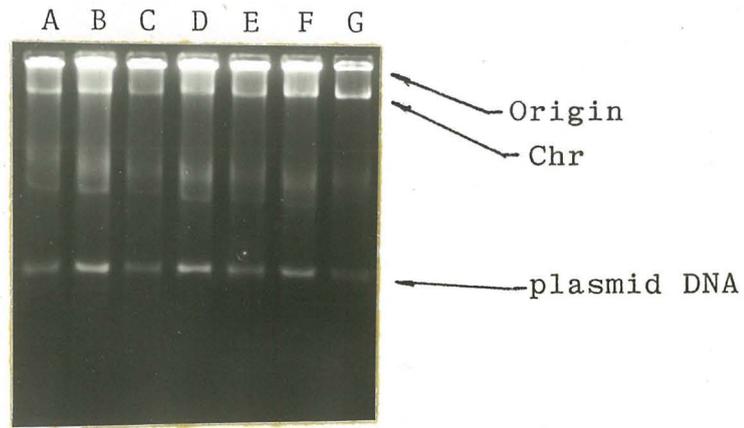


FIGURE 9: Size comparison of pQLBfr, p1HBfr, p9Bfr and p61181.

Track A: p61181, B: p61181 + p1HBfr,
 C: p1HBfr, D: p1HBfr + p1HBfr, E: p9Bfr,
 F: p9Bfr + pQLBfr, G: pQLBfr.

FIGURE 10: *Hae III* digest patterns of 5.6 kb oral plasmids and 5.8 kb plasmids from hospital isolates:

N.B. Photographs of the five gels have been cut to remove irrelevant tracks, and bring relevant tracks closer together for easier comparison.

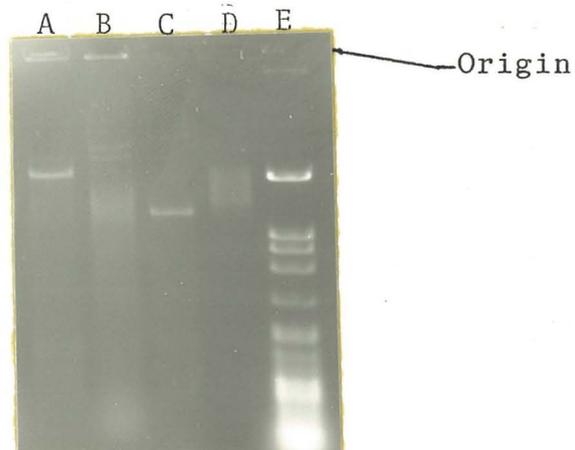


FIGURE 10(a): Track A: *Hae III* digest of pR5B10,
 B: *Hae III* digest of pR5B11, C: pR5B10,
 D: *Alu I* digest of pR5B10, E: *Hae III* digest
 of Lambda.

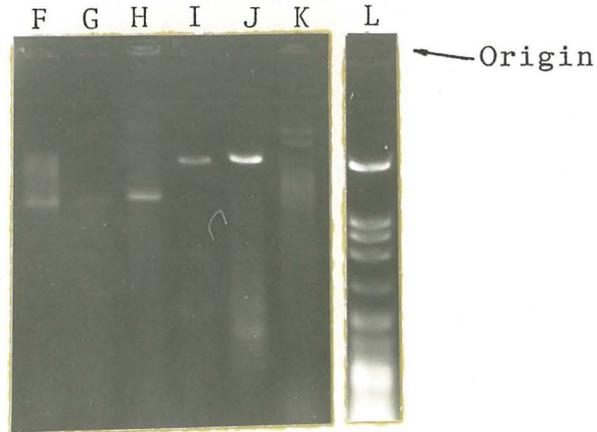


FIGURE 10(b): Track F: pR5B11, G: pR5B12, H: p9Bfr, I: *Hae III* digest of p9Bfr, J: *Hae III* digest of pR5B12, K: *Hae III* digest of pR5B11, L: *Hae III* digest of Lambda.



FIGURE 10(c): Track M: *Hae III* digest of Lambda, N: p1HBfr (covalently closed and nicked open circular plasmid bands visible), O: *Hae III* digest of p1HBfr, P: *Hae III* digest of pQLBfr, Q: *Hae III* digest of pR5B10.

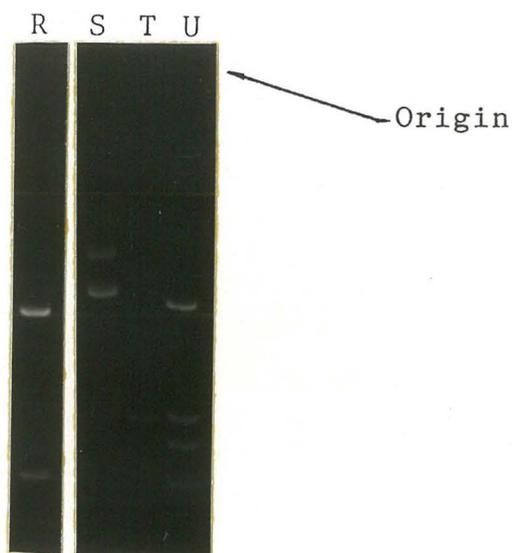


FIGURE 10 (d): Track R: *Hae III* digest of pQLBfr, S: *Hae III* digest of p1HBfr, T: *Alu I* digest of p1HBfr, U: *Hae III* digest of Lambda.

The gel in Figure 10(d) was electrophoresed for longer than the others, so that DNA fragment bands would travel further and separate more. This allowed more accurate estimation of the size of these fragments.

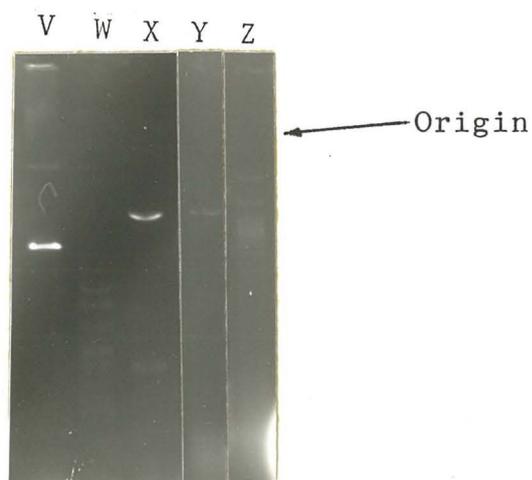


FIGURE 10(e): Track V: p 1BBfr (Covalently closed and nicked open circular plasmid bands visible), W: *Alu I* digest of p1BBfr, X: *Hae III* digest of p1BBfr, Y: *Hae III* digest of p61181, Z: *Hae III* digest of p1HBfr.

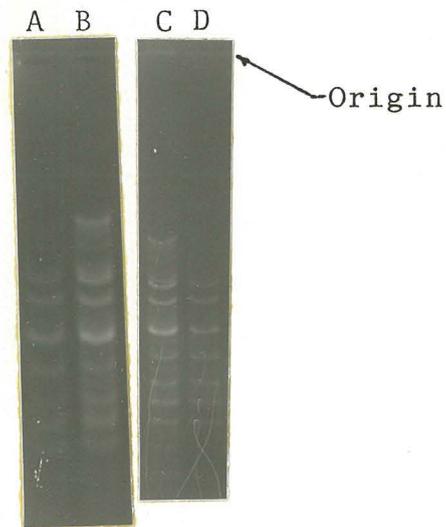


FIGURE 11: *Alu I* digestion patterns of four plasmids harboured by hospital isolates:
 Track A: *Alu I* digest of 5.0 kb p789b,
 B: *Alu I* digest of 5.2 kb pOL9a, C: *Alu I* digest of 2.7 kb pOL9c, D: *Alu I* digest of 4.0 kb pOL7.

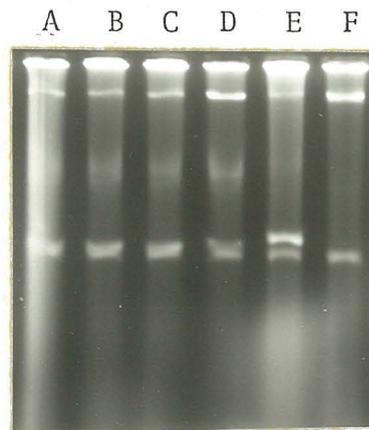


FIGURE 12: 5.6 kb oral plasmid pR5B10 compared with 5.8 kb plasmids from hospital isoaltes.
 Track A; p61181 + pR5B10, B: p1HBfr + pR5B10, C: p9Bfr + pR5B10, D: pQLBfr + pR5B10, E: 6.0 kb p62963 + pR5B10, F: pR5B10.

SECTION 4: CONJUGATION AND TRANSFORMATION

Two isolates of *B intermedius* (R6B1 and R6B2) from the same dental abscess (sample R6), and probably of common origin, harboured a 6.6 kb plasmid, and were resistant to 80 ug/ml of penicillin. R6B2 was used as a donor in conjugation experiments, and a purified preparation of the 6.6 kb plasmid (prepared in the same way as for restriction enzyme analysis) was used in transformation experiments. In neither set of experiments could penicillin resistant *E coli* be recovered.

Two isolates of *B fragilis* (PN9 and QLBfr) and one of *B thetaitomicron* (136Bth), all resistant to 48 ug/ml of tetracycline and harbouring plasmids, were also used in conjugation and transformation experiments. PN9 harboured four plasmids of 43, 5.1, 4.1 and 2.8 kb, QLBfr had a 5.8 kb plasmid, and 136B had three plasmids of 60, 8.6 and 3.4 kb. No tetracycline resistant *E coli* were recovered after conjugation experiments. However *E coli* showing a small decrease in sensitivity to tetracycline (an MIC of 6.5 as compared to 2.0 ug/ml) were recovered at high frequency after transformation with the 43 kb plasmid from PN9 (pPN9a) and the 60 kb plasmid from 136Bth (p136a).

There was no bacterial growth on selection plates spread with *E coli* transformed with sterile distilled water, and tetracycline resistant transformants were found to be non-lactose fermentative (HB101 phenotype) Gram-negative rods with the same cellular and colony morphology as *E coli* HB101. The small increase in resistance shown by the transformants was confirmed twice by determining the MIC of both control *E coli* HB101 and transformants which had grown in tetracycline free media as 2.0 and 6.5 ug/ml respectively.

The resistance shown by the transformed *E coli* appeared to be inducible to some extent. To quantify this observation the experiment outlined in section 4 of materials and methods was carried out. It was found that the induced bacteria grew on the tetracycline

plates when diluted 1:100, while the uninduced bacteria grew only when undiluted. The MIC for tetracycline of the induced and uninduced bacteria was also determined. There was no difference, the MIC being between 6.0 and 7.0 on two occasions for induced and uninduced bacteria of both sets of transformants. These results are further explained in Table XI.

Plasmid DNA could not be demonstrated in either set of transformed *E coli* HB101. *E coli* V517 and *B fragilis* PN9 were used as positive controls (Figure 13).

TABLE XI
Low level, inducible tetracycline
resistance in *E coli*
HB101 transformants^a

Pre treatment	DNA for transformation ^b	Dilution giving growth on 6 ug/ml of tetracycline ^c
Overnight growth on W/C agar	pPN9a p136a	1:1 1:1
Overnight growth on W/C agar + 6 ug/ml tetracycline	pPN9a p136a	1:100 1:100

- a The recipient *E coli* HB101 had an MIC of 2 ug/ml tetracycline and the donor *Bacteroides* strains each had an MIC of 48 ug/ml tetracycline.
- b The plasmids isolated from *B fragilis* strain PN9 and *B thetaiotamicron* strain 136Bth were 43 kb and 60 kb respectively.
- c Transformants, from the W/C agar 6 ug/ml tetracycline, were suspended in L broth to an optical density equal to 0.5 McFarland standard number one (Appendix III), and 100 ul aliquotes of serial ten fold dilutions plated on W/C agar with tetracycline at 6 ug/ml.

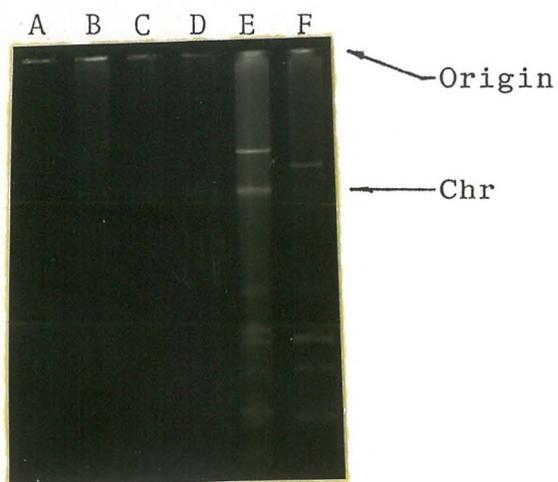


FIGURE 13: Absence of plasmid DNA in tetracycline resistant *E coli* transformants.

Tracks A-D: Lysates of transformants with no plasmids, E: eight plasmids of *E coli* V517, F: four plasmids of *B fragilis* PN9.

DISCUSSION

SECTION I: ISOLATION AND IDENTIFICATION

The samples from which bacteria were isolated in this survey were taken from oral infections by two dental surgeons in their Palmerston North surgeries (Table IV). Sample collection was not strictly controlled to prevent contamination with uninvolved salivary bacteria. The microbiota of dental abscess has been investigated using special sample collection techniques and controlled conditions to ensure samples for analysis were taken from the exact site of active disease, and that as little contamination with uninvolved bacteria occurred as possible (6,15,44,46). These investigations and studies of the ecological relationships of bacteria in mixed anaerobic infections (5,40,50), have implicated the Gram-negative anaerobes of the genera Bacteroides and Fusobacterium as the active pathogens in these infections. In this study no attempt was made to try and correlate various species of bacteria with types of infection. The established pathogens were isolated from samples using selective media and identified by simple biochemical tests (Tables II and III). This was done so that the antibiotic sensitivity survey and plasmid DNA analysis could be carried out.

The 14 isolates of Veillonella parvula were included because they were easily isolated and identified, are often found associated with oral infections (2,12), and little has been reported of the plasmid DNA of these bacteria. The veillonellae have been reported to be resistant to penicillin (29), and metronidazole (1,2).

From several samples yielding anaerobic bacteria in this study (i.e. B13, B21, B23, R5, Table IV) multiple isolates of what may be the same bacteria were made. This occurred initially because on primary isolation plates, the black pigmented Bacteroides, in particular, showed

variations in colony size and time for pigment to develop. Since there are 10 species of black pigmented Bacteroides recognised, all variants seen on primary isolation plates were subcultured. Most of these were later identified as B asaccharolyticus, but B melaninogenicus was also detected and this may have been missed if large numbers of isolates had not been taken. Also, there are three species of asaccharolytic black pigmented Bacteroides now recognised, B asaccharolyticus, B endodontalis, and B gingivalis. There is no doubt however, that some of these isolates are simply different clones of the same strain.

For the results of this research to be more reliably representative of the antibiotic sensitivity patterns and plasmid DNA content of Gram-negative anaerobic oral bacteria in Palmerston North, more people should have been sampled. All suitable patients visiting the two dentists over the two years of the survey were included however.

SECTION 2: ANTIBIOTIC SENSITIVITY SURVEY

The sensitivity levels of the hospital isolates in this survey are comparable with recently published results for the B fragilis group. However the high levels of resistance to cefoxitin and clindamycin, reported elsewhere in small proportions of isolates of this group, were not encountered. The oral bacteria were generally more sensitive than isolates of the same species reported on in the literature (Tables V-X) (14,18, 61,62,71).

The B fragilis group are intrinsically resistant to penicillin (70), and the hospital isolates were expected to be resistant. Only two strains of the black pigmented Bacteroides were resistant to penicillin, as were one non pigmented Bacteroides and one Fusobacterium isolate (Table V). In 1986, 30 percent of a group of pigmented and non pigmented bile sensitive Bacteroides

isolates from community hospitals in the United States were reported resistant to penicillin (18). Sensitivity surveys published since 1974 have consistently reported resistance to penicillin among the bile sensitive Bacteroides, fusobacteria, and veillonellae isolated in hospitals around the world (1,7,14,17,26,42,49,60,61,71).

In 1981 it was reported that prior treatment with penicillin significantly increased the prevalence and proportions of penicillin resistant Bacteroides and veillonellae in the subgingival microbiota (29). Four of the infections from which anaerobic bacteria were isolated in this survey had been treated with *B*-lactam antibiotics; only one of these (B23) yielded one moderately resistant Bacteroides isolates (Table V).

Only one hospital isolate was resistant to cefoxitin (Table VI). Recently published sensitivity surveys of this group in the United States indicate 10 to 15 percent of *B fragilis* group isolates from hospitals are resistant, some to levels as high as 128 (14,18,62), and 256 ug/ml (61). A high degree of variability between and within hospitals as regards cefoxitin resistant Bacteroides has been noted however (62). High level resistance to cefoxitin in low proportions of bile sensitive Bacteroides and fusobacteria has also been reported (60).

Metronidazole and ornidazole are 5-nitroimidazoles with the same antibacterial spectrum and activity, the difference being that ornidazole has a longer serum half life (30). Metronidazole resistance was reported in significant proportions and to fairly high levels (32 ug/ml) in earlier sensitivity surveys of the *B fragilis* group (1,56), and *B fragilis* strain AM24 has an MIC for metronidazole of 150 ug/ml (4). Resistance to this antibiotic has not been encountered in more recently published surveys of the *B fragilis* group, (26,49,61,71), including a nationwide survey of the group in the United States involving more than 500 new isolates in each of 1981, 1982, and 1983 (62). Metronidazole resistance has been reported in bile sensitive Bacteroides and

fusobacteria (1,60). No isolates resistant to the 5-nitroimidazoles were encountered in this survey (Tables VII and VIII).

The proportion of tetracycline resistant hospital isolates (Table IX) is comparable with that reported in some recently published surveys (1,3,49), and only about half that reported for the *B fragilis* group in others (7,60,62). Proportions of bile sensitive Bacteroides and fusobacteria reported resistant to tetracycline are generally several times higher than were found among the oral isolates (Table IX cf 1,3,7,49,60,62). The tetracycline resistant Bacteroides isolates of different species from samples B17 and R3 had no detectable plasmid DNA, and tetracycline resistance could not be correlated with any particular size or group of plasmid among the hospital isolates. Tetracycline resistance in the *B fragilis* group is often carried on transposons, which may or may not be plasmid borne (52).

High levels of resistance to clindamycin (MIC > 256 ug/ml) have been reported in very low numbers of isolates of the *B fragilis* group (18,61,71), and low proportions of bile sensitive Bacteroides and fusobacteria resistant to clindamycin have also been reported (7,61,71). Clindamycin resistance was not observed in this survey however (Table X).

Comparisons between sensitivity surveys of anaerobes can be misleading (70). Variation in testing methodology including medium used, additives, size and age of inoculum, and time and type of incubation can affect results (70). Bacteroides thetaiotamicron ATCC strain 29741 was included several times as an internal control in this survey. It consistently gave results in agreement with sensitivity levels previously determined for this strain, when it was suggested as a standard for use in antibiotic sensitivity surveys of anaerobes (33). Testing too few strains, not using recent clinical isolates, and placing various diverse species of bacteria into heterogenous groups for the presentation and analysis

of results can also be misleading (70). The various species of bacteria were put into five groups for the presentation of results in Tables V to X, but there were no notable differences in sensitivity patterns between species of bacteria in these groups, and their heterogenous nature was not forgotten during analysis of results.

This survey was on a small scale, the number of B fragilis group isolates was low, as was the number of patients from which oral bacteria were isolated. The low proportion of oral anaerobes resistant to penicillin and tetracycline may be due to the sample of 17 people from which they were isolated not being truly representative of the wider population of Palmerston North, or it may be due to reasons suggested by others for why the published sensitivity surveys of anaerobes might be unreliable for predicting the sensitivity patterns of anaerobes isolated in unique situations such as dental surgeries.

Regional differences in anaerobic sensitivity patterns have been noted overseas (17,18,72), and this phenomenon alone may explain the low number of penicillin and tetracycline resistant oral anaerobes.

Sensitivity levels of anaerobic bacteria at university and research hospitals have been found to differ from the sensitivity levels of the same bacteria isolated from community hospitals. It has been suggested that this may be due to different patient populations and antibiotic usage (17,18). It follows that anaerobic bacteria isolated in dental clinics may have different sensitivity patterns to those isolated in hospitals.

There is a suggestion that anaerobic bacteria resident at, and causing infections at specific anatomical sites, may have unique sensitivity patterns that are masked when sensitivity surveys group bacteria from sites all over the body together on the basis of their taxonomy alone (2,26). Immunochemical differences between oral and non-oral strains of B asaccharolyticus have been reported (39). When bile sensitive Bacteroides isolates

were taken exclusively from female genital tract infections, a higher proportion were found to be resistant to penicillin than would have been predicted from published sensitivity surveys (26). Bacteroides species isolated from oral sites have previously been reported to include lower proportions of isolates resistant to penicillin and tetracycline than the same species isolated from other anatomical sites (2,35).

SECTION 3: PLASMID ANALYSIS

The proportion of the hospital isolates found to be harbouring plasmids (54%), and their sizes, generally in the range 2.7 to 8.5 kb, are comparable with what has been reported of plasmids in the B fragilis group in the literature. In 1979, 43% of 121 strains examined were found to harbour plasmids (8), 25% of 32 strains had plasmids in 1981 (67), and in 1983 59% of 34 strains of the B fragilis group were found to have plasmids (8). Most of the plasmids found in the B fragilis group are small, being generally less than 5.0 M daltons (7.6 kb), although plasmids from 23 to 100 M daltons (35 to 150 kb) are demonstrable (8).

There was a notable difference in the proportions of oral Bacteroides isolates and hospital B fragilis group isolates with plasmids (13% compared to 54%). The significance of this difference can only be speculated upon.

The four base sequence specific restriction enzymes, *Hae III* and a *AluI*, used in this survey cleaved some plasmids into too many small fragments to be clearly visualized in agarose gels. The six base specific enzymes *Eco RI* and *Hind III* were used initially in digestions of plasmids, but it was found that these enzymes did not cut the plasmids they were incubated with at all. Other researchers have found plasmids isolated from

Bacteroides species to be inert to as many as 12 different restriction enzymes (51). Four base sequence specific restriction enzymes have previously been used to examine Bacteroides plasmids (8).

The B fragilis group has been suggested as a source of antibiotic resistance genes for other pathogenic bacteria (51,67,76). It has also been suggested that these anaerobes may obtain resistance genes from facultative bacteria (3). These suggestions have been supported by successful transfer of resistance genes from B fragilis group bacteria to E coli, with subsequent expression of resistance (23,36,37,51,52,68,76). The inert nature of Bacteroides plasmids to restriction enzymes (51), and the presence of resistance genes in these bacteria which only function in facultative bacteria (52), further suggest a role in the dissemination of resistance genes.

The 5.6 kb plasmids harboured by the oral Bacteroides isolates from sample R5 (pR5B10, pR5B11, pR5B12; (Figure 6) and the 5.8 kb plasmids found in hospital isolates (pQLBfr, p9Bfr, p61181, and p1HBfr; Figure 9) appear related, as their *Hae III* digest patterns are very similar (Figure 10). The size difference of the two groups could be accounted for by the presence of an insertion sequence in the hospital isolate plasmids (Figure 12). Rearrangement of plasmid DNA by some mechanism such as inversion, to bring two *Hae III* sites close together, and the presence of two populations of plasmid with one *Hae III* site at slightly different positions in a digest, could account for the *Hae III* restriction pattern of the plasmids pR5B11 and p1HBfr (Figure 10). Two very small fragments of linear DNA created in such a digest would not be visible; only the two large fragments would be seen. Multiple copies of an 850 base pair insertion sequence, IS160, have been shown to be responsible for the observed rearrangement of the 59 kb plasmid pBP16 (45). The rearrangements of this plasmid to form its derivatives included inversions between copies of IS160, and deletions of IS160. Similarly physical characterization of the B fragilis resistance plasmid

pBF4 has revealed the presence of multiple copies of two sets of inverted repeats, of 200 and 75 base pairs (24). This 27 kb plasmid shares sequence homology with many other Bacteroides resistance plasmids (68,24).

SECTION 4: CONJUGATION AND TRANSFORMATION

Penicillin resistance could not be transferred from B intermedius (R6B2) to E coli (HB101) by conjugation or transformation. Penicillin resistance in this isolate may reside on the chromosome as it often does in B fragilis (3,23). Penicillin resistance has been transferred to E coli by transformation with 1.98 M dalton (51), and 3.0 M dalton (76) B fragilis plasmids.

Low level inducible resistance to tetracycline was transferred to E coli HB101 by transformation with plasmid DNA isolated from a B fragilis strain (PN9), and a B thetaiotamicron strain (136Bth). Tetracycline resistance in B fragilis has often been reported to be inducible, and due to modification of antibiotic transport mechanisms (3). Several research groups have reported the transfer of tetracycline resistance from B fragilis to E coli (36,37,51), but E coli can mediate tetracycline resistance by mechanisms other than those that operate in B fragilis (52). The small increase in resistance shown by the E coli HB101 transformed with plasmids pPN9a and p136a (to 6 ug/ml cf. 48 ug/ml in the donor Bacteroides strains) may be due to alteration of some minor uptake mechanism or some other relatively ineffective means, leaving these bacteria still fairly sensitive to the antibiotic.

One group of researchers who reported tetracycline resistance transferred from B fragilis to E coli in 1977 used a tetracycline concentration of 4.0 ug/ml to select for transconjugates. The actual level of tetracycline resistance or MIC of the transconjugatants was not given (37). Others have used concentrations of 10 (52), 16 (51), 5 (36) and 25 (23) ug/ml.

The plasmids used to transform E coli HB101 could not be demonstrated in the transformants. Failure to demonstrate B fragilis plasmid DNA in E coli transformants and transconjugants has been reported before (3,37,76).

Plasmids smaller than 10 M daltons (15 kb) are not normally self-transmissible (51), but pPN9a and 136a which are 43 and 60 kb respectively, are large enough that conjugation experiments might be expected to yield tetracycline resistant transconjugants. The recipient strain used in these experiments (E coli HB101) may not have been compatible with the donors, other researchers have been unsuccessful in transferring tetracycline resistance from B fragilis isolates containing large plasmids to E coli (22,37).

The other tetracycline resistant B fragilis isolate, QLBfr, has a 5.8 kb plasmid. This plasmid is the same size and has the same restriction enzyme digestion pattern as plasmids carried by other relatively tetracycline sensitive hospital isolates, so it would have been surprising if transfer of this plasmid to E coli had conferred resistance on the recipient.

CONCLUSIONS

A lower proportion of the oral bacteria were resistant to tetracycline and penicillin than isolates of the same species isolated from different anatomical sites in different clinical settings and reported in the literature. Penicillin resistant oral Bacteroides are present in Palmerston North, as evidenced by isolates R6B1 and R6B2 (Table V).

A much lower proportion of oral anaerobic bacteria than B fragilis group bacteria harboured plasmids. This may be related to the higher antibiotic sensitivity shown by the oral bacteria.

The group of three isolates from sample R5 (Table IV) with a 5.6 kb plasmid suggest that the oral Bacteroides may exchange plasmids between species. The similarity between these and the slightly larger plasmids isolated from hospital isolates indicates that the oral Bacteroides may have access to the pool of genetic material available on plasmids harboured by the B fragilis group of bacteria.

APPENDIX I

MEDIUM 1

ANAEROBIC TRANSPORT MEDIUM (32)

Peptone or Gelysate, pancreatic digest of gelatin (Difco)	1.00 g
Yeast extract (Difco)	1.00 g
Glucose (Fisher)	1.00 g
Resazurin solution	0.04 ml
Distilled water	100.00 ml
L-Cystein-HCl (Sigma)	0.05 g
Salts solution	4.00 ml

All ingredients except cystein were boiled until colourless, allowed to cool to 40°C, then cystein was added. The pH was then adjusted to 6.8 with 20% NaOH, and the medium dispensed before autoclaving at 121°C for 15 minutes. The medium was fully reduced in the anaerobic chamber before bottles were sealed.

Resazurin solution: 25 mg of resazurin (Sigma) were dissolved in 100 ml of distilled water.

Salts solution:

CaCl ₂ anhydrous	0.2 g
MgSO ₄ 7H ₂ O	0.48 g
KHPO ₄	1.0 g
KH ₂ PO ₄	1.0 g
NaHCO ₂	10.0 g
NaCl	2.0 g

The CaCl and MgSO₄ were mixed in 300 ml of distilled water until dissolved. Then 500 ml of distilled water

added and the remaining salts added slowly while swirling the solution until all salts were dissolved. Then another 200 ml of distilled water was added, mixed, and the solution stored at 4°C.

MEDIUM 2

RINGER DISPERSION SOLUTION (59)

Ringer crystals (Oxoid)	0.356 g
Resazurin solution	0.40 ml
L-Cystein hydrochloride (Sigma)	0.05g
Sodium metaphosphate	0.1 g
Distilled water	100 ml

All ingredients except cystein were boiled until colourless, then cooled to 40°C. Cystein was then added, and the pH adjusted to 7.0 with 20% NaOH. The solution was then autoclaved at 121°C for 15 minutes.

MEDIUM 3

RINGER DILUTION SOLUTION (59)

Ringer crystals (Oxoid)	0.356 g
Resazurin solution	0.4 ml
L-Cystein-HCl (Sigma)	0.05 g
Distilled water	100 ml

Prepared as for Ringer dispersion solution.

MEDIUM 4SUPPLEMENTED BLOOD AGAR (59)

Brain heart infusion broth (Difco)	18.5 g
Yeast extract (Difco)	2.5 g
Davis agar	6.0 g
Distilled water	500 ml
Vitamin K (10 mg/ml)	0.5 ml
Haemin solution	2.5 ml

Ingredients were boiled to dissolve agar, then autoclaved at 121°C for 15 minutes. The agar was then cooled to 50°C, and 25 ml of defibrinated whole sheep blood added and mixed before pouring into petri dishes.

Vitamin K solution: Vitamin K (Serva, Heidelberg) was weighed out on aluminium foil and dissolved in absolute alcohol to give a final concentration of 10 mg/ml. This was filter sterilized and stored in a tightly closed bottle protected from light. The stock was further diluted for use in sterile distilled water. Vitamin K was used as a medium supplement in a final concentration of 0.1 ug/ml for agar media.

Haemin solution: One gram of haemin (Sigma; equine type III) was dissolved in 10 ml of 1N NaOH, then the volume adjusted to 100 ml with distilled water. The solution was then autoclaved at 121°C for 15 minutes. Haemin solution was used as a medium supplement at a final concentration of 5.0 ug/ml. Stock solution = 10 mg/ml.

MEDIUM 5BACTEROIDES SELECTIVE AGAR (6)

Brain heart infusion broth (Difco)	18.5 g
Yeast extract (Difco)	2.5 g
Davis agar	6.0 g
Vitamin K solution	0.5 ml
Haemin solution	2.5 ml
Distilled water	500 ml

These were boiled to dissolve the agar, then autoclaved at 121°C for 15 minutes, then cooled to 50°C before adding the following:

Neomycin (filter sterilized, 10mg/ml)	5.0 ml
Vancomycin (filter sterilized, 25mg/ml)	0.2 ml
Defibrinated laked sheeps blood	.25 ml

MEDIUM 6BACTEROIDES gingivalis SELECTIVE AGAR

As for Bacteroides selective agar, with vancomycin omitted.

MEDIUM 5

BACTEROIDES SELECTIVE AGAR (6)

Brain heart infusion broth (Difco)	18.5 g
Yeast extract (Difco)	2.5 g
Davis agar	6.0 g
Vitamin K solution	0.5 ml
Haemin solution	2.5 ml
Distilled water	500 ml

These were boiled to dissolve the agar, then autoclaved at 121°C for 15 minutes, then cooled to 50°C before adding the following:

Neomycin (filter sterilized, 10mg/ml)	5.0 ml
Vancomycin (filter sterilized, 25mg/ml)	0.2 ml
Defibrinated laked sheeps blood	25 ml

MEDIUM 6

BACTEROIDES gingivalis SELECTIVE AGAR

As for Bacteroides selective agar, with vancomycin omitted.

MEDIUM 7FUSOBACTERIUM SELECTIVE AGAR (66)

Trypticase (BBL)	5 g
Yeast extract (Difco)	2.5 g
NaCl	1 g
L-tryptophan (Difco)	0.1 g
Crystal violet (5mg/ml)	0.5 ml
Davis agar	6 g
Distilled water	500 ml

These were boiled to dissolve the agar, then autoclaved at 121°C for 15 minutes, then cooled to 50°C before adding the following:

Erythromycin (filter sterilized 1mg/ml)	4 ml
Defibrinated whole sheep blood	25 ml

MEDIUM 8WILKENS-CHALGREN AGAR (73)

Trypticase (BBL)	10 g
Gelysate (BBL)	10 g
Yeast extract (Difco)	5 g
D-Glucose (Fisher)	1 g
NaCl	5 g
L-Arginine - free base (Sigma)	1 g
Pyruvic acid - sodium salt	1 g
Davis agar	12 g
Distilled water	1000 ml
Haemin solution	5 ml
Vitamin K solution	1 ml

Ingredients were boiled to dissolve the agar, autoclaved at 121°C for 15 minutes, then cooled to 50°C before dispensing.

MEDIUM 9LURIA BROTH (38)

Tryptone (BBL)	10 g
Yeast extract (Difco)	5 g
NaCl	5 g
Distilled water	1000 ml

The broth was dispensed, then autoclaved at 121°C for 15 minutes.

APPENDIX II

PROCEDURES FOR MEDIA REAGENTS USED IN
IDENTIFICATION OF BACTERIA

1. CARBOHYDRATE FERMENTATION (59)

Broth - PY medium

Peptone (Difco)	1 g
Yeast extract (Difco)	1 g
Resazurin solution	0.4 ml
Vitamin K solution	2 ul
Distilled water	100 ml
Salts solution	4 ml
L-Cystein - HCl (Sigma)	0.05 g

All ingredients except cystein were boiled until colourless, then cooled to 40°C. Cystein was then added, and the pH adjusted to 6.8 with NaOH. The medium was stored in the anaerobic chamber.

Tubes of PY broth containing various carbohydrates (1g/100ml) were inoculated. After good growth was obtained, pH was measured with a pH meter.

Results were interpreted as follows:

pH below 5.5 = acid, 5.5-6.0 = weak acid,

pH above 6.0 = negative.

Uninoculated tubes were used as controls, their pH should be 6.2-6.4.

2. INDOLE PRODUCTION (59)

Indole broth: PY broth as for carbohydrate fermentation was used with 0.5 g of trypticase (BBL) added before autoclaving.

Ehrlicks reagent: Two grams of para-dimethylamino-benzaldehyde were dissolved in 190 ml of 95% ethanol, then 40 ml of concentrated hydrochloric acid were slowly added. The reagent was stored at 4°C in a dark bottle.

Indole broth was inoculated with culture and incubated at 37°C in the anaerobic chamber. Twenty four hours after good growth, indole production was tested for: One ml of Xylene was added to 2 ml of indole broth and mixed, then let stand for at least 2 minutes. Ehrlicks reagent (0.5 ml) was then slowly added down the side of the tube without shaking. Development of a pink or fuchsia ring within 15 minutes was considered positive, a yellow ring negative.

3. BILE GROWTH (2)

Bile Medium: One gram of glucose and 2 g of oxgall (Difco) were added to 100 ml of PY broth (see carbohydrate fermentation before autoclaving.

PYG broth: One gram of glucose was added to PY broth before autoclaving.

Bile medium and PYG broth were inoculated and incubated anaerobically at 37°C for 2 days. Growth in the two media were then compared. Results were recorded as inhibited (no growth in bile, growth in PYG), growth (some growth in bile), or stimulated (better growth in bile than PYG).

4. ESCULIN HYDROLYSIS (27)

Esculin broth:

Brain heart infusion broth (Difco)	2.5 g
Esculin	0.1 g
Bacto agar	0.1 g
Distilled water	100 ml

Ingredients were dissolved by boiling, then the pH adjusted to 7.0 before autoclaving at 121°C for 15 minutes.

Esculin broth was inoculated and incubated anaerobically at 37°C. After good growth several drops of 1% ferric ammonium citrate solution were added. Development of a black colour within 15 minutes was considered a positive reaction.

5. NITRATE REDUCTION (27,59)

Nitrate medium:

Trypticase (BBL)	2.0 g
Yeast extract (Difco)	0.5 g
Peptone (Difco)	0.5 g
Potassium nitrate	0.5 g
Vitamin K solution	2 ul
Distilled water	100 ml

Ingredients were mixed and autoclaved at 121°C for 15 minutes.

Nitrate A reagent: 0.5 g of sulphanilic acid was dissolved in 30 ml of glacial acetic acid, then 120 ml of distilled water was added. The reagent was stored at 4°C protected from light.

Nitrate B reagent: 0.2 g of 1,6-Cleve's acid (5-amino-2-naphthalenesulphoric acid) was dissolved in 30 ml of glacial acetic acid, then 120 ml of distilled water was added. The reagent was stored at 4°C protected from light.

Nessler reagent (32):

Solution A:

Mercuric chloride	1 g
Distilled water	6 ml

Solution B:

Potassium iodide	2.5 g
Distilled water	6 ml

Both solutions were dissolved completely then mixed.

Solution C:

Potassium hydroxide	6 g
Distilled water	6 ml

Solution C was dissolved completely then added to the mixture of A and B. Thirteen ml of distilled water was then added and mixed well. The reagent was filtered before use.

Nitrate medium was inoculated and incubated anaerobically at 37°. After good growth, 1 ml was removed to a separate tube, to which 0.2 ml of nitrate reagents A and B were added. Development of a pink or red colour indicates nitrate has been reduced to nitrite. If no colour developed within 5 minutes, a small amount of zinc dust was added. Development of colour after addition of zinc indicates nitrate was not reduced. If no colour develops after zinc addition, nitrate was reduced beyond nitrate (positive test), and the remaining nitrate culture was tested for ammonia by adding a few drops of Nessler reagent. A deep orange colour indicates a positive reaction.

6. GELATIN LIQUIFICATION (27)

Gelatin medium:

Peptone (Difco)	1 g
Yeast extract (Difco)	1 g
D-Glucose	0.1 g
Resazurin solution	0.4 ml
Salts solution	4.0 ml
Vitamin K solution	2 ul
Distilled water	100 ml
L-Cystein - HCl (Sigma)	0.05 g

The ingredients except cystein were boiled until colourless, then cooled to 40°C. Cystein was then added and pH adjusted to 6.8 with NaOH. The medium was then dispensed in 5 ml aliquotes into universals containing 0.6 g gelatin (Difco), and autoclaved at 121°C for 15 minutes.

The medium was inoculated and incubated anaerobically at 37°C for 2 days. After incubation, the inoculated universals, plus 3 uninoculated controls were chilled at 4°C until the controls solidified. Then all universals were removed to room temperature. Tubes which were not solidified were positive. Tubes that liquified at room temperature in less than half the time it took the controls were considered weakly positive.

7. KANAMYCIN DISC (59)

Kanamycin stock solution: 1 g of kanamycin base activity was dissolved in 10 ml of sterile phosphate buffer (pH 8.0). This stock was stored at 4°C for up to one year.

Stock solution was spotted onto discs in 10 ul aliquotes, autoclaved at 121°C for 15 minutes, and allowed to dry for 72 hours before use. Discs were placed on agar immediately after inoculation. A zone of 10 mm or less was considered resistant.

8. CATALASE TEST (59)

Culture was removed from an agar plate using a cool wire loop and placed in 15% hydrogen peroxide. Evolution of bubbles or foam was taken as a positive reaction.

APPENDIX III

REAGENTS AND BUFFERS

1. MCFARLAND NEPHELOMETER STANDARD (59)

0.1 ml of a 1% aqueous solution of barium chloride was combined with 9.9 ml of a 1% aqueous solution of sulphuric acid. This is a McFarland number one standard. The standard was diluted with 10 ml of distilled water and compared with broth cultures in bottles or tubes of the same type.

2. TRIS BORATE BUFFER (38)

Tris base (Sigma)	10.8 g
EDTA (BDH)	0.93 g
Boric acid (BDH)	5.5 g
Distilled water	1000 ml

The solutes were dissolved completely, then the pH adjusted with NaOH or HCl, and ethidium bromide added

Ethidium bromide (25mg/ml)	20 ul
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3. TRIS ACETATE BUFFER (28)

Tris base (Sigma)	0.48 g
EDTA (BDH)	0.074 g
Distilled water	100 ml

pH was adjusted to 7.9 with glacial acetic acid.

4. LYSIS MIXTURE (28)

Tris base (Sigma)	0.61 g
Sarkosyl (Sigma)	3 g
Distilled water	100 ml

pH was adjusted to 12.6 with NaOH immediately before use by adding 0.4 ml of freshly made 2N NaOH to 10 ml of lysis mixture.

5. ECKHARDT SOLUTIONS (13)Solution 1:

Tris borate buffer pH 8.0 (see above 2)	10 ml
Ficoll (Sigma)	2 g
Bromophenol blue	0.005 g
RNase (Sigma)	added to 0.01 mg/ml
Lysozyme (Sigma)	added to 0.2 mg/ml

RNase - 10 mg/ml in 0.4 M NaAcetate pH 4.0. RNase was digested at 98°C for 5 minutes and stored frozen.

Lysozyme - 100 mg/ml in distilled water. Stored frozen.

Solution 2:

Tris borate buffer pH 8.0	10 ml
SDS (Sigma)	0.02 g
Ficoll (Sigma)	1 g

Solution 3:

Tris borate buffer pH 8.0	10 ml
SDS (Sigma)	0.02 g
Ficoll (Sigma)	0.5 g

6. MEDIUM SALT RESTRICTION BUFFER (10)

NaCl	50 mM
Tris (Sigma) pH 7.4	10 mM
MgSO ₄	10 mM
Dithiothreitol (Sigma)	1 mM

7. *A1uI* STORAGE BUFFER

K ₃ PO ₄	20 mM
KCl	50 mM
EDTA (DH)	0.1 mM
Dithiothreitol (Sigma)	10 mM
Glycerol	50% v/v
pH 7.5	

8. *Hae III* STORAGE BUFFER

Tris HCl (Sigma)	20 mM
NaCl	400 mM
EDTA (BDH)	0.1 mM
2-mercaptoethanol (Sigma)	10 mM
Glycerol	50% v/v
pH 7.5	

9. TRIS EDTA BUFFER

Tris base (Sigma)	6.06 g
EDTA (BDH)	7.49 g
Distilled water	1000 ml

10. STOP BUFFER

SDS (Sigma)	0.1 g
Glycerol	5 ml
1 M EDTA solution	1 ml
Bromophenol blue (Sigma)	0.05 g
Distilled water	4 ml

1 M EDTA - 37.224 g of EDTA (BDH) was dissolved in 100 ml of distilled water, and the pH adjusted to 8.0 with NaOH.

11. ANTIBIOTIC STOCK SOLUTIONS

Penicillin G - 600mg (10000,00 units)
were dissolved in 3 ml of sterile distilled water.

Stock solution = 200 mg/ml

Cefoxitin - 0.48 g were dissolved in 10 ml of
sterile distilled water.

Stock solution = 48 mg/ml

Metronidazole - 0.064 g were dissolved in
10 ml of sterile distilled water.

Stock solution = 6.4 mg/ml

Ornidazole - same as for metronidazole.

Tetracycline - 0.24 g were dissolved in 10 ml
of methanol.

Stock solution = 24 mg/ml

Subsequent dilutions were made in sterile distilled
water.

Clindamycin - same as for metronidazole.

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