

**THESIS TITLE:**  
**THE EFFECTS OF VARIOUS PERIODONTAL PROCEDURES ON THE  
DETECTION OF BACTERIA IN THE BLOODSTREAM**

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## **PREFACE**

The work described in this thesis was carried out at the University of Glasgow Dental Hospital and School, from August 1999 to July 2000, under the supervision of Professor Denis Kinane and Dr Marcello Riggio.

This research represents original work carried out by the author and has not been submitted in any other form to any other university.

**DECLARATION:**

This thesis is the original work of the author

Katie Fraser Walker

December 2000

## **ABSTRACT:**

**Background :** Transient bacteraemias have been detected following dental manipulation for many years. Infective endocarditis can arise in susceptible individuals and antibiotic prophylaxis is required for certain procedures considered to be 'at risk'. Evidence is emerging that periodontal disease may contribute a significant risk factor for the development of certain diseases and CVD could be detrimentally influenced by the repeated entry of bacteria into the bloodstream. It is possible that antimicrobial mouthwashes such as chlorhexidine, could influence the subgingival microflora, reducing the levels of bacteraemia induced by instrumentation.

**Method:** A single blind parallel study lasting two weeks. A baseline blood sample was obtained from 38 volunteers with untreated periodontal disease following which a periodontal probing depth chart was collected. A blood sample was taken following this procedure. Patients were assigned to a mouthwash by a random block assignment chart by the order in which they attended. Following 2 weeks of either chlorhexidine or fluoride mouthwash use twice daily a blood sample was taken. Each subject rinsed with their mouthwash for 1 minute and performed toothbrushing for 2 minutes. A blood sample was taken following this procedure. A full mouth cavitron scaling was performed and a final blood sample was taken. Blood samples were analysed by conventional microbiology, Polymerase Chain Reaction and the LAL assay.

**Results :** Incidence of bacteraemias detected by conventional culture were : cavitron scaling (13.33%), periodontal probing (13.16%), toothbrushing (3.33%). By PCR : cavitron scaling (20%), toothbrushing (13.3%), probing (10.8%). The LAL assay was not considered to be a robust technique for LPS detection. Sample numbers were too small to determine any mouthwash effects.

**Conclusions :** These findings would suggest that dental bacteraemias induced by periodontal procedures are of a low level and pose further questions on the clinical significance of bacteraemia induced by dental manipulation.

## **SUMMARY:**

Evidence is emerging that oral health, in particular periodontal disease, may contribute a significant risk for the development of certain diseases namely coronary heart disease in addition to a number of other systemic medical problems including premature birth, septicaemia, lung and brain abscesses. It is hypothesised that cardiovascular disease, a multifactorial disease, would be detrimentally influenced by the repeated entry of bacteria into the bloodstream. Transient bacteraemias have been demonstrated following toothbrushing and periodontal treatment. Surgical manipulation in particular has been linked to infective endocarditis in susceptible individuals. It is therefore feasible that antimicrobial mouthwashes such as chlorhexidine, could influence the subgingival microflora, reducing the level of bacteria entering the bloodstream, and thus decrease the risk of developing serious systemic disorders. Furthermore, active and regular brushing with a dentifrice designed to prevent the onset of gingivitis (and ultimately periodontitis) could also reduce the level of bacteraemia post toothbrushing.

This single blind parallel study lasted two weeks. The study population consisted of 38 volunteers with untreated adult periodontal disease from the consultant clinics of Glasgow Dental Hospital Periodontology Department. The aims of the study were to test if periodontal manipulations could produce bacteraemias and to test if a mouthwash could influence the bacteraemia induced. After obtaining written informed consent, a baseline blood sample was taken on enrolment into the study. Following periodontal probing, a second blood sample was taken to determine whether this procedure would cause a bacteraemia. Each subject was either given a chlorhexidine or placebo mouthwash. Following a two week interval of twice daily mouthwash use, a baseline blood sample was taken at the beginning of their second visit. Subjects rinsed with their assigned

mouthwash for 1 minute and brushed their teeth for 2 minutes. A second blood sample was taken. The patients teeth were then scaled with a cavitron and a final blood sample was taken following this procedure.

The analyses of bacteraemias were performed in three ways:

1) Blood samples were cultured using Bactec culture bottles with positive bottles undergoing subsequent conventional microbiological analysis to aid identification of organisms isolated. 2) The molecular biological technique of Polymerase Chain Reaction was utilised with the use of 16S primers on frozen EDTA blood samples. However, PCR could not be performed on the Bactec culture bottles due to the presence of the inhibitor SPS (sodium polyanetholesulphonate). 3) The LAL assay for the detection of lipopolysaccharide was utilised on subjects 11-38 plasma samples and LPS levels calculated for each subject and compared between subjects.

No significant difference was observed in the number of positive cultures detected at 15 minutes following cessation of a procedure compared to immediately following a procedure. Positive sample results were compared for the three detection methods and between patients and between each sampling point. There was little correlation between the three detection methods and the detection of bacteraemia. PCR detected more samples positive for bacteria compared with conventional microbiology although samples which were both PCR and culture positive were not the same with the exception of 2 samples. 4/38 (10.8%) of samples were detected positive by PCR following periodontal probing, 4/30 (13.3%) were positive following toothbrushing with 6/30 (20%) positive by PCR following cavitron scaling. Unlike conventional culture, baseline samples were positive by PCR (visit 1, 3/38; visit 2, 1/30) prior to any periodontal manipulation.

This may indicate an increased sensitivity with molecular techniques in addition to the greater number of positive bacteraemia results found by PCR. PCR will also detect dead organisms which would not be culture positive.

By conventional microbiology, periodontal probing yielded a high bacteraemia incidence (5/38, 13.16%) as did cavitron scaling (4/30, 13.33%) with toothbrushing giving the lowest incidence (1/30, 3.33%). However, periodontal probing was performed at visit 1 before a mouthwash had been allocated and not on the same day as the cavitron scaling and thus are not strictly comparable. Positive samples did not correlate with those patients who had the greatest severity of disease, as determined by mean pocket depths and bleeding on probing and therefore it could not be predicted which patients would produce a detectable bacteraemia.

The LAL assay was used to determine LPS levels compared to mean patient pocket depths and for an average pocket depth of 3mm a value of 38831.38EU was observed. For 4 mm pockets this increased to 45053.83EU, and a further increase was observed for pockets greater than 4mm (55468.00EU). This suggests that the greater the pocket depth the higher LPS levels following periodontal probing. However, given the large standard deviations the LAL assay was not considered a robust technique for LPS detection.

An additional part of this project was an attempt to measure the effects of a proven antibacterial agent (chlorhexidine gluconate) on levels of bacteria in the bloodstream. It was anticipated that if this study was successful, follow up studies using other antimicrobial agents (e.g. Triclosan) would also be tested, to determine whether antigingivitis toothpaste would also have an effect on levels of bacteria in the bloodstream. Due to the small subject sample, no statistically significant conclusions could be drawn. However, comparing the two mouthwash groups using LPS levels as determined by the

LAL assay at baseline visit 1 and baseline visit 2, the chlorhexidine group at baseline visit 1 was 49021.43EU which increased slightly at baseline visit 2 (following 2 weeks of mouthwash use) to 53520.50EU. The fluoride mouthwash group at baseline 1 was 45211.14EU. At baseline visit 2 the LPS level almost doubled to 82243.18EU. This suggests that following no instrumentation, chlorhexidine mouthwash can lower the increase in LPS observed with the fluoride mouthwash. However, as mentioned previously, the LAL assay findings should be interpreted with caution.

It is evident that bacteraemias do arise following periodontal manipulation. Procedures which were previously regarded as innocuous and therefore not covered by prophylactic antibiotics such as periodontal probing gave high bacteraemia frequency. The levels of bacteraemia detection in this study were lower than that quoted by previous authors. Such low levels of bacteraemia pose further questions on the frequency, extent, duration and clinical significance of bacteraemia induced by dental manipulation.



***CHAPTER 1***  
***INTRODUCTION***

## ***PART 1 : GENERAL INTRODUCTION***

As defined by Bagg *et al* (1999), bacteraemia is said to be present when bacteria enter the bloodstream transiently and can be detected by laboratory blood culture techniques.

For more than 50 years it has been known that manipulation of the structures in the oral cavity results in the entry of oral bacteria into the bloodstream. Okell and Elliot demonstrated in 1935 that dental extractions released bacteria into the bloodstream, since when many studies have been performed to determine the interaction between dental procedures and bacteraemias. Due to awareness of high patient risk and medicolegal considerations, infective endocarditis in particular, has been associated with bacteraemias following dental treatment.

The Endocarditis Working Party of the British Society for Antimicrobial Chemotherapy currently recommend that antibiotic prophylaxis be given only for dental extractions, dental scaling, or periodontal surgery, although there is some evidence that other dental procedures may occasionally cause bacteraemias in adults. There is however, a paucity of concrete evidence that bacteraemias triggered by dental procedures are a primary cause of infectious endocarditis. (Oakley 1981, Van der Meer *et al.* 1992).

The human oral cavity is colonised by more than 300 different bacteria which are traditionally considered non pathogenic provided they are confined within the mouth. This number increases from 300-400 in the crevice of periodontal patients (Debelian *et al* 1994). Relatively atraumatic manipulations such as periodontal scaling, toothbrushing, and chewing produce a detectable bacteraemia (Schlein *et al.* 1991). Thus bacteraemias involving oral microorganisms may occur in dentate patients on a daily basis such as

during standard oral hygiene procedures. Generally, most episodes of bacteraemia are brief and transient and in the healthy patient pose very little threat to health due to efficient clearance by the host immune system (Schlein *et al* 1991). However, there is a marked tendency for certain organisms to preferentially infect damaged tissues and prosthetic structures, such as compromised or artificial heart valves (Freidlander *et al* 1994). In many patients, whose resistance is defective owing to disease such as valvular heart disease, diabetes, and acquired immune deficiency syndrome, or drug use (therapy with steroids or cytostatics or after anti-neoplastic radiation in the region of the jaw) bacteraemia is associated with an increased risk of serious complications (Hall *et al* 1994). In addition, infection can spread very quickly and may even become life threatening (Fonseca *et al* 1998). Dental surgical treatment in these patients must be carried out under antibiotic protection, although as stated previously the recommendations for procedures requiring antibiotic prophylaxis are vague and have little scientific basis (Oakley 1981, Van der Meer *et al.* 1992).

A particularly high rate of bacteraemia after dental treatment is found among those with poor oral hygiene, or no oral hygiene at all like that found among many mentally ill patients (Messini *et al.* 1999). It is likely, therefore, that periodontal patients with poor oral hygiene and a subsequent high oral bacterial load may have an increased preponderance to bacteraemia. It is unclear what effect patients with good oral hygiene and deep pocketing may have.

So far it has not been verified at what stage and for how long microorganisms remain in the blood and little is known about the nature and incidence of bacteraemias after basic periodontal procedures. The answers to these questions would permit determination of the duration and kind of antibiotic treatment for the patient at risk from bacteraemias.

In 1926 it was suggested that viridans *Streptococci* associated endocarditis developed secondarily to gingivitis or dental abscesses and the need for good oral hygiene was emphasised (Thayer *et al*). Indeed, the first patient death due to infective endocarditis developing after dental extraction was documented in 1930 (Kelson *et al*). Since these early times many case studies have confirmed that dental procedures have the potential to induce bacteraemia and serious complications.

### ***1.1 : STERILITY OF BLOOD : a product of the immune system***

The skin and mucous membranes covering the body are presumed to form an impenetrable physical barrier to the hundreds of billions of microbes that reside on their surfaces (Loesche *et al* 1997). Otherwise, the underlying tissues would not be bacteriologically sterile, as is usually found when standard bacteriologic procedures are used to culture blood or other tissues. Hence when an infection is encountered within the tissue, such as infective endocarditis, some breach in the barrier is presumed to have preceded the infection. The barrier breach hypothesis is supported by the isolation of skin organisms from endocarditis found in drug addicts (Lacassin *et al* 1995), and by the isolation of non-enterococcal *Streptococci* of oral origin such as *Streptococcus sanguis*, occasionally found in patients who have a history of dental surgery.

The assumption that blood is sterile has rarely been evaluated with bacteriologic techniques that will detect very low numbers of bacteria per millilitre of blood or that which detects anaerobic bacteria. This is confounded by laboratories who seek out only aerobic and facultative bacterial species, such as *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae* and *Escherichia coli*, that are well-known medical pathogens (Organon Teknika). When efforts are made to detect anaerobes, the incidence of positive cultures increases (Debelian *et al* 1995). In addition, a possible difficulty with

existing blood culturing techniques is the inability of single cells to grow in broth as opposed to agar surfaces. This is partly caused by the presence of inhibitory substances in the blood, but it could also reflect bacterial predilection for growth on solid agar. Culturing techniques that use membrane filters, and therefore solid surfaces yield higher incidences of bacteraemia (Heimdahl *et al* 1995). Loesche *et al* (1997) adapted the membrane filter procedure so that it could be performed within an anaerobic chamber. These researchers cultured blood from 40 asymptomatic individuals who were having their third molars electively extracted. From each of these patients 5mL of blood were inoculated into a thioglycollate broth and another 5mL were processed using the anaerobic membrane filter technique. The broth cultures were positive in 1 of the 40 blood samples, whereas the duplicate samples processed on the filters were positive in 31 of the 40 blood samples. They then collected blood from asymptomatic blood bank donors after they had donated 500mL of blood. They reasoned that if the positive blood cultures in oral surgery patients were a result of any contamination from the plug of skin punched out by the needle, that this plug and bacteria dislodged from it should have been washed out by the passage of about 500mL of blood through the tubing. In two separate series, 16 of 20 and 18 of 29 donors blood samples had a detectable bacteraemia. They concluded that under normal circumstances very small numbers of indigenous bacteria from the skin, intestine and oral cavity are constantly gaining access to the bloodstream. These numbers are low enough to be dealt with readily by the host's immune system.

The number of bacteria that inhabit a single periodontal pocket ( $10^8$  cfu/mg plaque) (Moore *et al* 1994) are greater than the number that come in contact with any other epithelial surface in the body. The host defends against this load by secreting GCF which is able to flush out soluble noxious waste products and most non-adherent particulate

matter (Cimasoni *et al* 1983). Large numbers of polymorphonuclear leucocytes exist in the GCF, where they quickly encounter bacteria, phagocytose them, and mount an oxidative burst (Loesche *et al* 1988). Specific antibodies to plaque species, complement and antibacterial molecules such as  $\alpha^2$ -macroglobulin, are found in the GCF (Schenkein *et al* 1982) and these assist the polymorphonuclear leucocytes in their antibacterial activities. In addition the gingival epithelial cells have a high turnover rate sloughing away their bacterial loads with them. These defences more than adequately protect the host from frank invasion, but can nonetheless cause an inflammatory response. This inflammation, can in turn, cause tissue loss when challenged by excessive bacterial penetration of a particularly reactive bacterial component, such as LPS of Gram-negative species or by an antigen (other than LPS) to which the immune system is particularly reactive. Under normal conditions, bacteria that penetrate the gingival epithelium are destroyed, but their cell components can act both as antigens and as stimuli for inflammation and periodontal disease (Page *et al* 1991). The immunologic response to bacterial species residing in the subgingival plaque is unusual. Because many of these species are unique to dental plaque, the presence of antibodies to them most likely results only from an antigenic challenge occurring in the periodontium. Although the numbers of bacteria that are in relatively constant contact with the gingival epithelium are high, the actual weight of the plaque on all of the teeth is approximately 10 to 30mg : a small amount considering that 99% or more of this antigenic load never enters the tissue or contacts the immune system. The importance of these plaque antigens in stimulating the immune system is demonstrated by the finding that high serum titres of serum antibody to *Pg* and *Aa* are reduced simply by debriding the tooth surfaces of visible plaque (Schenck *et al* 1987, Nakagawa *et al* 1990). Another possible explanation for the body's ability to protect against oral bacterial invasion is that the host has an extremely prime immune mechanism in the gingival tissue

and responds quickly to bacterial penetration. The presence of elevated titres to many periodontopathic species when there is periodontal disease (Ebersole *et al* 1982) indicates that the system is highly responsive to bacterial loads on the teeth. The above cited decrease in titres to *Pg* and *Aa* after debridement is further evidence of this responsiveness of the host to plaque antigens.

### **1.2 : Oral microbes**

Two distinct microbial ecosystems co-exist on the teeth, forming the supragingival and subgingival plaque communities. The supragingival plaque is dominated by facultative, quickly dividing, adherent saccharolytic organisms which in the presence of sucrose can give rise to dental caries. The subgingival plaque is dominated by anaerobic, relatively slow dividing, loosely adherent assacharolytic organisms which provoke an inflammatory response giving rise to periodontal disease. These plaque communities if they expand as a result of poor oral hygiene, have different potentials for contributing to medical diseases.

Microorganisms present in the oral cavity include facultative and anaerobic *Streptococci* (most often the viridans group), *Veillonellae* and *Diphtheroids*, making up 80% of the viable count; *Lactobacilli*, *Staphylococci* and filamentous forms each generally being 1% or less of the total; with *Spirochetes*, *Bacteroides*, *Candidae* and *Mycoplasma* present in minor quantities.

The main causative organisms of dental caries are Gram-positive oral *Streptococci* (*Streptococcus mutans* and *Streptococcus sanguis*). In gingivitis, *Actinomyces* species and Gram-negative anaerobic rods such as *Fusobacterium nucleatum* and *Bacteroides* species are also important pathogens. When gingivitis progresses to periodontitis the proportion of spirochetes and Gram-negative anaerobic rods particularly *Bacteroides*

*melaninogenicus*, *Fusobacterium nucleatum*, *Capnocytophaga* and *Actinobacillus actinomycetemcomitans* increases (Soames and Southam).

It was hypothesised that, bacteria isolated from the bloodstream following periodontal instrumentation would perhaps be a mixture of both plaque and periodontal organisms.

### ***1.2.1 : Differences in subgingival plaque composition in health and periodontal disease***

Because of the differences in plaque composition in health and periodontal disease it could be hypothesised that this difference would be reflected in the organisms found in the bloodstream following instrumentation between periodontally healthy and diseased individuals.

Slots (1979) and Socransky (1982) showed the microbial composition of subgingival plaque samples obtained from areas with healthy and diseased periodontal tissues displays marked differences. In the sparse subgingival microflora found at a clinically healthy periodontal site, most of the bacteria are facultatively anaerobic, and only a minor portion is made up by anaerobic bacteria. Furthermore, the cultivable microflora from such a site are generally dominated by Gram-positive cocci and rods. In contrast, cultivation of bacterial samples obtained from the abundant subgingival microflora present in areas with advanced periodontal disease discloses a predominance of Gram-negative anaerobic rods. Furthermore dark field analysis of samples from periodontitis sites shows substantially elevated proportions of motile rods and spirochetes as compared to healthy sites Listgarten & Helldén (1978).



### **1.3 : Bacterial access to the bloodstream**

Although it is evident that the mouth abounds with microorganisms, it is thought that only a few may make their way into the bloodstream. It is estimated that in adults with healthy, shallow gingival pockets there are 4cm<sup>2</sup> of gingival crevicular epithelium. In the presence of gingivitis and periodontal disease, this surface area can increase dramatically such that a dense mat of organisms approximate and are easily forced into this richly vascularised and ulcerated tissue. In addition, it has been shown that some organisms can invade the interstitial space of the epithelium to the depth of the periodontal fibres (Saglie *et al.* 1986).

Many investigators have discussed the actual source of the microorganisms found in dental bacteraemia and some have concluded that the gingival crevice is the primary reservoir (Jones *et al* 1970). It is evident that this may be true for the so-called clean mouth, but in the patient demonstrating periodontal disease with purulent exudate, the gingival crevice would be only one source of microorganisms.

The American Heart Association accepted the hypothesis that poor oral hygiene, periodontal and periapical infections may induce bacteraemias in the absence of dental procedures. With gingival inflammation, the connective tissues become highly vascularised allowing the invading microorganisms to readily enter the bloodstream (AHA 1990). The basement membrane of the gingival sulcus acts as an important barrier to bacterial invasion of the body from the oral cavity and thus has to remain intact through early treatment and avoidance of periodontal disease (Daly *et al* 1997). The positive fluid flow of GCF also has a protective part to play. Inflammation leaves an open door for microbes to enter the blood and hence dental prophylaxis should precede any dental treatment (AHA 1990). Indeed, further work is required to investigate the effect of

lowered oral bacterial loading and improved tissue integrity in reducing the prevalence of bacteraemia of dental origin.

With extraction, the route of bacterial entry into the blood is obviously through the open vascular bed. However, ulcerations and other disruptions in the basement membrane are known to occur commonly in periodontitis. Sites which bleed or suppurate indicate areas where ulceration of the periodontal epithelium has occurred, providing a portal of entry into the bloodstream. In the presence of inflammation bacteria may penetrate through disruptions of the epithelium into the connective tissues adjacent to the gingival sulcus. After entering the tissue, these organisms could access the bloodstream readily within the well vascularised gingiva. The inoculation of organisms from bacterial dental plaque is presumably followed by the breaking up and dissemination of clumps of bacteria in the pulmonary circulation which has many extremely small blood vessels. By the time the blood containing the bacteria reaches the antecubital fossa where sampling is taking place, the small bolus of bacterial dental plaque has been broken up into many smaller fragments. The removal of the organisms from the blood is effected by the reticuloendothelial system, which in immunocompetent patients is rapid (Palmer *et al* 1939).

### ***1.3.1 : Bacterial access in the absence of bleeding***

Roberts *et al.* (1997) suggested how organisms would gain entry to the circulation if overt bleeding was not present. It is hypothesised that the initial passage of bacteria into the blood vessels is probably due to sudden negative pressures occurring as a result of dentogingival manipulation which causes microscopic damage to the gingival blood vessels. This negative pressure occurs as part of a cycle of intermittent positive and negative pressures created within the gingival blood vessels during the first few moments

of any dentogingival manipulation. It is conceivable that the pressure variations which occur during the cyclical activity of chewing are similar. Associated with these pressures are modest amounts of microscopic or suboptical damage to the gingival blood vessels. It is during these first few seconds of tooth movement that the organisms probably gain entry to the circulation, hence Roberts stressed the necessity for sampling and cultivation of organisms from blood taken within 10 seconds of a dental extraction. It is interesting to note that in this study, toothbrushing caused a small amount of bleeding but had positive cultures on 38% of occasions compared with multiple extractions which caused a large amount of bleeding but gave only positive cultures on 54% of occasions.

There is an implied assumption in the American Heart Associations recommendations for chemoprophylaxis, that bacteraemia after dental procedures is caused by direct invasion of the blood stream. The committee specifies "all dental procedures that are likely to result in gingival bleeding" are at risk of infective endocarditis (AHA 1990). However, consideration is not given to patients with periodontal disease and gingival inflammation where any manipulation may result in bleeding. Indeed, there is no evidence from human or animal observations that bacteraemia occurs by direct invasion of blood vessels. The bacteria involved in infective endocarditis are not motile and capillaries present a positive pressure gradient relative to atmospheric pressure that would flush bacteria away from capillary leaks. The lymphatics appear to be the only channel for 'physiologic' entry into the bloodstream, and bleeding is less relevant than mastication, which, like muscle contraction generally enhances lymphatic flow.

### ***Comment***

It is generally accepted that the route of bacterial entry to the bloodstream is via the gingival crevice but in periodontal patients with inflamed gingival and periodontal tissues a larger surface area of pocket is available for bacterial entry and the basement membrane is not intact.

The data on post-procedure bleeding and bacteraemia show that there is no relationship between bleeding and bacteraemia (Roberts *et al* 1997). Significant bacteraemia can occur in the absence of clinically discernible bleeding. Bleeding therefore is not an important predictor of dental bacteraemia despite the procedures indicated to be 'at risk' in the current infective endocarditis prevention guidelines.

#### ***1.4 : Microbiological variables influencing the incidence of bacteraemia***

The incidence of dental bacteraemia varies widely from one study to another. The interpretation of such results from available studies of different oral procedures is complicated since patient selection, the volume of blood sampled, the time in which the blood samples were taken and in particular, the microbiological techniques used to isolate and identify the microorganisms are different. Many microbiological variables have an influence.

Fastidious microorganisms requiring special conditions or nutrients may not survive or grow in many common blood culture systems (Shanson *et al* 1985). The rapid growth of some microorganisms may inhibit growth of some, or compete for nutrients with other bacteria (Tunkel *et al* 1992). In addition, phagocytic cells and antimicrobial substances in blood may prevent the growth of microorganisms in blood cultures. Antimicrobial agents in blood from patients on antimicrobial treatment, even in low concentrations may inhibit the growth of susceptible organisms (Goldenberger *et al* 1997). Different methods have

been used to overcome these problems. Indeed antibiotic binding devices such as charcoal added to Bactec blood culture bottles allows the growth of organisms from the bloodstream from those patients with an acute infection, previously started on antimicrobial therapy (Organon Teknika).

A large proportion of bacteraemia is caused by fastidious organisms, and difficulty in the culturing of anaerobes may account for some of the differences reported (Hoen *et al* 1995). One study claims to demonstrate a total absence of bacteraemia in children following cleaning of the teeth, but the investigators did not use anaerobic cultures and samples were small volume due to the study population comprising children (Speck *et al* 1971). Hence, the availability of anaerobic culture cabinets and the generally improved anaerobic culture handling facilities assists in the detection of bacteraemia, especially in small samples where low inoculum density can give false negatives (Hutchinson *et al* 1992). Indeed, increased numbers of microorganisms isolated from patients with transient post-operative bacteraemia have been reported after increased volume of blood samples (Washington *et al* 1989, Hall *et al* 1993, Loesche *et al* 1997). Extended incubation times may also result in increased bacteraemia detection as many oral isolates are slow growing (Smith *et al* 1996).

Problems with detection were highlighted by Harty *et al.* (1994) who indicated 32 cases of infective endocarditis caused by *Lactobacilli* had been reported but the number of cases involving their isolation from blood culture has steadily increased in recent years. However, *Lactobacillus* endocarditis may still be under-diagnosed due to inadequate microbiological techniques as *Lactobacilli* grow poorly if at all on nutrient or blood agar with multiple blood cultures and incubation for extended periods not being uncommon. This difficulty may lead to their not being recognised or alternatively being misinterpreted

as minor contaminants. *Lactobacilli* may be implicated in 12.6% of culture negative cases reported in a recent review of 1989 cases of endocarditis caused by uncommon bacteria.

### ***1.5 : Bacterial isolation techniques***

Traditionally, conventional blood culture procedures have been used to detect bacteraemia based upon the isolation of microorganisms from blood which are then transferred directly to liquid and solid media.

Lysis filtration has been claimed to increase the recovery of microorganisms isolated from patients blood samples with transient bacteraemia and give a high yield of isolates (Heimdahl *et al.* 1990) as has the 'pour plate' method utilised by Coulter *et al.* (1990)

In addition, molecular biological techniques have recently been developed and can be utilised for the detection of bacteraemia.

#### ***1.5.1 : Conventional blood culture***

A study by Hutchinson *et al.* (1992) compared Bactec with Oxoid Signal blood culture systems for the detection of streptococcal and anaerobic bacteraemias following dental extraction. Contamination rates were shown to be 4% for the Bactec system which was lower than Oxoid Signal (7.5%). Of 24 test strains of *Streptococci*, 15 were isolated from Bactec bottles and 12 from the Signal system. This was not statistically significant, but it may have been possible that a much larger study may have shown a difference. However, of 19 strains of test anaerobes the Bactec system detected 5 and the Signal 14. This suggests that growth of anaerobes may not be sustained by the culture media in the Bactec bottles possibly leading to an underestimation of the true levels of bacteraemia. However, it could also be an indication of the smaller blood volume used in the Bactec system (5ml) as opposed to the 10ml sample in the Signal bottle. It is unclear why the authors used only

5ml of blood as the amount of blood that should be inoculated into the Bactec bottles as recommended by the manufacturer is 10ml.

However, a further study by Hutchinson (1992) compared the performance of the Sentinel blood culture system with two other systems, (one of which was Bactec) for the recovery of *Streptococci* and anaerobes. Blood cultures were taken from 55 patients one to two minutes after dental extraction. The samples were tested by the Bactec, the isolator 10 which works by lysis centrifugation, and Sentinel; a fully automated system. Positive samples were subcultured and *Streptococci* and anaerobes were further identified. Sentinel was comparable with Bactec (regarded as the gold standard), with Sentinel recovering 20 *Streptococci* and 14 anaerobes, and Bactec isolating 26 *Streptococci* and 15 anaerobes. The recovery of *Streptococcus sanguis* was significantly better from the Bactec system than from Sentinel. Contaminants were recovered from 10% of isolator 10, 7.2% of Sentinel bottles with the lowest contamination level of 2.7% from the Bactec system.

### ***1.5.2 : Pour plate method***

Traditional techniques of culturing organisms from blood involved direct inoculation of the blood sample onto agar plates. This pour plate technique is relatively sensitive and is useful where the volume of the blood sample to be cultured is small. It is less expensive than the Bactec system, and can also facilitate estimates of bacteraemia intensity. However, the pour plate technique may not be sensitive enough to produce growth due to the very small numbers of bacteria that enter the bloodstream. By utilising culture bottles which allows an extra amplification stage, the initial very small numbers of organisms multiply thus enhancing the potential for organism growth and detection on agar plates when sub-cultured. This suggestion is highlighted in a study by Baltch (1988) who

investigated quantitative bacterial pour plates. 29.4% were found to be positive - a lower value than the 61% found with conventional culture.

However, a further study by Coulter *et al.* (1990) gave conflicting evidence. Of the 54 samples cultured by both the pour-plate and Bactec broth methods, 16 (64% of positive cultures) were detected only by the pour-plate culture, two (8%) by Bactec only and seven (28%) were detected by both methods. Coulter thus claimed that the pour-plate technique gave significantly higher results than the Bactec system although other methodological variations could account for these differences e.g. pour plate method facilitating the growth of anaerobes, while only *Streptococci* were isolated from the broth culture. It is also possible the chance of contamination is higher with the pour plate method.

### ***1.5.3 : Lysis-filtration***

Lysis filtration as performed by Heimdahl *et al.* (1990) is the procedure where a blood sample is injected into a bottle containing a lysing solution and subsequently vacuum filtrated under a continuous flow of nitrogen, placed onto an agar plate and incubated. This results in a higher observed incidence of bacteraemia as it produces a concentration of organisms on the cellulose filter. Thus it facilitates the recovery and cultivation of microorganisms even if they were present in the blood culture in small numbers. Moreover, it prevents the rapid growth of certain microorganisms e.g. facultatively anaerobic organisms that otherwise might inhibit the growth of or outgrow the fastidious, strict anaerobic organisms in broth. However, a high frequency of contamination has been reported with blood culture systems which involve lysis of blood.

Heimdahl *et al.* (1990) studied patients with bacteraemia after dental extraction, third molar surgery, dental scaling, endodontic treatment, and bilateral tonsillectomy by means of lysis-filtration of blood samples with subsequent aerobic and anaerobic incubation.



Samples were taken before during and 10 minutes after treatment. Bacteraemia was observed in 100% of patients after dental extraction, 55% of patients after third-molar surgery, 70% of patients after dental scaling, 20% of patients following endodontic treatment and 55% of patients after bilateral tonsillectomy; higher values than those previously cited in the literature.

#### ***1.5.4 : Additives to culture bottles : Sodium polyanetholsulphonate***

Blood phagocytes remain active for 24 hours. Bender *et al.* (1958) showed that adding an inhibitor, sodium polyanetholsulphonate which is a polyanionic anticoagulant; to neutralise the natural antibacterial activity of the blood and to inhibit phagocytosis, complement, and lysozyme can increase the sensitivity of the blood culture techniques for the detection and recovery of bacteraemia. Most conventional modern blood culture bottle systems contain SPS.

#### ***1.6 : Atmospheric requirements / culture media***

Historically, blood culture procedures have been based on the direct transfer of isolated microorganisms from blood to conventional solid and liquid media and exposure to aerobic conditions. Fastidious microorganisms requiring special atmosphere or nutrients may not have survived or grown in many blood culture systems previously used. It has been shown that the addition of a papain digest ox liver to brain heart infusion cysteine broth (Organon Teknika) can enhance bacterial growth. The use of multiple culture media and cultivation in an anaerobic and aerobic and CO<sub>2</sub> rich atmosphere also provides a greater opportunity to culture a larger variety of bacterial species.

An increase in the incidence of Gram-negative bacteraemia and infective endocarditis caused by these organisms have been reported over the last 30 years (Hall *et al* 1993). This may be due to improved laboratory techniques in identifying Gram-negative organisms. Laboratory cultures of slow growing fastidious Gram-negative bacteria which are often found in the human oral periodontal flora and occasionally infective endocarditis are correctly identified only after incubation of 2 weeks or longer. This may in addition explain why 5% of infective endocarditis cases are culture negative (Tunkel *et al* 1992).

Messini *et al.* (1999) investigated bacteraemia in the mentally handicapped who in addition to having poor oral hygiene, can have a variety of associated cardiac defects. On sampling, following fillings, scaling and extractions, 83% of the handicapped patients had a detectable bacteraemia with 53% of these blood cultures containing only aerobic or microaerophilic bacteria, 20% contained strict anaerobes. 53% of positive cultures isolated only one organism. The majority of organisms were *Gemella morbillorum* followed by the viridans *Streptococcus* group. The remainder were Gram-positive cocci and from the anaerobes *Porphyromonas gingivalis* and *Peptostreptococcus* species were common isolates.

Horstkotte *et al.* (1997) determined the spectrum and quantities of isolates following dental intervention. During dental, periodontal and oropharyngeal interventions, bacteraemias were seen regularly (extractions 80-90%, periodontal surgery 60-70% surgery for impacted teeth 50-70%) or frequently (scaling 20-40%, surgery for impacted teeth 10-20%) the largest group of isolates again, being *Streptococci* (49%), *Peptostreptococci* (15.9%) *Corynebacteria* (14.5%) *Propionobacterium* (9%) *Pasteurella* (4.8%), *Bacteroides* (4.1%) and *Neisseria* (2.8%). Both studies indicate a variety of bacteria can be isolated if atmospheric and nutritional requirements are fulfilled.

Huffman *et al.* (1974) and Wank *et al.* (1976) showed that there was a predominantly anaerobic bacteraemia following dental manipulation. A more recent study by Lockhart *et al.* (1996) found Gram-positive cocci to be the most common isolate with 24% of patients yielding polymicrobial organisms following extraction. The former finding is supported by Hall *et al.* (1993) who found that cases of bacteraemia due to anaerobes outnumbered those due to aerobes after dental extraction in incidence and magnitude and reflects the normal oral microflora at the site of surgery. This is in agreement with the findings of most studies of bacteraemia after oral procedures, although some studies indicate high frequencies of *Staphylococcus* species (Guntheroth *et al.* 1984). Although as discussed later, there is a potential for this older finding to be due to skin contamination.

Hall *et al.* (1993) found bacteraemias resulting from dental extractions demonstrated that anaerobic strains of bacteria (*Actinomyces*, *Peptostreptococcus* and *Veillonella*) are isolated twice as frequently as aerobic strains. The most frequently isolated aerobes belong to the viridans *Streptococci* family of bacteria (*S.intermedius*, *S. mitor*, *S.mutans*, *S.sanguis*).

In an older study of 29 patients over the age of 65 years with infective endocarditis, dental manipulations were responsible for almost 20% of cases (Weinstein and Rubin 1973). In patients with dental manipulation-induced infective endocarditis, blood cultures invariably recovered the normal inhabitants of the oral cavity, namely the anaerobes and aerobes previously mentioned. However, in these cases it is unlikely a blood sample would be taken prior to manipulation before infective endocarditis was diagnosed. This gives support to the physiological bacteraemia as a factor which is discussed in greater depth in a later section. *Streptococci* and *Staphylococci* are the most prevalent bacteria reported as causative organisms in infective endocarditis. Nevertheless when blood cultures are properly obtained with skin and laboratory contamination avoided, *Staphylococci* and

*Enterococci* are rarely isolated from patients with native valve endocarditis arising from dental treatment (Tuazon *et al.* 1986).

Coulter *et al.* (1990) found that of 84 colonies recovered from extraction bacteraemia samples, 59 (70%) were grown anaerobically the rest aerobically. This, again supports the predominance of anaerobic isolates which was not shown in earlier studies. These colonies yielded 88 separate bacterial strains, further highlighting the importance of satisfying nutritional and atmospheric conditions to support bacterial growth. When characterised, these 88 strains were made up of 44 aerobes and facultative anaerobes, mainly *Streptococci*, and 22 strict anaerobes and 16 micro-aerophilic cultures, mainly *Actinomyces*. A total of 31 *Streptococcal* species, predominantly *S. sanguis* and *S. mitior*, were isolated from 20 of the 29 patients with bacteraemia. *Bacteriodes* and *Veillonellae*; both anaerobic species were also cultured but present in a smaller number of samples. When the anaerobes were present, they were often in samples with large detectable bacteraemias and in association with other anaerobic and facultative species. In contrast, *Streptococci* were isolated as a single species in a third of the positive blood culture bottles.

The apparent bias towards anaerobic / facultative isolates is sensible as analysis of organisms associated with the 2 most common dental diseases reveals predominantly Gram-negative anaerobes as putative pathogens in periodontal disease and Gram-positive *Streptococcus mutans* as the most important bacteria in dental caries (Asikainen & Alaluusua 1993). The majority of organisms cultured in blood after dental extractions or other dental procedures were Gram-positive cocci with viridans *Streptococci* and alpha-haemolytic pyogenic *Streptococci* as the most predominant organism (Everett &

Hirshmann 1977, Lockhart 1996). They are potentially pathogenic bacteria and have been found responsible for septicaemia, endocarditis, pneumonia, meningitis, urine infection, infection of dental prosthesis (*Streptococci*, *Staphylococcus epidermidis*, *Gemella sp*, *Aerococci*, *Lactococci*, *Corynebacterium sp*, *Propionibacterium sp*) as well as infections of wounds, bones, joints and surgical infections (*Peptostreptococci*, *Porphyromonas gingivalis*, *Staphylococcus epidermidis* (Isenberg & D'Amato 1995).

*Streptococcus sanguis* is the most frequently isolated organism in dental plaque, including subgingival sites affected by periodontitis (Loesche *et al*). While not considered to be a periodontal pathogen, this microorganism likely enters the circulation by contact with chronically inflamed and ulcerated periodontal tissues (Herzberg *et al* 1996). In severe periodontitis, the complex bacterial plaque can be exposed to wounded gingiva with aplanar surface area of up to 50cm (Loesche *et al* 1997). If untreated this large chronic wound may contribute over many years to recurrent bacteraemias. While dental infections such as periodontal disease appear to contribute directly to the frequency of clinically detectable bacteraemias, the actual polymicrobial dose is not known. A mg wet weight of dental plaque contains about  $10^8$  to  $10^9$  bacteria. Of these bacteria about 30% will be *S sanguis* (Moore *et al* 1994). Alpha-haemolytic *Streptococci* was the most common group of organisms cultured from infective endocarditis. *Streptococcus bovis*, viridans *Streptococci* (*sanguis* and *mitis*) and *Streptococcus mutans* were also often cultured from patients with infective endocarditis (Lacassin *et al* 1995). Geraci and Wilson (1982) described a group of fastidious antibiotic-sensitive cocco-bacilli responsible for Gram-negative infective endocarditis. They called this group of bacteria the HACEK group because it includes genera *Haemophilis*, *Actinobacillus*, *Cardiobacterium*, *Eikenella* and *Kingella*. These Gram-negative bacteria are often suspected to originate from the oral cavity. *Eikenella corrodens* and *Aa* are described as being significantly elevated in areas

of active periodontal destruction and in case studies of infective endocarditis among patients with juvenile, pre-pubertal or refractory periodontitis they have been found to be the causative organism.

These studies highlight that many organisms can be found in the bloodstream following oral manipulation, indeed several papers have shown more than 25 organisms in blood cultures from patients following dental procedures, thus there is a problem in the choice of a single antibiotic to cover the oral flora (Coulter *et al* 1990, Heimdahl *et al* 1990, Lofthus *et al* 1991, Messini *et al* 1999).

### ***1.7 : Types of isolates : plaque***

As stated earlier, dental plaque contains one of the most concentrated accumulations of microorganisms in the body. More than 350 bacterial species have been encountered in marginal periodontitis of which a considerable number is strict anaerobic, concentrated especially in proximal and subgingival plaque (Messini *et al* 1999).

The healthy gingival sulcus harbours a microflora dominated by Gram-positive facultative anaerobic species. Spirochetes and motile rods are also present but in small numbers. In periodontal disease the proportion of anaerobes (90%) including Gram-negative organisms (75%) and spirochetes (56%) increases significantly. *Aa* may be particularly pathogenic because of its ability to invade periodontal tissues. *Pg* is a major species in severe forms of adult periodontitis where it can constitute more than 40% of the cultivable flora. *Prevotella intermedia* was considered to contain two genotypes and serotypes, of which one is allegedly associated with forms of gingivitis and the other with various forms of periodontitis (Moore *et al*).

### ***1.7.1 : Which oral organisms are likely to enter and survive in the bloodstream ?***

As has been discussed, most members of the indigenous flora are anaerobes. Anaerobes are likely to penetrate epithelium and given the relatively large surface area of combined periodontal pockets, more anaerobes are likely to enter the underlying connective tissue than facultative organisms. It has been previously claimed by Loesche *et al.* (1997) that because of the high  $pO_2$  and  $Eh^1$  of these tissues, anaerobes are contained and rarely appear in the blood stream. Indeed, these organisms find the highly aerobic host cells so unfavourable that they rarely survive to enter the bloodstream. However, because most of these anaerobes are Gram-negative, their lipopolysaccharide can initiate events that may increase the circulating levels of cytokines, with possible adverse effects for the host (Beck *et al* 1998).

Despite it being generally regarded that blood is a highly oxygenated medium in which oral anaerobes can not survive for any length of time (Loesche *et al* 1995), it should be realised however that organisms reaching the blood from an oral site will have been transported in the bloodstream to peripheral sites of the body within 1 minute (Roberts *et al* 1992). Furthermore, some of the strictest anaerobes e.g. *Clostridium haemolyticum* are not killed when exposed to atmospheric oxygen on the laboratory bench top until after 10 minutes or longer. Moderate obligate anaerobes tolerate oxygen for much longer times. Anaerobes such as *Bacteroides fragilis* also have enzymes such as catalase and superoxide dismutase that are protective against toxic oxygen reaction products. Therefore anaerobes are probably better able to survive in the bloodstream than previously thought.

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<sup>1</sup> oxygen concentration is considered the main factor limiting the growth of obligately anaerobic bacteria. In the majority of microbial habitats it is the commonest and most readily reduced electron acceptor and its presence results in the oxidation of the environment. Anaerobic species require reduced conditions for their normal metabolism; therefore it is the degree of oxidation-reduction at a site that governs the survival of these organisms. This oxidation-reduction level is usually expressed as the redox potential.

Scattered among the anaerobic species among the indigenous flora are various facultative species (Loesche *et al* 1995). These lack the overt virulence factors of the classic pathogens. More of these facultative species, such as the *Streptococci* and *Actinomyces* are found in the oral cavity and in dental plaque (where air can pass) than anywhere else in the body. Many of the facultative species in supragingival plaque, when they breach the physical barrier of the epithelium, are not susceptible to the electrical barrier created by the high tissue oxidation-reduction potentials and if they were not quickly scavenged by tissue monocytes would survive in the bloodstream eventually gaining access to the peripheral circulation. Thus facultative species such as *S. sanguis* and *S. faecalis* are more likely to survive within the tissue. This factor is probably why facultative species are invariably found in asymptomatic bacteraemias, in addition to infected heart valves and prosthetic replacements. If their entry into the host is frequent, then elevated antibody titers should be found and possibly elevated levels of leucocytes (Kweider *et al* 1993).

Using a sensitive lysis-filtration technique, Loesche *et al.*,(1977) showed that 34 of 49 blood samples taken from blood bank donors after they gave a pint of blood yielded low numbers of facultative species. They concluded that under normal circumstances very small numbers, i.e. 1 to 10 cfu/5ml of indigenous bacteria gain access to the blood. These numbers are so low that the hosts surveillance systems are easily able to dispose of them. Roberts *et al.* (1999) often isolated facultative species indigenous to plaque such as *Streptococcus sanguis*, *Streptococcus milleri*, and *Streptococcus mutans*.

Periodontal pathogens are capable of invading the periodontal tissues, but loss of epithelial integrity within the periodontal pocket also allows direct bacterial translocation and bacteraemia. In chronic infection such as periodontitis, recurrent transient bacteraemias with Gram-negative periodontal pathogens is anticipated. This would result in repeated systemic exposure to Gram-negative bacteria, LPS and other bacterial products. It is likely



the more severe the periodontal inflammatory state the greater the haematogenous bacterial exposure, in terms of bacterial counts and duration.

### ***1.7.2 : True isolates/ contaminants***

In dental bacteraemia studies, elimination of *Staphylococcal* skin contaminants is desirable. Roberts *et al.*(1997) found the percentage of positive cultures from these potential contaminants was 8.6%. Their attempt to control for skin contamination was made by culturing the 0.5mL of blood that was first drawn from the cannula and 'discarded' into Bactec culture bottles. 5.9% positive cultures were obtained from the discarded 0.5mL samples. Roberts believed that the percentage of positive isolates for *Staphylococci* of 8.6% represents a true level of bacteraemia due to transient oral colonisation by *Staphylococci*. The 5.9% known contamination from the discarded 0.5mL still leaves a minimum true bacteraemia of 2.7% due to *Staphylococci*.

Whenever a blood sample is taken there is always the possibility of inadvertent contamination. The risk of contamination can be reduced by utilising a strict aseptic procedure for venepuncture and careful handling of microbiological samples. Contamination during the sampling procedure can be avoided by preparation of the patients antecubital fossa with alcohol and ensuring all blood letting equipment is sterile and only opened directly prior to the procedure with avoidance of touching components which would be coming in contact with the patients blood. Contamination can also arise in the laboratory and careful handling must be performed to avoid contamination of the agar plates. Heimdahl *et al.* (1990) suggest reducing the number of examinations of the plates. Should contamination occur, colonies produced are frequently skin contaminants (e.g. *Staphylococcus aureus*) and frequently lie outwith the regions of innoculum.

### ***1.7.3 : Are bacteraemia isolates of oral origin?***

The prolific resident and transient oral flora is an important source of spontaneous bacteraemias associated with acute or chronic oral/odontogenic infections. These infections may represent a far greater cumulative risk for the development of endocarditis than occasional health care procedures administered in a professional setting.

Interesting studies by Debelian *et al.* (1992), (1995) attempted to characterise oral microorganisms believed to have spread from the root canal into the blood stream during and after endodontic therapy of teeth with apical periodontitis, performing lysis filtration on the samples obtained. In addition, Debelian *et al.* examined biochemical profiles, antibiotic susceptibility tests and electrophoresis of soluble proteins, revealing that *Propionibacterium acnes*, usually considered to be a skin contaminant, isolated from both the root canal and blood samples were identical within patients but varied between patients. This suggested since the bacteria recovered from the blood stream was similar to the corresponding isolates from the root canal, the root canal was the source of the infection.

Thus the use of molecular biological techniques have been utilised to show that strains isolated from the blood are essentially identical to strains found at the same time in the patients mouth thus indicating the mouth is the portal of entry. Debelian *et al.* used phenotypic and genetic methods to trace microorganisms released into the bloodstream during and after endodontic treatment back to their presumed source - the root canal. Phenotypic methods used for characterisation and tracing of microorganisms in blood and root canals were biochemical and antimicrobial susceptibility tests, SDS-PAGE of whole cell soluble proteins and gas chromatography of cellular fatty acids. Phenotypic data were verified by DNA restriction patterns and corresponding ribotypes of the root canal and blood isolates by using a computer-assisted system for gel analysis. It is therefore

undoubted that *P.acnes* is a true isolate given the close congruity between organisms isolated from the root canal and associated blood cultures.

Oral *Streptococci* are highly specific colonisers of tooth and oral mucosal surfaces. They do not colonise other parts of the body and so a bacteraemia of *Streptococcus sanguis*, except in unusual circumstances, uses the mouth as a portal of entry to the blood circulation.

Proof that dental bacteraemia is directly linked to infective endocarditis has been demonstrated in rabbits with experimentally induced endocarditis that subsequently were subjected to exodontic and periodontic manipulation. The same *Streptococci* that were seeded in the mouth were isolated in the endocardium. Supportive clinical evidence of this causal relationship between infective endocarditis and a specific periodontal microbe, *Actinobacillus actinomycetemcomitans*, which resides in diseased periodontal pockets is given by Van Winkelhoff *et al* (1992). He stated that this Gram-negative cocco-bacillus has been detected in more than 37 reported cases of endocarditis since its identification as a taxonomic entity.

Oral species routinely enter the bloodstream during dental treatment and cause an asymptomatic bacteraemia. There are several anecdotal reports associating brain, prosthetic joint or heart valve infections with dental treatments (Doerffel *et al* 1997, Larkin *et al* 1994, Kaplan *et al* 1989). However, some of these reports undoubtedly indicate that by ribotyping, cell wall analysis of the isolates, or phenotypic profile, the identity of the blood isolate is the same species as that isolated from saliva, plaque or from root canals. It cannot be debated that these organisms are oral but it is uncertain whether dental treatment caused the bacteraemia as asymptomatic bacteraemias involving oral

bacteria may be a daily event. As mentioned previously, it may not be dental treatment but rather dental disease that contributes.

### ***1.8 : Clinical variables influencing incidence of bacteraemia***

It has been claimed the size of the bacterial inoculum entering the bloodstream is influenced by the degree of trauma associated with a particular manipulative procedure (Bender *et al* 1963). Indeed, it is apparent that different types of procedures result in differing bacteraemia incidences. It has been previously assumed that there is a direct relationship between the degree of manipulative trauma to a contaminated operative site and the magnitude of the resultant bacteraemia. Additionally, several authors have gone further to state the number of teeth extracted at a given time is an important factor in curtailing bacteraemia (Bender *et al* 1963, Robinson *et al* 1950, Okabe *et al* 1995). This has been disputed (Speck *et al* 1976). It can be hypothesised the longer a procedure lasts the greater the bacteraemia produced (Okabe *et al* 1995). It is also assumed that dental procedures such as surgical endodontics, i.e. the instrumentation of inflammatory periapical lesions, can expose a patient to bacteraemia while it was previously assumed routine non-surgical endodontic procedures cause no detectable bacteraemia (Lavelle *et al* 1996). To the contrary, there are a number of papers in the literature relating routine endodontic therapy to bacteraemia (Debelian *et al* 1992, 1995, 1998). However, it may also be hypothesised that clinical variables such as instrumentation through the apex would influence the production of a bacteraemia.

In addition to these, a variety of different culturing techniques used by varying authors influence the detection rate of bacteraemia and have been discussed previously. A variety of host factors will be considered in a later section.

### ***1.8.1 : Duration of procedure***

It is not known what influence the duration of the procedure has on the risk of bacteraemia but it is likely that it reflects both the volume and nature of organisms that originally gain entrance into the circulation. As extractions can be a particularly traumatic procedure and can have a prolonged operating time, this may explain the high bacteraemia incidences indicated by authors. King *et al.* (1988) took blood samples from healthy adult patients requiring extractions of at least 5 erupted teeth before and 1.5 - 3 minutes after their extractions and then before and 1.5 - 3 minutes after suture removal. Following the extractions, 87.5% of the subjects had an incidence of bacteraemia. However, only 5% of subjects yielded bacterial growth at the time of suture removal suggesting the length of the procedure and indeed trauma of the procedure has an effect.

However, Lockhart *et al.* (1996) observed an increase in positive blood cultures with shorter surgery times (less than 3 minutes) and therefore with simpler extractions. This may suggest that these teeth are in a more septic and hyperaemic environment of soft tissue and there is therefore a greater propensity for organisms to be forced into the circulation through a diseased or missing mucosal barrier. This opposes the findings of Roberts *et al* (1999) who found reduced levels of bacteraemia in children who usually have shorter operating times. On the other hand, Lockhart *et al* (1996) additionally found an increase in incidence of bacteraemia with more prolonged and therefore difficult extractions whose surgery time was longer than 6 minutes.

Differences in populations sampled could also influence bacteraemia levels found. It has been claimed that bacteraemia in children is usually of a greater magnitude than in adults, although this claim is not based on a study of dental bacteraemias (Carey *et al* 1984). Extractions in this group of patients are generally simple, although it is likely there is a

smaller volume and less variety of organisms in the mouths of children, a lower incidence and severity of disease around their teeth and smaller volumes of blood taken for culturing. It could be hypothesised therefore, that adult periodontal patients whose oral bacterial profile is vast, would exhibit bacteraemia frequently on instrumentation.

### ***1.8.2 : Effect of blood volume taken***

It can be hypothesised that the larger the volume of blood taken following each potential bacteraemia-inducing procedure, then the greater the chance of isolating bacteria. As stated previously, it is likely the smaller volumes of blood taken in paediatric studies would play a part in the lower levels of bacteraemia quoted. Indeed Bender *et al.* (1989) found that blood samples of 16ml had increased sensitivity in the detection of bacteraemia compared to 8ml samples.

Hall *et al.* (1993) compared 5ml and 10 ml blood volume samples in 50 ml and 100ml soybean casein digest broth culture bottles. A larger number of microorganisms were isolated with significant growth of Gram-negative bacilli in the 100ml bottles. Loesche (1997) also advocated increasing the volume of blood samples to increase the isolation of bacteria. Tenney *et al.* (1982) found microorganism yield from 7ml samples to be 29% greater than from 2 ml samples. This supports the theory that the volume for blood cultured is critical in the detection of bacteraemia. Indeed, Washington (1989) suggested that the volume of blood is relatively more important than the medium or the atmosphere of incubation in the detection of sepsis.

Concerning the culturing conditions, Salventi *et al.* (1979) recommended a blood to broth ratio of 1:5 to 1:10. A blood to broth ratio between 1:2 and 1:5 may result in an overall decreased number of positive cultures according to Reller *et al.* (1995).

### ***1.8.3 : Site of blood removal***

Beeson *et al.* (1945) compared various sites of blood sampling in patients with infective endocarditis to determine the effect this would have on levels of bacteria isolated from the bloodstream. This was an attempt to improve on the detection of bacteraemia levels as it is known that many cases of infective endocarditis are shown to be negative by conventional culture. Quantitative arterial and venous blood cultures were taken from the following peripheral locations: the femoral artery and vein and the antecubital vein as well as the right auricle, vena cavae, hepatic and renal veins. The femoral vein had appreciably less bacterial invasion and the hepatic vein had the smallest bacterial count. Blood collected from the antecubital fossa had colony counts only slightly lower than arterial blood. Thus the authors showed that blood collection from the antecubital fossa would provide researchers with a close to ideal sample for bacteraemia detection.

### ***1.8.4 : Extent of procedure***

Vargus *et al.* (1959) determined that the greater extent of surgery as measured by area of surgery or quadrants, showed a tendency to yield more positive blood cultures, although this data was not statistically significant. Several studies have suggested that the chances of detecting bacteraemia increase with the degree of invasiveness of the oral surgical procedure, although this relationship was not found by Debelian *et al.* (1998). Bender *et al.* (1958) found a higher incidence of bacteraemia following multiple extractions. Roberts (1998) also found that the prevalence following multiple extractions is greater than for a single extraction. They investigated 207 children divided into 4 groups; Group 1 comprised no surgical intervention and produced a positive culture incidence of 11%, group 2 after a single tooth extraction produced 43% culture positive, group 3 underwent multiple tooth extraction with 54% culture positive and group 4 had a mucoperiosteal flap

elevation from which 43% had positive isolates. The suggested trend of increased bacteraemia with increased trauma is supported however, the differences observed were not statistically significant.

Conflicting evidence is provided by Coulter *et al.* (1990) who found no correlation between either the incidence and intensity of bacteraemia and the number of teeth extracted. Heimdahl *et al.* (1990) also found that bacteraemia was not related to the extent of surgery since a single extraction produced a higher incidence of bacteraemia than for third molar surgery and bilateral tonsillectomy. Heavy colonisation of the tooth surfaces with aerobic and anaerobic microorganisms in combination with the forceful pushing movements used in dental extraction may have been of importance.

Previous studies indicate that the type of surgical treatment performed results in variation in the amount of transient bacteraemia produced. Indeed it is generally believed that bacteraemias are influenced by the invasiveness and duration of an oral surgical procedure and that dental extractions are the most likely of dental procedures to cause a bacteraemia, ranging from 10% to 92% according to different studies.

King *et al.* (1988), claimed they did not find a positive correlation between the amount and number of manipulations and the incidence of bacteraemia. The authors based this conclusion on individual cases which are not sufficiently powered to conclude that no correlation exists. Their findings of one patient who had nine teeth extracted had ten different isolates cultured was compared with another who underwent 17 extractions had no bacterial growth reported from blood culture. The authors claimed that organisms in the blood were possibly not present in great enough numbers to be detected by the culture techniques used or the individual host mechanisms may have removed the particular organisms before they could be detected. Nevertheless, 14/16 patients yielded positive blood cultures following tooth extractions and one of 20 yielded a positive blood culture



following suture removal which does support the concept of greater invasiveness of the procedure leads to a greater bacteraemia incidence.

Roberts *et al.* (1998) found the prevalence of bacteraemia following elevation of a mucoperiosteal flap to be low, which is surprising considering the extensive dentogingival trauma induced. However, most of the children studied were having elective surgery prior to orthodontic extractions and their standard of oral health was good whilst in other studies surgery was usually to relieve pain or infection associated with oral neglect. This may be indicative that level of disease may have an influence i.e. suppurating, swollen lesions as compared to non inflamed areas.

### ***1.9 : The influence of dental disease on incidence of bacteraemia***

#### ***Effect of oral hygiene***

It can be assumed that patients demonstrating marked oral infections are more likely to produce bacteraemia than those demonstrating better oral hygiene. Since it has been claimed the incidence and magnitude of bacteraemia following dental manipulations is proportional to the severity of the gingival inflammation (Okabe *et al* 1995), it is self-evident that good oral health will reduce the likelihood of infective endocarditis in an at-risk patient. It is alleged that with excellent oral hygiene, the prevalence of bacteraemia after extractions is approximately the same as when chemoprophylaxis is employed as oral sepsis doubles the risk of bacteraemia (Guntheroth 1984). However, the old view of conventional chemoprophylaxis is still empirically held.

In an early study, Cobe *et al.* (1954) felt that there was no significant difference in the incidence of bacteraemias between the periodontally diseased mouth and the healthy mouth. Conflicting evidence was provided by Sconyers *et al.* (1973) who reported that

16.7% of periodontal diseased patients developed bacteraemia after toothbrushing. In an unpublished study of dental students with healthy gingivae, performed by the same methods and cultured by the same personnel, no bacteraemias could be detected.

More recently, Lockhart *et al.* (1996) found the severity of odontogenic disease did not correlate with the results of the blood cultures. Schlein *et al.* (1991) investigated the effect of toothbrushing and transient bacteraemia in patients undergoing orthodontic treatment. 20 patients were selected and blood samples of 20 ml were drawn before and 5 minutes after brushing. 5 of the 20 patients (25% of the sample) had positive blood samples after brushing. Interestingly, those patients who were found to have a bacteraemia did not display poor oral hygiene.

Moreillon *et al.* (1988) induced endocarditis in rats more often in the presence of periodontal disease than with healthy gingiva, but there was no direct correlation between the number of bacteria circulating one minute following the extraction and the incidence of infective endocarditis.

Roberts *et al.* (1999) found no relationship between the incidence or intensity of bacteraemia and either the amount of plaque around the gingival margin or the gingival condition. Most of the children in their study had a detectable amount of plaque around the gingival margin and slightly inflamed gums that did not bleed when probed. This could be explained by the small study population (155) being young children who did not have periodontal disease. Roberts calculated that a much larger sample of 1,564 subjects would be required to observe a statistically significant association between plaque, gingivitis and bacteraemia. They concluded that home care procedures should be practiced thoroughly and frequently to reduce the risk of bacteraemia from toothbrushing and all children at risk from infective endocarditis even require prophylactic cover prior to tooth cleaning with a rubber cup and scaling which is in line with the AHA guidelines.

Roberts *et al.* found plaque scores for children with a bacteraemia to be slightly higher than those for culture negative cases although the difference did not reach significance. A highly statistically significant relationship was determined between gingival inflammation/gingival bleeding and bacteraemia. This may be explained by visible plaque not having been present for long enough to provoke a level of inflammation that would render the underlying tissues susceptible to microbial ingress whereas overt inflammation indicates more established disease which allows readier access of crevicular organisms to the bloodstream. Much of the plaque is too superficially coronally placed on the tooth surfaces to be readily carried into the crevicular and subgingival tissues during surgery.

Lockhart *et al.* (1996) also looked at clinical parameters in association with incidence of positive blood cultures and found a higher incidence of blood cultures positive for organisms 1 minute following extraction in patients with widened periodontal ligament spaces.

### ***1.9.1 : The effect of plaque***

Otten *et al.* (1987) have shown the relationship of microorganisms isolated from the bloodstream reflect both quantitatively and qualitatively the microflora of the local apical periodontitis or marginal periodontitis by looking at 3 groups of patients. In group 1 extractions were performed due to caries, periodontal disease or apical periodontitis producing 74% bacteraemia with a high proportion of anaerobes. Group 2 comprised patients with intact partially impacted third molars which were extracted producing 40% bacteraemias with isolates representative of the healthy gingival sulcus. In group 3, osteosynthesis plates to repair fractures were surgically removed with no organisms detected in the patients bloodstreams. These findings suggests that it is the plaque on the teeth which is the main source of dissemination of organisms into the bloodstream. This

supports findings by Heimdahl *et al* (1990) that a single extraction results in a greater bacteraemia than the extraction of wisdom teeth and bilateral tonsillectomy.

### ***1.9.2 : The effect of abscess***

Roberts *et al.* (1998) determined there was no relationship between the presence of a positive blood culture and the presence of an abscess. Conflicting evidence was provided by Speck *et al.* (1971) who studied the incidence of bacteraemia in a group of children undergoing dental extraction of both normal and abscessed teeth. 30% of the post extraction cultures were positive with viridans *Streptococci* isolated in all cases. As would be expected, bacteraemia was more common after the extraction of diseased teeth. Interestingly, there was no relationship between bacteraemia and the number of teeth removed. However, bacteraemia was demonstrated in a notable number of children who underwent extraction of normal teeth for orthodontic reasons.

Elliott and Dunbar (1968) have shown that the incidence of bacteraemia in healthy children after dental extraction was 36%, and they were able to isolate only viridans *Streptococci*. They concluded that positive blood cultures were more common in older children and after multiple extractions of diseased permanent teeth. In comparison to Speck *et al.*(1971), these investigators were unable to document bacteraemia in a small group of children who had sound permanent teeth extracted for orthodontic reasons. However, it should be noted that this older study may have used less sensitive detection methods and may not have used anaerobic culture media.

In a similar study, Coulter *et al.* (1990), investigated bacteraemia in children following dental extraction and the effects of the number and type of teeth extracted, oral hygiene, gingival health, presence of abscess and antibiotic prophylaxis. They found that antibiotic prophylaxis reduced the incidence of bacteraemia from 63% to 35%. The intensity of

bacteraemia was 2 cfu/mL of blood or less in 80% of the children. An agar pour plate method of broth culture was significantly more effective than broth in culturing the small volumes of inoculum. Of 83 bacterial strains characterised, 39 were strict anaerobes or microaerophilic with the remainder mainly *Streptococci* (*mitior* and *sanguis*). No direct association was demonstrated between the plaque and gingival indices and incidence of bacteraemia or between the number of teeth extracted, the presence of an abscess and the incidence or intensity of bacteraemia.

### ***1.9.3 : The effect of teeth with chronic periradicular disease***

The assumption that teeth with periradicular regions of radiographic rarefaction can induce bacteraemia without manipulation is not valid. Comparative bacteraemia studies in patients with asymptomatic teeth with or without periapical radiographic lesions show no difference in bacteraemia incidence (Lavelle 1996). In a study of 300 patients none were bacteraemic when blood samples were taken as controls before various dental manipulations. If bacteraemia could occur in patients with periradicular disease without manipulation, then some of the pre-operative control blood cultures would have been positive for growth. However, it should be remembered that an acute abscess has little to no radiographic appearance and radiographic lesions of rarefaction are not areas of infection harbouring bacteria, but rather quiescent lesions that have been identified mainly as radicular cysts or granulomas. Bacterial cultures from these lesions have been proven to be 85-100% negative (Soames and Southam 1992).

A study by Hall *et al.* (1993) supports this theory, as none of their patients studied were bacteraemic before surgery despite the majority of them having chronic dentoalveolar pathology.

#### ***1.9.4 : The effect of bleeding***

The common perception is that bacteraemia occurs only when dental procedures cause bleeding and does not occur when there is no bleeding. Hence procedures such as extractions and scaling require antibiotic prophylaxis, whereas procedures such as placement of simple dental fillings do not. However, a grey area must exist as some large fillings may induce bleeding if the gingivae is traumatised and placement of matrix band can certainly induce bleeding. Indeed consideration should be made to any procedure where gingivitis exists as bleeding would be a likely outcome.

Moreover, it has been suggested in earlier literature that the injection of adrenaline at the surgical site might reduce the blood flow and the uptake of organisms, although this was later shown to have no effect on the entry of organisms into the circulation (Eldirini *et al.* 1968).

Roberts *et al.* (1997) looked at the percentage of positive blood samples following a variety of dentogingival manipulative procedures by the presence or absence of bleeding to the naked eye. For all 14 groups of procedure for which there was the possibility of 'bleeding' or 'no bleeding', there was no significant difference in the presence or absence of bacteraemia. When groups in which bleeding would always occur were included in the analysis there was still no significant difference from the effects of bleeding on the detection of bacteraemia. However, the number of organisms isolated from blood where bleeding was present was statistically greater than when there was no bleeding. The trend was similar for oral *Streptococci*, with greater numbers being cultured from blood where bleeding was present.

### ***Comment***

Postoperative bacteraemias have been an established clinical finding for many years. There are many variables which make comparisons of bacteraemia levels impossible between studies. As will be discussed later, different procedures have been investigated and even studies which looked at similar procedures had many others factors which make them incomparable : different blood volumes, differing sampling times following a procedure, differing duration and extent of procedure not to mention differing culture techniques. The percentage of positive bacteraemias produced have varied as much as the procedures that were used to produce bacteraemias and the methods used to record the presence of microorganisms following the procedures.

It is a long-standing and widely held belief that there is a relationship between bacteraemias and indicators of dental disease. However the literature does not support this relationship when the study design is controlled for variables. Lockhart *et al.* (1996) found there was a lack of significant correlation between any of the indicators of dental disease and the results of blood cultures from many studies raises questions as to the relationship between dental disease, invasive procedures and the risk of sequelae such as endocarditis. Lockhart concluded since the overall incidence of infective endocarditis is low and bacteraemias are a routine and naturally occurring phenomenon, other factors such as the hosts immune response and the nature of organisms gaining entrance into the circulation may be of far greater importance than dental procedures.

It is assumed dental procedures that involve bleeding may carry with them a risk of bacteraemia. The American Heart Association (AHA) recommends chemoprophylaxis of at risk patients for “dental procedures known to induce gingival or mucosal bleeding

including professional cleaning”. But it recommends against chemoprophylaxis for “injection of local intraoral anaesthetic (except intraligamentary injections)”. Injections of local anaesthetic obviously causes bleeding but it is assumed does not cause bacteraemia. The British Society for Antimicrobial Chemoprophylaxis (BSAC) goes much further and recommends chemoprophylaxis only for extractions, scaling and periodontal surgery (but not for any other dental procedures). They claim the reason for this more conservative approach is that most other dental procedures, if they cause bacteraemia at all, are associated with such a low level bacteraemia as not to be a significant risk.

Studies indicate that patients who practice scrupulous oral hygiene, have no gingival bleeding and a low bacterial count have a markedly low bacteraemia incidence after dental manipulation. This is important as home-care procedures and dental manipulations that cause bleeding are often the source of blood-borne bacteria. The magnitude of the bacteraemia may be influenced by the level of oral hygiene. As most of the population has gingival bleeding, they are subject to frequent bacteraemia. As periodontal patients have pockets which are inaccessible by the patient to hygiene practices it was thought this group of patients would exhibit extensive bacteraemia and the organisms isolated would reflect the extent of the disease e.g. putative periodontopathogens.



### **1.10 : Duration of bacteraemia : How long does a bacteraemia last?**

The frequency of detection of bacteraemia varies with the time that blood is taken after a dental procedure. It is important to determine the optimum time for sampling as any variation that occurs can confuse the assessment of levels of bacteria detected.

Several authors have reported on the duration of a bacteraemia. However this cannot be answered accurately due to numerous variables influencing the time interval of a transient bacteraemia. Animal and human experiments have proved that varying times may be recorded even by the same group of investigators. Indeed intersubject variability undoubtedly has an effect - bacteraemia may be detected more readily in some patients than others due to perhaps to less efficient clearance by the hosts immune system. Furthermore, it may be difficult to isolate and culture bacteria from some patients due to the production of inhibitors in their blood.

The length of a bacteraemia is believed to be on average only 15 minutes but a recent study on children showed a high level of bacteraemia at 10 minutes and with extrapolation suggested that the length of bacteraemia is approximately 45 minutes (Roberts *et al.* 1999). This is supported by data from adults in studies that are often overlooked in the cardiology / bacteraemia literature which shows the length of bacteraemia may be almost 60 minutes.

#### **1.10.1 : Early studies**

Early studies by Schottmüller (1925) found the duration of positive blood cultures after an invasive procedure (septic uterine curettage) to be only 15 minutes. A longer time interval was reported by Elliott (1939) who investigated tonsillectomy and found positive bacterial cultures up to 80 minutes following the procedure. This was supported by Rise *et al.* (1969) who found in isolated instances, bacteria can exist in the bloodstream for up to an hour. Cobe (1954) found that intravenous inoculations of  $\alpha$ -haemolytic *Streptococci* in

rabbits were recovered in blood cultures taken by means of cardiac puncture at 1 minute and 3 minutes. Minimal amounts were detected at 5 minutes and none after 10 minutes, highlighting the efficient removal of bacteria from the bloodstream by the hosts reticuloendothelial system. Similarly, Northrup and Crowley (1949) reported positive cultures from 13% of their patients immediately following an extraction, with only one positive culture obtained after 10 minutes (which they attributed to contamination). This 'dose response' was supported by Taran (1944) who found 49% of 400 patients had positive cultures immediately after extractions; on repeat culture 30 minutes later, only 4 were positive. Using the same procedure, Robinson *et al.* (1950) found positive blood cultures only up to 10 minutes. Merrill *et al.* (1951) found only 3% of bacteraemias persisted longer than 30 minutes. Thus from these initial studies the suggested duration of bacteraemia after dental extraction is relatively brief, i.e. less than 30 minutes. This is supported by Baltch *et al.* (1982) who studied the rate, type and magnitude of bacteraemia in 56 patients undergoing dental cleaning with and without penicillin prophylaxis. 61% of patients were bacteraemic 5 minutes following the procedure. Although a significant decrease in detectable bacteraemia occurred in the patients receiving penicillin prophylaxis, the recovery of *Streptococci* was not significantly different in the two groups. At 30 minutes a significant decline in bacteraemia occurred in the patients who did not receive penicillin. Polymicrobial bacteraemia (2-6 bacterial strains) was detected most frequently 5 minutes following the procedure and in patients not receiving penicillin prophylaxis. A significant decrease in the number of patients with polymicrobial bacteraemia occurred at 30 minutes in patients receiving no penicillin prophylaxis, again indicative of the removal of bacteria by the hosts immune system

### ***1.10.2 : Recent evidence***

More recent studies indicate that procedure related bacteraemias are short lived with the frequency of blood cultures being highest in the first 30 seconds after tooth extraction (Roberts *et al.* 1997). Roberts *et al.* (1992) claimed a sampling time starting 45 seconds after the extraction of teeth was too long an interval to leave from the start of the bacteraemic stimulus as it is well known that the time for one complete circulation of the blood is of the order of ten seconds. This is demonstrated by the rapid onset of anaesthesia following an intravenous injection of a hypnotic drug.

Previous studies have found that the greatest incidence of bacteraemia occurred between 0.5 and 3 minutes with a duration of 10 minutes. Silver *et al.* (1975) showed that peak bacteraemia occurred 30-60 seconds after introduction of bacteria into the circulation into dogs, but others have found this to vary from 1-3 minutes. Lockhart *et al.* (1996) considered bacteraemia following a single tooth extraction in adults and the antibacterial effect of rinses with chlorhexidine hydrochloride. 94% of the control patients and 89% of positive control patients had blood cultures positive for microorganisms at the 1 minute or 3 minute mark following the initiation of surgery respectively. However, no statistically significant difference was observed in the incidence of blood cultures positive for microorganisms at both shorter and longer surgery times. Lockhart *et al.* (1996) found that bacteria can be detected for up to 10 minutes from the time of oral surgery, but the retrieval of microorganisms drops off sharply between 10 and 30 minutes.

Berger *et al.* (1988) showed that blood cultures taken immediately after extraction were 81.4% positive but decreased to only 48.8% in cultures taken 10 minutes after extraction. Bender *et al.* (1989) observed bacteraemia immediately after light scaling and 10 minutes afterwards. Initial cultures were positive for 30% of subjects tested but after 10 minutes only 5% of cultures were positive. The same researchers also found positive cultures in 48

cases in which instruments had extended beyond the apex during endodontic therapy. Immediately after manipulation 31.2% of the subjects had positive cultures but cultures taken 10 minutes later were all negative.

Supporting this evidence, Heimdahl *et al.* (1996) revealed the incidence of bacteraemia in samples taken during different surgical procedures were higher than that taken 10 minutes after the procedure. The authors also found that anaerobic organisms were more often observed after 10 minutes, possibly because of their greater magnitude in blood during surgery or more probable, because these particular microorganisms may grow preferentially in culture bottles. Ten minutes after treatment, the frequency as well as the magnitude of bacteraemia showed a pronounced reduction. Elimination of microorganisms from blood was rapid and after 10 minutes the viridans group of *Streptococci* were eliminated in 42 of 46 patients, which is consistent with results from similar studies by Sweet *et al.* (1978).

However, since modern culturing methods have increased the sensitivity of overall detection of organisms, it is possible the duration of the bacteraemia may be longer than previously reported. More recently, Messini *et al.* (1999) performed a bacteraemia study among handicapped patients who do not perform oral hygiene because of the severe grade of their illness and are suspected to be at higher risk for bacteraemia under dental therapy. Indeed, during dental treatment, bacteraemia could be shown for at least 30 minutes in contrast to the shorter intervals indicated by other researchers (Bender *et al* 1963, Berger *et al* 1974).

### ***1.10.3 : Timed dose response***

Roberts *et al.* (1992) investigated the optimum sampling time for detection of dental bacteraemia in children following the extraction of a single tooth. 458 blood cultures were taken in total. Each 6ml sample was taken starting at one of the following times: 10s, 30s, 60s, 90s, 120s, 180s, 600s over 2.5 mins. The samples were cultured in the Bactec culture system and when positive, the bacteria isolated were speciated. From these, 37% of children gave positive cultures. They found at 30s, 56% of the samples were positive, which reduced to only 38% of positive samples at 90s and 28% at 600s. This indicates bacterial levels reduce in a time dependent manner. Over 50% of the organisms found were viridans *Streptococci* which account for approximately 40% of the cases of infective endocarditis (Everett *et al* 1977).

The same authors claimed previously that the optimum sampling time in children was less than 2 minutes after dental treatment although only a small number of samples were examined. Roberts *et al.* (1999) concluded that in studies designed to test the efficacy of systemic and local prophylaxis, blood should be taken within the first 2 min, preferably starting at 30s.

### ***Comment***

Various authors have commented on the duration of procedure induced bacteraemias. It is generally agreed that older studies may have underestimated the length of bacteraemias due to the previous use of less sensitive detection techniques, and the actual length of a bacteraemia can be almost 60 minutes.

It is obvious however, that a timed dose response exists, dependent on the initial dose of the bacterial inoculum and the efficiency of the hosts immune system. The optimum time

for sampling and thus detection is best performed within the first 2 minutes, preferably as soon as possible following the beginning of a dental manipulative procedure.

### ***1.11 : Procedures causing bacteraemia***

The close anatomic relationship of the oral microflora to the bloodstream can facilitate bacteraemia and systemic spread of bacterial by-products and immunocomplexes. A variety of clinical procedures including tooth extraction, periodontal and endodontic treatment may cause translocation of microorganisms from the oral cavity to the bloodstream.

#### ***1.11.1 : Specific periodontal procedures***

##### ***Periodontal probing***

Daly *et al.* (1997) investigated bacteraemia caused by periodontal probing in 30 patients with untreated periodontitis. A positive bacteraemia was recorded for three of the patients prior to probing. Following probing, 13 patients (43%) exhibited bacteraemia of oral origin. Their results thus indicated that periodontal probing can cause bacteraemia in patients with periodontitis. No association was found between the severity of periodontitis as determined by the deepest pocket found for each patient and the occurrence of bacteraemia following probing. Thus the severity of periodontitis did not appear to predict which patients might experience bacteraemia following periodontal probing.

The spread of plaque organisms into the bloodstream during periodontal probing is most likely due to the periodontal probe tip penetrating the epithelial lining of the pocket into the underlying vascular connective tissue. Listgarten *et al.* (1976) demonstrated that the probe tip penetrates the epithelial lining when inflammation is present, and bleeding on

probing is an indication of an inflammatory lesion in the connective tissue. Thus, it might be expected that the chance of bacteraemia would increase with an increasing number of pockets which bled on probing.

Gram-negative bacteria have been reported to account for 60% of plaque organisms in periodontitis (Rawlinson *et al.* 1993). However, Daly *et al.* (1997) found only 15% of their post-periodontal probing blood samples contained Gram-negative organisms, which is similar to the incidence of Gram-negative bacteria found after subgingival irrigation but substantially less than the 38.5% incidence reported after root planing of single periodontal pockets (Waki *et al.* 1990). These findings could be indicative of a barrier to Gram-negative bacterial entry to the bloodstream. As probing gave similar Gram-negative bacterial incidence in blood to that of subgingival irrigation but significantly less than that produced with root planing of single periodontal pockets, it may be hypothesised that root planing is more traumatic to the tissues than probing or subgingival irrigation and perhaps Gram-negative bacteria enter the bloodstream when there is greater trauma. However, this may more likely reflect difficulties in culturing these organisms in Daly's study. This is supported by the same authors who found that 80% of the bacteria found in the positive blood samples were aerobic/facultative microorganisms thought to be associated with supragingival plaque. Nonetheless, this finding suggests that periodontal probing could be of concern for all patients who are at risk of infective endocarditis irrespective of their periodontal status.

### **1.11.2 : Mobility**

In a full periodontal charting, values of mobility are one indication of the severity of periodontal disease. It is of interest that Roberts *et al.* (1999), found certain conservative dentistry procedures to cause bacteraemias significantly more often than the baseline value. These were, in addition to polishing teeth (24.5%), and intraligamental injection (96.6%); rubber dam placement (29.4%), and matrix band with wedge placement (32.1%). Rubber dam placement and matrix band with wedge placement both involve forcing the teeth apart and cause local pressure changes that may push plaque onto damaged gingival tissue. It is therefore not surprising that this combination of movements carries a high risk of bacteraemia. It is likely that teeth with chronic periodontal disease, which exhibit some degree of mobility, will experience this type of stimulus and would perhaps, for example, cause a bacteraemia on chewing.

In studying the effect of rocking of teeth in sockets prior to extraction, Elliott (1977) found the incidence of blood cultures to be greater in the presence of “gingival disease” in 86% of the cases, while those subjects with marked periodontal disease showed bacteraemia, with viridans *Streptococci* isolated in nearly all cases.

Contradictory to these studies, Vargus *et al.* (1959) attempted to determine factors influencing the occurrence of a bacteraemia. They revealed that tooth mobility in addition to depth of gingival pocket depth were not important.

### **1.11.3 : Toothbrushing**

The oral cavity is a reservoir for microorganisms, thus by reducing their number through optimal care certain patient groups, such as those who are immunocompromised, may decrease their chance of a life-threatening systemic infection from an oral source. Many bone marrow transplant teams believe that toothbrushing increases the risk of bacteraemia



and bleeding, and advocate the discontinuation of oral hygiene with a toothbrush particularly during neutropenic periods. However, problems are more likely to arise when immunocompromised patients are not compliant with good oral hygiene habits since toothbrushing is the most effective means to remove plaque and to reduce gingival inflammation (Fonseca, 1998).

Various studies in the literature have documented the incidence of bacteraemia following toothbrushing. Rise *et al.* (1969), found that a bacteraemia could be produced by simply brushing the teeth. A group of 50 dental students were used in the experiment and 26% showed a positive bacteraemia in what were considered “clean mouths”. Other studies have shown similar values of bacteraemia incidence.

Cobe *et al.* (1954) looked at 305 patients from which 74 (24%) produced a positive culture, in a smaller study Berger *et al.* (1974) investigated 30 patients, 8(27%) of which produced positive bacteria growth. Rosmans and App (1971) looked at 30 patients from which 2 (7%) yielded a positive culture, a lower value than indicated previously. Indeed, Felix *et al.* (1971) investigated 30 patients, 15 (50%) provided positive cultures, a higher value than that formerly quoted. These differing values may have been due to alternative culture techniques and detection systems employed. Accumulating these values from a total of 445 patients, 112 (25%) produced a positive culture following toothbrushing. Alternatively, Speck *et al.* (1971) showed that minor dental trauma in the form of a standardised oral prophylaxis regimen was not associated with a detectable bacteraemia in a healthy population of children less than 10 years of age. Highlighting the importance of the timing of blood sampling, in a more recent study by Roberts *et al.* (1997), blood samples were obtained 30s after each of 13 dental operative procedures in 735 children. Toothbrushing alone caused a bacteraemia on 38.5% of occasions, a high percentage of

positive bacteraemia samples following a seemingly innocuous dental procedure carried out on a daily basis.

#### ***1.11.4 : Scaling***

It has been known for some time that routine dental cleanings which may disrupt the gingival crevicular epithelium around all teeth should also be considered a frequent source of bacteraemias (Rise *et al* 1969).

In 1949 Lazansky *et al.* produced a detectable bacteraemia by scaling the lower mandibular incisors for 10 minutes. In addition, Peterson (1976) recovered bacteria in patients treated routinely with scaling and root planing. Korn and Schaffer (1962) summarised data from 100 blood samples taken from 43 patients to evaluate post-operative bacteraemia produced by periodontal therapy. They determined routine root planing produced a bacteraemia incidence of 83.8%. Rogosa *et al.* (1960) reported a bacteraemia frequency of 88% following similar periodontal procedures on 33 patients. They believed that more refined culturing methods with larger blood samples were responsible for the high incidence of bacteraemias in their study.

Waki *et al.* (1990) investigated the effect of subgingival irrigation on bacteraemia following scaling and root planing in 54 periodontal maintenance patients. Following a baseline sample of which 2 were positive (3.7%), a second blood sample was taken 2 minutes following the initiation of scaling and root planing which itself took approximately 30-90 seconds. An overall incidence of bacteraemia following this procedure in periodontal maintenance patients was 10/54 (18.5%). Lucartorto *et al.* (1992) investigated post-scaling bacteraemia in HIV-associated gingivitis and periodontitis: a group of patients who may be at a particular risk from bacteraemia due to immune suppression. Blood was taken from subjects 15 and 30 min after the initiation of routine

dental scaling and root planing. 7 of 22 HIV-gingivitis patients were positive for colony forming units (cfu.) at 15 minutes but not at 30, with 7 of 22 HIV-periodontitis patients having positive cfu. Baseline samples, however were not taken therefore it could be disputed that isolates were present in the bloodstream prior to manipulation. Contaminants were eliminated by species identification i.e. 1 of the HIV gingivitis samples was discounted as it contained *S. epidermidis*.

Previous studies investigating the incidence of bacteraemia following subgingival scaling with either hand or ultrasonic instruments have reported an incidence of up to 88% (Rogosa *et al* 1960). However, very few papers report on bacteraemia following ultrasonic scaling. When the incidence of bacteraemia following ultrasonic and hand scaling was compared in 24 patients using a split mouth design, it was reported that blood cultures were positive in 79.2% of the patients who received ultrasonic scaling and 66.6% of the patients who underwent hand scaling. Therefore it would seem ultrasonic scaling produces a higher incidence of bacteraemia compared with hand scaling.

#### ***1.11.5 : Other oral manipulations***

##### ***Dental extractions***

A great deal of the historical bacteraemia literature involves oral surgical procedures namely extractions, most likely due to the high degree of trauma generated and the assumed resultant high bacteraemia values. It follows that there would be an increased infective endocarditis risk in at risk patients for these types of procedures. Indeed, it has been previously speculated that the incidence of bacteraemia after tooth extraction would perhaps be more predictable in patients who underwent multiple extractions (Bender *et al* 1963, Robinson *et al* 1950) or who had associated periodontal disease (Daly *et al* 1997). Published values of bacteraemia vary widely, and it should be noted there are different

detection techniques employed in each study and other variables which influence these figures:

<i>Research group</i>	<i>no. of subjects positive for bacteraemia and % of positive cultures</i>	
Okell & Elliott (1935)	84/138	(61%)
Burket and Burn (1937)	11/92	(12%)
Northrup & Crowley (1943)	12/96	(13%)
Taran (1944)	197/400	(49%)
Mc Entegart & Porterfield (1949)	108/200	(54%)
Rhoads <i>et al.</i> (1950)	26/68	(38%)
Robinson <i>et al.</i> (1950)	106/404	(26%)
Merril <i>et al.</i> (1951)	22/65	(34%)
Cobe (1954)	96/274	(35%)
Keosian <i>et al.</i> (1956)	27/100	(27%)
Cooley and Haberman (1957)	20/51	(39%)
Bender <i>et al.</i> (1956)	79/93	(85%)
Rogosa <i>et al.</i> (1960)	28/34	(82%)
Khairat (1966)	64/100	(64%)
Elliot and Dunbar (1968)	42/117	(36%)
Hurwitz <i>et al.</i> (1971)	0/32	(0%)
Berry <i>et al.</i> (1973)	15/23	(65%)
Peterson and Peacock (1976)	39/80	(49%)
Speck <i>et al.</i> (1976)	11/36	(31%)

**Table 1:** *table showing research groups, no. and percentage of subjects with positive bacteraemia samples following dental extraction.*

Interestingly, accumulating these values, following dental extractions from a total of 2403 patients, 937 provided a positive blood culture, a rate of 40% which was not significantly different from the 38% of positive cultures found after chewing.

Okabe *et al.* (1995) investigated the factors affecting the occurrence of bacteraemia associated with tooth extraction in 183 patients. Blood samples were taken within 2 minutes of the extraction procedure with bacteraemia found in 72.1% of patients who had one or more teeth extracted for various reasons. More recently, the findings by Lockhart *et al.* (1996) and Hall *et al.* (1993) of a 94% incidence of bacteraemia among a group of

patients suggests that dental extractions produce bacteraemias in nearly all cases. Lockhart attributed these findings, in addition to the 24% incidence of cultures yielding polymicrobial organisms and the 14% incidence of strict anaerobes, to improved culturing techniques and the earlier timing of the blood drawings than in other studies. These variables have been further discussed in an earlier section.

#### ***1.11.6 : Minor procedures***

Less invasive procedures may still produce a bacteraemia. Richards (1932) recovered viridans *Streptococci* from the bloodstream of patients whose gingival tissue had simply been massaged for 10 minutes. McLaughlin *et al.* (1996) investigated the incidence of bacteraemia following orthodontic banding using the Bactec culture bottle system. From 30 patients, three cases of bacteraemia were detected post operatively. Murray and Moosnick (1941) found that the chewing of paraffin for 30 minutes resulted in 55% incidence of bacteraemia in 336 patients who had varying degrees of oral pathology including caries and periodontal involvement. In addition, King *et al.* (1988) supported the suggestion that bacteraemia may occur spontaneously in patients with oral disease and following minor oral manipulations such as suture removal.

### ***1.12 : Rheumatic fever / infective endocarditis sequelae***

Rheumatic fever is a disease of disordered immunity characterised by inflammatory changes in the heart and the joints and in some cases, associated with neurological symptoms. Most patients have a definite history of a previous sore throat, classically a Lancefield group A  $\beta$ -haemolytic streptococcal infection. In rheumatic fever, there is a strong antibody response to streptococcus and this may cross react with antigens in the connective tissues, particularly in the joints and the heart. Heart changes include valvulitis, myocarditis, and pericarditis with valvular stenosis and/or incompetence are long term complications. In addition, rheumatic fever predisposes to infective endocarditis.

Infective endocarditis is an acute or chronic disease resulting from infection of a focal area of the endocardium. A heart valve is usually involved, but the process may affect the mural endocardium of the atrium or ventricle, or a congenital defect such as a patent ductus arteriosus or coarctation of the aorta (Underwood 1992).

In particular but not exclusively, elderly dental patients are at particular risk of developing infective endocarditis due to age-related impairment of the immune system and degenerative cardiac valvular defects from previous rheumatic fever. In addition, increased longevity is also associated with a greater incidence of periodontal disease (Kilmartin *et al* 1994). Degenerative and/or calcific lesions tend to partially obstruct the flow of blood between the chambers of the heart. As blood traverses these obstructions, velocity increases and turbulence is created. The turbulent flow of blood damages the endocardial endothelium. The exposed subendothelial connective tissue with its collagen fibres promotes platelet and fibrin deposition, thus forming a sterile thrombus. In the presence of a bacteraemia, the circulating microorganisms adhere to the thrombotic lesion, colonise and replicate. The bacteria stimulate aggregation of additional platelets and

become incorporated into the platelet-fibrin vegetation. This process provides the bacteria with protection from removal and destruction by phagocytes, as well as from the microbiocidal effects of antibiotics and host antibodies. This is also true among individuals who have prosthetic heart valves and are subsequently at a higher risk for endocarditis, which may be associated with substantial morbidity and mortality (Doerffel *et al* 1997). The formed vegetation, if untreated, can cause functional stenosis with further obstruction of blood flow, and can be a source of emboli that produce end organ damage (e.g. stroke), sepsis and seeding in other tissues and organs (Christensen *et al* 1993).

In addition to valve characteristics and cardiac haemodynamic factors, the pathogenesis of infective endocarditis is dependent on the number of circulating microbes, the microbial endocardial adherence characteristics, and the virulence of the organism (Barco *et al* 1991).

A high degree of interest has been focused on dental procedures because they provide the highest incidence of bacteraemia and are considered a frequent predisposing factor to infective endocarditis (Drangsholt *et al* 1998). However, the trauma associated with routine oral hygiene procedures at home can produce bacteraemia, with the frequency of exposure to daily related bacteraemia being higher than medical procedures (Guntheroth *et al* 1984). A report on failure of endocarditis prophylaxis concluded that 92% of reported cases of endocarditis followed manipulation (Barco 1991). This was limited mainly to extractions and periodontal procedures. It was suggested that a more complete medical history recording the inclusion of such procedures as periodontal probing, home care practices and the status of oral hygiene would have probably reflected a higher percentage of infective endocarditis associated with dental bacteraemia than was previously reported.

However, reviewing the literature, only a small percentage of all cases of endocarditis appear to arise from procedures classically thought of as 'bacteraemia-prone', and the administration of appropriate antibacterial prophylaxis is not always protective (Pallasch *et al* 1989, Hall *et al* 1993). Consequently alternative approaches to prevention must be investigated and also the real risk estimated (Seymour *et al* 2000).

### ***1.13 : Physiologic bacteraemia***

As stated previously, spontaneous bacteraemias associated with acute or chronic oral/odontogenic infections may represent a far greater cumulative risk for the development of endocarditis than do occasional health care procedures administered in a professional setting (Guntheroth *et al* 1984).

It is assumed that infective endocarditis results from the seeding of circulating bacteria onto damaged valves, and various dental and surgical procedures have been proved to induce bacteraemia in up to 80% of cases (Everett *et al* 1977). The relationship between dental procedures and infective endocarditis has been supported by anecdotal reports since 1935 (Okell *et al*) and experimental data (Wannamaker *et al*) which led to recommendations in 1965 for its prevention world-wide. However, a clear relationship between such procedures and the development of infective endocarditis has never been adequately demonstrated. In work by Van der Meer *et al.* (1992), the authors suggested that the majority of cases of infective endocarditis are the consequence of spontaneous bacteraemia.

Drangsholt *et al.* (1998) highlighted that there are over 1000 case reports or small case series that have been published between 1930 and 1996 reporting 1 to 9 consecutive cases of infective endocarditis occurring shortly after a dental procedure. Of these, there are at least 100 that describe a clinical scenario of weeks and months of either an untreated



periapical or periodontal infection, followed by the dental extraction of the affected tooth, and thereafter followed shortly by symptoms of endocarditis.

Further to this, Drangsholt claims most cases of bacteraemia and infective endocarditis of oral origin are not caused by dental procedures, but rather by poor oral hygiene leading to inflamed gingiva which are susceptible to bleeding. To determine the cause of cases of endocarditis, physicians often ask patients if they received any dental treatment in the past 6 months, and following a positive response the dental treatment is usually blamed for the endocarditis. But the period between bacterial invasion and the onset of symptoms of streptococcal endocarditis is relatively short - from a few days to 2 weeks in the vast majority of cases. Guntheroth and Bayliss *et al.* (1984) both stressed proper oral hygiene was a far more important preventive measure than chemoprophylaxis against endocarditis. Nissen *et al.* (1992) studied 132 cases of native valve infective endocarditis in a population of 930,000 in Denmark during a 10 year period and found that none had a dental source. The authors concluded that the reason for the lack of a dental source was probably the generally high level of dental hygiene in Denmark, and not the high compliance with antibiotic prophylaxis.

Concerning bacteraemia studies, pre-operative blood samples must be obtained in order to estimate the true post-operative bacteraemia. In several studies, low levels of bacteraemia could be detected pre-operatively in a few patients with especially poor oral hygiene (Daly *et al.* 1997) and although this could have been potentially due to skin contamination, physiological bacteraemia may be implicated.

Interestingly, Terezhalmay *et al.* (1997) attempted to determine the oral disease burden in patients undergoing mechanical or bioprosthetic heart valve implantation, by performing a comprehensive clinical and radiographical regional examination on 156 consecutive patients with emphasis on identifying acute and chronic oral/odontogenic infections and conditions. The mean number of remaining teeth in the cohort was 19.32; of these 1.07 were carious involving a mean number of 2.51 tooth surfaces. In addition, 15.38% of these patients had evidence of acute or chronic periapical abscesses and 43.6% of the patients had moderate to advanced periodontitis. Furthermore, Smith *et al.* (1993) reported a dental health awareness survey on 81 at-risk patients attending a cardiology outpatient clinic. 45% of the total number of patients examined were dentally unfit with a high prevalence of periodontal disease existing in the group, with four having periapical infections related to non-vital teeth. A vast number of patients in the dentally unfit group were thus shown to have periodontal disease which had not been recognised to be a risk factor by the cardiologists.

The evidence that several patients with viridans streptococcal endocarditis did not have dental extractions in the preceding months has been evident from case studies dating back at least to 1909. Okell and Elliott found that 11% of their patients with alveolar pyorrhoea had viridans *Streptococci* in their bloodstream before extractions; rocking a loosened tooth in these patients produced 86% positive cultures. Interestingly, the authors did not specify how long the tooth was manipulated for. More important in terms of cumulative exposure was the significant study by Murray and Moosnick (1940), who took blood cultures from patients with dental disease after they chewed paraffin for 30 minutes; 185 of 336 patients had positive cultures with Gram-positive *Streptococci*. Cobe *et al.* (1954) in a similar

study looked at 205 patients and 35 (17%) produced a positive culture. Diener *et al.* (1964) from 50 patients, 11 (22%) produced a positive culture following chewing.

A study by Guntheroth (1984) investigated the importance of dental procedures as a cause of infective endocarditis. When 18 paediatric patients with infective endocarditis were reviewed for failure of chemoprophylaxis, none had previous dental treatment. Surprisingly, published reports reveal a similarly low prevalence of dental extractions preceding infective endocarditis, only 3.6% for 1322 cases. Although extractions have been repeatedly shown to cause bacteraemia, (bacteraemia was associated with 40% of 2403 reported extractions), it was also found in 38% of patients after mastication and in 11% of patients with oral sepsis when no intervention was performed (Robinson *et al.* 1950).

The surprisingly low prevalence of prior dental extraction or procedures in 1322 patients with infective endocarditis is in contrast to inferences from anecdotal sources. Kelson and White (1945) asserted a 25% prevalence rate, whereas their documented prevalence was one-tenth of that. In more than 96% of 1322 patients, infective endocarditis developed without dental procedures.

### ***1.13.1 : Cumulative exposure***

Dentist induced bacteraemias are usually low grade in intensity and transient in duration with the blood becoming sterile in 15-30 minutes after cessation of the procedure (Everett *et al* 1977, Loesche *et al* 1977, Guntheroth *et al* 1984). Of considerable importance is the incidence and severity of so-called spontaneous bacteraemias. The incidence determined in various studies (Murray *et al* 1941, Everett 1977, Loesche 1977, Bender 1984, Durack 1984, Guntheroth 1984) is toothbrushing 0-26%, dental flossing 20-58%, chewing paraffin and normal mastication 17-51%. Defaecation has been a suspected cause of enterococcal endocarditis, and six cases of viridans endocarditis have been reported in association with mucosal damage in edentulous patients (von Reyn *et al* 1981). With sophisticated anaerobic techniques, it is possible to detect bacteraemias in 60-80% of persons at any given time (Everett *et al* 1977, Loesche *et al* 1977).

Guntheroth (1984) calculated the relative risk of bacteraemia from self-administered oral hygiene procedures versus bacteraemia due to invasive dental procedures. In a 1-month period for a patient with dental disease culminating in an extraction, the relative exposure from random bacteraemia, mastication, brushing teeth and tooth extraction can be calculated. The cumulative monthly exposure is 5,376 minutes, only 6 of which are attributable to the extraction itself. The odds that any given endocardial infection was seeded physiologically rather than from the extraction is nearly 1000 to 1. Even if infective endocarditis is diagnosed after an extraction, to attribute a causal relation is post hoc reasoning, unless a blood culture was sterile before the extraction.

A colony forming unit establishing on the endocardium and potentially leading to infective endocarditis is a random phenomenon and it is possible that the infrequent bacteraemia of 5.9 cfu/ml associated with extractions and occurring very infrequently is of much less impact than a slightly less intense inoculum of 3.6 cfu/ml occurring 8760 times per year

(Guntheroth, 1984). It is possible for a dental extraction to cause a bacteraemia that may lead to infective endocarditis but this is less likely than the baseline or background bacteraemia. Also it is relatively probable that this may be the explanation for so-called failures of prophylaxis. The size of inocula in “healthy” versus “inflamed” gingival tissues needs to be explored, since there is evidence that the prevalence of bacteraemia is greater in adults with discernible levels of gingival inflammation following dental flossing (Bader, 1998).

Roberts *et al.* (1999) disputed the role of dental extractions in the aetiology of infective endocarditis and questioned whether to abandon antibiotic prophylaxis intended to prevent the damaging effects of professionally induced odontogenic bacteraemia. “It is ineffective and potentially harmful and (*sic*) dental bacteraemia is of low probability as a cause of endocarditis.” They follow this by stating “On reflection it is difficult to understand how the medical and dental experts can so confidently advise the use of antibiotic prophylaxis when there is no evidence that dental surgical procedures *per se* are the cause of the bacteraemia that lead to endocarditis. It is much more likely that it is everyday bacteraemia with the compelling accumulation of asymptomatic bacteraemia that leads to the majority of cases of infective endocarditis.”

As common oral hygiene practices each morning produce low-level bacteraemias with high frequency, it is intriguing to note that episodes of myocardial ischaemia and infarction occur most frequently within an hour after awakening than throughout the remainder of the day. This epidemiological observation could be explained by the thrombogenic potential of dental bacteraemias. Thus dental bacteraemia may contribute, in addition, to the development of vascular inflammation and atherosclerosis and also acute thrombotic episodes.

### ***1.13.2 : Studies***

Guntheroth *et al.* (1984) first hypothesised cumulative exposure to bacteraemia by comparing estimates of bacteraemia from “background”, “chewing” and “brushing teeth” with bacteraemia from a “single extraction”. As stated earlier, it was estimated that over a period of a month, the cumulative exposure was 5370 minutes of “physiologic” bacteraemia compared to only 6 minutes of “surgical” bacteraemia (a multiplication factor of 895). The concept of cumulative bacteraemia is important in assessing the likely impact of dentogingival manipulative procedures other than dental extractions.

Roberts *et al.* (1999) reviewed cumulative exposure data for a variety of dentogingival manipulative procedures and showed that the everyday procedure baseline without sepsis for the low estimate was 5,640,585 greater than that of a deciduous molar extraction. Similarly, toothbrushing twice a day is 154,219 times greater than that of an extraction. From these data it is clear that there is a low risk of cumulative bacteraemia from dental surgical procedures compared to everyday procedures usually by a factor of many hundreds, thousands or even millions. Roberts *et al.* also state that there is a comparatively high risk of bacteraemia from simple professional cleaning procedures. Even a simple dental examination carries 48 times greater risk than a simple dental extraction, whereas simple polishing of teeth and scaling carry 390 and 31 times greater risk. Local anaesthetic procedures, even a simple infiltration, are a considerably greater risk than deciduous molar extractions, indeed the conventional intraligamental analgesia is among the list of banned procedures for cardiac patients. Procedures for conservative dentistry also carry a considerable risk for bacteraemia. The application of rubber dam carries the greatest risk at 2,110,341 times greater than that of a single extraction, slow and fast drills carry a risk of 24 and 1195 times that of an extraction. Multiple extractions

cause a significantly greater risk than a single extraction. Mucoperiosteal surgery which involves the cutting of an extensive length of the gingival crevice, causes even greater cumulative exposure than multiple extractions. Thus the procedures most often regarded as requiring antibiotic prophylaxis as indicated in the literature do not carry the greatest risk of cumulative bacteraemia.

### ***Comment***

Daily activities such as toothbrushing, flossing and chewing may produce transient bacteraemias similar to those produced by dental procedures. It is impossible to predict which will cause a bacteraemia and which will develop an endocarditis. One logical approach to endocarditis prophylaxis would be to decrease the number of bacteria in the oral cavity prior to dental manipulation, thereby reducing the number of organisms that may be introduced into the bloodstream.

### ***1.14 : Antibiotic prophylaxis***

As mentioned previously, although bacteraemia does not lead to any complication in healthy individuals, serious problems may occur in high risk patient groups. In individuals with congenital or acquired heart disease or those fitted with a valvular prosthesis, circulating bacteria may reach the defective endocardium and cause infective endocarditis. Such patients must be given antibiotic prophylaxis for certain dental procedures in order to prevent bacteraemia. For this purpose, various agents and methods of prophylaxis have been recommended but the results have not always been sufficient.

Minimising the occurrence of postoperative bacteraemia by administration of a prophylactic drug has been considered to be an important protective mechanism in the prevention of infective endocarditis. Prophylactic antibiotics are believed to act by interfering with three of the major stages in the pathogenesis of infective endocarditis: 1) bacteraemia, by reducing the number of microorganisms in blood; 2) adherence, by decreasing the affinity of microorganisms for heart valves; 3) multiplication of microorganisms on the heart valves, by interfering with the metabolic activity of the microorganism. This assumption is based on the results of several clinical studies that have shown a marked reduction in bacteraemia when prophylactic antimicrobial drugs have been administered.



### **1.14.1 : Penicillin V, amoxicillin**

Hall *et al.* (1993) investigated the incidence of bacteraemia in sixty healthy patients randomised to receive placebo, penicillin V or amoxicillin one hour before dental extraction was performed. Blood samples for microbiological investigation were collected before, during (i.e. during the period of maximum levels of bacteria in the blood when attachment of microorganisms to damaged heart valves is most likely to occur) and 10 minutes after surgery, and were processed by lysis filtration under anaerobic conditions. There was no statistical difference among patients in the placebo group, the penicillin V group and the amoxicillin group in terms of incidence or magnitude of bacteraemia due to viridans *Streptococci* or anaerobic bacteria during extraction or 10 minutes after the procedure. This finding is in contrast to those of earlier clinical studies that reported a decrease in frequency of bacteraemia when prophylactic penicillins were used. This could possibly be a false reduction due to a less sensitive detection technique.

The overall incidence rates of bacteraemia after dental extraction were 95%, 90% and 85% respectively for the three groups. For patients in the placebo group, a reduction in the incidence of bacteraemia from 90% during extraction to 80% 10 minutes after extraction was observed. A reduction in incidence from 90% to 70% was noted for the penicillin V group while that for the amoxicillin group was 85% to 60%.

*Streptococcus intermedius* was the most common species isolated and was also found to have the highest number of organisms per ml of blood in all three groups. Other frequently isolated viridans *Streptococci* were *Streptococcus mitior*, *Streptococcus mutans* and *Streptococcus sanguis*. The incidence of bacteraemia with viridans *Streptococci* was 70% in the placebo and penicillin V groups and 55% in the amoxicillin group. Aerobic

species other than viridans *Streptococci* were isolated in small numbers, (only a few strains of *Staphylococci* and *Corynebacteria* were recovered) with anaerobic strains isolated twice as frequently as aerobic strains. Species of *Actinomyces*, *Peptostreptococcus* and *Veillonella* were the most commonly recovered organisms from patients with anaerobic bacteraemia. The overall incidence of anaerobic bacteraemia after dental extraction was 85% in both the placebo and penicillin V groups. In the amoxicillin group, 75% of the patients had bacteraemia due to anaerobes.

It was hypothesised that the protective effect of prophylactically administered penicillins must be due to interference with crucial steps in the development of endocarditis (other than the transient bacteraemia that occurs initially). This study also confirmed that administration of penicillin in this manner does not affect microorganisms in the oral cavity in such a way that a change in incidence or magnitude of post extraction bacteraemia is observed. This finding is in agreement with those of earlier studies on the influence of penicillins on the oral microflora.

#### ***1.14.2 : Cephalosporin***

In a similar study, Hall *et al.* (1996) investigated the effects of prophylactic administration of 'cefactor' on bacteraemia after dental extraction. Cephalosporins have been shown to be effective in the treatment of upper respiratory tract infections, urinary tract infections and skin infections and have a broad range of activity against Gram-positive and Gram-negative bacteria. The use of cephalosporins has also been proposed for the treatment of streptococcal endocarditis in penicillin-allergic patients. 39 patients were randomly assigned to receive either 1g cefaclor (19 patients) or placebo (20 patients) 1 hour prior to dental extraction. Blood samples for microbiological investigation were collected before,

during and 10 min after surgery, and were processed by lysis filtration under anaerobic conditions. The incidence of bacteraemia with viridans *Streptococci* was 79% in the cefaclor group and 50% in the placebo group during extraction. No difference in the incidence or magnitude of bacteraemia was observed when the two patient groups were compared. Post-extraction bacteraemia was characterised by a clear dominance of Gram-positive strains in both patient groups. Strains of Gram-negative species were found in low numbers as were aerobic Gram-positive isolates such as *Corynebacterium*, *Micrococcus* and *Staphylococcus*. The incidence of positive cultures during dental extraction was 79% in the cefaclor group and 85% in the placebo group. Ten minutes after extraction, the incidence had decreased to 53% and 47%, respectively. The incidence of bacteraemia with viridans *Streptococci* during extraction was 79% in the cefaclor group and 50% in the placebo group. Ten minutes after extraction the incidence was 26% and 30%, respectively. Strains of *Streptococcus intermedius* were most frequently isolated, followed by strains of *Streptococcus sanguis* and *Streptococcus mitis*, in both patient groups.

The incidence of anaerobic bacteraemia during and 10 minutes after dental extraction was 74% and 47%, respectively, in the placebo group. *Actinomyces sp.* were the most commonly recovered strains while Gram-negative strains of *Veillonella* and *Prevotella* were only isolated from single patients. Statistically, cefaclor did not decrease the overall incidence or magnitude of bacteraemia, bacteraemia with viridans *Streptococci* or bacteraemia with anaerobic bacteria at any sampling time, in comparison to placebo.

These observations indicate the same lack of immediate bacterial killing in the blood as observed after prophylaxis with other cell wall-active antibiotics such as penicillin V and amoxicillin in the previous study by the same authors. The authors also highlighted the importance of a blood culture technique with high sensitivity (lysis filtration) for detection

of transient poly-microbial bacteraemia, and implied that low levels of residual antimicrobial substances in blood may otherwise result in false-negative cultures in the laboratory.

The authors could not prove the lack of reduction of bacteraemia after prophylactic administration of cefaclor was due to microbial resistance to cefaclor, since for all but 2 of the 120 stains isolated from the patients blood the minimum inhibitory concentrations were below the serum concentrations measured during the dental extractions. While bacteraemia is rapidly cleared by the reticuloendothelial system (as can be shown by the reduction in bacteraemia seen at 10 minutes in all groups), the period of exposure of microorganisms to the antibiotic in blood was presumably too short to make an immediate reduction of the bacteraemia possible.

Viridans *Streptococci* from the mouth are the single most frequent isolates accounting for almost half the cases of infective endocarditis. The specific action of viridans *Streptococci* on heart valves and the intima of the myocardial tissue is due to the organism's dextran production which is important for these bacteria to adhere to platelets. But strains of *Staphylococci*, not viridans *Streptococci* are most commonly cultured from joint infections (Little *et al* 1994). Since transient bacteraemia resulting after dental manipulation is primarily streptococcal in nature, penicillin, especially penicillin V, would be the best agent to provide protection against viridans *Streptococci*. A broad spectrum, anti-staphylococcal agent such as cephalosporin may be ineffective against a haematogenously seeded viridans streptococcal endocarditis.

### ***1.14.3 : Action of antibiotic prophylaxis***

Baltch *et al.* (1988) showed a 4.5% incidence of bacteraemias at 5 minutes following extraction with the use of prophylactic vancomycin. However, Hall *et al.* (1996) showed that systemic antibiotics do not reduce the incidence of bacteraemia, but suggested that they may affect the bacteria in other ways to reduce growth. It is likely the elimination of bacteria occurs in the systemic circulation, since systemic antibiotics do not reach high enough concentrations in the gingival crevice and the antibiotic may be destroyed by  $\beta$ -lactamase producing organisms located there.

A study by Hess *et al.* (1983) involved an examination of the incidence of post-extraction bacteraemia following prophylactic penicillin coverage in children with heart disease. Results revealed that 21% of children had post extraction bacteraemias and of the 32 isolates from these children, 24 organisms were sensitive to penicillin. None of the patients had infective endocarditis following the extractions. This supports the concept that antibiotics do not prevent bacteraemias and suggests that host mechanisms such as reticuloendothelial system, complement, platelets and neutrophils may be involved in the prevention of infective endocarditis through removal of particular organisms before the organisms can adhere to the endocardium. Alternatively, Hill-Smith and Schuman (1983) suggested that the purpose of endocarditis prophylaxis is to destroy organisms in the blood stream, prevent multiplication and destroy organisms adhering to the endocardium before the fibrin layer can protect the organisms from phagocytosis. In contrast, Hess *et al.* (1983) suggested it is far more important to have sufficient blood levels of antibiotics after the manipulations, which will eradicate microorganisms lodging on the endocardium than to try and prevent the development of transient bacteraemias.

#### ***1.14.4 : Problems with conventional antibiotic prophylaxis***

##### ***a) Compliance***

Wahl *et al.* (1994) discussed dental-induced endocarditis and highlighted the fact that despite the 1990 AHA recommendations for the prevention of infective endocarditis being simple, many physicians and dentists still fail to comply with them. Examples of non-compliance include overprescribing or underprescribing antibiotics, errors in dosage and timing, improper choice of antibiotics, prescribing antibiotics for low-risk patients or procedures and not prescribing antibiotics for at-risk patients or procedures. These AHA guidelines are not based on any controlled studies; there has never been a placebo-controlled human study on antibiotic prophylaxis for the prevention of infective endocarditis. The barriers to conducting a controlled study on antibiotic prophylaxis include the ethical problems of knowingly exposing control patients to the risks of endocarditis and the logistic problems in finding a large enough study population (estimated to be at least 6000 at-risk patients) for such a rare disease. Some authorities are calling for controlled studies, however, saying that it may be unethical to continue using antibiotic prophylaxis without more evidence of its benefits versus its risks and costs. The current guidelines not only reflect concern for the prevention of infective endocarditis but also for the prevention of problems with the antibiotics used.

Several studies support Wahl's findings and show patient compliance with AHA guidelines on antibiotic prophylaxis to be very low. The compliance rate among physicians has been shown to be even lower. A 1989 survey of practising dentists and physicians found an overall compliance rate of only 39% for responding dentists and 27% for responding physicians who specialised in cardiology, cardiovascular surgery, family practice and internal medicine, further dramatising the low compliance rate. In Britain there are many errors in dosing, timing and duration of prophylaxis as well as selection of

procedures, but compliance among dentists and physicians in Britain has been higher than in the US. Patients often receive incomplete information regarding their valvular abnormalities and their significance to dental treatment. This makes it difficult for the dentist to comply with the regulations without a physicians consultation.

#### ***1.14.5 : Problems with prophylaxis***

The American Heart Association previous 1984 recommendations for intramuscular or intravenous chemoprophylaxis were impractical, with the discomfort and inconvenience of antibiotic administration impeding good dental care. The committee still implies that gingival bleeding allows bacterial access to the bloodstream, whereas experimental studies establish the lymphatics as the only access. Although oral chemoprophylaxis for major dental procedures appears prudent, the British regimen of a single dose of amoxycillin administered orally is much simpler and probably more effective. However, scrupulous oral and dental hygiene is undoubtedly superior in preventing infective endocarditis than any chemoprophylaxis regimen.

The AHA reviewed the prevalence of previous dental extractions in patients with infective endocarditis. Out of a total of 1322 patients, only 47 patients (3.6%) had extractions within 2 months of the onset of symptoms. This suggests even if all extractions were adequately protected with chemoprophylaxis, only a small reduction in bacterial infective endocarditis would result. This is supported by the fact there has been no significant incidence reduction in Great Britain in the modern antibiotic era.

A further problem with prophylaxis is that in most patients with endocarditis the portal of the organism cannot be determined. Furthermore, recent studies have reported a shift in the bacterial spectrum of childhood endocarditis with a significant increase in uncommon organisms, raising issues regarding which antibiotic is most appropriate.

#### ***1.14.6 : Adherence to antibiotic prophylaxis regimens : periodontal considerations***

In a Scandinavian study (Gutschik, 1990) 108 dentists were selected with patients in their care who had undergone cardiac surgery and insertion of prosthetic heart valves. These dentists were questioned regarding antibiotic prophylaxis. Two-thirds were well aware that their patients had prosthetic heart valves and the vast majority of dentists believed that antibiotic prophylaxis is justified in connection with certain dental procedures. However, only 15 dentists prescribed prophylactic antibiotics before scaling. The dosage, route, frequency and duration of antibiotic administration used by more than half of the dentists was not in accordance with modern principles of antibiotic prophylaxis to prevent infective endocarditis. These researchers recommended the topical use of antiseptics e.g. chlorhexidine in connection with dental treatment. However the authors also highlighted that administration of chlorhexidine for several days before a dental procedure increases the risk for selection of *Streptococcus sanguis* which is the most frequent organism in streptococcal endocarditis.

Cetta *et al.* (1995) investigated adults with congenital heart disease and examined their knowledge of endocarditis prophylaxis. Of 100 patients, 60 knew the name of their heart disease. 50 patients correctly defined endocarditis but only 43 knew hygiene procedures could prevent endocarditis. 96 knew that they needed to take a medicine before dental procedures and 76 knew that an antibiotic was necessary. Nguyen *et al.* (1994) highlighted the very wide range of dosages and duration's of antibiotic prophylactic regimes from those of the current AHA use of amoxycillin. Some replies of orthopaedic surgeons questioned varied from a few hours before a procedure to a few days after completion of the procedure. Surgeons did not have a common explanation as to why they preferred their specific regimen. Some chose a type of antibiotic because they were familiar with it from their training. Each survey answer was different and was based



mostly on past experiences and/or personal instinct. None of the surgeons could specifically explain why they preferred their regimen. Opinions differed about the proportion of patients in whom endocarditis develops as a consequence of a medical or dental procedure.

As stated earlier, infective endocarditis antibiotic prophylaxis is not based on controlled clinical trials. This concern does not diminish the ethical problems of knowingly exposing control patients to the risks of endocarditis in addition to the logistics of evaluating a significant number of at-risk study patients. There are also growing concerns for the emergence of resistant clones.

Although antibiotics may significantly reduce the mortality of infective haemolytic streptococcal endocarditis, the increasing prevalence of previously less common microbial infections conflicts with the presumed reliability of antibiotic prophylaxis. This is important since as stated previously approximately 150 bacterial species have been encountered with apical periodontitis and 350 with marginal periodontitis. Anaerobic strains predominate and factors produced by polymicrobial infections interfere with intracellular phagocytosis and killing. This further complicates the pathogenesis of pulpal and periodontal infections. The polymicrobial environment of infective endocarditis is similarly complex, although their correlation with pulpal or periodontal infections remains largely conjectural.

Confusing the issue further, Van der Meer *et al.* (1992) remarked that the problem of non-compliance i.e. not using any antibiotic prophylaxis may not be a problem at all since an antibiotic may do more harm than good.

#### ***1.14.7 : Side effects of antibiotic prophylaxis***

All drugs carry certain risks with no exception, therefore prophylaxis needs to be balanced against the side effects of the antibiotic. The risks associated with antibiotics range from allergy to anaphylactic shock, blood dyscrasias, gastrointestinal upsets to pseudomembranous colitis, colonisation of resistant and/or fungal strains, cross-reactions with other drugs (e.g. birth control pills) nephrotoxicity and ototoxicity to death. These risks should be weighed against the benefits of the chemoprophylaxis. For example, penicillin prophylaxis for all patients with mitral valve prolapse (MVP) would cause more than three times as many deaths from anaphylaxis than no prophylaxis would cause cases of endocarditis. As a result, the AHA no longer recommend chemoprophylaxis for all patients with MVP but rather only for those with MVP with valvular regurgitation, i.e. those patients considered to be at high risk. Previously, the recommendation of antibiotics by Durack and Petersdorf (1995) for prophylaxis before dental procedures by the higher risk parenteral route, and the subsequent endorsement by a committee of the AHA, added a substantial hurdle in obtaining dental care for patients with heart disease. The inconvenience and discomfort of antibiotic administration intravenously was mentioned previously, in addition, relatively few practising dentists were able to provide parenteral treatment in their surgery. As the recommendations of the AHA for oral chemoprophylaxis are rarely observed, one can imagine the further potential for disaster with IV antibiotics. The AHA now also recommends oral regimens as the standard route of administration for dental procedures, even for high risk patients, due to the higher allergenicity of parenteral regimens.

The present recommendation in Great Britain is to simplify the prophylaxis for dental procedures to a single dose of 3 grams amoxycillin for an adult, administered ½ to 1 hour before the procedure. One half of that dose is recommended for children younger than 10

years, although weight-related dosages would be more appropriate. Oakley and Somerville (1981) argued that blood levels are substantially better with amoxycillin than with penicillin and the therapeutic blood level will persist for 8 to 10 hours, much longer than the 30 minutes of documented bacteraemia in patients after extractions. Indeed, as serum levels of amoxycillin are still bactericidal for most oral *Streptococci* 10 hours after the first dose, this makes the previous recommendation of a second dose 6 hours after the first dose even more unnecessary.

The occurrence of a random, spontaneous bacteraemia of oral origin is an important consideration in infective endocarditis and emphasises the need for patients at risk of infective endocarditis to ensure excellent dental health. If a medicated low risk patient dies from anaphylaxis or becomes pregnant as a result of taking the antibiotic, a lawyer will probably use these reports to show the risk in medicating was unacceptably high. On the other hand, if a non-medicated low risk patient contracts endocarditis, the clinician can cite these reports as reasons not to medicate.

Another argument used to minimise the potential benefits of dental prophylaxis emphasises the negligible impact it would have on the overall frequency of infective endocarditis. Even if prophylaxis were 100% effective, it would reduce the frequency of BE by <10% since so few cases of infective endocarditis (IE) are potentially attributed to dental procedures. Thus some physicians have come to believe that it is not worth giving prophylactic antibiotics to patients at risk before dental procedures. However, as Simmons *et al.* (1992) discuss, even if such a reduction in frequency were to occur it would be almost impossible to detect. This does not mean however, that prophylactic antibiotics are not worth giving.

From the standpoint of health care costs, administration of antibiotics and employment of other measures to prevent IE are expensive because the indications for dental prophylaxis

are common, but the risk of developing IE is low. These findings have led some investigators to suggest that administration of prophylactic antibiotics with the aim of preventing IE is not cost-effective, but the cost to the individual patient is not excessive. In view of the impossibility of conducting a prospective clinical trial of administration of prophylaxis for IE, evaluation of prophylaxis has been directed at clinical phenomena that might be predictive of a satisfactory prophylactic effect.

#### ***1.14.8 : Which procedures require prophylaxis***

On the basis of the work of several groups, one might conclude that antibiotic prophylaxis for dental extractions is unlikely to reduce the prevalence of infective endocarditis and that the practice should be abandoned; conversely, the conclusion could be made that prophylaxis should be extended to cover all dental procedures. In which case should prophylaxis be extended to cover the physiological bacteraemia induced by patients toothbrushing? It is obvious, especially with regard to the bacterial ecology in patients receiving penicillins, that this is inadvisable given the complete change from normal observed in patients who are prescribed long-acting antibiotic therapy, which produces a similar flora to that of immunocompromised patients (Drucker *et al* 1976).

Freidlander *et al.* (1994) gave antibiotic prophylaxis in elderly patients for a wide variety of procedures including intraligamentary injections, oral prophylaxis, oral surgery, periodontal probing, scaling and surgery, conventional and surgical endodontics (including rubber dam and clamp placement) dental impressions, subgingival preparation of teeth for reception of crown restorations, placement of retraction cord matrix bands and wedges. This list of procedures is more extensive than that advocated by other clinicians (Bender and Barkan 1989; Giglio *et al.* 1992; Hay 1993; Stankewitz *et al.* 1980; Tan and Gill 1992). However, Freidlander *et al.* believed it is appropriate in older individuals with

degenerative changes in native heart valves, possible age-related impairment in host resistance and with periodontal disease because of less than meticulous oral hygiene. Though unproven, a combination of these age-related factors may increase the relative risk for infective endocarditis.

#### **1.14.9 : Endodontic therapy**

Most forms of endodontic therapy are associated with little or no bacteraemia although there has been recent evidence to the contrary. Lavelle *et al.* (1996) investigated whether antibiotic prophylaxis was required for endodontic treatment and Debelian *et al.* (1995) investigated anaerobic bacteraemia in patients undergoing endodontic therapy. In Debelian's study all subjects had apical periodontitis. In the first group of 13 patients who had their root canal instrumented deliberately 2mm beyond the apex, 54% yielded cultivable microorganisms from the bloodstream, and of these, 6 yielded anaerobes. In the treatment of the 13 remaining patients whose instrumentation ended 1mm short of the apex, 31% had bacteria that could be cultured from the bloodstream, 2 of which were anaerobes.

Where the AHA guidelines recommend antibiotic prophylaxis prior to rubber dam clamp placement or vital pulp extirpation if associated with bleeding, the BSAC recommendations are more generic. Neither the AHA or BSAC provide specific guidelines for antibiotic prophylaxis in patients with prior antibiotic therapies for periapical or other infections. The primary association between endodontic procedures and endocarditis depends on the oral flora and inflammatory response in the adjacent tissues (e.g. apical and marginal periodontium), but ignores spontaneous bacteraemias and those associated with chewing and oral hygiene. Yet these would jeopardise the efficacy of antibiotic prophylaxis if the bacteraemias proved a greater cumulative risk than the

occasional endodontic procedure. Hence where conventional antibiotic prophylaxis recommendations are vague, chlorhexidine mouthwash as a measure to reduce bacteraemia would be useful if proven to be effective.

#### ***1.14.10 : Failure of prophylaxis / antibiotic resistance***

There have been numerous cases of failure of AHA antibiotic regimens, including parenteral regimens to protect against endocarditis, both for antibiotic-sensitive and antibiotic-resistant organisms. In a 1988 study of parenteral penicillin prior to oral surgery or periodontal prophylaxis, Baltch *et al.* found that 14.7-16.1% of patients still had a bacteraemia at 5 min and 3.1-9% at 30 min after the procedure.

Indeed, some cases of antibiotic-resistant endocarditis may have been indirectly caused by antibiotic prophylaxis, which encouraged the colonisation of resistant strains of bacteria. One study showed that 85% of patients harboured some penicillin-resistant microorganisms and 21% harboured some erythromycin-resistant microorganisms as part of their normal oral flora. Two, three dose erythromycin regimens one week apart can cause resistant streptococci for as long as 43 weeks. Four single dose amoxycillin regimens one week apart can cause significantly increased streptococcal resistance. Durack *et al* (1995) reported that 92% of failed prophylactic antibiotic regimes were associated with dental procedures in susceptible patients. 75% of these failures were specifically related to orally derived *Streptococci*. Roberts *et al.* (1998) found their bacterial isolates were susceptible to most of the antibiotics recommended for antibiotic prophylaxis but erythromycin, gentamycin, penicillin G, and teicoplanin were only 80% or less effective in their efficacy while chlorhexidine, amoxycillin, clindamycin and vancomycin were between 92 and 100% effective. To minimise the potential of resistant

strains, the AHA recommends completing as much dental treatment as possible at one sitting and spacing appointments at least one week apart.

### ***1.15 : Chemoprophylaxis : methods of protection***

As indicated previously, even with adequate chemoprophylaxis the reduction in infective endocarditis would be small. It is interesting to consider what reduction might be expected from maintenance of excellent oral and dental health. Judging from bacteriologic studies after extractions with and without oral sepsis, the presence of infection appears to at least double the risk for IE. The reduction is the same order of magnitude of the reduction in prevalence of bacteraemia after extractions when chemoprophylaxis is used.

Thus the most effective methods of preventing infective endocarditis are good oral and dental hygiene and aggressive dental management. Cardiologists, primary care physicians and dentists should emphasise the importance of scrupulous care of the teeth and gums.

Historical studies by Theyer (1926) suggested that poor oral hygiene was more important in the aetiology of infective endocarditis than invasive surgical procedures, and the real risk for endocarditis comes from poor hygiene and chronic bacteraemias during normal activities (e.g. chewing food) rather than dental procedures. This is an important concept relevant today when you consider the data collated by Drangsholt *et al* (1998) who investigated 450 case series of endocarditis cases located from the years 1930 to 1996. Precipitants of the disease were recorded and mean percentage of cases exposed were: dental procedures 7.5%, dental infections and disease 7.6%, medical procedures 14.5%, non-oral infections and diseases, 15.5% and intravenous drug abuse 4.5%. In the majority of cases (52.5%) the precipitating cause could not be determined.

#### ***1.15.1 : Infective endocarditis : a sequelae of dental manipulation?***

As stated previously, it is evident that most patients do not acquire infective endocarditis as a direct consequence of an invasive oral, surgical or medical procedure. Indeed, it



would be interesting to determine the normal incidence of infective endocarditis without dental intervention. A direct relationship between an oral procedure and the development of infective endocarditis has been reported in 3.6%-14% of all cases. This finding indicates that antibiotic prophylaxis would protect only a minority of all susceptible patients from developing the disease. A recent report by Van der Meer *et al.* (1992) claimed an overall protective efficacy of 6% among patients with native-valve endocarditis and challenged the cost-benefit of antibiotic prophylaxis. However, it is generally agreed that patients who are susceptible to infective endocarditis should receive antimicrobial prophylaxis prior to undergoing invasive procedures in the oral cavity.

As there have been no controlled clinical trials of the efficacy of prophylactic antibiotic regimens in preventing the disease in susceptible patients, the present recommendations are based on indirect data from clinical experience and clinical studies of bacteraemia, studies with experimental animal models, pharmacokinetic studies and *in vitro* studies.

#### ***1.15.2 : Animal studies***

In the 1970s experimental studies suggested that high doses of bactericidal antibiotics could prevent endocarditis whereas bacteriostatic agents could not. Several studies have subsequently revealed that the situation is more complex. During the 1980s, Glauser *et al.* (1987) presented a number of reports on antimicrobial prophylaxis for endocarditis in the experimental rat model; these studies showed that prophylaxis can be achieved in the absence of bacterial killing in the blood. Recent observations with experimental animals have also challenged the role of reduction of bacterial adherence as an important prophylactic mechanism. Inhibition of bacterial growth after attachment of the organisms to the heart valves may be a more important aim for successful antimicrobial prophylaxis for endocarditis. Animal studies demonstrated the efficacy of prophylaxis but showed that

it had no effect on the number of circulating bacteria. These data led the authors to state that “the determination of the magnitude of bacteraemia after certain procedures alone might not provide reliable information on the risk of subsequent development of infective endocarditis”.

### ***1.15.3 : Problems with using bacteraemia studies***

Most studies of human bacteraemia measure viable bacteria available in venous blood at some time after possible infusion without knowledge of the actual inoculated dose. The percentage recovery of plaque bacteria from venous blood is unknown. Furthermore, the human innoculum would contain fragments of the complex dental plaque biofilm. Many investigators have attempted to validate the use of prophylactic antibiotics by demonstrating a diminution in the occurrence of post-extraction bacteraemia. As stated previously, the low incidence of bacteraemia after antibiotic prophylaxis reported in early studies, which was not found in more recent studies such as that by Hall *et al.* (1993), might be due to low sensitivity of the blood-culture methods used. Remnants of antimicrobial agents in the blood sample may also inhibit bacterial growth in the laboratory. This phenomenon was observed by Malinverni *et al.* (1984) in an experimental endocarditis model in rats given antimicrobial prophylaxis. These investigators used a lysis-centrifugation process that resulted in a significantly higher recovery of microorganisms from blood (due to concentration of the microorganisms and thus dilution of inhibitory factors) compared with conventional blood-culture systems. Thus previous studies may have overestimated the reduction in the intensity of bacteraemias (the number of organisms in the blood) because traces of antimicrobial agents remained in the blood sample. The paper by Hall *et al.* measured the efficacy of antibiotic prophylaxis in relation to post-extraction bacteraemia. When the authors used a

lysis-filtration technique previously shown to increase the yield of bacteraemia in studies of prophylaxis in animals, they were unable to demonstrate a prophylactic effect of antibiotics during or 10 minutes following dental extraction in the case of bacteraemia due to viridans *Streptococci*. Since this is a short time, it may not have been sufficient to demonstrate an antibiotic killing effect. However, post-extraction bacteraemia generally lasts no longer than 15 minutes after a procedure, which allows ample time for viable bacteria to colonise a vegetation despite the use of prophylactic antibiotics. Thus if a protective effect of prophylactic administration of penicillin exists, it is probably mediated by interference with microorganisms at a later stage in the development of infective endocarditis rather than during the initial period of transient bacteraemia.

Bender *et al.* (1956) first highlighted the fact that in all previous attempts to prevent post-extraction bacteraemia, antibacterial agents were introduced parenterally as a means of attacking the organisms after they had entered the bloodstream. No attempt was made to eliminate the organisms at their source in the gingival crevice by local antimicrobial treatment before intravascular entry. Bender also suggested the most effective treatment in the prevention of post-extraction bacteraemia was a combination of systemic and local therapy which offers a higher degree of protection against dental bacteraemia by reducing not only the size of the bacterial inoculum entering the bloodstream, but also the number of different species of microorganisms, some of which are antibiotic resistant.

#### ***1.15.4 : Local measures***

##### ***Significance of reducing size of inoculum***

Local disinfection has the added benefit of reducing the size of the bacterial inoculum. Experimental rabbit models have shown that although erythromycin uniformly failed to protect the animal from infective endocarditis, greater prophylactic efficacy occurred when a lower streptococcal inoculum was introduced (Garrison *et al* 1970). When fewer bacteria gain entry into the bloodstream, both the normal defence mechanisms and the antibiotics clear the bacteria more rapidly, reducing the likelihood of colonising the diseased heart tissue.

##### ***1.15.5 : Irrigation of the gingival crevice***

Numerous studies show that disinfecting the mouth or gingival crevice causes a statistically significant reduction in the incidence of bacteraemia, the condition that initiates infective endocarditis. Although sterilisation of the gingival sulcus is impossible, numerous methods have been used to reduce the number of microorganisms in and around this area. Anti-microbial solutions used as oral rinses or locally applied into the gingival sulcus are effective in reducing the number of bacteria that enter the circulation.

In one study, a solution of iodine-glycerol prevented bacterial growth completely in samples from the gingival sulcus (Scopp *et al.* 1971). The local application of 0.5% chlorhexidine solution in the region of the gingival sulcus has also been investigated and in one study was more effective than the other anti-microbials in reducing bacteraemia (Fine *et al.* 1996). As anti-microbial agents applied to the sulcus directly are superior to oral rinses, it has been suggested that the application of iodine or chlorhexidine within the gingival sulcus is an excellent substitute for antibiotics applied in the same manner

(Greenstein *et al.* 1986). These particular anti-septics are even more effective because there are no resistant species as those which can arise with antibiotics.

The local anti-microbial agents kill Gram-negative, Gram-positive, aerobic and anaerobic microbes, in addition to antibiotic resistant strains. However, topical antiseptic treatment is not intended to replace prophylaxis by systemic antibiotics nor to obviate the need for a high standard of oral hygiene in patients at risk from bacteraemia-producing procedures.

Although the preventive effect of local irrigation has been demonstrated in previous studies, the concept is still somewhat controversial. There are reports which suggest that irrigation of the gingival sulcus of teeth with antiseptic solutions before extraction, could decrease the frequency of bacteraemia by half (Fine *et al.* 1996). On the other hand, antiseptic solutions have also been found to be ineffective for prevention of bacteraemia, and indeed sulcus irrigation has been claimed to induce bacteraemia itself (Rahn, 1993).

Macfarlane *et al.* (1984) conducted a double blind study on 60 patients to assess the efficacy of irrigating the gingival crevice with iodine, chlorhexidine or sodium chloride on the incidence of bacteraemia following tooth extraction. After local anaesthetic, the gingival crevice was irrigated with 10ml of solution that was retained in the mouth for 2 minutes before rinsing. Blood samples were taken immediately prior to and 30 seconds following the extraction. 16/20 positive post-extraction blood cultures were found in the control group. In the chlorhexidine and povidone iodine groups post-extraction blood cultures were positive in 5 and 8 samples, respectively. A significant difference was found between control and chlorhexidine groups and control and povidone iodine groups. However, there was no significant difference between the chlorhexidine and povidone iodine groups. 46 bacterial isolates were identified among the 29 patients with positive

post-extraction blood cultures. Although 24 of the isolates were *Streptococci* no comment was made regarding the number of isolates obtained from each patient, or whether or not patients treated with chlorhexidine or povidone iodine had fewer isolates than those receiving placebo.

However, positive post-extraction blood samples were still present in 40% of the patients treated with povidone iodine and 25% of those treated with chlorhexidine, indicating that although irrigation can reduce bacteraemia, it is not completely eliminated and there is still the potential for inducing infective endocarditis.

Yamalik *et al.* (1992) investigated the effect of local irrigation of the gingival sulcus, with different antiseptic solutions on the frequency of bacteraemia after tooth extraction to compare the efficacies of the three antiseptics. The patients were grouped into a) the control group in which no irrigation was performed prior to extraction, b) the gingival crevice of the tooth irrigated with 10ml 1% povidone iodine, c) 10ml 3% hydrogen peroxide and d) 10ml 0.02% chlorhexidine. After local irrigation the patients were instructed to rinse the mouth with the given antiseptic solution for 2 min, following which the teeth were extracted. In the control group 14 of 20 (70%) post-extraction bacteraemias were positive. The most effective agent was found to be povidone iodine of which 7 of the culture samples were positive with a bacteraemia frequency of 35%. In the chlorhexidine group the frequency of bacteraemia was 40% and in the hydrogen peroxide group the bacteraemia frequency was 50%.

Rhan *et al.* (1995) investigated the prevention of post extraction bacteraemia comparing topical povidone iodine and chlorhexidine in 120 patients either to receive an interligamentary injection or an elective extraction of a molar. Bloods were taken 2, 4 and 6 minutes after the procedure was complete. Bacteraemia was detected in the blood

cultures of 52.5% of control subjects, 27.5% of povidone iodine patients and 45% of chlorhexidine patients. The bacteraemia-reducing effect of chlorhexidine was shown to be significantly lower than that of povidone iodine, supporting Yamalik's findings, which suggests that chlorhexidine has a lower antibacterial activity. They found a statistically significant difference between the incidence of viridans *Streptococci* bacteraemia in the povidone iodine group and the chlorhexidine group with respect to the control subjects, which is of note as viridans *Streptococci* are the main organisms historically implicated in infective endocarditis.

Rhan *et al.* also implied that the most effective method of antiseptic therapy to reduce bacteraemia following dental procedures is the irrigation of the gingival sulcus prior to treatment, a procedure which does not induce a bacteraemia.

#### **1.15.6 : Mouthwash**

As stated previously, the principal of prophylaxis for infective endocarditis is to minimise the number of bacteria invading the blood circulation. Since the source of microorganisms in bacteraemia that occurs after dental manipulation is supragingival and subgingival plaque, any technique that reduces the number of microorganisms in plaque may reduce bacteraemia.

Loe and Schiott (1970) claimed that rinsing twice daily with 10ml of 0.2% chlorhexidine solution for one minute resulted in 'complete plaque elimination'. This apparent eradication of plaque persisted during the 22 days of the monitored study period. A later paper by Loe *et al.* (1976) reported that reduced plaque levels were maintained for 2 years using daily 0.2% chlorhexidine mouthrinses. Using the same oral rinse, Flotra *et al.* (1972) monitored 50 subjects for 4 months. They reported a 66% reduction in plaque and 24% decrease in gingivitis. The authors noted that the test group which had subgingival

scaling demonstrated an 88% reduction of plaque and 43% decrease of gingivitis. These findings emphasised the benefit of subgingival plaque removal to reduce gingivitis.

A potentially effective method for controlling daily bacteraemia arising from home oral hygiene practices is the use of an oral rinse such as 0.2% chlorhexidine solution. As stated previously this has been shown to inhibit bacterial plaque formation, the precursor to gingival inflammation. Studies suggest that bacteraemia is more likely to occur with gingival inflammation. Plaque reduction with mouthwash can lower inflammation. Thus, the inflammatory conditions and the associated bacteraemia can be controlled by two daily rinses of 0.2% chlorhexidine solution without home oral hygiene procedures that would cause bleeding. Alternatively, other authors state that when used alone as a mouthwash chlorhexidine has been shown to be only partially effective in reducing the prevalence of dental bacteraemia.

Lockhart *et al.* (1996) considered bacteraemia following a single tooth extraction in adults and the antibacterial effect of rinses with chlorhexidine hydrochloride. These authors determined there was no statistically significant difference in either the incidence of blood cultures positive for organisms or in the nature of organisms identified between the chlorhexidine and placebo groups.

#### ***1.15.7 : Pre-procedural mouthwash***

In a controlled clinical study by Fine *et al.* (1996) the authors examined the effect of subgingival irrigation and rinsing with an antiseptic mouthrinse before ultrasonic scaling of a quadrant containing inflamed gingivae. The results showed that pre-procedural subgingival irrigation and rinsing can significantly reduce the level of bacteraemia



associated with ultrasonic scaling. These results support the AHA's recommendation of adjunctive subgingival irrigation prior to invasive procedures in patients at risk of developing infective endocarditis. This recommendation was empirical at the time and not derived from experiments that simulate clinical situations. Fine *et al.* (1996) found that, overall, the antiseptic mouthrinse resulted in aerobic counts that were 92.3% lower than those with the control mouthrinse and anaerobic counts that were 87.8% lower. These differences were statistically significant.

#### ***1.15.8 : Reduction of oral loading***

In addition to the use of conventional prophylaxis regimes Freidlander *et al.* also advocated the use of a chlorhexidine mouthwash for one minute, five minutes before initiating therapy. They claim that topical chlorhexidine reduces the bacterial inoculum and the likelihood of endocarditis.

Freidlander (1994) recommends for those having exodontia, chlorhexidine should be painted on the isolated and dried gingiva four minutes prior to extraction, and the gingival sulcus irrigated with the same solution using a blunt needle, just prior to initiating surgery. Effectiveness of these regimens are not absolute and endocarditis may occur despite apparently appropriate antibiotic prophylaxis.

Rise *et al.* (1969) performed bacteraemia inducing procedures on 50 patients at 2 sittings; the first with no mouthrinsing prior to the procedure, the second with 15cc of buffered sodium peroxyborate monohydrate administered prior. Blood samples were taken 15 minutes following the oral surgical procedures. 28 positive cultures were obtained from the 50 patients. Following the rinsing 14 were positive. Therefore the buffered sodium

peroxyborate monohydrate used prior to these procedures did reduce the transient bacteraemias induced.

Efficacy comparisons between antimicrobial substances are complicated by the fact that most drugs are offered in different concentrations and are used with different exposure times. Rahn *et al.* (1995) examined the antimicrobial effects of povidone iodine and hexitidine in comparison to sterile water. Starting with a concentration of bacteria of log 5 to log 6 cfu/ml, the povidone iodine solution produced a reduction of 2-3 log levels within 30 seconds. Efficacy was dose and exposure time related. The efficacy of hexitidine was obviously less; even a longer exposure time led to a reduction of only 1.5 log levels.

Povidone iodine has been shown to significantly reduce the number of bacteria around teeth although it is not clear whether this results in a reduction in the incidence, nature or duration of bacteraemias following invasive procedures. However, Witzemberger *et al.* (1982) showed no reduction in bacteraemias from gingival scaling following a one minute rinse and three minute sulcus irrigation with 10% povidone iodine.

### ***Summary***

Although antiseptics mentioned in the previous studies reduced the frequency of bacteraemia to various degrees, povidone iodine was the most effective, followed by chlorhexidine. However, povidone iodine is not used routinely due to problems of sensitisation. The most successful route of administering these antiseptics is pocket irrigation; mouthwash has a limited effect due to problems of penetration, despite it being the most compatible route for patient self administration.

### ***1.15.9 : Combination therapy of conventional antibiotic prophylaxis in conjunction with chlorhexidine***

Due to the limited spectrum of effectiveness of antibiotics, it has also been suggested to reduce the bacterial concentration in the surgical area by combining antibiotic application with antiseptics.

Human bacteraemia studies indicate that the lowest incidence and duration of bacteraemia occur when parenteral penicillin and streptomycin are administered one hour before the procedure with local disinfection just before manipulation (Bender *et al.* 1958). Local disinfection combined with the parenterally administered antibiotics provided the most significant reduction in incidence of bacteraemia (controls 85%, penicillin 53%, penicillin and streptomycin 26% and local disinfection with penicillin and streptomycin 17%). Although the results of this study were published in 1958, recommendations by the AHA for the use of penicillin and streptomycin were not adopted until 1977 as a more protective regimen for patients at risk for infective endocarditis and local measures are still not used routinely.

Another study investigating bacteraemia associated with tooth extractions gave similar results after using oral penicillin plus local disinfection of the gingival sulcus with povidone iodine. The bacteraemia incidence in the controls was 86%; after oral penicillin prophylaxis it was 40%; after prophylaxis and local disinfection of the gingival sulcus, bacteraemia incidence was 5%.

Scopp and Orvietto (1971) reported a 50% decrease in bacteraemia with antiseptic application. They have also demonstrated that prophylaxis with systemic penicillin administration had a similar effect and that the combined systemic and local prophylaxis method decreased the frequency of bacteraemia from 87% to 5%.

The local administration of antiseptic solutions such as chlorhexidine could be recommended as an adjuvant to systemic antibiotic prophylaxis as neither method alone completely eliminates bacteraemia.

Using chlorhexidine as a patient administered mouthwash to determine whether levels of bacteraemia can be reduced, if proven to be effective, could be an ideal preventive measure for those at risk.

### ***1.16 : Chlorhexidine***

In the late 1940's, scientists seeking to develop antimalarial agents formulated a group of compounds called polybiguanides which demonstrated a broad antimicrobial spectrum. Chlorhexidine, one of the drugs created is commonly used as an antiplaque agent. Its structural formula consists of two symmetric 4-chlorophenyl rings and two biguanide groups connected by a central hexamethylene chain.

Chlorhexidine is a base and is stable as a salt. The most common oral preparation, chlorhexidine digluconate, is water soluble and at physiological pH readily dissociates releasing the positively charged chlorhexidine component. The bactericidal effect of the drug is due to the cationic molecule binding to extra microbial complexes and negatively charged microbial cell walls, thereby altering the cells' osmotic equilibrium. At low concentrations, low molecular weight substances will leak out, specifically potassium and phosphorous. At higher concentrations, precipitation of cytoplasmic contents occurs resulting in cell death. Rolla and Melsen (1975) suggested that chlorhexidine also functions to inhibit plaque formation by the following mechanisms: a) by binding to anionic acid groups on salivary glycoproteins, thus reducing pellicle formation and plaque colonisation and b) by binding to salivary bacteria and interfering with their adsorption to teeth.

### **1.16.1 : Spectrum of activity**

Chlorhexidine is bactericidal and effective against Gram-positive, Gram-negative and yeast organisms. Rinsing with 10ml of 0.2% chlorhexidine (20mg) solution results in suppression of new plaque deposits. Salivary bacterial counts taken immediately after rinsing with a 0.2% chlorhexidine solution demonstrated an 80% to 90% reduction of organisms. Subsequent to cessation of drug application, plaque reappears and salivary counts of organisms return to baseline values within 48 hours.

Hennessey (1973) reported that Gram-positive organisms are more sensitive than Gram-negative microbes and *Streptococci* were more affected than *Staphylococci*. Chlorhexidine is also effective against *Candida albicans in vitro*; *in vivo* studies in man have confirmed its efficacy against fungal infections.

Antimicrobial mouthrinses have been recommended for patients undergoing either cancer chemotherapy or radiotherapy before invasive oral procedures and as a prophylactic measure to control bacterial and fungal overgrowth and to reduce inflammation. A particularly useful application of oral antiseptics is the treatment of inflammatory periodontal diseases, where a causal treatment is necessary. The mechanical removal of dental plaque is required and the additional use of antiseptics is recommended for the healing of inflammatory changes.

Periodontal diseases are plaque induced infections and therapeutic strategies should be directed at eliminating periodontopathic organisms. However it is still unclear which microbes cause gingivitis and periodontitis. Without knowing which bacteria are overt pathogens clinicians have resorted to the non-specific removal of all microorganisms. That may be counter-productive because certain organisms are inhibitory for other species. For example, in the presence of *Streptococcus sanguis*, *Actinobacillus*

*actinomycescomitans*, an overt pathogen associated with aggressive periodontitis, does not colonise.

The implication of this reported decrease in mucositis, plaque accumulation, and candidiasis with chlorhexidine use is that there will be a corresponding decrease in bacteraemias. This benefit may be offset by the risk of emergence of resistant or more virulent organisms normally held in check by the predominant flora of the oral cavity. It has been reported that chlorhexidine mouthrinses can encourage the overgrowth of *Streptococcus sanguis*, an organisms known to cause endocarditis.

### ***1.16.2 : Adsorption***

Chlorhexidine derives its unusual anti-plaque efficacy from its ability to adsorb to anionic substrates (hydroxyapatite, pellicle, salivary glycoproteins and mucous membranes). Bonesvoll *et al.* (1974) using radioactively labelled chlorhexidine, determined that approximately 30% of the drug was retained after a patient rinsed with 10ml of 0.2% chlorhexidine solution for one minute. The bound chlorhexidine was subsequently released over an 8-12 hour period and weak concentrations could be found in saliva for 24 hours. The slow release of the drug from retention sites provides a prolonged bactericidal effect.

### ***1.16.3 : Metabolism and toxicity***

Poor absorption of chlorhexidine is a factor in its low toxicity. Experiments conducted with radiolabelled chlorhexidine rinses indicated that mucosal and gingival penetration was minimal and it was poorly absorbed from the gastrointestinal tract. Bonesvoll (1974) reported that when oral chlorhexidine rinses were used, 4% of the solution and all of the adsorbed drug was eventually swallowed. 90% of the retained drug was excreted in the

faeces and the remainder was eliminated via the urinary tract. Studies monitoring chlorhexidine also determined that none accumulated in the body or was metabolically altered into potentially harmful by products. In particular, parachloroaniline was not detected in the urine of animals or man (Winrow *et al* 1973).

#### ***1.16.4 : Side effects***

The most common side effect of chlorhexidine is the formation of a yellow-brown stain that develops in the gingival third and interproximal of affected teeth. This occurs on approximately 50% of the patients within several days. Staining of the tongue has also been reported. The precise staining mechanism has not been determined, but it has been postulated that there is a dietary aetiology. Rolla *et al.* (1975) suggested stain was formed by precipitation of iron sulphide that requires professional removal. Discolouration of pits and fissures, cemento-enamel junction, proximal surfaces and composite restorations are commonly observed.

There have been reports of occasional dulling of taste sensation for several hours, parotid swelling and desquamative lesions associated with chlorhexidine application. However, these are uncommon sequelae and cessation of drug therapy results in a return to normal.

#### ***1.16.5 : Long term effects of chlorhexidine application***

In 1976 investigators reported the effects of two years of daily chlorhexidine application. A test group applied 10ml of 0.2% chlorhexidine digluconate daily in conjunction with brushing, while a control group only brushed. Plaque gingivitis, changes in microbiota, systemic and local side effects were monitored. It was found that chlorhexidine was unable to penetrate subgingivally and emphasises that subgingival debridement is a needed adjunct. Schiott *et al.* (1970) found long-term therapy resulted in a 30-50% reduction of

salivary bacteria without any detectable shifts in ratios of bacteria. Upon cessation of chlorhexidine use, the microbial population returned to pre-treatment levels. Inspection for changes in salivary flora sensitivity revealed there was a slight shift towards organisms that were less affected by chlorhexidine, which returned to normal after completion of therapy.

After two years use various medical parameters were investigated and revealed that there were no systemic side effects attributed to chlorhexidine application.

#### ***1.16.6 : Possible development of bacterial resistance to chlorhexidine application***

Bacterial resistance to certain drugs appears as the result of selection of mutants that develop due to chromosomal alterations or through transfer of genetic information by conjugation. Antimicrobials usually do not cause mutations, but instead participate in the selection by providing an environment conducive to growth of the less susceptible microbes. Chlorhexidine has been reported to cause mutations; however this does not occur often (a mutation frequency of 0.014% when *Salmonella typhimurium* was exposed to low concentrations of chlorhexidine).

The development of resistant strains is a frequently encountered phenomenon associated with antibiotic therapy. Kornman (1986) and others monitored patients who received long-term systemic tetracycline and reported that subgingival plaque developed resistance to drug therapy. This has not been clearly demonstrated with chlorhexidine and Gjermeo (1970) postulated that human bacteria recovered by investigators after therapy did not develop resistance, but rather represented strains that were less sensitive to the drug.



### ***1.16.7 : Effect of chlorhexidine on oral microflora***

For various reasons it is not possible to eliminate microorganisms from the oral cavity. A temporary quantitative reduction of the bacterial count may be required for the treatment or prophylaxis of oral infection and this may be achieved by the application of oral antiseptic agents. In contrast to the disinfection of the skin, there are particular problems in the antiseptics of the oral mucosa. The bacterial colonisation of the oral cavity is very high; for example, the number of colony forming units in the saliva range from log 5 to log 6 per ml, and in the gingival sulcus up to log 12 cfu may be present. The efficacy of oral antiseptics is affected by the dilution effects as well as inactivation due to salivary proteins. On the other hand, an increase of antiseptic concentration is limited by local irritation and a high absorption rate with the risk of systemic intoxication.

Bollen *et al.* (1998) investigated the effect of a one stage full-mouth disinfection on different intra-oral niches. 16 patients with severe periodontitis were randomly allocated to a test and control group. The patients from the test group received full-mouth disinfection consisting of scaling and root-planing of all pockets in two visits within 24h, in combination with tongue brushing with 1% chlorhexidine gel for one min, mouth rinsing with a 0.2% chlorhexidine solution for two min and subgingival irrigation of all pockets with 1% chlorhexidine gel. In addition to oral hygiene, the test group rinsed twice daily with 0.2% chlorhexidine and sprayed the tonsils with 0.2% chlorhexidine for two months. Plaque samples were taken at baseline and after two and 4 months and changes in probing depth attachment level and bleeding on probing were reported. The full mouth disinfection results in a statistically significant additional reduction / elimination of periodontopathogens, especially in the subgingival pockets but also in the other niches. The number of cfu/ml remained relatively constant with time in the control group, a more

significant reduction ( $p < 0.05$ ) was observed in the test group especially at month two and four.

For pathogenic bacteria the changes in the number of cfu/ml for several pathogenic organisms (*Aa*, *Pg*, *Pi*, *Fn*, *Pm*, *Cr*) in the control group were negligible whereas patients from the test group showed significant reductions with the exception of *Aa*. For some patients in the test group several bacteria were even no longer detectable by means of culturing, especially at the two months recall. *Porphyromonas gingivalis* for example was initially detected in 10 samples from the test group and six samples from the control group. After therapy there was no change in the control group but in the test group only four samples remained positive. In the test group a small reduction in the number of cfu from saliva samples was detected. Significant reductions in the amount of pigmented anaerobic *Porphyromonas* and *Prevotella* species could be reported for all niches in the test group.

Several papers have indicated that most periodontopathogens (with the exception of *spirochetes*) can colonise besides the periodontal pockets different other niches in the oral cavity: the dorsum of the tongue, the buccal mucosa, the tonsils and the saliva. Research on partially edentulous patients rehabilitated with implant supported bridges, suggested the existence of an intra-oral transmission (translocation from one niche to another) of these periodontopathogens. The relative importance of the intra-oral niches in the development and maintenance of periodontal infections is very questionable. Danser *et al.* (1994) showed that extraction of all the remaining teeth in an oral cavity resulted in a significant reduction in the proportion of pathogenic bacteria in all the remaining niches. This suggests a predominant role for periodontal pockets in the contamination of the oral cavity. It is difficult to conclude however, whether the improvement in bacterial reduction

is due to the full mouth approach or due to the intensive mouth rinsing. Flotra *et al.* (1972) showed the effect of oral rinsing on the subgingival flora to be negligible.

#### ***1.16.8 : Chlorhexidine-specific effects on periodontopathogens***

An important factor to be considered in relation to the presence of periodontopathogens, is the clinical benefit of disinfection. Rinsing of the mouth before invasive oral procedures, for example with chlorhexidine, did not significantly alter the number of positive blood cultures or the nature of organisms as shown by Lockhart (1996). On the other hand Bollen *et al.* (1998) reported a statistically significant additional reduction/elimination of periodontopathogens in patients with severe periodontitis by full mouth disinfection. However, these findings are limited to investigated plaque samples. Blood samples were not performed. In epidemiological studies, prophylaxis has been given varied importance. Imperiale and Horowitz (1990) concluded that prophylaxis gave 91% protection while Van der Meer *et al.* (1992) believed it to give only 49%.

#### ***1.16.9 : Microbiologic effect of irrigation and debridement***

Wennström *et al.* (1987) evaluated the microbiological effects of repeated subgingival irrigation of deep periodontal pockets as a single measure of treatment combined with mechanical debridement. The pockets in the various quadrants were randomly assigned to professionally performed subgingival irrigation with 0.2% chlorhexidine gluconate, 3% hydrogen peroxide, saline or to non-irrigation. During the first phase of treatment, the pockets were periodically irrigated and no subgingival mechanical debridement was performed. During the second phase, subgingival scaling and root planing were carried out with adjunctive subgingival irrigation of the pockets. Patients plaque control was carefully supervised. The results showed that periodic subgingival antimicrobial irrigation

*per se* had only limited and transient effects on the subgingival microflora. Repeated irrigation with chlorhexidine or hydrogen peroxide did not induce any changes in total viable counts, anaerobes, rods, Gram-negative anaerobic rods, black pigmented *Bacteroides*, *B.gingivalis* or motile rods and spirochetes that were different from those of control sites irrigated with saline. Wennström *et al.* (1987) did not reveal any clinical effects of therapeutic significance by professionally performed irrigation with chlorhexidine or hydrogen peroxide when used alone or in combination with thorough mechanical debridement. Furthermore optimal supragingival plaque control did not induce any qualitative changes of the subgingival microflora, findings which are consistent with the results presented by Kho *et al.* (1985).

Similar, although not equally pronounced changes were observed following irrigation with saline. The microbiological results of Wennström *et al's* (1987) study extended their previous findings by showing that apart from the effects on spirochetes and motile rods no specific effects on the composition of the subgingival plaque flora were obtained by repeated irrigation with chlorhexidine or hydrogen peroxide.

The effect of irrigation may be attributed to a washing effect rather than to an antimicrobial effect of the irrigants used. It may be possible that an active treatment effect on subgingival microflora may be obtained by the use of more frequent and longer application periods and / or more effective antimicrobial substances.

Professionally performed irrigation in combination with mechanical debridement (thorough scaling and root planing) failed to demonstrate any significant effect of the adjunctive irrigation therapy on the total viable counts, motile rods and spirochetes, Gram-negative anaerobic rods or *Bacteroides*.

It has been suggested that mechanical debridement may act synergistically to local antimicrobial treatment by allowing the agent to penetrate and retain in the deeper parts of

the pocket thereby exerting a prolonged effect (Rosling *et al.* 1983) However the observation by Wennström that similar reductions of the various microbiological variables were obtained by adjunctive irrigation with saline as with chlorhexidine and hydrogen peroxide does not support this hypothesis. In addition the non-irrigated control sites showed a similar reduction pattern as the irrigated sites. This may arise if the mechanical debridement performed was close to optimal and consequently any additive effect of the irrigation treatment would not be expected.

#### ***1.16.10 : Ultrasonic scaling in conjunction with chlorhexidine : effects on bacteraemia***

A pilot study by Allison *et al.* (1993) to investigate whether the incidence of bacteraemia following subgingival ultrasonic scaling and root planing could be reduced by the use, pre and intra-operatively of an irrigant containing 0.12% chlorhexidine. Patients with a minimum of seven sites per quadrant having 4mm pockets with bleeding on probing were entered into the study. The patients gingival crevices were then irrigated with either chlorhexidine or placebo. 10 minutes later, ultrasonic scaling and root planing was performed with a continuous flow of either the chlorhexidine or control solutions. Blood samples were taken pre-operatively, while post-operative samples were taken 1 minute after completing the scaling of each quadrant and then 10 min after scaling the second quadrant. Routine aerobic and anaerobic bacterial culture methods were used to identify viable blood borne bacteria. Results showed that the incidence of bacteraemia was 70% in the control quadrants and 30% in the experimental quadrants which was a statistically significant difference. The predominant isolates were viridans *Streptococci*. These findings suggest that the incidence of bacteraemia following subgingival ultrasonic scaling and root planing can be reduced by using an irrigating solution containing 0.12% chlorhexidine.

Another study investigated bacteraemia in patients who did not receive penicillin prophylaxis prior to ultrasonic scaling. Of those investigated, 61% of patients demonstrated positive blood cultures five minutes after the procedure compared with an incidence of only 10.7% among patients who did receive penicillin.

Rahn *et al.* (1995) reported a 30% reduction incidence of bacteraemia for chlorhexidine experimental sites compared with sterile water control sites in patients treated by means of an ultrasonic device with treatment time being 15 minutes.

#### ***1.16.11 : Why mouthwash as a route of administration of antimicrobials is not effective***

##### ***: Problems of subgingival penetration***

A study by Pitcher *et al.* (1980) may explain why mouthwash as a route of administration of antimicrobials is not effective. They investigated the access to subgingival plaque by disclosing agents using mouthwash and direct irrigation. Teeth which were to be extracted and exhibited periodontal pockets had a disclosing agent applied as either a mouthrinse or by direct irrigation at the entrance of the pocket. After extraction, the extent of apical penetration of the solution was measured. The tooth was then stained enabling similar measurements from the reference point to the level of the apical plaque border and the coronal level of the residual periodontal ligament. The results showed that neither mouthrinsing nor direct irrigation routinely achieved penetration of the solution to the apical plaque border. Possibly the inflammatory crevicular fluid released in pockets creates an osmotic gradient which prevents solutions from penetrating. The lack of total penetration to the apical plaque border may have also been due to the presence of subgingival calculus or of a band of more tightly adapted pocket epithelium immediately coronal to the epithelial attachment. While mouthrinsing failed to achieve any significant penetration of pockets, the direct irrigation technique was partially effective.

In addition a study to support this finding, Greenstein *et al.* (1987) showed that antimicrobial rinses and irrigations do not permeate more than 3mm into the gingival sulcus, thus why efforts to sterilise the sulcus with medications have failed. Pocket depths less than 3mm are not considered definitely pathological and therefore the delivery of antimicrobials in this manner, will not permit it to reach the area of importance where bacteria are thought to gain entrance to the systemic circulation.

Hardy *et al.* (1982) suggest that deeper penetration of the syringe needle or solution may result in irrigation to the apical plaque border. However, it is clear that such procedures themselves may result in bacteraemia and case reports exist of infective endocarditis caused by irrigation devices (Bender *et al* 1984).

#### ***1.16.12 : Problems of sufficient contact time/concentration***

Subgingival application of antimicrobial agents has been suggested to be an effective adjunct to the mechanical debridement of deep periodontal pockets (Keyes *et al.* 1978, Mazza *et al.* 1981). It is assumed that microorganisms left behind during mechanical debridement of a periodontal pocket could be eliminated by subsequent subgingival irrigation with an antimicrobial agent. However, when considering the potential effect of subgingival irrigation or indeed mouthwash use, it becomes necessary to evaluate whether in fact this mode of administration has the ability to establish and maintain effective concentrations of the used antimicrobial agent within the pocket. An ideal antimicrobial agent should require only a very short contact time with the remaining subgingival microbiota in order to kill the bacteria or to inhibit their growth, since the time normally used for irrigation of a periodontal pocket is relatively short. Also, will such a short contact time allow the establishment of sufficient concentration of the agent used to be effective ?

Southard *et al.* (1989) addressed the efficacy of irrigating deep pockets with 2% chlorhexidine as a supplement to oral hygiene and root debridement. The authors reported that bleeding scores, pocket depths and spirochetal counts were reduced after non-surgical periodontal therapy with or without antimicrobial irrigation. Investigators concluded that biweekly or daily irrigation with 2% chlorhexidine for 24 weeks did not enhance treatment of patients with effective oral hygiene who underwent supra and subgingival root planing.

#### ***1.16.13 : Mouthrinse studies***

Studies on mouth rinses are difficult to control and data is conflicting due to inconsistencies in study design that often lack adequate sample size, randomisation, blinding of examiner and control over other variables.

#### ***1.16.14 : Subgingival irrigation with chlorhexidine : superior method of administration***

Subgingival irrigation may be classified as intentional localised irrigation of a gingival crevice or pocket. With the advent of effective antimicrobial agents already shown to significantly lessen existing plaque and gingivitis, researchers have investigated their adjunctive use as irrigating solutions. Studies show the efficacy of these medicaments is enhanced to include reductions in suspected periodontopathic, subgingival bacteria (Bender *et al* 1984). Manual toothbrushing and rinsing are capable of accessing 1-3mm subgingivally while subgingival irrigation reaches 4-5mm into periodontal pockets. The outdated “non-specific plaque hypothesis” considered all plaque a homogenous mass with only the quantity of the plaque being detrimental. However, in the last 10 years, research has indicated that the primary cause of periodontal infections is specific bacterial activity (Socransky *et al* 1977, Page *et al* 1976, Listergarten *et al* 1978, Newman *et al* 1985). Investigators have determined that although several hundred types of microbiota may be



present in the oral cavity, only a few singly, or in combination, are responsible for periodontal tissue destruction (Williams *et al* 1990). This newer concept, “specific plaque hypothesis” emphasises the importance of the plaque and its bacterial ecology. This theory recognises that certain bacteria are associated with various types and stages of periodontal diseases. The pathogenicity of plaque may vary from one person to another and even between sites within that person (Lindhe *et al* 1980). This results in individuals exhibiting various host responses probably related to their own immunological or host response. At healthy sites, the Gram-positive aerobic organisms such as *Streptococci* predominate. In periodontitis the disease associated microflora are generally Gram-negative; anaerobic strains found in the deeper subgingival plaque and the layer of loosely attached plaque which lies in direct contact with the junctional epithelium. These bacteria species which have been identified as periodontal pathogens are *Porphyromonas gingivalis* and *Prevotella intermedia* associated with adult periodontitis and *Actinomyces actinomycetemcomitans*, which may prove to be the principal microorganism responsible for juvenile periodontitis (Christersson *et al* 1987).

#### ***1.16.15 : Comparison of irrigation and oral rinsing***

In 1975 Tempel and colleagues examined fractions of solution that irrigation removed after brushing and flossing, and that which was expectorated following oral rinsing. The volumes of soluble and insoluble matter were compared. The results showed that water irrigation proved to be more effective than oral rinsing in removing particulate debris and soluble bacterial products. Researchers have since examined the ability of oral irrigation to qualitatively alter sufficient subgingival microorganisms to create a shift toward health in the plaque mass. Supragingival irrigation therapy has been examined for its influence on subgingival plaque, since the disease associated flora thrive deep within the gingival

crevices, accessing these areas is essential to prevent, or inhibit the periodontal destructive process. It has previously been established that a mechanised pulsed water stream can access on average approximately half the depth of the pockets and adversely effect plaque (Eakle *et al* 1986). A six week study was conducted to evaluate that ability of powered irrigation at depths up to 6mm on various bacteria (White *et al* 1988). Gingivitis did not improve in the control group but in the experimental group where participants performed supervised water irrigation five days a week during the study, gingivitis decreased in shallow and deep pockets. In addition, the deep pockets exhibited a reduction in spirochetes, motile rods and a count reduced by half in *Bacteroides melaninogenicus* and *Prevotella intermedia* in the water irrigation group a reduction in bleeding on probing was also seen (Aday *et al* 1982).

Other investigators examined 16 irrigated versus untreated control sites in patients diagnosed with advanced chronic adult periodontitis (Cobb *et al* 1988). Using both scanning and transmission electron microscopy the excised periodontal pockets were examined immediately following one irrigation treatment with sterile saline. The irrigated pockets harboured significantly less bacteria than the non-irrigated sites. These results were most apparent up to 4 mm.

As stated earlier, oral irrigation has proved to be superior when compared to rinsing for delivery of antimicrobial compounds (Lang *et al* 1981, Brownstein *et al* 1990, Flemming *et al* 1990). Many researchers have reported an enhanced effect of delivery by irrigation. Brownstein *et al.* (1990) showed that irrigation with 0.06% chlorhexidine proved to be an effective means of treating naturally occurring gingivitis. These studies and others

(Greenstein 1987) imply irrigation may increase the access of solutions below the gingival margin and improved tissue health may result.

An ultrastructural examination of an untreated periodontal pocket immediately following irrigation showed no tissue damage (Cobb *et al* 1988). Studies as early as 1969 have been conducted on supragingival irrigation effects regarding bacteraemia. Tamini (1975) evaluated bacteria in blood samples of subjects displaying varying degrees of periodontal health. The participants who used oral irrigation in addition to toothbrushing, exhibited no greater bacteraemia levels than those in the toothbrushing group. This study demonstrated that transient bacteraemias are not necessarily produced by oral irrigating devices, contradictory to Bender *et al* 1984 findings.

Much information exists which supports the efficacy of root planing to eliminate subgingival deposits. However, most therapists fail to totally cleanse root surfaces where probing depths are in excess of five millimetres. Surgical procedures are often required to gain access and despite this more invasive therapy, only 50% of root surfaces in pockets greater than 6mm are effectively cleaned. Areas such as grooves, furcations and the cemento-enamel junction retain most residual calculus (Waerhaug *et al* 1978, Cafeese *et al* 1986).

Daily self administered subgingival irrigation may prove to be an important component of a home oral hygiene program for these patients who cannot maintain periodontal sites which are inaccessible to brushing and flossing. In the early 80's a group of periodontal patients participated in a study which was designed to determine if self applied subgingival irrigation was a viable approach for a safe and effective adjunct in home periodontal

maintenance care (Soh *et al* 1982). The test group first received scaling and polishing and then instruction in pocket irrigation. After four weeks of daily self-irrigation, it was concluded that the technique itself caused no discernible injury and further with the addition of chlorhexidine, subgingival irrigation proved to be an affective method in reducing periodontal inflammation and controlling subgingival plaque. Thus this technique may have proven more effective in reducing subsequent bacteraemia than mouthwash usage.

### ***1.17 : Systemic disease caused by oral microorganisms***

Recent progress in oral microbiological classification, identification, and the possibility of tracing organisms in extra-oral sites back to the oral cavity have led to renewed interest in and concern for bacteraemia and its systemic risks. The likelihood that treatment activities induce bacteraemia depends on the amount and complexity of the plaque present on the teeth and on the clinical state of the gingival tissues. Human endodontic and periodontal infections are associated with complex microfloras. These infections are predominantly anaerobic with Gram-negative rods being the most common isolates.

The microorganisms that gain entrance to the blood in relatively high numbers and circulate throughout the body are usually eliminated by the reticuloendothelial system within a few minutes (transient bacteraemias) and lead to no other clinical symptoms than possibly a slight increase in temperature (Van Velzen *et al* 1984). However the disseminated microorganisms, if finding favourable conditions, will tend to localise at a given spot and after a certain time lag will begin to multiply (Nord *et al* 1990).

There have been several case reports of systemic diseases caused by oral microorganisms. Larkin *et al.* (1994) reported on an unusual case of metastatic paraspinal abscess and

paraplegia secondary to dental extraction with *Streptococcus mutans* being the main isolate. Indeed, this is not solely limited to extractions. Doerffel *et al.* (1997) report a severe prosthetic valve-related endocarditis following dental scaling.

### ***1.17.1 : Periodontal organisms in systemic disease***

We do not normally think of the teeth affecting the rest of the body, with the exception of an acute abscess. But the teeth as the only non-renewable surface in the body, harbour large numbers of bacteria ( $10^8$  to  $10^9$  cfu per mg plaque are in intimate contact with the gingival epithelium). Bacteria and bacterial by-products constantly provoke the underlying tissue to the extent that some degree of inflammation is considered normal. It may be possible that the plaque flora does more than just challenge the gingival epithelium.

Slots (1998) recently discussed an old theory of focal infection, first suggested by Hippocrates to explain various inflammatory diseases. It was thought that foci of infection, which themselves might go unnoticed because of lack of symptoms, initiated the seeding of pathogenic microorganisms or their products to distant body sites. Dental focal infection must also be assumed in other types of infectious heart disease, intracranial infections, thoracic infections and various medical infections that contain organisms indigenous to the oral cavity (e.g. *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* van Winkelhoff (1993) and Slots 1999). The late 1980's witnessed a resurgence of interest in the focal infection concept, provoked by epidemiological studies from Finland. The Finnish studies found an association between 'overall' dental condition (periodontitis, periapical lesions, dental caries, missing teeth, and pericoronitis) and acute cerebral infarction in young males (Syrjanen *et al.* 1989) and coronary heart disease including myocardial infarction, coronary atherosclerosis and

ischaemia (Mattila *et al.* 1989). A recent paper by Loesche *et al.* (1997) pointed out the relationship between oral parameters such as elevation of specific bacteria and number of missing teeth in a population of elderly US veterans, with coronary heart disease. In this population group, the association for oral parameters was at least as strong as serum cholesterol, body mass and smoking, with the risk of heart disease related to periodontitis stated to be in the same area as that for a chronically elevated cholesterol score.

Later epidemiological studies in the US demonstrated a positive relationship between alveolar bone loss and stroke and coronary heart disease (DeStefano *et al.* 1993). Recent studies have also found relationships between periodontal disease and pre-term low birth weight (Offenbacher *et al.* 1996), the results of one study involving pregnant women showed that those with periodontal disease were eight times more likely to deliver low-birth-weight babies. A great deal of circumstantial evidence has been amassed linking untreated periodontal disease to endocarditis and lung infections in patients with chronic obstructive pulmonary disease (Loesche and Lopatin 1998). Furthermore it has been known for some time that periodontal infection can affect the stability of insulin dependent diabetics and certainly can play a role in weakening the host immune system.

A growing amount of clinical and experimental evidence suggests a link between infection and atherosclerotic diseases including both myocardial and cerebral infarction (DeStefano *et al.* 1993, Beck *et al.* 1996). A prime example is a greatly increased risk of stroke in septicaemic patients (Syrjänen *et al.* 1989). Controlled clinical studies have recently shown however, that certain other milder bacterial infections are also a risk factor for infarction (Contreras *et al.* 1998). A preceding febrile respiratory infection was a major risk factor for stroke in young and middle aged patients (Syrjänen *et al.* 1988). So far however, little clinical evidence is available to suggest that by controlling infection the risk of infarction or development of atherosclerotic lesions might be reduced except in

patients with endocarditis, where the risk of thromboembolic complications rapidly diminish when the infection is controlled with antimicrobial therapy.

Several cases have been reported of *Actinobacillus actinomycetemcomitans* causing systemic disease (van Winkelhoff *et al* 1999). *Aa* can be found in the oral cavity of 15% to 60% of healthy individuals. However, *Aa* is looked upon as a key pathogen in juvenile and prepubertal forms of periodontitis and in severe adult periodontitis (Listergarten *et al* 1976). This microorganism is of particular interest to periodontists because mechanical and surgical treatment often does not eliminate this bacterium from the periodontal lesions (Bragd *et al* 1978). Invasion of the soft tissue has been shown and may be the reason for the lack of success of mechanical treatment (Saglie *et al* 1985). A case of symptomatic *Aa* bacteraemia in a patient with an implanted pacemaker is reported by van Winkelhoff *et al.* (1993). Oral examination revealed a mild form of periodontitis with *Aa* isolated from several oral sites. Thirty minutes following extraction of periodontally compromised teeth, blood was cultured with samples positive for *Aa*. DNA fingerprinting of strains from the blood and the oral cavity showed identical profiles. This finding strongly suggests that the oral cavity was the primary source of *Aa* bacteraemia in this case.

Christensen *et al.* (1993) reported an instance of septic pulmonary embolism where the usual predisposing factors were absent; an antecedent toothache and periodontal disease all suggested the gingiva as the source of the infection. Cultures yielded a pure growth of *Streptococcus intermedius*. The authors hypothesised that periodontal infection led to bacteraemia, seeding of the lungs and multiple anaerobic pulmonary abscesses, similar to reported instances of infective endocarditis from dental foci without any prior dental procedures. In an analysis of patients with various anaerobic lung infections which included results of dental evaluations, Bartlett (1987) and Finegold and Wexler (1988)

noted evidence of periodontitis in roughly two thirds of patients. However since periodontal disease is prevalent among the population, this may be purely coincidental.

Epidemiological research can identify relationships but can not prove causation. Since most periodontal diseases are considered random, independent infections of the periodontium, periodontal disease has been suggested as being the cause of various systemic diseases. However, if some types of periodontal disease merely constitute an oral component of a systemic disorder or have aetiological features in common with medical diseases, periodontal and medical diseases might frequently occur together without indicating a cause-effect relationship.

It cannot be disputed that infection is a risk factor for atherogenesis and thromboembolic events (Syrjänen *et al* 1989, Mackenzie *et al* 1963, Nery *et al* 1987). This has been recognised for decades as Gram-negative bacteria or the associated lipopolysaccharide (LPS) when presented as a systemic challenge in animal models, can induce inflammatory cell infiltration into major blood vessels, vascular smooth muscle proliferation, vascular fatty degeneration, and intravascular coagulation (Osler *et al* 1908, Marcus *et al* 1993). The remarkable similarities of bacterial-induced vascular pathology and the natural history of atherogenesis have led certain investigators to suggest that, in addition to genetic and dietary influences, infections of unknown origin may contribute to the observed cardiovascular pathology (Syrjänen *et al* 1989, Umino *et al* 1993).

Additional evidence indicates that periodontal pathogens themselves may play an aetiological role. Herzberg and colleagues (1998) have conducted studies that relate infection with *Streptococcus sanguis*, a supragingival plaque organism, (appropriately named because it is often isolated from the bloodstream and is also associated with endocarditis) to increased platelet aggregation by the presence of a virulence factor platelet



aggregation associated protein (PAAP). This virulence factor causes extensive vegetative growth on heart valves. The potential significance of oral Gram-positive bacteria including streptococcal species (*sanguis*) and periodontal pathogens which may also possess PAAP (*Porphyromonas gingivalis*) in mediating thromboembolic events is yet to be determined fully. In addition, the identification of pathogens associated with periodontal disease in atheromas (Chui *et al.* 1999) lends support to Herzberg's findings.

Animal studies by Beck *et al.* (1998) examined the effects of *P.gingivalis* infection and high-fat diets on atheroma formation in C57B6 mice which are genetically susceptible to cardiovascular disease. These atherosclerosis-prone mice were assigned to high-fat and low-fat diets. The high-fat diet produced the atherosclerosis phenotype, the low-fat diet does not as would be expected. The mice were on the diet two months prior to priming with a dose of heat killed *P. gingivalis*; a similar group of mice were sham primed with media only. The mice were challenged 21 days later with live *P.gingivalis*, performed to mimic a chronic *P.gingivalis* infection. Sloughing of a chamber was the result of an intense inflammatory reaction to the infectious agent. The high fat groups in both figures lost about the same percentage of chambers during the follow up period but the group primed with heat-killed oral bacteria lost their chambers more quickly. These data suggested that high-fat diets in these susceptible mice lead to an enhanced inflammatory response to *P.gingivalis* challenge. It remains to be determined in these experiments whether the inflammatory response also promotes atheroma formation.

Mattilla *et al.* (1989) found that conventional risk factors for strokes and heart attacks could not be explained by age, social class, smoking, serum lipids or diabetes when included in statistical analysis. NHANES (National Health and Nutrition Examination Study) (DeStefano *et al* 1993) using a prospective cohort study confirmed the conclusions of this study linking coronary artery disease and periodontal disease. The most noteworthy

finding was that periodontal disease and poor oral hygiene are stronger indicators of risk of total mortality than of coronary heart disease. There is additional support to mortality data as the mortality values for gingivitis were intermediate between those of no disease and periodontitis. Considering edentulous patients lost their teeth because of dental decay and / or periodontal disease, and that these patients would represent the accumulation of a disease process about the teeth, this could explain why they were 2.6 times more likely to die by the time of subsequent examinations than the individuals with no periodontal disease.

The best documented microbe linking chronic infections to cardiovascular disease has been *Chlamydia pneumoniae*, which has actually been detected in coronary arterial fatty streaks and atheromatous plaques. Thus it would not be unlikely to find that a chronic periodontal infection could also play some role in cardiovascular diseases. Dental infections may also contribute to the risk of atherosclerosis and myocardial infarction. A recently published study (Chiu *et al.* 1999) indicates that atherosclerotic plaques are commonly infected with Gram-negative periodontal pathogens, including *Aa* and *Pg*. While the prevalence of bacteraemia secondary to pulmonary infection with *C. pneumoniae* is not known, as stated previously, dental surgical procedures cause detectable bacteraemias in more than 80% of patients. Simple oral hygiene procedures applied at home cause bacteraemia in at least 40% of individuals. It is likely therefore that exposure to dental microorganisms over a lifetime occurs far more frequently than to any other atherosclerosis associated microbes.

### ***1.17.2 : Prosthetic joints : a consideration***

Infection around a large prosthetic orthopaedic joint can necessitate replacement or result in a total loss of the prosthesis (Coiffe *et al* 1988). Improved modern operative techniques and the perioperative use of antibiotics have helped to reduce early infections which are associated with contamination at the time of surgery (Jaspers *et al* 1985). However, late infections, generally occurring after three months or more, are believed to be either delayed early infections or represent haematogenous spread from a distant source (Blackburn *et al* 1991, Thyne *et al* 1991). Delayed infection is caused by delayed growth of bacteria implanted during the operation. It is distinguished from haematogenous infection, in which there is a potential source of infection distant from the prosthetic joint such as chronic dental infection (Blackburn *et al* 1991, Jaspers *et al* 1985, Coiffe *et al* 1988, Jacobson *et al* 1991). Haematogenous infection is diagnosed when the same strain of bacteria is cultured from the joint, from a primary focus and from blood. Joint infection from a transient bacteraemia secondary to dental treatment has yet to be established as a aetiological factor and for this reason there are no guidelines for antibiotic prophylaxis for patients with prosthetic joints (despite those existing for prosthetic heart valves). Limited clinical data reported in the literature has substantiated this fact. Although few orthopaedic surgeons believe that transient bacteraemias from dental treatment can cause deep infections at a prosthetic site, many orthopaedic surgeons believe premedication with antibiotics is advisable prior to dental treatment (Jaspers *et al* 1985).

The long incubation period for late orthopaedic prosthesis infections from 3-60 months makes it impossible to prove that bacteraemia resulting from dental treatment was the causative and only bacteraemic event in that period of time (Blackburn *et al* 1991). As stated previously with regard to infective endocarditis, the risk of random, spontaneous

bacteraemias associated with daily normal brushing and chewing is calculated to be much greater than from professional dental treatment.

Thus it may be rational to suggest that orthopaedic patients who suffer acute oral bacterial infection or chronic, advanced periodontal problems should be treated vigorously by both conventional and adjunctive antibiotic therapy to reduce even the remote chance of haematogenous spread of the organism.

### ***1.17.3 : Effect of microbial toxins on systemic disease.***

Certain Gram-positive bacteria have the ability to produce diffusible proteins, or exotoxins with specific pharmacologic actions (Van Heyningen *et al* 1970). The exotoxins are considered some of the most powerful poisons known. Endotoxins are part of the outer membrane of the cell wall of Gram-negative bacteria and they are mainly released after cell death (Boivin *et al* 1935). Endotoxin is compositionally a lipopolysaccharide (LPS) that when introduced to the host gives rise to a large number of pathological manifestations. It appears that microbial toxins especially LPS play an important role in focal infection. Syrjanen *et al.* (1989) suggest that components of causative bacteria of oral infections especially LPS may promote atherosclerosis, affect blood coagulation, the function of platelets, and prostaglandin synthesis. These are functions important in thrombus formation which may lead to cerebral and myocardial infarction.

### ***1.17.4 : How are bacteraemias dealt with by the host ?***

Researchers are only beginning to determine causal relationships involved between periodontal disease and other serious sequelae. Periodontal disease is a bacterial plaque induced infection and regardless of the recent advances in altering the host response to the effects of the Gram-negative anaerobic pathogens, the only management for periodontal

disease is directed at reducing plaque levels sub and supragingivally. Control of plaque reduces the inflammatory response and lowers the risk of destructive periodontal infection with its attendant loss of supporting structure. According to current thinking we may in addition, reduce the risk of several potentially fatal diseases.

Body surfaces may be colonised by over  $10^{13}$  bacteria and some small proportion of these bacteria gain access to our underlying tissues and are quickly dispatched, most likely via an inflammatory reaction and immune response (Loesche *et al* 1997). These penetrating bacteria may prime our immune system giving rise to the production of antibodies to most members of the normal flora. This can be viewed as beneficial if these antibodies can cross react with true pathogens (Loesche *et al* 1997).

However, with poor oral hygiene the bacterial load on the teeth increases 10 fold with perhaps 50 cfu/ml blood during asymptomatic bacteraemia which in turn stimulates the immune system having potentially harmful effects of increased WBC, fibrinogen levels, cytokine levels ( $\text{TNF}\alpha$  and  $\text{PGE}_2$ ) and autoimmune reactions to heat stress proteins (Kweider *et al* 1993, Offenbacher *et al* 1996). Any bacteraemia that can cause the host to mount a leukocyte response could trigger a series of events that affect platelet aggregation and changes in blood coagulation (Valtonen *et al* 1991). If the anaerobes persisted long enough in the tissues to cause inflammation, then a battery of LPS mediated events could occur in the vascular system and affect clotting times and platelet aggregation (Dennison *et al* 1998). This then initiates the events on the walls of blood vessels that result in the formation of atheromas. Indeed, established risk factors for myocardial infarction, which may be chronic contributors in addition to lack of exercise, poor diet, high stress, lipid metabolism and smoking includes infections with Gram-negative-LPS containing bacteria. It should also be remembered that periodontal disease and cardiovascular disease share several common aetiologic pathways (Lowe *et al* 1998). Both are more likely to occur in

persons who are older, male, of lower educational status, having fewer financial resources, and who smoke, are hypertensive, stressed and socially isolated.

LPS as a systemic trigger can activate a cascade of inflammatory cytokines that are capable of eliciting most of the vascular and coagulation complications associated with atherosclerosis / CHD (Offenbacher *et al* 1996). The fact that vascular inflammation occurs in the apparent absence of infection has lead several investigators to look for an infection of unknown origin with dental diseases mentioned as a possible component of the disease. Data by Offenbacher among others suggest that there are marked differences in the host response to challenge with bacteria. An exaggerated host response to LPS mediated by the presence of hyper responsive monocytic cells may be implicated in relation to cardiovascular disease. Certain patients with early onset periodontitis, refractory periodontitis and insulin dependent diabetes have peripheral blood monocytes that secrete 3-10 fold greater amounts of PGE<sub>2</sub> interleukin 1 $\beta$  and TNF $\alpha$  when exposed *in vitro* to LPS. Beck *et al.* (1998) have proposed that the individuals with the hyper-reactive monocyte phenotype are at risk for both atherosclerosis, CHD and periodontitis. The M $\phi$ + trait appears to be under both genetic (monocytic hyper-responsiveness to LPS has been genetically mapped tentatively to the arm of HLA-DR3/4 which is the region where increased susceptibility to insulin dependent diabetes mellitus has been proposed to reside) and environmental influence.

A large number of observations demonstrate the critical regulatory role of the LPS-M $\phi$ -mediator activation pathway in the pathogenesis of periodontal diseases. Levels of these mediators increase within the periodontal tissues and gingival crevicular fluid with increasing disease severity and during active periods of disease progression. Increasing evidence suggests that the LPS-M $\phi$ -mediator pathway also plays a critical role in

infection-associated atherogenesis and thromboembolism. Thus it may be that the presence of a hyperresponsive MØ phenotype places certain individuals at risk for both atherosclerosis / CHD and periodontitis. These individuals would overreact to the penetration of Gram-negative bacteria from the subgingival plaque into the periodontium. These and other findings suggest that the wide inter-individual diversity in the host's response to a given bacterial challenge is a direct consequence of multiple factors. These include the functionality of the first line host defence cells (neutrophils), the bacteria infecting the host (pathogenicity) the environment in which the bacteria exist (deep anaerobic pockets allowing for continuous challenge to the host), and the genes which regulate the T-cell monocyte response.

Kweider *et al.* (1993) showed that blood taken from periodontal patients had significantly higher white blood cells and fibrinogen levels than blood taken from 50 similarly aged control patients who did not have periodontal disease. This supports the role of dental infections as risk factors in cardiovascular disease because elevated serum fibrinogen and white blood cell counts are known risk factors for coronary heart disease, in particular myocardial infarction. However, there were more smokers in the periodontal group (78%) than in the control group (26%) so these results must be interpreted with caution.

Levels of certain bacteria such as *S. sanguis* (a supragingival plaque organism), increase when there is poor oral hygiene. If *S. sanguis* entered periodontally inflamed tissue it might penetrate the barrier because of its facultative nature and cause a low level bacteraemia.

Thus, periodontal infections may directly contribute to the pathogenesis of atherosclerosis and thromboembolic events by providing repeated systemic vascular challenges with LPS and inflammatory cytokines.

Researchers from the University of Minnesota have indicated a possible causal relationship between certain plaque bacteria and aberrant blood platelet activity (Herzberg *et al* 1998). It was concluded that such an interaction could result in an increased risk for blood clots, which are associated with myocardial infarction. *Porphyromonas gingivalis* which is one of the putative periodontal pathogens has been suggested as one of these causative organisms.

#### ***1.17.5 : The association of smoking with bacteraemias***

The well established association of smoking with cardiovascular disease may in part be mediated by poor oral hygiene and periodontal disease. Smoking may increase the frequency and magnitude of the bacterial invasion of the gingival tissue and poor oral hygiene would increase the size of the inoculum. Smoking would alter the white blood cell response of the host thereby facilitating the entrance of some bacteria into the blood stream. It could be that these associations are coincidental reflecting a lifestyle of neglect, which predisposes to both heart disease and dental disease. However, it is possible that missing teeth and periodontal disease are additional risk factors for heart disease by predisposing the individual to chronic low-grade infections. Smoking is a major risk factor for periodontal disease and tooth loss (Grossi *et al* 1995). Smoking, by causing a vasoconstriction in gingival blood vessels, could promote bacterial invasion of the periodontium by mostly Gram-negative bacterial species (Loesche *et al* 1995). This, in turn could initiate a series of LPS mediated events that might affect clotting times and platelet aggregation.



### **1.18 : Bacterial toxins : lipopolysaccharide (LPS)**

Bacterial toxins are a special class of noxious substances, which can be differentiated from classical poisons such as strychnine, by virtue of their bacterial origin, their high molecular weight and their ability to be toxic to sensitive hosts when administered in relatively small amounts. The first bacterial toxins to be discovered were the classical exotoxins of diphtheria by Roux and Yersin in 1887, tetanus by Faber in 1889 and botulinus by van Ermengem in 1896. These were protein toxins which were shown to be solely responsible for the pathogenicity of the bacterium which produced them. At the beginning of the twentieth century bacteriologists assumed that toxins were responsible for the pathogenicity of all bacteria, but this has not proved to be the case. In 1936 another class of bacterial toxins, endotoxins, were described by Boivin and Mesrobian. Unlike the previous protein exotoxins which were excreted into the extracellular environment by the bacteria producing them, these endotoxins were structural components of the bacterial cell wall and were chemically complex. The bacteria which produced them were Gram-negative whereas the other toxins were produced by Gram-positive bacteria, e.g. *Clostridium tetani*, which produces tetanus toxin.

The term endotoxin is used synonymously with lipopolysaccharide (LPS). Both terms refer to the high molecular weight ( $0.2 \times 10^6$  to  $1.0 \times 10^6$ ), heat stable ( $100^\circ\text{C}$ ) substances which contain a highly characteristic lipid, usually linked to long polysaccharide chains, and present in the outer membrane of Gram-negative bacteria only. Gram-negative bacteria are ubiquitous, being present in large numbers in the environment, food and the normal commensal flora of man. It would appear that all bacteria which stain Gram-negative produce LPS.

The main functions of LPS probably relate to the maintenance of the structural integrity and selective permeability of the outer layer of the bacterial cell wall. The role of

endotoxin in Gram-negative bacterial infections remains to be determined (McCartney *et al* 1986).

The main interest in the biological activities of LPS has focused on their effects after parenteral administration to mammals. Endotoxins were originally recognised for their capacity to induce fever (Siebert *et al* 1923), but pyrogenicity is only one of their numerous biological activities. Although not all substances that cause fever are of bacterial origin, there was an increasing awareness at that time that most pyrogenic episodes associated with intravenous therapy and many bacterial infections were due to endotoxin. Consequently the terms pyrogen and endotoxin are now often used interchangeably, particularly in the pharmaceutical industry.

The basic mechanisms by which LPS exerts its endotoxic activities involve both cellular and humoral systems, and the complex inter-relationship of these systems makes it difficult to define and distinguish the primary, secondary and any incidental effects of LPS in the experimental animal (McCartney *et al* 1986). Although the mode of action of endotoxin at the molecular and cellular levels has not yet been fully elucidated, much work has been performed on the cellular and biochemical reactions which form the basis of the endotoxin response. Many of the biological activities of endotoxin have been used as assays. However, many workers have claimed that the LAL assay has great advantages of sensitivity, convenience, reliability and cost, in comparison with other *in vivo* and *in vitro* methods.

### **1.19 : Molecular techniques : Polymerase Chain Reaction (PCR)**

Conventional laboratory methods historically used to identify bacterial pathogens involve their isolation and propagation in culture, followed by serological, morphological or biochemical tests to determine their identity. Unfortunately, closely related bacterial species may share interdependencies for growth and therefore cannot be isolated in pure form. Additionally, in certain microbial populations, a large percentage of bacterial cells will not form colonies on agar plates. In cases where an infectious bacterial population can be isolated, its characterisation may take days and sometimes longer if an organism is particularly slow growing (Salo *et al* 1999). These problems inevitably make the identification of bacterial pathogens difficult or impossible in many cases, leading to an uncertain clinical diagnosis and treatment protocol (Spencer *et al* 1988).

Although blood culture has remained the gold standard in aetiological diagnosis, the sensitivity may be limited, because patients may not be bacteraemic at the time of sampling or they may have had antibiotics before sampling (Smith *et al* 1996). In addition, particular bacteria such as *Pneumococci* lyse very easily due to the function of pneumococcal autolysin, which may reduce the amount of viable *Pneumococci* present in blood and thus decrease the sensitivity of culture (Isaacman *et al* 1998). Due to the fact that most bacteraemias have very low viable counts (Isaacman *et al* 1998), PCR offers a sensitive and specific alternative. The advantage of PCR over culture is that PCR detects non-viable and non-culturable organisms (Ward *et al* 1990) and antibiotic treatment should hence not affect the sensitivity of the method. Furthermore, the sample volume required is small and sample preparation is quick and rapid (Isaacman *et al* 1998).

PCR is a sensitive and specific method for identification of microorganisms based on the detection of unique segments of DNA. This method has permitted the identification of fastidious bacteria that are difficult to grow via culture (Shanker *et al* 1991, Barker *et al*

1992). A number of recent investigations have applied this technology towards the identification of common bacterial pathogens (Smith *et al* 1996, Isaacman *et al* 1998, Heininger *et al* 1999). Although the use of PCR has offered promise for the rapid identification of serious bacterial infections, little has been done to assess its utility in the detection of dental bacteraemia.

### **1.19.1 : Basis of PCR**

PCR is a technique which is used to exponentially amplify copies of a specific region of DNA, producing sufficient DNA to be visualised on an agarose gel. This technique can be used to identify with very high probability, disease-causing viruses and bacteria. In order to use PCR, the exact DNA sequences which flank a gene or any sequence of interest must be determined. The nucleotide sequences of many genes and flanking regions of genes of many organisms are known, and despite the DNA of different organisms being distinct, some genes may be the same, or very similar. By identifying those genes which are different, and therefore unique, this information can be used to identify a specific organism.

The building block sequence of a gene is the precise order of appearance of 4 different deoxribonucleotides (adenine, thymidine, cytosine and guanine) within a stretch of DNA. The arrangement of the components comprise a gene sequence and the number of bases in the sequence may vary widely depending on the gene.

As DNA is double stranded (with the exception of some viruses), the two strands pair in a very precise way e.g. adenine always pairs with thymidine and cytosine with guanine.

The primers are extended on a single-stranded denatured DNA template by a DNA polymerase in the presence of deoxyribonucleotide triphosphates (dNTPs), with the concomitant synthesis of new DNA strands complementary to the template strands. Further strands synthesis can be achieved by heat denaturation of double stranded DNA, annealing of primers and primer extension by DNA polymerase. Four strands of DNA now exist where originally there were two. Repeating the procedure will produce eight strands, then 16 therefore theoretically about 20 cycles will produce approximately one-million copies of the original sequences ( $2^{20}$ ). Thus, with this amplification potential there is enough DNA in one tenth of one millionth of a litre of human saliva which contains a small number of shed epithelial cells to use PCR to identify a genetic sequence as having come from a human being. Consequently only a very tiny amount of an organisms DNA need be available originally (Qiagen).

### ***1.19.2 : The PCR technique***

Unknown DNA is heated causing the paired strands to separate thus making them accessible to primers. A large excess of primers relative to the amount of DNA being amplified is added. The reaction mixture is then allowed to cool to allow double-strands to form again. Because of the large excess of primers the two strands will always bind to the primers instead of with each other.

An enzyme is added to the mixture of all the deoxyribonucleotides which can read the opposing strands sequence and extend the primers sequence by linking dNTPs together in the order in which they pair across from one another. This particular enzyme is called a DNA polymerase as it makes DNA polymers. *Taq* polymerase, was originally isolated from a bacterium that can live in hot springs, therefore can withstand the high temperature

necessary for DNA strand separation and thus can be left in the reaction. The enzyme thus synthesises new DNA in opposite directions but only in this particular region of DNA.

After one cycle, more primers and dNTPs are added and the cycle repeated. The primers will bind to the old sequences as well as the newly synthesised sequences. The enzyme will again extend primer sequences. There will be many copies of just the particular region of interest. By using different primers which represent flanking regions of different genes of various organisms in separate experiments, it can be determined whether any DNA has been amplified. If it has not, then the primers did not bind to the DNA of the sample, and it is therefore highly unlikely that the DNA of an organism which a given set of primers are specific for, is present. Alternatively appearance of DNA by PCR will allow precise identification of the source of the amplified material (Qiagen).

### **1.19.3 : 16S rRNA**

The bacterial 16S ribosomal RNA (rRNA) molecule is involved in the conversion of genomic DNA sequences into functional proteins, a process that is remarkably similar in all living organisms. The 16S rRNA molecule must maintain certain structural characteristics to preserve its function. Not surprisingly, the DNA sequence that encodes the 16S rRNA is highly conserved, and structural constraints dictate where mutations can occur that will not disrupt the function of the 16S molecule. Therefore, mutations in the 16S rRNA genes have accumulated at a slow but constant rate over hundreds of millions of years of evolution, and these mutations are usually located in characteristic divergent regions. This pattern of conservation can be exploited to determine the identity of a bacterial pathogen in clinical samples and can also lead to the identification of previously uncharacterised bacterial pathogens. By sequencing these divergent regions in the 16S rRNA genes and comparing these sequences to a database of known 16S rRNA sequences, identification of a bacterial isolate can be achieved successfully and accurately.

Protocols based on 16S rRNA sequencing have been devised (Ley *et al* 1998). In addition to determining the source of a bacterial pathogen, it is necessary to design broad-range PCR primers to amplify bacterial 16S rRNA genomic regions (Greisen *et al* 1994). The choice of appropriate primers is somewhat empirical, and a number of forward and reverse primer pairs have been described that hybridise to conserved 16S rRNA gene sequences but include variable divergent diagnostic sequences. Critical parameters in this determination include choosing pairs that are spaced relatively closely and avoiding choosing primers that are both homologous to eukaryotic 16S-like rRNA sequences. Additionally, implementation of this method in the clinical laboratory necessarily involves a set up of separate areas for tissue processing, PCR set up and PCR product manipulations to minimise contamination.

Knowledge of the 16S rRNA sequences of bacterial species has utility beyond clarifying bacterial phylogeny and taxonomy. The sequence data is also ideal for designing DNA probes for rapid detection and enumeration of oral or any bacterial species in clinical environment samples without the need for *in vitro* cultivation. Since 16S rRNA sequences for over 2000 bacterial species are now available, highly specific probes are relatively easy to design. Aligned 16S rRNA sequences are compared to identify the signature regions of 20 to 30 bases in the sequences that are unique for each target organisms or group of organisms. Oligonucleotides generally 20 to 30 bases in length, which are complementary to these signature regions serve as the specific DNA probes for hybridisation reactions. When oligonucleotide DNA probes are used, a single base mismatch in the target region is often sufficient to differentiate target species from closely related species. With other types of DNA probes, such as genomic whole and cloned, cross-reactivity is often observed with closely-related species.

The 16S rRNA molecule has been the focus as this molecule is present in all organisms performing exactly the same function, its sequence must be conserved sufficiently that regions of homology can be clearly established and, it must be sufficiently large that its information content is adequate for making phylogenetic inferences. The 16S rRNA molecule has proven the best candidate molecule identified to date for phylogenetic analysis. Over 4000 sequences have been determined for bacteria and are available from the Ribosomal Database Project, Genbank and other sequence databases. Work on other molecules such as 23S rRNA, RNA polymerase and elongation factor G support the phylogenetic inferences made using the 16S rRNA.



#### **1.19.4 : Primers**

Bacteria specific primers that target nearly all bacteria are available. The 27f and the 1492r are the most widely used primers for PCR amplification and cloning of 16S rRNA genes as they target nearly all bacterial species (Lane *et al* 1991).

27f sequence            5'AGAGTTTGATCMTGGCTCAG3'

1492r sequence        5'TACGGYTACCTTGTTACGACTT3'

Only the reverse primers anneal to rRNA, both the forward and reverse primers anneal to the rRNA genes (M=C:A, Y=C:T).

#### **1.19.5 : Accuracy of PCR studies**

Isaacman *et al.* (1998) assessed the accuracy of a PCR based assay for the detection of pneumococcal bacteraemia in children compared with conventional blood culturing methods. 21/480 patients of the study group had *Pneumococcus* detected by blood culture. PCR was positive in 12 (57%) of the 21 cases in which *Streptococcus pneumoniae* was isolated from the blood culture and in 206 (45%) of the 459 cultures that showed no growth of this organism. If blood culture is used as the gold standard for defining the presence or absence of bacteraemia, PCR showed a sensitivity of 57% and a specificity of 55%. 16% of the 103 control test children tested positive for pneumococcal DNA by the PCR test. The frequency of PCR positive/culture negative cases in this population was significantly lower than that of the study population, 16/103 (16%) versus 206/459 (45%). The authors claimed that although the assay showed excellent *in vitro* activity for the detection of *S. pneumoniae* in whole blood, they were unable to achieve similar sensitivity and specificity when using this assay on clinical specimens.

Rudolph *et al.* (1993) used a nested PCR assay that showed excellent sensitivity using an *in vitro* preparation. When their assay was tested on 16 clinical specimens from patients with culture proven pneumococcal bacteraemia, their assay showed moderate sensitivity (75%) when applied to the buffy coat fraction and poor sensitivity when used on whole blood. This difference in sensitivity using differing fractions of blood can be explained as most clinically significant bacteraemias in humans are characterised by low numbers of circulating bacteria, which is possibly due to phagocytosis. The clearance of viable and non-viable bacteria from blood depends on many factors, including opsonisation and phagocytosis. Consequently, isolated and thus concentrated phagocytic cells should provide an excellent source of bacterial pathogen and target DNA.

Salo *et al.* (1999) attempted the detection of *Pneumococcus* by PCR and found that more samples were detected by PCR than by culture in both the whole blood and buffy coat groups in the early phase bacteraemia samples. However, the lower sensitivity in the buffy coat compared to whole blood samples which did not agree with Rudolph's findings may be due to the loss of target DNA during the purification of leucocytes. Phagocytosis may also be incomplete at this early time point after challenge and thus decrease the sensitivity when the buffy coat fraction was used. The serum samples yielded fewer positive PCR findings at this time point compared to the whole blood or buffy coat samples. These studies by Salo *et al.* have thus shown that the PCR method is capable of detecting *Pneumococci* even in culture-negative blood samples and thus warrants further research. This study also showed clearly whole blood to be the best source for the amplification of bacterial DNA.

Cursons *et al.* (1999) looked at the use of PCR to detect septicaemia in critically ill patients using two different DNA extraction techniques sequentially. Using the initial extraction method (n=101) ten patients were positive by both PCR and blood culture, eight

were PCR positive and blood culture negative and seven were negative by both methods. From the clinical criteria, PCR detected at least six true positives that had been missed on blood culture. An improved extraction procedure was then utilised in which ten patients were positive by PCR and blood culture, 29 patients were PCR positive and blood culture negative and two patients were PCR negative and culture positive.

Most work that has been published on bacterial PCR techniques has concentrated on the detection of a few specific organisms such as *Pneumococci* or *Meningococci* (Isaacman *et al*, 1998; Salo *et al*, 1999; Zhang *et al*, 1995). Little clinical work has been published regarding use of PCR of conserved nucleotide sequences as a generic screening test to detect the possible presence of a wide range of bacterial species (i.e. universal primers for nearly all bacterial species).

#### ***1.19.6 : Sensitivity of PCR versus conventional blood culture***

A large proportion of patients who appear clinically septic have negative blood cultures (Pizzo *et al*, 1993; Bodey *et al*, 1986; Riikonen *et al*, 1993). Patients may be bacteraemic but for a variety of reasons, the organism does not grow in the culture medium. As mentioned previously, this could be attributable to the transient nature of bacteraemia, antibiotic treatment, an insufficient volume of blood in the sample, known and unknown bacteriostatic factors i.e. phagocytic, complement or antibody activity in the blood sample (Yagupsky *et al* 1990) or fastidious organisms which lose viability in the culture medium.

Blood culture (BC) is regarded to be of sufficient sensitivity to detect between one and ten viable bacteria per millilitre of blood. PCR has a similar sensitivity although it is speculated that in samples which are PCR positive and blood culture negative, PCR has detected bacteraemic episodes of organisms that may or may not have been non-viable and

intracellular phagocytosed bacteria (Kane *et al* 1998). Under ideal conditions, PCR can detect a single copy of a target DNA sequence in a given sample. Mariani *et al.* (1998) found that the PCR technique was much more sensitive than conventional culture for the detection of bacteria in synovial fluid, virtually doubling the number of positive results. Indeed Cursons *et al.* (1999) looked at the use of PCR to detect septicaemia in critically ill patients and found that the use of PCR to amplify a conserved region of the 16S rRNA gene appeared to reliably detect the presence of bacteria in the bloodstream in a significant number of patients who were missed using routine blood culture techniques. They found the yield could be increased almost fourfold.

Zhang *et al.* (1995) investigated the detection of *Streptococcus pneumoniae* in blood by PCR. To evaluate the effectiveness of the PCR as a diagnostic test, a blinded prospective clinical trial in which 36 blood specimens were evaluated by culture and PCR for evidence of bacteraemia was conducted. These results showed a sensitivity of 80% (4 of 5 culture positive specimens were PCR-positive) and a specificity of 84% (26 of 31 culture-negative specimens were PCR-negative) for the PCR when compared with blood culture as the gold standard. Zhang *et al.* (1995) found only one of their specimens to be blood culture-positive and PCR-negative, while five of the specimens were PCR-positive and culture-negative. The clinical data on the PCR-positive culture-negative discordant cases indicate that the PCR-based assay may be more sensitive in the detection of *S. pneumoniae* bacteraemia.

BC is considered the gold standard for detecting microorganisms in the blood. Nevertheless, many clinicians and microbiologists are concerned that BC e.g. for critically ill patients receiving antimicrobial chemotherapy could indicate lower rates of bacteraemia than are actually present. Antibiotic binding devices such as the Bactec Plus F system were developed in response to these concerns. However, the rate of detection of bacteria

in blood specimens has not changed substantially. Heininger *et al.* (1999) investigated *Escherichia coli* detection by PCR and blood culture and found that after bacterial challenge the rate of detection of *E. coli* by the three methods decreased in a time dependent manner as found in previous studies using conventional culture. Even after 210 min the *E. coli* PCR was positive for 61% of the 13 blood samples whereas BC with the Bactec system and the direct plating method were positive for 53 and 8% of the samples, respectively. No significant difference was observed between PCR and BC for the detection of *E. coli* at any of the examination times but the rate of detection by the direct plating method was noticeably lower than that by either PCR or BC. The difference in detection rate between PCR and BC as described by Heininger *et al.* (1999) was not due to higher sensitivity of PCR than BC. Regardless of whether PCR or BC is used the ability to detect bacteraemia depends on the presence of at least one microorganism in the blood being sampled. When tested *in vitro* both methods detected as little as 1 cfu of *E. coli* per ml of blood, a volume which is normally used for diagnosing bacteraemia. Minimal detection levels of BC systems *in vitro* have yet to be reported. Since the number of pathogens is less than 1 microorganism per ml of blood in 62% of all adult patients with *E. coli* bacteraemia and can be less than 0.04 organism per ml of blood, large blood volumes (20-30ml) have to be used in order to avoid false-negative results. Thus even though the PCR method is designed for smaller blood volumes, it may have to be adapted accordingly. This problem may be solved with the rapidly increasing clinical use of PCR technology to detect pathogens which could not be isolated by conventional methods.

### ***1.19.7 : Detection of bacteraemia in association with antimicrobial agents***

Heininger *et al.* (1999) also found the sensitivity of BC decreased significantly during experimental *E. coli* bacteraemia in rats receiving doses of cefotaxime. When blood was taken 15 min after cefotaxime was administered IV, only 10% of the samples were found to be positive by BC whereas 100% of the samples were found to be positive by PCR. This finding that BC is far less efficient than PCR in detecting bacteraemia during antimicrobial treatment is most probably due to the killing of *E. coli* by cefotaxime; killed bacteria are not detectable by BC while PCR detects bacterial DNA independently of viability. In addition, the higher sensitivity of PCR compared to conventional BC techniques is due to the fact that intracellular or phagocytosed microorganisms are also detectable by PCR. The direct plating method was used to quantify bacterial numbers in *E.coli*-challenged blood. This method corroborated the results obtained by PCR and BC with respect to the rapid clearance of *E.coli* from the bloodstream. The lower sensitivity of the direct plating method compared with PCR or BC may be due to the smaller sample volume and a lack of dilution of bacterial growth-inhibiting factors in the blood which are known to influence bacterial recovery rates in BC. In addition, BC acts as an additional amplification step thus direct plating from the BC bottle would not be worthwhile to determine cfu.

Ley *et al.* (1998) used PCR in the detection of bacteraemia in patients with fever and neutropenia using a broad diagnostic approach (eubacterial) 16S rRNA gene amplification and sequencing to augment cultural diagnosis of bacteraemia. 111 patient episodes of fever and neutropenia were evaluated during the study, of which 17 were associated with positive cultures. PCR detected bacterial DNA in 9/11 blood culture positive episodes for which a sample was available for PCR. On species identification by sequence analysis, isolates were identical to those derived from the conventional identification of the

cultured organisms. Bacterial DNA was detected in 20 episodes associated with negative blood cultures, of which 18 occurred in patients who were receiving antibiotics at the time of sample collection.

The finding that following antimicrobial therapy, despite killing most of the bacteria in the bloodstream, bacterial DNA still persists may be of importance. Extracellular bacterial DNA has been considered to be immunologically inert in mammals, however it has been recently shown that bacterial DNA has substantial immunostimulatory properties comparable to those of endotoxin and that its presence can cause sepsis-like symptoms in mice. Thus methods for detecting DNA might improve our understanding, and PCR could prove to be a useful adjunct tool supplementing conventional BC techniques in diagnosing bacteraemia.

#### ***1.19.8 : Which blood component should be used for PCR?***

A major barrier to the direct use of body fluids in PCR is the presence of inhibitory agents in the samples. Numerous approaches have been taken to overcome this problem. Ni *et al.* (1992) demonstrated a sensitivity and specificity of 91% in the detection of *Neisseria meningitidis* by PCR after boiling of clinical cerebral spinal fluid (CSF) samples. This approach of using heat denaturation of proteinaceous inhibitors has been widely employed in the preparation of non-blood body fluids for PCR analyses. Direct detection of human cytomegalovirus has been performed with urine samples from which the PCR inhibitor has been removed by either dialysis or ultrafiltration. However, these examples do not involve whole blood specimens, and it has been repeatedly demonstrated that certain components of whole blood, particularly the porphyrin ring of the haemoglobin molecule, can inhibit the PCR amplification process by binding to the *Taq* DNA polymerase. Unlike the case for CSF, which can constitute up to 10% of the reaction mixture without significantly

inhibiting the PCR process, final blood concentrations of less than 1% have been shown to be strongly inhibitory to the PCR process. The majority of PCR based detection methods for blood borne pathogens have used only selected fractions from whole blood in order to overcome this problem. Intracellular pathogens can be detected by PCR when using only leucocytes. Likewise, true extracellular pathogens can be detected by using serum or plasma. However, for complete recovery of low-copy-number pathogens which may be present in multiple blood fractions, the use of a single blood component will reduce the sensitivity of the assay.

### ***Comment***

It was hypothesised that improved techniques for diagnosing bacteraemia are needed and that amplification of bacterial DNA by PCR could overcome some of the problems associated with conventional BC techniques. The potential of PCR technology to recover intracellular or non-growing microorganisms may be useful for diagnosing bacteraemia. Although PCR has already been successfully applied in the detection of fastidious microorganisms, in particular for bacteraemia a direct comparison of BC techniques with PCR during bacteraemia has not been performed *in vivo*.

To obtain complete recovery of microbial DNA we used a method for the preparation of DNA samples from whole blood (Qiagen whole blood DNA extraction kit). In this process no blood components were eliminated prior to the DNA purification.



### **1.20 : Lipopolysaccharide detection : limulus amoebocyte lysate assay (LAL)**

Limulus amoebocyte lysate (LAL) is an aqueous extract of the blood cells of the horseshoe crab, *Limulus polyphemus*. The original test was based on the fact that when bacterial endotoxins are incubated at 37 °C with LAL, a firm clot or gel is formed. This technique was commonly referred to as the LAL test and was used to detect and quantitate bacterial endotoxins.

The clotting of the *Limulus* blood was first described in 1885 by W.H. Howell. The amoebocyte is the sole element in the blood of *Limulus polyphemus*. It is a nucleated cell, the cytoplasm of which is packed with granules. Although the important role of amoebocytes in the coagulation of the blood of *Limulus* had been recognised for many years, the important discovery which resulted in the LAL test was made by Frederick Bang in 1956. He observed that the blood of the horseshoe crab clotted *in vivo* when the crabs were injected with live or heat killed marine Gram-negative bacteria. He also demonstrated the gelation of *Limulus* serum *in vitro* when treated with Gram-negative bacterial or lipopolysaccharides (endotoxin) extracted from their cell walls.

Amoebocyte lysate, prepared from washed amoebocytes which have been disrupted by either freezing and thawing or by distilled water, will form a gel on incubation with bacterial endotoxin. The rate of reaction is dependent upon the concentration of endotoxin present. From their original observations, Levin and Bang postulated an enzymatic method for the gel formation produced by the endotoxin-LAL interaction. Further studies have confirmed this hypothesis. Endotoxin in the presence of divalent calcium ions activates a pro-clotting enzyme to produce an active enzyme which is a serine protease. The activated enzyme acts on a clottable protein (coagulogen), resulting in limited proteolysis of this protein. This is followed by polymerisation and gel formation.

More recently the LAL assay has been modified for use with chromogenic substrate. Where the gel-clot assay was subjective and at best semiquantitative, the chromogenic assay is both objective and quantitative. The modification is based on the principle that endotoxin-activated lysate releases from the colourless substrate yellow *p*-nitroaniline (pNA) which can be measured spectrophotometrically. The intensity of colour generated is proportional to the quantity of endotoxin present. This assay permits the detection of 0.2pg endotoxin per ml in water and 1pg endotoxin per ml in plasma.

Similarities have been demonstrated between the mammalian coagulation system and the precoagulant enzyme of the horseshoe crab, and have supported the suggestion that blood coagulation in the horseshoe crab is an example of a primitive system from which human blood coagulation has developed. Furthermore it is interesting to speculate that the reaction of *Limulus* blood to endotoxins may be a defence mechanism, which has enabled the horseshoe crab to survive infection over the millions of years of its existence in an aquatic environment which has been shown to have a large population of Gram-negative bacteria. This is of particular interest regarding the recent association of LPS of periodontal organisms with cardiovascular disease.

Both rabbit and man exhibit a similar threshold dose of 0.1-1ng/kg for a pyrogenic response (Greisman *et al* 1967). Hence the current officially recognised procedure in the European Pharmacopoeia for detection of endotoxin is the rabbit pyrogenicity test. This test is mandatory for all fluids and drugs intended for parenteral administration to man. This test however, has been criticised for its high cost and poor precision (Bangham *et al* 1971). The *Limulus* test is approximately 5 to 100 times as sensitive as the rabbit pyrogen test (Tomasula *et al* 1977, Müller *et al* 1982).

### ***1.20.1 : Uses of the LAL assay***

In contrast to the pharmaceutical applications, the clinical and experimental uses of the assay are not as well established. The LAL assay has been used to detect endotoxin in a variety of body fluids namely urine, CSF, urethral and cervical exudates, ocular exudates and blood. However, there are problems associated with the measurement of endotoxin in blood, owing to the presence of inhibitors in plasma. These endotoxin inhibitors influence endotoxin-LAL activity and have to be inactivated. Esterases, complement, antibody, lipoprotein and  $\alpha$ -globulin have all been implicated. Removal of these interfering substances can be achieved by several different procedures, of which the most favoured is dilution and heating of the plasma sample (Du Bose *et al* 1980). Accidental contamination with exogenous endotoxin is possible at every stage of the assay procedure, from venepuncture to the termination of the assay with acetic acid. Thus it is essential to use pyrogen-free equipment and procedures throughout.

### ***1.20.2 : Which type of blood sample to use***

Several authors do not find significant differences in LAL assays using platelet rich plasma (PRP) and platelet poor plasma, although there is experimental evidence that PRP improved detection of endotoxin in plasma (Das *et al* 1973). Endotoxin has also been shown to bind to platelets (Herring *et al* 1963). Thus the use of PRP may allow estimation of low levels of endotoxin, which would otherwise remain undetected.

### ***1.20.3 : Assessment of the LAL assay***

In a paper by Berger *et al.* (1988) the clinical usefulness of the LAL test was evaluated. Even after phenol-water extraction of serum, which completely eliminates serum constituents inhibiting or amplifying the test, reliable results could not be achieved.

Furthermore, the insufficiency of heat inactivation, dilution with pyrogen-free water and precipitation with perchloric acid as recommended in the manuals of the tests, was obviously demonstrated. Neither of the procedures was able to eliminate the serum factors disturbing the test. However, the authors concluded that the LAL test represents a reliable scientific instrument for measuring endotoxin blood levels and for elucidating the role of endotoxin in septic disease.

A study by Elin *et al.* (1975) investigating the effectiveness of the LAL assay showed that 48 plasma samples out of 237 yielded positive results. Variation in the sensitivity of different batches of lysates was noted. Out of the 29 blood cultures which were positive, 12 of these cultures were associated with a positive test with 17 positive blood cultures associated with negative tests. In addition, 36 plasma samples yielding positive tests were associated with sterile blood cultures. Studies on the LAL assay on human plasma have focused on the relation of the test results and positive blood cultures containing Gram-negative bacteria. Assuming that a greater concentration of endotoxin is more likely to produce a positive test, a relationship should exist between the concentration of endotoxin in the blood and the probability of a positive test result. The authors hypothesised that there is probably not a sufficient amount of endotoxin from the bacteria alone to give a positive test result in Gram-negative bacteraemia. Quantitative studies of Gram-negative bacteraemia indicate that most patients have fewer than 100 organisms per millilitre of blood. The authors concluded that since more than 1000 gram-negative bacteria per millilitre are required for a positive LAL test and the endotoxin bound to bacteria and free endotoxin have similar levels of activity in the test, it seems unlikely that the amount of endotoxin present in the blood in Gram-negative sepsis could produce a positive Limulus test. Therefore, a positive test on human plasma, if it is due to endotoxin, is probably caused by endogenous liberation of endotoxin into the blood or a combination of

endogenous endotoxin and Gram-negative bacteraemia. The authors also suggested since endotoxin injected intravenously into an intact animal is cleared very rapidly by the liver, liver impairment may be a prerequisite for a positive Limulus test. Previous studies have shown positive test results without septicaemia in the presence of impaired liver function. Stumacher *et al.* (1973) assessed the diagnostic value of the test to evaluate the role of endotoxin in infections due to Gram-negative bacteria. Limulus assays were performed on 694 specimens collected during 344 episodes of suspected bacteraemia and compared with qualitative and quantitative blood cultures. Positive assays were obtained from 28 of 65 patients (43%) with bacteraemia due to Gram-negative bacilli and in 11 out of 43 patients (26%) with localised infections due to Gram-negative organisms. However the occurrence of positive assays in eight of 22 patients (36%) with infections due to Gram-positive bacteria who had no concomitant infections with Gram-negative organisms, casted doubt on the diagnostic value of the assay. In addition, no correlation was found between positive assays and the number of circulating Gram-negative bacilli or the occurrence of shock or death.

In addition Elin *et al.* (1975) revealed a clear difference among lysates prepared in different laboratories. These early studies reported that inconsistencies in the clinical value of endotoxin tests might be partially explained by variations in sensitivity of LAL-batches, variability of gel formation and the presence of inhibitors in plasma. It should be remembered, however, that Elin's assessment of the LAL assay was based on the gel test, not the newer test using chromogenic substrate. Also, different methods for the removal of these inhibitors were used such as chloroform extraction or dilution and heating. Evidently the quantity of endotoxins usually was not sufficient to result in a positive LAL test in most Gram-negative bacteraemias. However, the sensitivity of the endotoxin test was enhanced by the introduction of chromogenic assay methods. Such assays are

potentially of clinical relevance because of their short assay time compared with blood cultures, thus allowing earlier diagnosis of Gram-negative bacteraemia. Also, these tests theoretically may detect a clinically relevant endotoxaemia in the absence of circulating intact Gram-negative bacteria. They revealed that 4 lysate preparations were unable to detect about 75% of the positive samples. This may have been due to these lysate preparations not being frozen or lyophilised as they are now, and it may be that changes in the physical state of a lysate decrease its sensitivity.

Studies by Levin (1975) who developed the assay suggested that the test was of diagnostic value in infections with Gram-negative bacilli. However, they found that patients with fungaemia provided a positive assay, and negative assays could arise in patients with bacteraemia due to Gram-negative organisms. During 344 episodes of illness prompting collection of blood for cultures, 694 concomitant venous-blood specimens were obtained for culture and Limulus assay. Among the 65 patients with bacteraemia caused by Gram-negative bacilli, the Limulus assay was positive in 28 (43%) and negative in 37(57%). There were 43 localised Gram-negative infections not associated with bacteraemia. The assay was positive in 26% and negative in 74% of these patients. Nine other patients had either fungaemia or bacteraemia with Gram-positive cocci in addition to clinically apparent localised infections caused by Gram-negative bacilli. Seven of these patients also had positive Limulus assays. Of greater importance, however, was the finding of a positive Limulus assay in 36% of patients with bacteraemia caused by Gram-positive cocci in whom there was no clinical or cultural evidence of concomitant infection due to Gram-negative bacilli. In addition, positive assays occurred in specimens obtained from 21 patients in whom no infection could be detected during hospitalisation, despite intensive investigation. A positive Limulus assay was obtained in 75 of the 344 patients studied and

216 patients had both negative blood cultures and Limulus assays. Positive assays occurred in 30 to 40% of bacteraemias due to Gram-negative organisms. However, a similar prevalence of positive assays was observed in bacteraemias caused by *Streptococci* and coagulase-positive *Staphylococci*. Concomitant quantitative blood cultures and Limulus assays were performed on specimens from 57 patients with bacteraemia due to Gram-negative bacilli. It was possible to recognise approximately one bacterium per 5 or 10ml of blood in routine cultures whereas quantitative cultures indicated bacterial numbers in excess of 10 bacilli per millilitre of blood. The frequency of positive Limulus assays bore no relation to the number of Gram-negative bacilli per millilitre of blood during bacteraemia, and patients with more than 250 bacilli per millilitre of blood did not have positive assays more often than those with less than 10 bacilli per millilitre. The lack of correlation between the number of bacteria in quantitative blood cultures was most apparent among patients with bacteraemia of the greatest magnitude. Three of the four patients whose blood contained 10,000 or more Gram-negative bacilli per millilitre of blood had negative assays. Previous problems with the LAL assay were concerned with different investigators interpretations of what was considered a definitively positive reaction as it was a purely subjective decision with the old test. With one study, the authors classified a positive test as 'definite gelation' compared with another group of investigators who relied on a definite increase in viscosity and turbidity before inferring a positive result.

The finding of positive assays in 36% of patients with bacteraemia due to Gram-positive organisms unassociated with infection due to Gram-negative organisms and positive assays in a small proportion of patients with no detectable infections, however, casted considerable doubt on the test's diagnostic value. Again it should be remembered that this study was based on the older gelation test rather than the chromogenic assay. 39% of

positive assays occurred in patients with no detectable infection or in patients with bacteraemia due to Gram-positive bacilli without associated infection with Gram-negative organisms. The occurrence of false-positive reactions with an assay as sensitive as this technique might be considered by some to result from endotoxin contamination and negative controls would eliminate this.

Earlier studies have also reported positive assays in subjects with localised infections due to Gram-negative organisms without bacteraemia and negative assays in patients with bacteraemia due to Gram-negative bacilli. These findings have been attributed to the release of endotoxin into the blood, but retention of Gram-negative bacilli within the local lesion in one report, and escape of Gram-negative bacilli and retention of endotoxin in the other. Although such explanations appear justifiable on an individual basis, it is difficult to comprehend how release of endotoxin but retention of bacteraemia (endotoxaemia without bacteraemia) and release of bacteria with retention of endotoxin (bacteraemia without endotoxaemia) could both result from similar focal infections.

A study by Cardis also observed positive Limulus assays in patients without detectable infections. However they still considered the assay to reflect endotoxaemia. They attributed these false positive reactions to endotoxin that had been released from the gastrointestinal tract and could not be cleared by a poorly functioning reticuloendothelial system. Since no other substances have yet been identified that will induce a positive Limulus assay, and since endotoxin release from the gastrointestinal tract has been demonstrated experimentally, the explanation that endotoxaemia may result from a variety of causes other than infections due to Gram-negative organisms is conceivable.

A comparative study investigating the LAL assay with blood culture was performed by Thomas *et al.* (1984) in 51 critically ill patients. Of 400 samples tested, the endotoxin



assay and bacterial culture were both negative for 342 samples. In 21 samples from 15 patients, Gram-negative aerobic microorganisms were cultured. Corresponding endotoxin assays were positive in 14 samples, and Gram-positive bacteria were isolated from 23 samples. The associated endotoxin assays were all negative. 12 samples were found to be endotoxin-positive without a corresponding Gram-negative bacterial culture. In 7/12 of these positive endotoxin assays, a laboratory or clinical explanation for these positive tests could be provided e.g. patients receiving antibiotic therapy. The authors determined that with the high sensitivity, specificity and predictive values obtained, the endotoxin assay was a useful clinical adjunct for both the detection and exclusion of Gram-negative septicaemia.

Quantitative studies in Gram-negative bacteraemia (Finegold *et al* 1969) had shown that most blood samples contained less than 100 bacteria per millilitre. Since more than 1000 Gram-negative results were formerly required for a positive endotoxin test (Elin *et al* 1975, Jorgensen *et al* 1973), it was considered unlikely that endotoxin could be detected in most Gram-negative bacteraemias (Elin *et al* 1975). Bacterial quantification in a total of 233 samples showed only four samples to be culture-positive and endotoxin-positive. Up to 12 bacteria were found, but clearly the small number of culture-positive results did not allow any definite conclusions regarding the number of circulating Gram-negative bacteria required for a positive endotoxin assay. To determine the clinical relevance of the chromogenic endotoxin assays for the detection of Gram-negative septicaemia, these assays are always compared with the blood culture. It is questionable whether this is realistic. Endotoxaemia may cause clinical symptoms suggestive of Gram-negative septicaemia in the absence of detectable bacteraemia. This endotoxaemia may be the result of bacteria outside the circulation generating endotoxins, which subsequently gained access to the circulation. E.g. intestinal hypoxia (Cuevas *et al* 1973) and vasoactive

substances may promote transmural escape of endotoxins from the colon. Moreover, bacteraemia is a transient phenomenon (Levin *et al* 1982). This may explain why endotoxaemia may be observed with negative blood cultures in patients with clinically evident septicaemia. The opposite can also be observed i.e. positive blood cultures and negative endotoxin tests in one third of positive cultures. It is likely a low number of circulating bacteria would not produce an endotoxin level above the detection level of the assay.

### **1.21 : Aims**

It is obvious from the preceding section that the literature concerning dental bacteraemia is vast. As can be concluded from the literature review, many factors influence the detection of bacteraemia, few studies are comparable and a proportion of the literature is outdated. Most studies are involved with oral surgical procedures which are assumed to give high bacteraemia values. Few papers have been concerned with periodontal disease, its treatment and bacteraemia incidences resulting from instrumentation.

The aims of this study were:

1. To determine the effect of timing of blood sampling on detection of bacteraemia and to determine the incidence of bacteraemia following various routine periodontal procedures, namely; periodontal probing, toothbrushing and cavitron scaling.
2. Detection of bacteraemia would be detected by three means : Conventional blood culture using the Bactec culture bottle system (Organon Teknika) which is regarded as the gold standard, and further identification of these isolates using conventional microbiology. Molecular biology, specifically Polymerase Chain Reaction was performed on these samples to compare and contrast the two detection methods and their relative sensitivity and specificity. Given the recent interest in periodontal pathogens, LPS and their roles in systemic disease, the Limulus Amoebocyte Lysate assay was also utilised to measure the levels of LPS in the blood samples obtained.
3. A further aim of the study was to compare chlorhexidine with fluoride mouthwash to determine whether any reduction in bacteraemia between patients could be elicited. It was unlikely a statistically significant result would be achieved due to the low number of subjects recruited, but any observed trends could indicate the feasibility of a larger study in the future.

***CHAPTER 2***  
***SUBJECTS, MATERIALS AND METHODS***

## ***PART 1 : SUBJECTS***

### ***2.0 : ETHICAL APPROVAL***

Ethical approval for the study was obtained from the North Glasgow University Hospital NHS Trust. Volunteers were invited to participate in the study after reading an information sheet and giving written informed consent. The study was designed and conducted according to guidelines for Good Clinical Practice.

### ***2.1 : PATIENT SELECTION***

Male and female subjects (n=40) over 18 years of age were selected from University of Glasgow Dental School Department of Periodontology consultant clinic referral pool.

#### ***2.1.1 : INCLUSION AND EXCLUSION CRITERIA FOR SELECTION OF SUBJECTS***

Patients diagnosed as suffering from adult periodontitis were selected for inclusion into this study. Further inclusion / exclusion criteria were:

- patients were at least 18 years of age to give informed, written consent to participate
- were willing to give informed consent and conform with the protocol
- had veins suitable for blood samples as assessed by the investigator
- possessed a minimum of 20 teeth to allow sufficient instrumentation to induce a bacteraemia
- exhibited previously untreated moderate to severe chronic adult periodontal disease (CPITN 3 or 4) as defined by the referral letter from the GDP, radiographs or previous periodontal charting. This was confirmed by clinical examination at visit 1. Any

patients who on examination did not display this level of disease were withdrawn from the study.

Participation in the study was not allowed if the subject:

- would be put at risk by participating in the clinical study (e.g. subjects with abnormal haematological profile or those at risk from dental bacteraemia)
- had a medical or dental condition which may interfere with the clinical assessments
- was taking medication which may interfere with the clinical assessments
- had a history of infectious disease (e.g. hepatitis, HIV) drug abuse or allergy to dental products
- had an incompatible dentition e.g. orthodontic bands, partial dentures or teeth unsuitable for extensive cavitation scaling.

## ***PART 2 : MATERIALS AND METHODS***

### ***2.1.2 : CLINICAL PROTOCOL***

#### ***Visit 1***

At the initial visit, verbal and written explanation of the background to the study was given with its objectives and subject involvement highlighted. If they wished to participate, following the collection of written informed consent and a detailed medical history from the subjects, a baseline blood sample was obtained (sample 1). Following a general oral examination, a full mouth periodontal probing depth chart was then taken using a PCP 12 periodontal probe. Periodontal probing depth scores were recorded in addition to bleeding on probing scores. 6 points on each tooth were examined as described by Jenkins and Allan : mesiobuccal, mid-buccal, distobuccal, mesiolingual, mid-lingual and distolingual. Mobility scores were recorded by rocking each tooth between an instrument handle and index finger in a buccolingual direction (and mesiodistal direction when no adjacent tooth was present). The amplitude of tooth movement of the crown tip from its most extreme buccal (or mesial) position to its most extreme lingual (or distal) position was observed : Grade 1 mobility - visible horizontal mobility up to 1mm, Grade 2 - visible horizontal mobility between 1 and 2mm and Grade 3 mobility - visible horizontal mobility greater than 2mm or rotation or vertical mobility. Gingival recession and loss of attachment level scores were also recorded.

A blood sample was taken following this procedure (sample 2). Each subject was allocated a mouthwash according to a random block assignment chart in the order in which they attended. The subjects were assigned one of two commercially available mouthwashes, either Boots regular fluoride mouthwash 500 ml or the positive control (chlorhexidine 0.12% 300ml). Boots regular fluoride mouthwash was used as it is a proprietary brand mouthwash not containing chlorhexidine. It is known that chlorhexidine

is the most effective plaque inhibiting mouthwash and is regarded as the gold standard chemical of anti-plaque agents. In this proof of principle experiment to determine the effects chlorhexidine may have on bacteraemia, a mouthwash not containing chlorhexidine such as a regular fluoride was used as an adequate control. The mouthwashes were packed in their original containers but overwrapped and coded differentially. The mouthwash product was allocated by a dental nurse so blindness was maintained. The subjects were advised to use 10ml of their allocated mouthwash twice daily for 14 days in addition to normal oral hygiene. It was highlighted to the subjects that they were to use the mouthwash at least two hours before or after toothbrushing. Subjects were also given supplies of toothpaste (Signal Unilever) and a toothbrush to use during the study.

It was reinforced to the subjects that they were not allowed to use any other mouthwash or toothpaste other than those supplied for the duration of the study, but may brush or floss their teeth as usual. Subjects beginning a course of medication during the study were asked to inform the clinical assessor so that their continued eligibility to participate could be assessed.

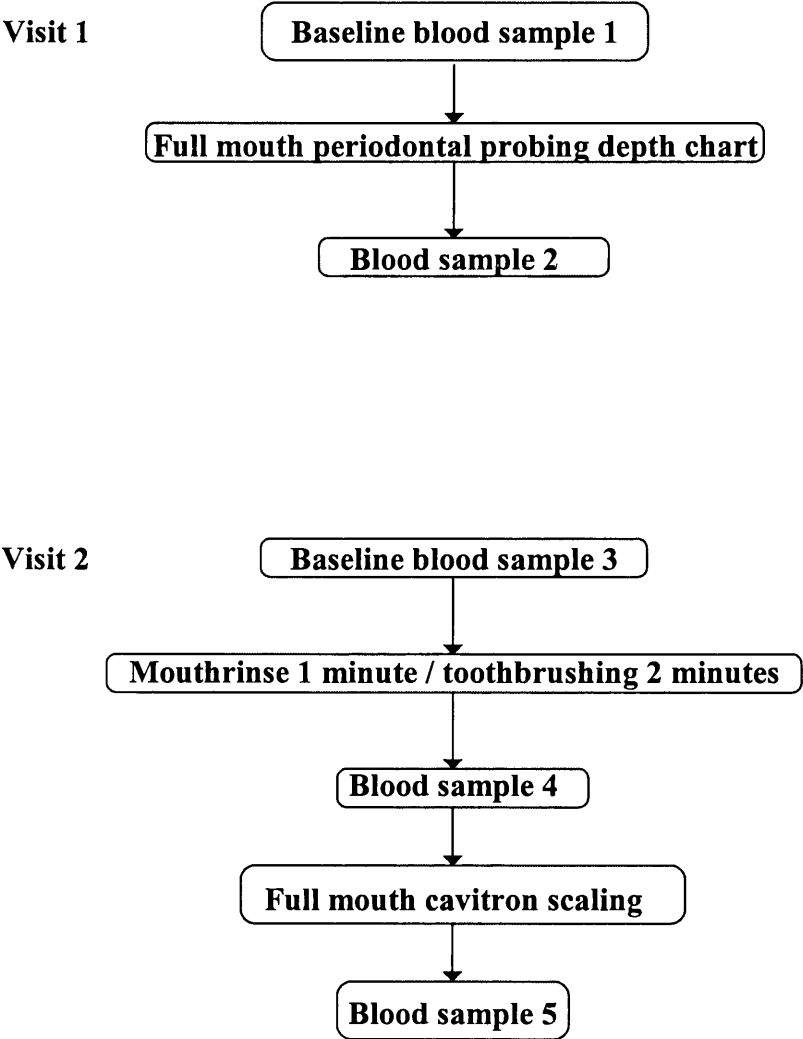
### *Visit 2*

At visit 2 compliance with the protocol was checked verbally and in addition, the volume of mouthwash remaining was measured by a nurse to give a quantitative indication of how well the protocol was adhered to. If non-compliance was detected (by less than 280ml of mouthwash use, or the subject admitting to deviating from the protocol) the blood samples were not taken and the subject was excluded from the study. If compliance was shown, a baseline blood sample was taken (sample 3). The patient was then asked to rinse with their assigned mouthwash for 1 minute, following which supervised toothbrushing for 2 minutes was undertaken. A blood sample was then taken (sample 4). A full mouth



cavitron scaling was then performed following which the final blood sample was taken (sample 5).

**Diagram 1: Summary of Clinical Protocol**

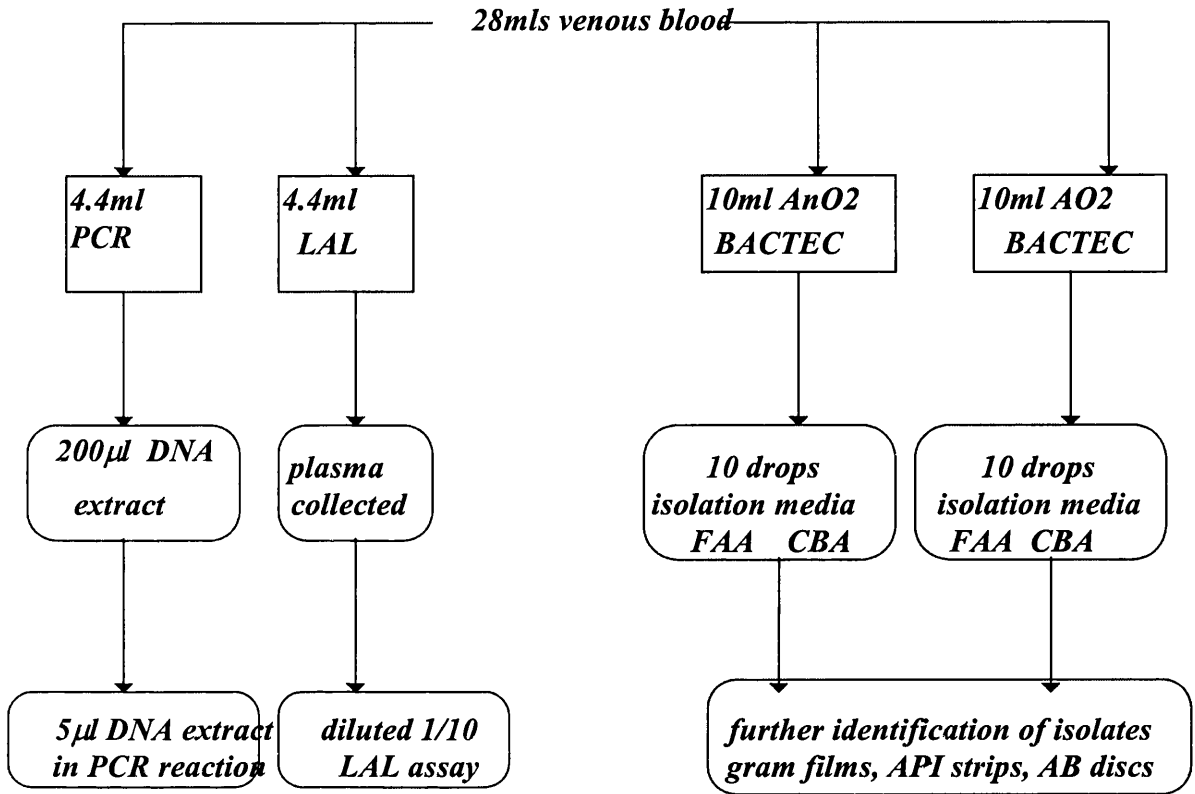


### **2.1.3 : BLOOD SAMPLES**

Blood was obtained from veins in the antecubital fossa. Prior to each sampling the site was wiped with alcohol to minimise the number of potential skin contaminants. Each sample comprised 28ml of blood obtained using a 19 gauge butterfly and safety lock blood collection set (0.8x19mmx178), 20ml plastipak syringe and vacutainer holder which were all attached to a Connecta TH three way stopcock (*Figure 1*). Two 4.4ml hemogard vacutainer EDTA tubes (Becton Dickinson, Oxford, UK) were used for the collection of samples required for PCR and LAL analyses. Whole blood was used for the DNA extraction procedure prior to PCR. It was noted that the EDTA concentration in the vacutainer collection tubes was below the level indicated in the literature (4mM+) to be inhibitory to PCR. Plasma was obtained for the LAL assay by centrifugation for 15 minutes at 1700rpm. Both samples were subsequently frozen and stored at -70°C. In addition, 20ml of venous blood withdrawn into the syringe was used to inoculate 2 Bactec bottles (Organon Teknika Cambridge, UK): 10ml inoculated into an FAN aerobic (1041) and FAN anaerobic culture bottle (1042) by means of Microlance 3 needle. The Bactec bottles were then transported to the Bacteriology Department, Glasgow Western Infirmary for incubation and continuous monitoring for the presence of microorganisms. The BacT/Alert Microbial Detection system utilises a colorimetric sensor and reflected light to monitor the presence and production of CO<sub>2</sub> that is dissolved in the culture medium. If microorganisms are present in the sample, CO<sub>2</sub> is produced as the organisms metabolise the substrates in the culture medium. When growth of the microorganisms produces CO<sub>2</sub>, the colour of the bottle sensor changes from green to yellow. Samples indicated positive were then transported back to Glasgow Dental Hospital for subsequent analysis. Negative bottles were also collected and it was hoped by testing both positive and negative bottles by PCR, the sensitivity of conventional BC and PCR could be estimated.

Diagram 2 :

**BLOOD SAMPLES**



## 2.2 : CONVENTIONAL MICROBIOLOGY : Bactec culture bottles

Bactec® FAN® Aerobic and Anaerobic culture bottles were utilised in conjunction with the BacT/Alert Microbial detection system for recovery and detection of both aerobic and anaerobic microorganisms from blood. The Bac T/Alert Microbial Detection System can be utilised to determine if microorganisms are present in blood or other normally sterile body fluid samples taken from a patient suspected of having a bacteraemia/fungaemia. The BacT/Alert System and culture bottle provide both a microbial detection system and a culture media with suitable nutritional and environmental conditions for organisms commonly encountered in blood infections. In particular, growth performance for the anaerobic and aerobic bottles was performed by the manufacturers using NCCLS recommended strains of particular commonly isolated medical pathogens:

<b>TEST ISOLATES: BACTEC ANAEROBIC BOTTLES</b>
<i>Bacteroides fragilis</i> ATCC 25285
<i>Bacteroides vulgatus</i> ATCC 8482
<i>Clostridium perfringens</i> ATCC 13124
<i>Escherichia coli</i> ATCC 25922
<i>Peptostreptococcus asaccharolyticus</i> ATCC 14963
<i>Streptococcus pneumoniae</i> ATCC 6305
<i>Staphylococcus aureus</i> ATCC 25923

**Table 2**

<b>TEST ISOLATES BACTEC:AEROBIC BOTTLES</b>
<i>Candida albicans</i> ATCC14053
<i>Escherichia coli</i> ATCC 25922
<i>Micrococcus luteus</i> ATCC 4698
<i>Neisseria meningitidis</i> ATCC 13090
<i>Pseudomonas aeruginosa</i> ATCC 27853
<i>Staphylococcus aureus</i> ATCC 25923
<i>Streptococcus faecalis</i> ATCC 29212
<i>Streptococcus pneumoniae</i> ATCC 6305
<i>Streptococcus pyogenes</i> ATCC 19615
<i>Xanthomonas maltophilia</i> ATCC 13637.

**Table 3**

The Bactec FAN bottles contain 40mls of media and activated charcoal, the presence of which is to inhibit any antibiotic activity which the patient may have been prescribed which would otherwise inhibit the growth of any isolates in the blood sample. An internal sensor is present which detects the presence of carbon dioxide as an indicator of microbial growth. The recovery and detection of different types of microorganisms is dependent on the use of the appropriate culture medium. FAN bottles contain brain heart infusion solids (2.8%), sodium polyanetholesulfonate (0.05%), pyrooxidine HCl (0.001%), menadione (0.00005%), hemin (0.0005%), an activated charcoal (8.5%), L-cysteine and other complex amino acids and carbohydrate substrates in purified water. The anaerobic bottles are prepared with an atmosphere of nitrogen and CO<sub>2</sub> under vacuum. Aerobic bottles are prepared with an atmosphere of oxygen under vacuum.

### ***2.2.2 : Subculturing***

Samples from positive bottles were inoculated by means of sub-venting culture units (Organon Teknika, Cambridge) onto two fastidious anaerobic agar (FAA) plates which were incubated in an anaerobic chamber, two Columbia blood agar (CBA) plates, one incubated in an aerobic environment and one incubated in a CO<sub>2</sub> rich chamber, each for 14 days. These different environments, it was hoped, would provide ideal growth conditions for any isolates and also aid identification if certain organisms were shown to grow preferentially in a particular environment.

Further subculturing was performed to obtain pure growth of a single organism if several differing colonies were shown to grow. This would facilitate further analysis.

Gram films were performed on all isolates to aid identification of organisms in addition to colonial morphology and atmospheric requirements data. For anaerobicity testing, metronidazole discs were utilised on FAA in an anaerobic chamber. Areas of inhibited growth of an organism indicated a true anaerobe and facilitated further testing. The use of API commercial identification kits were used to definitively identify species isolated.

Following identification, all isolates were stored in protect beads and frozen for cryovial storage (Bioconnection, Leeds, UK). This system uses coloured beads in a 'cryovial' containing cryopreservative fluid. After inoculation and storage a single bead can be removed to inoculate culture media and allow regrowth at a later date.

Prior to disposal of negative Bactec bottles, Gram films were performed directly from the bottle. However, Gram filming from Bactec bottles proved problematic due to the presence of charcoal which made bacterial detection troublesome.

### **2.2.3 : Identification of isolates : Gram stain**

1. A heat fixed bacterial smear on a glass slide was covered with crystal violet.
2. After 1 minute, the crystal violet was washed off with water.
3. The bacterial smear was covered with iodine.
4. Following 1 minute, the iodine was washed off with water.
5. The slide was briefly washed for 1 second with ethanol and then the ethanol was washed off with water.
6. The slide was then counterstained with safranin.
7. The smear was washed again, blotted dry and then examined microscopically using oil immersion.

### **2.2.4 : API system**

Identification of pure cultures was performed according to established microbiological methods and taxonomic schemes. Anaerobic isolates were identified using API 20A, streptococcal species were identified using rapid ID 32 Strep (Bio Merieux). A suspension of the bacterial species to be identified is made and inoculated into each of the API wells. Each system uses various standardised and miniaturised enzymatic and biochemical tests (reduction of nitrates, indole production, alkaline phosphatase, arginine arylamidase, proline arylamidase, leucyl glycine arylamidase, pyroglutamic acid arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase, glutamyl glutamic acid arylamidase, serine arylamidase, urease, arginine dihydrolase,  $\alpha$ -galactosidase,  $\beta$ -galactosidase,  $\beta$ - galactosidase 6-phosphate,  $\alpha$ - glucosidase,  $\beta$ -glucosidase,  $\alpha$ -arabinosidase,  $\beta$ -glucuronidase,  $\beta$ -N-acetyl-glycosaminidase, mannose fermentation, raffinose fermentation, glutamic acid

decarboxylase,  $\alpha$ - fucosidase) with a specifically adapted computerised system for analysis of the results.



### ***2.3 : Molecular biology***

#### ***Polymerase Chain Reaction : DNA extraction***

##### ***Blood and body fluid spin protocol : QIAamp™ DNA purification from whole blood***

Qiagen QIAamp™ DNA blood mini kit was purchased from Qiagen Ltd, Crawley, UK. DNA extraction and preparation of the PCR reaction mixture were carried out in separate class II laminar flow cabinets using filter protected pipette tips (Aerogard; Alpha Laboratories, Croydon, UK)

#### ***Preparatory***

Samples were equilibrated to room temperature and a water bath or dry bath heated to 56°C.

#### ***Buffer reconstitution:***

***Buffer AW1*** : Stored at room temperature (15-25°C). 25ml ethanol was added before using for the first time.

***Buffer AW2*** : Stored at room temperature. 30ml ethanol was added before using for the first time.

***QIAGEN protease*** : Stored at -20°C. To avoid repeated freezing and thawing, aliquots of Qiagen protease were stored.

## ***PROTOCOL***

- 1 20 $\mu$ l Qiagen protease was pipetted into the bottom of a 1.5ml microcentrifuge tube.
- 2 200 $\mu$ l of each EDTA whole blood sample was added to the microcentrifuge tubes.
- 3 200 $\mu$ l buffer AL (lysis buffer) was added to each sample. This was mixed by vortexing for 15 secs.
- 4 An incubation at 56°C for 10 mins was performed in a water bath.
- 5 The 1.5ml microcentrifuge tube was briefly centrifuged to remove fluid from the inside of the lid.
- 6 200 $\mu$ l ethanol (96-100%) was added to the sample, and mixed by vortexing for 15 sec. After mixing, the microcentrifuge tube was briefly centrifuged to remove fluid from the inside of the lid.
- 7 The mixture was carefully applied from step 6 to the QIAamp spin column (in a 2ml collection tube) without wetting the rim, the cap closed, and centrifuged at full speed 6000xg (8000 rpm) for 1 min. The QIAamp spin column was placed in a clean 2ml collection tube, and the tube containing the filtrate discarded.
- 8 The QIAamp spin column was carefully opened and 500 $\mu$ l Buffer AW1 added without wetting the rim. The cap was closed and the spin column centrifuged at

6000xg (8000rpm) for 1 min. The QIAamp spin column was placed in a clean 2ml collection tube, and the collection tube containing the filtrate was discarded.

**9** The QIAamp spin column was carefully opened and 500 $\mu$ l Buffer AW2 was added without wetting the rim. The cap was closed and centrifuged at full speed (20000xg, 14000 rpm) for 3 min.

**10** The QIAamp spin column was placed in a clean 1.5ml microcentrifuge tube and the collection tube containing the filtrate was discarded. The QIAamp spin column was carefully opened and 200 $\mu$ l Buffer AE was added. Buffer AE was incubated at room temperature for 1 min, and then centrifuged at 6000xg (8000 rpm) for 1 min.

**NB** 200 $\mu$ l of whole blood yields 3- $\mu$ g of DNA.

Purified DNA samples were run on a 0.8% gel to check the DNA extraction procedure was successful prior to performing PCR (*Figure 19*).

## ***2.4 : Polymerase Chain Reaction***

### ***2.4.1 : Oligonucleotides***

Broad range primers targeting a conserved region of both Gram-positive and Gram-negative bacterial DNA that codes for 16S rRNA were used. The primers were selected to give the broadest specificity, and hence a universal application in the detection of eubacterial DNA. These were synthesised by MWG Biotech (Milton Keynes, UK).

The upstream primer sequence was :

27f : 5'- AGA GTT TGA TC (AC) TGG CTC AG -3'

The downstream sequence was:

1492r : 5'- TAC GG(CT) TAC CTT GTT ACG ACT T -3'

These primers generated a 1540-base pair amplification product.

### ***2.4.2 : PCR amplification preparation***

Prior to performing a PCR reaction all plasticware items (filter tips (Alpha Labs, Eastleigh, UK), 1.5ml centrifuge tubes (Sarstedt, Leicester, UK), ultrapure water and wax (Dynawax™, Flowgen, Lichfield UK) were UV irradiated in a class II microbiological safety cabinet for 13 mins.

Samples were collected from -20°C storage and placed in the fridge to thaw.

All reactions were set up on ice.

### 2.4.3 : PCR protocol

PCR amplification was carried out in a reaction volume of 50 $\mu$ l consisting of 5 $\mu$ l sample and 45 $\mu$ l of reaction mixture containing 1xPCR buffer (10mM Tris-HCl pH8.8, 1.5mM MgCl<sub>2</sub>, 50mM KCl, 0.1% Triton X-100), 2 units of *Taq* DNA polymerase (Pharmacia Biotech Ltd, Milton Keynes, UK), 0.2 mM dNTPs and 50pmol of each primer.

Lower and upper master mix tubes (1.5ml) were set up as follows.

LOWER (for 1 reaction vol in $\mu$ l)		UPPER (for 1 reaction vol in $\mu$ l)	
25Mm MgCl <sub>2</sub>	3	27F	0.15
10xbuffer	5	1492R	0.15
20mM dNTP's	0.5	Ultrapure water	19.7
<i>Taq</i> DNA Polymerase	0.2	TOTAL	20.0
Ultrapure water	16.33		
TOTAL	25.0		

Master mixes were vortexed and then briefly centrifuged.

25 $\mu$ l of lower master mix was added to each 0.5ml microcentrifuge tube. Wax was melted in a waterbath heated to 70°C and 40 $\mu$ l of wax added to each reaction tube to separate the upper and lower mixtures. 20 $\mu$ l of upper master mix was added.

Thawed samples of DNA extract were added to PCR reaction tubes in an isolated Template Tamer (Appligene Oncor, Oxford, UK) by positive displacement pipettes to minimise sample-to-sample contamination. 5 $\mu$ l of each sample was added to the corresponding upper master mix with 5 $\mu$ l of water added to the negative control and 2 $\mu$ l of known bacterial DNA acting as a positive control.

The reagent (negative) control (all PCR reagents without DNA) was run to evaluate the success of amplification, the specificity of amplification, the specificity of the reaction and the purity of the reagents. Samples were then placed into an Omnigene thermal cycler (Hybaid, UK) and PCR performed.

#### ***2.4.4 : PCR amplification***

The primary PCR amplification consisted of an initial denaturation step at 95°C for 5 minutes; 35 cycles at 95°C for 30s, annealing at 50°C for 1 minute, and extension at 72°C for 1 min; and a final elongation step at 72°C for 10 min.

Two rounds of PCR were performed using 5µl of the first round PCR product as a template for the second round.

#### ***2.4.5 : Agarose gel electrophoresis***

4g agarose (Promega, Southampton, UK) was dissolved in 200ml 1xTBE (50mM Tris, 50mM H<sub>3</sub>BO<sub>3</sub>, 1mM EDTA) (Sigma Chemical Co Ltd, Poole, Dorset, UK), buffer with 30µl ethidium bromide (Sigma) to produce a concentration of 2% w/v by heating in a microwave for 3 mins.

The cooled mixture was poured into a gel electrophoresis tank, with the end plates and well forming comb inserted and allowed to set for 30 mins. Once the gel had solidified the plates and comb were carefully removed. The gel slab was then submerged in 1 x TBE buffer.

#### ***2.4.6 : Visualisation***

In 1.5ml microcentrifuge tubes 1.5µl of bromophenol blue tracking dye (Sigma) was added to 10µl PCR product and loaded into the wells of the gel. The gel required a run time of 45 minutes at 70V for good resolution of a size marker (100bp DNA ladder, Promega)

The gel was examined under UV illumination for the presence of 1540-bp band and photographed using a VDS Image Master gel documentation system (Amersham, Pharmacia Biotech, St. Albans, UK). The sample was declared positive if a 1540-bp fragment was visualised. The sample was declared negative if the same fragment was not detected with the internal negative control, with the target product visualised with the internal positive control. This indicated the reaction had successfully amplified control DNA and hence no sample DNA was present.

#### ***2.4.7 : Contamination control***

GDH&S Molecular Biology Laboratories follow The National Committee for Clinical Laboratory Standards (NCCLS) approved guidelines for contamination control. Briefly, all reagents are prepared in a reagent laboratory by dedicated personnel not involved in PCR testing and in a physical location distinct from the PCR areas. Specimen preparation, amplification and product detection are performed in physically distinct areas of the building. Work is unidirectional and lab coats, gloves and equipment are not carried from one area to the next. Positive and negative controls were included with each run.

***Bactec inhibition protocols:***

***2.4.8 : Phenol : chloroform extraction***

A phenol : chloroform extraction was performed on 200µl of the inoculated Bactec culture medium with an equal volume of P:C (1:1) 100µl:100µl (Appligene Oncor, Oxford, UK) vortex mixed, then centrifuged at 13000 for 3 minutes. The upper aqueous layer was retained in a fresh tube. An equal volume of chloroform was then added, mixed well and centrifuged with the upper layer retained. This was then incorporated in a PCR reaction mixture.

***2.4.9 : Bovine serum albumin***

Bovine serum albumin 10µg/ml 0.01%-0.1% (w/v) (Promega, Southampton, UK) was added to the upper master mix of the PCR reaction.

***2.4.10 : Millipore centrifugal filter devices***

1. The sample reservoir was inserted into the filtrate vial.
2. The solution was added to the sample reservoir (2mL maximum volume) being careful not to touch the membrane with pipette tip. A covered rotor was used in order to minimise sample evaporation and the device was sealed by attaching the retentate vial to the sample reservoir.
3. The covered device was placed into the centrifuge rotor and the filtrate vial attached; this was counterbalanced with a similar device.
4. The centricon YM-3 centrifugal devices were spun at 4000-7500 x g (with retentate vial in place) until the desired concentration was achieved.
5. The centrifugal filter assembly was removed from the centrifuge and the filtrate vial separated from membrane support base, retaining the filtrate.



Samples were stored between 15°C and 30°C as recommended by the manufacturers.

#### **2.4.11 : GeneClean protocol**

Following the Qiagen extraction procedure:

1. 100µl of eluted DNA extract was measured with a pipette.
2. 3 volumes (300µl) of NaI solution was added, maintaining the final concentration of NaI above 4M. The tube was placed in a 45-55°C water bath. After one minute, the contents of the tube were vortex-mixed then returned to the water bath. The incubation was continued for approximately 5 minutes.
3. GLASSMILK was added: GLASSMILK was resuspended by vortexing for 1 minute. The final volume was 400µl, therefore 5µl glassmilk was added. The amount of GLASSMILK suspension was determined according to the following considerations

Final volume	Max. Amount of DNA	GLASSMILK
<50µl	<5µg	5µl
500-1000µl	<7.5µg	10µl
1ml	<12.5µg	20µl
3ml	<50µg	100µl

The amount of GLASSMILK required is based on the amount of DNA and the volume of NaI solution. (1µl of GLASSMILK will bind 1-2µg of DNA).

### ***Binding DNA to glassmilk :***

After the glassmilk was added to the solution, this was mixed and incubated at room temperature for 5 minutes to allow binding of the DNA to the silica matrix.

This was mixed every 1-2 minutes to ensure that glassmilk stayed suspended.

4. The silica matrix was pelleted with the bound DNA.

This was then centrifuged for approximately 5 seconds at 13000 r.p.m. The supernatant was retained in case all the DNA did not bind to the glassmilk.

5. The pellet was washed 3 times with prepared (ethanol added) NEW wash. 10-50 volumes (200-700 $\mu$ l) of prepared NEW wash was added to the pellet. The pellet was resuspended in the wash by pipetting back and forth while digging into the pellet with the pipette tip. After it was resuspended, it was centrifuged for 5 seconds and the supernatant discarded. The wash procedure was repeated twice. After the supernatant from the third wash had been removed, the tube was centrifuged again for a few seconds to allow removal of residual liquid (with a small bore pipette tip) to avoid diluting the elute with NEW wash. The pellet was air dried. As residual ethanol can interfere with many downstream reactions (i.e. PCR) it was removed from the glassmilk as thoroughly as possible. The cap was left open for 5-10 minutes at room temperature or 55°C.

6. Elution of DNA from glassmilk. The pellet was resuspended in a volume of water equal to the volume of glassmilk. This was then centrifuged for about 30 seconds to make a solid pellet. The supernatant containing the eluted DNA was carefully removed and placed in a new tube. Approximately 80% or more of the bound DNA that will elute does in this first step. A second elution can be done, resulting in an additional 10-20% recovery of eluted DNA. A third elution normally does

not add more than 1% to the total DNA yield. White pellets can be discarded after eluting DNA.

#### ***2.4.12 : Mec A detection from Bactec blood culture bottles protocol***

##### ***Concentration of organisms from Bactec bottles***

An aliquot (0.6mL) of well-mixed blood and broth containing bacteria was spun at 1500rpm for 3 minutes in a microcentrifuge. 200 $\mu$ L of the supernatant fluid was removed to another microfuge tube to be spun at 10,500rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 200 $\mu$ L of 10mM Tris-10 mM NaCl pH 8 (TN). After repeating the spin and discarding the supernatant, the pellet was resuspended in 50 $\mu$ L TN.

##### ***Organism lysis***

3  $\mu$ L of achromopeptidase (Sigma England) solution (20U/ $\mu$ L in 10mM Tris, 1 mM EDTA pH 7.0) were added to the suspension from a Bactec bottle. The suspension was incubated in a 56 °C water bath for 30 minutes, followed by 5 minutes in boiling water. The organism suspension was visibly cleared at this point and it was hoped contained the template DNA suitable for PCR amplification.

#### ***2.4.13 : Benzyl alcohol - guanidine hydrochloride organic extraction***

A total of 0.1ml of the inoculated medium was added to 0.1ml of lysis buffer and briefly mixed with a vortex mixer. Lysis buffer consisted of 5.0M guanidine hydrochloride / 100mM Tris (pH 8.0) in sterile water. A total of 0.4ml of water was added, followed by the addition of 0.8ml of 99% benzyl alcohol (Sigma Chemical Co.) and the sample was mixed again by vortexing. The sample was centrifuged at 7000xg

for 5 min. A total of 0.4ml of the aqueous supernatant was removed and placed into a new microcentrifuge tube.

A total of 40 $\mu$ l of 3.0M sodium acetate was added, followed by the addition of 0.44ml of isopropanol, and the sample was centrifuged at 16000xg for 15 min at 4°C. The precipitated DNA was washed with 70% ethanol and the pellet was air-dried. The DNA was resuspended on 0.1ml of 10mM Tris-0.1mM EDTA buffer (pH 8.5).

## ***2.5 : LIMULUS AMOEBOCYTE LYSATE ASSAY (LAL)***

The Limulus amoebocyte lysate assay was purchased from Biowhittaker UK Ltd, Cambrex Berkshire UK.

### ***2.5.1 : E. Coli Endotoxin***

Reconstituted - 1ml of LAL water was added warmed to room temperature.

Vortex mixed for 15 mins

Prior to each use the endotoxin was warmed to room temperature and vortex mixed for 15 minutes.

### ***2.5.2 : Chromogenic substrate***

Reconstituted by adding 6.5ml of LAL water 2mM

Protected from light exposure by covering vial in tin foil.

### ***2.5.3 : Limulus Amoebocyte Lysate***

Reconstituted immediately before use with 1.4ml of reagent water and swirled gently to avoid foaming.

Reconstituted lysate was stored at -10°C and was thawed and used only once.

### ***2.5.4 : Stop reagent***

SDS solution 10g/100ml in water.

### **2.5.5 : Specimens**

pH of sample 7-8 (adjusted using pyrogen free NaOH, HCl)

### **2.5.6 : Bloods**

Blood products induce or give false positive or negative results. Friberger, Knos and Mellstam have reported the removal of non-specific inhibition in blood products by diluting the test sample 1:10 in LAL water and heating at 70°C for 5 mins.

Thus plasma samples were diluted 1:10 with LAL water and heated at 70°C for 5 minutes to remove endogenous inhibitors.

### **2.5.7 : Equipment required**

All materials and equipment coming into contact with the specimen or test reagents were pyrogen-free. (Materials may be rendered pyrogen-free by heating at 180°C for 4 hours).

Dilution tubes 13x100ml

Pipettes and tips

Assay tubes 10x 75mm

Dry bath / multiblock heater @ 37°C

Tube blocks for heater

Stopwatch

Vortex mixer

Spectro or filterphotometer 405-410nm

### ***2.5.8 : Preliminary reagent preparation***

Potency of endotoxin = 20 EU / vial

Initial dilution is 1/20

That is, 0.1ml of endotoxin stock into 1.9ml of LAL reagent water.

1. 1.0 EU/ml solution of endotoxin was prepared by diluting 0.1ml of endotoxin stock with 1.9ml of LAL reagent water. This was vigorously vortexed for 1 minute.
2. 0.5ml of this 1.0 EU/ml solution was transferred into 0.5ml of LAL reagent water and labelled 0.5 EU/ml. This was vigorously vortexed for 1 min before use.
3. 0.5ml of the 1.0 EU/ml solution was transferred into 1.5ml of LAL reagent water and labelled 0.25 EU/ml. This was vigorously vortexed for 1 min before use.
4. 0.1ml of the 1.0 EU/ml solution was transferred into 0.9ml of the LAL reagent water and labelled 0.1 EU/ml. This was vigorously vortexed for 1 min prior to use.

### **2.5.9 : Increased sensitivity**

By extending the first incubation to 30mins it was possible to measure concentrations between 0.01 and 0.10 EU/ml. To prepare the endotoxin standards between 0.01 and 0.10 EU/ml, we began with the 0.10 EU/ml solution prepared from the 1.0EU/ml solution as described previously. (Diluted 0.2ml of the 1.0EU/ml solution into 1.8ml LAL reagent water)

Endotoxin concentration EU/ml	Endotoxin Standard Solution 0.1EU/ml	LAL Reagent Water
0.05	0.5ml	0.5ml
0.025	0.5ml	1.5ml
0.01	0.1ml	0.9ml

### **2.5.10 : Test procedure**

	sample	blank
Test sample / standard 20-25°C	50µl	-
LAL reagent water	-	50µl
LAL	50µl	50µl
Mix and incubate 37°C	10min	10min
Substrate solution 37°C	100µl	100µl
Mix and incubate 37°C	6min	6min
Stop reagent	100µl	100µl
Mix immediately		



### ***2.5.11 : Microplate method***

The substrate solution was removed from the fridge (4°C) and pre-warmed to 37°C

1. The microplate (Nunc pyrogen free Elisa plate, Fischer scientific UK, Lifescience, Loughborough, Leicestershire) was pre-equilibrated at 37°C in the heating block adaptor. While leaving the microplate at 37°C, 50µl of sample or standard was carefully dispensed into the appropriate microplate well.

2. Each series of determinations included a blank and 4 endotoxin standards run in duplicate. Blank tubes contained 50µl of LAL reagent water instead of sample.

All reagent times / incubations were identical

3. At T=0, 50µl of LAL was added to the first microplate well.

Timing began as LAL was added to the first well. It was important that consistency was maintained in the order of reagent addition from well to well and row to row, and in the rate of pipetting. Once the LAL had been dispensed into all the microplate wells containing the sample or standards, the microplate was briefly removed from the heating block and the side of the plate was repeatedly agitated to facilitate mixing. The plate was returned to the heating block adaptor and the cover replaced.

4. At T=15 mins (NB increased time from 10 mins to increase the sensitivity), 100µl of substrate solution pre-warmed to 37°C was added. Constant order and rate of pipetting was ensured. Again the microplate was agitated to assure mixing.

5. Following 6 mins 100µl of SDS stop reagent was added maintaining the same order as previously. Again the microplate was agitated to assure mixing.

6. The absorbance of each reaction well was read at 405-410 nm in an Elisa plate reader using distilled water to adjust the photometer to zero absorbance.

### ***2.5.12 : Calculation of endotoxin concentration***

Standard conditions absorbance at 405-410nm is linear in concentration range of 0.1-1.0EU/ml endotoxin. The mean absorbance of the blank was subtracted from the mean absorbance value of the standards and samples to calculate the mean change in absorbance.

### ***2.5.13 : Graphic method***

Mean absorbance for 4 standards was plotted on the y axis versus corresponding endotoxin concentration in EU/ml on the x axis. The best fit line between these points was drawn and endotoxin concentrations of samples were determined graphically.

### ***2.5.14 : Linearity***

Within the concentration range used to predict endotoxin values should be verified.

No less than 4 standards should be arranged along with a blank in quadruplicate.

***CHAPTER 3***

***RESULTS***

### ***3.0 : PATIENT RECRUITMENT***

Male and female subjects over 18 years of age were selected from the University of Glasgow Dental School Department of Periodontology consultant clinic referral pool. 38 in total were recruited, with 8 patients who did not attend for visit 2 and thus were withdrawn from the study. 30 patients completed the study. Of these 30 individuals 18 were male and 12 were female with a mean age of 42.3 years (range 24.8-64.6 years).

The primary outcome variable was the number of positive cultures. Identification of the specific organisms isolated was also performed. Mean detection scores were compared between treatment groups for each procedure and differences in detection between the various clinical procedures was also analysed.

Secondary outcome variables were levels of LPS in the blood samples (measured by LAL assay) and the presence of bacterial DNA in the bloodstream measured by PCR using general 16S primers.

The code was broken following the analysis. J52 was the placebo (fluoride) mouthrinse and G27 was the positive control (chlorhexidine) mouthrinse.

#### ***Statistical tests :***

Statistical analyses were performed on Minitab PC statistical package. Cross tabulations and Chi-square tested were applied. Statistical tests were applied as appropriate and  $p < 0.05$  was taken as the level of statistical significance.

### 3.0.1 : CLINICAL POCKET CHART DATA

Table 4 : Table showing subject number, mouthwash product allocation code J52 (fluoride) mean pocket depth, mean number of pockets greater than 5mm, mean mobility scores and mean bleeding on probing scores for each subject.

Control group :

<b>SUBJECT NO.</b>	<b>PRODUCT CODE</b>	<b>MEAN POCKET DEPTH</b>	<b>MEAN NO. OF POCKETS&gt;5mm</b>	<b>MEAN MOBILITY</b>	<b>MEAN BOP</b>
1	J52	4.68	0.57	0.38	0.75
4	J52	3.57	0.25	0	0.49
5	J52	2.73	0.13	0	0.29
9	J52	2.84	0.14	0	0.48
10	J52	2.98	0.16	0	0.36
11	J52	2.93	0.2	0.19	0.43
14	J52	2.98	0.15	0	0.18
16	J52	4.18	0.15	0.17	0.53
18	J52	3.07	0.13	0	0.33
21	J52	4.01	0.2	0	0.94
24	J52	3.65	0.24	0.18	0.68
26	J52	3.09	0.12	0.39	0.3
28	J52	6.25	0.63	0.42	0.8
30	J52	3.99	0.37	0.04	0.8
31	J52	3.6	0.2	0.04	0.67
32	J52	3.83	0.25	0.04	0.79
34	J52	4.93	0.46	0.31	0.87
36	J52	4.28	0.34	0	0.88
37	J52	3.17	0.13	0	0.74

**TABLE 5 : Table showing subject number, mouthwash product allocation code G96 (chlorhexidine) mean pocket depth, mean number of pockets greater than 5mm, mean mobility scores and mean bleeding on probing scores for each subject.**

**Test group :**

<b>SUBJECT NUMBER</b>	<b>PRODUCT CODE</b>	<b>MEAN POCKET DEPTH</b>	<b>NUMBER OD POCKETS&gt;5mm</b>	<b>MEAN MOBILITY</b>	<b>MEAN BOP</b>
2	G96	3.36	0.23	0	0.64
3	G96	3.53	0.2	0	0.56
6	G96	2.23	0.05	0	0.21
7	G96	3.96	0.33	0	0.72
8	G96	4.57	0.46	0.18	0.87
12	G96	3.58	0.26	0	0.38
13	G96	2.93	0.07	0	0.45
15	G96	3.14	0.11	0	0.37
17	G96	2.98	0.17	0	0.27
19	G96	3.48	0.22	0.08	0.65
20	G96	2.86	0.12	0	0.4
22	G96	3.28	0.2	0.18	0.63
23	G96	4.57	0.44	0.28	0.59
25	G96	4.45	0.39	0	0.85
27	G96	4.38	0.34	0.25	0.92
29	G96	3.92	0.17	0.04	0.67
33	G96	2.8	0.09	0	0.59
35	G96	2.93	0.08	0	0.51
38	G96	3.5	0.24	0	0.65

Differences between the two groups were not statistically significant (cross tabulations and the Pearson Chi-square test) indicating severity of disease as determined by pocket depth, number of pockets greater than 5mm, mobility and BOP scores were balanced between the two mouthwash groups.

### ***3.1 : Conventional microbiology : Timing of sampling***

The timing of blood sampling following the bacteraemia-inducing procedure was investigated. The length of a bacteraemia is believed to be on average only 15 minutes. However, a recent study on children showed a high level of bacteraemia at 10 minutes and with extrapolation suggested that the length of bacteraemia is approximately 45 minutes (Roberts *et al* 1999). In contrast other studies have indicated that procedure related bacteraemias are short lived with the frequency of blood cultures being highest in the first 30 seconds after tooth extraction (Roberts *et al* 1997).

To investigate this further 20 subjects, (10 chlorhexidine, 10 fluoride mouthwash groups) blood samples were obtained 15 minutes following cessation of each procedure. In comparison, blood samples from a further 10 subjects were obtained immediately following cessation of the procedure. No difference was found in the number of positive samples at 15 mins compared with immediately after completion of the procedure. Interestingly, there were a higher number of false positives with the samples obtained 15 minutes following the procedures; therefore, in the study, samples were taken immediately following the procedure.

### *Timing of sampling*

Tables showing the comparison between positive blood samples for bacterial growth obtained by conventional microbiology at two separate time intervals: T=15 mins and T=0 mins following each periodontal manipulation.

**Table 6 : Samples T=15 mins positive by Bactec system (negative results not shown)**

<b>procedure</b>	<b>group</b>	<b>result on subsequent subculture</b>
baseline	control	no growth
post-probing	control	<i>Propionibacterium acnes</i>
		<i>Neisseria pharyngis</i>
		<i>Streptococcus viridans</i>
		<i>Micrococcus</i>
		<i>Staphylococcus albus</i>
post-cavitron	control	<i>Actinomyces naeslundii</i>
baseline	control	no growth
post-probing	control	no growth
post-probing	chlorhexidine	Anaerobic <i>Streptococci</i>

**Table 7 : Samples T=0 mins positive by Bactec system (negative results not shown)**

<b>procedure</b>	<b>group</b>	<b>results on subsequent subculture</b>
post-cavitron	chlorhexidine	<i>Streptococcus parasanguis</i>
post-cavitron	chlorhexidine	<i>Streptococcus parasanguis</i>
		<i>Actinomyces naeslundii</i>
		<i>Eubacterium spp.</i>
		<i>Eubacterium limosum</i>
post-probing	control	<i>Micrococcus</i>
post-cavitron	chlorhexidine	<i>Propionibacterium acnes</i>



**Culture results :**

Tables showing positive Bactec culture bottle samples and subsequent isolate identification on subculture for each procedure sampled :

**Table 8 : POSITIVE BACTEC CULTURE BASELINE SAMPLES**

subject	subculture
1	no growth
4	no growth

*NB baseline sample taken at visit 1 prior to mouthwash allocation*

**Table 9 : POSITIVE BACTEC CULTURE POST-PERIODONTAL PROBING**

subject	group	subculture
1	control	<i>Propionobacterium acnes</i>
		<i>Neisseria pharyngis</i>
		<i>Streptococcus viridans</i>
		<i>Micrococcus</i>
		<i>Staphylococcus albus</i>
4	control	no growth
8	chlorhexidine	<i>Anaerobic Streptococci</i>
14	control	<i>Micrococcus</i>
26	control	<i>Prevotella intermedia</i>
		<i>Actinomyces naeslundii</i>
35	chlorhexidine	<i>Haemophilus aphrophilus</i>
		Coagulase -ve <i>Staphylococci</i>

**Table 10 : POSITIVE BACTEC CULTURE POST-TOOTHBRUSHING**

subject	group	subculture result
21	control	<i>Gemella haemolysans</i>

**Table 11 : POSITIVE BACTEC CULTURE POST-CAVITRON SCALING**

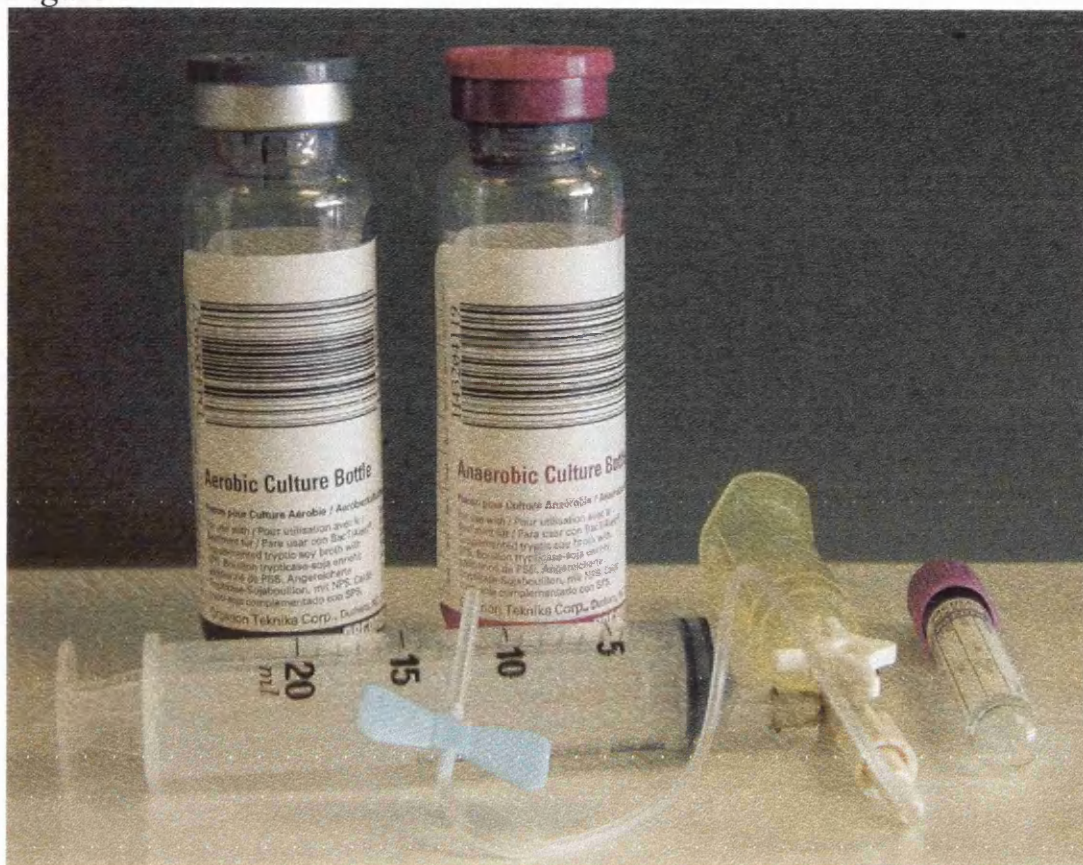
subject	group	subculture result
1	control	<i>Actinomyces naeslundii</i>
13	chlorhexidine	<i>Streptococcus parasanguis</i>
13		<i>Streptococcus parasanguis</i>
		<i>Actinomyces naeslundii</i>
		<i>Eubacterium spp</i>
		<i>Eubacterium limosum</i>
19	chlorhexidine	<i>Propionobacterium acnes</i>

*(N.B. negative culture results are omitted from these tables)*

**LEGEND:**

***Figure 1 : Blood sampling apparatus - Bactec aerobic and anaerobic culture bottles, EDTA vacutainer, 20ml syringe with 3 way stopcock, vacutainer attachment and butterfly.***

Figure 1



### 3.2 : PCR : Whole blood EDTA samples

The technique of Polymerase Chain Reaction was performed on the whole blood EDTA samples according to protocol 2.4.3, which previously had DNA extracted using the Qiagen blood and body fluid spin protocol (2.3).

In order to increase PCR sensitivity, two rounds of PCR were performed, that is, 5µl of the first round PCR product was used as a template for the second round (Figure 22) since all samples were negative following a single round of PCR (Figure 21). This was indicative of the low levels of bacteria present in the samples.

Samples which were PCR-positive following 2 rounds of PCR were as follows:

**TABLE 12 : POSITIVE PCR SAMPLES**

***control mouthwash group :***

subject	procedure
10	baseline 1
24	baseline 2
5	post-probing
10	post-probing
16	post-probing
9	post-toothbrushing
31	post-toothbrushing
14	post-cavitron
28	post-cavitron

**TABLE 13 : POSITIVE PCR SAMPLES***test mouthwash group :*

<i>subject</i>	<i>sample</i>
7	baseline 1
2	baseline 1
7	post-probing
38	post-probing
3	post-toothbrushing
27	post-toothbrushing
4	post-cavitron
13	post-cavitron
27	post-cavitron
29	post-cavitron
38	post-cavitron

## PCR LEGEND

*Fig 20 : 0.8% agarose check gel showing extracted DNA following Qiagen DNA extraction procedure on whole blood EDTA samples*

*Fig 21 : 2% agarose gel showing first round PCR on EDTA whole blood extracted DNA samples. All samples were negative, lanes 12 and 28 100-bp DNA ladder, lane 13 positive control showing successfully amplified product, lanes 14 and 29 negative control.*

*Fig 22 : 2% agarose gel showing two rounds of amplification. Successfully amplified products are shown lanes 2, 4, 5, 6, 8 and 9 positive control lane 11, 100-bp ladder lanes 12 and 28, negative control lane 13.*

*Fig 23 : 2% agarose gel lane 3 positive sample by PCR when spiked with *Streptococcus sanguis* previously negative by culture. Successfully amplified products lane 5 weak positive by PCR previously culture positive frozen EDTA blood sample, lane 7 positive sample by PCR when spiked with *Streptococcus sanguis* and incorporating a mechanical lysis procedure previously negative by culture, lane 9 100-bp marker, lane 11 positive control, lane 13 negative control.*

*Fig 24 : 2% agarose showing inhibited PCR samples from Bactec bottles. No positive signal shown, lane 10 positive control, lane 11 negative control, lane 12 100-bp marker.*

Figure 20

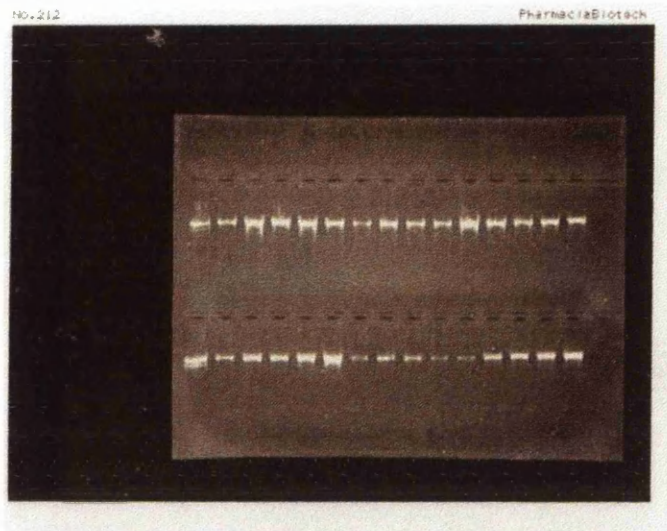


Figure 21

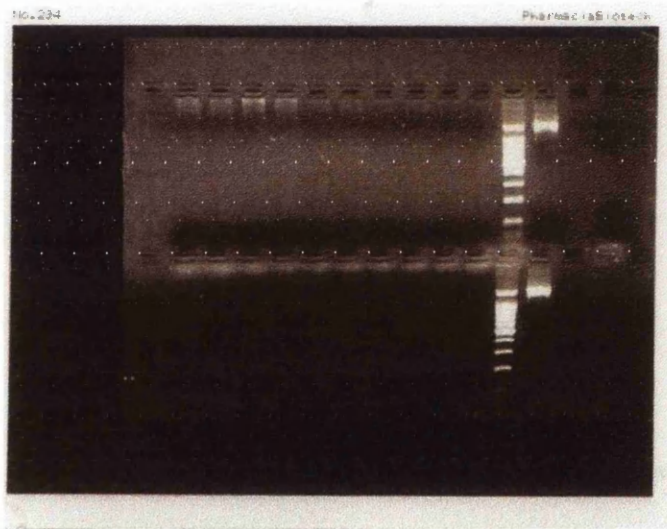


Figure 22



Figure 23

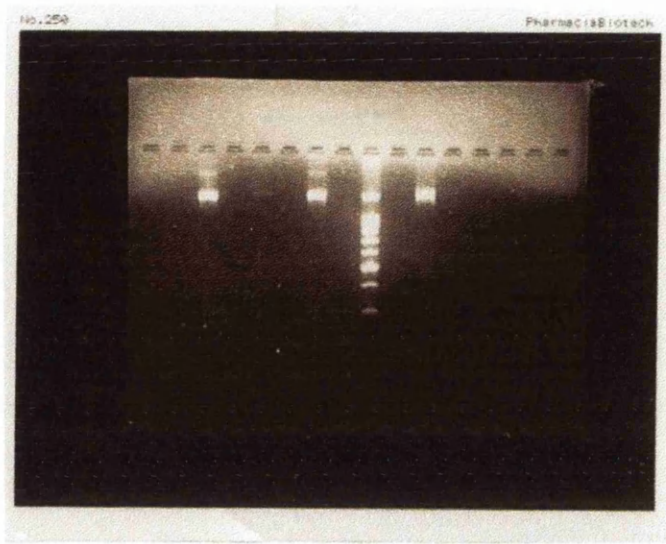
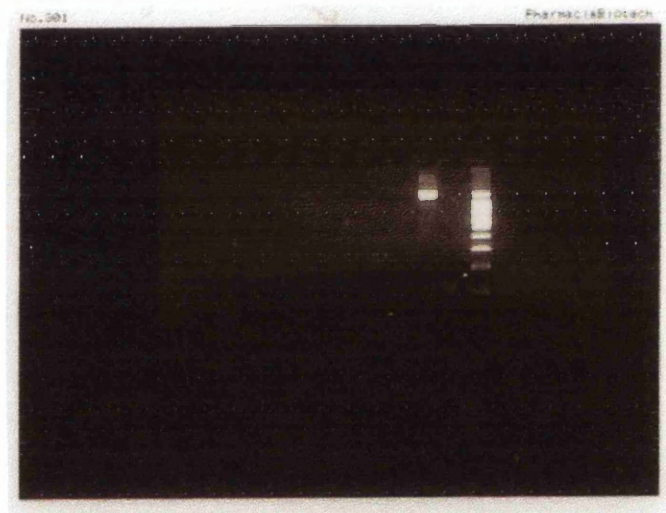


Figure 24





## ***Polymerase Chain Reaction performed on Bactec culture bottle samples :***

***(Figure 24)***

PCR was attempted on the Bactec culture bottle blood samples. DNA was obtained by various extraction protocols as follows in an attempt to remove the presence of an inhibitor to the PCR reaction:

### ***3.3.1: Qiagen QIAamp DNA extraction procedure for whole blood***

No PCR product was obtained from the Bactec culture bottles with 2 rounds of PCR.

### ***3.3.2 : Phenol : chloroform extraction***

No PCR product was obtained even when combined with the QIAmp extraction procedure.

### ***3.3.3 : Bovine serum albumin***

This did not remove the inhibition and despite a spiked sample, no positive band was produced.

### ***3.3.4 : Millipore centrifugal filter devices***

The inhibitor was not removed by this filtration procedure.

### ***3.3.5 : GeneClean protocol***

The inhibitor was not removed by this procedure.

### ***3.3.6 : Mec A detection from Bactec blood culture bottles protocol***

The inhibitor was not removed by this procedure.

### ***3.3.7 : Benzyl alcohol - guanidine hydrochloride organic extraction***

The inhibitor was not removed using this protocol.

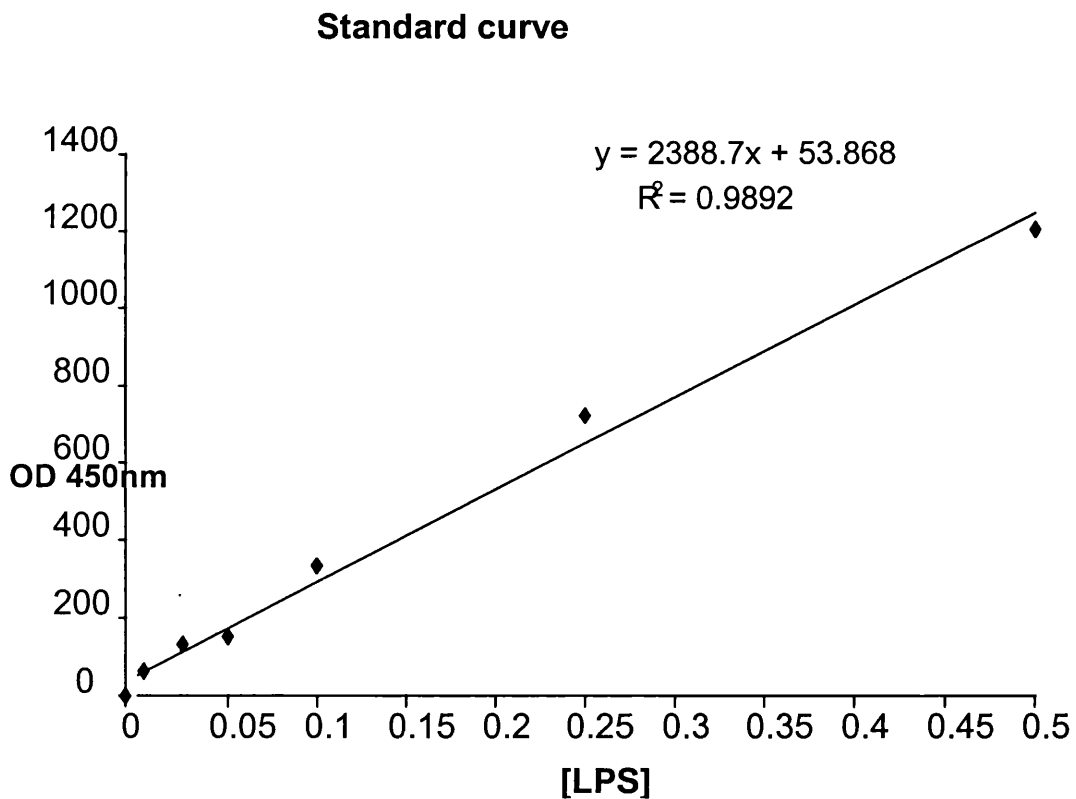
***Limulus Amoebocyte Lysate Assay Results:***

Plasma was obtained by centrifugation of EDTA blood samples and was heated and diluted (2.5.6) to facilitate lipopolysaccharide analysis using the LAL chromogenic assay. Endotoxin standards were used to produce a standard curve, from which endotoxin concentration could be calculated in samples with corresponding optical densities (OD).

***Graph 1 :***

***Standard curve showing mean absorption for standards (y axis) versus corresponding endotoxin concentration in EU/ml (x axis).***

***N.B. Standards were included for each plate run.***



**LAL RESULTS:**

<b>subject</b>	<b>procedure</b>				
	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>
<b>EL</b>	0.032	0.022			
<b>JMcC</b>	0.033	0.029	0.031	0.031	0.030
<b>JB</b>	0.057	0.036	0.035	0.032	0.034
<b>JK</b>	0.033	0.030	0.028	0.032	0.028
<b>FJH</b>	0.053	0.031	0.038	0.053	0.044
<b>EE</b>	0.059	0.059	0.034	0.037	0.033
<b>LC</b>	0.045	0.039	0.050	0.035	0.039
<b>LMcC</b>	0.060	0.030	0.029	0.031	0.066
<b>LL</b>	0.028	0.025			
<b>MB</b>	0.039	0.025			

*Table 5 : mean LPS levels detected for each patient following each procedure*

<b>subject</b>	<b>procedure</b>				
	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>
<b>RM</b>	0.037	0.063			
<b>ND</b>	0.088	0.077	0.068	0.061	0.081
<b>HM</b>	0.052	0.046	0.045	0.658	0.046
<b>WL</b>	0.047	0.059	0.048	0.056	0.048
<b>JS</b>	0.049	0.049	0.050	0.059	0.050
<b>IM</b>	0.024	0.034			
<b>MM</b>	0.025	0.023	0.023	0.029	0.022
<b>CC</b>	0.026	0.020	0.160	0.030	0.028
<b>DR</b>	0.040	0.030	0.025	0.021	0.028
<b>AD</b>	0.038	0.055	0.029	0.030	0.027

*Table 6 : mean LPS levels detected for each patient following each procedure*

<b>subject</b>	<b>procedure</b>				
	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>
<b>SM</b>	0.038	0.023	0.029	0.029	0.028
<b>AH</b>	0.030	0.034	0.025	0.026	0.030
<b>CD</b>	0.032	0.028	0.047	0.042	0.035
<b>KS</b>	0.039	0.050	0.032	0.031	0.029
<b>CJ</b>	0.063	0.081	0.072	0.075	0.083
<b>SCD</b>	0.068	0.080	0.053	0.083	0.088
<b>KR</b>	0.063	0.073	0.076	0.073	0.080
<b>CG</b>	0.092	0.070	0.072	0.065	0.063

*Table 7 : mean LPS levels detected for each patient following each procedure*

*All LPS levels measured in endotoxin units (EU)*

*Procedure 1: baseline visit 1, Procedure 2 :periodontal probing*

*Procedure 3: baseline visit 2, Procedure 4 : toothbrushing*

*Procedure 5 : cavitron scaling*

### ***3.4 : Clinical results***

Comparing all the subjects pocket depths, 1 subject had an average pocket depth of 2mm, 17 had an average pocket depth of 3mm, 15 had an average pocket depth of 4mm and 5 had an average pocket depth of greater than 4mm.

24 subjects were considered for statistical purposes to have bleeding on probing (mean>0.50) with 14 subjects regarded as not having BOP (mean<0.50). No samples were positive for bacterial growth by conventional microbiology at sample 1 baseline. 5 were positive following periodontal probing. At visit 2 no samples were positive for bacterial growth at baseline with only 1 positive following toothbrushing and 3 positive following cavitron scaling. None of these differences were statistically significant using cross tabulations and the Pearson Chi-square tests given the small sample size.

Comparing the average pocket depths between the two mouthwash groups using cross tabulation and the Pearson Chi-Square tests as previously, shows they are reasonably balanced with 19 subjects in each group (before withdrawals) and an even distribution of pocket depths between the two groups. For bleeding on probing again the distribution between the two mouthwash groups was relatively even with 13 subjects (54.2%) having mean BOP>0.50 in the chlorhexidine mouthwash group and 6 with mean BOP<0.50 (42.9%) with 11 subjects (45.8%) in the fluoride mouthwash group having mean BOP>0.50 and 8 with mean BOP<0.50 (57.1%).

Following two weeks mouthwash use 15 subjects remained in each mouthwash group. At baseline sample 3 no conventional microbiology samples were positive. Following toothbrushing 1 sample was positive for bacterial growth belonging to the fluoride

mouthwash group. No samples for bacterial growth were found from those subjects in the chlorhexidine mouthwash group. Following cavitron scaling 2 subjects were positive for bacterial growth in the chlorhexidine group whereas 1 was positive in the fluoride group. In total, 4 samples were positive following mouthwash use, 2 each for both the mouthwash groups. A far larger sample size would have been necessary to provide sufficient power to detect any statistical difference between the mouthwashes.

***CHAPTER 4***  
***DISCUSSION***

#### ***4.1.: RATIONALE FOR SAMPLE SIZE***

The aim was to complete the study with 20 subjects having used both the placebo and the positive control mouthwash. As this was a pilot study, we had no reliable indicator of the necessary sample size. However, it was anticipated that this number would be sufficient to show any differences in clinical indices within the three methods utilised to detect bacteraemia. The three different bacteraemia detection methods would correlate and possibly provide insight into the various ongoing bacteraemic processes. It was also hoped differences between a proven antimicrobial agent and placebo would be shown.

##### ***4.1.1 : Timing of sampling***

Bactec bottles which indicated a positive culture presence occasionally provided no growth on subsequent subculture. It was thought that perhaps the organisms in these cases were uncultivable on CBA and FAA, which would have provided a positive sample with PCR. Alternatively, the manufacturers suggest that high levels of white blood cells in the blood sample can give a false positive result with the Bactec culture bottles. However, because of the difficulties with inhibition of the Bactec bottles when PCR was performed this could not be proved. As stated previously, the samples which provided no growth (not baseline) were those taken 15 minutes following the procedure and perhaps elevated levels of leukocytes may be present at this time following a bacteraemia-inducing procedure (Organon Teknika).



Many dental bacteria are particularly slow-growing. The culture bottles remained in the Bactec incubator for 7 days as routine at the Western Infirmary, at which point a microbiological report was provided. Subsequently, samples were placed in a hot room for a further 7 days until collection. It is for this reason (i.e. longer incubation) that several bottles become positive and provided positive growth on subsequent subculture during this time interval, despite an initial negative report.

### ***Comment***

*Propionobacterium*, *Neisseria*, *Eubacterium*, *Streptococci* and *Actinomyces* have been isolated in low number in fissure plaque. Gingival crevice plaque has higher levels of obligately anaerobic bacteria. Many different species can be found : Gram-negative spirochetes and anaerobic *Streptococci* are isolated almost exclusively from this site. The ecology of this crevice is influenced by the anatomy of the site and the flow and properties of the GCF. *Streptococcus oralis* and *Actinomyces naeslundii*, are associated with the healthy gingival crevice. *Prevotella intermedia* has also been recovered from this site.

Despite both culture media sustaining growth and meeting the atmospheric requirements of all of the species tested by the manufacturers, it is known that several species of bacteria (e.g. *Campylobacter*) will not be supported by the Bactec medium (Hutchinson *et al* 1992) and it is therefore likely particular fastidious dental isolates may not be sustained. Molecular techniques could be beneficial in the detection of such species.

## ***4.2 : Molecular biology : PCR***

### ***4.2.1 : Discussion***

3/38 (7.9%) samples were positive by PCR at baseline sample 1. This may be indicative of PCR being more sensitive than conventional microbiology in detecting bacteria present without any manipulation.

Following periodontal probing 4/38 (10.8%) samples were PCR-positive. At baseline visit 2, 1 (3.3%) sample was PCR positive in the fluoride mouthwash group. Following toothbrushing 4 (13.3%) samples were PCR positive with 2 in each of the mouthwash groups. Following cavitron scaling 6 (20%) samples in total were positive which were evenly found within the mouthwash groups.

Samples which were positive by PCR were not necessarily the same samples to those which had been positive by conventional microbiology. It should be remembered that the actual blood samples tested by molecular biology and conventional microbiology were not the same. This was not technically feasible as there were inhibitors in the Bactec bottles which prevented PCR being performed on these samples. It could be that if the frozen EDTA blood samples had been cultured prior to freezing they may have indicated positive by conventional culture also. Conversely, the same may have been true if PCR could have been performed on the Bactec bottles. This would allow a greater correlation between PCR positive and culture positive samples. There was no apparent correlation between samples which were PCR positive and those which were culture positive.

Comparing average pocket depths with those samples which were PCR-positive at visit 1, no samples were positive with 2mm pockets, 2 (11.8%) samples were positive with 3mm pockets and 1 (6.7%) sample was positive with 4mm pockets.

Following periodontal pocketing no samples were positive in subjects with an average of 2mm pockets, 1 (6.3%) sample was positive with 3mm pockets, with 3 (20%) positive with an average of 4mm pockets. At baseline visit 2, only 1 (7.1%) sample was positive by PCR in a subject with an average pocket depth of 3mm. This may indicate that patients are at risk of physiological bacteraemia irrespective of their periodontal pocket depths. Following toothbrushing, 4 (13.3%) samples were PCR positive in total, 1 (7.1%) which was positive for a subject with an average of 3mm pockets and 3 (27.3%) positive with 4mm pockets. Following cavitron scaling 6 (20%) samples were PCR positive, 2 (14.3%) positive with 3mm pockets, 3 (27.3%) positive with 4mm pockets and 1 (100%) positive with pockets greater than 4mm.

A greater number of samples were positive by PCR compared with conventional microbiology indicating greater sensitivity with this molecular technique compared with conventional microbiology. Indeed, several samples were positive at baseline supporting the theory of physiological bacteraemia without manipulation. The samples which were PCR positive had a similar trend to those which were positive with conventional microbiology. Cavitron scaling provided the most positive results by PCR (6) followed by periodontal probing (4) and toothbrushing (4). As these values are similar to the conventional microbiology findings, it supports that these are true positives and not contaminated samples.

4 samples which were positive by PCR were those prior to any clinical manipulation or baseline samples (1 or 3). This could be indicative of a low level transient bacteraemia without any manipulation. It could be suggested that these positives may be caused by background contamination although as stated previously, contamination control was stringent and was not a problem in this study.

As already stated, on comparing the culture and PCR samples, no apparent correlation exists. Only two samples were both PCR- and culture-positive; JK5 and CG 2. It would be expected that several more samples which were culture-negative should be PCR-positive as PCR is far more sensitive than conventional culture. In addition, several organisms may be uncultivable and therefore only detected by PCR. Indeed, 16 non-baseline samples were positive by PCR alone, compared to 10 blood culture samples which provided an identifiable organism with conventional microbiology on subculture.

Moreover, a small volume of blood (200 $\mu$ l) was used in the DNA extraction, of which 5 $\mu$ l of sample extract was used in the PCR reaction. This means that the very small number of bacteria may not have been present in the small volume of blood taken for PCR. However, by using two rounds of PCR the sensitivity is increased so that it would detect even small numbers of bacteria, hence it is unlikely that significant numbers of bacteria are present, if at all.

Despite the 27f/1492r PCR primer pair used being able to detect most bacterial species, it has been noted in other studies that *Actinomyces* species are detected relatively infrequently with these primers. This would help to explain why three of the samples culture-positive for *Actinomyces* species were negative by PCR. In addition, many of the isolates were Gram-positive and it was initially thought that the lysis stage

was not adequate to break the thick bacterial cell wall. Our lysis stage in the DNA extraction procedure was fully tested and shown to be adequate (Figure 23).

If the organisms identified by PCR were culturable, we need to explain why the blood cultures remained negative. Some of the positives detected by PCR may be non-fermentive and the BacT/Alert system detects organisms by the colorimetric detection of CO<sub>2</sub> produced by growing organisms. In addition, the clinic was located at a site distant from the laboratory, there may have been a delay in Bactec blood culture samples reaching the laboratory. As early processing is known to be important, this short delay may have reduced the detection rate using this blood culture system. From the previous literature, it is possible that the number of positive blood culture samples is an underestimate and some of the bacteria in the bottles which were found to be positive by Bactec but provided no subsequent growth on subculture may simply have exhausted the nutrients in the medium and died before subculture was attempted. It should be highlighted that PCR will detect dead and moribund organisms which may be present in the bloodstream, which should result in more positive PCR results than conventional blood culture.

#### **4.2.3 : PCR inhibition**

When PCR was performed on positive Bactec bottles from which bacterial isolates had been cultured (using DNA extracted by the Qiagen silical column purification method) no products were obtained. Furthermore, despite inoculating bottles with *Streptococcus mitis* no signal was obtained (Figure 24).

#### 4.2.4 : *Bacterial considerations*

When PCR was attempted directly from DNA extracted from Bactec bottles, no positive signal could be obtained despite the culture bottles yielding culturable bacteria. As mentioned previously, Gram-positive organisms have a thicker peptidoglycan cell wall than Gram-negative bacteria. Since many of the organisms isolated from the Bactec bottles were Gram-positive, it was initially anticipated Gram-positive organisms maybe more troublesome to lyse and the Qiagen extraction procedure lytic enzyme may not have been sufficient, possibly explaining why positive bands were not produced with the DNA extracted from positive Bactec bottles.

In order to test this hypothesis, a mechanical lysis stage was incorporated using glass beads which were vortexed with the 200 $\mu$ l blood sample, pulse centrifuged and then 200 $\mu$ l of the supernatant used in the QIAamp extraction procedure. This was shown to be unnecessary as the negative samples which had both been spiked with *Streptococcus sanguis* were both positive and gave a similar signal strength whether the glass bead mechanical lysis stage had been incorporated or not (Figure 23).

The sample which was shown to be positive by culture gave a very weak positive signal by PCR on the EDTA whole blood sample which had been frozen at -70°C. It should be remembered that this sample, although taken at the same time as the Bactec bottles, had not undergone an amplification stage so very small numbers of bacteria would be present that may not be detected by PCR. It was for this reason that 2 rounds of PCR, that is, using the first round product as the template for the second PCR reaction, were performed (Figures 21 and 22). However, this could not explain the negative signals obtained from the Bactec following two rounds of PCR (Figure 24).

#### ***4.2.5 : Special aspects relating to blood samples***

With PCR templates prepared from blood, the major inhibitor of PCR is thought to be the haem component derived from haemoglobin. The concentration of haemoglobin in blood is approximately 160mg/ml. Citrate, heparin, and potassium EDTA (K-EDTA) are common anticoagulants used for blood samples. Heparin has been reported to inhibit PCR; heparin inhibits PCR completely at a concentration of 0.15 iU per ml corresponding to 1 $\mu$ l of anticoagulant-treated blood in a 100 $\mu$ l PCR volume. In contrast, 1mM citrate or 0.04mM K-EDTA - equivalent to the addition of 1 $\mu$ l of appropriately treated blood - does not affect PCR performance.

#### ***4.2.6 : Inhibition of Bactec samples***

A common technical problem with PCR is failed amplification due to the presence of PCR inhibitors. As mentioned previously PCR inhibitors include haem compounds found in blood, in addition to aqueous and vitreous humours, heparin, EDTA, urine, polyamines and plant polysaccharides. To deal with this problem, PCR inhibitors must be diluted, inactivated or removed from the sample. In initial experiments bacteria were inoculated into the blood culture media and the DNA extracted for use as a target in a broad range 16S rRNA gene PCR assay. These assays repeatedly failed to give a PCR product due to the presence of a substance inhibitory to the PCR. Multiple attempts to extract the DNA failed to remove the inhibitor.

Phenol, used for the removal of proteins such as proteases and nucleases, decreases the yield of PCR product when present at a final concentration of 0.2%. At 0.5% phenol, PCR products are undetectable. Ethanol has an inhibitory effect at concentrations greater than 1%, with isopropanol appearing to be a slightly stronger inhibitor than

ethanol. Sodium acetate decreases the yield of PCR product at final concentrations >5mM. EDTA can inhibit PCR when present at concentrations >5mM.

With the QIAamp method, DNA absorbs to the silica in the presence of a chaotrope, is washed with buffer and eluted from the column in TE buffer. It was thought the charcoal contained in the bottles was perhaps an inhibitory factor so an additional centrifugation and wash step was incorporated. This had no effect and the PCR was still inhibited. Alternative BacT/Alert bottles were tested which did not contain charcoal. These contained a pancreatic digest of casein (1.95%), papaic digest of soybean meal (0.3%), sodium polyanetholesulfonate (0.035%), menadione (0.00005%), hemin (0.0005%), reducing agents and other complex amino acid and carbohydrate substrates in purified water. Changing the Bactec bottles to charcoal-free types had no effect and it was thought that perhaps complex carbohydrates and proteins, present in the broth mixture, may be acting as inhibitors. Several amendments to the protocol were carried out to attempt to overcome this inhibition as follows:

#### ***4.2.7 : Phenol chloroform extraction:***

A phenol chloroform extraction (2.4.8) was performed as it was hoped if complex carbohydrates and proteins were acting as an inhibitor they would be eliminated by this method. However, this did not remove the inhibitor.

#### ***4.2.8 : Bovine serum albumin:***

The addition of bovine serum albumin (2.4.9) 10µg/ml 0.01%-0.1% (w/v) (Promega, Southampton, UK) to tissue DNA samples increases the amount of DNA generated by



neutralising many deleterious factors found in tissue samples which can inhibit PCR. It is claimed concentrations of up to 0.8 $\mu$ g/ $\mu$ l may increase the efficiency of a PCR reaction much more than DMSO or glycerol. BSA was added to the upper master mix of the PCR reaction with 5 $\mu$ l inhibited DNA extract sample and had no effect in removing the inhibitor.

#### ***4.2.9 : Millipore centrifugal filter devices:***

Centricon Centrifugal Filter Devices (Millipore, Watford, UK) (2.4.10) claim to provide fast, efficient concentration and desalting of macromolecular solutions by ultrafiltration through low-adsorption, hydrophilic membranes. Designed for use in centrifuges with fixed-angle rotors, they can provide up to 80-fold sample enrichment with minimal solute loss by adsorption.

Concentration is achieved by ultrafiltering the sample solution through an anisotropic membrane. Centrifugal force drives solvents and low molecular weight solutes through the membrane into the filtrate vial. Retained macrosolutes are above the membrane inside the sample reservoir. As the sample volume is diminished, retained solute concentration increases.

#### ***Retention and Recovery***

The membranes used in the centricon are characterised by a nominal molecular weight cut-off, i.e. their ability to retain molecules above a specified molecular weight. Solutes with molecular weights close to the membrane cut-off may only be partially retained. Membrane retention depends on the solutes molecular size and shape. The inhibitor was not removed by this filtration procedure.

#### ***4.2.10 : GeneClean protocol***

The GeneClean™ kit is claimed to be a rapid and efficient method for purifying DNA. The procedure is useful as a general method to clean DNA so that subsequent applications are more efficient (2.4.11).

The methodology is that DNA binds to silica in high concentrations of chaotropic salt and elutes in low salt concentrations. The mechanism of DNA binding in high salt concentrations has not been described, but may involve chaotropic salt disruption of the water structure around negatively charged silica, allowing a cation bridge to form between it and the negatively-charged phosphate backbone of DNA. When the salt is removed, rehydration of the silica matrix breaks the attraction between the matrix and the DNA. The fact that DNA binds in high salt concentration and elutes in low salt concentration makes this method especially useful as a purification procedure. Since the DNA is eluted with either water or a low salt buffer, it can be used immediately in subsequent reactions without precipitation or other further manipulation. This is unlike ion-exchange methods that require binding in low salt concentration and elution in high salt concentration and require precipitation or other means of removing salt before the DNA can be used.

The inhibitor was not removed following this protocol.

#### ***4.2.11 : Mec A detection from Bactec blood culture bottles protocol***

The differential centrifugation technique on blood culture bottles described by Ubukata was utilised. This was used in conjunction with the adapted Murakami and Minamide's Achromopeptidase lysis procedure (2.4.12), however inhibition still persisted.

#### ***4.2.12 : Benzyl alcohol - guanidine hydrochloride organic extraction***

This protocol of Fredericks *et al* (1998) designed particularly for purification of DNA from blood culture media did not remove the inhibitor (2.4.13)

Various combinations of the above failed to eliminate the inhibitor from the Bactec bottles. Using the QIAamp extraction procedure initially, which was known to adequately extract DNA, followed by the above procedures as further clean-up steps still had no effect. It was therefore decided to abandon PCR analysis of the inhibited Bactec bottles.

All attempts at amplification of the bacterial 16S rRNA gene from inoculated Bactec blood culture media failed. The inhibitor persisted despite numerous attempts to purify the DNA, and was identified as sodium polyanetholesulfonate (SPS), a common additive to blood culture media. Like DNA, SPS is a high-molecular-weight polyanion that is soluble in water but insoluble in alcohol. Accordingly, SPS tends to copurify with DNA.

The final extraction method designed by Fredricks *et al* (1998) for purification of DNA from blood culture media and removal of SPS was attempted. Blood culture media (Bactec both with and without charcoal) spiked with *E. coli* and *Streptococcus*

*sanguis* were subjected to an organic extraction procedure with benzyl alcohol. Successful amplification of the extracted 16S rRNA gene should be achieved by adding 5 $\mu$ l of undiluted processed sample DNA to a 50 $\mu$ l PCR mixture. Fredericks *et al* (1998) claimed that with other purification methods, the inhibitory effect of SPS could be overcome only by dilution of these samples. The blood culture additive SPS is a potent inhibitor of PCR that is resistant to removal by traditional DNA purification methods, but Fredericks claimed could be removed by a benzyl alcohol extraction. We did not find this using their protocol and inhibition persisted. An SPS concentration of 100ng/ml in the 50 $\mu$ l reaction mixture is inhibitory, whereas use of a mixture with SPS at 10ng/ml Fredericks claimed resulted in successful amplification of the target. The concentration of SPS in Bactec aerobic and anaerobic blood culture media is 0.05% or 500 $\mu$ g/ml. Thus unprocessed blood culture media would have had to be diluted more than 5000-fold in order to amplify microbial DNA in our assay.

### ***Summary***

Although blood is known to possess substances inhibitory to PCR, many DNA purification methods are successful in eliminating these inhibitors. The QIAamp kit is specifically utilised for extracting DNA from blood samples. The PCR inhibitor present in commercial blood culture media tends to copurify with DNA and was identified as sodiumpolyanetholesulphonate (SPS). SPS is added to blood culture media for its anticoagulant and anti-complementary activities which are believed to increase the level of growth of most microbes. Given the chemical similarities between DNA and SPS, it is not surprising that they co-purify. Both are high molecular weight polyanions that are soluble in water but insoluble in alcohols. Accordingly, this explains why even phenol-chloroform fails to extract SPS. Alcohol

precipitation merely precipitates SPS along with DNA. SPS binds to silica in the presence of chaotropes and elutes with water, just like DNA, thus explaining why the QIAamp columns did not eliminate SPS. Ultrafiltration in centricon-100 columns concentrates SPS because it is too large to pass through the membrane. Washing of cell pellets by centrifugation and resuspension are variably successful in removing SPS. Because SPS may bind to haemoglobin and erythrocyte membranes (which also pellet in blood culture media) and it is inhibitory at extremely low concentrations, excessive wash cycles are required for successful removal.

#### ***4.2.13 : Cases positive by PCR but negative by culture***

As PCR has a higher detection rate it is likely that some samples which were negative by culture due to their presence in low numbers or uncultivability would be positive by PCR. It is possible that even baseline samples could be truly PCR-positive rather than simply contamination due to low levels of bacteraemia occurring physiologically (Ley *et al* 1998). Possible causes for false-positives by PCR include clinical contamination sources such as the skin during venepuncture or through contamination during DNA extraction or indeed during the PCR laboratory procedure itself. However, contamination was not a problem in the currently reported studies as the contamination protocols used were stringent.

False-positive results are a recognised problem with the PCR technique, both in research and hospital laboratories. There are two recognised explanations. The most common problem is carryover of amplicons from previous reactions. Another source of false-positive PCR results is cross-contamination with bacterial DNA isolated from positive clinical samples or the positive control sample during the processing procedure. The frequency with which these problems occur is difficult to document. The routine use of positive and negative controls is necessary to reliably track laboratory contamination. Contamination resulting from poor sample collection is difficult to exclude except when based on the usual clinical criteria that are used to interpret the results of blood culture. These are: a) consistent positivity from multiple blood samples; b) species not commonly existing as commensals and; c) the clinical context.

Positive PCR samples where the culture has been negative may indicate background contamination from organisms obtained during specimen collection, lack of specificity of the primers used if looking for a specific organism (Greisen *et al* 1994), or

subclinical bacteraemia or DNA-aemia that may represent killed fragments of bacteria in the bloodstream (Heininger *et al* 1999). The use of reverse transcriptase PCR may also be useful to identify bacteria that are actively replicating and thus clinically important. This is a molecular tool based on the Temin and Baltimore findings of the enzyme reverse transcriptase that retroviruses use to produce DNA copies of their RNA genome. This enzyme can be used to construct a DNA copy, called complementary DNA (cDNA), of any RNA. Thus genes, or major portions of genes can be synthesised from mRNA.

Because of the extreme sensitivity of the PCR technique, any contamination occurring during collection of the blood specimen or in the laboratory processing will lead to false positives (Victor *et al* 1993).

#### ***4.2.14 : Cases positive by blood culture but PCR-negative***

It is possible that problems with DNA extraction prior to PCR amplification could result in failure to detect certain organisms thus generating false negative results e.g. a problem of inadequate lysis of the bacterial cell wall of Gram positive organisms (Cursons *et al* 1999, Isaacman *et al* 1999). A further consideration is that non-viable bacterial fragments are cleared from the circulation relatively quickly and may be missed if only one sample is taken (Isaacman *et al* 1999). In addition, although it is unlikely, certain organisms may not be detected by the PCR primers due to for example, incomplete homology (Kane *et al* 1998, Isaacman *et al* 1999).

Causes for poor sensitivity or false-negatives by PCR can also be related to the low density of organisms in patients with occult bacteraemia (Isaacman *et al* 1998). As mentioned earlier, since DNA from only a small aliquot of blood can be analysed in a single PCR reaction, it is possible in cases with only a few bacteria per ml that no



bacteria were present in the aliquot used for the PCR reaction. This finding may also limit the usefulness of PCR in other clinical situations in which the density of infection is low. Conversely, if PCR cannot detect organisms particularly when two rounds of amplification have been used, then it could be argued that they may be present in such low numbers that they cannot be of clinical significance. Furthermore, blood and its components may affect the PCR results by inhibiting the function of *Taq* DNA polymerase, thus yielding false-negative results. Indeed, proteins may be synthesised in affected hosts that are inhibitory to the PCR reaction (Salo *et al* 1999).

### ***4.3.1 : LAL discussion***

Due to circumstances beyond our control the first 10 subjects samples could not be processed using the LAL chromogenic assay due to contamination of the plasma samples with haemoglobin. Subjects 11-38 samples were however, analysed using the LAL assay.

To facilitate statistical analyses the values in the LAL assay were transformed i.e. multiplied by 100000.

Mean LPS scores for the 28 LAL subject samples at baseline visit 1 were 47116.29EU (SD 19559.78). This level dropped to 43218.68EU (SD 19024.42) following periodontal probing which is unexpected. If bacteria were being released into the bloodstream following probing the LPS level should be higher than baseline. At baseline visit 3 this level was higher than at baseline visit 1 and following periodontal probing (67153.09EU, SD 98196.17). LPS levels increased again following toothbrushing as would be expected (82380.26EU, SD 138901.57) but fell following cavitron scaling (55326.70, SD 52382.24). This last result is unexpected and is perhaps explained by a cavitron tip not being able to penetrate into deeper pockets without the administration of local anaesthetic. This was not performed in this study as injections themselves can induce a bacteraemia. However, it could be argued that the cavitron was able to penetrate to the base of the pocket and perhaps the cavitron removed the LPS out into the mouth rather than pushing it into the bloodstream

It could be argued that the LPS levels vary independently from instrumentation and that a number of other factors play a more important role e.g. liver function. It is possible that LPS measured by the LAL assay is extremely variable across different

days or that the Hawthorne effect of reduced plaque levels in patients following enrolment into the study.

None of these differences were statistically significant and as can be seen the standard deviations are extremely high and variable. It is for this reason that the LAL assay was not regarded an accurate and reliable measure of LPS, further analyses was disregarded and the results should be interpreted with caution.

## ***CHAPTER 5***

### ***OVERALL DISCUSSIONS AND CONCLUSIONS***

## **5.1 : OVERALL DISCUSSIONS AND CONCLUSIONS**

**Hypothesis :** As the subjects were untreated periodontitis patients it was expected that they should have a high incidence of bacteraemia following periodontal procedures, and that the isolates would reflect the complex anaerobic flora found in periodontal pockets.

It was impossible to predict which dental procedure would cause a bacteraemia to be detected or which patient would be affected as there was no apparent association between severity of disease and detection of a bacteraemia. Subjects with the deepest pocketing were not necessarily those in whom bacteria was detected in the bloodstream. There was not a single procedure which allowed the detection of a bacteraemia in all subjects.

The procedures which caused the detectable bacteraemias by conventional microbiology among subjects were periodontal probing (5 subjects/38 13.16%) cavitron scaling (4 subjects/30 patients 13.33%) and toothbrushing (1 subject/30 patients 3.33%). Cavitron scaling was the procedure which induced bacteraemia on most occasions which can be appreciated given the high tissue trauma induced by mechanical instrumentation and water spray. Periodontal probing also resulted in a high bacteraemia level which is logical as previously suggested by Roberts, bacteria are being readily carried into crevicular and subgingival tissues with this procedure. This is lower than values quoted by Berger *et al* 1988 following dental extractions, which supports the idea that less trauma results in lower bacteraemia levels.

The bacteraemia levels found in our study are lower than that quoted in the literature: Waki *et al* 1990 quoted 18.5% following scaling. Interestingly, Bender *et al* 1989 quoted 30% bacteraemia immediately following scaling which reduced to 5% 10

minutes later. Our lower levels may be indicative of the sampling time being too long after the start of the procedure. Our lower value taken immediately following the procedure is similar to Bender's finding at 10 minutes. In addition as Roberts *et al* 1997 highlighted there may have been too long a gap between cessation of a manipulative procedure, application of a tourniquet and blood sampling time. As Heimdahl *et al* 1996 suggested, optimum sampling may be best performed during a procedure to yield more positive culture samples which would require the assistance of another trained person. Our finding may also be unique to periodontal patients whose immune systems readily and regularly have to deal with bacteraemia through the periodontal pocketing. This is opposed to our original hypothesis that periodontal patients would produce very high bacteraemia levels on instrumentation due to the high bacterial load found in periodontal pockets. However, it is possible, that our conventional microbiological detection levels were not sensitive enough.

The majority of samples positive by subculture yielded one isolate (50%) which agrees with the findings of Roberts *et al* 1998 in a bacteraemia study following oral surgical procedures. 20% yielded two isolates, 20% yielded 4 and 10% yielded 5. In addition, supporting the previously mentioned study by Roberts, *Streptococci* were a predominant isolate, isolated on three occasions from different patients, as was *Actinomyces naeslundii* isolated on three occasions, *Propionibacterium acnes*, *Staphylococci* and *Micrococcus* isolated twice. It has been reported that contamination with skin commensals such as *P. acnes* may occur in up to 3% of all blood specimens even when the skin over the venepuncture site is prepared carefully. However the *P. acnes* isolated from our samples only grew anaerobically. No growth was exhibited aerobically or in microaerophilic atmospheres therefore it is highly unlikely to simply

be a skin contaminant. In addition Debelian *et al* 1992 also isolated *P. acnes* from the blood of patients undergoing endodontics.

The only putative periodontal pathogen isolated was *Prevotella intermedia* with the non-pathogenic *Haemophilus aphrophilus* isolated on one occasion, although there is current interest in the possibility of *Eubacterium* species being implicated in periodontal disease.

This is of particular significance as non-streptococcal bacteria such as those found in the periodontal pocket are now reported as causing infective endocarditis, and in addition these bacteria are not uniformly susceptible to the antibiotics recommended for prophylaxis. Animal studies such as that by Glauser *et al.* (1987) indicate that periodontal disease does increase the incidence of infective endocarditis and that the number of microbes entering the bloodstream may not be as important in the production of infective endocarditis as other qualities, such as the microbes ability to adhere.

The incidence of bacteraemia appears to depend on several factors including clinical manipulation and on microbiological laboratory technique. This may also include the level of gingival inflammation and bacterial load, however the low bacteraemia levels isolated from this group of periodontal patients suggest the opposite. As suggested earlier however, it may be that our conventional microbiological culturing technique was not sensitive enough to detect all bacteraemia and if the study was to be repeated, a technique of lysis filtration could prove beneficial. The magnitude of bacteraemia may indeed be affected by a variety of factors, such as the procedure itself or the health status of the gingivae. It is more logical that the incidence of bacteraemia relates to the amount of plaque present and the extent to which organisms are introduced into the

tissues. However, as all patients in this study were untreated periodontal patients one would expect a higher bacteraemia level than that found. A greater number of teeth manipulated should result in a greater area of disruption and facilitate entry of microorganisms. However, although some patients had lost teeth due to periodontal disease it was ensured all subjects taking part in the study had a minimum of 20 teeth.

As Roberts *et al* 1999 states, a much larger study population is required to show significant associations with bleeding on probing, pocket depth etc. if such a relationship exists.

As mentioned previously, the timing of sampling may have been too long an interval following the onset of a procedure. Sampling during manipulation may allow optimum bacterial detection. However, it could be argued that the optimum detection of bacteria may not be as important as those isolates found to persist in the bloodstream some time following manipulation. Such microorganisms may display bacterial resistance to removal by the hosts immune system and therefore a greater propensity to cause disease.

There were too few patients in the study to assess the effect of a mouthwash especially since not all patients yielded a detectable bacteraemia. Only 5 patients produced a bacteraemia at visit 1 following periodontal probing. At visit 2, only 1 of these 5 subjects produced a bacteraemia, the remaining 4 did not and it would be illogical to suggest this was purely the effect of the mouthwash.

Mouthwash effects were difficult to assess at visit 2. Periodontal probing was not performed at this visit therefore a direct comparison could not be made to establish what effect the procedure actually had. e.g. if the cavitron scaling at visit 2 produced a larger bacteraemia than the periodontal probing at visit 1, but the mouthwash lowered the possible bacteraemia by enhancing tissue integrity or reducing bacterial load; too



many variables make this impossible to interpret. However, comparing the initial baseline samples at visit 1 and 2 should show potential effects, if any, of the mouthwash.

It may be possible that by placing patients on any mouthwash would result in a Hawthorne effect or placebo response which would raise the patients awareness and increase their attention on oral hygiene practices giving a false reduction in bacteraemia inducing procedures and any plaque reducing effect a mouthwash may have.

PCR results did not correlate with conventional microbiology results. If PCR could have been performed on the inhibited Bactec culture bottles perhaps the molecular and conventional microbiology samples would be more comparable. It is likely given the greater number of positive samples detected by PCR than with conventional culture that PCR is more sensitive and can detect the presence of bacteria at baseline samples. However, PCR also detects bacteria which are moribund which may not be of clinical significance.

It is evident that bacteraemia does arise following periodontal manipulation. Procedures which were previously regarded as innocuous and therefore not covered by prophylactic antibiotics such as periodontal probing allowed the detection of bacteraemia almost as frequently as cavitron scaling.

The frequency of bacteraemia detection in this study was lower than that quoted by previous authors. However, it is possible that patients with adult periodontitis present a unique patient base whose immune systems are highly primed with periodontal bacteria, hence when a bacteraemia is induced it is quickly and efficiently cleared by the patients reticuloendothelial system.

Such low levels of bacteraemia present further questions on the clinical significance of bacteraemia induced by dental manipulation in a professional setting.

***CHAPTER 6***  
***FURTHER WORK***

## **6.1 : FURTHER WORK**

A larger number of blood samples per patient and indeed a larger volume of sample would perhaps increase the sensitivity of the detection of the bacteraemia by conventional blood culture. However, as indicated by the literature the optimum blood volume was used with the Bactec culture bottles. Indeed other culture techniques e.g. lysis filtration, and including the use of selective culture media may allow a higher degree of detection. The bacteraemia detected may be underestimated due to the use of non-selective culture media that may have not sustained the growth of some organisms. It is possible that the use of selective media, particularly those designed to support the growth of more fastidious periodontal pathogens may result in both quantitative and qualitative differences in future findings.

As mentioned previously the timing of sampling may have been too long following the onset of the procedure but the isolates of clinical significance may be those which persist in the bloodstream.

Observations made on single cases have only limited scientific value but certainly illustrate important inter-individual variations in periodontal patients. Although the patients examined in the study were selected for their similar levels of periodontal disease clinically (CPITN values), there were obviously marked differences between the subjects with respect to composition of subgingival and indeed supragingival plaque. There were obviously differences in the levels of BOP which may indicate differences in the inflammatory reaction in the gingival tissues which could influence whether a bacteraemia is more readily produced. As in Wennstrom's study (1987) it may be suggested that in addition to clinical variables, microbiological parameters

should be included as criteria in the selection of comparable patients/pockets to be studied in order to test the effect of certain therapies.

It is apparent that not all patients develop a detectable transient bacteraemia following invasive dental procedures. Therefore it would be useful to have a screening process to identify qualifying bacteraemia-susceptible subjects prior to testing the efficacy of a mouthwash or the bacteraemia inducing potential of any procedure.

It is recognised that ideally the method for measuring periodontal probing depths should be reproducible and a probe of constant force or Florida probe should be used.

In addition plaque and gingivitis indices should be essential. The O'Leary plaque index (O'Leary *et al* 1972) records the presence or absence of plaque in contact with the gingival margin and the Gingival bleeding index (Ainamo and Bay 1975) records the presence or absence of bleeding from gingival margin elicited by a periodontal probe.

It was evident that too many variables were attempted to be investigated in the study. A more simple design investigating fewer clinical procedures before and after mouthwash use may have been more appropriate.

It was evident from the high standard deviation values that the LAL assay was not ideal and ELISA may be a more robust technique for LPS detection for future studies.

Instead of fluoride mouthwash it would have been more appropriate to have a true placebo mouthwash i.e. the same chlorhexidine mouthwash product without the active

chlorhexidine ingredient. This would eliminate the possible effect fluoride or other constituent may have had on reducing bacteraemia incidence.

PCR primers for specific periodontopathogens could be utilised in the detection of bacteraemia. This would remove the possible element of detected bacteraemia being actual contamination when using 16S primers. However, it is acknowledged that the majority of isolates found in this study were not classical periodontopathogens.

A further molecular biological technique that is, sequencing the isolates from the cultures could be used to check if the results of different techniques agree in the determination of the species. The PCR primers used in the present study were also used by Cursons *et al* who discovered a compromise between using the small, highly conserved DNA sequences required for a sensitive and broad-spectrum PCR test and using longer DNA sequences that enable better organism identification, but are less sensitive. An extraction technique which would allow the combination of both amplifications would be ideal.

The problem of inhibited Bactec culture bottles when PCR was performed needs to be investigated further. An extraction technique which would allow removal of the inhibitor would be ideal. Otherwise the culture bottles would need to be changed to those which do not contain SPS. This has the added complication of possibly reducing the number of isolates found by conventional culture given the antiphagocytic properties of blood would not be inactivated.

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## ***APPENDIX***

## *APPENDIX 1 : patient selection*

### *Periodontitis classification : 1999 international workshop for a classification of periodontal diseases and conditions*

The primary features of periodontitis include clinical attachment loss, alveolar bone loss, periodontal pocketing and gingival inflammation. In addition, enlargement and recession of the gingiva and increased mobility, drifting and tooth exfoliation may occur. With few exceptions most forms of periodontitis are chronic inflammations that may progress continuously or by bursts of activity.

The histopathological characteristics of periodontitis include periodontal pocketing, location of junctional epithelium apical to the cemento-enamel junction, loss of collagen fibers subjacent to pocket epithelium, bone loss, numerous neutrophils in the junctional and pocket epithelium and dense inflammatory infiltrate with plasma cells, lymphocytes and macrophages.

Bacterial infection is implicated as the primary cause of periodontal disease. Several bacterial species residing in biofilm on tooth surfaces related to dental plaque have been closely associated with periodontitis. These include *Porphyromonas gingivalis*, *Actinobacillus actinomycescomitans*, *Bacteriodes forsythus*, non-classified spirochetes, *Prevotella intermedia*, *Campylobacter rectus*, *Eubacterium nodatum*, *Treponema denticola*, *Streptococcus intermedia*, *Prevotella nigrescens*, *Peptostreptococcus micros*, *Fusobacterium nucleatum* and *Eikenella corrodens*. It appears that various complexes of putative periodontal pathogens can initiate and perpetuate the disease in a susceptible host (Armitage, 1999).

### ***Chronic periodontitis : new classification (consensus report 1999)***

Adult periodontitis has been classified as a form of periodontitis that usually presents after the age of 35. Bone resorption progresses slowly and predominantly in the horizontal direction. Well known local environmental factors are of major aetiological importance whereas major abnormalities in host defence have not been found. However the scientific basis for using the patients age of disease onset as a classification criteria is lacking and as the form of periodontal disease previously called 'adult periodontitis' can occur over a wide range of ages, and can be found in both the primary and secondary dentition, the term adult periodontitis has been discarded and 'chronic periodontitis' is used instead. Chronic periodontitis can be divided into localised and generalised. Some of the features and characteristics of chronic periodontitis are:

- Most prevalent in adults, but can occur in children and adolescents.
- Amount of destruction is consistent with the presence of local factors.
- Subgingival calculus is a frequent finding.
- Associated with a variable microbial pattern.
- Slow to moderate rate of progression but may have periods of rapid progression.
- Can be further classified on the basis of extent and severity.
- Can be associated with local predisposing factors (e.g. tooth-related or iatrogenic factors).
- May be modified by and/or associated with systemic diseases (e.g. diabetes mellitus, HIV infection).

- Can be modified by factors other than systemic disease, such as cigarette smoking and emotional stress.

Chronic periodontitis is recognised as the most frequently occurring form of periodontitis. Its onset may be at any age but is most commonly detected in adults. The prevalence and severity of the disease increases with age, it may affect a variable number of teeth and has various rates of progression. Chronic periodontitis is initiated and sustained by bacterial plaque, but host defence mechanisms play an integral role in its pathogenesis. The progressive nature of the disease can only be confirmed by repeated examinations and it is reasonable to assume that the disease will progress further if treatment is not provided.

Chronic periodontitis can be further characterised by extent and severity. Extent is the number of sites involved and can be described as localised (<30% of sites affected) or generalised (>30% of sites affected). Severity can be categorised on the basis of the amount of clinical attachment loss slight 1-2mm, moderate 3-4mm and severe >5mm.

