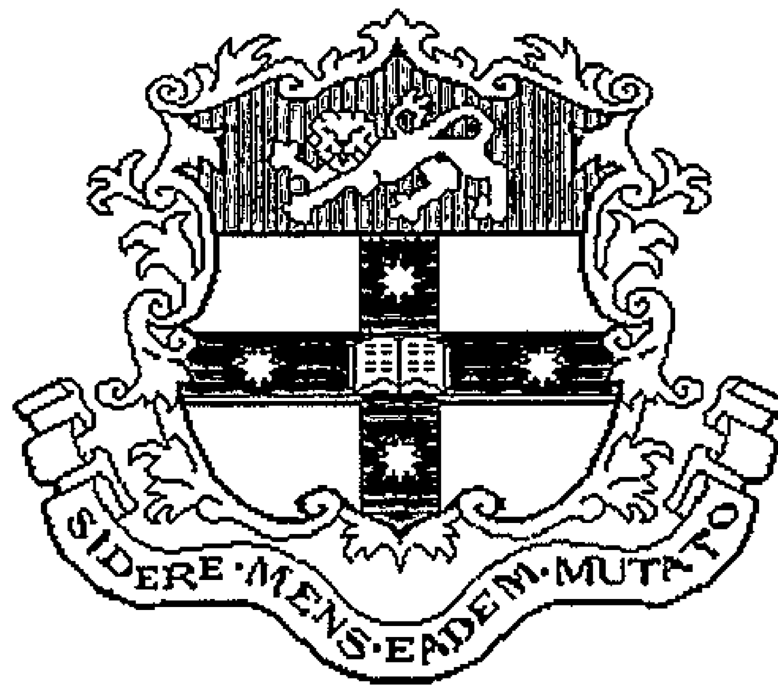
ANALYSES OF MICROBIAL POPULATIONS ASSOCIATED WITH CARIOUS PULPITIS



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BDS (Honours)

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A thesis submitted in fulfilment of the

requirement for the degree of

Doctor of Philosophy

Institute of Dental Research

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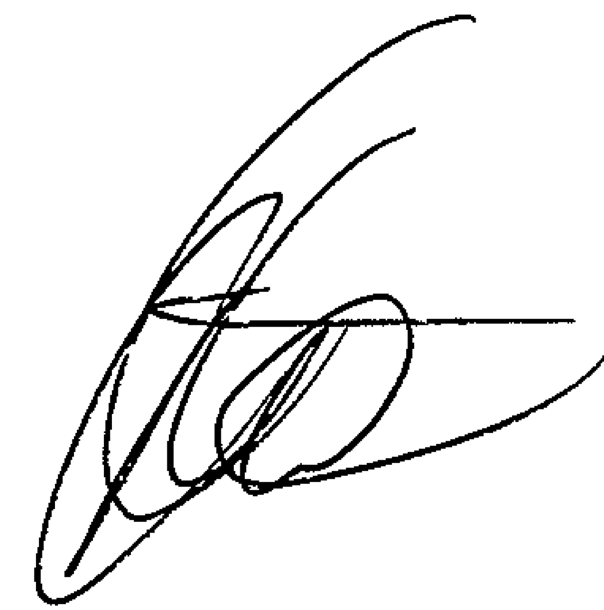
STATEMENT OF AUTHORSHIP

The experimental work presented in this thesis was undertaken at the Institute of Dental Research. All work presented in this thesis was performed by the candidate except where stated otherwise. This work has not been submitted in whole or in part for any other degree, or at any other University.



Fjelda Elizabeth Martin
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PUBLICATIONS

The following papers contain results described in this thesis.

Nadkarni MA, Martin FE, Jacques NA, Hunter N. Determination of bacterial load by real-time PCR using a broad range (universal) probe and primers set. *Microbiology* 2002; 148: 257-266.

Martin FE, Nadkarni MA, Jacques NA, Hunter N. A quantitative microbiological study of human carious dentine by culture and real-time PCR: Association of anaerobes with histopathological changes in chronic pulpitis. *Journal of Clinical Microbiol* 2002; 40: 1698-1704.

Martin FE, Hunter N. Histopathological changes in chronic human pulpitis; *in preparation.*

Nadkarni MA, Martin FE, Jacques NA, Hunter N. Quantitative extraction and protection of DNA from a complex mixed anaerobic/microaerophilic bacterial flora; *in preparation.*

Paper describing studies peripheral to the thesis.

Nguyen K-A, DeCarlo A, Paramaesvaran M, Nadkarni MA, Martin FE, Yun PW, Hunter N. Clinical relevance of *Porphyromonas gingivalis* proteases (gingipains) and haemoglobin binding receptor in adult periodontitis. *Infection and Immunity, revised manuscript in preparation.*

LIST OF ABBREVIATIONS

%	percentage
±	plus or minus
≥	greater than or equal to
≤	less than or equal to
°C	degrees Celsius
Rn	fluorescence signal
µm	micrometre
16S rDNA	16 small subunit ribosomal deoxyribonucleic acid
16S rRNA	16 small subunit ribosomal ribonucleic acid
A_{260}	absorbance at 260nm
ALP	alkaline phosphatase
ANOVA	analysis of variance
bp	base pair
CFAT	cadmium fluoride acriflavin tellurite
CFU	colony forming units
C_T	threshold cycle
d	day
dNTP	mixture of four types of deoxynucleotides: deoxyadenosine 5″-triphosphate (dATP); deoxyguanosine 5′-triphosphate (dGTP); deoxycytidine 5′-triphosphate (dCTP) and deoxythymidine 5′-triphosphate (dTTP)
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediamine tetra-acetic acid

EGTA	ethylene glycol-bis (β-aminoethyl ether)-*N, N, N′, N′*,-tetra-acetic acid
ETSA	enriched trypticase soy agar
FAM	6-carboxyfluorescein
h	hour
IL	interleukin
IPC-BT	internal positive control-*Bactrocerra tryoni*
KVA	kanamycin vancomycin agar
min	minute
MES	2-[N-morpholino] ethane sulphonic acid
MHC	major histocompatibility complex
OD	optical density
PCR	polymerase chain reaction
PMNL	polymorphonuclear leukocyte
RNase	ribonuclease
RTF	reduced transport fluid
s	second
SEM	standard error of the mean
SD	standard deviation
SDS	sodium dodecyl sulphate
TAMRA	6-carboxy-tetramethylrhodamine
Taq	Thermus aquaticus
t_d	bacterial doubling time
T_m	melting temperature of DNA
TNF	tumour necrosis factor
UNG	uracil-*N*-glycosylase
VIC	proprietary buffer supplied by Qiagen

These abbreviations were used for frequently mentioned terms; otherwise normal chemical nomenclature was used throughout this thesis.

ABSTRACT

Dental caries continues to be a significant public health problem affecting mankind in many parts of the world. Microbial activities induce the progressive localised destruction of teeth that without treatment, would eventually result in infection of the dental pulp and surrounding periapical tissues. Although the bacteria responsible for caries initiation and early caries progression have been extensively studied, the microbiology of dentine caries is reported to show considerable diversity and has not yet been fully identified. Few studies have analysed the microbiology of deep caries or examined the relationship between the microflora and the histopathology of chronic pulpitis in symptomatic teeth.

Matched carious dentine samples and dental pulps were obtained from teeth without evidence of periodontal disease but with coronal caries and symptoms of pulpitis. Bacteria were cultured from the carious dentine samples under both anaerobic and microaerophilic conditions. Real-time polymerase chain reaction (PCR) technology was also used to identify and enumerate the bacteria. Development of techniques for the efficient extraction of bacterial DNA from both Gram-negative and Gram-positive bacteria found in carious dentine was an essential prerequisite for molecular analysis. In addition, the dental pulps were processed and categorised into one of four groups on the basis of the dominant pathology of the tissue (minimal inflammation, soft tissue degeneration, hard tissue degeneration and inflammatory degeneration).

Analysis of the culture data indicated a predominance of Gram-positive bacteria, particularly lactobacilli, while Gram-negative bacteria were also present in significant numbers with *Prevotella* species the most numerous anaerobic group cultured. Real-time PCR indicated a greater anaerobic microbial load than that determined by colony counting. The total number of anaerobes detected by PCR was 41-fold greater, while *Prevotella* spp. and *Fusobacterium* spp. were 82-fold and 2.4-fold greater respectively. PCR also identified the presence of *Micromonas*

micros, Porphyromonas endodontalis and *Porphyromonas gingivalis* in 71%, 60% and 52% of carious dentine samples, respectively. Correlation matrices from the real-time PCR data revealed significant multiple associations involving *Fusobacterium* spp. in combination with *P. endodontalis, M. micros* and/or *Prevotella* in the tissue response categories of minimal inflammation, soft and hard tissue degeneration. A positive correlation was also observed between *M. micros* and *P. endodontalis* for the category of inflammatory degeneration of the dental pulp. These anaerobes have been strongly implicated in endodontic infections that occur as sequelae to carious pulpitis. Accordingly, the data suggest that the presence of threshold levels of these bacteria in carious dentine may be indicative of irreversible pulpitis. Knowledge of the microbial predictors associated with irreversible pulpitis creates potential for the development of a diagnostic tool, and for restorative materials with antimicrobial properties.

INTRODUCTION

The problem

Despite the recognised advantages of fluoride in reducing tooth decay in the community, dental caries and associated pulpal symptoms continue to be a major public health problem. The development of dental caries requires certain prerequisites including the presence of cariogenic microorganisms, fermentable carbohydrates, susceptible tooth surfaces and time. The microorganisms involved in caries initiation and early caries development have been well documented and those most frequently implicated include streptococci, particularly the mutans group, and lactobacilli. Through the production of lactic acid, these species promote an acidic environment capable of demineralising enamel and exposing the dentine tubules. This phase is followed by the invasion of more opportunistic microbes such as veillonellae, neisseria and eubacteria that utilise lactic acid to produce less acidic products. The altered environment then favours the obligate anaerobes including fusobacteria, prevotellae and the porphyromonads. These organisms produce enzymes and toxic by-products with potential to degrade the pulpal matrix, attack immune components and interfere with repair.

Despite this knowledge, it is not known which microorganisms drive the carious process culminating in pulpal infection or what key interactions exist between microbes to assist this process. Additionally, the available literature provides no understanding of the relationship between the microbial profile of caries, pulpal histopathology and patient symptoms. Although the microbial populations of dental caries are known to be highly complex and variable, they have not been fully identified and it is possible that the organisms primarily responsible for pulpal infection have not yet been found. This problem is further exacerbated by the small number of studies that have investigated the microbiology of carious dentine and the single report on the relationship between the dentinal microflora and the histopathology of chronic pulpitis. As a result there is no reliable evidence to assign an aetiological role for caries pulpitis to a particular microbial species.

Aim of the study

This study will extend the results of previous work (Massey *et al.*, 1993) by examining the microbiology of carious dentine using traditional culture techniques. In addition, molecular techniques (polymerase chain reaction) will be used to enumerate the more fastidious anaerobic species. Data from both sources will then be correlated with the histopathology of chronic pulpitis from human teeth in an attempt to identify microbial predictors associated with pulpal infection.

Research approach

To achieve this aim, an overview of the early theories of dental caries as well as a description of the dental structures, components and processes involved in the development of dental caries is presented in Chapter 1. A review of the significance of anaerobic infections including pathogenic attributes associated with dental caries and associated periapical disease is covered in Chapter 2. In Chapter 3, the protective and destructive aspects of the inflammatory and immunological reactions of the dental pulp to the carious process are examined. Chapters 4 to 7 encompass the experimental work. In Chapter 4, sample collection of carious dentine from extracted teeth, microbiological identification and enumeration using traditional culture methods are described. The detection of bacterial DNA by polymerase chain reaction (PCR) is presented in Chapter 5. The first part of the chapter includes a review of PCR technology as a method of identifying and enumerating bacteria. The remainder of the chapter contains experimental work divided into two stages; the first stage describes the development and testing of the universal amplicon, designed to detect the total bacterial load from samples. The second stage uses the universal amplicon to examine different methods of bacterial DNA extraction and protection in preparation for PCR. In Chapter 6, PCR technology is used to identify and enumerate anaerobic bacteria from the carious dentine samples. In Chapter 7, the histopathology of the dental pulps is reported and the results correlated with the microbial quantitation from both the culture data (Chapter 4) and PCR data (Chapter 6). The final chapter evaluates the achievements of the studies, identifies limitations and includes suggestions for the direction of future research resulting from this work.

CHAPTER 1

THE DEVELOPMENT OF DENTAL CARIES

1.1 Introduction

Dental caries is one of the most prevalent diseases affecting mankind. It is a unique disease in that humans have life-long susceptibility as the presence of carious teeth does not confer protective immunity (Bowen, 1999). Although caries rarely endangers life, it accounts for substantial pain and loss of masticatory function. Despite an increase in recent years in understanding of the pathogenesis of caries, knowledge is incomplete and dental caries continues to be a major public health problem.

Dental caries is described as a "dieto-bacterial" disease related to the pathogenic activities of bacteria in dental plaque. It involves the localised, progressive destruction of teeth by organic acids produced as a result of the fermentation of carbohydrates by oral bacteria. It is a chronic disease, which progresses very slowly, is seldom self-limiting and in the absence of treatment usually results in destruction of the tooth (Figure 1.1a), with subsequent infection of the dental pulp and surrounding periapical tissues (Figure 1.1b).

Dental caries is an ancient disease as interpreted from skeletal remains that provide a permanent record of caries through the ages. A pattern of sporadic caries has been documented from the beginning of the Iron Age (500BC) to the end of the medieval period (1500AD). In the seventeenth century the pattern of caries changed to one of increasing prevalence. These changes were associated with the establishment of the New World sugar industry and improvements in flour milling. Sugar consumption in Britain then remained stable until the mid 1800's when the removal of sugar import duties and repeal of the Corn Laws resulted in a rapid increase in consumption, especially by the lower socio-economic groups in industrial areas. This was accompanied by an increased incidence of gross damage to tooth structure

Figure 1.1a. Cavitation of the occlusal surface of the first permanent molar indicating gross clinical caries involving destruction of enamel and dentine.

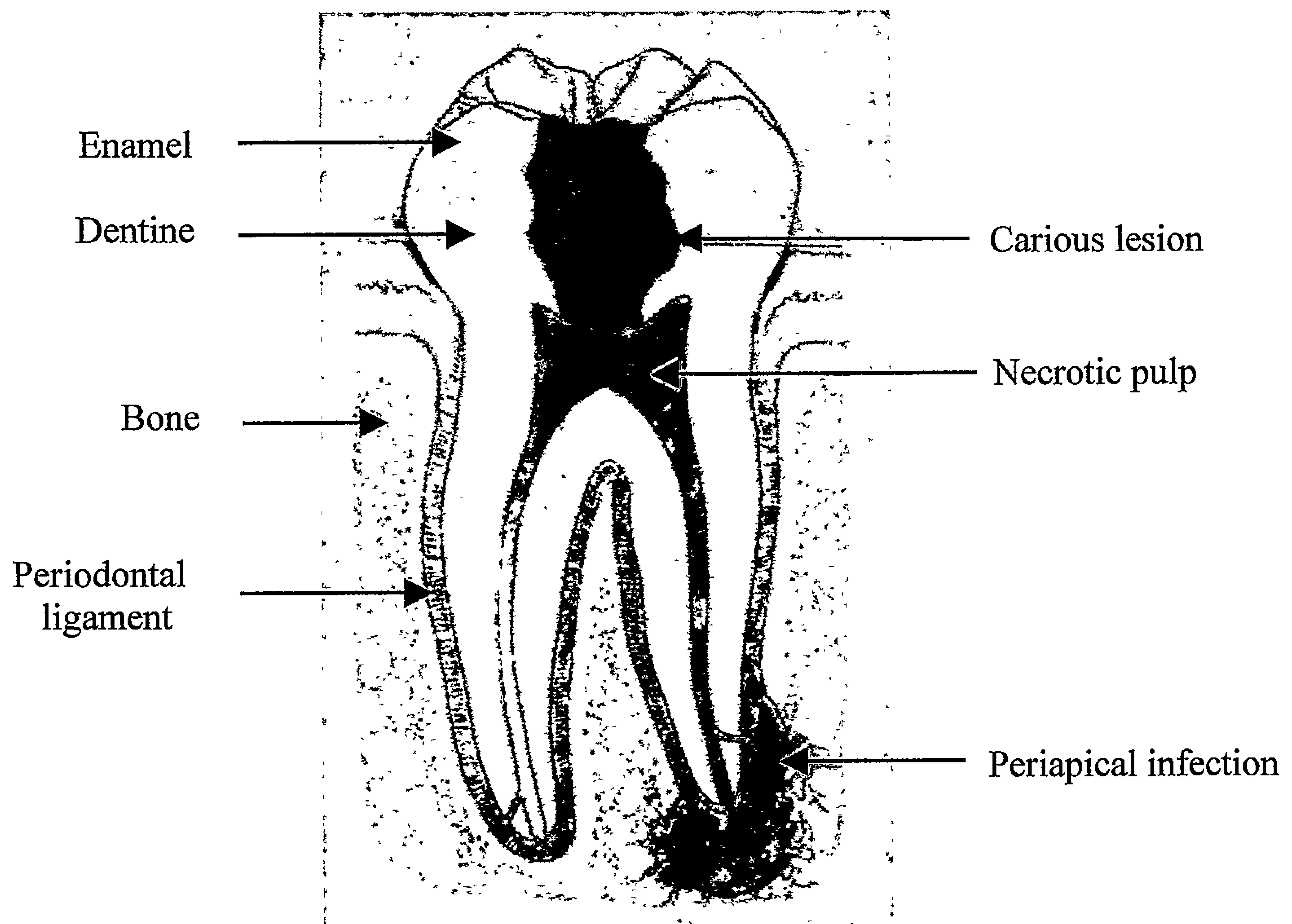


Figure 1.1b. Diagram illustrating the spread of infection from the necrotic dental pulp to the surrounding periapical tissues.

resulting from caries (Corbett and Moore, 1976). By the 1950's dental caries had reached epidemic proportions with 90-95% of the population in the developed world affected (Clarkson, 1999).

Historical evidence indicates the importance of sugar in caries aetiology. Sucrose is probably the most cariogenic sugar because it more readily facilitates the generation of organic acids and as a result of its low cost and ubiquitous availability in the diet of people in developed countries. In addition, sucrose is unique as a substrate for the production of extracellular storage of polysaccharides and insoluble matrix polysaccharides. Thus favouring the colonisation of teeth by microorganisms, increasing the retentiveness of plaque to the tooth surface and providing a reservoir of energy for continued cariogenic metabolism (Thylstrup and Fejerskov, 1994).

Descriptions of the carious process were recorded as early as the 1500's. There have been many theories advanced over the years to explain the aetiology of dental caries. Some of these have included worms, imbalance of body humours, the vital theory, chemical, parasitic, proteolytic and chelation theories, and autoimmunity. More recently, G.V. Black and P. Fauchard noted the association between caries and the acid derived from food particles. The basic concept which remains unchanged today was that proposed by WD Miller in 1890, the chemico-parasitic theory. According to observations by other researchers and work carried out by Miller in the 19th century, dental caries is caused by acid attack on the enamel resulting from the fermentation of dietary carbohydrates by oral bacteria (Hardie, 1982).

These early workers reported the presence of masses of microorganisms on the tooth surface, now known as dental plaque, and studied the effects of acids produced by different oral bacteria on enamel. In addition, they demonstrated the presence of bacteria within the dentinal tubules of more advanced carious lesions. Results from studies on experimental enamel lesions indicated that dental plaque acted as a diffusion limiting membrane, influencing the passage of ions into and out of the enamel surface and limiting the access of salivary buffers to the site of acid attack, thereby producing sub-surface demineralisation.

Direct evidence for the aetiological role of bacteria came from experimental studies with animals carried out by Orland and colleagues in the 1950's. They found that germ-free rats failed to develop caries even when fed a sucrose-rich diet. Inoculation of oral bacteria into these animals resulted in the development of caries. Many researchers have now confirmed the finding that bacteria are essential to both the initiation and development of caries. It has also been shown that caries can be transmitted from infected to non infected individuals. Young children without teeth usually do not carry *Streptococcus mutans,* a species implicated in the development of dental caries, however, following tooth eruption the ecological conditions for colonisation are established and the child acquires the bacteria from the primary care-giver, usually the mother (Caufield, 1997).

Dental caries has been described as a multifactorial disease. As with any other infectious process, the severity and occurrence of the disease depend on a balance between the microbial challenge, the host defences and environmental conditions (Hardie, 1982). The host defence mechanisms that operate in the mouth are complex. These include the chemical and structural composition of the teeth, their morphology and position in the mouth; factors that are critical in determining caries susceptibility. Other considerations include the buffering capacity and antibacterial components of saliva, the behavioural factors of the host, and the presence of restorations and appliances. One of the major environmental factors known to affect dental caries is the frequency of consumption of fermentable sugars. Another important consideration is the amount of fluoride that has been incorporated into tooth structure and the amount available locally. Dental caries can be prevented by adequate oral hygiene, reduced frequency of sugar consumption and the use of fluoride supplements.

The caries preventive measures aimed at controlling bacterial build-up include mechanical removal of plaque with a tooth brush or floss, chemical methods using topically applied solutions and gels, such as fluoride and chlorhexidine and development of a vaccine to protect against caries using *S. mutans* as the immunising agent. Although these measures have been shown to be effective in controlling the disease, dental caries persists and the core of

traditional dental practice involves the removal of carious dentine and its replacement with a restorative material.

1.2 Enamel-dentine-pulp complex

The hard tissue barrier of enamel, dentine and cementum provides strong mechanical support and protection to the loose connective tissue of the dental pulp. The harmful influences of the oral environment will not affect the pulp while the hard tissue barrier remains intact.

1.2.1 Enamel

Tooth enamel is a highly mineralised tissue containing, by weight, 96% mineral (hydroxyapatite) and 4% organic material and water. The mineral phase is made up of densely packed hydroxyapatite crystals arranged to form cylindrical rods with an average width of 5μm. The crystal orientation within the rod varies and those crystals distant from the centre flare laterally as they approach the periphery; this forms the inter-rod enamel. It is the susceptibility of these crystals to dissolution by acid that provides the chemical basis for dental caries (Figures 1.2a and 1.2b). The organic material forms a fine, lacy network between the enamel crystals. The bulk of this organic material consists of tyrosine-rich amelogenin polypeptide tightly bound to the hydroxyapatite crystals. Microscopic spaces filled with water separate the crystals (Ten Cate, 1998).

1.2.2 Dentine

Dentine is an avascular, mineralised connective tissue forming the bulk of the tooth. It is characterised by the presence of multiple closely packed dentinal tubules that radiate from the pulp and contain the cytoplasmic extensions of the dentine-forming odontoblasts. Mature dentine consists of approximately 70% inorganic material, 20% organic material and 10% water, by weight. The inorganic component consists mainly of hydroxyapatite and the organic phase is type I collagen with minor amounts of glycoproteins, proteoglycans and phosphoproteins (Ten Cate, 1998).

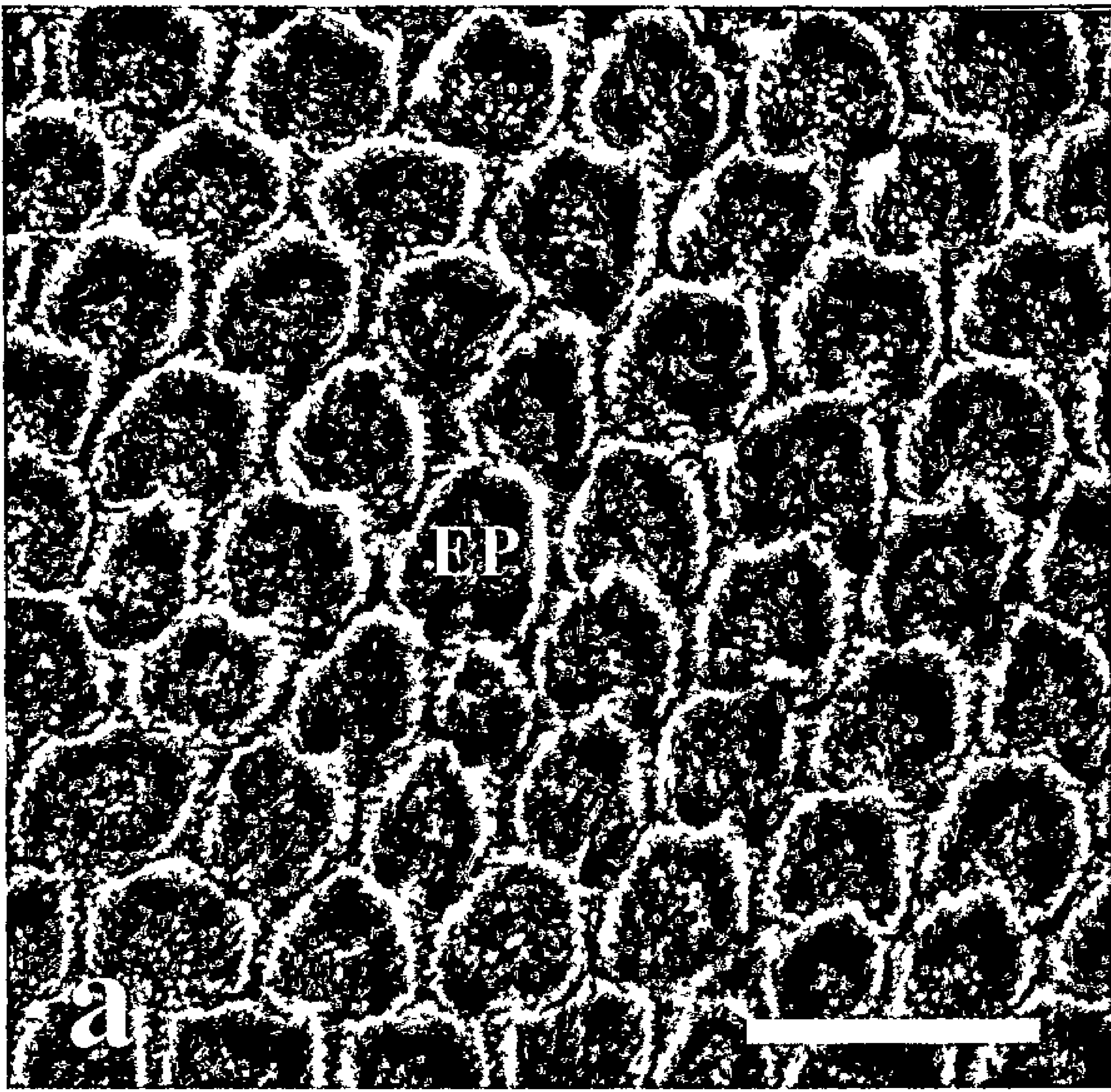
Dentine tubules are responsible for the permeability of dentine. They are tapered structures approximately 2.5μm in diameter near the pulp, 1.2μm mid-dentine and 0.9μm at the dentino-

enamel junction (DEJ) (Figures 1.3a and 1.3b). However, the cross-sectional areas of the tubules differ markedly due to variations in the amount of peritubular (intratubular) dentine. There are approximately 35,000 dentine tubules/mm^2 at the DEJ with approximately double that number/mm^2 at the pulp surface. This increase per unit area is a result of crowding of the odontoblasts at the pulp surface compared with the greater surface area available at the DEJ. Dentine tubules branch to form a profuse anastomosing canicular system of side branches noted to be more numerous in root dentine than in the coronal portion. It is speculated that dentinal tubules contain the process of an odontoblast bathed in tissue fluid, nerve fibres and types I and V collagen fibrils. The tissue fluid has been described as a gel containing proteoglycans, glycoproteins including fibronectin and the plasma proteins albumin and transferrin (Ten Cate, 1998).

1.2.3 Dental pulp

The dental pulp is a loose connective tissue encased in a hard tissue shell. Histologically, four distinct zones can be distinguished: the odontoblastic zone at the pulp periphery, the cell-free zone of Weil beneath the odontoblasts, the cell-rich zone and the pulp core (Figure 1.4). The pulp is composed of cells, extracellular matrix, blood vessels and nerves. The principal cells of the pulp include the odontoblasts, fibroblasts, putative undifferentiated mesenchymal cells, macrophages and other immunocompetent cells (Ten Cate, 1998).

The odontoblasts are responsible for the development of dentine, and form a distinctive lining of columnar cells around the periphery of the pulp with processes extending into dentine (Figure 1.5). Fibroblasts are the most numerous cells in the pulp and particularly in the coronal portion where they form the cell-rich zone. The function of the fibroblasts is to establish and maintain the pulp matrix consisting of collagen and ground substance. Undifferentiated mesenchymal cells represent the pool of cells from which connective tissue cells of the pulp are derived. Little is known about the immunocompetence of the dental pulp, however, small numbers of macrophages, T lymphocytes and antigen-expressing dendritic cells have been demonstrated in healthy pulp tissue (Pulver *et al.*, 1977; Jontell *et al.*, 1987).



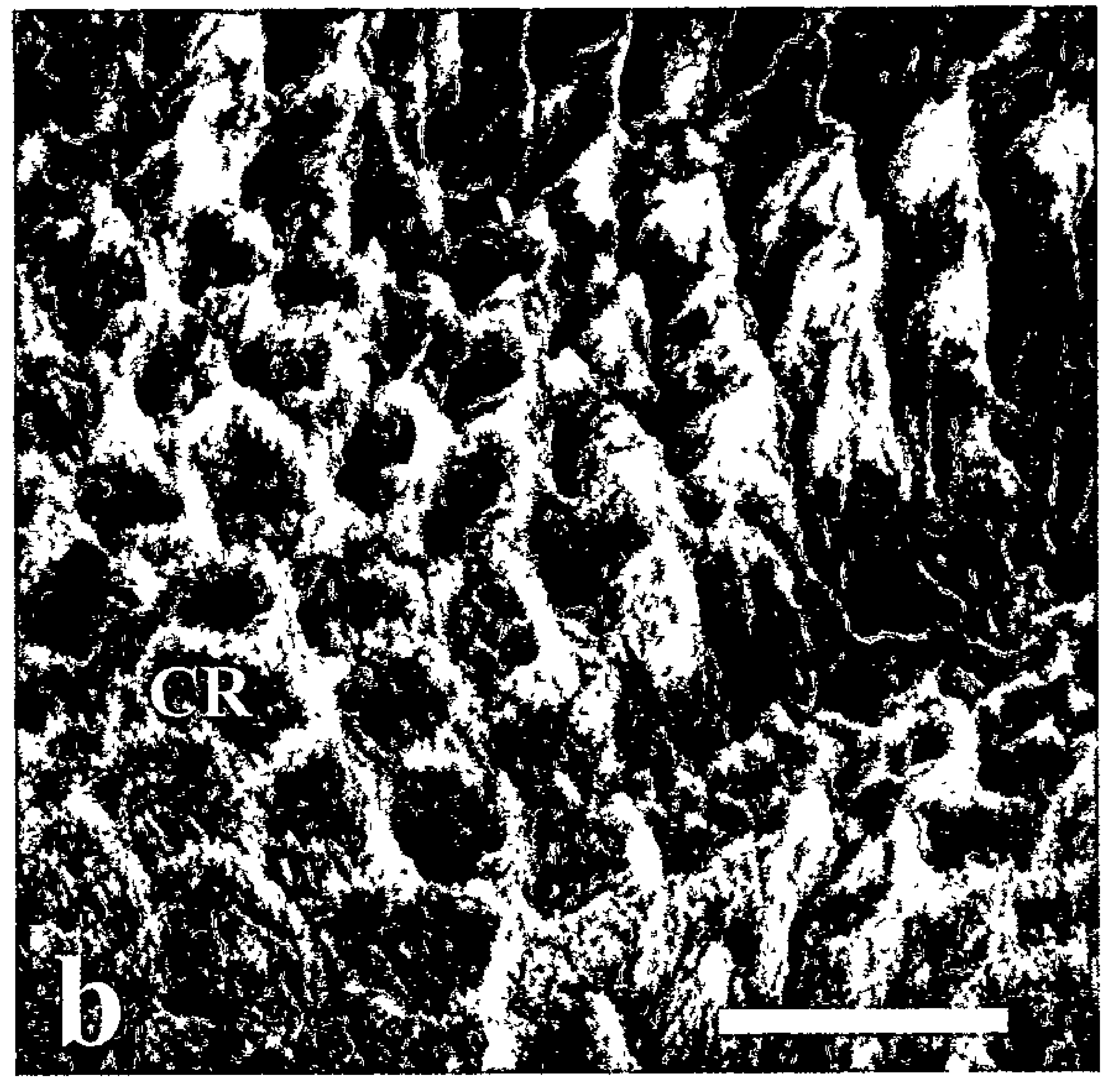
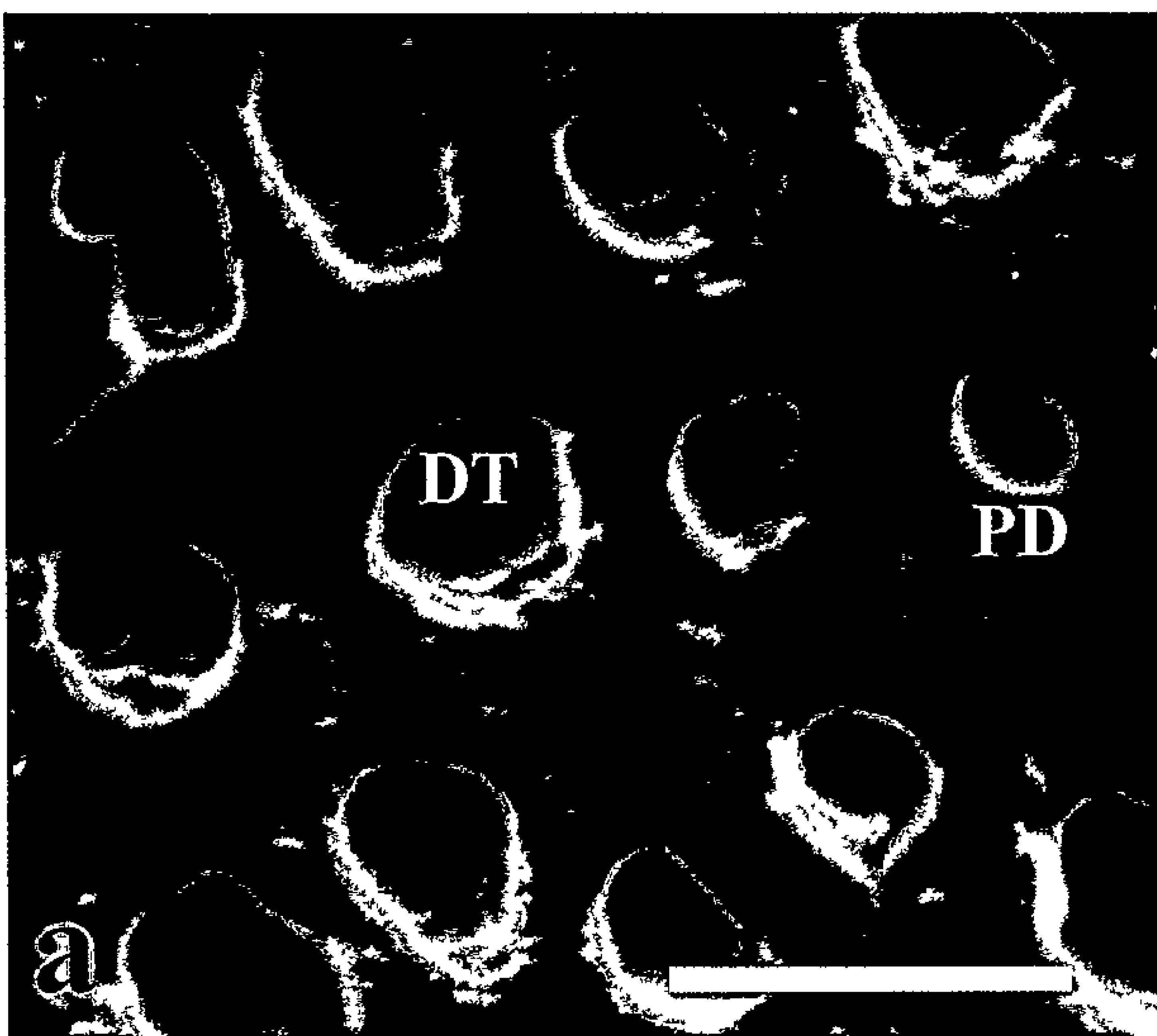


Figure 1.2a and 1.2b. Scanning electron micrographs of enamel structure following acid etching. Note the characteristic keyhole pattern of enamel prisms (EP) with preferential dissolution of the enamel crystallites in the central region (CR) of the prisms. Scale bars indicate 10μm.



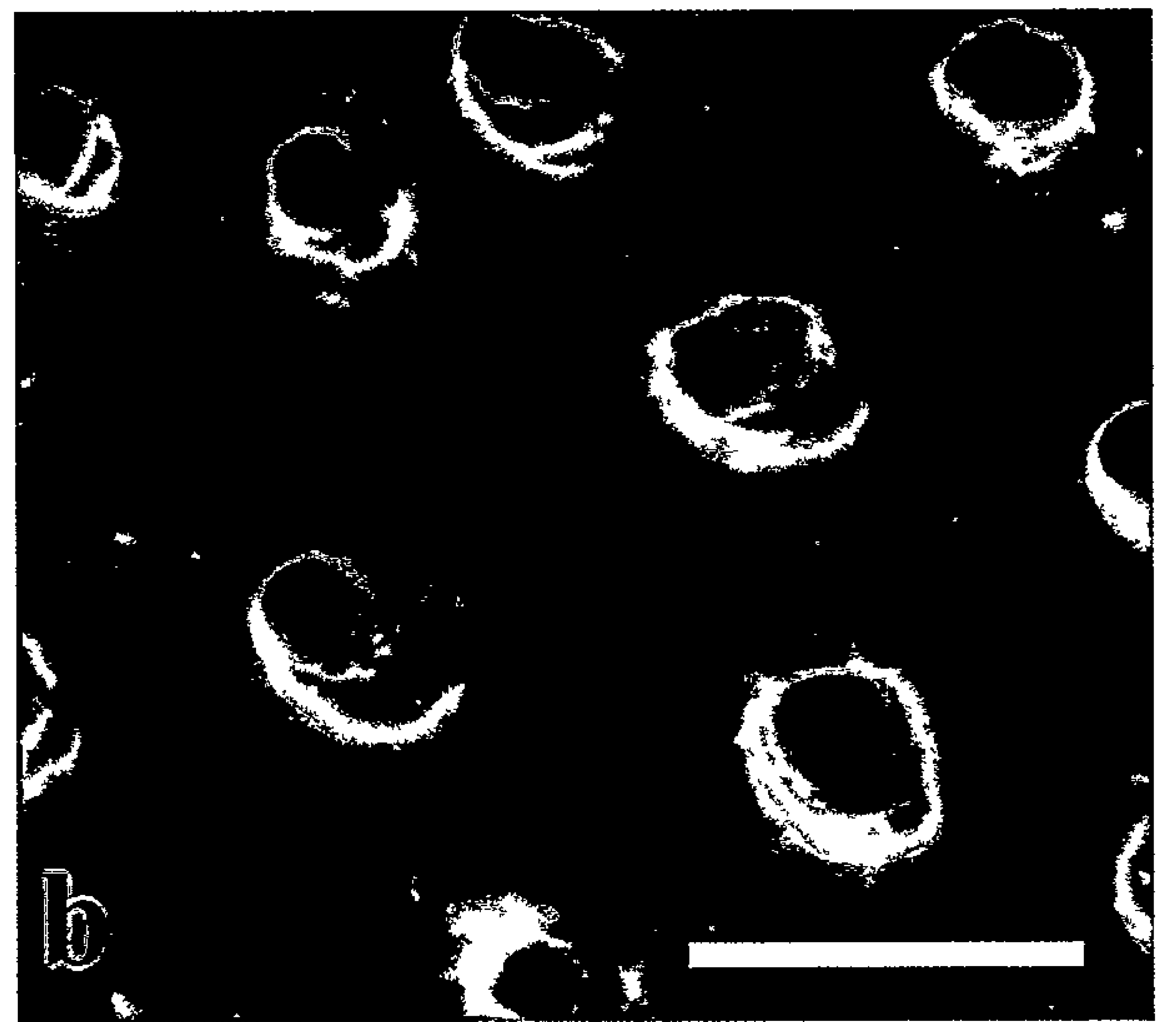


Figure 1.3a and 1.3b. Scanning electron micrographs of dentine. The structure, following acid etching, shows the dentine tubules (DT) surrounded by peritubular dentine (PD). Figure 1.3a has been prepared from dentine near the dental pulp. Figure 1.3b has been prepared from dentine near the dentino-enamel junction. Scale bars indicate 5μm.

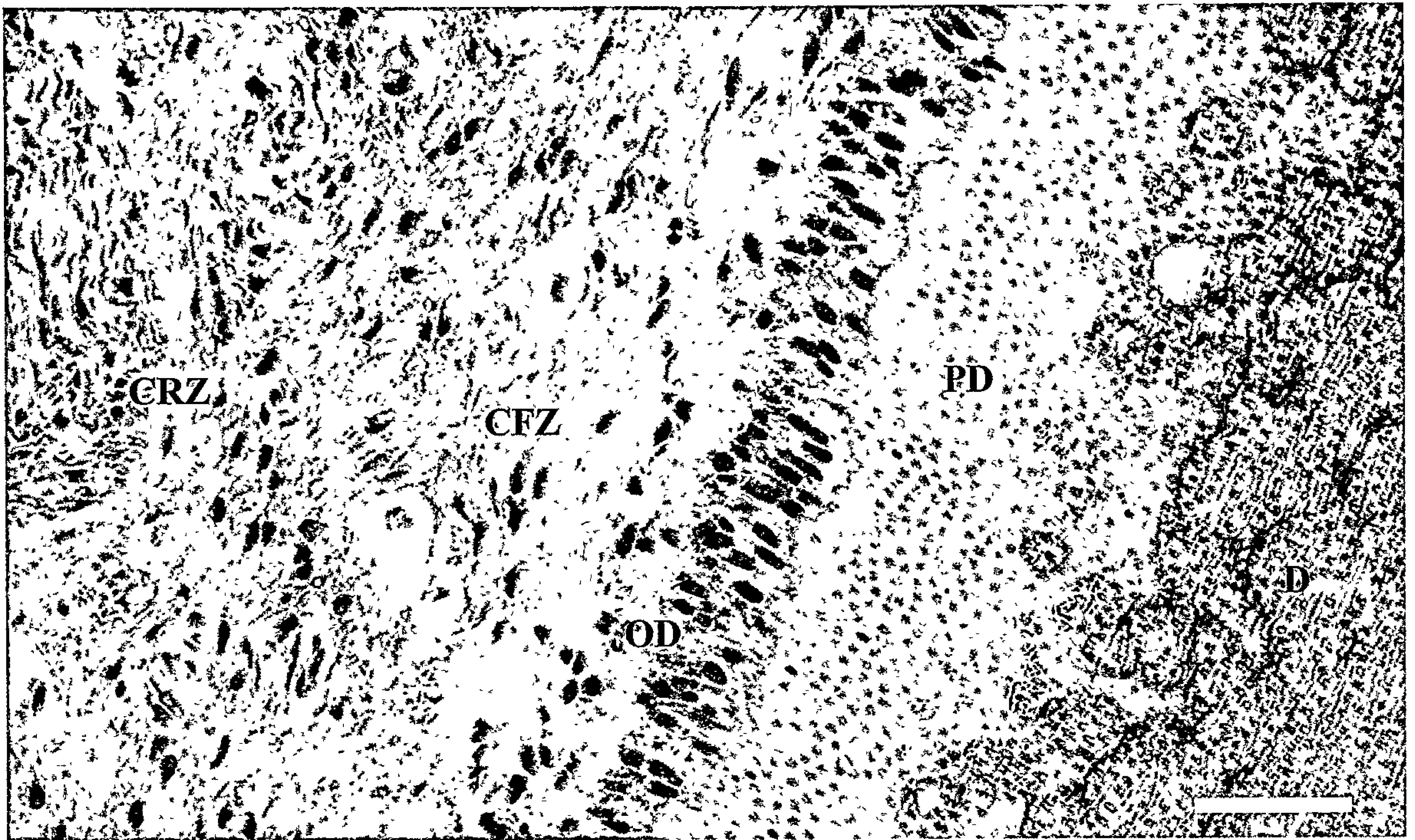


Figure 1.4. Dentine-pulp interface showing dentine (D) and pre-dentine (PD) with subjacent odontoblastic layer (OD), cell-free zone of Weil (CFZ) and the underlying cell-rich zone (CRZ). Scale bar indicates 100μm.

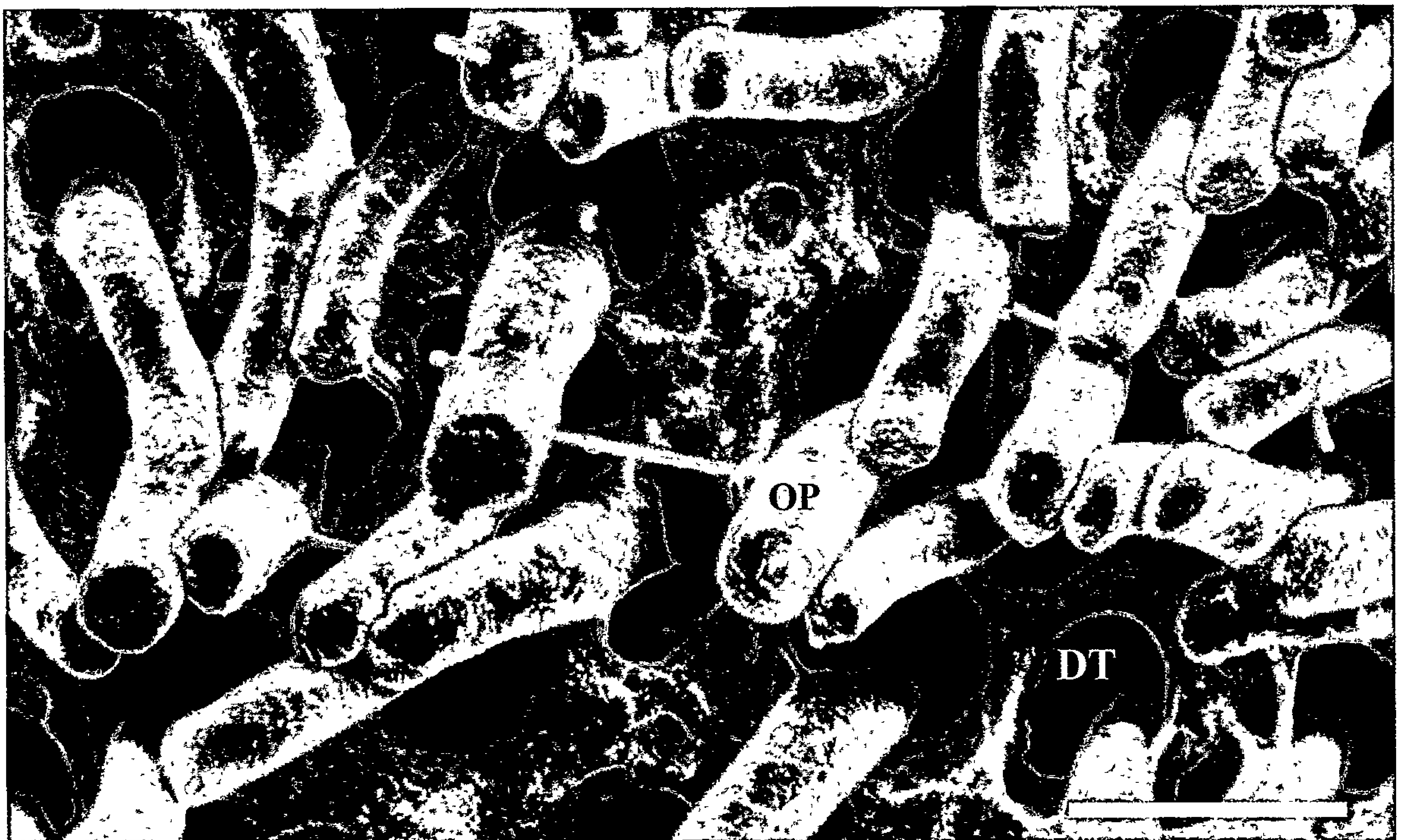


Figure 1.5. Scanning electron micrograph showing the structure of dentine in cross-section. Note the dentinal tubules (DT) and cut odontoblastic processes (OP). Scale bar indicates 5μm.

The matrix of the pulp consists of ground substance and collagen fibres. The fibres are principally type I and type III collagen, present in a ratio of 55:45 which remains constant throughout life. The amount of collagen increases with the age of the pulp, but the ratio remains stable. The ground substance is similar to that of any other loose connective tissues and is composed of glycosaminoglycans, glycoproteins and water. It functions to support the cells and provides nutrient and metabolite transfer between the vasculature and the cells. With increasing age the composition of the matrix alters producing metabolic changes, reductions in cellular function and irregular mineral deposition (Ten Cate, 1998).

The blood vessels of the pulp enter and exit through the apical and accessory foramina accompanied by sympathetic nerve fibres that form a plexus around the blood vessels and innervate the smooth muscle in the arterial walls. These nerves play a significant role in regulating pulpal blood flow. The main vessels are thin-walled arterioles and venules that branch as they move coronally through the pulp. Further branching extends into the sub-odontoblastic area creating an extensive vascular capillary network with terminal loops and anastomoses between venules, as well as arterio-venous anastomoses. Lymphatic vessels are also present in the pulp and originate as small, blind, thin-walled vessels in the coronal pulp and between the odontoblasts. These drain into collecting vessels with numerous interconnections and exit apically as larger vessels accompanying blood vessels and nerve fibres, finally draining into lymph vessels in the periodontal ligament (Bernick, 1977).

Nerves enter the pulp through the apical and accessory foramina and are generally accompanied by blood vessels forming the neurovascular bundle. The nerves travel coronally, branching and rebranching to ultimately form an extensive plexus of nerves in the cell-free zone (subodontoblastic plexus of Raschkow) just beneath the odontoblasts in the crown of the tooth. The nerve bundles contain both myelinated and unmyelinated sensory nerve fibres. The myelinated axons are primarily A delta fibres associated with sharp, localised pain; these are described as fast conducting with a low stimulation threshold. The unmyelinated C fibres are

associated with dull more diffuse pain; these fibres are slow conducting with a high stimulation threshold. As the nerve bundles move coronally, the myelinated axons gradually lose their myelin sheath resulting in an increase in the numbers of unmyelinated axons in the coronal part of the tooth. While most of the unmyelinated nerve bundles terminate in the subodontoblastic plexus, a small number of axons pass between the odontoblast cells and enter the dentine tubules in close proximity to the odontoblastic processes. The presence of intratubular nerves has been shown primarily in coronal dentine extending for only for a limited distance within dentine (Ten Cate, 1998).

1.3 Saliva

Saliva is made up of fluid produced by six major salivary glands and 200-400 minor glands with different loci in the mucosa of the mouth (Thylstrup and Fejerskov, 1994). The functions of saliva include lubrication for swallowing and speech, solvent action for those substances with taste properties, defence mechanisms against microorganisms and protection of the oral cavity against damaging pH changes by salivary buffering. Saliva is a highly complex fluid containing a variety of both organic and inorganic components. The organic components comprise proteins and carbohydrates. The inorganic components include calcium, phosphate, fluoride, sodium, potassium, chloride, hydrogen ion and bicarbonate. Saliva is normally supersaturated with regard to hydroxyapatite, but contains proteins such as statherin and proline-rich proteins that prevent spontaneous precipitation of the calcium phosphate salts, thus encouraging a protective and reparative environment important to maintain the integrity of the tooth surface. Numerous cellular elements are also present which include desquamated epithelial cells from mucous membranes, neutrophils and microorganisms.

Saliva plays a significant role in maintaining an appropriate balance within the oral environment. While saliva provides proteins for development of the pellicle, which subsequently allows the formation of plaque and glycoproteins that provide essential bacterial nutrients, other features assist the elimination of microbes. These include an adequate saliva flow, which allows for debris and food to be cleared from the mouth along with the removal of

microorganisms by protein agglutination from tooth surfaces and mucous membranes. Saliva and crevicular fluids both contain neutrophils, polymorphonuclear leukocytes and immunoglobulins (IgA, IgG and IgM) which assist bacterial removal. In addition, components such as salivary peroxidase, lactoferrin, lysozyme and histidine-rich and proline-rich peptides may reduce the numbers of bacteria in plaque or diminish colonisation within the mouth (Thylstrup and Fejerskov, 1994).

Once plaque has formed on the tooth surface, the buffering effect of the bicarbonate and phosphate components of saliva control the pH of superficial plaque. Other alkaline salivary components contribute to this effect; for example sialin stimulates base production by oral bacteria and encourages the early cessation of glycolysis. Another component is urea, which is transformed by urease into carbon dioxide and ammonia. These components limit the demineralisation of tooth structure and by raising the pH of plaque reduce the advantage of the aciduric bacteria. Other positive effects of saliva include the potential of the inorganic components to increase the resistance of hydroxyapatite to acidic dissolution and the ability to remineralise early carious lesions (Thylstrup and Fejerskov, 1994).

The importance of saliva in controlling dental caries is demonstrated most effectively by the aggressive progression of disease when salivary flow is reduced or absent (xerostomia). Salivary dysfunction may be caused by lifestyle factors, disease or the side effects of medications. Lifestyle factors include stress, fluid balance problems such as strenuous exercise or excessive caffeine intake, smoking and the use of addictive drugs. Medical factors include diabetes mellitus, autoimmune disease such as Sjorgren's syndrome, chronic renal failure, alcoholism and pathology of the salivary glands including infection, neoplasm or prior radiation treatment. With regard to medication, there are several hundred medications known to induce xerostomia; some of these include psychotropic agents, diuretics, antihistamines, anticonvulsants, anti-emetics and anti-Parkinson's disease drugs (Walsh, 2000).

1.4 Dental plaque

The oral cavity is the only site in the body where hard non-shedding surfaces are colonised

by microorganisms. This unique feature allows the accumulation of large masses of bacteria, known as dental plaque, on the tooth surfaces. This plaque is a highly organised structure of bacteria that adheres to the pellicle of the tooth surface (Figures 1.6a and b). Plaque forms on all exposed tooth and restoration surfaces especially where it is not disturbed. The plaque may vary in thickness from a few bacterial cells up to a millimetre thick in areas of stagnation and has been described as an open structure with fluid filled voids and channels extending through its entire thickness (Wood *et al.*, 2000). Microorganisms do not adhere directly to the apatite crystals of tooth enamel. Following cleaning of the tooth surface, salivary proteins almost instantaneously adsorb onto the enamel forming a protein coating described as the pellicle. This film varies in thickness from 1-10μm and plays an important role in microbial colonisation by providing receptors for the adherence of specific bacteria. The major constituents of the pellicle are salivary glycoproteins, phosphoproteins and lipids. Development of the plaque biofilm occurs with the attachment of bacteria to the pellicle covered enamel surface via a number of mechanisms. Initially there is a loose association of bacteria to the tooth surface resulting from the action of van der Waal's forces which counteracts the negative electrostatic charges exerted by the bacteria and tooth surfaces. Bacterial attachment then occurs through selective inter-molecular interactions via lectin-like or hydrophobic ligands of the bacteria called "adhesins"; these bridge the space and form bonds with the carbohydrate groups in the pellicle glycoprotein (Gibbons, 1984). Examples include *Streptococcus sanguinis* (formerly *Streptococcus sanguis*) and *Streptococcus oralis* which bind specifically to terminal sialic acid residues in the glycoprotein, and *Actinomyces viscosus* and *Actinomyces naeslundii* which adhere to proteins such as proline-rich protein and statherin (Thylstrup and Fejerskov, 1994). Amylase, lysozyme, albumin and immunoglobulins have also been identified in the acquired pellicle, as well as types I and IV collagen which can act as ligands for a range of oral bacteria (Socransky and Haffajee, 1991; Marsh and Martin, 1992). Most bacteria exhibit multiple binding sites allowing attachment to a range of surfaces (Gibbons, 1984; Whittaker *et al.*, 1996).

The microbial flora of the oral environment is complex and may contain up to 500 different

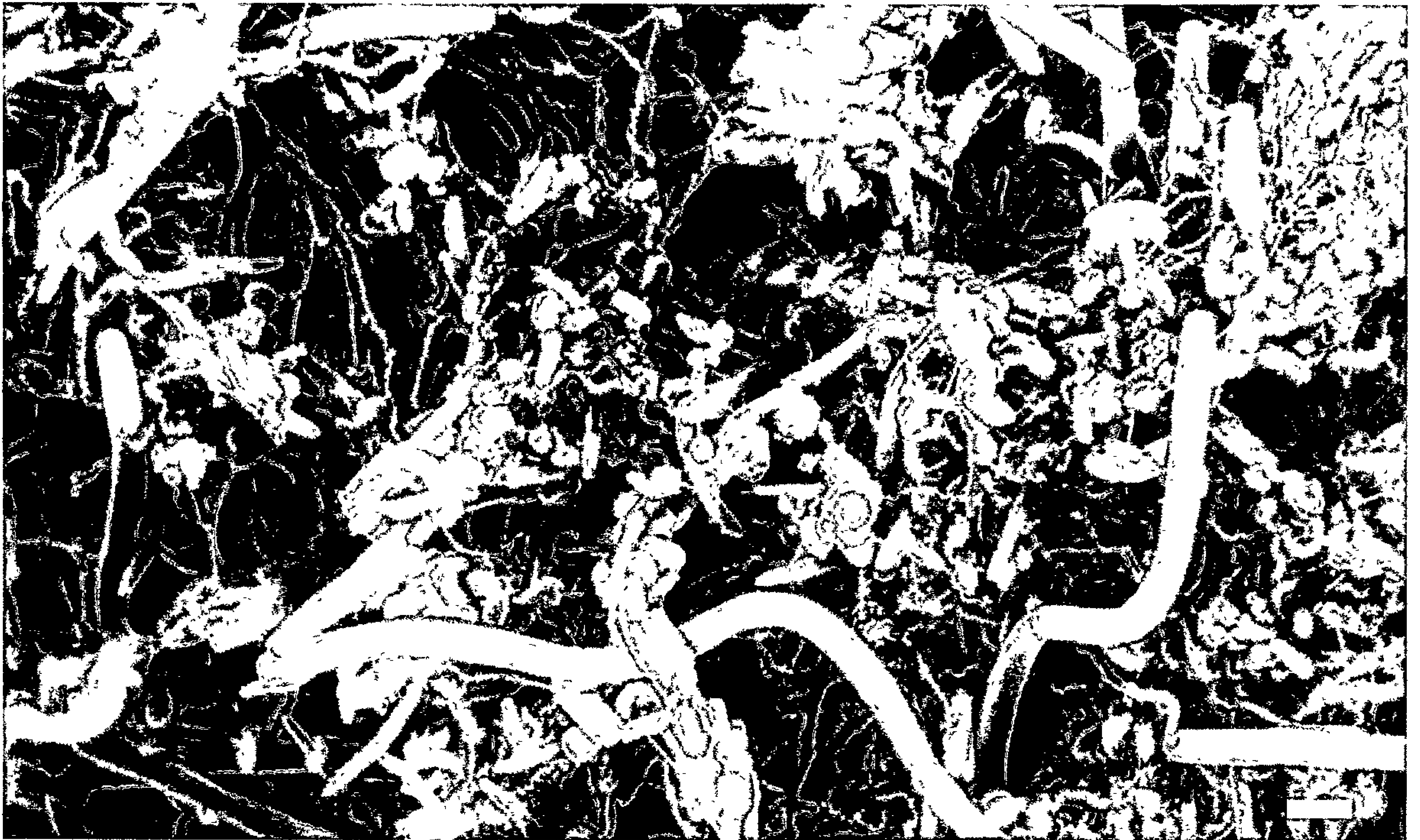


Figure 1.6a. Scanning electron micrograph showing the structure of dental plaque depicting a mixture of bacteria including long and short rods, bacilli and cocci. Scale bar represents 1 μm.

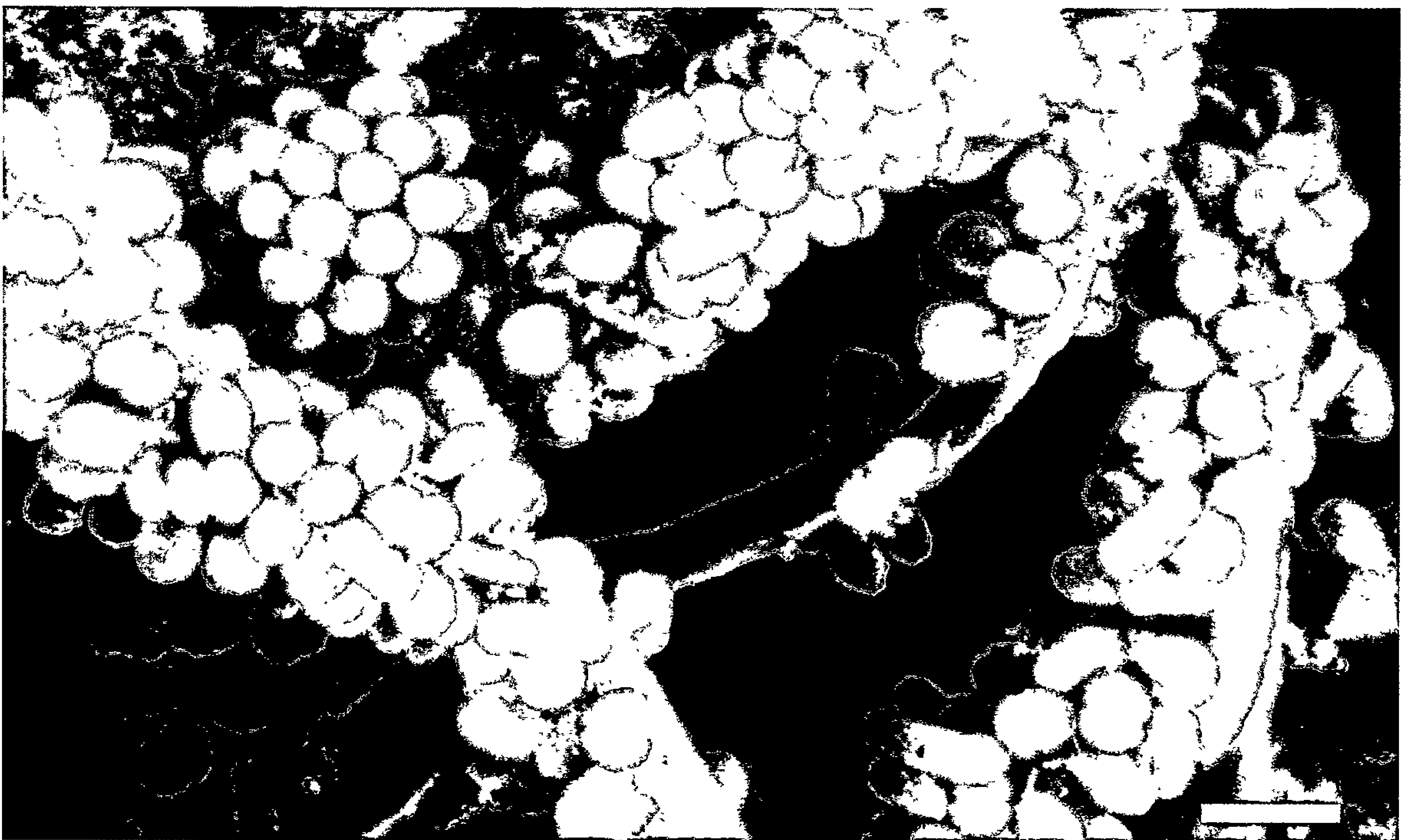


Figure 1.6b. "Corn-cob" arrangement of bacteria found in plaque illustrating the coaggregation of smaller cocco-bacilli organisms with long rods. Scale bar represents 1 μm.

bacterial species, with any individual harbouring between 150-200 different species (Socransky and Haffajee, 1994; Paster *et al.*, 2001). The bacterial content of plaque varies depending on its age and location. In newly formed plaque, the Gram-positive facultative organisms dominate; these include *S. sanguinis*, *Streptococcus mitis*, *Streptococcus gordonii* and *Actinomyces viscosus* which elaborate adhesins for saliva coated and mineralised surfaces. Both the streptococci and actinomycetes groups are facultatively anaerobic and their doubling times for microbial growth during initial plaque development are less than one hour. As plaque develops into a biofilm, metabolism of the initial bacterial species creates conditions suitable for the colonisation of species that grow more slowly and have more fastidious growth requirements (Whittaker, *et al.*, 1996). Other bacteria then colonise plaque through attachment to existing bacteria (coaggregation) using specific partnerships mediated by complementary surface structures on the partner cells. Each newly adherent cell type then becomes a nascent surface and bridge for additional cells. Some specific interactions include the attachment of *S. mutans* to *A. viscosus* and *S. sanguinis* (Lamont *et al.*, 1991), *Prevotella intermedia* to actinomycetes (Nesbitt *et al.*, 1993), *Porphyromonas gingivalis* to *A. viscosus* and *S. sanguinis* (reviews in Hamada, 1998 and Lamont *et al.*, 1992), *Actinomyces israelii* to *Cytophaga* spp. (Kolenbrander and Celesk, 1983) and *S. sanguinis* to *Fusobacterium nucleatum* creating a "corn-cob" configuration of bacterial coaggregation (Figure 1.6b) (Kaufman and DiRenzo, 1989).

Of particular importance are the anaerobic fusobacteria that, as a group, coaggregate with some strains of all oral bacteria and act as a bridge to connect the early with the late colonisers (Kolenbrander and London, 1993). Each strain of fusobacteria coaggregates with only a certain set of partners and does not exhibit intrageneric coaggregation. Fusobacteria coaggregate with strains from a number of species of early colonisers including streptococci, actinomycetes, veillonellae and capnocytophaga. The late colonisers that favour coaggregation with fusobacteria include eubacteria, *Haemophilus actinomycetemcomitans* (formerly *Actinobacillus actinomycetemcomitans*), treponemes and strains of *Prevotella* and *Porphyromonas* spp. (Kolenbrander *et al.*, 1993; Kolenbrander and London, 1993).

Further plaque development relies on the multiplication of existing organisms and coaggregation of additional bacteria to produce confluent growth and biofilm formation (Marsh and Martin, 1992). Thus, as plaque matures and becomes thicker there is a lowering of the oxygen concentration causing a shift in the diversity of the microflora towards increasing numbers of filamentous, Gram-negative and anaerobic bacteria (Thylstrup and Fejerskov, 1994).

The structure and development of plaque differs depending on its location. The plaque found in fissures is generally less complex, being composed mainly of Gram-positive cocci, particularly *Streptococcus* spp.; it also contains some rods and less inter-bacterial matrix. The fissure site tends to provide a more severe environment with a limited range of nutrients. Plaque that forms in the gingival crevice contains a greater diversity of bacterial species with higher levels of the obligately anaerobic bacteria, many of which are Gram-negative. This reflects the anatomy of the area and the flow and properties of gingival crevicular fluid. Many of the organisms found in this location are proteolytic and derive their energy sources from the degradation of host proteins and the catabolism of amino acids. Microorganisms commonly found in this region include representatives of the genera *Actinomyces, Capnocytophaga, Fusobacteria, Prevotella* and *Porphyromonas* (Marsh and Martin, 1992).

To cope with the fluctuating nutritional conditions and environmental variations within the mouth, the microflora must be biochemically flexible. The primary source of nutrients comes from the proteins and glycoproteins in saliva and gingival crevicular fluid. In addition, the diet provides carbohydrates and proteins. Carbohydrates such as glucose, sucrose and lactose easily diffuse into plaque and are readily metabolised. Carbohydrates can be transported directly into the cell and used to synthesise intracellular polysaccharides as carbohydrate storage for subsequent energy production when dietary supplies are low. Many species of oral bacteria are also able to synthesise extracellular polysaccharides from carbohydrates. These polysaccharides are polymers of glucose or fructose and form a considerable bulk of the acellular matrix of plaque. This binds the plaque together, aids its adherence to the tooth

surface and provides a ready store of energy for bacterial metabolism, thus enhancing the pathogenicity of plaque (Thylstrup and Fejerskov, 1994).

The production of acid by acidogenic and aciduric bacteria in dental plaque is an absolute prerequisite for the development of dental caries. The bacterial species within plaque capable of fermenting carbohydrates to produce acid include *Streptococcus* (particularly *S. mutans*), *Lactobacillus* spp. and *Actinomyces* species. Diet is the primary source of carbohydrate available to plaque bacteria and excessive amounts, of particularly glucose and sucrose, result in sustained acid production, low plaque pH and an increased production of extracellular polysaccharides.

1.4.1 Plaque metabolism

Most saccharolytic oral bacteria catabolise sugars via the Embden-Meyerhof glycolytic pathway to generate energy and the precursors for cellular material. In addition, many organisms are also able to utilise the pentose phosphate shunt pathway. The main end-product of glycolysis is pyruvate, which can then be processed into a range of organic acids depending on the particular microorganism and the availability of oxygen. When available glucose levels are high, many bacteria convert the glucose to lactate. When glucose levels are low a greater proportion is converted to a variety of acids including formic, acetic, butyric, propionic and succinic acids, and ethanol. The acid end-products may be further degraded by other oral microorganisms, for example, *Veillonella* spp. consume lactic acid as an energy source and produce propionic and acetic acids, and *Eubacterium alactolyticum* converts acetic acid into butyric and caproic acids. The consumption of lactic and acetic acids with the subsequent production of butyric, propionic and caproic acids results in a significant decrease in the acidity of plaque (Thylstrup and Fejerskov, 1994).

Following the ingestion of carbohydrates, the pH of plaque decreases rapidly within the first few minutes which is followed by a slow increase to approach baseline levels after 30-60 minutes (Stephan, 1944). The extent and duration of the pH drop depend on the type of bacteria and the age of the plaque. The pH drop becomes more pronounced with increasing age

of the plaque. The resting pH of plaque has been estimated to be approximately 5.6-7.0, however, following a sucrose challenge Stephan curves show a pH drop to 5.5 in 2 day old plaque and to pH 4 in 6 day old plaque (Imfeld and Lutz, 1980).

In contrast to the large amount of information available regarding the fermentation of carbohydrates, far less is understood about the metabolism of nitrogenous components by oral bacteria. Amino acids may be required not only as building blocks in cellular macromolecules, but also as an energy source for bacterial metabolism. Some oral species demonstrate peptidase or urease activities, for example *S. sanguinis* has been shown to degrade casein and *A. viscosus* and *Streptococcus salivarius* can metabolise urea into carbon dioxide and ammonia; the latter can then be converted into a number of amino acids. Many of the periodontopathogens are assacharolytic and hence proteolytic, and their growth depends on an ability to utilise nutrients from gingival crevicular fluid. These bacteria also produce enzymes that are capable of degrading host molecules associated with the pocket epithelium, thus providing peptides and amino acids. Further, amino acid utilisation might also be an important mechanism whereby oral microorganisms counter the extreme pH changes caused by the metabolism of carbohydrates and urea (Marsh and Martin, 1992).

ter Steeg and van der Hoeven (1989) studied the succession of bacterial species during batch growth of subgingival plaque organisms in human serum and distinguished three phases of growth. The first phase involved consumption of carbohydrates by rapidly growing saccharolytic organisms such as the *Streptococcus* spp. with the subsequent production of lactic and formic acids. In the second growth phase proteins were hydrolysed with some amino acid fermentation taking place, the remaining carbohydrates were metabolised and the lactate and formate consumed. This stage of growth was dominated by *Prevotella, Porphyromonas, Veillonella* and *Fusobacterium* species. The final phase was characterised by extensive amino acid fermentation and progressive protein degradation predominantly by *Peptostreptococcus* and *Eubacterium* species. At this stage numerous toxic products including sulphide, ammonia, butyric acid and other fatty acids had accumulated.

1.5 Dental caries

Caries is a condition in which the dental hard tissues are demineralised by organic acids produced by bacteria following the metabolism of carbohydrates. The formation of dental plaque is essential to the process of enamel dissolution as it controls the diffusion of acids away from the enamel surface and limits the buffering action of saliva. This tissue loss leads to the collapse of the inorganic matrix and gradually a cavity is formed.

Early enamel caries involves the formation of subsurface holes or spaces within the enamel. This occurs with acid dissolution of individual hydroxyapatite crystals resulting in an enlargement of the intercrystalline spaces. The change in structure causes an increase in tissue porosity observed clinically as a whitish, opaque area of enamel due to alterations in the optical properties. In the initial stages the outer 20-50μm of enamel remains relatively unaffected. Although understanding of the demineralisation process is incomplete, there is speculation that the protective role of the salivary proline-rich proteins and statherin may initially inhibit surface demineralisation. Also, the surface enamel in close proximity to the plaque could be protected by the dynamics at the solid/solution interface (Ten Cate, 1998).

Enamel caries is a slowly progressing, phasic disease that is partially reversible with appropriate plaque and dietary control. However, with progress of the lesion, increasing porosity occurs along the direction of the rods towards the dentino-enamel junction, forming a conical shaped lesion with its base at the enamel surface. The previously intact surface enamel develops defects, cracks and micro-cavities before finally collapsing upon cavitation.

On reaching the relatively lightly mineralised dentino-enamel junction (DEJ) the process of demineralisation spreads laterally. The lesion follows the direction of the dentinal tubules towards the pulp and develops a conical shape with its base at the DEJ. At this stage the enamel surface is still usually intact. The enamel is avascular and acellular and cannot respond to injury, whereas the dentine and odontoblasts are integral components of the pulpo-dentinal organ and as such possess specific defence reactions that respond to external insults. Thus, when the surface dentine becomes demineralised, the odontoblasts react to the diffusion of

acids, metabolites and breakdown products from the microorganisms with two main responses, tubular sclerosis and formation of reactionary dentine.

Tubular sclerosis is the most common defence reaction of the pulp and involves the deposition of mineral within the dentinal tubules. Mineral in the form of apatite is laid down to form a mineralised barrier to protect the underlying odontoblasts from the advancing front of the carious lesion. It has been suggested that tubular sclerosis is probably an accelerated form of peritubular dentine formation, which occurs in response to mild stimulation, but progresses until the tubules become completely obliterated (Pitt Ford, 1992). In addition to the presence of these intratubular hydroxyapatite crystals, large rhombohedral crystals have been observed and identified as whitlockite crystals (Frank and Vogel, 1980). When examined under transmitted light this sclerotic zone is seen as translucent dentine because the mineral content within the tubules reduces the scattering of light causing the tissue to appear homogeneous. The second type of response is the formation of reactionary (reparative) dentine laid down at the surface of the pulp chamber by those odontoblasts under the caries-affected tubules. The structure of this tissue may resemble regular reactionary dentine or severely dysplastic tissue, depending on the severity of the stimulus (Thylstrup and Fejerskov, 1994).

Once cavitation occurs, bacteria are able to penetrate the demineralised dentine via the dentinal tubules. It is inevitable that bacterial succession will occur during development of the carious lesion, with the microflora responsible for the initiation of the lesion differing from those causing progression. Positive associations between microflora and those lesions that progress into dentine include lactobacilli and actinomycetes, and veillonellae and mutans streptococci (Marsh and Martin, 1992). This phase is followed by the invasion of a more diverse group of microorganisms including the Gram-negative anaerobes. Examination of the acid profiles of carious dentine by Hojo *et al.* (1991, 1994) found that lactic, propionic and acetic acids accounted for approximately 90 per cent of the total acid found in dentine caries. They reported that active carious lesions that had a low pH (4.9) and demonstrated a high lactate production were associated with the presence of *Lactobacillus, Streptococcus* and

Actinomyces species. Where there was restricted diffusion of both carbohydrates and acids in deep caries, arrested caries or under restorations, there was a shift in the bacterial profile. In these locations lactate was subsequently converted to acetate and propionate by a group of bacteria including *Eubacterium, Propionibacterium, Arachnia* and *Veillonella* species. Other acids, including butyrate, valerate and caproate were also found, indicating an environment with a higher pH (5.7) dominated by collagen-decomposing and amino acid fermentative bacteria. This is in agreement with earlier studies indicating an alkaline or neutral pH of carious dentine that the authors attributed to the presence of ammonia (Mäkinen *et al.*, 1969).

Progression of the carious lesion requires bacterial invasion via the dentinal tubules (Figure 1.7). Studies *in vitro* have reported the bacterial invasion of root dentine by Gram-positive bacteria including *Enterococcus faecalis, S. gordonii, S. sanguinis* and *Actinomyces* spp. (Akpata and Blechman, 1982; Perez *et al.*, 1993; Siqueira *et al.*, 1996). However, the penetration of Gram-negative rods in monoculture *in vitro* has not been recorded. Akpata and Blechman (1982) noted that *Bacteroides melaninogenicus* ssp. *melaninogenicus* (*Prevotella melaninogenica*) did not invade root canal dentine after three weeks, while Perez *et al.* (1993) could not detect any dentine penetration by *Prevotella intermedia* after 28 days using either light microscopy or scanning electron microscopy. A scanning electron microscope study by Siqueira *et al.* (1996) reported that *Porphyromonas endodontalis* and *Porphyromonas gingivalis* had penetrated only a small number of dentine tubules after 21 days incubation. In contrast, studies *in vitro* investigating the ability of mixed cultures to invade dentine have shown that the penetration of *Lactobacillus casei* was improved when co-cultured with *Streptococcus sobrinus* or *A. viscosus* (Nagaoka *et al.*, 1995), and the invasion of *P. gingivalis* was enhanced in mixed culture with *S. gordonii* (Love *et al.*, 2000). It has been proposed that the invasion of dentinal tubules by *P. gingivalis* was facilitated by its ability to bind to the SspA or SspB polypeptides expressed on the surface of *S. gordonii* which is capable of dentine penetration through collagen binding (Love, *et al.*, 2000).

Figure 1.7. Scanning electron micrograph illustrating the attachment of cocci-shaped bacteria to the walls of the dentinal tubules. Scale bar represents 1μm.

Once formed, the dentinal lesion can be divided into three zones.

i) The zone of destruction which is an area of softened, discoloured dentine where the basic architecture has been destroyed by the action of acids and proteolytic enzymes from bacteria. Bacterial invasion is extensive and microorganisms are found occupying areas where dentine has been destroyed. The soft dentine represents a combination of necrotic tissue and the bacterial biomass.

ii) The zone of penetration which is the region within the lesion that has been penetrated by bacteria. *Lactobacillus* spp. predominate because of their ability to survive the acidic conditions and are found occupying the dentine tubules. The basic structure of dentine is intact although many tubules become confluent and distended as a result of demineralisation. The intertubular dentine in this zone is extensively demineralised, with evidence of collagen breakdown interspersed with remnants of densely mineralised tissue.

iii) The zone of demineralisation results from acids produced by the biomass of anaerobic and aciduric microorganisms. The tubules in this zone are essentially normal, although there is some loss of peritubular dentine and crystals can be found within the tubules (Trowbridge, 1981). This is the deepest part of the lesion and is reportedly free of bacteria although the dentine may be partially demineralised (Pitt Ford, 1992).

The pH of the tooth environment is therefore not only influenced by the number and type of microorganisms present, but also by the buffering capacity of saliva, its flow rate and viscosity, the diffusion characteristics of plaque, the presence of fluoride, the type of diet and frequency of sugar ingested. Microbial deposits on tooth surfaces are constantly metabolically active, producing a variety of acidic and alkaline components even in the absence of a continuous supply of dietary substrate. It is the net result of the balance between these different processes that will determine the rate of progression of the carious lesion (Thylstrup and Fejerskov, 1994).

1.6 Pulp defence mechanisms

The pulp tissue reacts to caries before microorganisms penetrate the pulp chamber. When demineralisation of the dentine is 0.5 to 1 millimetre from the pulp, inflammatory reactions appear in the subodontoblastic layer. It has been proposed that this inflammation results from the diffusion of biologically active substances including bacterial enzymes and peptides, endotoxins, polysaccharides, somatic antigens, antibodies, immune complexes, chemotaxins, chemotaxigens, complement proteins, organic acids, products of tissue destruction and ammonia (Trowbridge, 1981). Numerous studies have shown that bacteria and their products produce severe pulpal reactions. Bacterial substances from dental plaque (Bergenholtz and Lindhe, 1975), lyophilised sonicates of plaque bacteria (Bergenholtz, 1977; Warfvinge and Bergenholtz, 1986), material from carious dentine (Mjör and Tronstad, 1972) and high molecular weight complexes of bacterial cell walls (Warfvinge *et al.*, 1985) are capable of initiating acute inflammatory lesions in the pulp within 8-32 hours after their application to cut dentine surfaces.

The earliest reaction of a pulp to dental caries can be found in the odontoblastic layer underlying the lesion. Observations include a reduction in the size and number of odontoblasts, and a change in their shape from tall/columnar to flat or cuboidal. These changes precede obvious inflammation of the pulp. As the zone of demineralisation advances, reparative dentine is produced as a defensive reaction to the injury. The formation of reparative dentine is restricted to that portion of the pulp adjacent to the tubules communicating with the lesion and its elaboration encroaches on the cell-free zone. Reparative dentine is poorly calcified, contains irregular dentinal tubules and is unpredictable in the amount formed. Therefore, it cannot provide an impervious barrier but does afford some degree of protection to the pulp. The success of this defence, and therefore survival of the pulp, will depend on the rate of progression of the carious lesion, as more reparative dentine is formed in response to a chronic rather than acute lesion (Trowbridge, 1981).

The formation of sclerotic or reparative dentine is not the only factor that determines the defence capabilities of the pulp. Clinically the dental pulp has been shown to demonstrate healing and repair despite being continuously exposed to the oral environment and studies have shown the disappearance of inflammatory cell infiltrates before the appearance of reparative dentine (El-Kafrawy and Mitchell, 1963; Bergenholtz, 1990). Results of experiments by Pashley *et al.* (1983) indicated that dentine permeability decreased significantly within hours after the dentine was exposed. It has been proposed that dentinal fluid contains high molecular weight proteins, such as fibrinogen, which could reduce the functional radius of the tubules (Pashley *et al.*, 1984). Other possible contributions to tubular occlusion could include the retention of immunoglobulin molecules (Hahn and Overton, 1997) and binding of penetrating bacterial antigens forming immune complexes (Okamura *et al.*, 1980; Ackerman *et al.*, 1981), the deposition of crystalline precipitates or the presence of large collagen fibrils within the tubules (Pashley, 1996). In addition, the outward flow of plasma fluid into the dentinal tubules in response to injury would provide a further line of defence against the inward diffusion of noxious substances.

As caries progresses, the defence reactions of the pulp become less effective. Sclerotic dentine is lost by the action of acids and enzymes from the invading bacteria. The body of the lesion then consists of zones of penetration and destruction, containing a large biomass of bacteria. Lateral spread of the lesion along the dentino-enamel junction undermines much of the adjacent enamel, which is prone to fracture with the potential to create a large open lesion. The pulp does not become inflamed until caries has involved the reparative dentine and wide areas of dentine have become demineralised. When microorganisms enter the pulp tissue acute inflammation supervenes with an influx of polymorphonuclear leukocytes followed by the formation of micro-abscesses. Infection of the pulp is a relatively late event in the carious process and its spread through the radicular portion of the pulp can lead to subsequent infection of the periapical tissues (Pitt Ford, 1992).

1.7 Carious dentine microflora

The microbial populations of dental caries are known to be highly complex and variable and have not yet been fully identified, although characteristic organisms are found. The microbes involved in caries initiation and early caries development have been well studied and are primarily Gram-positive. The organisms most frequently implicated include *Streptococcus* spp., especially the mutans streptococci group, and lactobacilli, in addition to the Gram-positive pleomorphic rods, including *Actinomyces* spp. (van Houte and Kent, 1994). The main Gram-negative bacteria identified are *Veillonella* spp., *Bacteroides* (*Prevotella* and *Porphyromonas* spp.) and *Fusobacterium* spp. (Brown *et al.*, 1986; van Houte and Kent, 1994). While it is commonly accepted that early carious lesions contain a high proportion of aerobic microorganisms, progression of the lesion changes the physiological environment including the pH and oxygen tension causing a shift in the bacterial population from aerobic to anaerobic species. A number of studies have shown the predominance of anaerobic microorganisms within caries (Loesche and Syed, 1973; Edwardsson, 1974; Hoshino, 1985; Torii *et al.*, 1986; Hahn *et al.*, 1991; Massey *et al.*, 1993).

In deep caries the Gram-positive microorganisms again dominate, with rods, especially *Lactobacillus* spp. being numerically dominant followed by the Gram-positive cocci (Hahn, *et al.*, 1991). As the lesion progresses in depth the number of Gram-positive cocci, particularly mutans streptococci decreases while the anaerobic cocci such as *Peptostreptococcus* spp. and *Peptococcus* spp. increase. The numbers of lactobacilli are reportedly high in both superficial and deep lesions with the Gram-positive facultative and pleomorphic species actinomycetes, propionibacteria, eubacteria, arachnia and bifidobacteria also present (Hahn, *et al.*, 1991; Massey, *et al.*, 1993). The Gram-negative anaerobic rods prevotellae, porphyromonads and fusobacteria have been isolated from deep carious dentine in greater numbers than from shallow caries, although they constituted only a minor component of the flora in some studies (Ando and Hoshino, 1990; Hahn, *et al.*, 1991). In contrast, Massey, *et al.* (1993) reported that the Gram-negative microbes comprised a significant percentage of the total flora. Carious

dentine, therefore, presents as a mixed infection with representative bacteria from aerobic, facultative and anaerobic groups which display a high degree of complexity and variability. However, as few studies have investigated the presence of anaerobic bacteria in dental caries, their true profile is, as yet, undetermined and little is known of their role in the pathogenicity of this disease process. The next chapter will examine the role of anaerobes in infections, with particular reference to dental caries and periapical disease.

CHAPTER 2

ANAEROBIC INFECTIONS

2.1 Introduction

Prior to 1970, a significant proportion of samples cultured from the sites of post-operative wound infection produced no pathogens, yet Gram staining indicated the presence of microbes. This inability to identify the causative pathogens ultimately resulted in incorrect treatment choices, and thus failure of therapy (Nichols and Smith, 1994). It is now known that many of these unrecoverable microbes were endogenous anaerobic bacteria that constitute the predominant human microflora, and can be found on the skin, mucous membranes of the oral cavity and upper respiratory tract, the intestinal tract, vagina and skin near the anus. It is reported that anaerobes outnumber aerobes in those regions above the diaphragm (including the oral cavity) by 100:1, and below the diaphragm by 1000:1 (Goldstein, 1999). Although anaerobes are part of the normal flora, they have the capacity to cause serious human infections as demonstrated in early clinical studies of patients with empyema and aspiration pneumonia where anaerobes were cultured from 76% and 93% of patients, respectively, compared with aerobes from 65% and 54% of patients, respectively (Bartlett *et al.*, 1974a and 1974b). Anaerobes have been associated with a variety of infections including brain abscess, meningitis, sinusitis, peritonitis, appendicitis, diverticulitis and bacteremia (Goldstein, 1999). Sometimes these infections are associated with significant morbidity and mortality; therefore, inadequate or improper treatment can have dire consequences (Rosenblatt, 1997).

Despite many studies citing anaerobes as the predominant microorganisms there is widespread opinion among wound care practitioners that aerobic and facultative organisms are the primary pathogens involved in delayed wound healing (Bowler *et al.*, 2001). This opinion was formed on the basis of numerous studies over the last two decades where little or

no attempt was made to isolate anaerobes, while studies involving detailed microbiological analyses of wound samples revealed that anaerobes constituted, on average, 38% of the total number of isolates (Bowler, *et al.*, 2001). In this context, endogenous anaerobic bacteria have been reported to be the likely cause of postoperative infection when wound specimens failed to yield bacterial growth on culture (Nichols and Smith, 1994).

Failure to recognise the importance of anaerobes in infections may be the result of a number of factors: they are overlooked because it is assumed they are not detrimental, specimen collection and transport requires care and the culture, isolation and identification of anaerobes is more laborious, time-consuming and expensive than that required for aerobic and facultative organisms. As a result of these difficulties, the cultivation of anaerobes is often regarded as too demanding for diagnostic laboratories (Bowler, *et al.*, 2001). Of concern are results of surveys of anaerobic culturing from hospital microbiology laboratories which indicate a low interest in anaerobic microbiology, lack of standardisation of procedures involving specimen collection through to identification and limited technological resources (Goldstein *et al.*, 1995; Botta and Arzese, 1997). Additional problems have included the pressure of cost containment with a tendency for laboratories to cut back on procedures regarded as unusual or costly. Anaerobic culture and identification could be regarded by some to fall in this category (Rosenblatt, 1997). Indeed, Goldstein *et al.* (1995) reported that 12% of microbiology laboratories had considered stopping or reducing anaerobic bacteriology and susceptibility testing, and suggested that educational efforts be directed towards emphasising the relevance of anaerobes in infection and increasing the performance of anaerobic bacteriology.

Even with appropriate anaerobic culturing and identification, the role of anaerobes in infections may be difficult to estimate because anaerobic infections are typically polymicrobial, also involving aerobic and facultative organisms. Therefore it is difficult to differentiate between pathogenic and non-pathogenic organisms within the infected site. Bowler and Davies (1999) reported a significantly increased number of anaerobes (particularly *Prevotella* and *Peptostreptococcus* spp.) within infected leg ulcers compared with non-infected ulcers, and

proposed that the role of microbial synergistic interactions in chronic wound infection may be more important than the presence of any specific pathogen. A common group of anaerobic organisms have been reported in chronic infections found in diverse locations. These include *Bacteroides, Prevotella, Porphyromonas, Peptostreptococcus, Fusobacterium, Veillonella, Clostridium* and *Eubacteria* spp. (Chaudhry *et al.*, 1998; Durmaz *et al.*, 1999; Goldstein, 1999; Bowler, *et al.*, 2001). A similar group of organisms have also been reported to be associated with oral infections (Tanner and Stillman, 1993).

2.2 Isolation and identification of anaerobes

Where anaerobes are likely to be present, careful attention must be applied to several key areas involving their isolation and identification including: collection of appropriate specimens of adequate size or volume, and transport to the laboratory and processing of samples using optimal media and atmospheric conditions to ensure anaerobic growth from the specimen. The most appropriate sampling method for the culture of the anaerobic pathogens involves the aseptic collection of abundant amounts of purulent fluid or excised tissue samples not contaminated by normal flora (Bowler, *et al.*, 2001). Generally, collection of a sample using a swab is not adequate, because the sample is either insufficient or contaminated (Rosenblatt, 1997). Once collected, tissue samples can be placed in jars previously gassed with carbon dioxide or into a pre-reduced, non-nutritive transport medium to protect the anaerobes from exposure to oxygen and from dessication. Despite the perception that anaerobes die in air, a number of studies have shown that many of the common wound colonisers will survive in air for several days and that the sampling and transport methods might be less critical than the microbiological methods required to isolate the organisms (review in Bowler *et al.*, 2001). The ability to survive for a period in the presence of air has been attributed to the reducing environment of the necrotic tissue in which they reside.

Ideally, specimens should be stored correctly and processed as rapidly as possible after collection to maintain microbial viability and preserve the relative proportions. A variety of media are required to ensure optimal recovery for both aerobic and anaerobic organisms. These

should be carefully selected, freshly made, and reduced prior to use (Peterson, 1997). Non-selective media is used to assess total growth from the sample; however, none of the media tested in one study grew all anaerobes when inoculated at low density (Rosseel and Lauwers, 1997). Additionally, selective or differential media are considered essential to allow the rapid detection and separation of anaerobes from facultative organisms. Anaerobic culture can be achieved using an anaerobic chamber for large-scale processing or, on a smaller scale, jars with chemically generated anaerobic systems. However, manipulation of anaerobes in air prior to anaerobic incubation can compromise optimal results (Cox *et al.*, 1997). Also, quality control procedures must ensure that the jar lids and catalysts are functioning adequately (Rosenblatt, 1997). A study by Doan *et al.* (1999) assessed the recovery of periodontal pathogens using two different chemically generated anaerobic systems and an anaerobic chamber, and found that the recovery of bacteria by the different systems varied considerably from sample to sample. In addition to these variations anaerobes grow at different rates; although some anaerobes will grow within 24 hours, the more fastidious species, such as *Porphyromonas*, will take 5-7 days to grow adequately before they can be further investigated, and require up to 14 days for pigmentation (Petit *et al.*, 1993). Further identification involves Gram stain, the use of antimicrobials, biochemical kits to detect the presence of enzymes and gas-liquid chromatography (Rosenblatt, 1997). The value of Gram staining for anaerobes is limited unless staining can be carried out within the anaerobic chamber, as exposure to oxygen compromises the cell wall integrity of some Gram-positive anaerobes causing them to stain as Gram-negative (Johnson *et al.*, 1995a).

2.2.1 Major groups of anaerobic bacteria

Many changes in the taxonomy of anaerobic bacteria have occurred in recent years. Following the introduction of nucleic acid analyses for the identification of bacteria, new species have been found and old species have been renamed. The nucleic acid-based methods include DNA-DNA hybridisation, determination of GC content, and 16S and 23S rDNA sequencing. The use of 16S rDNA sequencing has provided a revolutionary breakthrough in

both taxonomy and bacterial identification, especially for those species that have not yet been cultured. The 16S rDNA sequencing method classifies bacteria according to their phylogenetic relatedness. Those bacteria with a similar evolutionary history will have nucleic acid sequences that are closely related (Pace, 1997). However, the phylogenetic approach does not necessarily correlate with phenotypic characteristics such as Gram-staining, morphology and growth requirements that are accepted as established methods of bacterial identification.

Of the main anaerobic bacterial groups, most changes have occurred in the taxonomy of the Gram-negative rods, particularly the genus *Bacteroides*. The genus *Prevotella* now includes most of the saccharolytic pigmented and non-pigmented species from *Bacteroides* and the former *Mitsuokella dentalis* and *Hallela seregens.* The genus *Porphyromonas* includes the asaccharolytic species from *Bacteroides.* Organisms from the genera *Prevotella* and *Porphyromonas* are found as part of the oral flora, and also in the gastrointestinal and urogenital tracts and are recovered as components of a mixed flora from infections related to those sites. The genus *Fusobacterium* contains a number of recently added species and subspecies that are found as indigenous flora in the oral cavity, respiratory, gastrointestinal and urinary tracts. *Fusobacterium nucleatum* is the species most commonly detected in clinical infections in humans. This organism comprises a number of subspecies that share similar nucleic acid sequences. Members of the genus *Bacteroides* are found in the normal flora of the gastrointestinal and genital tracts, and the oral cavity. They are isolated from infections of the head and neck, intra-abdominal and urogenital sites, and also from soft tissue and bone infections. The genus *Campylobacter* now includes the organisms *Wolinella recta* and *Wolinella curva.* Strains of *Campylobacter* have been isolated from infections of the head and neck, lungs and appendix. (Finegold and Jousimies-Somer, 1997; Jousimies-Somer, 1997).

The most clinically important group of anaerobic Gram-positive cocci is represented by the genus *Peptostreptococcus,* members of which have been cultured from a wide variety of sites, particularly abscesses and infections of the mouth, other soft tissues, bone, upper respiratory and female genital tract. Data from surveys of anaerobic infections indicate that they account

for 25-30% of all anaerobic isolates (Murdoch, 1998). Other Gram-positive cocci found in clinical infections include species of streptococci, particularly the *S. milleri* group which are associated with purulent infections. Although not strictly anaerobic, these microaerophilic bacteria are often isolated by anaerobic culture (Jousimies-Somer, 1997).

Other anaerobic Gram-positive organisms include the genera *Clostridium, Actinomyces, Eubacterium* and two new groups, *Atopobium* and *Pseudoramibacter.* Although none of the organisms in the genus *Actinomyces* is strictly anaerobic, many of the species are associated with chronic suppurating infections with a tendency to form draining sinuses in sites including the soft tissues, urinary tract, oral cavity, prostate, chest, eyes and ears. The genus *Eubacterium* presently includes more than 40 species and is phenotypically heterogeneous. Species have been isolated from oral infections, and abscesses of the rectum, brain, pelvis and lungs. The genus *Clostridium* is currently undergoing a phylogenetic reorganisation that will subsequently result in relocations and changes to nomenclature. These organisms are often isolated from significant infections in humans and also from wounds and soft tissue infections, although not from the oral cavity (Finegold and Jousimies-Somer, 1997).

Of the new species, *Pseudoramibacter* now includes *Pseudoramibacter alactolyticus* (formerly *Eubacterium alactolyticus*) which has been isolated from patients with periodontal disease, root canal infections and oral abscesses in addition to other infections such as brain, intestinal and lung abscesses, cellulitis and post-operative wound infections. The new genus *Atopobium* was created to include a new species, *Atopobium rimae,* a renamed *Lactobacillus* species (*L. minutum)* and a renamed *Streptococcus* species (*S. parvulus).* The clinical significance of these oral organisms is at this stage poorly defined (Finegold and Jousimies-Somer, 1997; Jousimies-Somer, 1997).

2.3 Dental caries

The following review outlines the development of dental caries as an example of a mixed infection initiated by aerobic and microaerophilic bacterial colonisation of the enamel surface which evolves into a complex infection dominated by anaerobes. Of the approximately 500

bacterial species that reside in the oral cavity only a small percentage may play an important role in the pathogenesis of dental caries, although it is not known which organisms drive the carious process or cause pulpal death. Despite the fastidious nature of the organisms identified in dental caries, they possess pathogenic attributes that enable colonisation of the tooth surface, survival in plaque, invasion and destruction of dentine and pulpal tissue, and evasion of host defence mechanisms.

2.3.1 Bacterial colonisation

As described earlier, bacterial colonisation is facilitated by bonding through adhesins found on filamentous appendages and outer cell membranes or vesicles of bacteria. Initially the early colonisers, streptococci and actinomycetes, attach to pellicle covered tooth surfaces via adhesins. In the presence of dietary sucrose the mutans streptococci synthesise complex mixtures of extracellular polysaccharides which further assist in the early colonisation of the enamel surface (Gibbons, 1984). The later colonisers coaggregate primarily through fusobacteria which provide a bridge between the aerobic and anaerobic microbial constituents of plaque. This is supported by Bradshaw *et al.* (1998) who showed that coaggregation-mediated interactions between *F. nucleatum* and *Prevotella* spp. or *Porphyromonas* spp. facilitated survival of the black-pigmented anaerobes in an aerated environment.

During the development and maturation of dental plaque, densely packed mixtures of different bacterial species produce many bacterial interactions. Successful interactions are important in the promotion of virulent strains and may be found where one species aids the attachment or provides growth factors for another. Reports of the progression of caries via bacterial invasion of dentine tubules illustrate further favourable interactions between microaerophilic and anaerobic bacteria (Love, *et al.*, 2000). Other relationships are antagonistic due to competition for binding sites or nutrients, or the production of substances toxic to another species. For example, some strains of streptococci elaborate organic acids or hydrogen peroxide which inhibit the growth of Gram-negative anaerobes (Hillman *et al.*, 1985; Mashimo *et al.*, 1985). The black-pigmented Gram-negative anaerobic bacteria in turn limit the growth of

streptococci and other Gram-positive bacteria including actinomycetes, by the elaboration of bacteriocins (Hammond *et al.*, 1987; Hillman, *et al.*, 1985) and haematin (Takazoe *et al.*, 1984). Selective habitats, such as the deep carious lesion, that favour a shift in the bacterial population towards more anaerobic microflora are also accompanied by changes in the environmental and nutritional requirements of the predominant organisms.

2.3.2 Environmental requirements

Maturation of plaque over time has been associated with a decline in the number of aerobic organisms and an increase in the number of anaerobes with an accompanying lowering of the oxidation-reduction potential. From this observation evolved the concept, published in the 1960's, that describes the survival of obligate anaerobes in plaque as dependent on the presence of microaerophilic or facultative organisms to maintain a low redox potential. More recent information indicates that obligate anaerobes can survive in the presence of oxygen and that it is the ability to defend against the toxic effects of reactive oxygen by-products that determines survival (review in Bowden and Hamilton, 1998). Survival of organisms against oxidative stress is dependent on an ability to metabolise oxygen to harmless components or to remove toxic oxygen by-products, such as superoxide anion, hydrogen peroxide and hydroxyl radicals which may damage cell membranes, proteins, DNA and other nucleic acids. Removal of the toxic by-products occurs through the action of defensive enzymes including superoxide dismutase, peroxidase and catalase. Aerobic and facultative microorganisms utilise oxygen to produce hydrogen peroxide and water which lowers the oxygen concentration and redox potential in plaque. They are also well equipped with defensive enzymes against oxidative stress, whereas the anaerobes lack the ability to metabolise oxygen and possess limited defensive enzymes. Bowden and Hamilton (1998) reported that a number of oral anaerobes colonising humans including the porphyromonads, prevotellae and bacteroides showed some defensive enzyme activity involving superoxide dismutase and NADH oxidase, although not catalase or peroxidase.

An additional modulating factor is the pH of the environment. Marsh (1994) proposed

that a low pH, rather than the availability of carbohydrates, was the main factor involved in the selection of potentially cariogenic bacterial species. At a pH below 5, the predominant population was reported to be the more aciduric bacteria including *S. mutans*, *L. casei* and *Veillonella dispar*. However, as the pH increased to 7 or higher, anaerobic *Porphyromonas* and *Prevotella* spp. dominated. This transition is enhanced by the metabolism of lactic and acetic acids to less acidic products (Thylstrup and Fejerskov, 1994), and the breakdown of organic material in deep carious lesions, root canals and periodontal pockets by proteolytic enzymes producing amino acids for utilisation by anaerobic organisms (ter Steeg and van der Hoeven, 1989; Sundqvist, 1994). A combination of these effects results in a less acidic environment favouring anaerobic microorganisms.

2.3.3 Nutritional requirements

Bacteria in dental plaque receive nutrients from saliva and dietary components, with a significant group utilising carbohydrates as the main energy source resulting in the production of powerful acids capable of demineralising enamel. As plaque matures and the carious lesion advances into dentine, the more fastidious bacteria utilise serum proteins, haemin and degraded dentine components. This transition requires the development of favourable nutritional relationships that benefit fastidious anaerobic bacteria and include the production of various components by co-habiting bacteria (Loesche, 1968). *Micromonas micros*, for example, makes amino acids and peptides available from the breakdown of serum glycoproteins and connective tissue components for use by other anaerobes as energy sources (Sundqvist, 1994). Some oral organisms have specific nutritional requirements that are provided by other bacteria; examples include *Campylobacter rectus* which is reliant on the presence of proteolytic and formate-producing organisms such as *P. melaninogenica* for its growth (Grenier and Mayrand, 1986; Sundqvist, 1994) and *Porphyromonas gingivalis* which utilises protohaem produced by *C. rectus* in situations of haemin limitation (Grenier and Mayrand, 1986).

Favourable bacterial interactions have also been reported in animal models where the pathogenicity of one bacterial species was enhanced by the presence of other species. An

example of this includes *P. melaninogenica* which in pure culture is not pathogenic, but mixed with plaque bacteria produces abscess formation (MacDonald *et al.*, 1963). Similar interactions have been reported for *F. nucleatum* with *P. intermedia* or *P. gingivalis* (Baumgartner *et al.*, 1992) and *P. endodontalis* with *P. intermedia* or *P. nigrescens* (Siqueira *et al.*, 1998).

2.3.4 Destructive bacterial elements

Although the mechanism of pulpal infection is not known, microorganisms from superficial and deep caries produce enzymes and organic acids capable of inducing both defensive as well as destructive responses in the pulpal tissue, effectively compromising the immune and repair mechanisms of the pulp. Those substances that have the potential to induce tissue damage can be broadly classified into three overlapping groups: chemicals that injure tissue cells, chemicals that cause cells to release biologically active factors and those that affect the intercellular matrix (Socransky and Haffajee, 1991). A wide variety of bacterial metabolic by-products produced by carious microorganisms have been shown to adversely affect the growth and metabolism of mammalian cells. Butyric, succinic and propionic acids produced by *Porphyromonas* spp. are toxic to human cells (Singer and Buckner, 1981; Rotstein *et al.*, 1985). Other toxic products include indole, ammonia, hydrogen sulphide and methyl mercaptan produced by strains of fusobacteria, actinomycetes, eubacteria, veillonellae, prevotellae and porphyromonads (MacDonald *et al.*, 1963; Mink *et al.*, 1983; Persson *et al.*, 1990).

The cell walls of Gram-negative bacteria contain lipopolysaccharide (LPS/endotoxin), a major virulence factor, which exerts a range of biological effects resulting in the amplification of inflammatory reactions. Lipopolysaccharide is capable of activating the complement cascade via the alternate pathway, degranulating mast cells, activating platelets, basophils and endothelial cells, and inducing the release from cells of collagenase, tumour necrosis factor (TNF), interferons (α, β, γ), prostaglandins and interleukins (IL-1, IL-6, IL-8). It also enhances neutrophil-mediated injury with the resultant release of oxygen radicals and proteases (review in Seltzer and Farber, 1994). Studies have demonstrated that administration of endotoxin to experimental animals (Hirafuji and Shinoda, 1994) and topical application to cut dentine

(Warfvinge, *et al.*, 1985) provokes a pulpal inflammatory reaction. Dahlen and Bergenholtz (1980) documented the presence of endotoxin in necrotic pulps and found that higher levels of endotoxin were associated with greater numbers of Gram-negative bacteria. Further, increased amounts of endotoxin were associated with symptomatic teeth (Khabbaz *et al.*, 2000), teeth with apical radiolucencies and with exudate associated with root canal therapy (Horiba *et al.*, 1991).

Gram-positive bacteria are also capable of influencing inflammatory reactions via cell wall components that include peptidoglycans and lipoteichoic acids. These components are known to initiate and sustain a chronic inflammatory reaction. The peptidoglycans can induce the secretion of IL-1, TNF α, lymphokines and prostaglandins, in addition to nitric acid, oxygen radicals and eicosanoids from macrophages and lymphocytes. Both cell wall components have been implicated in activating the complement cascade via the alternate pathway with the subsequent release of C3a which is chemotactic for neutrophils (reviews in Farber and Seltzer, 1988 and Seltzer and Farber, 1994).

Both Gram-positive and Gram-negative microorganisms from carious dentine and infected root canals have been shown to produce a range of enzymes that contribute to pathogenicity by either direct or cytotoxic effects (Slots and Genco, 1984). Proteinases, such as keratinase, collagenase, fibronectin degrading enzymes, trypsin-like enzymes, gelatinase and other enzymes including neuraminidase, phospholipase A, DNase, RNase, catalase, superoxide dismutase and hyaluroidase have been associated with *Prevotella* and *Porphyromonas* spp. (Socransky and Haffajee, 1991; Seow *et al.*, 1992; Sundqvist, 1993). Of the Gram-positive species, *S. gordonii* and *P. micros* produce a number of proteinases (Rams *et al.*, 1992; Juarez and Stinson, 1999). These enzymes have been shown to evoke a wide range of potentially harmful activities including dysregulation of the complement cascade, degradation of immunoglobulins and bactericidal proteins, alteration of neutrophil antimicrobial activity and hydrolysis of collagen (Travis *et al.*, 1997).

Other pathogenic characteristics of *Prevotella*, *Porphyromonas*, *Fusobacterium* and

Lactobacillus spp. include the ability to bind fibronectin and type I and V collagen, and to degrade fibrinogen (Falkler *et al.*, 1982; Harty *et al.*, 1994; Haraldsson and Holbrook, 1998). Some of these microorganisms also have the ability to haemagglutinate erythrocytes, aggregate platelets and release haemolytic enzymes. Hillman *et al.* (1993) frequently recovered haemolytic bacteria from sites with active periodontal disease where haemolysin production was regulated by the availability of environmental haemin. However, it has been suggested that haemolysins may be capable of lysing a number of cell types other than erythrocytes, which may provide a means for bacterial invasion (Beem *et al.*, 1998) and interference with tissue repair (Haraldsson and Holbrook, 1998).

To establish an infection in host tissue, bacteria must also be able to survive the host defence mechanisms. This can be achieved by disturbing the inflammatory response, preventing opsonisation, evading phagocytosis or killing the phagocyte. *Prevotella* and *Porphyromonas* spp. produce several proteolytic enzymes that are capable of degrading or inactivating human plasma proteins involved in host defences, such as the immunoglobulins and complement components (Killian, 1981; Sundqvist *et al.*, 1985; van Winkelhoff *et al.*, 1992). Further, these organisms can resist phagocytosis through capsulation (Sundqvist *et al.*, 1991) or by altering the shape of neutrophils (Seltzer and Farber, 1994), and can avoid destruction by interfering with the killing mechanisms of polymorphonuclear leukocytes (Rotstein *et al.*, 1989; Yoneda *et al.*, 1990). Other microorganisms are able to produce leukotoxins capable of killing leukocytes (Taichman *et al.*, 1980). These effects result in paralysis of the immune system of the host, thus facilitating the penetration and spread of potentially toxic substances, enzymes and antigens released by microorganisms.

As described, pulpal tissue damage can be elicited by components of microorganisms permeating the dentine or, in more advanced caries, causing direct effects on pulpal cells. Progression of the carious lesion has been reported to be episodic in nature in relation to a changeable, diverse and complex flora. The initial bacterial attack by streptococci and lactobacilli becomes modified as the environmental conditions alter to produce a mixed

bacterial infection. The defence reactions of the pulp initially involve the formation of reparative dentine and occlusion of the dentinal tubules by plasma proteins (Bergenholtz, 1990). These reactions may slow the rate of carious attack and the inflammatory reaction dissipates if the causative microorganisms are removed with restoration of the tooth. However, if allowed to continue, the carious lesion develops more anaerobic populations with increasing depth, eventually invading reparative dentine. Continued progression of the lesion results in development of acute inflammation and micro-abscess formation as microorganisms directly attack the pulp tissue, culminating in pulpal death with possible extension to involve the periapical tissues.

2.4 Periapical disease

As the dental pulp is contiguous with the periapex, bacterial invasion of the pulp as a result of untreated caries can extend to involve the periapical tissues. Periapical inflammation develops in response to irritants from microorganisms, their by-products, pulp breakdown products and inflammatory mediators from within the root canal. The host defence reaction initiated by irritants emanating from the root canal induces numerous inflammatory mediators from a number of cell types. These processes operate in an attempt to limit invasion of pathogens, however the host response may account for much of the resultant tissue damage. The sequelae of pulpal infection and necrosis produce two types of lesions: periapical abscess or periapical granuloma. The periapical abscess is characterised histologically by an accumulation of polymorphonuclear leukocytes and clinically by the rapid development of pain, swelling and elevation of the tooth from its socket. The periapical granuloma represents a mass of chronic inflammatory cells typically including lymphocytes, macrophages, mast cells, plasma cells and fibroblasts that have developed in response to inflammatory stimuli from the root canal. The types and pathogenicity of the bacteria involved as well as the response of the host determine which type of lesion develops. It has been speculated that more virulent organisms are responsible for abscess development, whereas granulomas are formed by less virulent microorganisms (Trowbridge and Stevens, 1992).

Fortunately, serious complications arising from periapical infections are rare. However, in some cases, infection spreads to adjacent areas creating abscesses in the soft tissues of the face, the submandibular or sublingual tissues, and the parapharyngeal, submaxillary, or infratemporal spaces. Extension of the infection into the maxillary sinuses and then to the nervous system can cause serious complications. The finding of anaerobic bacteria in dentine caries and periapical infections is of importance because these organisms have been associated with serious infections arising from dental foci including bacteraemia, endocarditis, meningitis, subdural and pulmonary empyema (Brook *et al.*, 1991).

Reports of microbiological studies from teeth with infected root canals indicate that the majority of cases involve polymicrobial and predominantly anaerobic flora. The principal isolates include fusobacteria, porphyromonads, prevotellae, peptococci, peptostreptococci and eubacteria, with facultatively anaerobic streptococci found in the coronal part of the canal. This group represents a limited selection of the total oral flora and is similar to that found in the periodontal pocket, although less complex. Development of this restricted group of predominantly anaerobic microorganisms occurs as a result of synergistic microbial inter-relationships and selective environmental conditions. Environmental factors contributing to the creation of a selective habitat include the lack of oxygen and development of a low reduction-oxidation potential with increasing time. Conditions within the root canal support anaerobic bacteria capable of fermenting amino acids and peptides from tissue fluid and disintegrating connective tissue, whereas bacteria that rely on the fermentation of carbohydrates as an energy source will be restricted by a lack of nutrients. Microbial interrelationships include the production by some bacteria of a wide range of proteolytic enzymes that assist the supply of nutrients and the production of haemin and vitamin K, which are essential nutrients for the black-pigmented species (Sundqvist, 1994).

In the majority of cases a mixed infection was reported to be responsible for the development of clinical symptoms, with an increase noted in both the bacterial load and the diversity of species found in cases with symptoms compared with those that presented

symptom-free (Sundqvist, *et al.*, 1989; Gomes *et al.*, 1994). However, there is no documented evidence for the role of a single organism or group of microorganisms in the aetiology of periapical disease. Equally, attempts to relate the presence of certain bacteria in root canal infections with the development of clinical symptoms have been unsuccessful, as few studies have compared the flora from symptom-free with symptomatic teeth (Seltzer and Farber, 1994). In some studies the presence of particular microorgansims has been implicated in the development of acute symptoms. For example pain, swelling, tenderness and exudate have been associated with peptostreptococci, prevotellae, fusobacteria, eubacteria, *P. endodontalis* and *P. gingivalis* (Yoshida *et al.*, 1987; Haapasalo, 1989; Hashioka *et al.*, 1992; van Winkelhoff *et al.*, 1992; Gomes *et al.*, 1996). However, the presence of *Prevotella* and/or *Porphyromonas* spp. in a microbial mix provides no certainty of acute symptoms. This is illustrated in a study by Sundqvist *et al.* (1989) where *Prevotella* and *Porphyromonas* spp. were isolated from 22 teeth with apical periodontitis; 16 of the teeth presented with symptoms, the remaining six were asymptomatic. Difficulties in developing associations have also arisen from a lack of specificity in the terminology used to describe the clinical problem and from difficulties in earlier studies of accurately culturing and identifying clinically important organisms. In addition, organisms previously identified have now been reclassified and there is continuing description of new organisms and taxa (Sundqvist, 1994).

The infected root canal has been reported to be inhabited by a relatively low number of species comprising mainly anaerobes. Periapical lesions induced in rats following exposure of the pulp to oral fluids showed a change in the anaerobic bacterial counts infecting the root canal of 24% at 7 days to 47% after 15 days (Tani-Ishii *et al.*, 1994). Published studies indicated that obligate anaerobes have been cultivated in 80 to 100% of cases with between one and 11 different species found in each sample (Oguntebi *et al.*, 1982; Lewis *et al.*, 1986; Fukushima *et al.*, 1990; Baumgartner and Faulkler, 1991; Brook *et al.*, 1991; Sundqvist, 1992; Lana *et al.*, 2001). More recent reports involving molecular methods of identification have extended the number of species in individual canals to 17 (Siqueira *et al.*, 2000). The most

frequently isolated species include the previously listed anaerobes and facultative anaerobic streptococci, in addition to clostridia, lactobacilli, eubacteria, actinomycetes, veillonellae and treponemae (Baumgartner and Falkler, 1991; Sundqvist, 1992; Xia *et al.*, 1999; Lana *et al.*, 2001). The strains of *Prevotella* spp. most commonly found include *P. nigrescens, P. intermedia, P. melaninogenica* and *P. tannerae* (Sundqvist, *et al.*, 1989; Bae *et al.*, 1997; Xia, *et al.*, 1999). Of the *Porphyromonas* group, *P. endodontalis* has been identified in infected root canals and associated with periapical pathoses more frequently than *P. gingivalis* (van Winkelhoff *et al.*, 1985; Haapasalo, 1989; Sundqvist *et al.*, 1989; Siqueira *et al.*, 2001).

The anaerobic microorganisms in polymicrobial root canal infections are likely sources for the production of endotoxins and the up-regulation of cytokines and enzymes resulting in inflammation, pain and bone resorption associated with periapical infections. The peptostreptococci are of particular interest as their capacity to produce proteolytic enzymes may play an important role in the pathogenesis of periapical infections (Seltzer and Farber, 1994). *Prevotella intermedia, P. endodontalis* and *P. gingivalis* are also capable of degrading a number of glycoproteins from serum to yield amino acids and peptides for utilisation by these and other species such as *Fusobacterium* and *Eubacterium* spp. which possess insufficient proteolytic activity (Sundqvist, 1992).

It is unlikely that any single virulence factor is responsible for the tissue damage noted in dental caries or apical periodontitis. For these conditions a range of virulence factors have been implicated at different stages of the infection. Colonisation and inter-bacterial aggregation form the first stages of disease initiation, followed by specific bacterial successions in favour of the development of anaerobic microorganisms as the environment alters. Nutritional interactions then represent a second stage in the population shift. As most bacteria from the oral cavity are only weakly virulent on an individual basis, inter-bacterial cooperation is essential to provide the necessary components for a species to become pathogenic. Thus, various combinations of microorganisms have a synergistic pathogenic effect not possessed by any individual species. Apart from supplying nutrients and anaerobic conditions, the different bacterial species

contribute to a pool of virulence factors which include enzymes, toxic products and mechanisms that are capable of inactivating or resisting host defence mechanisms. The response of the dental pulpal and periapical tissues to the microorganisms and their pathogenic effects is reviewed in the next chapter.

CHAPTER 3

INFLAMMATORY REACTIONS IN RESPONSE TO DENTAL CARIES

3.1 Introduction

As the dentine and pulp form an integral unit through the extension of odontoblastic processes into dentine, any insults affecting the dentine will directly affect the pulp. These insults may take the form of caries, restorative procedures or trauma. Each of these interactions has the potential to cause pulpal inflammation, cell damage or even pulp death. The typical response of the pulp to any physical, chemical or bacterial insult is inflammation. Generally, if the insult is minor the pulp will heal and return to normal, with the possible exception of sclerotic dentine development. With dentine caries the inflammatory reaction will resolve when the irritation is removed, although in some instances the pulp may heal despite constant bacterial irritation, which is probably related to changes in the permeability of the dentinal tubules. However, if the insult is more severe the pulp may become necrotic (Bergenholtz, 1981).

Massler (1967) documented the nature of caries as an intermittent and chronic process with periods of activity alternating with periods of stasis. As a result, the histopathology of the pulp tissue will vary depending on when the lesion is sampled; whether it is in an active or passive phase. As a consequence, it is difficult to predict how the pulp will respond to either caries progression or restorative treatment. Studies have reported a range of responses and have demonstrated pulpal inflammation associated with minimal carious lesions in newly erupted teeth (Massler, 1967), while others have shown no apparent pulpal change although caries had progressed to within 0.8mm of the pulp (Shovelton, 1968).

There is a lack of consensus in the literature regarding how early the pulpal response to caries can be detected. This is further confused by the finding of inflammatory cells in the pulps

of unerupted or newly erupted, clinically intact teeth (Massler, 1967). Unfortunately, no correlation has been found between any single clinical symptom and the degree of inflammation in the pulp. Dummer *et al.* (1980) used electric pulp tests, thermal tests, percussion tests and pain history and could not find any clear association between symptoms or tests and pulpal disease. In addition, Massler (1967) noted the occurrence of superficial lesions in association with sharp pain but without evidence of pulpal inflammation. These results contrasted with a report of partial necrosis and severe inflammation of the pulp without a history of pain (Langeland, 1987).

Despite these clinical and histopathological discrepancies, it is well documented that bacteria and their products play a major role in pulpal inflammation through dental caries and microleakage around restorations (Brännströmm, 1981). However, there have been relatively few detailed studies reporting the microbiology of carious dentine or the relationship between dentinal microflora and the histopathology of carious pulpitis. Therefore, our understanding of the microbiology of the carious lesion is limited and incomplete.

The response of the pulp to the advancing carious lesion reportedly involves both specific and nonspecific inflammatory reactions, with resultant healing and repair as well as irreversible tissue damage (Bergenholtz, 1981). These reactions are related to the host defence mechanisms including the complement cascade, the immune system and the action of phagocytic leukocytes. Although inflammation is usually a protective mechanism, it also has destructive aspects that may be responsible for further tissue damage. In contrast to other tissues in the body, cell damage in the pulp may not be as easily repaired due to limited access of appropriate repair-competent cells and the constraints imposed by the hard tissue encasement. As a result, pulpitis is an example of an infection where the host reaction may produce more damage than that caused by the effects of the microorganisms.

Despite decades of research, little is known of the response of the pulp to dental caries. One mechanism is proposed, whereby the initial reaction is one of a mononuclear infiltration dominated by lymphocytes, plasma cells and macrophages, with acute reactions involving the

mobilisation of polymorphonuclear leukocytes only when the pulp is in danger of imminent invasion by bacteria. This is in contrast to the accepted progression of inflammation where the acute phase generally precedes the chronic condition. Carious pulpitis represents a form of chronic inflammation described as an insidious, low-grade response also seen in some common and disabling human diseases such as rheumatoid arthritis, atherosclerosis and chronic lung diseases (Cotran *et al.*, 1999).

3.2 Pulpal inflammatory reactions

The earliest sign of pulpal inflammation involves a disturbance of the odontoblastic layer of the pulp with a reduction in the number and size of odontoblasts. Early inflammatory changes following this are subtle and difficult to detect with the light microscope. The first indication of more obvious inflammation appears as a diffuse infiltration of mononuclear chronic inflammatory cells including lymphocytes, plasma cells and macrophages. It has been suggested that this inflammatory infiltrate is a local immune response to the components or metabolites of bacteria, as the prime function of the plasma cell is to produce immunoglobulin (Torneck, 1974). Accompanying this infiltrate is an increase in vascularity with the proliferation of small vessels and tissue oedema. In addition, there is some degeneration of pulp fibroblasts and destruction of collagen. As the lesion advances, enlargement of arterioles and capillaries, as well as the dilation of venules becomes evident. The small blood vessels become congested and oedema increases. At the microscopic level, alterations to endothelial cell morphology and disruption of the basement membrane with an accompanying increase in local vascular permeability have been reported (Langeland, 1987).

As the carious lesion progresses towards the pulp, the intensity and character of the inflammatory infiltrate changes. Inflammation begins as a low-grade chronic response, and it is only in the late stages that acute inflammation has been demonstrated. As the microorganisms penetrate deeper and invade reparative dentine, the adjacent pulpal tissue becomes infiltrated by macrophages and polymorphonuclear leukocytes that have been shown to emerge from adjacent small blood vessels (Torneck, 1977). Polymorphs have also been identified entering

and migrating into dentinal tubules (Trowbridge, 1981). Other forms of pulpal damage include lytic changes affecting fibroblasts with disruption of the plasma membrane and release of cytoplasmic organelles. Diffuse oedema has been reported throughout affected pulp tissue appearing as vacuoles surrounded by electron dense material at the periphery (Torneck, 1981).

Following pulp exposure, the incidence and degree of vascular change increases with more frequent disruptions of vessel walls, thrombosis and haemorrhage. By contrast, damage to the nerve tissue occurs later than in adjacent tissues possibly due to the abundance of collagen, which is less susceptible to proteolytic activity, surrounding the nerve bundles (Torneck, 1974). Evidence of microbial penetration into the pulp tissue is found by the presence of rod and coccal-shaped microorganisms both extracellularly and intracellularly within polymorphs and macrophages. These inflammatory cells are attracted to the area of microbial penetration in response to chemotactic activity and following phagocytosis of the bacteria they disintegrate. This releases the engulfed bacteria (dead or alive) and lysosomal enzymes into the pulp producing destruction of pulp tissue and liquefaction necrosis (Page *et al.*, 1973; Langeland, 1987). An increase in the number of phagocytic cells in the pulp in advanced inflammation may be responsible for much of the cellular damage produced and be detrimental to the survival of the pulp (Torneck, 1977).

The progressive accumulation of polymorphs inevitably results in suppuration which clinically may become localised to form a small abscess within the coronal portion of the pulp. Surrounding this are chronic inflammatory cells found at a distance from the central area of irritation. The area of acute inflammation then becomes localised by the proliferation of fibrous tissue and small blood vessels to "wall-off" the affected region. At this stage, the remainder of the pulp may show no signs of pathology. If chronic inflammation persists for a long period, dystrophic mineralisation can occur with dead and dying inflammatory cells becoming mineralised through the deposition of calcium salts accumulating to form a mineralised mass. Although calcifications occur frequently, even in intact and unerupted teeth, the number and amount increases during the carious process and, therefore, they are regarded as pathological

entities as their presence reduces the ability of the pulp to respond to injury (Langeland, 1987).

Degeneration of the pulp occurs when the number of bacteria entering the pulp exceeds the ability of the pulp to withstand the insult. Whether or not the pulp then undergoes total necrosis depends on several factors including the virulence of the bacteria, the ability to offset an increase in intrapulpal pressure from tissue oedema and the resistance of the host. Host factors include the age and health of the pulp, which in turn influence the efficiency of the circulation and lymph drainage (Walton and Torabinejad, 1996).

Animal experiments have demonstrated the development of inflammation and abscess formation of surgically exposed pulps in control, but not germ-free animals (Kakehashi *et al.*, 1965). In other studies, bacterial plaque extracts placed on freshly cut dentine produced inflammatory changes in the pulp presumably from penetration of bacterial products through the dentinal tubules (Mjör and Tronstad, 1972; Bergenholtz, 1977; Bergenholtz and Warfvinge, 1982). It was speculated by Mjör and Tronstad (1972) that immunological reactions had also occurred. In this context, following sensitisation of experimental animals to antigens applied through the root canal system, there was a strong local immunological response with accumulation of plasma cells, accompanied by a systemic reaction (Barnes and Langeland, 1965; Okada *et al.*, 1967).

When the antigenic challenge is temporary, the inflammatory response is generally short-lived and self-limiting. However, where the process continues chronic pulpitis develops producing a predominantly mononuclear cell infiltrate (Torneck, 1977; Torneck, 1981). The relationship between depth of caries penetration and histopathology of the pulp has revealed that no significant pathology is noted until the carious lesion is approximately 1.1mm from the pulp. Once the carious process is within 0.3-0.5mm there is an increase in pathological changes with considerable inflammation (Reeves and Stanley, 1966). These pathological changes are considered to be the result of the activation of both non-specific inflammatory reactions as well as specific immunological responses.

3.3 Non-specific inflammatory reactions

Pulp injury involving cell damage and/or cell death is followed by the release of non-specific chemical mediators of inflammation. The release of histamine, interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor (TNF) is responsible for the earliest events in inflammation. Later effects are mediated by arachidonic acid metabolites, bradykinin, plasma-derived factors, other cytokines and the neuropeptides such as calcitonin gene related peptide and substance P (Walton and Torabinejad, 1996).

Histamine and serotinin are the two major vasoactive amines involved in early inflammatory reactions. Both occur in a variety of cells including mast cells, platelets and basophils. Histamine is considered to be the most important vasoactive amine and is released by inflammatory stimuli causing dilation of arterioles and increased vascular permeability. Mast cells are the main source of histamine; in addition they also release leukotrienes and platelet activating factor. Although histamine has been shown to be present in inflamed pulp tissue, controversy exists regarding the presence of mast cells in the human dental pulp. It has been reported that normal pulps lack mast cells, while small numbers have been found in inflamed pulps (Zachrisson, 1971; Miller *et al.*, 1978).

Cytokines are peptides produced by different cell types, such as activated lymphocytes, macrophages, endothelium, epithelium and connective tissue cells, that modulate the function of other cell types by binding to specific receptors on target cells. The major cytokines that mediate inflammation include IL-1 and TNFα and β. Secretion of these products can be induced by endotoxins, immune complexes, exotoxins, cell injury and various inflammatory stimuli. The most important inflammatory effects include endothelial changes; specifically inducing the synthesis of endothelial adhesion molecules and chemical mediators, producing molecules associated with matrix remodelling, and the priming of neutrophils (Cotran, *et al.*, 1999). The presence of elevated levels of the cytokines IL-6 (Barkhordar *et al.*, 1999), IL-8 (Huang *et al.*, 1999), IFN-γ, IL-4 and IL-10 (Hahn *et al.*, 2000) have been demonstrated in inflamed pulps from carious teeth.

The main products of arachidonic metabolism include prostaglandins and leukotrienes which are synthesised from cell membranes by the activation of phospholipases in response to inflammatory stimuli. These are described as short-range mediators that are rapidly formed by most cell types, exert their effects locally and are enzymatically destroyed or spontaneously decay. Of the various prostaglandins that are produced by different cell types in the body, some are powerful vasodilators that increase vascular permeability and potentiate the effects of other vasodilator agents. Prostaglandins have been identified in inflamed pulps and elevated concentrations of PGE_2 have been located in pulps from symptomatic teeth (Cohen *et al.*, 1985). Leukotrienes are also mediators of acute inflammation and are secreted by neutrophil polymorphs in inflammatory lesions. They are capable of inducing vascular dilation and of increasing venular permeability. In addition, they are potent chemotactic agents and can activate neutrophil responses such as adhesion to venular endothelium and the release of lysosomal enzymes (Anderson, 1985).

Three interrelated plasma-derived factors including complement, kinin and the clotting systems mediate a variety of activities in inflammation. The complement system consists of approximately 20 component proteins found in plasma. This system functions to provide defence by lysing microbes and facilitating increased vascular permeability, chemotaxis and opsonisation. Of the numerous steps involved in the cascade, the activation of C3 is probably the most critical and the most important inflammatory mediators are C3a and C5a (Cotran, *et al.*, 1999). Most of the activators of the classical and alternate pathways of the complement system have been identified in inflamed pulp tissue or in periapical lesions. Both C3 and C4 have been located in dentinal tubules, in odontoblasts and associated with bacteria isolated from human carious dentine (Pulver *et al.*, 1978). The kinin system generates vasoactive peptides from plasma proteins with the final release of bradykinin, a potent agent that increases vascular permeability.

Platelet-activating factor is another phospholipid derived mediator known to have multiple inflammatory effects. A variety of cell types elaborate platelet-activating factor,

including platelets, basophils, neutrophils, moncytes/macrophages and endothelial cells. The functions of this mediator, in addition to platelet stimulation, include vasodilation or vasoconstriction depending on concentration, increased venous permeability, chemotaxis and degranulation of leukocytes (Cotran, *et al.*, 1999).

Pulpal innervation, in addition to mediating pain perception, may also modulate some aspects of the inflammatory response through the secretion of neuropeptides. Neurogenic responses include an increase in blood flow, plasma extravasation and leukocyte accumulation (Stashenko *et al.*, 1998). Sensory nerve fibres containing calcitonin gene-related peptide and substance P have been shown to sprout into areas surrounding inflamed pulp tissue following injury (Byers, 1994). It has been proposed that these responses promote tissue healing by encouraging perfusion through angiogenesis, the removal of noxious substances by phagocytosis and by the close association of sprouting nerve fibres secreting calcitonin gene-related peptide at sites of reparative dentine formation. Support for this theory comes from experimental data reported by Byers and Taylor (1993) who demonstrated that innervated teeth with pulp exposures showed far less tissue necrosis than denervated teeth. In addition, Jontell (1998) has described an intricate network involving contact between nerve fibres and pulp micro-vessels via the cell processes of dendritic cells. These findings indicate that the nervous system and the pulp vasculature interact to recruit immunocompetent cells to the sites of tissue injury.

Phagocytosis of bacteria by polymorphonuclear leukocytes and macrophages is accompanied by the generation of reactive oxygen and nitrogen intermediates as the cells undergo a respiratory burst upon encountering invading microorganisms or other appropriate stimuli (Marton and Kiss, 2000). These cells also contain lysosomal enzymes which when released may contribute to tissue damage (Sandberg *et al.*, 1988). Contents of the primary and secondary granules of PMNL include myeloperoxidase, lysozyme, cationic proteins, neutral proteinases, lactoferrin, elastase and cathepsin G. These components contribute to both the elimination of bacteria as well as the destruction of structural components of host tissues (Trowbridge and Stevens, 1992). Confirmation of the presence of some of these enzymes in

inflamed pulps was reported by Rauschenberger *et al.* (1991) who noted elevated levels of elastase, cathepsin G and lactoferrin in moderate to severely inflamed pulpal tissue samples compared with normal pulps.

3.4 Immunopathological mechanisms within the pulp

Once the integrity of the hard tissue barrier of the tooth is broken, noxious substances gain entry to the pulp tissues potentially activating immune responses by acting as antigens. These immune responses involve specific interactions with invading antigens which result in dense aggregations of macrophages, lymphocytes and plasma cells in inflamed pulp tissue (Torneck, 1977; Torneck, 1981). Although the roles for cell mediated and humoral immune responses have been inferred from the presence of specific cell types within the injured pulp, it is not apparent how the immunopathological mechanisms are organised.

Contrary to the findings of Seltzer and Bender (1984), the normal dental pulp has been found to contain a variety of immunocompetent cells capable of immunological responses. Jontell *et al.* (1987) first reported the presence of class II major histocompatibility complex (MHC) antigen-expressing dendritic cells in healthy pulps. These cells were noted in the odontoblastic layer and the central pulp tissue, and are believed to be responsible for the initiation of immune responses by presenting foreign antigens to $CD4^+$ T lymphocytes. Inflammatory stimuli also promote the activation and differentiation of macrophages. The presence of resident macrophages in normal pulpal tissue has been documented from light and electron microscopic observations (Seltzer and Bender, 1984). Macrophages act primarily as scavengers by virtue of their phagocytic activity and are important for the elimination of antigen-antibody complexes. However, they also produce several bioactive substances including microbicidal enzymes, cytokines and growth factors for fibroblasts and endothelial cells. Class II MHC-expressing macrophages play a critical role in activating T lymphocytes (Jontell *et al.*, 1998) and later in the inflammatory process B cells require assistance from macrophages for optimal stimulation and differentiation (Tew *et al.*, 1989). Bergenholtz *et al.* (1991) reported a rapid and intense influx of MHC class II antigen expressing cells in

inflammatory lesions of the pulp induced by challenge with lipopolysaccharide from *P. gingivalis*. It is therefore reasonable to assume that macrophages and dendritic cells play a key role in the initiation of immune activities in the pulp in response to bacterial antigens.

Using monoclonal antibodies and indirect immunofluorescence, Pekovic and Fillery (1984) were the first to demonstrate the presence of T lymphocytes in inflamed pulpal tissue from teeth with advanced caries. Confirmation of this finding was reported by Hahn *et al.* (1989) using immunohistological staining with monoclonal antibodies. These investigators noted the presence of low numbers of T lymphocytes in normal tissue with increased numbers of T and B lymphocytes in inflamed pulpal tissue indicating the presence of immune reactions as part of the pathogenesis of the carious lesion. In early inflammation T lymphocytes play a crucial role in orchestrating immune responses following recognition of antigens. They secrete several cytokines that regulate the duration and intensity of immune reactions and are further classified into Th1 and Th2 cells depending on the cytokines released. The $CD4^+$ Th1 lymphocytes produce mainly IL-2 and IFN-γ and are involved in macrophage activation, whereas Th2 cells produce IL-4, IL-5 and IL-6 and stimulate the proliferation and differentiation of B cells (Jontell, *et al.*, 1998).

In contrast to T lymphocytes, B cells are rarely seen in normal dental pulp tissue. Researchers have failed to identify B lymphocytes using immunohistochemistry to detect cytoplasmic or surface immunoglobulin (Pulver, *et al.*, 1977; Pekovic and Fillery, 1984) or with monoclonal antibodies against B cell markers (Jontell, *et al.*, 1987), while others have observed only occasional B lymphocytes (Hahn *et al.*, 1989). These data suggest that B cells do not participate in the initial phase of the immune response in pulpal tissue.

Early inflammatory changes in the pulpal tissue show a response composed of predominantly T lymphocytes and monocytes (Izumi *et al.*, 1995). As the carious lesion progresses, chronic inflammatory infiltrates of macrophages, lymphocytes and plasma cells accumulate in pulpal tissues (Massler, 1967; Torneck, 1974; Torneck, 1977; Langeland, 1987). Carious exposure of the pulp produces an increase in the severity of the inflammatory reaction

with an elevation in the number of macrophages and polymorphonuclear leukocytes (Torneck, 1977; Torneck, 1981). The transition from an early cellular immune response induced by mainly T-lineage cells to an advanced phase showing a humoral response containing B cells and plasma cells, has been described by Izumi *et al.* (1995). Accompanying these changes is an increase in the numbers of IgG-containing cells in inflamed pulps (Pulver, *et al.*, 1977; Nakinishi *et al.*, 1995). Significant numbers of IgA immunocompetent cells were also observed, with few IgM immunocompetent cells noted. Both Falkler *et al.* (1987) and Hahn and Falkler (1992) support the finding of predominantly IgG in irreversibly inflamed pulpal tissue. In contrast to the increasing quantity of immune components found with inflammation, levels of the third component of complement (C3) reportedly become diminished in inflamed pulps compared with normal pulpal tissue, which may indicate that the inflammatory response is consuming C3 (Speer *et al.*, 1977; Okamura, *et al.*, 1980; Pekovic and Fillery, 1984;). One mechanism for complement removal during carious pulpitis is the cleavage of C3 and C5 into fragments by proteolytic activity from Gram-negative anaerobes such as *P. gingivalis, P. endodontalis* and *P. intermedia* (Sundqvist *et al.*, 1985). In conjunction with these changes, an increase in soluble bacterial products, components of the complement system and arachidonic acid metabolism products accumulate causing cellular destruction. These components are also chemotactic for polymorphs and macrophages, which on release of lysosomal enzymes cause further destruction of the pulpal tissue.

Studies over the last decade have further characterised the profile of cellular infiltrates using immunohistochemistry. Hahn *et al.* (1989) identified and enumerated the T and B lymphocytes in pulpal tissues from carious teeth that were clinically diagnosed with reversible or irreversible pulpitis. They found that $CD8^+$ T cells outnumbered $CD4^+$ T cells in normal pulpal tissue, which is in agreement with the findings of others (Jontell, *et al.*, 1987), although Mangkornkarn *et al.* (1991) detected greater numbers of $CD4^+$ T cells. In inflamed pulps, Hahn *et al.* (1989) reported increased numbers of $CD4^+$ helper cells, $CD8^+$ T cells and B lymphocytes. In the group with reversible pulpitis, more than 90% of the lymphocytes were identified as T

lymphocytes, with a $CD4^+/CD8^+$ ratio of 0.56, while the irreversible group was characterised by a higher number of all T lymphocytes, B lymphocytes, plasma cells and neutrophils. The $CD4^+/CD8^+$ T cell ratio in this group was 1.14. B cell numbers increased markedly with increasing inflammation, with B/T ratios of 0.04 (normal), 0.05 (reversibly inflamed) and 1.6 (irreversibly inflamed). These findings were confirmed by Izumi *et al.* (1995) who found numerous T lymphocytes in shallow dentine caries with an increase in B cell and plasma cell numbers in teeth with deep caries.

These findings, in addition to the observations of other authors (Torneck, 1981; Trowbridge, 1981), suggest that immunocompetent cells are important in the pathogenesis of carious pulpitis and emphasise the central role of T lymphocytes in regulating immune functions. Whether the inflammatory lesion has destructive or protective characteristics will depend on the regulatory functions of the T lymphocytes. A high ratio of $CD4^+/CD8^+$ T cells with a predominance of B lymphocytes in the irreversibly inflamed pulpal tissue could be explained by the regulation of the $CD4^+$ T cells on B cell activities. Despite the high numbers of $CD8^+$ T cells noted in these studies, a continuing influx of antigens from the carious lesion as well as excessive activation of the $CD4^+$ helper T cells, B cells and plasma cells, could create immunopathological changes caused by lymphokine release and immunoglobulin production.

The periapical lesion represents a local immune reaction subsequent to pulpal infection. Continuous flow of bacterial antigens and tissue breakdown products through the apex from the pulpal tissue causes either an acute or chronic response in the periapical tissues, depending on the balance between the microbial factors and the host defences. An acute response is characterised by an influx of polymorphonuclear leukocytes and monocytes into the periapical area. This is followed by more chronic cell infiltrates including T and B cells, macrophages, plasma cells, eosinophils and mast cells together with connective tissue cells. The chronic response is characterised by granulomatous tissue infiltrated by chronic inflammatory cells. Both $CD4^+$ and $CD8^+$ subsets of T cells have been identified in periapical lesions with $CD4^+$ cells dominating in the active phase of lesion expansion, followed by higher levels of the $CD8^+$

and plasma cells appearing as the lesion becomes more chronic. These changes suggest that $CD8^{+}$ T cells may function to dampen excessive immunoreactivity within periapical lesions (Stashenko and Yu, 1989; Marton and Kiss, 2000).

Studies using the rat model indicate that two days after pulpal exposure and infection, pulpal necrosis occurs, followed at seven days by periapical bone destruction. A period of rapid destruction then takes place between 7-20 days followed by a chronic phase characterised by slowed resorption of bone. The rapidity of bone destruction, often preceding total pulpal necrosis, suggests that many of the pathogenic effects are the result of soluble mediators such as cytokines rather than the necrotising effects of bacterial components acting directly on the tissues (Stashenko, *et al.*, 1998). Interleukin 1α has been identified as a pro-inflammatory cytokine associated with periapical bone destruction. It is produced by a variety of cells including macrophages, fibroblasts and endothelial cells in response to lipopolysaccharide. In addition, there is evidence that matrix metalloproteinases from host cells contribute to periapical tissue breakdown through collagen destruction and that bone resorption can also be induced by inflammatory factors and antibodies produced by B cells and plasma cells in the more chronic phase of infection (Takahashi, 1998).

The immune system has both a protective and destructive role. In a protective mode it is capable of mobilising protection of the pulp against injurious stimuli. Conversely, these mechanisms also have the capacity to cause damage during the inflammatory process by activation of complement, release of chemotactic agents and attraction of neutrophils to phagocytose immune complexes. Consequent release of lysosomal enzymes and inflammatory mediators from phagocytic cells and invading bacteria can cause considerable destruction to the surrounding tissue.

In an attempt to evaluate the role of microorganisms putatively involved in the aetiology and pathogenicity of carious pulpitis, thesis studies were designed to identify and enumerate bacterial species found in the carious dentine of vital human teeth with chronic pulpitis. In the first study traditional culture techniques were used to identify and enumerate the microbial

species retrieved. In the second study identification of microorganisms, from the same samples, involved molecular methods of identification using polymerase chain reaction technology. Finally, the histopathology of the pulps was examined and correlations were sought between the microbial type and number in the sampled carious dentine and the cellular responses of the dental pulp. Details of the first study form the basis for the next chapter.

CHAPTER 4

A CULTURE STUDY OF THE MICROBIOLOGY OF HUMAN CARIOUS DENTINE

4.1 Introduction

Dental caries has been recognised as a bacterially mediated disease since 1890 when Miller introduced the chemoparasitic theory of tooth decay. Despite many decades of research, the bacterial profile of dental caries remains incomplete and understanding of microbial effects on the dental pulp, inadequate. Therefore, dental caries continues to be a major public health problem in many parts of the world despite the recognised advantages of fluoride in reducing tooth decay at a community level.

While it is known that bacteria and their products are responsible for pulpal inflammation as a result of dental caries, few studies have investigated the microbiology of carious dentine or the relationship between dentinal microflora and the histopathology of chronic pulpitis. As a result there is no reliable evidence to assign an aetiological role for carious pulpitis to a particular microbial species. Massey *et al.* (1993) demonstrated a significant relationship between the number of *Prevotella* spp. in carious dentine and an advanced inflammatory pulpal change. This study expands on the results of previous work by examining the microbiology of carious dentine in human teeth using culture techniques.

4.2 Materials and methods

4.2.1 Sample collection

Sixty five vital carious teeth were obtained with informed consent from separate, randomly selected patients of either sex from the United Dental Hospital in Sydney, Australia. The age of the patients ranged from 17 to 75 years with an average age of 37 years. Patients were excluded from the study if they reported a history of significant medical disease or antimicrobial therapy within four months prior to tooth extraction. The patients presented with

clinical signs and symptoms of pulpitis and requested extraction to relieve symptoms. The nature of symptoms was noted at the time of sampling.

Anterior, premolar and molar unrestored teeth with coronal enamel and dentine caries were selected for the study on the basis of clinical diagnostic tests which indicated that they were vital, with clinical symptoms of reversible pulpitis (pain and heightened sensitivity to hot and cold stimuli). These teeth had no obvious exposure of the pulp tissue and periodontal pocket depths of less than 4mm. This study was approved by the Central Sydney Area Health Service Ethics Review Committee, Sydney, Australia (Reference No: 6/96).

4.2.2. Dentine processing

Immediately after extraction under local anaesthesia, each tooth was placed in a container of pre-reduced transport fluid (RTF) (Syed and Loesche, 1972) and transferred immediately to an anaerobic glove chamber containing 85%N_2, 5%CO_2, 10%H_2. Superficial plaque and debris overlying the lesion was first removed, the teeth were rinsed several times with RTF, then using sterile sharp curettes and excavators, all softened and necrotic dentine was collected as small fragments from the carious lesion of each tooth. In addition, shavings of dentine were taken from the stained but minimally decalcified deep layers of the lesion. Evidence of carious pulpal exposure was noted at this stage. Dentinal sampling was completed less than 20 minutes after tooth extraction.

The total sampled dentine from each tooth was transferred separately from the anaerobic chamber in an airtight container, weighed, and a standard solution of 10 mg wet weight dentine/ml in RTF was prepared for each tooth. The fragments of carious dentine were dispersed in the chamber by vortexing for 20 seconds before being homogenised by hand in a 2ml glass homogeniser for 30 seconds using a standardised technique of vertical and rotational movements.

Serial dilutions in the range 10^{-3} to 10^{-6} in RTF were prepared for plating. Plates were duplicated for each dilution and each medium then incubated in either the glove chamber for up to 2 weeks or, for microaerophilic conditions, in an anaerobic jar with a CO_2 gas pack (Oxoid,

Basingstoke, UK) at 37°C for 48 hours. Isolates representing five microbial genera: *Streptococcus, Lactobacillus, Actinomyces, Fusobacterium* and *Prevotella* were chosen for more detailed study on the basis of previous studies of carious dentine (Hoshino, 1985; Massey *et al.*, 1993). The following media and conditions were used to examine both selective and general growth as described by Massey *et al.* (1993). Total microbial load per mg wet weight of dentine was determined by the number of colony forming units (CFU) on Trypticase Soy agar (Oxoid) enriched with menadione, haemin, L-cysteine and horse blood (ETSA) under both anaerobic and microaerophilic conditions (US Department Health and Human Services–Centres for Disease Control). Mitis Salivarius (Oxoid) (Chapman, 1944) and Rogosa agar (Bacto Laboratories, Detroit, MI, USA) (Rogosa *et al.*, 1951) were used to study the growth of streptococci and lactobacilli respectively under microaerophilic conditions (95%N_2 and 5%CO_2). Cadmium fluoride acriflavine tellurite (CFAT) agar was used for the selective isolation of *Actinomyces* spp. and related Gram-positive filamentous organisms under anaerobic conditions (Zylber and Jordan, 1982). A kanamycin-vancomycin containing anaerobic blood agar (KVA) was used for identification of the obligately anaerobic *Prevotella* spp. (Dowell, 1975). Crystal violet erythromycin (CVE) agar was used under anaerobic conditions for the isolation of *Fusobacterium nucleatum* (Walker *et al.*, 1979).

An initial group of twenty specimens was examined to identify the main colony forms on selective media. One colony forming unit of each type seen was subcultured, Gram-stained and assessed with biochemical diagnostic test kits: RapID ANA II (Innovative Diagnostic Systems, Atlanta, GA, USA) for anaerobic bacteria; Rapid ID 32 Strep for streptococci and, api 50 CH and api 50 CHL medium for lactobacilli (bioMérieux, Marcy-l'Ètoile France). To confirm the accuracy of testing, strains of known species were employed: *Streptococcus mutans* LT11 (Tao *et al.*, 1993), *Lactobacillus acidophilus* ATCC 4356 (Institute of Dental Research Culture Collection, Westmead Centre for Oral Health, Westmead, NSW, Australia), *Actinomyces israelii* ATCC 12102, *Prevotella melaninogenica* ATCC 25845 and *F. nucleatum* ATCC 25586 (American Type Culture Collection, Rockville, MD, USA) were used as controls. Following this identification, plates from each of the clinical samples were examined and the numbers of each main colony

type recorded. The presence of *Streptococcus salivarius* colonies was used as an index of salivary contamination of the samples.

4.2.3. Statistical analyses

Non-parametric methods of statistical analysis were applied as preliminary scrutiny indicated that the data were markedly skewed. Basic descriptive statistics were applied to the microbial counts from the culture data including mean, standard error, median, minimum and maximum counts.

4.3 Results

The number of colony forming units per mg of carious dentine showed considerable diversity and inter-subject variability of the microflora (Table 4.1). Hundred fold differences in total microbial loads were apparent between samples and selective counts varied between teeth by up to three orders of magnitude. Data for selective microbial groups were noticeably skewed, with the mean values greater than the median values in each case. Anaerobic microorganisms were isolated from all samples and the numbers of colonies cultivated anaerobically, on non-selective plates, were approximately six times greater than those grown under microaerophilic conditions. Culture data showed a predominance of Gram-positive microorganisms, with *Lactobacillus* spp. cultivated in greatest numbers on selective media. Gram-negative organisms were also present in significant numbers with *Prevotella* spp. being the largest anaerobic group. These data showed 97% of teeth positive for *F. nucleatum* and *Streptococcus* spp. (63/65), 95% for *Lactobacillus* spp. (62/65), 91% for *Prevotella* and *Actinomyces* spp. (59/65) and 88% for *P. melaninogenica* (57/65).

On the basis of colony form, five main types were identified on CFAT medium. Biochemically, these resembled *A. israelii, Actinomyces odontolyticus, A. naeslundii, Bifidobacterium* and *Propionibacterium* species. Seven main colony types were evident on the KVA medium which were identified biochemically as *P. melaninogenica, P. intermedia, Prevotella buccae, Prevotella oris, Prevotella corporis, Capnocytophaga* spp. and *Lactobacillus* species. Of these, the black pigmented prevotellae were isolated in the greatest numbers. Three colony types were

evident using CVE media and biochemically they were identified as *F. nucleatum*. *Leptotrichia buccalis* was also noted.

Streptococci were studied on Mitis Salivarius agar. Four main colony types were distinguishable and biochemically these resembled *S. mutans*, *S. salivarius*, *Streptococcus anginosus* and *S. sanguinis*. *Lactococcus*, *Leuconostoc* and *Enterococcus* species were also present. Lactobacilli were grown on Rogosa agar and nine main colony forms were identifiable. These resembled *L. acidophilus*, *Lactobacillus paracasei*, *Lactobacillus rhamnosus*, *Lactobacillus fermentum* and *Lactobacillus plantorum* when tested biochemically. *Lactobacillus acidophilus* presented in five different colony configurations and was numerically dominant.

The use of biochemical tests to identify microorganisms from dentine caries provided variable results and indicated that microbes of different appearance could be identified as the same organism. One example is described in more detail; the appearance of *P. melaninogenica* varied from a 1mm circular mid-brown, shiny, pulvinate colony with a smooth translucent edge to a 2.5mm tan to dark brown umbonate colony with a ruffled edge. The different colony morphologies were identified by a range of codes using the RapID ANA II System (Table 4.2).

Table 4.1. Bacteria detected in carious dentine by colony counting.

Bacterium	CFU (mg dentine)$^{-1}$ [a]		
	Range	Median	Mean ± SEM
Anaerobic	$1.4 \times 10^5 – 3.7 \times 10^7$	55×10^5	$73 \pm 9.0 \times 10^5$
Microaerophilic	$2.5 \times 10^4 – 4.7 \times 10^6$	8.4×10^5	$12 \pm 1.0 \times 10^5$
Prevotella[b]	$0.0 – 5.1 \times 10^6$	1.1×10^5	$4.7 \pm 1.3 \times 10^5$
P. melaninogenica[c]	$0.0 – 5.1 \times 10^6$	1.0×10^5	$4.3 \pm 1.3 \times 10^5$
F. nucleatum	$0.0 – 1.0 \times 10^6$	0.8×10^5	$1.8 \pm 0.3 \times 10^5$
Actinomycetes	$0.0 – 5.3 \times 10^6$	0.7×10^5	$2.3 \pm 0.9 \times 10^5$
Lactobacilli	$0.0 – 1.9 \times 10^7$	5.2×10^5	$10 \pm 3.0 \times 10^5$
Streptococci	$0.0 – 3.1 \times 10^6$	1.7×10^5	$3.7 \pm 0.7 \times 10^5$

[a] Data collected from 65 samples.
[b] All *Prevotella* colonies grown on KVA
[c] Colonies identified biochemically as *P. melaninogenica*

Table 4.2. Different colony morphologies and identification codes for *P. melaninogenica* using the RapID ANA II System*.

Colony morphology	**ANA identification code**
Small, mid to dark brown, smooth or rough edge, pulvinate or umbonate	027340
Mid-brown, smooth edge, convex	037340
Small, shiny brown, smooth translucent edge, pulvinate	427340
Light to mid-brown, smooth, translucent edge, pulvinate or umbonate	437300
Mid to dark brown, ruffled or clean edge, wet surface, brown or tan coloured, umbonate	437340

*While the identification codes supported classification of the colonies as *Prevotella*, it should be noted that colony morphology was highly variable.

4.4 Discussion

The results of the present study are in agreement with others in finding a large variation in microbial counts between samples. There was also agreement that anaerobes comprised the majority of the organisms cultured and that the largest group of isolates were the facultative Gram-positive rods, although *Lactobacillus* spp. were isolated in lower frequency than in previous studies (Loesche and Syed, 1973; Hoshino, 1985; Hahn *et al.*, 1991; Massey, *et al.*, 1993) which may reflect the selection of carious, symptomatic teeth used in this study.

Compared with the data reported by Massey *et al.* (1993), CFU counts from this study indicated approximately a one hundred-fold reduction in the mean bacterial counts from non-specific and selective media. However, these values were within the range reported in previous studies (Loesche and Syed, 1973; Hoshino, 1985; Hahn, *et al.*, 1991) and may represent differences in the efficiency of liberating microorganisms from the carious dentine during homogenisation of the samples. There was also a considerable reduction in the peak incidence of some species noted in this study, with values of 14% for *Actinomyces* spp., 46% for *Lactobacillus* spp. and 18% for *Streptococcus* spp., which contrasted with the data from Massey *et al.* (1993) of 31%, 71% and 61% respectively. Low recovery of streptococci, particularly *S. mutans*, from carious lesions has been reported previously (Edwardsson, 1974; Hoshino, 1985; Hahn, *et al.*, 1991), with none recovered from pulpal and deep caries sites (Hahn, *et al.*, 1991). However, recovery of *S. mutans* may have been enhanced using a Mitis-Salivarius agar containing kanamycin or bacitracin (Kimmel and Tinanoff, 1991). Takazoe *et al.* (1973) showed minimal invasion of decalcified dentine by *S. mutans in vitro* unless proteinase was added, indicating the potential for invasion only after the organic dentine matrix had been partially decomposed. In addition, only those strains of *S. mutans* expressing adhesins specific for type I collagen were found to be capable of invading dentine tubules (Love *et al.*, 1997).

In agreement with Massey *et al.* (1993), Gram-negative bacteria comprised a significant percentage of the total cultivable flora in this study where, in some specimens, *Prevotella* spp. comprised at peak incidence up to 21% and *F. nucleatum* up to 12%. However, other authors noted few or no Gram-negative rods (Loesche and Syed, 1973; Edwardsson, 1974; Hoshino,

1985; Hahn, *et al.*, 1991) or did not investigate for anaerobic microorganisms (McKay, 1976). This may relate to a lack of awareness of anaerobes or to difficulties involving their islolation, especially in early studies where samples were exposed to air (Loesche and Syed, 1973) or processed without an adequate anaerobic environment (Edwardsson, 1974). Similar concerns have also been reported in the area of wound infection where studies during the last two decades have involved minimal or no isolation of anaerobes because of a tendency to regard aerobic or facultative microorganisms as the primary pathogens (Bowler *et al.*, 2001).

In a study of carious dentine from 29 human teeth with irreversible pulpitis, Hahn *et al.* (1991) found two main types of carious lesions. The first with high numbers of lactobacilli and low counts of streptococci and bacteroides (*Prevotella* and *Porphyromonas* spp.), the second with low lactobacilli numbers, numerous black-pigmented bacteroides, increased numbers of streptococci and non-lactobacilli Gram-positive rods including actinomycetes, propionibacteria, bifidobacteria, arachnia and eubacteria. Although this *Lactobacillus* spp. microbial pattern was not seen in the present study, examples of non-lactobacilli Gram-positive rods were recovered from the carious dentine samples and have been previously isolated from carious dentine (Massey, *et al.*, 1993), necrotic dental pulps (Lana *et al.*, 2001) and infected dentine of root canals (Ando and Hoshino, 1990).

Studies have demonstrated the development of bacterial succession in periodontal microflora (ter Steeg and van der Hoeven, 1989) and root canal microflora in rat periapical lesions (Tani-Ishii *et al.*, 1994). Both studies showed a transition from rapidly growing, microaerophilic, saccharolytic bacteria to an increasingly diverse group of isolates dominated by a Gram-negative, anaerobic microflora. It has been postulated that this transition in carious dentine may be associated with the development of pulpal necrosis (Hahn, *et al.*, 1991). This bacterial succession, presumably, results from changes in bacterial relationships due to alterations in the environment and nutritional interactions. Early bacterial coaggregation in dental plaque has been demonstrated between *Actinomyces* spp. and *Streptococcus* spp. (Crowley *et al.*, 1987) and is followed by late colonisers that include fusobacteria, eubacteria, prevotellae and porphyromonads (Kolenbrander and London, 1993). The decrease in

facultative organisms with time may be due to a restriction of available nutrients with a lowering in the oxidation-reduction potential of the surrounding environment which then favours the growth of anaerobes. In addition, anaerobes metabolise amino acids and proteins from tissue fluids, produce a number of factors which kill other microbial species (Socransky and Haffajee, 1991), assist colonisation and resist phagocytosis (Slots and Genco, 1984; Sundqvist, 1993), which enhances their dominance in this environment.

Prevalence of the Gram-positive microaerophilic bacteria following culture of the carious dentine may be a result of their high numbers or due to the relative ease with which these microbes can be cultivated compared with Gram-negative anaerobes. It is well recognised that obligately anaerobic bacteria are difficult to culture and the more fastidious varieties may be suppressed by the rapid growth of more robust species creating an inherent bias. During cultivation of the carious samples a number of inconsistencies were noted: in some samples plentiful growth of black-pigmented microbes on non-specific plates was not reproduced on the specific KVA plates, although it should be noted that the *Porphyromonas* species could have been inhibited by the presence of kanamycin; in other samples shown by Gram stain to contain a large number of Gram-negative rods or cocci, the number of Gram-negative anaerobes cultured was low; and variation was noted in the colony morphology and biochemical identification codes of a number of bacteria including *P. melaninogenica.* To clarify these anomalies it became apparent that additional, molecular, methods of bacterial identification were required. In the following chapter the application of PCR technology to microbial identification and enumeration is reviewed and experiments investigating the preparation of DNA for real-time PCR are described.

CHAPTER 5

DETECTION OF BACTERIAL DNA BY REAL-TIME POLYMERASE CHAIN REACTION

PCR is a rapid, sensitive and specific method for the detection of bacteria and is increasingly applied to the diagnosis of infectious diseases (White *et al.*, 1992; Tang *et al.*, 1997). It is an improvement on previous methods of bacterial detection and identification including cultivation, immunological and hybridization methods. As reported by Fredricks and Relman (1999), rather than growing an intact microbe one can now amplify a segment of its DNA, replacing culture media with the PCR reaction mix and an incubator with the thermal cycler. A recent advancement in this technology analyses the PCR product formed in real-time rather than the usual end point analysis where PCR components become limited and quantification inaccurate. One example of this technology is the Applied Biosystem ABI-PRISM 7700 (TaqMan) Sequence Detection System (Foster City, CA, USA) which uses the 5′ nuclease assay for real-time detection and quantification of PCR products with a dual-labeled fluorogenic probe (Heid *et al.*, 1996).

This chapter begins with a review of PCR technology and its application to bacterial identification and quantitation. Following this is the experimental section which is presented in two parts; the first involves development and testing of the universal amplicon, designed to detect the total bacterial load in a sample. Once achieved, this provided the basis for the second part which examined the issues of extracting DNA from microorganisms while providing protection against the destructive activities of nucleases.

5.1 Review

The commensal flora of the human body has the potential to cause disease when the host defences are impaired. However, in many instances of infection the aetiological agent cannot be identified. It is entirely possible that knowledge of the diversity of human microflora is as inadequate as that in other environments, where less than 0.4% of all bacterial species have

been identified. It, therefore, follows that an inability to cultivate many of the commensal flora may explain the failure in diagnosing and adequately treating related diseases (Relman, 1998).

Results of studies have frequently reported that direct microscopic counts from different environments exceed viable-cell counts by several orders of magnitude. It would appear that the majority of cells seen microscopically are viable but do not form visible colonies on agar. This could be due to the presence of known species unable to be grown under the conditions provided, because they had entered a nonculturable state or unknown species that have not been previously cultivated because of a lack of suitable conditions (Amann *et al.*, 1995). A culture-independent approach for the detection and identification of microbial pathogens is required to overcome these problems and has been achieved by the introduction of molecular techniques using gene sequences to characterise bacteria. This breakthrough was achieved by Carl Woese (1977, 1987) who by comparing ribosomal DNA (rDNA) sequences established a molecular sequence–based phylogentic tree that could be used to relate all organisms. The construction of a phylogenetic tree involves aligning pairs of rDNA sequences from different organisms and noting the number of nucleotide differences; these differences are determined to be a measure of the "evolutionary distance" between the organisms. The tree can be considered an approximate map of the evolution of the cell lines that constitute modern microorganisms. As molecular identification of organisms is based on gene sequences there is no requirement to capture and grow a functioning cell and therefore unculturable organisms can theoretically be identified and related to known organisms through the phylogenetic tree. As a result of this phylogenetic proximity, predictions regarding the behaviour and properties of the unknown organism can be made with reasonable confidence (Pace, 1997).

Certain features of a genetic sequence are required for it to be useful in identifying uncharacterised microorganisms. Ideally, the sequence must be conserved among a large group of organisms, its rate of change should be constant over long periods, the sequence should not be shared by different organisms through horizontal transmission and, finally, the sequence should be amenable to broad-range detection. The genetic sequences that are most useful as molecular chronometers are those that encode molecules with a highly conserved and essential

biological function. The sequence of the small subunit RNA molecule of the ribosome (16S rRNA) meets these requirements. This molecule has a length of approximately 1,500 nucleotides and folds in a precise way to form ribosomes; highly conserved structures found in all living cells with the function of protein synthesis (Relman, 1993; Relman, 1998). Large databases now exist containing the rDNA sequences for a multitude of organisms.

5.1.1 Development of the polymerase chain reaction

Advances in molecular biology have made it possible to study microbial communities by amplifying gene sequences from different environments followed by purification and sequencing. Initially cloning was used to produce multiple copies of DNA sequences of interest; however, this is a time consuming process and has more recently been replaced by polymerase chain reaction (PCR) technology. This technology, devised by Kary Mullis in 1983 and first published by Saiki *et al.* (1985) described a quick and efficient method for producing many copies of specific regions of DNA; a process that has revolutionised molecular genetics.

PCR exploits certain features of DNA replication. DNA polymerase uses single-stranded DNA as a template for the synthesis of a complementary new strand. The synthesis is directed from a starting point specified by a pair of closely spaced, chemically synthesised oligonucleotide primers that anneal to the template at certain points. The technique of PCR involves the process of repeated cycles to amplify the selected sequence. Each cycle consists of three steps: a DNA denaturation step, in which the double strands of DNA are separated; a primer annealing step, where the primers anneal to their target sequences and an extension step where the DNA polymerase extends the sequences between the two primers. At the completion of each cycle, the quantity of PCR product is theoretically doubled. Therefore, the mechanism of PCR is a primer-directed DNA synthesis, with the advantage that the process is repetitive and the number of copies produced increases exponentially, so that within a few hours (30 to 40 cycles) the original target DNA has been amplified a million-fold or more.

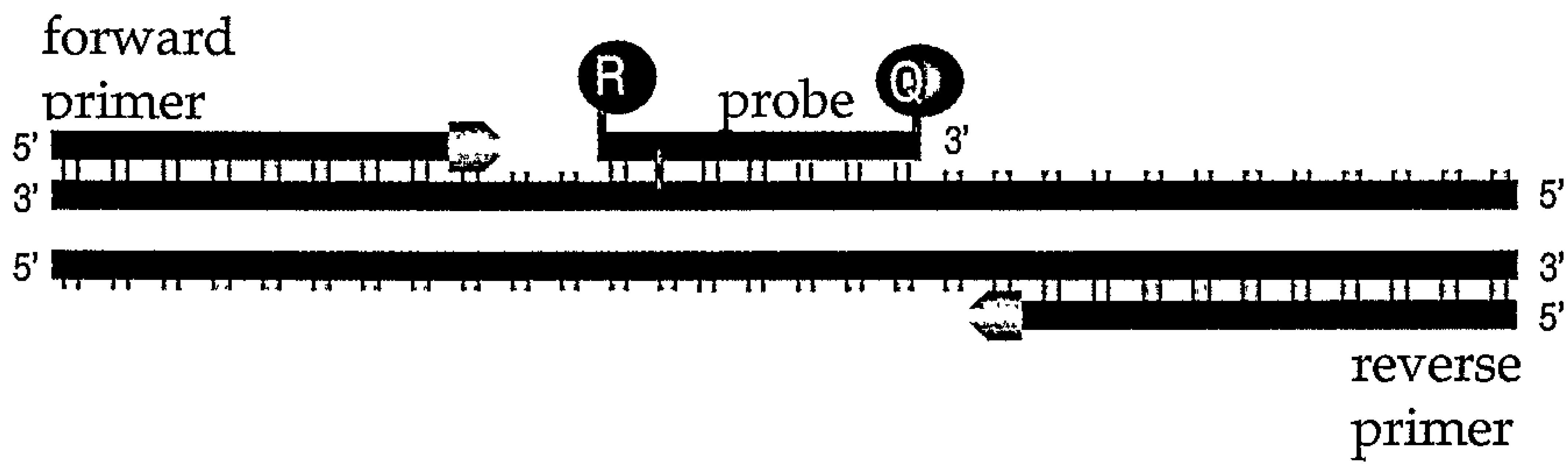
Originally, the Klenow fragment of *E. coli* DNA polymerase I was used to extend the annealed primers, however, this enzyme is heat-sensitive and becomes inactivated at temperatures required to separate double-stranded DNA, thus requiring the addition of fresh

enzyme for each cycle. With the discovery of thermostable DNA polymerases, for example *Taq* (*Thermus aquaticus*) polymerase, the enzyme can be added at the beginning and remains active throughout a complete set of amplification reactions. In addition, these enzymes are active at higher temperatures, thereby increasing the specificity and rate of DNA synthesis. Subsequent automation using dedicated thermal cyclers plus the simplicity of PCR has resulted in its widespread application through many scientific disciplines (O'Leary *et al.*, 1997).

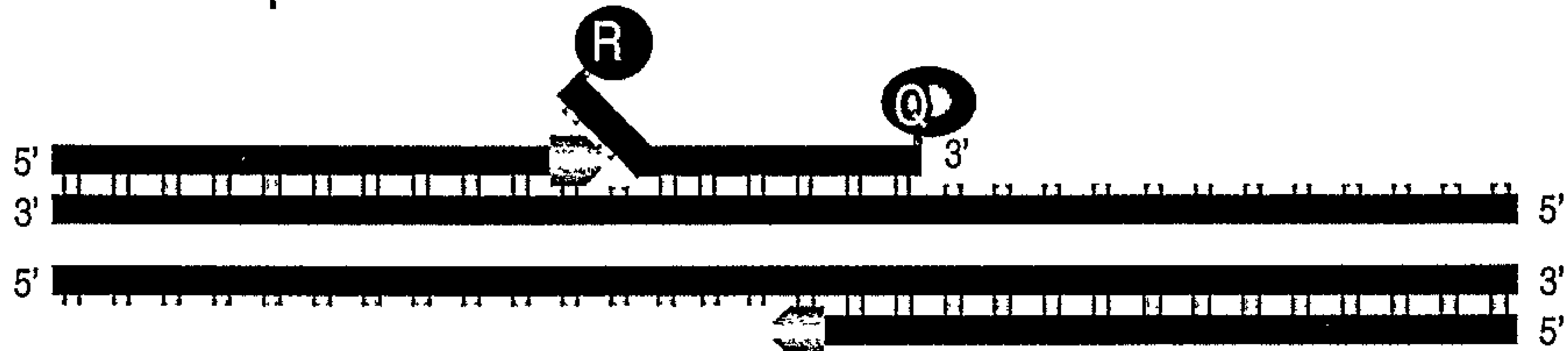
Although a powerful tool, PCR has some disadvantages: the process is labour intensive, post-PCR manipulation of the sample provides the potential for contamination of the amplified products, the quality of the PCR product at the end of the reaction could be affected by depletion of reagents and direct quantitation of the original sample is not possible. Holland *et al.* (1991) devised a method to overcome many of these problems whereby product detection occurs concurrently with target amplification and little or no handling of the post-PCR sample is required. This was achieved by introducing a labelled oligonucleotide probe designed to hybridise with the target DNA. During amplification the 5′→3′ exonuclease activity of *Taq* polymerase degrades the probe only when it has been hybridised to the specific target. The amount of probe degradation is therefore proportional to the amount of signal generated, which is proportional to the amount of original sample DNA.

Continued improvements in probe design made use of a probe labelled with two dyes, a fluorescent reporter dye and a fluorescent quencher dye. This TaqMan probe hybridises to an internal region within the amplicon between the primers (Figure 5.1). The reporter dye (6-carboxyfluorescein [FAM], tetrachloro-6-carboxyfluorescein [TET], or 2′,7′-dimethoxy-4′,5′-dichloro-6-carboxyfluorescein [JOE]) is covalently linked to the 5′ end and the quencher dye (6-carboxy-tetramethylrhodamine [TAMRA]) is attached at the 3′ end. The location of the quencher dye in close proximity to the reporter dye, when the probe is intact, dampens emission of the reporter dye. When hydrolysis of the probe occurs during amplification and the *Taq* polymerase cleaves the probe from its target sequence, separation of the quencher and reporter dyes allows emission of a fluorescence signal. This emission is quantitative for the initial amount of sample DNA (Bassler *et al.*, 1995; Bassam *et al.*, 1996).

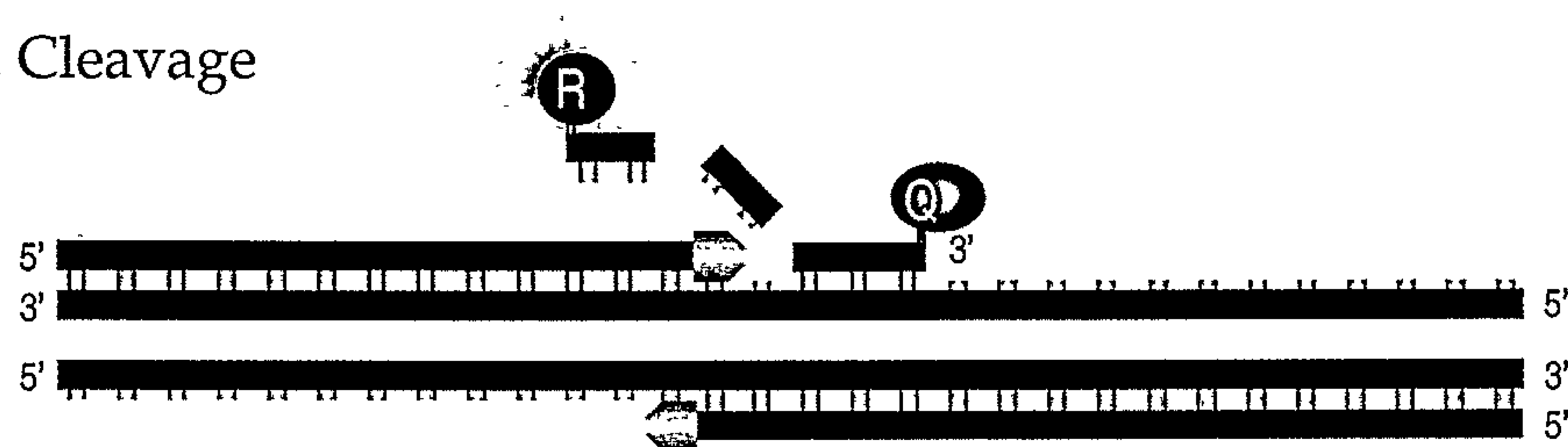
1. Polymerisation



2. Strand displacement



3. Cleavage



4. Polymerisation completed

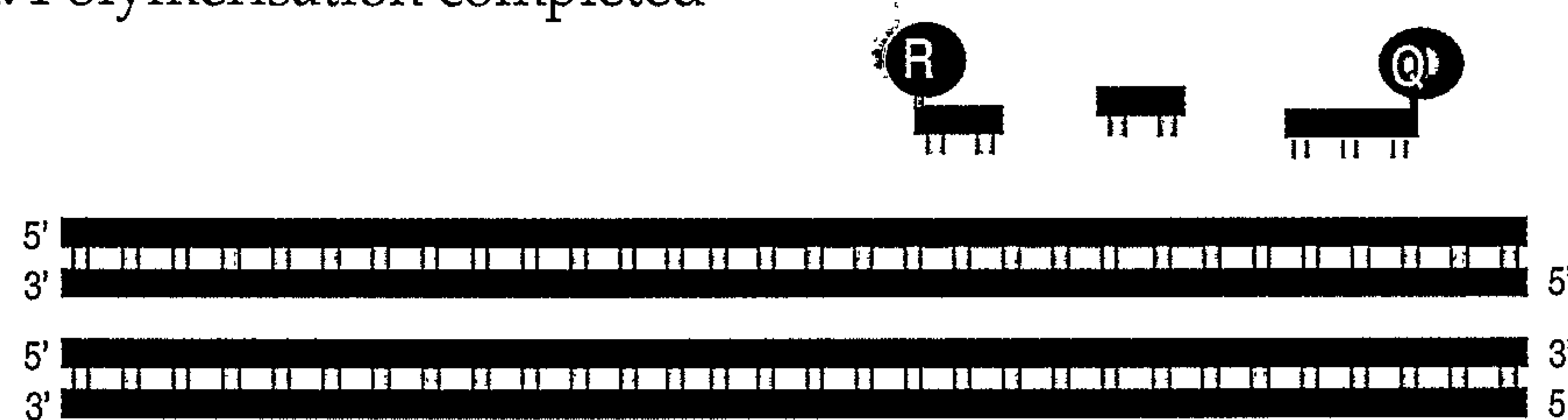


Figure 5.1. Diagram representing stages in the fluorogenic 5′ nuclease chemistry. Prior to polymerisation, the primers are annealed to the target DNA and the two fluorescent dyes (R-reporter and Q-quencher) are attached to the probe with the reporter dye emission quenched. During strand displacement the DNA polymerase extends the sequences between the primers with initial displacement of the probe. The extension cycle results in cleavage of the probe with separation of the reporter and quencher dyes resulting in emission of fluorescence from the reporter dye. Polymerisation is complete when new strands of DNA have formed. Diagram provided by Applied Biosystems, Foster City, CA, USA.

Further improvements in instrumentation now allow "real-time" monitoring of the fluorogenic signal as it is generated during amplification. The system integrates a thermal cycler, a laser light source, a fibre-optic network, a cooled CCD camera detector and a computer with dedicated software. Upon collection of the fluorescence signals, the software computes the contribution of each of the dyes to the spectrum and normalises the reporter dye against the standard ROX (6-carboxy-X-rhodamine) passive internal reference dye. Real-time monitoring of the reaction can define the point at which amplification of the PCR product is first detectable. This takes place when fluorescence generated by cleavage of the probe passes a fixed threshold above the baseline. It occurs during the exponential phase of product accumulation, early in the PCR when reagent availability cannot limit the reaction. The higher the original amount of DNA in the sample the fewer the amplification cycles required before a significant increase in fluorescence is recorded. Therefore, measurement of the initial amount of sample DNA is determined from the cycle at which it crosses the baseline (C_T) compared with a standard curve created from known amounts of target DNA (Bassam, *et al.*, 1996; Heid, *et al.*, 1996).

For optimal hybridisation reactions, further development has evolved the use of a "hot start" strategy for PCR. This prevents non-specific primer annealing and extension that might occur when the reaction components are mixed at room temperature. To achieve this, modified forms of the *Taq* polymerase enzyme have been created that are activated only after exposure to an elevated temperature, thus preventing the polymerase from functioning until thermal cycling begins (Birch *et al.*, 1996).

Therefore, current technology using real-time PCR allows amplification of a specific segment of rDNA from a background of mixed DNA in a closed-tube system which requires no post-PCR processing, thereby minimising contamination problems and decreasing processing times. Analysis is performed in the log phase of product accumulation and is consequently not limited by reagent depletion, is claimed to be highly reproducible and allows for a large dynamic assay range.

5.1.2 Detection of oral pathogens using PCR

As previously discussed, conventional diagnostic procedures are based on the ability to culture microorganisms or identify their presence using antibodies. The culture approach is limited as many microbes defy cultivation by standard methods, and of those that can be cultured, many are slow growing with fastidious growth requirements, while antibody testing is relatively insensitive. PCR now provides an effective alternative. Numerous investigators have reported more frequent detection of periodontal pathogens such as *P. gingivalis, P. intermedia, Bacteroides forsythus, Treponema denticola, H. actinomycetemcomitans* (Loesche *et al.,* 1992; Ashimoto *et al.,* 1996; van Steenbergen *et al.,* 1996; Mättö *et al.,* 1998) and endodontic pathogens, for example, *P. endodontalis* (Dymock *et al.,* 1996) from clinical samples using PCR compared with traditional culture techniques. Other studies have identified unknown species (Harper-Owen *et al.,* 1999) as well as unexpected species in oral environments for example, *T. denticola, P. gingivalis, Prevotella tannerae* and *B. forsythus* in endodontic infections (Xia *et al.,* 1999; Siqueira *et al.,* 2000; Siqueira *et al.,* 2001). Within the area of microbiology, PCR has permitted the detection of microorganisms from environments which harbour primarily slow growing obligate anaerobes with fastidious growth requirements and that are often difficult or impossible to culture. Other advantages include the ability to correctly identify bacteria otherwise not adequately identified by screening with phenotypic tests or commercial identification kits (Baumgartner *et al.,* 1999; Haraldsson & Hobrook, 1999) and the opportunity to quantify bacterial load, or particular species, within a mixed environment (Suzuki *et al.,* 2000). In addition, the ability to quantify load allows monitoring of the effectiveness of treatment regimes during therapy (Takamatsu *et al.,* 1999).

5.1.3 Limitations of PCR technology

Despite the obvious advantages already discussed, there are limitations that can interfere with accurate and efficient performance of the PCR, including the possibility of producing misleading or inaccurate data.

5.1.3.1 Design of primers and probes

Primers and probes for PCR are designed from sequences of microorganisms found in databases to target discrete regions of the rDNA gene. The rDNAs of different organisms vary in areas of sequence conservation so that by targeting areas of greater or lesser conservation, primers and probes can be designed to identify individual or groups of microorganisms. It is assumed that the rDNA database sequence entries from which primers and probes are designed are accurate. Unfortunately, there are reports of errors in many of the sequences. Two common problems exist; the first involves contamination by the inclusion of DNA from an unrelated organism following cloning and the second includes entries that contain errors or missing segments (Pennisi, 1999). Another confounding factor, which may compromise the accuracy of primer and probe design, is the presence of many relatively short sequences. Some published sequences are only 500bp long, which represents only one third of the entire 16S rDNA sequence. This may result in insufficient comparative information to allow correct placement in the phylogenetic tree (Hugenholtz *et al.*, 1998).

Certain guidelines must be considered when designing primers and probes to optimise efficient performance for real-time PCR. To assist probe hybridisation, the melting temperature (T_m) of the probe should be between 68-70°C, and 10°C higher than that for the primers (58-60°C). The probe length should ideally be 20-25 nucleotides in length; probes longer than 30 nucleotides have reduced synthesis efficiency and form structures that are likely to interfere with probe hybridisation and cleavage, and DNA amplification. Other design factors, specific for the Applied Biosystems 7700, include a GC content between 30-80%, no runs of greater than three consecutive Gs in either the probe or the primers, the 5′ end of the probe should not finish with a G, the last 5 nucleotides at the 3′ end of the primers should contain no more than 2 GCs, the probe should be selected from the strand that contains more Cs than Gs and the amplicon length should be between 50-150 base pairs (Bassam, *et al.*, 1996). Other important design considerations include the elimination of secondary structures, duplexes and primer-dimers that might result in the formation of non-specific amplification products. Careful adjustment of temperature, magnesium ion concentration and *Taq* polymerase concentration

within the reaction can eliminate the formation of non-specific PCR products. Software packages are available that provide guidelines and information regarding the design of primers and probes and the avoidance of spurious secondary structures.

Once designed, probe and primer sequences must be checked against database entries to ensure sequence specificity for individual microorganisms and to prevent cross-reactivity with closely related species. Following synthesis of the components and prior to testing with clinical samples, the primers and probe must first be optimised in order to select those concentrations that will provide the most efficient amplification. The primers and probe are then tested against DNA from known microorganisms to verify the specificity of the amplicon.

5.1.3.2 The polymerase chain reaction

The greatest problem affecting PCR usage is that of false positive results which occurs when the majority of molecules detected are from exogenous sources rather than from the test sample. Transfer of even minute quantities of material from a previous PCR, which could contain millions of amplicon copies, is capable of contaminating reagents, buffers, glassware and ventilation systems. To avoid amplicon carry-over, PCR laboratories are required to take special precautions including the use of disposable materials, pre-aliquoted reagents, positive displacement pipettes, premixing of all reagents, avoiding the creation of aerosols and adding DNA as the last step prior to dispensing into the reaction wells. In addition, preparation of reaction mixes should be carried out in a biohazard hood located in a separate area from that of sample preparation and PCR product analysis (Kwok & Higuchi, 1989).

Other techniques to avoid contamination include the use of real-time PCR, where amplification and analysis occurs in a sealed tube, and an amplicon sterilisation step within each PCR run. Amplicon sterilisation involves the substitution of dUTP for TTP in the amplification reaction mixture, resulting in the incorporation of U instead of T in the amplicon and providing a method of discriminating between the original target sequence and the PCR amplicon. The bacterial enzyme uracil-*N*-glycosylase (UNG) is added to the reaction mix and during a brief incubation step the UNG acts by cleaving all uracil residues, thereby

enzymatically degrading any amplicons from previous reactions. Heating the reaction mixture to 94°C then inactivates the UNG (Persing, 1991).

Another source of contamination when using a broad range or universal amplicon to detect total bacterial load comes from bacterial contamination of the *Taq* DNA polymerase. This occurs during manufacture of the polymerase enzyme which is commonly expressed as a recombinant protein in *Escherichia coli* followed by incomplete purification. In the same manner, use of the UNG enzyme will provide additional contamination (Böttger, 1990; Schmidt *et al.*, 1991; Corless *et al.*, 2000).

The use of controls in each PCR experiment is essential to monitor the efficiency of the reaction. False positive reactions are one of the greatest problems with PCR because of the sensitivity of the enzymatic amplification. False positives occur because the PCR amplifies contaminating DNA that may have been present in minute amounts. To detect false positive reactions the PCR should include a negative control or no-template-control. This control consists of all reagents except the DNA sample and will detect contamination in the PCR reagents such as buffers, water and *Taq* polymerase. It may also be necessary to include a no-enzyme-control to check for fluorescence contamination of the sample.

DNA extracted from clinical specimens may contain PCR inhibitors such as potassium ions, blood, urine, vitreous humor, sputum and saliva (Mättö, *et al.*, 1998; Fredricks & Relman, 1999). These inhibitors must be diluted, removed or inactivated to allow DNA amplification to proceed. Fortunately, DNA purification protocols remove most PCR inhibitors. However, unless the inhibitory components are identified and removed, a lack of amplification recorded during the reaction would not necessarily be due to an absence of target DNA, thus creating a false negative result. This can be overcome by monitoring the amplification of a second target nucleic acid during the reaction which serves as an internal control. A limited number of internal control molecules are added to the test sample and co-amplified with the target DNA, therefore, obtaining a positive signal from the internal control indicates successful amplification and can be used to monitor amplification and detection (Rosenstraus *et al.*, 1998). Other reasons for false negative reactions include failure of the DNA extraction procedure to release

DNA, loss of DNA during the purification process, or due to inadequate initial sample volume (Fredricks and Relman, 1999).

Another source of error with PCR is the formation of chimeric gene products. These may occur when genes from mixed bacterial populations are amplified and chimeric products form during the primer extension phase when some templates are incompletely copied. Therefore, during ensuing cycles the partial copies re-anneal to be further extended onto a second template, thus creating chimeric genes of regions copied from different templates. Fortunately, the formation of chimeric products is regarded as a rare event and most likely to occur as a result of low dNTP substrate concentrations in late cycles (Giovannoni, 1991).

5.1.3.3 Preparation of samples for PCR

Sample collection and processing can have a significant impact on the outcome of the PCR assay. Many different types of clinical samples can be used for DNA amplification including tissue, blood, semen, hair, pleural fluid, saliva and archival material, fixed and embedded in paraffin or resin. However, fixation of materials in formaldehyde damages the DNA, often resulting in a smaller sized product available for amplification. Other tissues and fluids require refrigeration and rapid processing or freezing to preserve the DNA and prevent deterioration.

Since quantification of the number of bacteria is entirely dependent on the amount of DNA measured, the extraction and purification protocol becomes a critical step in the procedure. Successful extraction of bacterial DNA from clinical samples often requires the concentration of small amounts of DNA, the inactivation of nucleases and purification to eliminate proteins. Ideally, the method chosen should be sensitive, simple, rapid and reproducible, requiring no specialised equipment or biochemical knowledge. Following extraction, the DNA should be sufficiently pure to allow enzymatic modifications and risk to personnel from any pathogens should be negligible (Boom *et al.*, 1990). Traditionally, the extraction and purification of bacterial DNA has been performed with large numbers of cells using standard procedures to lyse cells with phenol-chloroform followed by extraction with organic solvents to remove residual proteins and cell wall components, followed by precipitation of nucleic acids in ethanol to remove traces of the solvents. However, such techniques incur considerable loss of

DNA and are not applicable to small scale DNA purification. There are reports of various protocols for digesting and purifying clinical samples; some use mechanical, chemical or enzymatic means to break open the bacterial cell walls. Mechanical methods of sample preparation include freeze-thawing, boiling, sonication with or without glass beads, and crushing or homogenising. Other methods use enzymes such as lysozyme, mutanolysin, and proteinase K, chaotropes which include guanidine isothiocyanate, guanidine hydrochloride or hexadecyltrimethyl ammonium bromide (CTAB). In addition detergents, such as sodium dodecyl sulphate (SDS), can be used to disrupt and lyse the bacterial cell wall, digest proteins and inactivate nucleases. Some of these techniques work efficiently for particular microbes, however, there is no single method which optimally digests all bacteria (Fredricks and Relman, 1999).

Numerous commercial kits are also available for the extraction and purification of small quantities of DNA for use with PCR, although published data comparing these kits provides conflicting results. For example, the QIAamp Blood Kit (Qiagen, Clifton Hill, Victoria, Australia) was found by Dixon *et al.* (1998) to be optimal for extracting DNA from serum when comparing 13 different kits, whereas de Kok *et al.* (1998) described the same kit as having very poor isolation efficiency when only a small amount of the target DNA was present.

Correct handling and storage of samples for PCR can impact on the accuracy of bacterial quantitation. Wahlfors *et al.* (1995) found that one additional freeze/thaw cycle of DNA samples weakened the PCR signal obtained. They cautioned that the composition of the sample could be distorted by careless handling, additional freeze/thaw cycles and prolonged storage at inappropriate temperatures, and strongly recommended that analyses be carried out immediately after sample collection. Another study demonstrated that samples stored at 4°C after boiling showed degradation of the DNA (Nakajima *et al.*, 1994). These reports indicate a problem with degradation of sample DNA. Many of the microorganisms found in carious dentine are capable of deoxyribonuclease (DNase) activity. These enzymes have the ability to initially hydrolyse chromosomal DNA into small fractions, followed by total degradation. Although the functions of DNases are unknown, there is speculation regarding roles in

pathogenicity through the facilitation of tissue invasion, provision of nucleic acid precursors for bacterial growth and protection of bacterial integrity from foreign DNA (Rudek & Haque, 1976; Minion *et al.*, 1993; Azcárate Peril *et al.*, 2000). Other authors suggest roles in DNA recombination, replication and repair systems (Yanagida *et al.*, 1982). Nucleases have been found in a number of anaerobic species including fusobacteria, peptostreptococci, propionibacteria, veillonellae, prevotellae (Porschen & Sonntag, 1974) and porphyromonads (Mayrand *et al.*, 1984; Leduc *et al.*, 1995). DNase activity has also been detected in microaerophilic bacteria including lactobacilli (Miller *et al.*, 1971) and oral streptococci (Smith & Bodily, 1967). For most of these microorganisms the DNase is extracellular and does not require lysis of the cell for nuclease activation, however, *Lactobacillus plantarum* produces two nucleases, with one product found in the cytoplasm and membrane fractions of the bacterium (Caso & Suárez, 1997).

Nuclease activity from some bacteria requires the presence of magnesium and/or calcium and incubation at a temperature greater than 16°C. Nuclease activity can usually be controlled by storing bacteria at low temperatures (0-4°C) or exposure to high temperatures (>70°C for 15 minutes). Exceptions are the heat stable DNases of *Yersinia enterocolitica* where boiling is ineffective (Nakajima, *et al.*, 1994). Nuclease activity has also been reported to be inhibited in the presence of actin (Lazarides & Lindberg, 1974), $ZnCl_2$, ethylinediamine tetra-acetic acid (EDTA) (Leduc, *et al.*, 1995), ethylene glycol-bis (β-aminoethyl ether)-*N, N, N', N'*,-tetra-acetic acid (EGTA) and sodium dodecyl sulphate (SDS) (Bendjennat *et al.*, 1997).

5.1.3.4 Calculation of bacterial numbers following PCR

Quantitation of target DNA is generally accomplished by measuring the intensity of ethidium bromide staining of PCR products in an agarose or polyacrylamide gel which is compared with the band intensity from a known amount of identical bacteria. This procedure has been described as time-consuming and inaccurate (Gibson *et al.*, 1996). The calculation of cell numbers from sample DNA following quantitative PCR uses a standard curve comprising serial dilutions of known amounts of bacterial DNA run in parallel with the sample DNA. The amount of DNA from a reaction can then be determined by interpolation from the standard

curve (Desjardin *et al.*, 1998). The known DNA representing the standard might be made up of the specific bacterial species under study (Lyons *et al.*, 2000), an internal control which uses identical primer binding regions with a unique probe site compared with the target DNA (Rosenstraus, *et al.*, 1998) or an homologous competitor which utilises two primers forming a shorter amplicon than the target (Rupf *et al.*, 1999).

Each of these methods allows for quantitation of the amount of target DNA, although accurate quantitation of bacterial cell numbers requires knowledge of the size and number of the genomes within a cell, in addition to the number of rDNA operons for individual microorganisms. The size of the genome from different bacterial species may range from 600-13,000kb, the number of genomes may also vary while the number of ribosomal operons can range from 1 to 14 and operon copies may be located on two different genomes (Farrelly *et al.*, 1995; Fogel *et al.*, 1999). This information is available for only a limited number of organisms and is unknown for most oral microbes, with the problem further complicated by studying a mixed microbial population, as found in dental caries. In addition, bi-directional replication can further increase the numbers of a given rDNA operon depending on the number of replication forks and the location of the rDNA operon relative to the origin of replication. The number of replication forks is directly related to the generation time (t_d) which in turn depends on the metabolic status of the bacteria at the time of sampling (Neidhardt *et al.*, 1990; Klappenbach *et al.*, 2000). Not knowing the exact number of copies of 16S rDNA operons in any given species at the time of sampling, therefore, represents the main limitation to the absolute determination of bacterial numbers by real-time PCR based on 16S rDNA. However, where a complex multi-species population might be sampled along with impurities, or where bacteria are internalised within a matrix, such as dentine, other methodologies, such as direct microscopic cell counts or fluorescence-based enumeration, are likely to be far less sensitive.

5.1.3.5 Cost

PCR is expensive. The cost of reagents, equipment and the requirement for separate laboratory space is substantial. Real-time PCR requires the use of fluorescent labelled probes and the optimisation of both probes and primers adds considerably to the overall cost.

However, PCR-based tests offer speed and sensitivity that may offset the costs of microbiological tests, treatment and hospitalisation. In addition, miniaturised reactions/arrays and the use of robotics could assist in cost reductions with time (Fredricks and Relman, 1999).

5.2 Experimental sample preparation for real-time PCR

Bacterial analysis of a clinical sample requires not only identification of the microbes present but a determination of the total bacterial load to provide a baseline reference and allow assessment of treatment efficacy in clinical situations. To facilitate this, a universal probe and primers set was designed and used in this study. However, the quantitation of bacteria using nucleic acid-based technology is entirely dependent on the specificity of the designed probes and primers and the amount of DNA measured, therefore the extraction and purification protocol becomes a crucial part of the procedure. Accurate quantification of bacteria also relies on the efficient isolation of DNA from a mixed bacterial population that reflects the true bacterial profile. This is especially important when a low amount of target DNA is present, a limited amount of the clinical sample is available and when PCR inhibitors could be present.

Another factor detrimental to the reliable quantitation of bacteria is the presence of bacterial nucleases. Many of the microbial species found in carious dentine are capable of DNase activity whereby the chromosomal DNA becomes hydrolysed into component nucleotides (Minion, *et al.*, 1993; Leduc, *et al.*, 1995). In this study an internal positive control was used during sample processing and PCR to indicate deterioration of the PCR signal through nuclease activity, although it would not detect DNA hydrolysis following storage.

In the initial stage of the present study a universal amplicon was developed to determine the total bacterial load from samples. In addition, a probe and primers set was designed to enumerate *P. gingivalis*. Following successful testing of these amplicons the second stage of research assessed a series of different DNA extraction procedures for their ability to release DNA from bacterial cells and overcome the presence of nucleases and possible PCR inhibitors. Measurement of DNA was assessed by gel electrophoresis and/or PCR primarily using the universal probe/primers set. The design, optimisation and testing of the universal amplicon represented a conjoint effort involving Dr M Nadkarni and the author, with completion of the

amplicon design by Dr M Nadkarni which is reflected in the authorship of the associated publication (Nadkarni *et al.*, 2002). The experimental data presented in this chapter also represents a convergence of research and where Dr M Nadkarni had the prime role this is indicated in the text. Additionally, where data or gels produced by Dr M Nadkarni have been incorporated in the results, this has been indicated.

5.2.1 Materials and methods

5.2.1.1 Bacterial strains and culture conditions

Escherichia coli strains JM109 (Yanisch-Perron *et al.*, 1985) NM522 (Gough & Murray, 1983) and XL 1 blue (Stratagene, La Jolla, CA, USA) were available from the Institute of Dental Research Culture Collection (Westmead Centre for Oral Health, Westmead, NSW, Australia). *Staphylococcus aureus* strains ATCC 12600, ATCC 9144, ATCC 12598, ATCC BM 10458 and ATCC BM 10143; *Staphylococcus epidermidis* strains ATCC 35983 and ATCC 14990; *Staphylococcus hemolyticus* ATCC 29970 and *S. hemolyticus-infiltrative keratitis isolate; Staphylococcus schleferi* ATCC 43808; *Pseudomonas aeruginosa* strains ATCC 19660, ATCC 15442, 6294 and 6206; *Pseudomonas fluorescens*-infiltrative keratitis isolate; *Pseudomonas putida*-lens saline isolate; *Pseudomonas stutzeri*-infiltrate isolate; *Pseudomonas alcaligens* laboratory-isolate; *Pseudomonas* species and *Serratia marcescens* ATCC 274 were kindly provided by Dr. Mark Willcox, Co-operative Research Centre for Eye Research and Technology, The University of New South Wales, Australia. All *Escherichia, Staphylococcus, Pseudomonas* and *Serratia* species were grown in Luria Burtani (LB) broth (Miller, 1972) at 37^0C in a shaking incubator. *Stretococcus mutans* LT11, *S. gordonii* ATCC 10558 and *S sanguinis* (formerly *S. sanguis*) ATCC 10556 (obtained from the American Type Culture Collection, Rockville, MD, USA) were grown at 37^0C in Brain Heart Infusion broth (Oxoid) under 95% N_2 and 5% CO_2 (v/v); *F. nucleatum* ATCC 25586, *Fusobacterium necrophorum* ATCC 25286, *A. israelii* ATCC 12102 and *A. naeslundii* ATCC 12104 were obtained from the American Type Culture Collection and grown at 37^0C in Brain Heart Infusion broth in an anaerobic chamber (85% N_2, 5% CO_2, 10% H_2 [v/v/v]). *Porphyromonas gingivalis* ATCC 33277, *P. melaninogenica* ATCC 25845, *Micromonas micros* (formerly *Peptostreptococcus micros*) ATCC 33270 and *Peptostreptococcus anaerobius* ATCC

27337 were obtained from the American Type Culture Collection and grown at 37^{0}C in an anaerobic chamber in CDC broth [1% (w/v) trypticase peptone and 1% (w/v) trypticase soy broth (Difco, Becton Dickinson, MD, USA), 1% (w/v) yeast extract (Oxoid), 5mg NaCl ml^{-1}, 400µg L-cysteine ml^{-1} (Sigma Chemical Co., St Louis, MO, USA) containing 5µg haemin ml^{-1} (Sigma), 2µg menadione ml^{-1} (Sigma) and 2% (v/v) horse serum (CSL Biosciences, North Ryde, NSW, Australia)}. *Porphyromonas endodontalis* ATCC 35406, obtained from the American Type Culture Collection was also grown in an anaerobic chamber according to the method of Zerr *et al.* (1998). *Lactobacillus acidophilus* ATCC 4356 and *Lactobacillus rhamnosus* ATCC 7469 from the Institute of Dental Research Culture Collection (Westmead Centre for Oral Health, Westmead, NSW, Australia) were grown at 37^{0}C in MRS broth (Oxoid) under 95% N_2 and 5% CO_2 (v/v).

5.2.1.2 Sources of other bacterial DNA

DNA from *Legionella pneumophila* serogroup 4 ATCC 33156, serogroup 5 ATCC 33216, serogroup 6 ATCC 33215, serogroup 1 Knoxville-1 ATCC 33153, Philadelphia-1, as well as *Legionella anisa, Legionella bozemanii* serogroup-2, *Legionella londiniensis, Legionella maceachernii and Legionella waltersii* was provided by Mr Rodney Ratcliff, Infectious Diseases Laboratories, Institute of Medical and Veterinary Science, SA, Australia; and from *Mycobacterium tuberculosis* H37RV by Mr Greg James, Microbiology Laboratory, Westmead Hospital, NSW, Australia.

5.2.1.3 Design of primers and probes for real-time PCR

5.2.1.3.1 Universal primers and probe. (Dr M. Nadkarni)

The designed probe and primers set were based on regions of identity within the 16S rDNA following the alignment of sequences from most of the Groups of bacteria outlined in Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). The 16S rDNA bacterial sequences with GenBank accession number in parentheses include; *Bacteroides forsythus* (AB035460), *P. gingivalis* (POYRR16SC), *P. melaninogenica* (PVORR16SF), *Cytophaga baltica* (CBA5972), *Campylobacter jejuni* (CAJRRDAD), *Helicobacter pylori* (HPU00679), *Treponema denticola* (AF139203), *Treponema pallidum* (TRPRG16S), *Leptothrix mobilis* (LM16SRR), *Thiomicrospira denitrificans* (TDE243144), *Neisseria meningitidis* (AF059671), *Haemophilus actinomycetemcomitans* (formerly *Actinobacillus actinomycetemcomitans*) (ACNRRNAJ),

Haemophilus influenzae (HIDNA5483), *E. coli* (ECAT1177T), *Salmonella typhi* (STRNA16), *Vibrio cholerae* (VC16SRRNA), *Coxiella burnetii* (D89791), *L. pneumophila* (LP16SRNA), *P. aeruginosa* (PARN16S), *Caulobacter vibrioides* (CVI009957), *Rhodospirillum rubrum* (RR16S107R), *Nitrobacter winogradskyi* (NIT16SRA), *Wolbachia species* (WSP010275), *Myxococcus xanthus* (MXA233930), *Corynebacterium diphtheriae* (CD16SRDNA), *M. tuberculosis* (MTRRNOP), *Streptomyces coelicolor* (SC16SRNA), *A. odontolyticus* (AO16SRD), *Bacillus subtilis* (AB016721), *Staphylococcus aureus* (SA16SRRN), *Listeria monocytogenes* (S55472), *Enterococcus faecalis* (AB012212), *L. acidophilus* (LBARR16SAZ), *S. mutans* (SM16SRNA), *Clostridium botulinum* (CBA16S), *M. micros* (PEP16SRR8), *Veillonella dispar* (VDRRNA16S), *F. nucleatum* (X55401), *Chlamydia trachomatis* (D89067) and *Mycoplasma pneumoniae* (AF132741) were aligned using the GCG program Pileup (Wisconsin Package Version 8, 1994) accessed through the Australian National Genomic Information Service (ANGIS, http://www.angis.org.au).

The Primer Express Software provided by Applied Biosystems was of limited value in determining a universal probe and primers set as the primary selection criterion of the software is the length of the amplicon (50-150bp). Use of this software resulted in a series of best-fit suggestions for the universal primers and probe, leading to unsatisfactory sequence homology for many of the bacterial genera. As a result, the regions of identity within the 16S rDNA were assessed manually, with the Primer Express Software used to check for primer-dimer or internal hairpin configurations, melting temperate (T_m) and percentage GC values within possible primers/probe sets. The designed amplicon (Table 5.1) complied with six of the eight guidelines set by Applied Biosystems for the design of primers and probes. These included a T_m of the DNA between 58–60°C for the primers and 68–70°C for the probe; the G+C content between 30-80%; no runs of more than 3 consecutive Gs in either the primers or the probe; no G on the 5′-end of the probe and the probe selected from the strand with more Cs than Gs. The primers and probe set only deviated from the ideal in that the last 5 nucleotides of the 3′-end of the forward primer contained more than 2 GCs, and that the amplicon of 466bp (based on that generated between residues 331 to 797 on the *E. coli* 16S rRDNA gene, GenBank accession no. ECAT1177T) exceeded the 50-150bp recommended. The oligonucleotide probe was labeled

with the fluorescent dyes 5-carboxyfluorescein (FAM) at the 5′ end and 6-carboxy-tetramethylrhodamine (TAMRA) at the 3′ end.

The universal probe and primers were checked for possible cross hybridization with bacterial genes other than 16S rDNA as well as genes from Eucarya and Archea using the database similarity search program BLAST (Altschul *et al.*, 1990) accessed through ANGIS. The BLAST search results showed only one significant hit; that of a specific breast cancer cell line (BT029) which was detected only by the reverse primer. However, the universal primers did not amplify the human DNA sample supplied by Applied Biosystems in their Beta-actin Detection Kit probe set, thus confirming the specificity of the probe and primers set for the 16S rDNA of the Domain *Bacteria.*

5.2.1.3.2 P. gingivalis probe and primers

For the species-specific quantification of *Porphyromonas gingivalis,* a primers-probe set was designed from the variable region of the 16S rDNA sequences reported in Genbank (AB035455, AB035456, AB035457, AB035458, AB035459, AF287987, PGRRNA, POYRR16SC) and accessed through ANGIS. The *P. gingivalis* primers-probe set (Table 5.1) generated a 150bp amplicon spanning nucleotides 590 to 739 (inclusive) in the *P. gingivalis* 16S rDNA sequence (GenBank accession no. L16492) with an internal site for the dual-labelled (FAM and TAMRA) fluorogenic probe. The primers-probe set fulfilled all recommended guidelines set by Applied Biosystems. Once designed, the primers and probes were synthesised by Applied Biosystems.

5.2.1.3.3 Internal positive control. (Dr M. Nadkarni)

A chimeric plasmid was constructed to act as an internal positive control using DNA from the Queensland fruit fly, *Bactrocerra tryoni* (M Nadkarni, unpublished data). A primers-probe set was created to enable the detection of the exogenously added internal positive control (IPC-BT) and was designed from the sequence of the *dsX* gene of *B. tryoni* using Primer Express software (Applied Biosystems). The primers-probe set amplified an 89bp region spanning nucleotides 37 to 126 on the *dsX* gene. The probe sequence for the IPC-BT was labelled with the reporter fluorescent dye VIC at the 5′ end to differentiate it from the species-specific and

Table 5.1. Primers and probes for detection of the total bacterial load (universal), *P. gingivalis* and *B. tryoni* (internal positive control, IPC-BT).

Primers or probe	Sequence (5′-3′)	T_m (^0C)
Universal forward primer	TCCTACGGGAGGCAGCAGT	59.4
Universal reverse primer	GGACTACCAGGGTATCTAATCCTGTT	58.1
Universal probe	[6-FAM]CGTATTACCGCGGCTGCTGGCAC [TAMRA]	69.9
P.gingivalis forward primer	TCGGTAAGTCAGCGGTGAAAC	58.8
P.gingivalis reverse primer	GCAAGCTGCCTTCGCAAT	58.7
P.gingivalis probe	[6-FAM] CTCAACGTTCAGCCTGCCGTTGAAA [TAMRA]	68.8
B. tryoni forward primer	GGAAGGTAAGTTGCATTTCAGCA	59.3
B. tryoni reverse primer	GCGTACTTATCATGGTAAATTAAGTCAATT	58.6
B. tryoni probe	[VIC] TCCCGTTACAAAATCGTGTTTACATCGTATACTCG [TAMRA]	69.1

universal probes which were labelled at the 5′ end with the fluorescent reporter dye FAM (Table 5.1). Due to software limitations, the VIC probe cannot be used to quantify amounts of DNA, therefore results are expressed in terms of C_T values only.

5.2.1.4 Conditions for real-time PCR

Precautions were taken at all stages to avoid contamination and the possibility of false positive PCR results. These included the use of disposable materials where possible with UV sterilisation prior to use, pre-aliquoted reagents, preparation of all reaction mixes for PCR in a biohazard hood located in an area remote from DNA preparation and the addition of DNA as the last component to the reaction mix. Amplification and detection of DNA by real-time PCR used the ABI PRISM 7700 Sequence Detection System with a 96-well plate format. PCR was carried out in duplicate or triplicate reactions, in a 25µl reaction volume containing 300nM of each of the universal primers, and 100nM of the universal probe and 100nM each of the primers and probe for the Internal Positive Control (IPC-BT) and *P. gingivalis* set using the TaqMan Universal PCR Master Mix or the TaqMan PCR Core Reagents Kit (Applied Biosystems). The reaction conditions for amplification of DNA were 95^{0}C for 10min and 40cycles of 95^{0}C for 15s and 60^{0}C for 1min. Data were analysed using the Sequence Detection Software Version 1.6.3 supplied by Applied Biosystems.

5.2.1.4.1 DNA standards used for determining bacterial number by real-time PCR

Most experiments used *E. coli* DNA to generate a standard graph, within the range 238fg-2.38ng, for the determination of bacterial numbers by real-time PCR. *Porphyromonas gingivalis* was also used to generate a standard graph using DNA within the range 360fg-3.6ng. Standard graphs were always prepared from data accumulated at the same time as the test samples in order to act as internal controls.

5.2.1.4.2 Bacterial cultures used in isolation experiments

No attempts were made to standardise optical densities of the different bacterial cultures as comparison of DNA recovery from the various bacterial species with each other was not required within the experiments. Therefore, only relative assessments have been made regarding the DNA measured from each of the methods tested. It should be noted that it was not

possible to purify all the DNA isolated or quantify all the results by PCR because of the costs involved.

Unless stated otherwise all procedures reported for the isolation and quantitation of bacterial cultures were repeated at least once to confirm reproducibility of the results.

5.2.1.5 Extraction and protection of bacterial DNA

As a survey of the literature revealed no clear methodology for the extraction of bacterial DNA combined with appropriate protection against the action of nucleases, an incremental experimental approach was used to determine the most efficient procedure to address both aims. Initially different mechanical methods were used to facilitate the release of bacterial DNA from a Gram-positive bacterium *S. mutans* and a Gram-negative bacterium, *P. gingivalis.* These bacteria were selected to provide representative examples of microbes found in dental caries that provided difficulties in DNA extraction and nuclease control, respectively.

Following successful testing and optimisation of both the universal and *P. gingivalis* probe/primers sets, the universal amplicon was used to quantitate DNA from a number of experiments involving the extraction and protection of DNA to provide comparative data, while the *P. gingivalis* amplicon was used in an experiment assessing nuclease activity.

5.2.1.5.1 Mechanical extraction procedures

5.2.1.5.1.1 Freeze-boil method

P. gingivalis and *S. mutans* cells (~10^9 cells) were harvested by centrifugation (14,000 *g*, 2min, 18–20^{0}C) (Biofuge Pico, Heraeus Instruments, Sydney, Australia) and resuspended in 200µl of buffer containing 10mM sodium phosphate pH 6.7 and frozen at –20^{0}C (2-16h) before being boiled for 10min. After cooling to room temperature (18–20^{0}C) the samples were incubated at 37^{0}C for 10min following the addition of 1mg DNase-free RNase ml^{-1} (Sigma). The unpurified samples were diluted 100-fold and a 2.5µl aliquot quantified using the ABI PRISM 7700 Sequence Detection System with the universal primers-probe set (Table 5.1) and the TaqMan Universal PCR Master Mix. Triplicate measurements were taken based on a standard graph generated by known amounts of *E. coli* DNA.

5.2.1.5.1.2 Freeze-thaw method

P. gingivalis and *S. mutans* (~10^9 cells) were harvested as described and resuspended in 200µl of buffer containing 10mM sodium phosphate pH 6.7 and frozen at –20°C. After thawing and RNase treatment, the samples were diluted 100-fold for PCR quantitation as described above.

5.2.1.5.1.3 Sonication

P. gingivalis and *S. mutans* (~10^9 cells) were harvested and resuspended in 200µl of buffer containing 10mM sodium phosphate pH 6.7 prior to continuous sonication for 3, 6 or 9min at 75W using a Branson Sonifier (Model 250; Branson Ultrasonics Corporation, Danbury, CT, USA). Aliquots were collected at each time interval and following RNase treatment, samples were diluted 100-fold for PCR quantitation as described.

5.2.1.5.2 Nuclease detection

Different experiments were designed to assess whether the loss of *P. gingivalis* DNA noted following mechanical treatments was the result of shearing or DNase activity.

5.2.1.5.2.1 Isolation of P. gingivalis DNA in the presence of the internal positive control (IPC-BT) (Dr M. Nadkarni)

P. gingivalis cells (~2.5 x 10^8) were harvested by centrifugation (14,000*g*, 2min, 18 – 20°C) and the pellet resuspended in 100µl of buffer containing 10mM sodium phosphate pH 6.7 and 1µl (620pg/µl) IPC-BT DNA. Following the freeze/thaw and freeze/boil procedures, described previously, the samples were diluted 100-fold with 2.5µl aliquots used for real-time PCR. Quantitation of DNA used the *P. gingivalis* and IPC-BT primers-probe sets with the TaqMan PCR Core Reagents Kit. Duplicate measurements were taken from a standard curve generated from known amounts of *P. gingivalis* DNA.

5.6.1.5.2.2 Detection of nuclease activity in P. gingivalis

Exogenous *P. gingivalis* DNA (300–400ng), prepared using the ATL method (5.2.1.5.3.10) and purified using the QIAamp DNA Mini Kit (Qiagen) was added to samples containing approximately 300–400ng DNA prepared using the freeze/thaw, freeze/boil, 3 and 6 min sonication procedures, prior to incubation at 50°C for 30min. The presence of DNA was

determined visually following electrophoresis of samples in 1% (w/v) agarose gels in TAE and stained with ethidium bromide.

5.2.1.5.3 Chemical DNA isolation procedures

Further experiments were conducted to establish a protocol for DNA extraction and isolation designed to release the maximum amount of polymeric DNA and inhibit nuclease activity from an expanded group of representative Gram-positive and Gram-negative bacteria using a variety of enzymes and chemicals.

5.2.1.5.3.1 Enzymatic, $ZnCl_2$ and EDTA methods

Actinomyces israelii, F. nucleatum, L. acidophilus, P. gingivalis, P. melaninogenica and *S. mutans* cells (~2.5 x 10^8 of each bacterial species) were harvested by centrifugation (14,000*g*, 2min, 18–20°C) and resuspended in 100µl of 10mM sodium phosphate buffer pH 6.7 containing 1mg lysozyme ml^{-1} and 2mg mutanolysin ml^{-1} (lysis buffer), with and without the addition of 5mM $ZnCl_2$ or 100mM EDTA. After incubation at 60°C for 30min, the bacteria were lysed in the presence of 1%SDS and incubated at 37°C for 10min following the addition of 1mg RNase ml^{-1}. Quantitation of unpurified DNA using real-time PCR used 2.5µl aliquots of 100-fold diluted samples with the universal primers-probe set and the TaqMan Universal PCR Master Mix. Triplicate measurements were taken from a standard graph generated from known amounts of *E. coli* DNA.

5.2.1.5.3.2 Enzymatic, EDTA, EGTA, DEPC and combination methods

Actinomyces israelii, F. nucleatum, L. acidophilus, P. gingivalis, P. melaninogenica and *S. mutans* cells (~5 x 10^8 of each bacterial species) were harvested by centrifugation (14,000*g*, 4min, 18–20°C) and resuspended in 200µl of 10mM sodium phosphate buffer pH 6.7 containing 1mg lysozyme ml^{-1} and 2mg mutanolysin ml^{-1}, with and without 100mM EDTA, 100mM EGTA, 20mM diethylpyrocarbonate (DEPC), 100mM EDTA plus 20mM DEPC and 100mM EGTA plus 20mM DEPC. After incubation at 60°C for 30 min, the bacteria were lysed in 1% SDS, incubated at 56°C for 10min with 2mg proteinase K ml^{-1} (Qiagen) and finally incubated at 37°C for 10min following the addition of 1mg RNase ml^{-1}. The samples were purified using the QIAamp DNA Mini Kit and eluted into AE buffer (proprietary buffer,

Qiagen). DNA was quantified using 2.5μl aliquots of a 1:100 dilution of purified DNA, in triplicate, with the universal primers-probe set as described.

5.2.1.5.3.3 Extended incubation method

Attempts were made to improve the recovery of DNA from the Gram-positive bacteria *A. israelii, L. acidophilus* and *S. mutans* while controlling the nuclease activity of *P. gingivalis* by incorporating proteinase K into the buffer and extending the incubation period. Cells of *A. israelii, F. nucleatum, L. acidophilus, P. gingivalis, P. melaninogenica* and *S. mutans* (~5 x 10^8 of each) were harvested by centrifugation (14,000*g*, 4min, 18–20°C) and resuspended in 200μl of 10mM sodium phosphate buffer pH 6.7 containing 2mg lysozyme ml^{-1}, 2mg mutanolysin ml^{-1}, 2mg proteinase K ml^{-1} and 20mM DEPC, with and without the addition of 100mM EDTA and 10mM $MgCl_2$. After a prolonged incubation of 150min at 56°C the bacteria were lysed in 1% SDS. The presence of DNA was determined visually following electrophoresis of samples in agarose gels.

5.2.1.5.3.4 MES buffer method

Further attempts to improve the recovery of DNA from the Gram-positive microorganisms were based on the methods described by Pollock *et al.* (1987). Cells of *A. israelii, F. nucleatum, L. acidophilus, P. gingivalis, P. melaninogenica* and *S. mutans* (~5 x 10^8 of each), and mixtures of (~2.5 x 10^8) of *L. acidophilus* plus *S. mutans*, and *P. gingivalis* plus *S. mutans* were harvested by centrifugation (14,000*g*, 4min, 18–20°C) and resuspended in 200μl of 25mM MES (2-[N-morpholino] ethane sulphonic acid) buffer pH 5.2 containing 2mg lysozyme ml^{-1} and 2mg proteinase K ml^{-1}, with and without 20mM DEPC, 100mM EDTA and 100mM DEPC plus 100mM EDTA. Following incubation for 120min at 37°C, 10mM $NaHCO_3$ and 100mM NaCl were added and incubated for a further 120min at 37°C. Aliquots were taken both before and after the addition of 1% SDS and the presence of DNA assessed by agarose gel electrophoresis.

5.2.1.5.3.5 Comparison of buffers

This experiment compared the sodium phosphate and MES buffers with a shorter incubation time. Cells of *A. israelii, F. nucleatum, L. acidophilus, P. gingivalis, P. melaninogenica* and *S. mutans* (~5 x 10^8 of each) and mixtures of (~2.5 x 10^8) of *L. acidophilus* plus *S. mutans,*

and *P. gingivalis* plus *S. mutans* were harvested by centrifugation (14,000*g*, 4min, 18–20°C) and resuspended in 200µl of buffer containing either 10mM MES pH 5.2 or 10mM sodium phosphate pH 6.7 plus 2mg lysozyme ml^{-1}, 2mg mutanolysin ml^{-1}, 2mg proteinase K ml^{-1} and 20mM DEPC. Following incubation for 15min at 60°C, 10mM $NaHCO_3$ and 100mM NaCl were added and the cells lysed with 1% SDS. The presence of DNA was determined visually following gel electrophoresis.

5.2.1.5.3.6 Phosphate lysis buffer with additional DNase inhibitors

Cells of *A. israelii, F. nucleatum, L. acidophilus, P. endodontalis, P. gingivalis, P. melaninogenica, M. micros* and *S. mutans* (~5 x 10^8 of each) were harvested by centrifugation (14,000*g*, 4min, 18–20°C) and resuspended in 200µl buffer containing 10 mM sodium phosphate pH 6.7, 2mg lysozyme ml^{-1}, 2mg mutanolysin ml^{-1} and 2mg proteinase K ml^{-1} with one of the following; 10mM ascorbic acid (reducing agent), 0.1mM aurin tricarboxylic acid (topoisomerase inhibitor), 50mM trisodium citrate (chelator), or a combination of 10mM ascorbic acid plus 50mM trisodium citrate, with and without 20mM DEPC. Following 20min storage on ice or 6min pulse sonication, the samples were incubated for 30min at 56°C, lysed in 1% SDS or purified using the QIAamp DNA Mini Kit. Aliquots were loaded into agarose gels to visualise the DNA recovered.

5.2.1.5.3.7 Varying the lysozyme concentration

Bacterial cultures (~5 x 10^8) of *A. israelii, F. nucleatum, L. acidophilus, P. endodontalis, P. gingivalis, P. melaninogenica, M. micros* and *S. mutans* were harvested by centrifugation (14,000*g*, 4min, 18–20°C) and resuspended in 144µl buffer (10mM sodium phosphate pH 6.7) containing 20mM DEPC. Cell suspensions were incubated on ice for 20min or sonicated in pulse mode for 6min at 75W (Branson Sonifier), followed by the addition of 56µl of 1mg or 5mg lysozyme ml^{-1}, 2mg mutanolysin ml^{-1} and 2mg proteinase K ml^{-1}. Another group was resuspended in the same buffer containing the lysis enzymes and 20mM DEPC, and stored on ice for 20min. The cell suspensions were then incubated at 56°C for 40min with intermittent vortexing for 10sec after every 10min. Cells were lysed with 1% SDS and 1mg RNase ml^{-1} was

added, followed by a further incubation at 37^{0}C for 10min. The unpurified DNA was visualised following gel electrophoresis.

5.2.1.5.3.8 Isolation of DNA in DEPC

Bacterial cultures (~5 x 10^8) of *A. israelii, F. nucleatum, L. acidophilus, P. endodontalis, P. gingivalis, P. melaninogenica, M. micros* and *S. mutans* were harvested by centrifugation (14,000*g*, 4min, 18–20^{0}C) and resuspended in 200µl of buffer containing 10mM sodium phosphate pH 6.7, 5mg lysozyme ml^{-1}, 2mg mutanolysin ml^{-1} and 2mg proteinase K ml^{-1}, with and without the addition of 20mM DEPC. The cell suspensions were incubated at 56^{0}C for 40min with intermittent vortexing for 10sec after every 10min. Cells were lysed with 1% SDS, 1mg RNase ml^{-1} was added, and the sample incubated for a further 10min at 37^{0}C. The DNA was purified using the QIAamp DNA Mini Kit. The presence and quality of the DNA was determined visually following electrophoresis of samples in agarose gels.

5.2.1.5.3.9 Isolation of DNA using a modified DEPC method

Bacterial cultures (~5 x 10^8) of *A. israelii, F. nucleatum, L. acidophilus, P. endodontalis, P. gingivalis, P. melaninogenica, M. micros* and *S. mutans* were harvested by centrifugation (14,000*g*, 4min, 18–20^{0}C) and resuspended in 144µl buffer (10mM sodium phosphate pH 6.7) containing 20mM DEPC. Cell suspensions were incubated on ice for 10min or sonicated in pulse or continuous mode for 6min followed by the addition of 56µl of 5mg lysozyme ml^{-1}, 2mg mutanolysin ml^{-1} and 2mg proteinase K ml^{-1}. The cell suspensions were incubated at 56^{0}C for 40min with intermittent vortexing for 10sec after every 10min. Cells were lysed with 1% SDS, 1mg RNase ml^{-1} was added, followed by further incubation at 37^{0}C for 10min. DNA was purified using a QIAamp DNA Mini Kit. Quantitation of DNA using the universal primers-probe set and the TaqMan PCR Core Reagents Kit was carried out in triplicate using 2.5µl aliquots of a 1:100 dilution of purified DNA, based on a standard graph generated from known amounts of *E. coli* DNA.

5.2.1.5.3.10 Isolation of DNA in ATL buffer from the QIAamp DNA Mini Kit

Bacterial cultures (~5 x 10^8) of *A. israelii, F. nucleatum, L. acidophilus, P. endodontalis, P. gingivalis, P. melaninogenica, M. micros* and *S. mutans* were pelleted by centrifugation (14,000*g*,

4min, 18–20^{0}C). Cell pellets were resuspended in 180μl ATL buffer (Qiagen) and 400μg proteinase K (Qiagen). The cell suspensions were incubated at 56^{0}C for 40min with intermittent vortexing for 10sec every 10min. Ribonuclease (1mg ml^{-1}) was added, followed by a further incubation at 37^{0}C for 10min. DNA was purified using the QIAamp DNA Mini Kit and quantitation of DNA using the universal primers-probe set was carried out as described previously. The presence and quality of the DNA was determined visually following electrophoresis of samples in agarose gels.

5.2.1.5.4 The effect of $ZnCl_2$ as a PCR inhibitor (Dr M Nadkarni)

In order to determine whether $ZnCl_2$ acted as an inhibitor of real-time PCR, *P. gingivalis* cells (~2.5 x 10^{8}) were harvested by centrifugation (14,000*g*, 2min, 18–20^{0}C) and resuspended in 100μl of 10mM sodium phosphate buffer pH 6.7 containing 1mg lysozyme ml^{-1} and 2mg mutanolysin ml^{-1} with the addition of 5mM, 50mM and 500mM concentrations of $ZnCl_2$. After incubation at 60^{0}C for 30min, the samples were either lysed in 1% SDS, or were purified using the QIAamp DNA Mini Kit following the manufacturer's instructions before being diluted, with 2.5μl aliquots used for real-time PCR. DNA preparations with and without purification were diluted to theoretically contain equivalent amounts of DNA prior to PCR. Quantitation of DNA, in triplicate, used the universal primers-probe set and a standard curve generated from known amounts of *E. coli* DNA, as described previously.

5.2.1.5.5 Additional extraction methods

Other extraction methods not described in detail in the preceding methods included: combinations of different chemicals with boiled or autoclaved suspensions; $ZnCl_2$/DEPC combination in phosphate buffer; the use of Triton X-100 to remove SDS which was found to interfere with the action of mutanolysin; the use of chloroform in the initial suspension; TRIS as an alternate buffer and the use of 10 and 100mM dipyridyl, dimethylsulphoxide (DMSO), $HgCl_2$, histidine, mannitol and actin in the initial suspension as nuclease inhibitors (Gottesfeld *et al.*, 1971; Pirt, 1975; Dhermy *et al.*, 1977).

5.2.1.6 Statistical analyses

Basic descriptive statistics were applied to the PCR results following DNA quantitation producing mean, standard deviation and standard error information.

5.2.2 Results

5.2.2.1 Sensitivity of the universal probe and primers in detecting E. coli rDNA

TaqMan technology determines the PCR cycle at which the increase in fluorescence of the reporter dye reaches a threshold cycle (C_T) (Figure 5.1a). The C_T is proportional to the log of the amount of target DNA and hence the log of the number of bacteria in the sample, provided there is only one copy of the reported sequence within the genome. The standard graph based on *E. coli* rDNA (Figure 5.1b) theoretically equates one *E. coli* cell to the detection of 4.96fg DNA, provided the seven copies of rDNA in each copy of the chromosome (Farrelly, *et al.*, 1995) are not taken into consideration. Using *E. coli* as a standard, between 238fg of *E. coli* DNA (corresponding to 48 *E. coli* cells) and 2.38ng of *E. coli* DNA (corresponding to 4.8 x 10^5 *E. coli* cells) was consistently detected. It should be noted that at extreme high and low C_T values, a two-fold error in the estimation of the relative amount of DNA can occur. C_T values below 0.1pg and above 1000pg DNA should therefore be avoided in calculating the amount of DNA in a sample provided alternative dilutions are practical (Fig. 5.1b).

Detection in the apparent range of 4.8 to 48 cells was limited by contamination from bacterial DNA in the commercially supplied reagents. The degree of contamination varied with different kits of the TaqMan Universal PCR Master Mix and TaqMan PCR Core Reagents Kit. To minimise this problem different lot numbers of TaqMan Universal PCR Master Mix and TaqMan PCR Core Reagents Kit supplied by Applied Biosystems were tested, and only those with minimum contaminating DNA were used. Although 40 cycles were theoretically available for the reaction, contamination of reagents manifest in the no-template-control restricted the sensitivity of the reaction to C_T values below 33–38 cycles depending on the batches of reagents used. In Figure 5.1a, the no-template–control is seen at C_T of 37.7. This contamination is not detected when using the species-specific probes and primers.

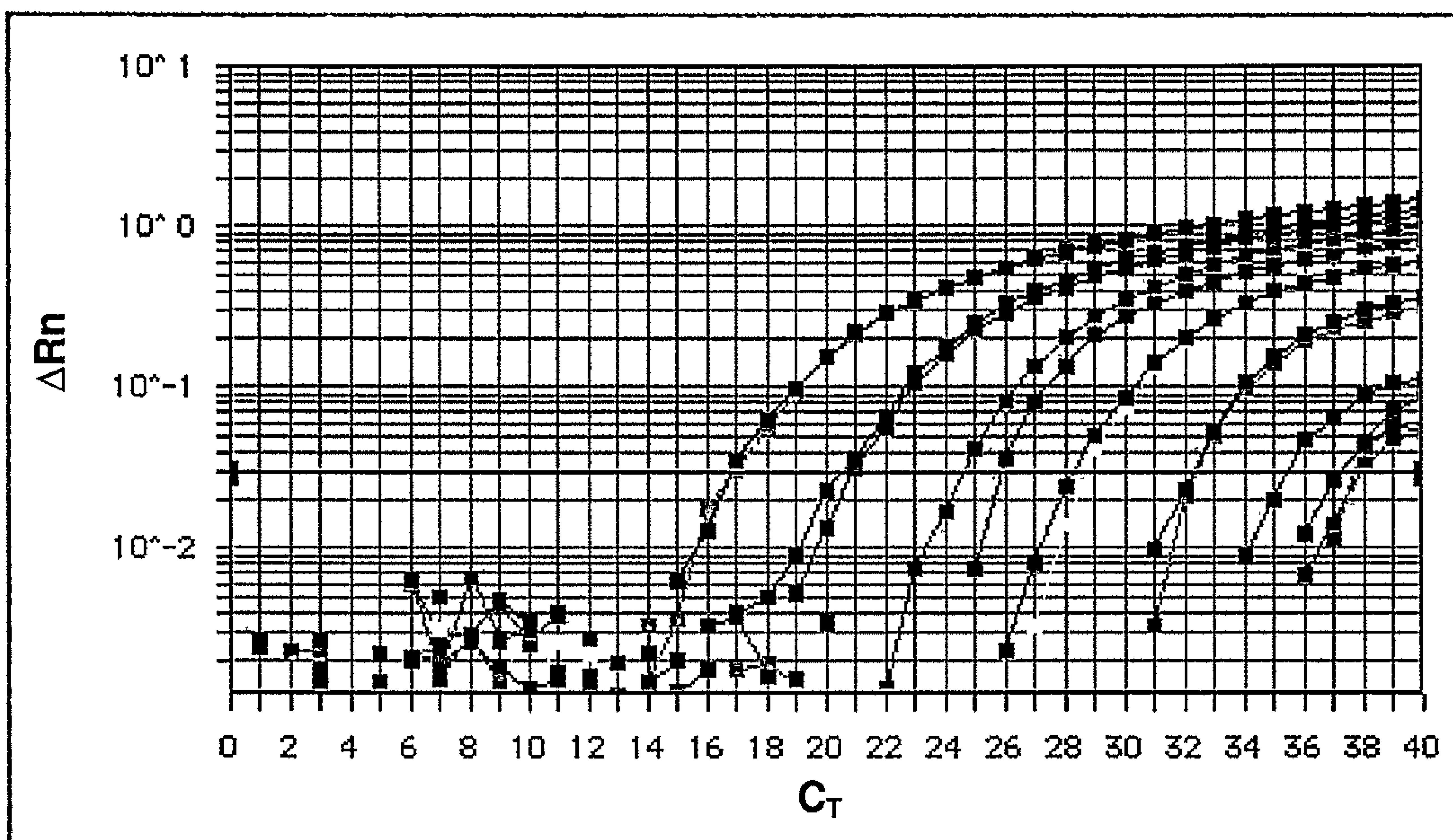


Figure 5.1a. Sensitivity of the universal probe and primers set in detecting *Escherichia coli* rDNA. Purified *E. coli* DNA was used as the template in quantities of 2380pg, 238pg, 23.8pg, 2.38pg, 238fg and 23.8fg, representing C_T values in the range 16.9-35.3 where the intercept of the magnitude of the fluorescence signal (ΔR_n) with the horizontal threshold line in bold represents the C_T value for a given sample. The fluorescence signal at C_T 37.7 corresponds to the no-template control and represents bacterial DNA contamination in the commercially supplied reagents.

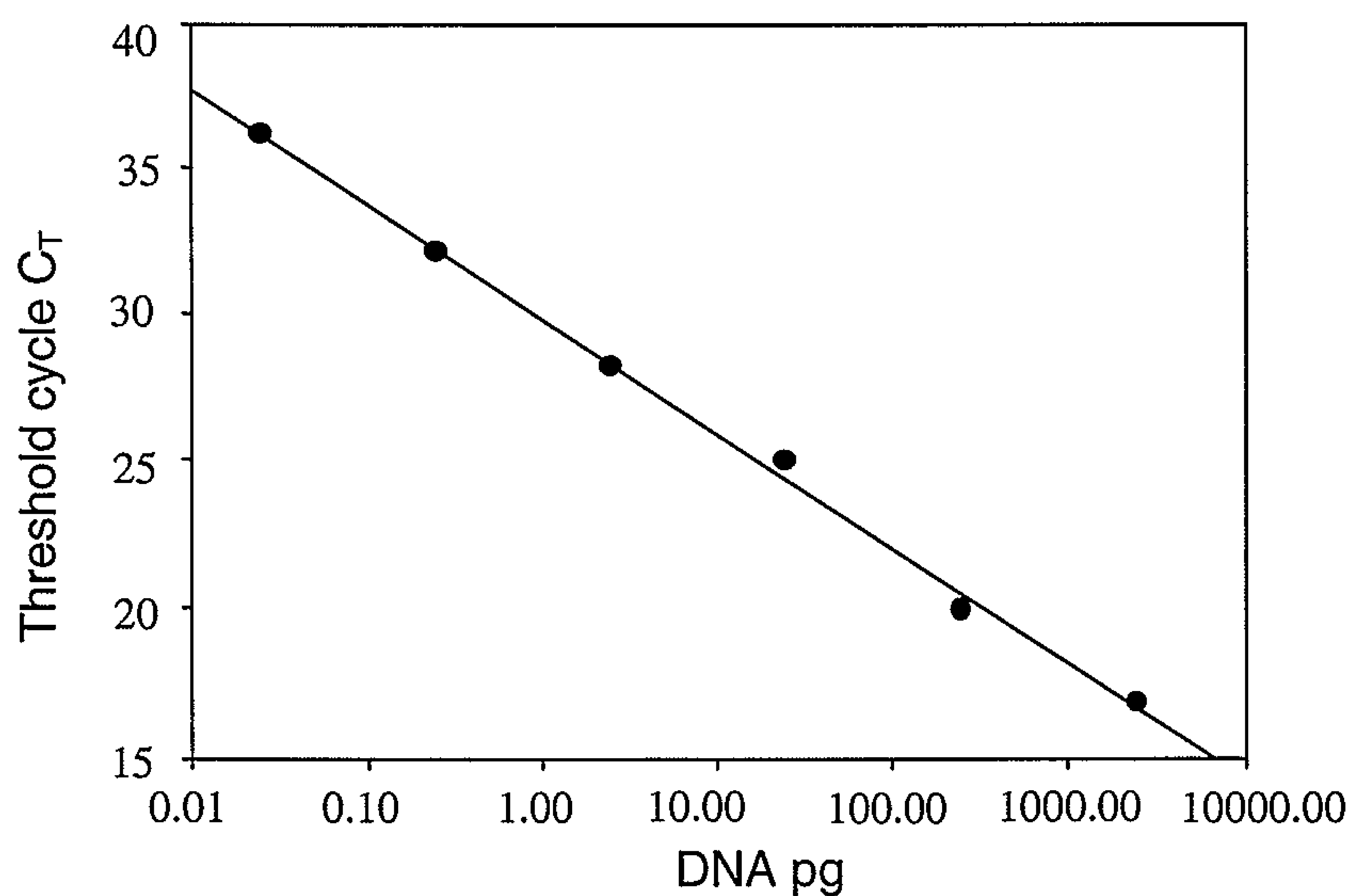


Figure 5.1b. Relation between the threshold cycle and the amount of *E. coli* DNA using TaqMan real-time PCR. Each point represents an amount of *E. coli* DNA corresponding to the C_T value using the universal probe and primers set. The correlation coefficient of the straight line, R^2, was 0.994.

5.2.2.2 Broad range detection of bacterial species by the universal probe and primers set

In order to determine the ability of the universal probe and primers set to detect a broad range of bacteria, samples of DNA extracted from 49 strains representing 34 different species from the major Groups of bacteria listed in Bergey's Manual of Determinative Bacteriology (Holt, *et al.*, 1994) were subjected to real-time PCR using the probe/primers set. All the selected species were detected within a C_T range of 17.05–34.00 (Table 5.2). For each species there was little variance in the value of 2.00×10^2 (range $1.98–2.06 \times 10^2$) *E. coli*-equivalent bacteria per pg DNA when *E. coli* DNA was used as a standard, indicating that the source of DNA was not influencing the level of detection and that the probe and primers set was equally efficient in detecting the DNA irrespective of the species from which it was extracted. Only in the case of *M. micros* was there a mismatch in identity between the probe and primers set and the 16S rDNA. This constituted a single nucleotide deletion in the 16S rDNA compared with the 5′-end of the forward primer. This sequence discrepancy was clearly tolerated during real-time PCR detection of *M. micros* DNA (Table 5.2).

5.2.2.3 Detection using the P. gingivalis and internal positive control probe and primers

Specificity of the *P. gingivalis* probe and primers set was tested against *S. mutans* LT11 (Tao *et al.*, 1993), *F. nucleatum* ATCC 25586, *F. necrophorum* ATCC 25286, *A. israelii* ATCC 12102, *A. naeslundii* ATCC 12104, *P. gingivalis* ATCC 33277, *P. melaninogenica* ATCC 25845, *M. micros* ATCC 33270 and *P. anaerobius* ATCC 27337, *P. endodontalis* ATCC 35406, *L. acidophilus* ATCC 4356 and human Beta-actin DNA (Applied Biosystems), and found to detect only *P. gingivalis* DNA.

The addition of a chimeric plasmid containing unique non-bacterial DNA to mixed bacterial samples allowed both a determination of the efficiency of DNA recovery following sample preparation and the detection of potential PCR inhibitors in the reaction mix during real-time PCR. The IPC-BT produced a fluorescence signal with real-time PCR, measured by the C_T reading, confirming an internal location for the probe within the amplicon (Fig. 5.2).

Table 5.2. Representative bacterial species detected by real-time PCR using the universal probe and primers set.

Bacterial Species*	C_T †
Gram negative aerobic bacteria †	
Pseudomonas aeruginosa ATCC 19660	18.14
Pseudomonas aeruginosa ATCC 15442	18.46
Pseudomonas aeruginosa 6294	18.26
Pseudomonas aeruginosa 6206	19.67
Pseudomonas fluorescens	19.19
Pseudomonas putida	22.35
Pseudomonas stutzeri	18.87
Pseudomonas alcaligens	19.33
Pseudomonas species	19.52
Legionella pneumophila knoxville-1 ATCC 33153	21.93
Legionella pneumophila serogroup 4 ATCC 33156 21	34.00
Legionella pneumophila serogroup 5 ATCC 33216	20.08
Legionella pneumophila serogroup 6 ATCC 33215	21.19
Legionella pneumophila philadelphia-1 ATCC 33152	25.18
Legionella anisa	24.02
Legionella bozemanii serogroup 2	21.46
Legionella londiniensis	20.50
Legionella macearchernii	22.97
Legionella waltersii	21.96
Gram negative facultative anaerobic bacteria	
Escherichia coli JM109	19.86
Escherichia coli NM522	28.22
Escherichia coli XL 1 blue	26.95
Serratia marsescens ATCC 274	20.96
Gram negative anaerobic bacteria	
Porphyromonas gingivalis ATCC 33277	23.50
Porphyromonas endodontalis ATCC 35406	22.05
Prevotella melaninogenica ATCC 25845	20.48
Fusobacterium necrophorum ATCC 252	23.15
Fusobacterium nucleatum ATCC 25586	21.05
Gram positive bacteria	
Staphylococcus aureus ATCC 12600	16.15
Staphylococcus aureus ATCC 9144	29.57
Staphylococcus aureus ATCC 12598	27.41
Staphylococcus aureus ATCC BM 10458	26.32
Staphylococcus aureus ATCC BM 10143	27.20
Staphylococcus epidermidis ATCC 35983	17.88
Staphylococcus epidermidis ATCC 14990	22.27
Staphylococcus hemolyticus ATCC 29970	21.14
Staphylococcus hemolyticus	22.28
Staphylococcus schleferi ATCC 43808	22.29
Streptococcus sanguis ATCC 10556	17.05
Streptococcus salivarius ATCC 25975	20.27
Streptococcus gordonii ATCC 10558	20.03
Streptococcus mutans LT11	18.78
Peptostreptococcus anaerobius ATCC 27337	22.36
Peptostreptococcus micros ATCC 33270	22.83
Gram positive asporogenous bacteria	
Lactobacillus acidophilus ATCC 4356	20.73
Lactobacillus rhamnosus ATCC 7469	24.53
Actinomycetes	
Actinomyces naeslundii ATCC 12104	24.32
Actinomyces israelii ATCC 12102	26.38
Mycobacterium tuberculosis H37RV	26.00

* Groups of bacteria based on Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994).

† DNA was either extracted from equivalent volumes of cultured bacteria or obtained from independent sources and diluted to be within the range of the threshold cycle (C_T) of the standard graph C_T vs *Escherichia coli* DNA. The data are the means of duplicate determinations. Variation in the duplicates was ≤3.7%, except where underlined the duplicates varied between 6.0 and 11.8%.

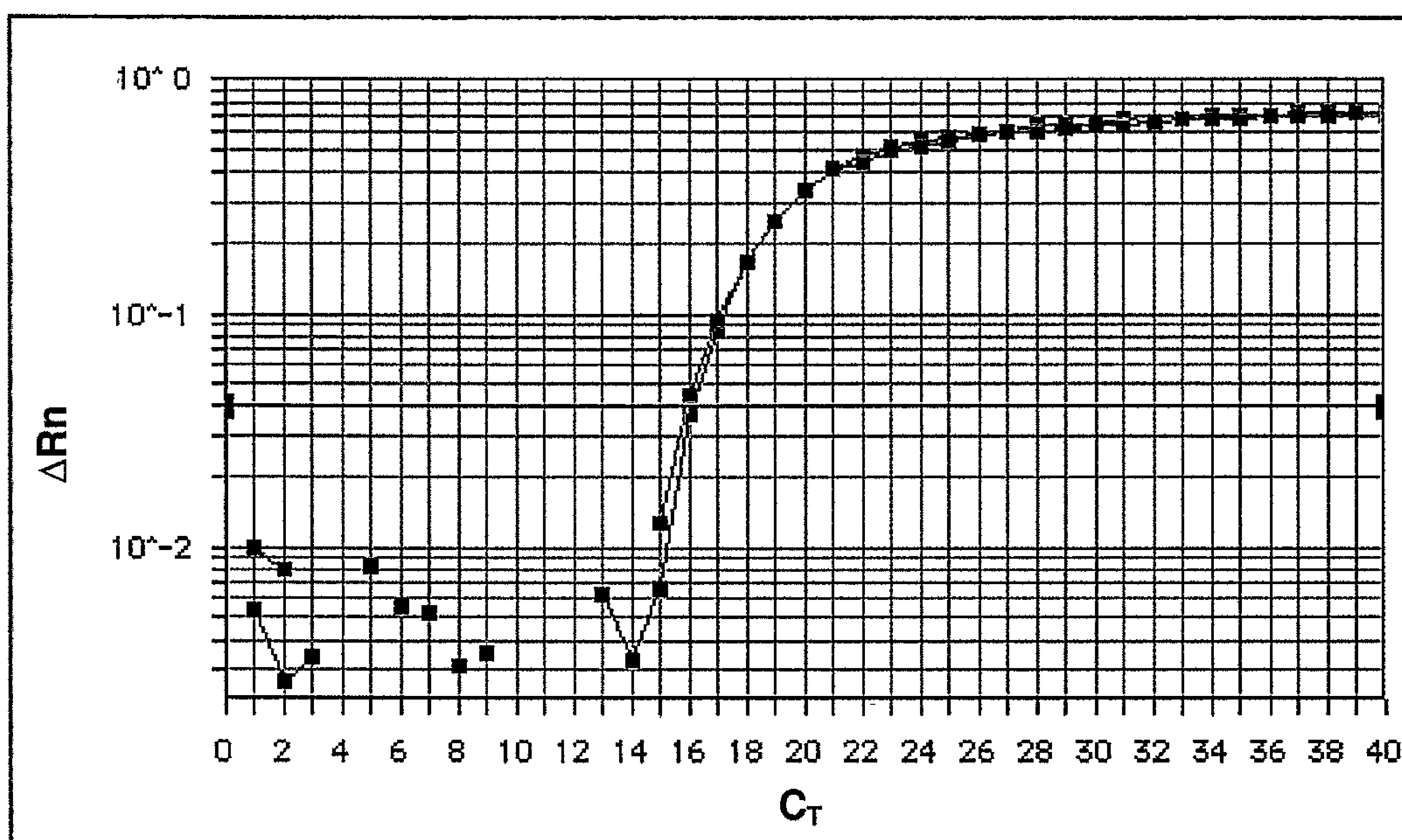


Figure 5.2. Real-time PCR amplification curve produced by the fluorescence signal of IPC-BT DNA using the IPC-BT primers and probe and showing an internal site for the probe within the amplicon.

5.2.2.4 Mechanical DNA isolation procedures.

These experiments were repeated three times due to variations in the amount of *P. gingivalis* isolated from different cultures. Overall results showed the largest amount of DNA measured for *P. gingivalis* following the freeze/boil procedure, with decreasing amounts of DNA measured following the freeze/thaw and sonication methods. In contrast, *S. mutans* showed more effective release of DNA following sonication, with less DNA quantified after the freeze/thaw and freeze/boil processes (Figure 5.3).

5.2.2.5 Nuclease detection

5.2.2.5.1 Isolation of P. gingivalis DNA in the presence of the internal positive control

When *P. gingivalis* DNA was processed together with the internal positive control (IPC-BT), the amount of DNA recovered after the freeze/thaw process was substantially reduced, as recorded by the C_T value for both the *P. gingivalis* and the IPC-BT probe and primers when compared with DNA recovered following the freeze/boil method (Table 5.3). Deterioration of both the *P. gingivalis* and the IPC-BT signal indicated the action of a DNA degrading agent which was inactivated by boiling the sample.

5.2.2.5.2 Detection of nuclease activity in P. gingivalis

The presence of nuclease activity was confirmed by agarose gel electrophoresis where purified exogenous *P. gingivalis* DNA was degraded by *P. gingivalis* DNA prepared by the freeze/thaw process and following 3min and 6min sonication times. A band of *P. gingivalis* DNA with smearing was noted following incubation with the freeze/boil prepared *P. gingivalis* culture (Figure 5.4), whereas no DNA was recovered following the other procedures.

5.2.2.6 Chemical DNA isolation procedures.

5.2.2.6.1 Enzymatic, $ZnCl_2$ and EDTA methods

The enzymes lysozyme and mutanolysin were added to the phosphate buffer to encourage cell wall lysis, particularly for the Gram-positive bacteria. Additionally $ZnCl_2$ and EDTA were incorporated as potential nuclease inhibitors. Following incubation, PCR results showed that similar amounts of *A. israelii* and *F. nucleatum* DNA were extracted by each procedure, whereas

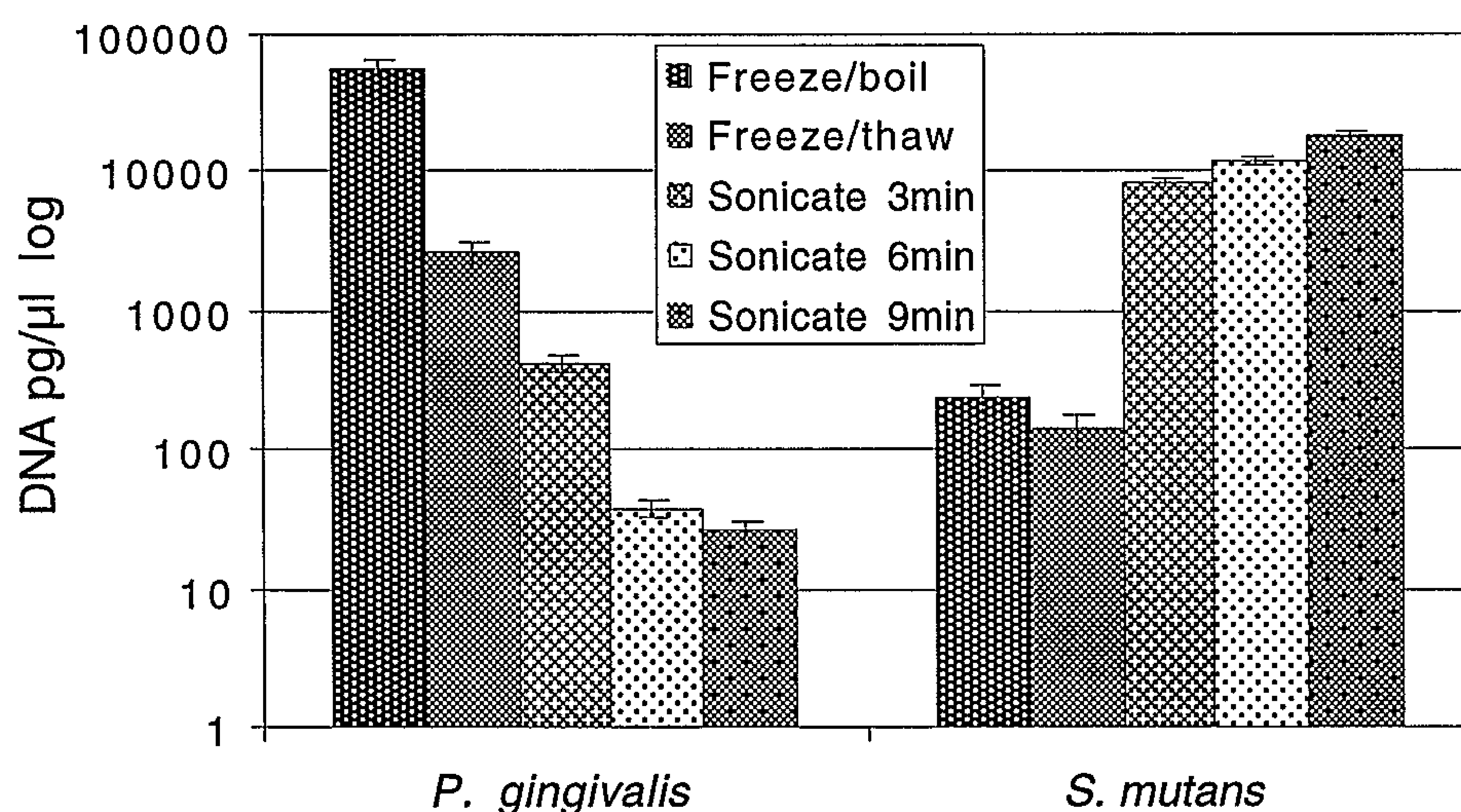


Figure 5.3. PCR quantitation of DNA from cultures of *P. gingivalis* and *S. mutans* isolated in 10mM sodium phosphate buffer and subjected to mechanical treatments including: freeze/boil, freeze/thaw and continuous sonication for 3, 6 and 9 min. Data shows one representative experiment of three, expressed as mean values from triplicate readings ± SEM.

Table 5.3. Isolation of *P. gingivalis* DNA in the presence of the internal positive control (IPC-BT). Quantitation used both the *P. gingivalis* and IPC-BT probe-primer sets utilising different fluorescent reporter dyes FAM and VIC, respectively. Data provided by Dr M Nadkarni.

DNA isolation method	*P. gingivalis* C_T (FAM) (St Dev)	IPC-BT C_T (VIC) (St Dev)
Freeze/boil	16.69 (0.06)	16.65 (0.30)
Freeze/thaw	22.40 (0.06)	24.40 (0.16)

Note: The higher the original amount of sample DNA, the lower the C_T value.

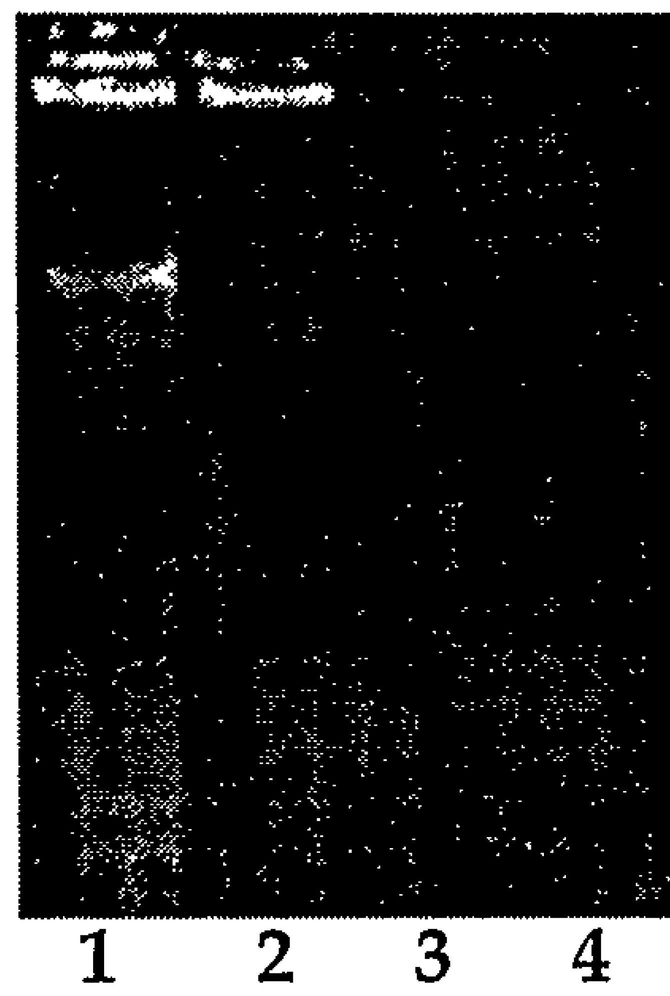


Figure 5.4 Agarose gel electrophoresis of DNA from *P. gingivalis*. Lane 1: purified exogenous *P. gingivalis* DNA incubated with freeze/boil prepared *P. gingivalis* DNA.; Lane 2: purified exogenous *P. gingivalis* DNA incubated with freeze/thaw prepared *P. gingivalis* DNA; Lane 3: purified exogenous *P. gingivalis* DNA incubated with 3 min sonicated *P. gingivalis* DNA; Lane 4: purified exogenous *P. gingivalis* DNA incubated with 6 min sonicated *P. gingivalis* DNA.

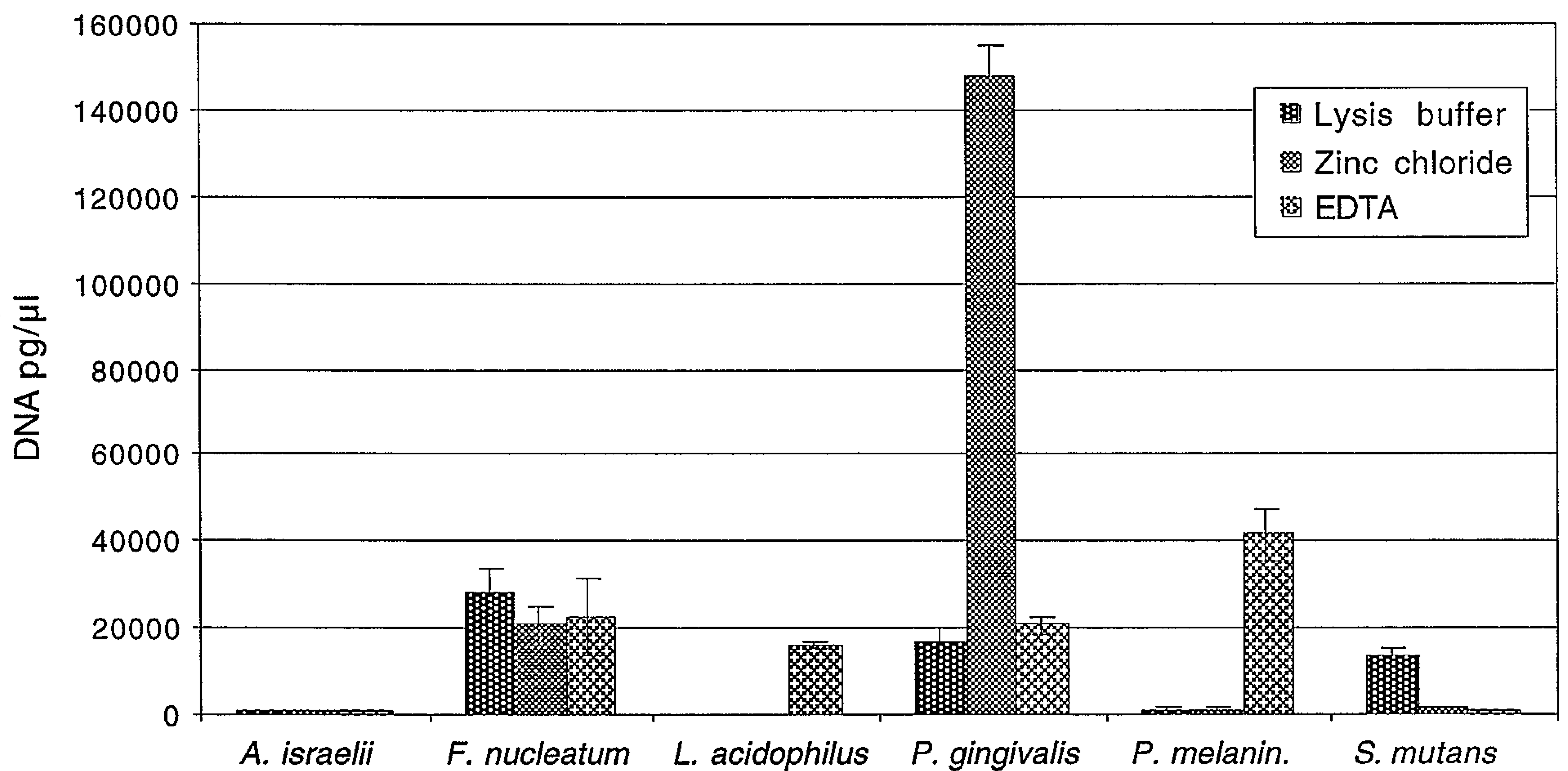


Figure 5.5. PCR quantitation of DNA from cultures of *A. israelii, F. nucleatum, L. acidophilus, P. gingivalis, P. melaninogenica* and *S. mutans* isolated in lysis buffer containing 10mM sodium phosphate, 1mg lysozyme ml^{-1} and 2mg mutanolysin ml^{-1}, with and without 5mM zinc chloride and 100mM EDTA. Data shows one representative experiment of two, expressed as mean values from triplicate readings ± SEM.

more DNA was recovered from *P. gingivalis* suspended in $ZnCl_2$, from *P. melaninogenica* and *L. acidophilus* in EDTA and from *S. mutans* in the lysis buffer (Figure 5.5).

5.2.2.6.2 Enzymatic, EDTA, EGTA, DEPC and combination methods

Following PCR most DNA was quantified from *F. nucleatum* suspended in the lysis buffer alone, and lysis buffer containing DEPC and EGTA/DEPC; for *L. acidophilus* the EDTA containing buffer; for *P. gingivalis* the DEPC containing buffer; for *P. melaninogenica* the EDTA/DEPC combination and for *A. israelii* and *S. mutans,* the lysis buffer (Figure 5.6).

Experiments from 5.2.2.6.1 and 5.2.2.6.2 were repeated three times as the DNA recovery from cultures of *A.israelii, P. gingivalis* and *L. acidophilus* varied depending on the culture batch used.

5.2.2.6.3 Extended incubation method

In an attempt to improve DNA recovery from the Gram-positive bacteria, while controlling nuclease activity, proteinase K was included in the lysis buffer and incubated for a prolonged period, with and without the addition of EDTA and $MgCl_2$. The gel image shows minimal DNA bands for *A. israelii* and obvious bands for *F. nucleatum, P. gingivalis* and *P. melaninogenica* for both procedures. However, when EDTA and $MgCl_2$ were absent a distinct band was seen for *S. mutans* but not for *L. acidophilus* (Figure 5.7a), whereas the reverse was seen when EDTA and $MgCl_2$ were present (Figure 5.7b).

5.2.2.6.4 MES buffer method

An acidic buffer (MES) with lysozyme and proteinase K was used in combination with $NaHCO_3$ and NaCl and an extended incubation to assist cell lysis of the Gram-positive microbes. The agarose gel images of the cultures prior to SDS treatment (Figure 5.8a) showed good recovery of DNA from only *S. mutans,* with less recovered from the mixture of *S. mutans* and *L. acidophilus,* and none recovered from *L. acidophilus*. However, following SDS treatment only minimal bands of DNA were noted for the Gram-negative bacterial cultures. The combination of *S. mutans* and *P. gingivalis* prior to SDS treatment produced a smeared image indicating a lack of *P. gingivalis* nuclease inhibition. Adding DEPC (Figure 5.8b) improved the recovery of *F. nucleatum, P. gingivalis* and the *S. mutans/P. gingivalis* combination prior to SDS

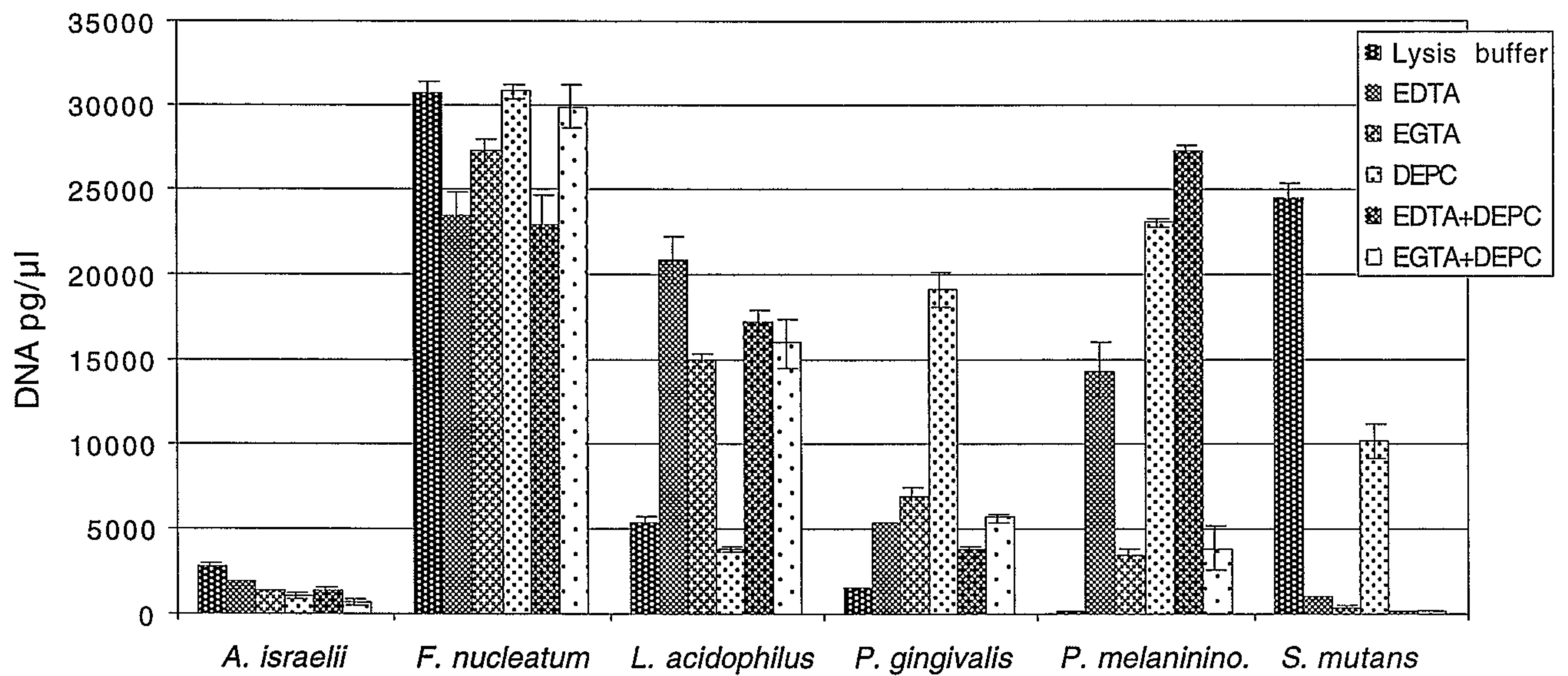
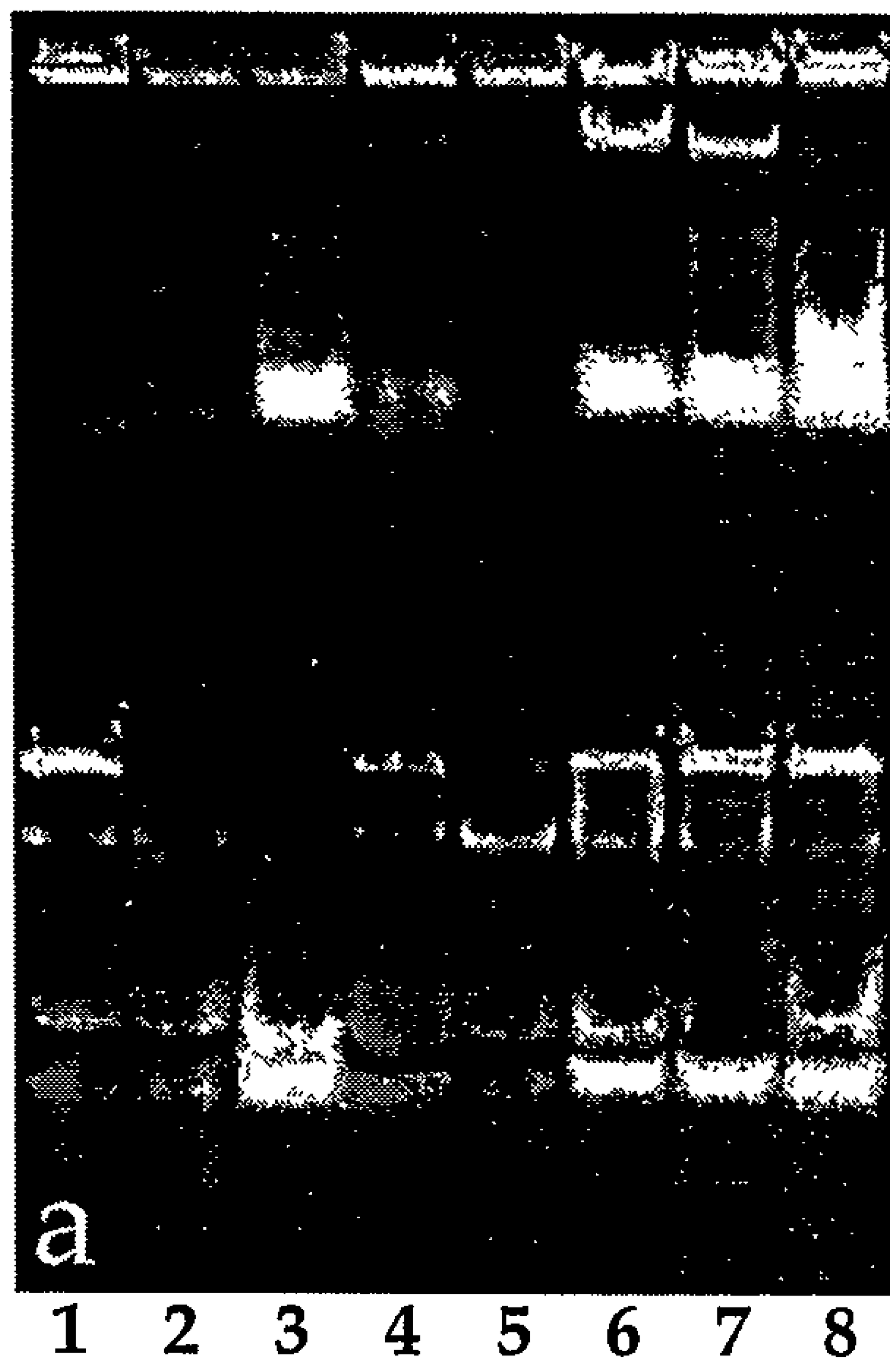
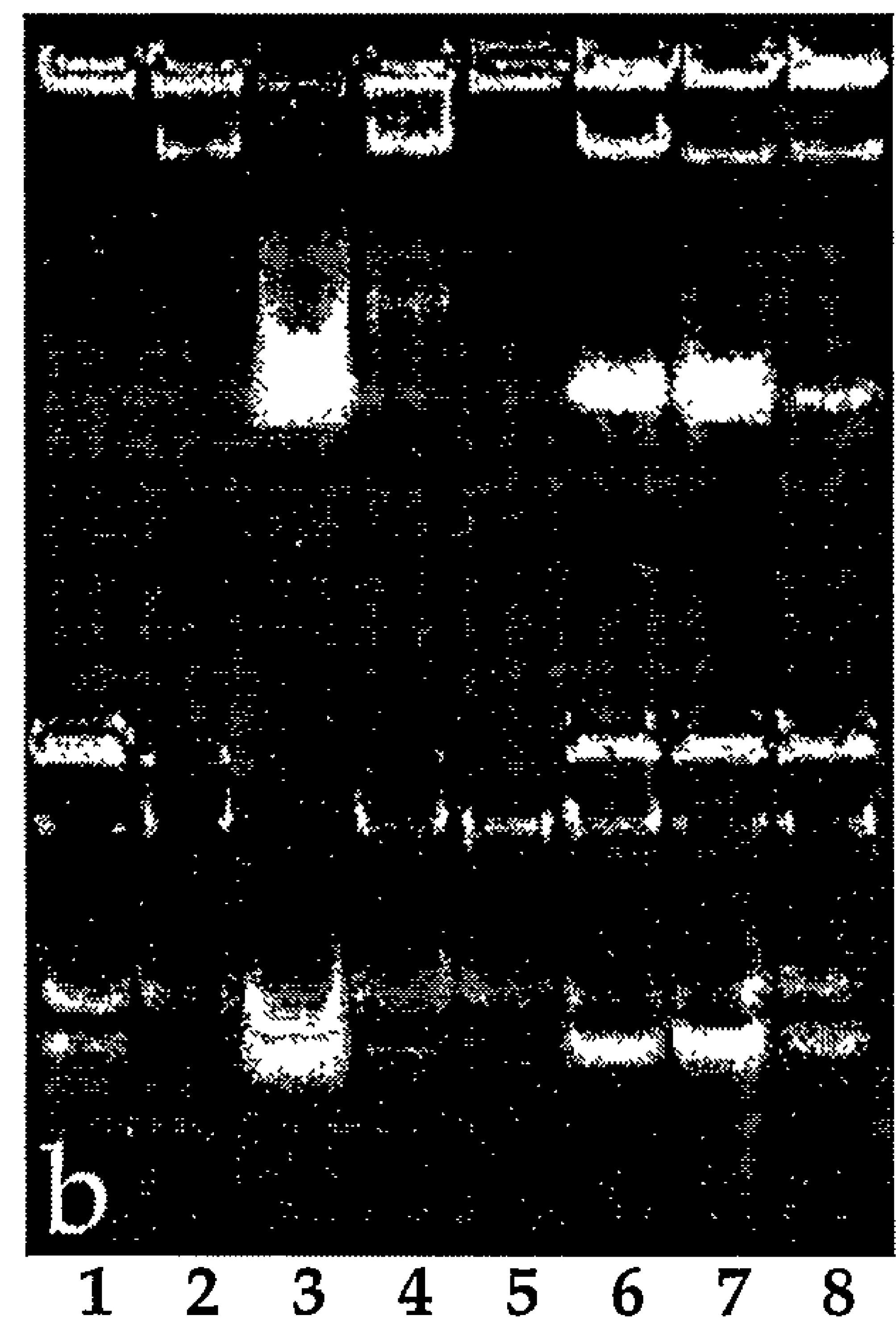


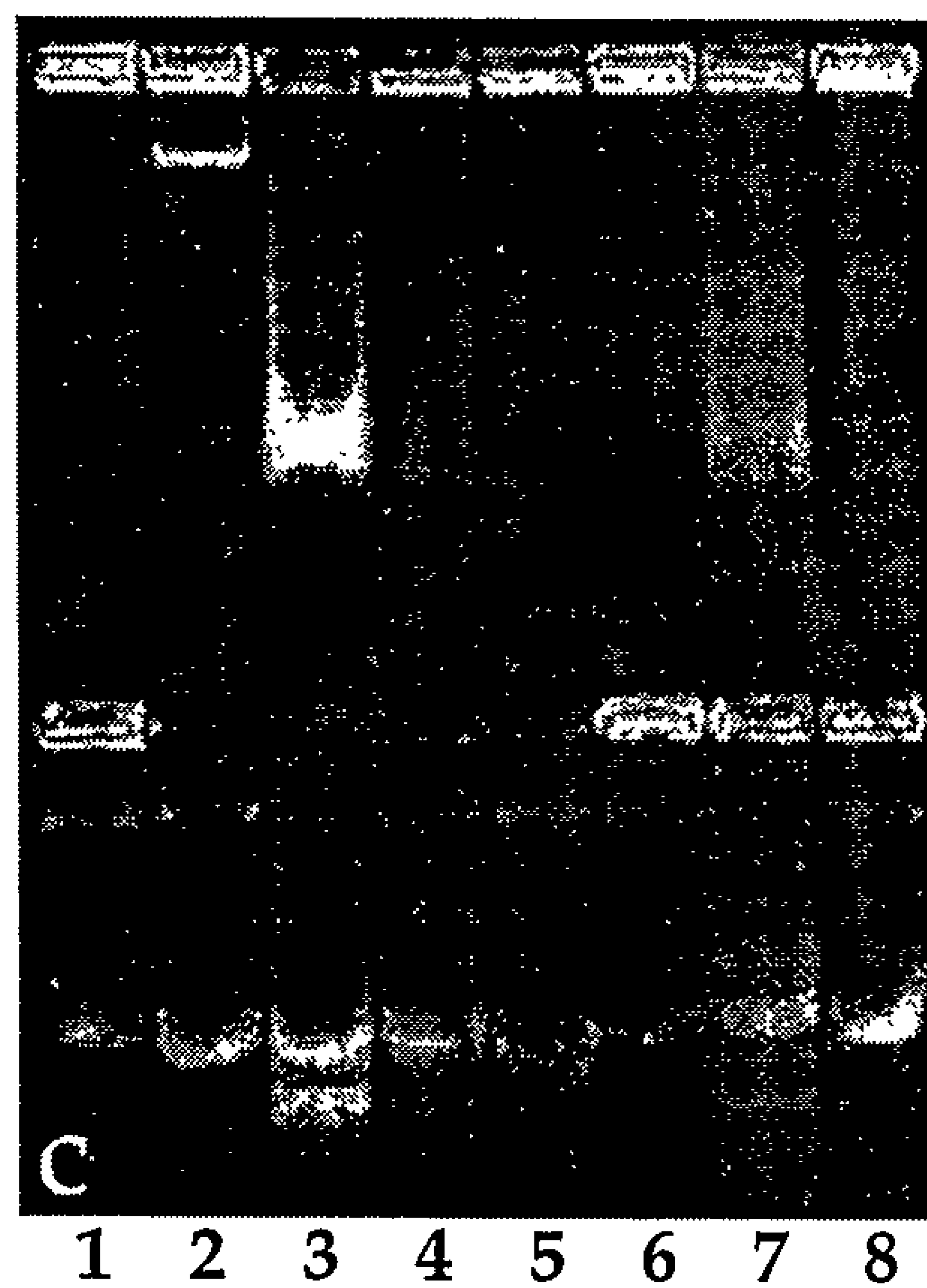
Figure 5.6. PCR quantitation of DNA isolated from cultures of *A. israelii, F. nucleatum, L. acidophilus P. gingivalis, P. melaninogenica* and *S. mutans* following incubation in lysis buffer containing 10mM sodium phosphate, 1mg lysozyme ml^{-1} and 2mg mutanolysin ml^{-1} with or without 100mM EDTA, 100mM EGTA, 20mM DEPC, 100mM EDTA/20mM DEPC or 100mM EGTA/20mM DEPC. Representative experiment of three, expressed as mean values from triplicate readings ± SEM.

Figure 5.7. Agarose gel electrophoresis of DNA from bacterial cultures isolated in 10mM sodium phosphate, 2mg lysozyme ml^{-1}, 2mg mutanolysin ml^{-1} and 2mg proteinase K ml^{-1} (Figure 5.7a) and with the addition of 100mM EDTA and 50mM $MgCl_2$ (Figure 5.7b).

Lane 1: *A. israelii;* Lane 2: *F. nucleatum;* Lane 3: *L. acidophilus;* Lane 4: *P. gingivalis;* Lane 5: *P. melaninogenica;* Lane 6: *S. mutans.*







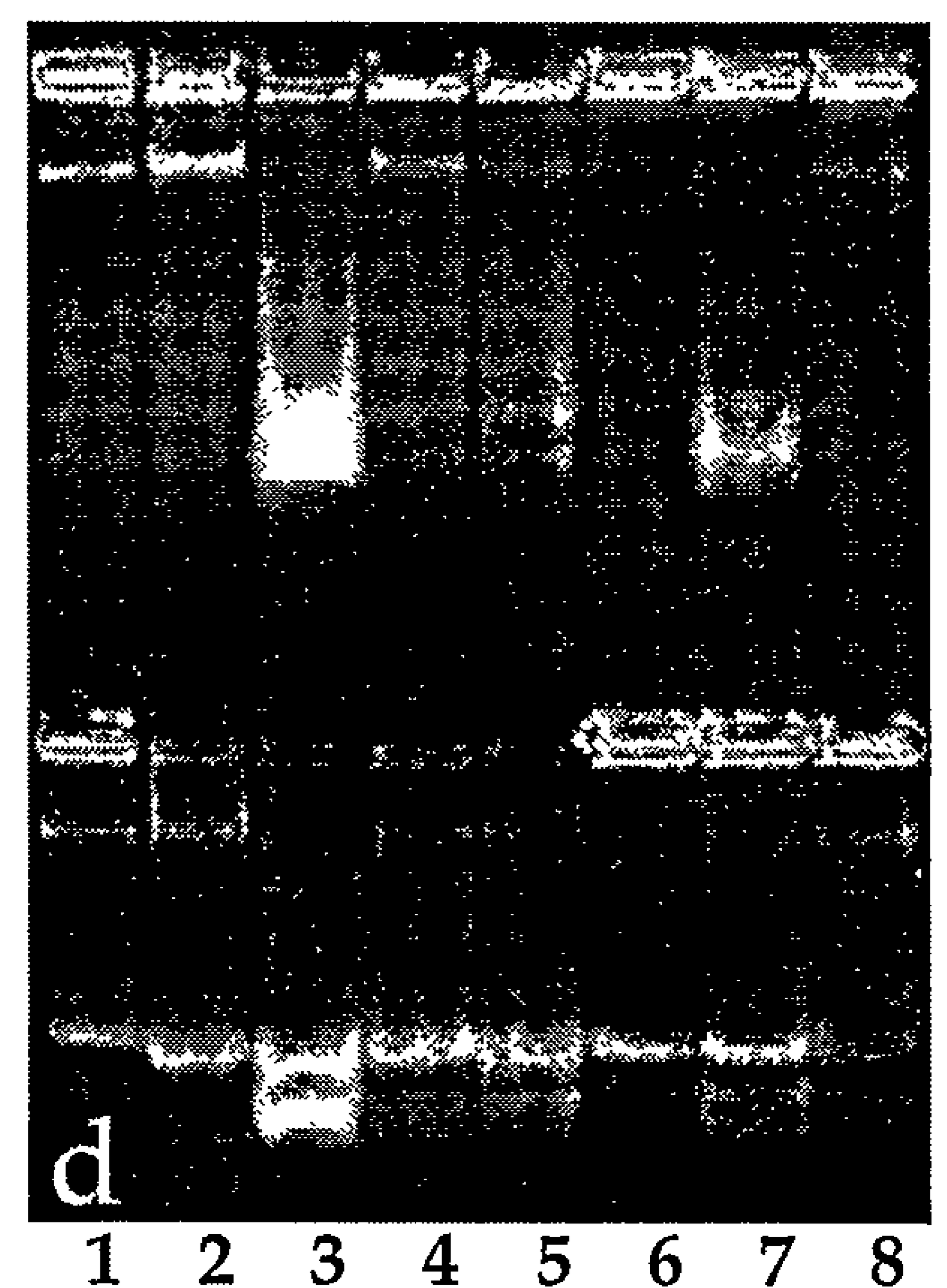


Figure 5.8. Agarose gel electrophoresis of DNA from bacterial cultures isolated in 25mM MES buffer containing 2mg lysozyme ml^{-1} and 2mg proteinase K ml^{-1} (Figure 5.8a), with the addition of 20mM DEPC (Figure 5.8b), 100mM EDTA (Figure 5.8c), or 20mM DEPC and 100mM EDTA (Figure 5.8d). The upper rows show DNA isolation prior to 1% SDS treatment, the lower rows show DNA isolation after SDS treatment..

Lane 1: *A. israelii;* Lane 2: *F. nucleatum;* Lane 3: *L. acidophilus;* Lane 4: *P. gingivalis;* Lane 5: *P. melaninogenica;* Lane 6: *S. mutans;* Lane 7; *L. acidophilus* and *S. mutans;* Lane 8: *P. gingivalis* and *S. mutans*. Figures 5.8c and 5.8d were provided by Dr M Nadkarni.

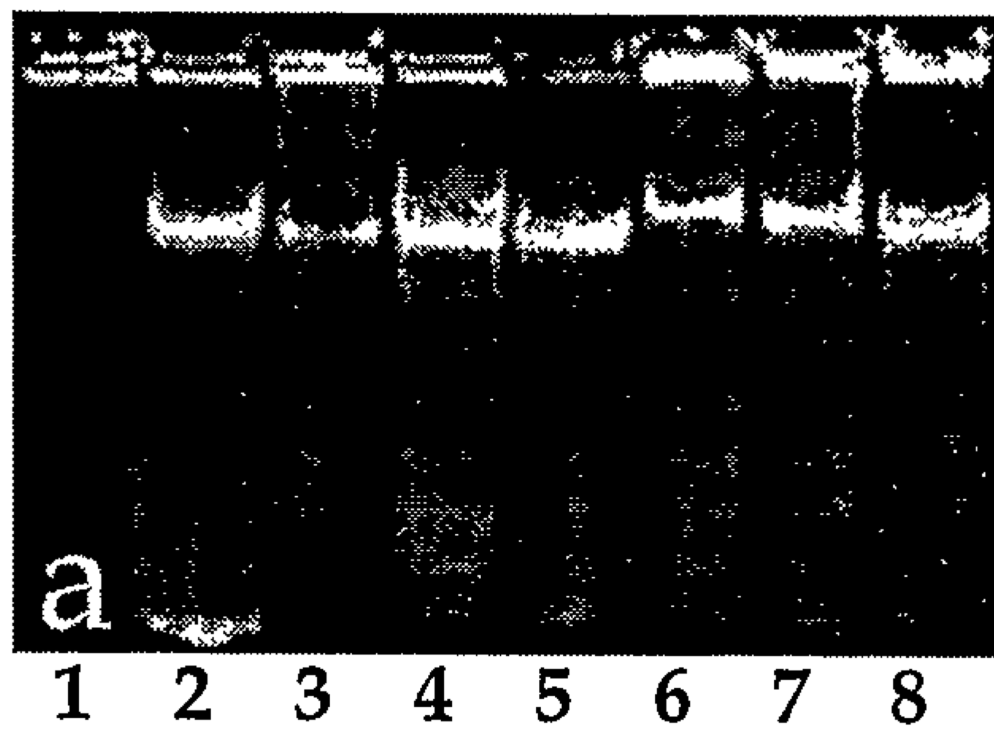
treatment. The addition of EDTA (Figure 5.8c) recovered only *F. nucleatum* and the addition of DEPC/EDTA (Figure 5.8d) assisted the DNA extraction of *A. israelii, F. nucleatum* and *P. gingivalis.* Minimal DNA was recovered from the remaining Gram-negative bacteria after SDS treatment and marked smearing of the DNA was noted with *L. acidophilus* for each method. This experiment was repeated three times as the results for *P. gingivalis* and *L. acidophilus* isolation varied with different culture batches.

5.2.2.6.5 Comparison of buffers

Using a modified form of the previous experiment including mutanolysin and DEPC, the action of MES was compared with phospate buffer with an incubation period of 15 min. Following incubation in phosphate buffer (Figure 5.9a), the agarose gel showed bands for all cultures except *A. israelii;* those bands for *L. acidophilus* and *S. mutans,* individually or combined, were less intensely stained than the bands of the Gram-negative bacteria. Following incubation in MES buffer the Gram-negative bacteria showed bands with intense staining, while those for the Gram-positive bacteria were quite faint (Figure 5.9b).

5.2.2.6.6 Lysis buffer with additional DNase inhibitors

DNA bands were seen in the agarose gel images for all cultures with each of the combinations (10mM ascorbic acid, 0.1mM aurin tricarboxylic acid and 50mM trisodium citrate), however, considerable smearing was noted for all except *A. israelii* and *M. micros* (Figures 5.10a-c). With the addition of DEPC (Figures 5.10d-g), the combinations showed increased staining intensity for all cultures except *A. israelii.* Following pulse sonication (Figures 5.10h-k), deterioration of the band intensities was noted for all combinations except ascorbic acid/trisodium citrate/DEPC (Figure 5.10k). Of the various combinations, ascorbic acid/DEPC and ascorbic acid/citric acid/DEPC/6 min pulse sonication showed the most intensely stained bands from each of the cultures. However, following purification of the samples, DNA from the Gram-negative bacteria appeared in the gels with marked smearing and from the Gram-positive bacteria as faint bands (Figures 5.10l, m).



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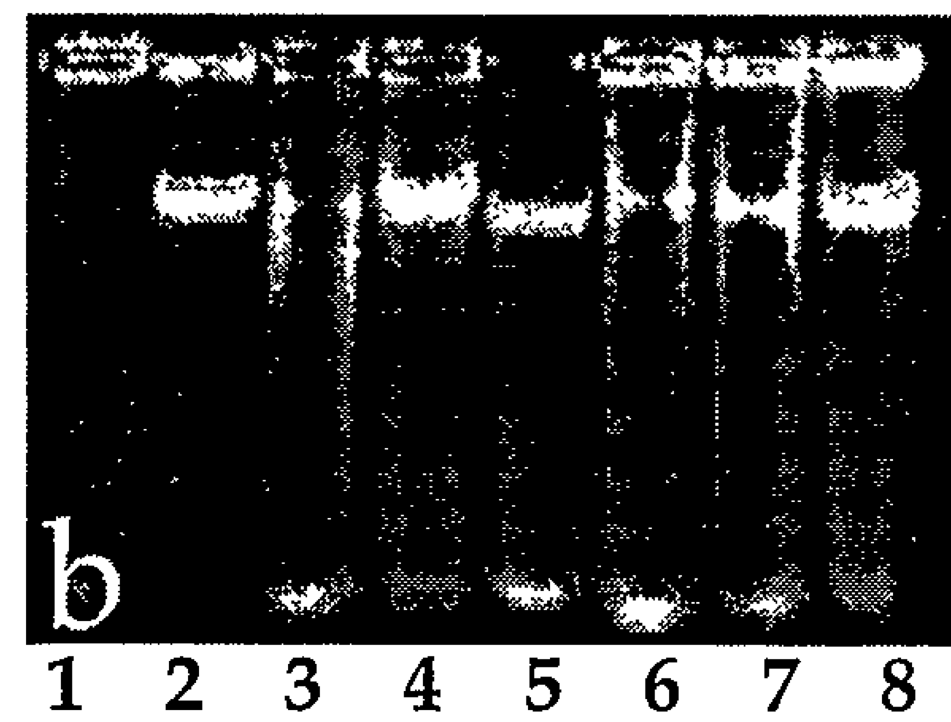
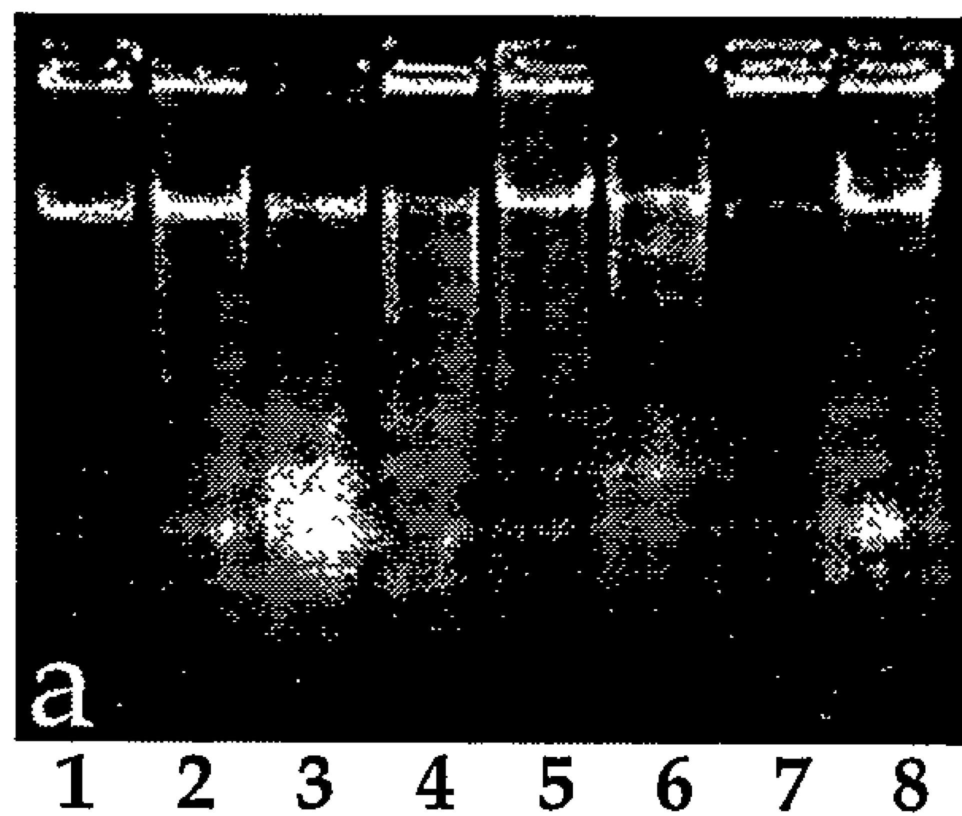


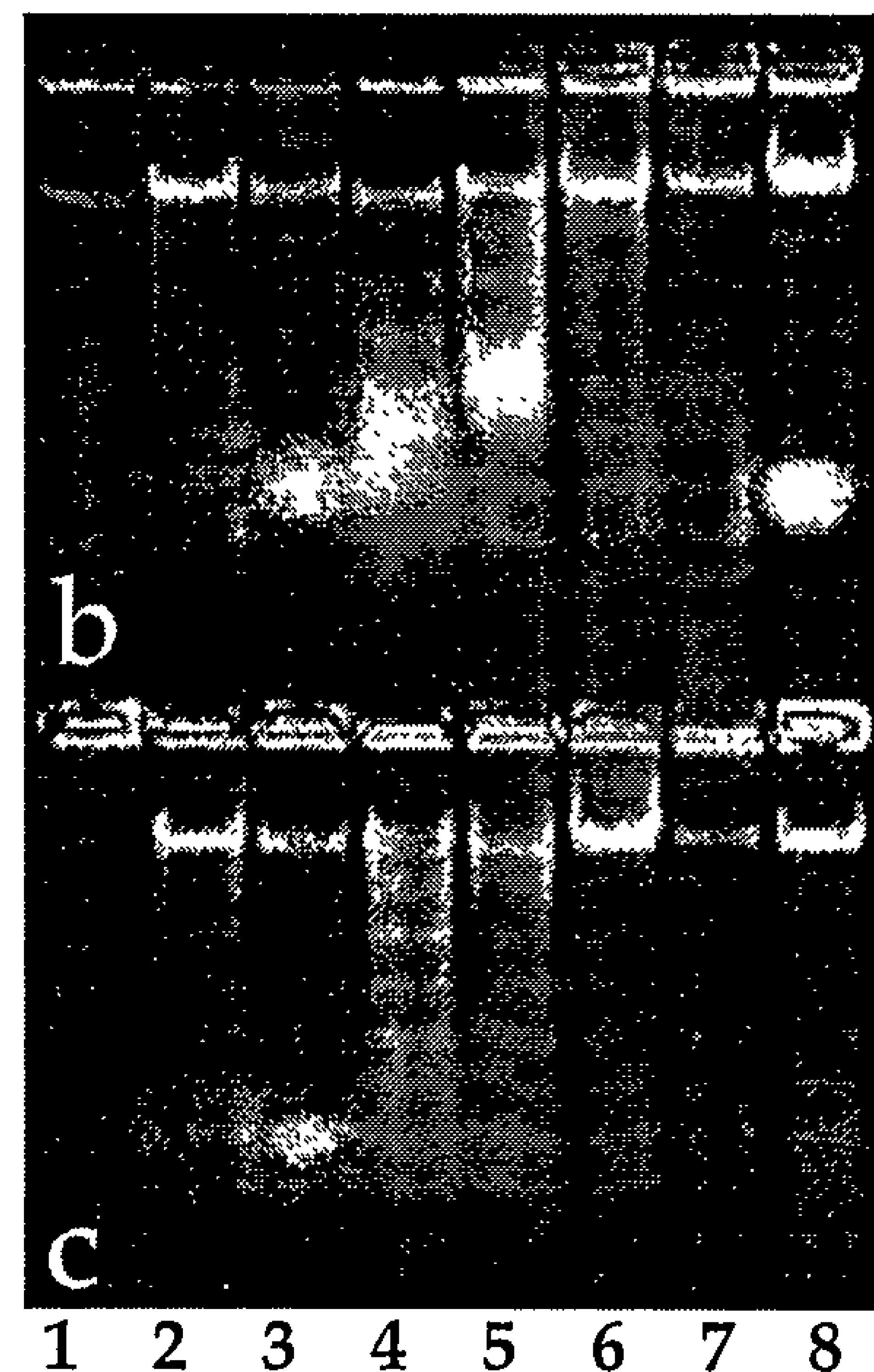
1 2 3 4 5 6 7 8

Figure 5.9. Agarose gel electrophoresis of DNA from bacterial cultures isolated in 10mM sodium phosphate buffer (Figure 5.9a) and MES buffer (Figure 5.9b) containing 2mg lysozyme ml^{-1}, 2mg mutanolysin ml^{-1}, 2mg proteinase K ml^{-1} and 20mM DEPC.

Lane 1: *A. israelii;* Lane 2: *F. nucleatum;* Lane 3: *L. acidophilus;* Lane 4: *P. gingivalis;* Lane 5: *P. melaninogenica;* Lane 6: *S. mutans;* Lane 7: *L. acidophilus* and *S. mutans;* Lane 8: *P. gingivalis* and *S. mutans.*



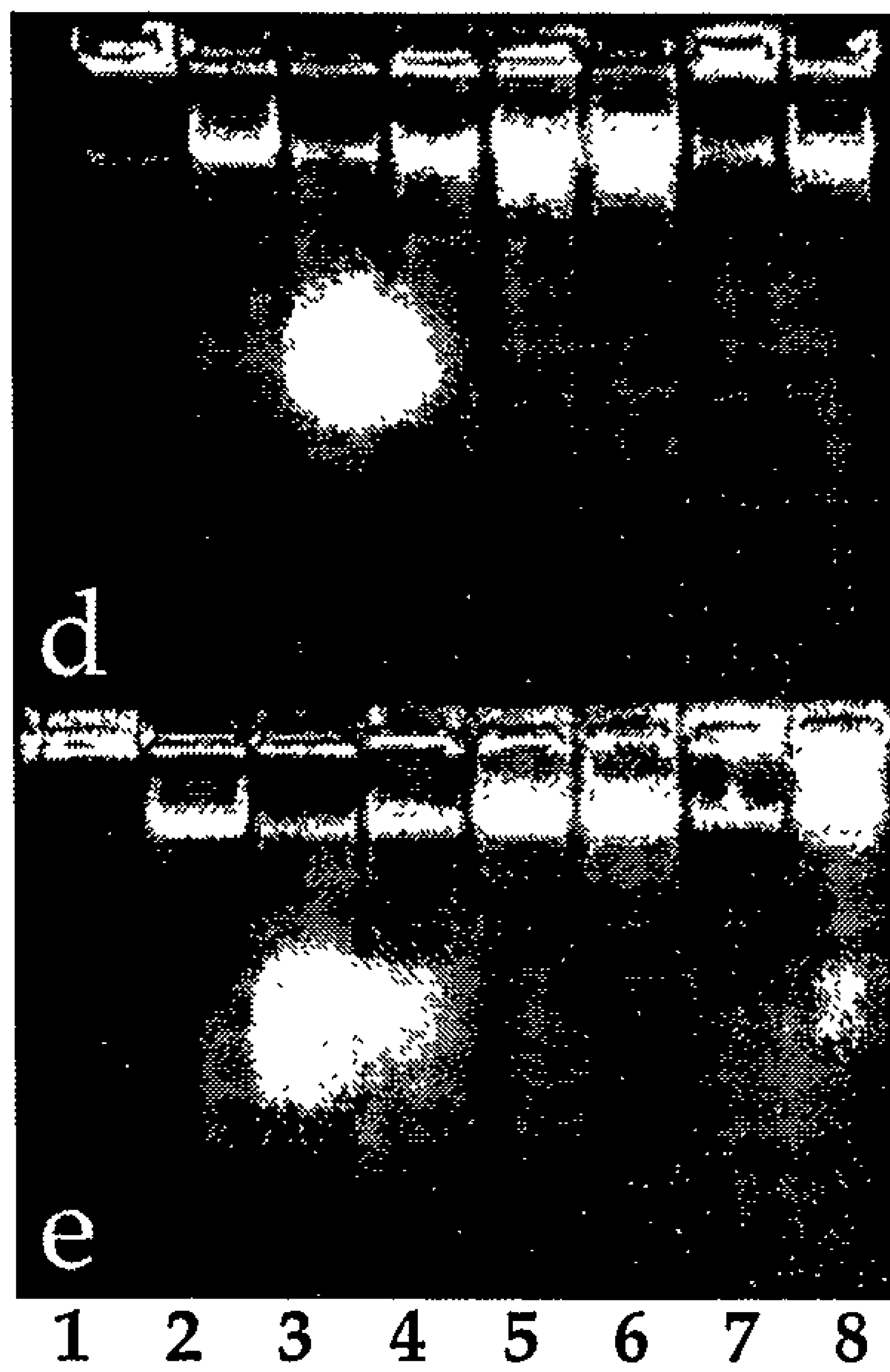
1 2 3 4 5 6 7 8



1 2 3 4 5 6 7 8

Figure 5.10a-c. Agarose gel electrophoresis of DNA from bacterial cultures isolated in 10mM sodium phosphate buffer containing 2mg lysozyme ml^{-1}, 2mg mutanolysin ml^{-1} and 2mg proteinase K ml^{-1} with the addition of 10mM ascorbic acid (Figure 5.10a), 0.1mM aurin tricarboxylic acid (Figure 5.10b) or 50mM trisodium citrate (Figure 5.10c).

Lane 1: *A. israelii;* Lane 2: *F. nucleatum;* Lane 3: *L. acidophilus;* Lane 4: *P. endodontalis;* Lane 5: *P. gingivalis;* Lane 6: *P. melaninogenica;* Lane 7: *M. micros;* Lane 8: *S. mutans.*



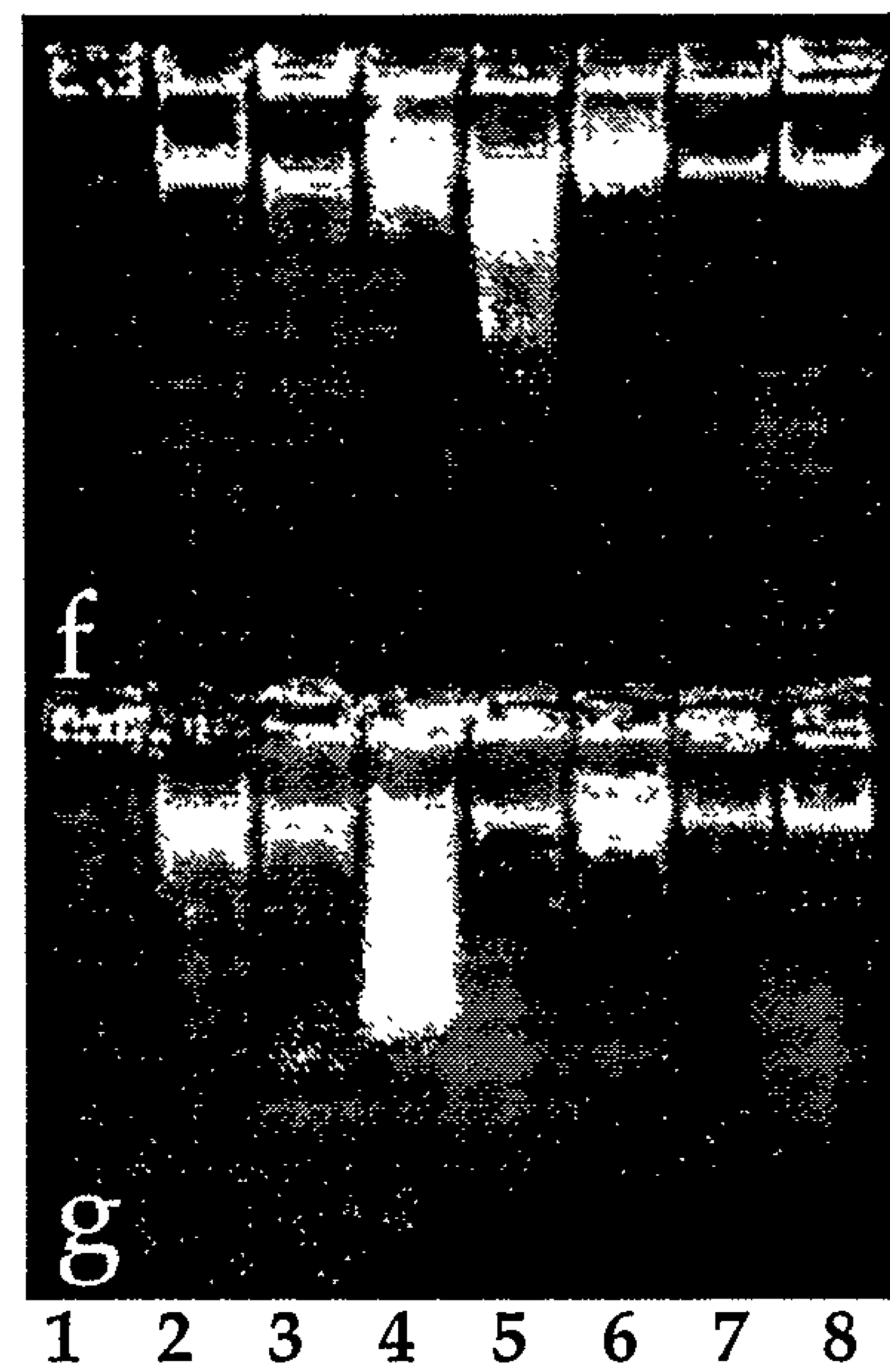
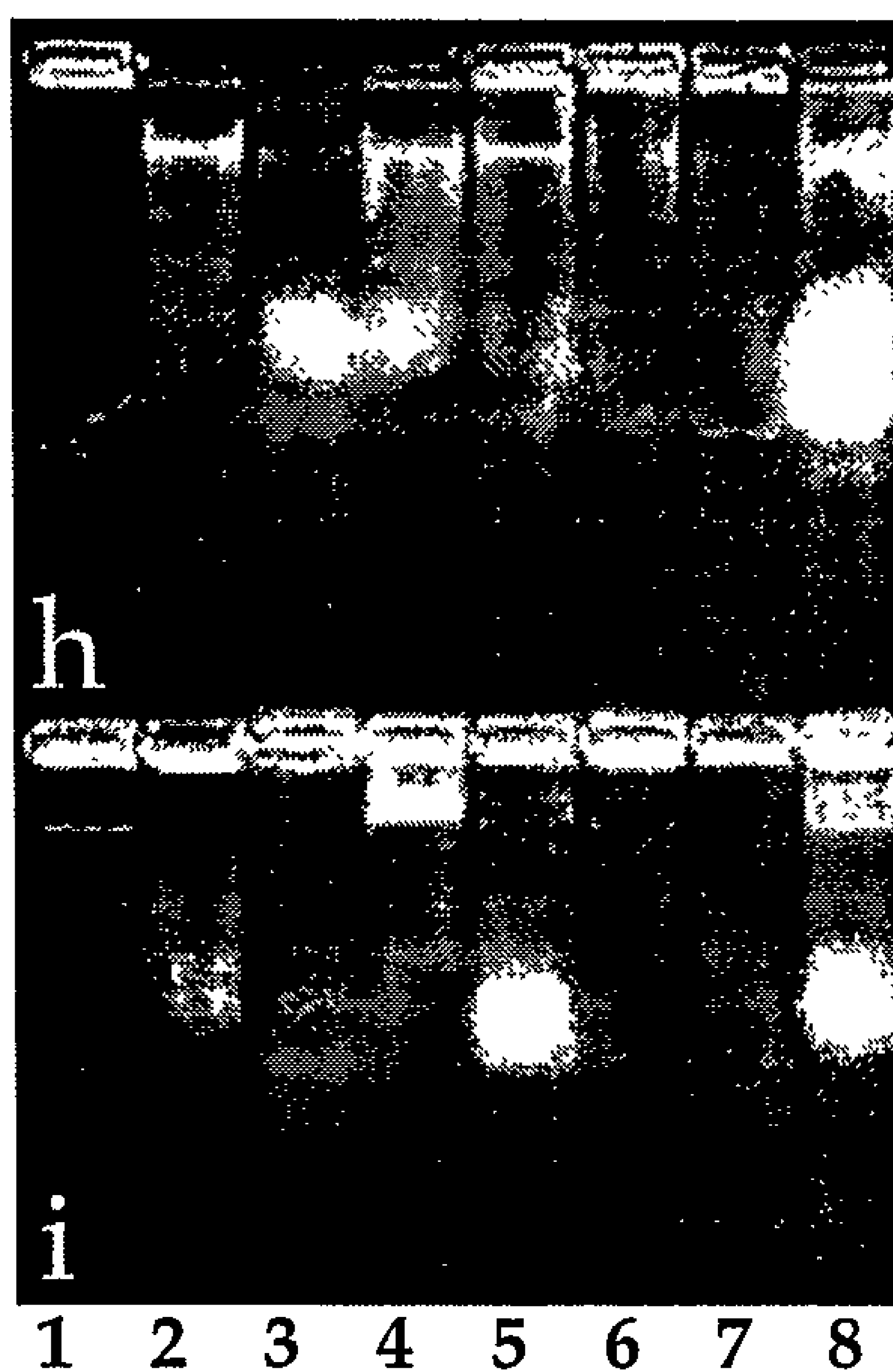


Figure 5.10d-g. Agarose gel electrophoresis of DNA from bacterial cultures isolated in 10mM sodium phosphate buffer containing 2mg lysozyme ml^{-1}, 2mg mutanolysin ml^{-1}, 2mg proteinase K ml^{-1} and 20mM DEPC with 10mM ascorbic acid (Figure 5.10d), 0.1mM aurin tricarboxylic acid (Figure 5.10e), 50mM trisodium citrate (Figure 5.10f) or 10mM ascobic acid plus 50mM trisodium citrate (Figure 5.10g).

Lane 1: *A. israelii;* Lane 2: *F. nucleatum;* Lane 3: *L. acidophilus;* Lane 4: *P. endodontalis;* Lane 5: *P. gingivalis;* Lane 6: *P. melaninogenica;* Lane 7: *M. micros;* Lane 8: *S. mutans.*



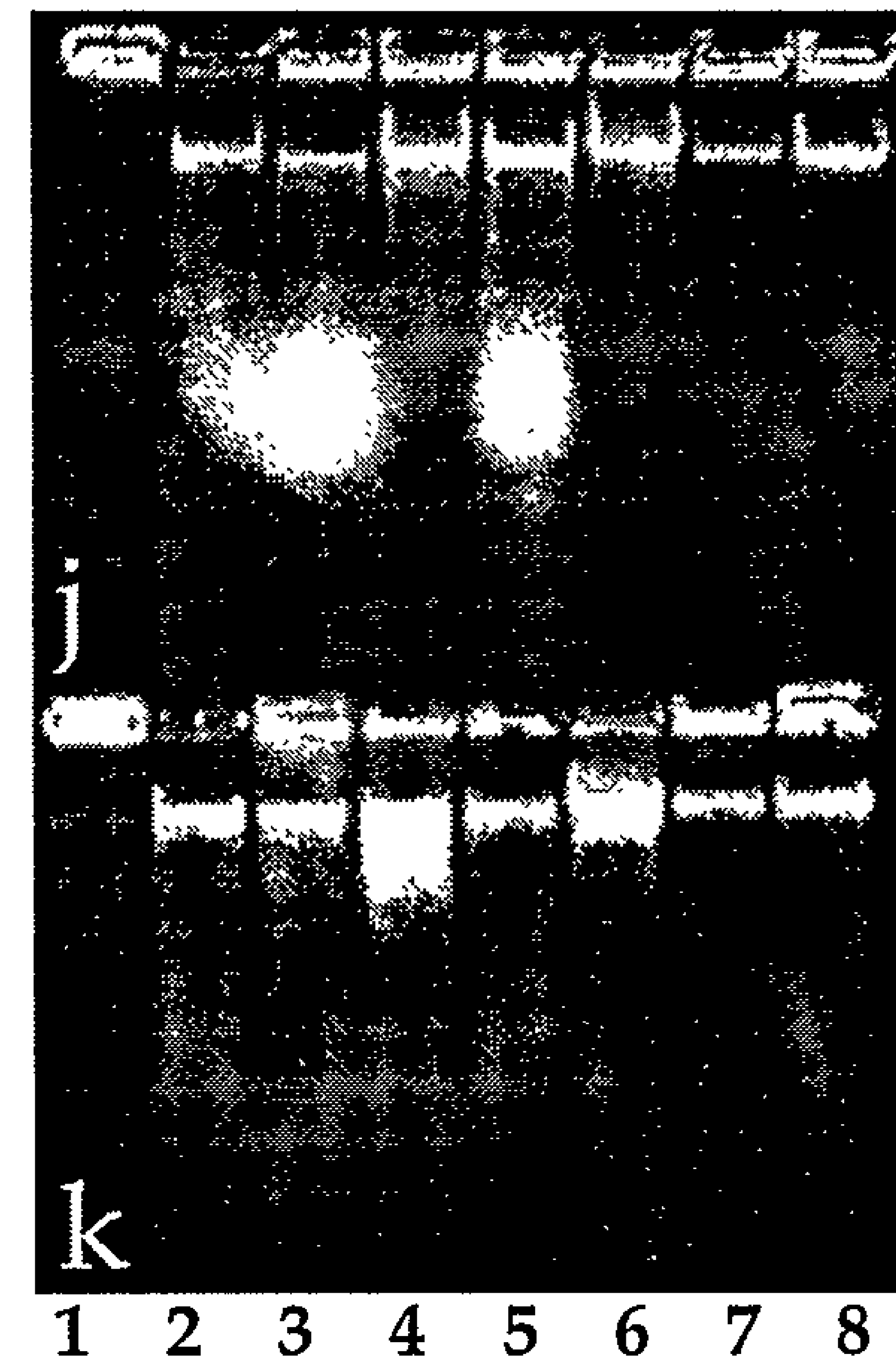
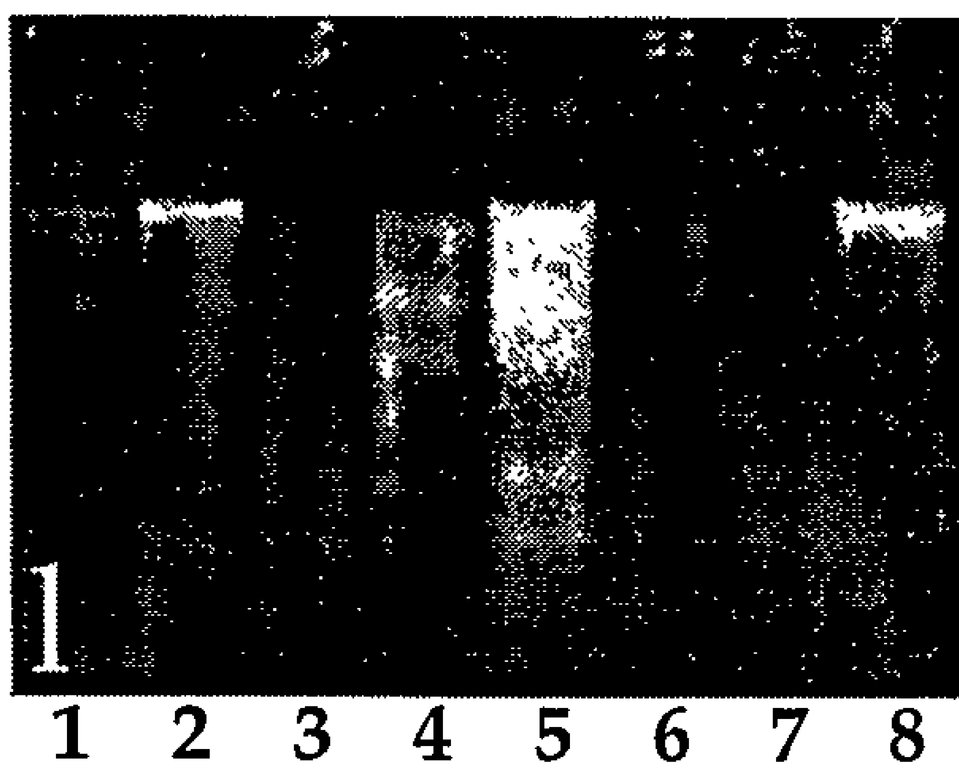


Figure 5.10h-k. Agarose gel electrophoresis of DNA from bacterial cultures isolated in 10mM sodium phosphate buffer containing 2mg lysozyme ml^{-1}, 2mg mutanolysin ml^{-1}, 2mg proteinase

K ml^{-1} and 20mM DEPC with 10mM ascorbic acid (Figure 5.10h), 0.1mM aurin tricarboxylic acid (Figure 5.10i), 50mM trisodium citrate (Figure 5.10j) for 10mM ascobic acid plus 50mM trisodium citrate (Figure 5.10k) with 6min pulse sonication.
Lane 1: *A. israelii;* Lane 2: *F. nucleatum;* Lane 3: *L. acidophilus;* Lane 4: *P. endodontalis;* Lane 5: *P. gingivalis;* Lane 6: *P. melaninogenica;* Lane 7: *M. micros;* Lane 8: *S. mutans.*



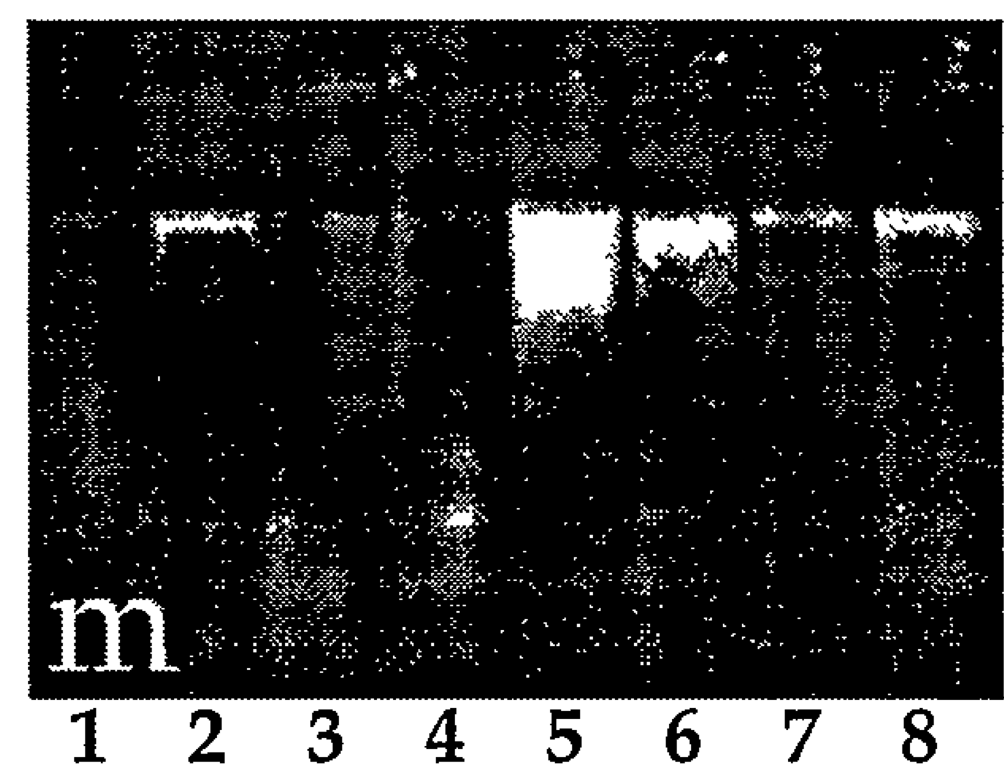


Figure 5.10l-m. Agarose gel electrophoresis of DNA from bacterial cultures isolated in 10mM sodium phosphate buffer containing 2mg lysozyme ml^{-1}, 2mg mutanolysin ml^{-1}, 2mg proteinase K ml^{-1} and 20mM DEPC with 10mM ascorbic acid (Figure 5.10l) or 10mM ascorbic acid plus 50mM trisodium citrate with 6min pulse sonication (Figure 5.10m) following purification.
Lane 1: *A. israelii;* Lane 2: *F. nucleatum;* Lane 3: *L. acidophilus;* Lane 4: *P. endodontalis;* Lane 5: *P. gingivalis;* Lane 6: *P. melaninogenica;* Lane 7: *M. micros;* Lane 8: *S. mutans.*

5.2.2.6.7 Varying the lysozyme concentration

In this experiment the concentration of lysozyme was varied and the procedure was separated into two parts; initially the culture pellets were suspended in buffer and DEPC, then following incubation or sonication the enzymes were added. To provide a comparison, another group of cultures was suspended in buffer with DEPC plus enzymes for 20min storage on ice. Results from the agarose gels showed an increased staining intensity of the DNA bands for those cultures isolated in buffer containing 5mg lysozyme ml^{-1}, particularly for *A. israelii, P. endodontalis* and *P. melaninogenica* compared with 1mg lysozyme ml^{-1} (Figures 5.11a-d). The results following storage of the cultures on ice with the later addition of the enzymes, showed an increase in the recovery for *A. israelii* and *M. micros* for both methods, and in 1mg lysozyme ml^{-1} an increased detection for *P. melaninogenica,* although with reduced detection of *P. endodontalis* (Figures 5.11a-d). Following sonication prior to the addition of enzymes (Figures 5.11e-h) the gel images showed a loss of intensity for *M. micros* following both pulse and continuous sonication, while for *F. nucleatum* (5mg lysozyme ml^{-1}) and *P. gingivalis* (1mg and 5mg lysozyme ml^{-1}) loss of staining intensity resulted from continuous sonication. The remaining sonicated cultures produced bands that appeared similar to those stored on ice for 20 min in the two-stage procedure.

5.2.2.6.8 Isolation of DNA in DEPC

Purified DNA preparations extracted in the absence of DEPC (Figure 5.12a) were visualised in an agarose gel and showed bands for *F. nucleatum, P. endodontalis,* and *S. mutans,* and faint bands were seen for *P. melaninogenica, M. micros* and *L. acidophilus.* DNA extracted in the presence of 20mM DEPC (Figure 5.12b) showed DNA bands evident for *F. nucleatum, P. endodontalis, P. gingivalis P. melaninogenica* and *S. mutans.* Without DEPC in the suspension, *P. gingivalis* could not be recovered, however, the inclusion of DEPC reduced the recovery of *F. nucleatum, M. micros* and *S. mutans* (Figures 5.12a and b).

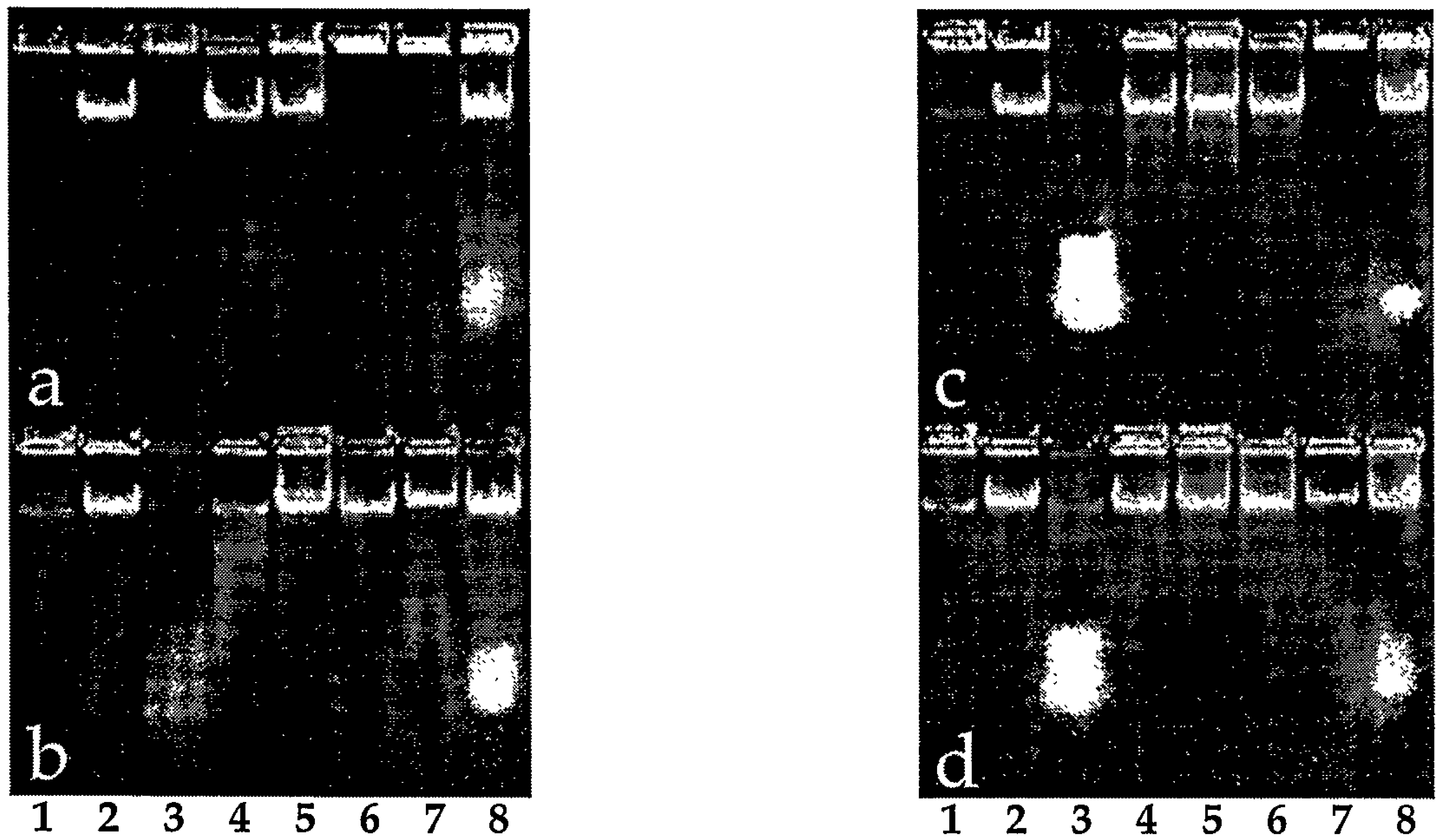
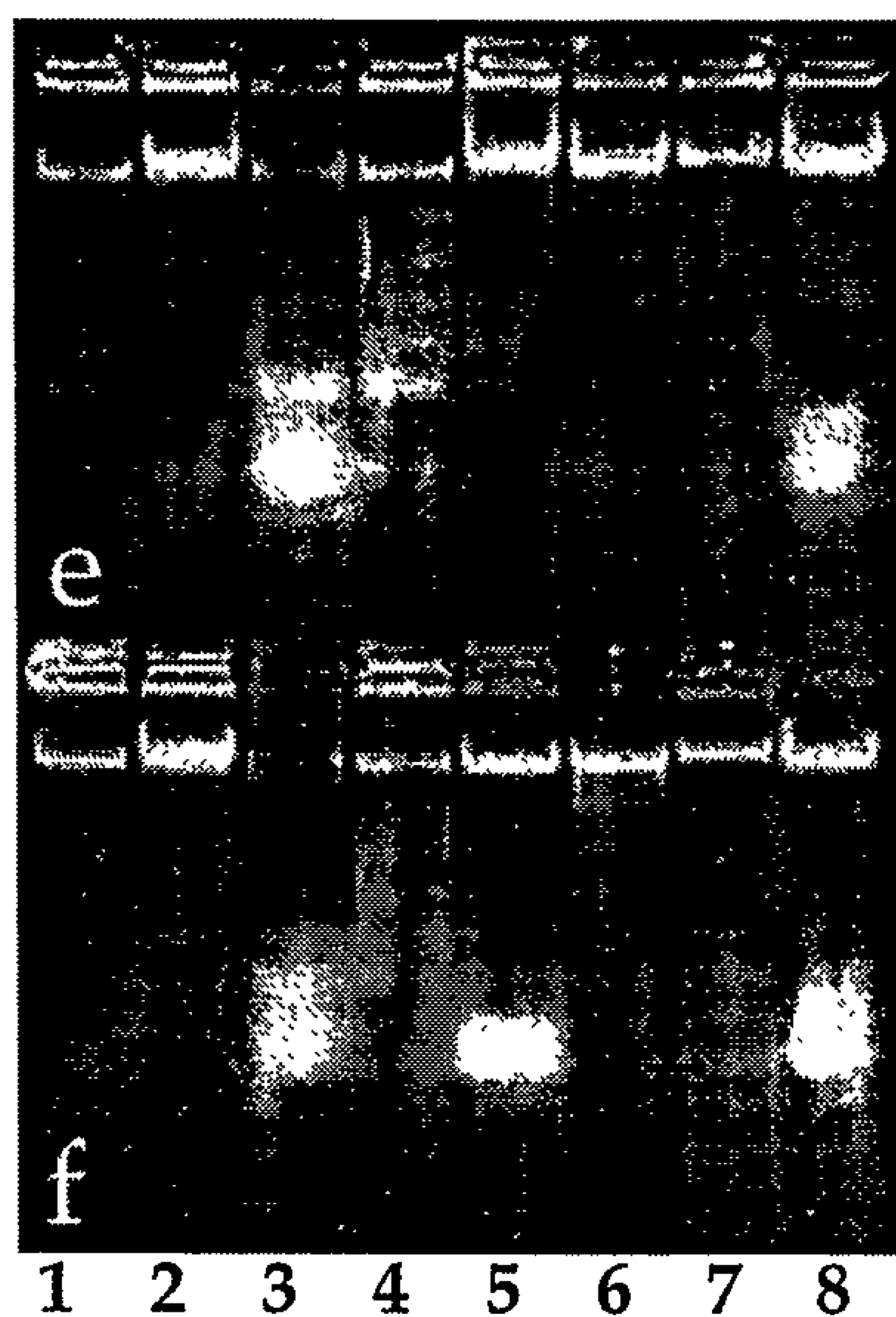


Figure 5.11a-d. Agarose gel electrophoresis of DNA from bacterial cultures isolated in 10mM sodium phosphate buffer containing, 2mg mutanolysin ml^{-1}, 2mg proteinase K ml^{-1}, 20mM DEPC with 1mg lysozyme ml^{-1} (Figure 5.11a, b) or 5mg lysozyme ml^{-1} (Figure 5.11c, d). The cultures from Figure 5.11a and Figure 5.11c were suspended in the lysis buffer and stored for 20min on ice, those cultures from Figure 5.11b and Figure 5.11d were suspended in 10mM sodium phosphate and 20mM DEPC and stored on ice for 20min prior to addition of the enzymes. Lane 1: *A. israelii;* Lane 2: *F. nucleatum;* Lane 3: *L. acidophilus;* Lane 4: *P. endodontalis;* Lane 5: *P. gingivalis;* Lane 6: *P. melaninogenica;* Lane 7: *M. micros;* Lane 8: *S. mutans.*



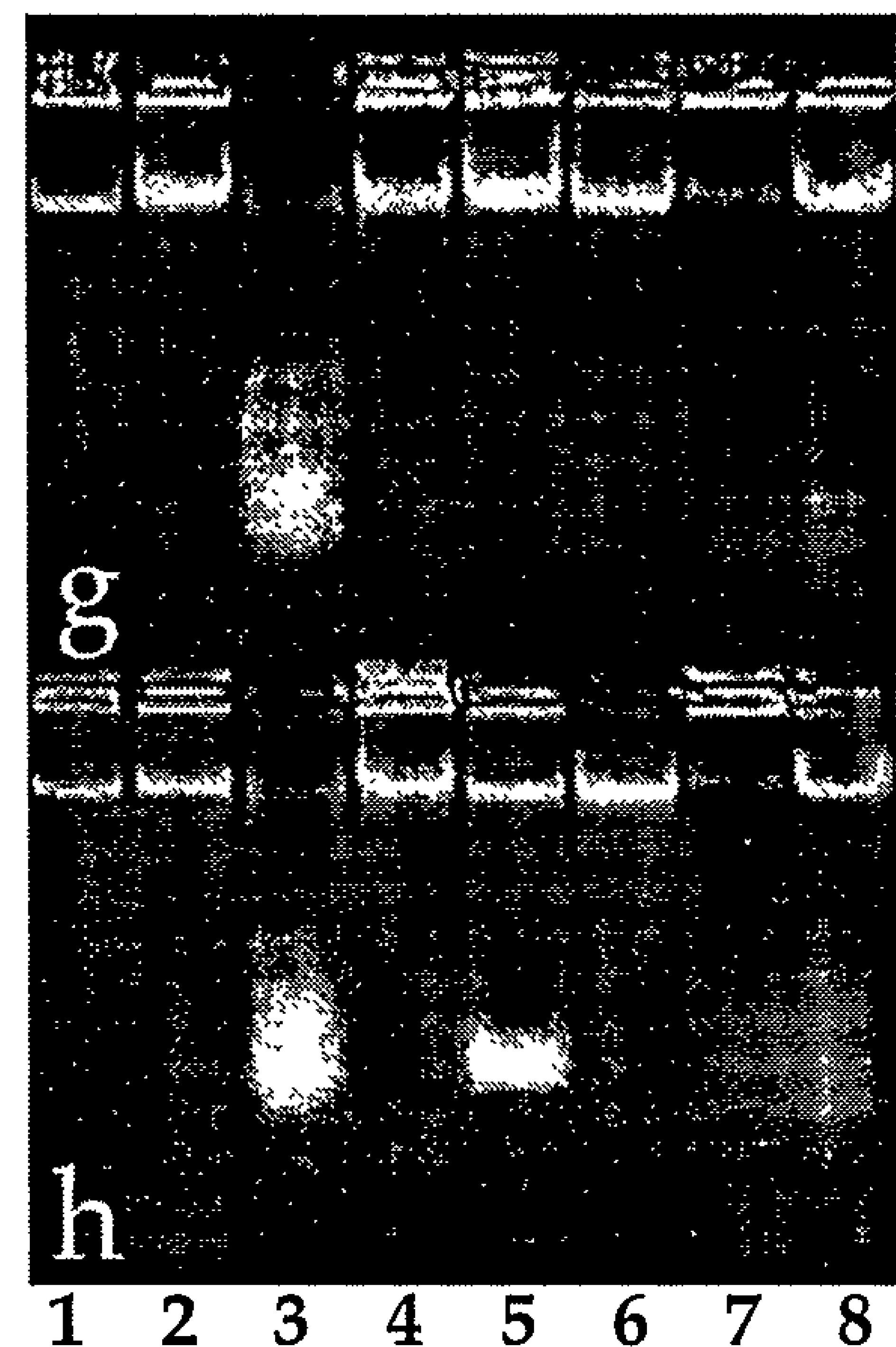


Figure 5.11e-h. Agarose gel electrophoresis of DNA from bacterial cultures isolated in 10mM sodium phosphate buffer containing, 2mg mutanolysin ml^{-1}, 2mg proteinase K ml^{-1}, 20mM DEPC with 1mg lysozyme ml^{-1} (Figure 5.11e,f) or 5mg lysozyme ml^{-1} (Figure 5.11g,h). The cultures from Figure 5.11e and Figure 5.11g were suspended in 10mM sodium phosphate and 20mM DEPC and sonicated in pulse mode for 6min prior to the addition of the enzymes, those cultures from Figure 5.11f and Figure 5.11h were suspended in 10mM sodium phosphate and 20mM DEPC and sonicated in continuous mode for 6min prior to addition of the enzymes.

Lane 1: *A. israelii;* Lane 2: *F. nucleatum;* Lane 3: *L. acidophilus;* Lane 4: *P. endodontalis;* Lane 5: *P. gingivalis;* Lane 6: *P. melaninogenica;* Lane 7: *M. micros;* Lane 8: *S. mutans.*

Figures 5.11f and 5.11h were provided by Dr M Nadkarni.

5.2.2.6.9 Isolation of DNA using a modified DEPC method

Quantitation of DNA using the DEPC method containing lysis buffer comparing 6 min pulse or continuous sonication with storage on ice showed similar DNA recovery following all procedures, except for *P. melaninogenica* where 6min pulse sonication provided higher DNA quantitation (Figure 5.13). The recovery of *A. israelii*, although improved following sonication, was still low compared with the other cultures. This experiment was repeated three times, and in separate instances, improved DNA recovery from *A. israelii* and *L. acidophilus* was achieved, but this data was not reproducible and varied with the culture batch used.

5.2.2.6.10 Isolation of DNA using ATL buffer from the QIAamp DNA Mini Kit

PCR quantitation of cultures incubated in ATL buffer prior to purification showed improved DNA recovery for all Gram-negative bacteria when compared with data from the same cultures following isolation in DEPC with different procedures (Figure 5.13). However, the amount of DNA retrieved from the Gram-positive cultures was almost negligible. Agarose gel images from DEPC 6min pulse prepared cultures showed an intensely stained band representing *S. mutans* with other preparations producing smearing, except for *A. israelii* and *L. acidophilus* which produced faint bands (Figure 5.14a). The ATL-treated cultures exhibited distinct DNA bands for the Gram-negative bacteria and a faint band for *M. micros* (Figure 5.14b).

5.2.2.6.11 Effect of $ZnCl_2$ as an inhibitor of PCR.

Cultures of *P. gingivalis* isolated in the presence of 5mM $ZnCl_2$ and diluted 100-fold (final concentration 0.05mM) prior to PCR showed no inhibition of the PCR reaction. However, with approximately the same number of cells in a final concentration of 0.5mM $ZnCl_2$, DNA quantitation was reduced by approximately 100-fold and in a concentration of 5mM $ZnCl_2$, DNA recovery was completely inhibited (Figure 5.15). Following purification with the QIAamp DNA Mini Kit, the inhibitory action of 0.5mM $ZnCl_2$ was controlled, however, the 5mM concentration still incurred some inhibition despite purification.

Figure 5.12. Agarose gel electrophoresis of DNA from bacterial cultures isolated in 10mM sodium phosphate buffer containing 5mg lysozyme ml^{-1}, 2mg mutanolysin ml^{-1} and 2mg proteinase K ml^{-1} (Figure 5.12a) and with the addition of 20mM DEPC (Figure 5.12b).
Lane 1: *F. nucleatum;* Lane 2: *P. endodontalis;* Lane 3: *P. gingivalis;* Lane 4: *P. melaninogenica;* Lane 5: *M. micros;* Lane 6: *S. mutans;* Lane 7: *L. acidophilus;* Lane 8: *A. israelii.* Gel images provided by Dr M Nadkarni.

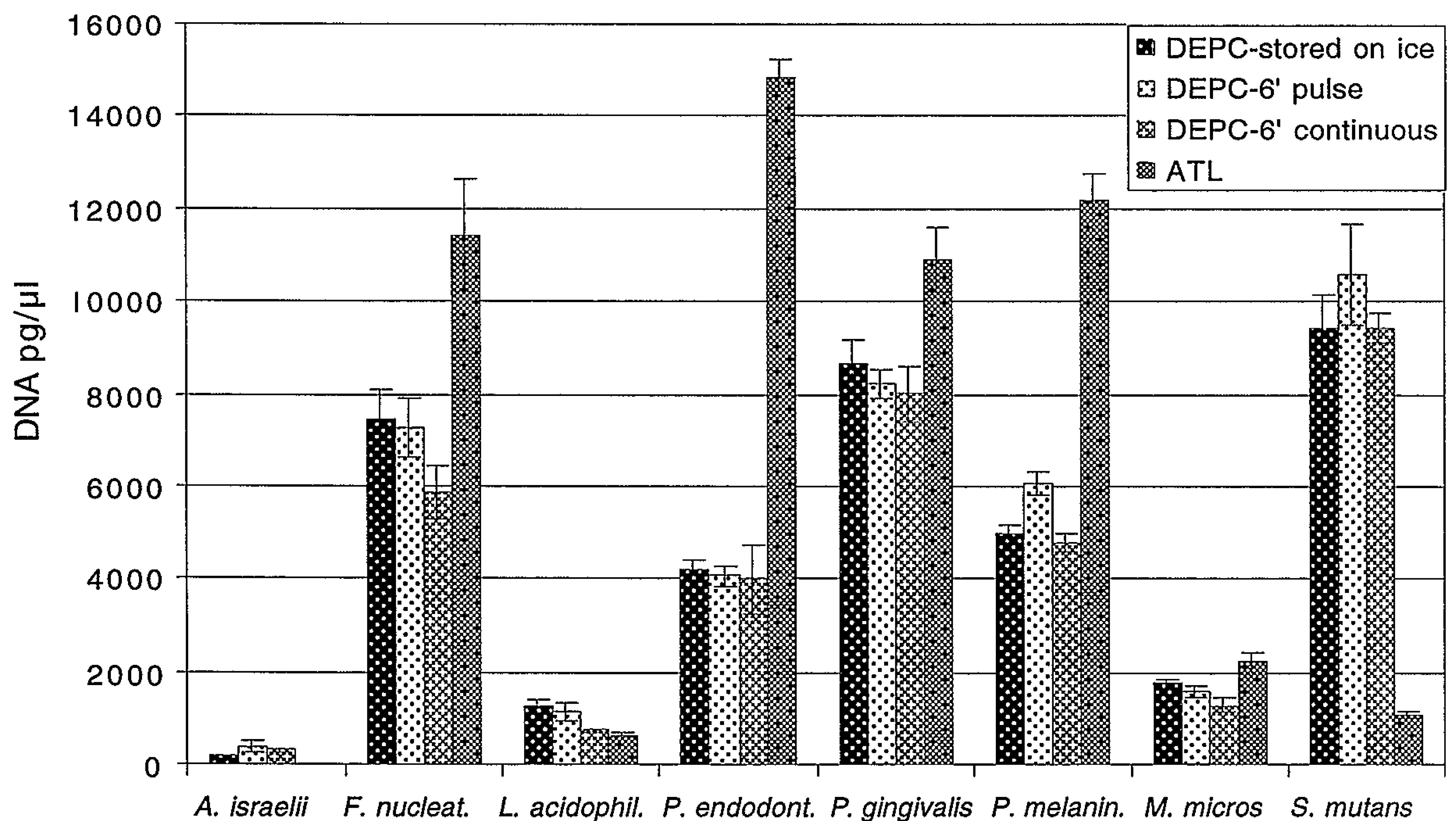


Figure 5.13. PCR quantitation of DNA isolated from cultures of *A. israelii, F. nucleatum, L. acidophilus, P. endodontalis, P. gingivalis, P. melaninogenica, M. micros,* and *S. mutans* suspended in 5mg lysozyme ml^{-1}, 2mg mutanolysin ml^{-1}, 2mg proteinase K ml^{-1} and 20mM DEPC, comparing storage of the culture on ice with 6min pulse and 6min continuous sonication, and isolation in ATL. Data shows one representative experiment of three, expressed as mean values from triplicate readings ± SEM.

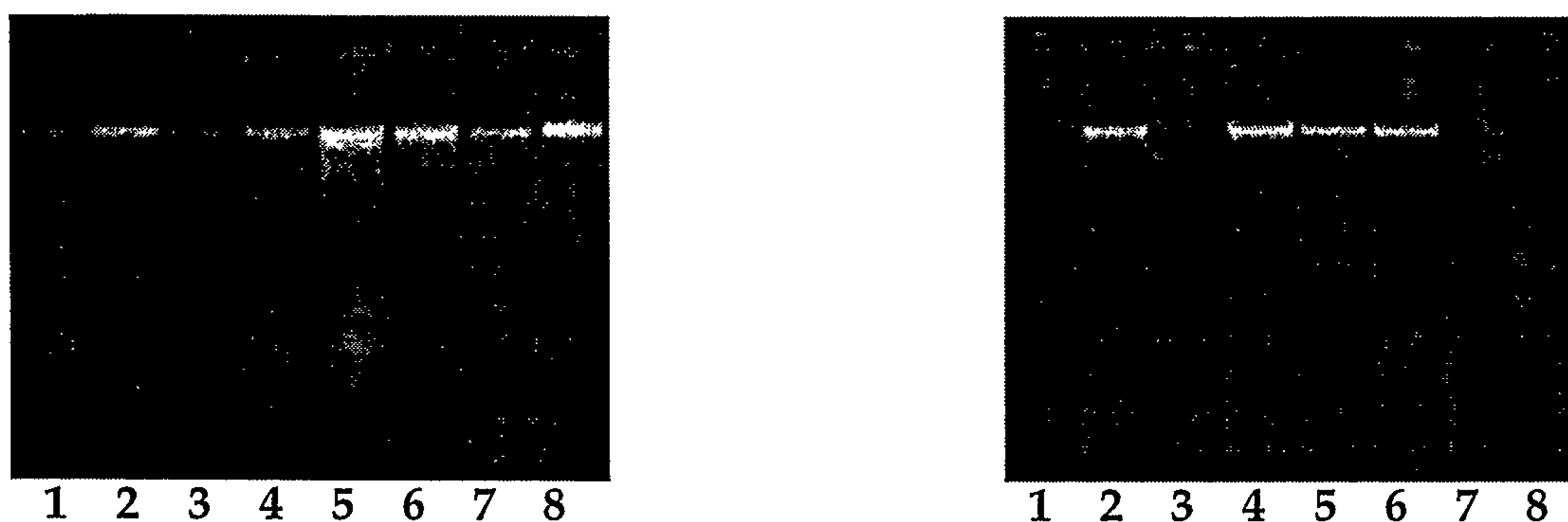


Figure 5.14. Agarose gel electrophoresis of DNA from bacterial cultures isolated in 10mM sodium phosphate buffer containing 5mg lysozyme ml^{-1}, 2mg mutanolysin ml^{-1}, 2mg proteinase K ml^{-1} and 20mM DEPC with 6min pulse sonication (Figure 5.14a) and using the ATL method (Figure 5.14b). Lane 1: *A. israelii;* Lane 2: *F. nucleatum;* Lane 3: *L. acidophilus;* Lane 4: *P. endodontalis;* Lane 5: *P. gingivalis;* Lane 6: *P. melaninogenica;* Lane 7: *M. micros;* Lane 8: *S. mutans.*

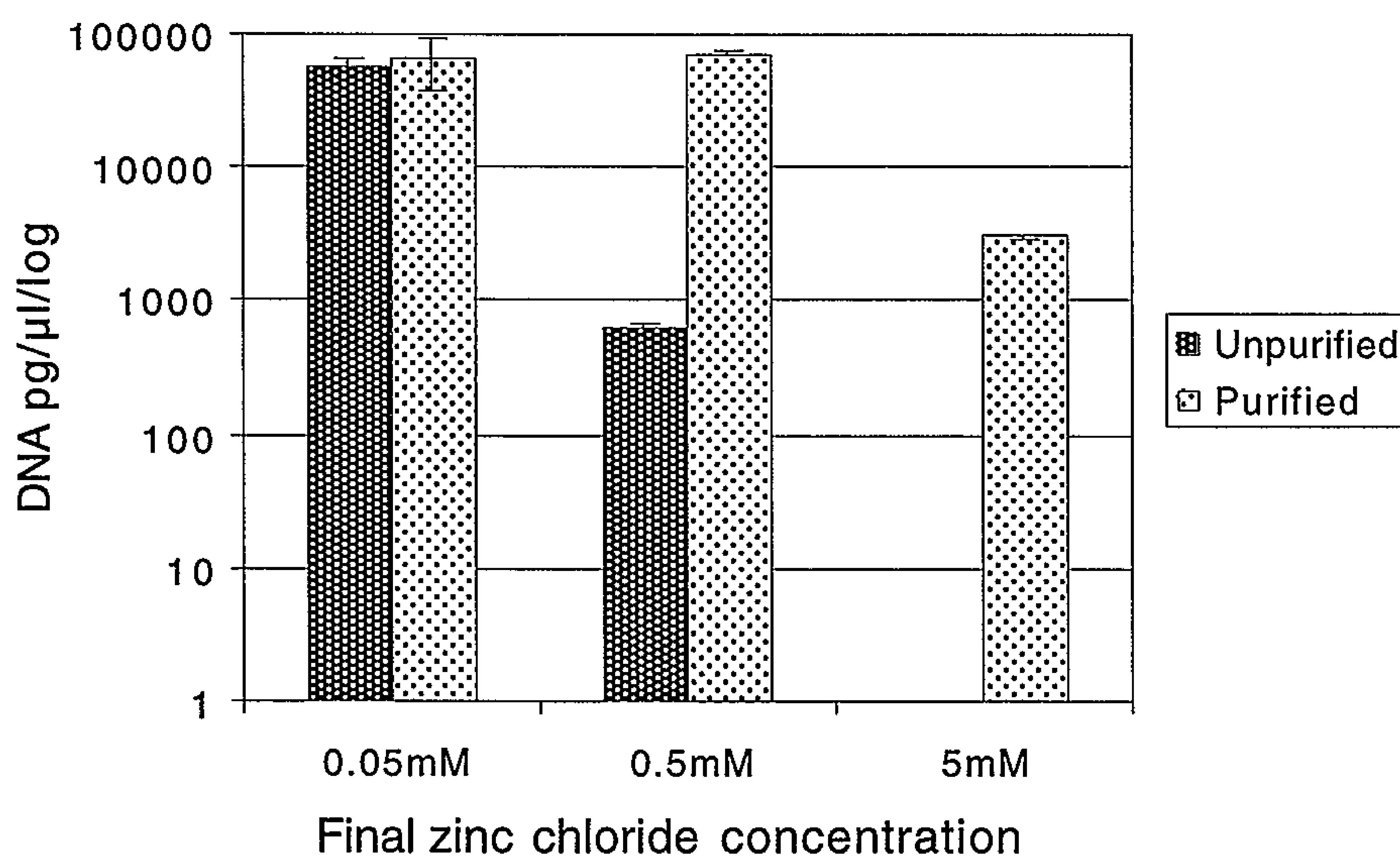


Figure 5.15. PCR quantitation of DNA isolated from cultures of *P. gingivalis* in lysis buffer containing 10mM sodium phosphate, 1mg lysozyme ml^{-1} and 2mg mutanolysin ml^{-1} with final concentrations of 0.05mM, 0.5mM and 5mM zinc chloride. Data are expressed as mean values from triplicate readings ± SEM. Data provided by Dr M Nadkarni.

5.2.3 Discussion

Approximately 500 bacterial species are estimated to inhabit the oral cavity. The composition of the oral microflora is extremely complex and diverse comprising aerobic and anaerobic Gram-positive and Gram-negative bacteria (Paster *et al.*, 2001). In recent years considerable changes have occurred in the taxonomy of anaerobic bacteria, primarily based on DNA sequence information, which has led to the introduction of many new species or renaming of old species (Jousimies-Somer & Summanen, 1999). Traditional methods of microbial detection are often unable to detect uncultured or fastidious microbes (Amann, *et al.*, 1995; Wade *et al.*, 1997; Hugenholtz, *et al.*, 1998; Kroes *et al.*, 1999; Spratt *et al.*, 1999; Paster, *et al.*, 2001) and as the cultivation of microbes often takes days to weeks for initial growth, phenotypic testing and identification, molecular techniques offer a considerable advance. The advent of sequence-based identification has not only overcome the laborious methods of identification by traditional culture but has also indicated that less than 1% of the microorganisms present in nature have been cultivated (Amann, *et al.*, 1995; Hugenholtz, *et al.*, 1998). In contrast, PCR-based methods allow both accurate identification and quantification of a small number of organisms in a substantially shorter time (White, *et al.*, 1992; Chen *et al.*, 1997; Wahlfors, *et al.*, 1995). The development of a rapid molecular testing method should allow the investigation of a mixed microflora associated with disease in its entirety without the inherent bias of culture (Amann, *et al.*, 1995; Parrish & Greenberg, 1995; Dymock, *et al.*, 1996; Relman, 1998; Conrads *et al.*, 1999; Harper-Owen, *et al.*, 1999; Kroes, *et al.*, 1999; Paster, *et al.*, 2001). These advances should improve the knowledge of disease diagnosis and treatment monitoring.

Primers with broad inter-species specificity have been designed to amplify 16S rDNA by PCR and have been used to determine bacterial numbers in complex communities (Wilson *et al.*, 1990; Greisen *et al.*, 1994; Hykin *et al.*, 1994; Marchesi *et al.*, 1998; Klausegger *et al.*, 1999; Suzuki, *et al.*, 2000;). A majority of these studies, however, report the use of more than a single set of primers to detect the bacteria of interest. In addition, some techniques, such as competitive PCR (Blok *et al.*, 1997; Rupf, *et al.*, 1999) are labour intensive and require the

analysis of results from multiple reactions for each test sample. In contrast, the universal primers and probe set used in this study (Nadkarni, *et al.*, 2002) provides an efficient method for determining the total bacterial load in a sample. Although larger than the 150bp limit set in the Applied Biosystems protocol, the 466bp amplicon was found to be uniformly successful in detecting a wide range of bacteria. Takai and Horikoshi (2000) reported an amplicon from the same region of the genome which effectively retrieved phylogenetically diverse prokaryotic rDNA, although designed with variable bases. Corless *et al.* (2000) have reported a universal primers-probe set as a tool for the rapid detection of bacteria by real-time PCR. However, analysis of their forward and reverse primers and probe showed multiple mismatches with most of the dental pathogens including *B. forsythus, P. gingivalis, P. melaninogenica,* and one or more mismatches with many other bacteria at the 5′- as well as the 3′-ends. Similarly, the universal primers-probe set described by Lyons *et al.* (2000) for the detection of total bacteria in dental plaque has one or two mismatches within the probe for the 16S rDNA of *S. aureus, C. jejuni, H. pylori, Wolbachia* species, *M. micros, F. nucleatum, M. pneumoniae, L. mobilis* and *T. denitrificans,* and their reverse primer has no corresponding 16S rDNA sequences in the databases for numerous Gram-positive and Gram-negative bacteria. Furthermore, their primers-probe set exceeds a number of the guidelines set by Applied Biosystems for TaqMan technology. Their probe was selected from the strand with more Gs than Cs, which could affect the amount of fluorescence measured. The T_m of 48.4^{0}C and 35.4^{0}C for their forward and reverse primers, respectively (calculated from their reported sequences using the Primer Express Software) contrasts with the 58^{0}C–60^{0}C set by Applied Biosystems and necessitated the authors using additional annealing steps at 52^{0}C for 1min and 72^{0}C for 2min to allow for the elongation of their longer amplicon (727bp). This extended the total reaction time by at least 70min when using the ABI-PRISM 7700 Sequence Detection System for real-time PCR. The total time taken for quantification was further increased by the requirement for an initial PCR reaction.

One limitation of any broad-range or universal probe is the sensitivity limitation created by contamination. This contaminating DNA is thought to be present in either the enzyme

preparations (*Taq* polymerase or UNG) from organisms used to prepare the enzyme (*Thermus aquaticus* or *E. coli*) or from bacterial or fungal growth in cultures, chromatography columns or buffers used during enzyme purification (Böttger, 1990; Rand & Houck, 1990; Schmidt, *et al.*, 1991; Maiwald *et al.*, 1994; Corless, *et al.*, 2000; Lyons, *et al.*, 2000); an observation verified in the current study by the presence of rDNA in reagent mixes and negative controls containing no added *E. coli* DNA (Fig. 5.1a). Corless *et al.* (2000) reported contamination from the no-template control appearing in the 24th cycle, severely limiting the sensitivity of their reaction. Contamination in this study was restricted to 33-38th cycles by careful choice of the reagents. Other authors have attempted to remove contamination from the reagents using various methods including DNase I treatment (Lyons, *et al.*, 2000), restriction enzymes followed by DNase I treatment (Corless, *et al.*, 2000), UV irradiation (Hitti *et al.*, 1997; Schmidt, *et al.*, 1991), UV treatment and pre-PCR uracil DNA glycosylase digestion (Niederhauser *et al.*, 1994) or UV irradiation in the presence of 5-methoxypsoralen (Klausegger, *et al.*, 1999). However, Corless *et al.* (2000) reported that it was not possible to eliminate endogenous contamination from the *Taq* polymerase without severely compromising the efficiency of the PCR with a resultant reduction in sensitivity.

Detection and enumeration of *P. gingivalis* in previous studies has involved the use of amplicons ranging in length between 197bp to 527bp, with most requiring post-PCR processing (Wahlfors, *et al.*, 1995; Ashimoto, *et al.*, 1996; García *et al.*, 1998; Rupf, *et al.*, 1999; Tran & Rudney, 1999). The designed *P. gingivalis* primers-probe set used in this study was shown to be not only specific for *P. gingivalis,* but its design fell within the guidelines suggested by Applied Biosystems to optimise bacterial quantification in generating a 150bp amplicon. This is considerably shorter than the 900bp *P. gingivalis* primers-probe set designed by Lyons *et al.* (2000) for real-time PCR which incorporates part of the interspacer region as well as the 16S gene.

Since quantitation of the number of bacteria is entirely dependent on the amount of DNA measured, the DNA extraction and purification protocol becomes a critical step in the procedure. At this stage there is no universal method to optimise the digestion of all microbes

in all tissues (Fredricks and Relman, 1999). There are reports of some DNA purification protocols working efficiently with samples designed to amplify one or a limited number of microbes (Desjardin, *et al.*, 1998; Higgins *et al.*, 1998; Sen, 2000). Complex microbial communities, such as carious dentine, harbour a wide variety of both Gram-negative and Gram-positive species and difficulties arise when applying strategies that will efficiently lyse Gram-positive bacteria without shearing the DNA of more fragile organisms. A further complication of DNA extraction concerns the presence of nucleases. Results from this study indicated a deteriorating PCR signal for *P. gingivalis* DNA suggestive of DNA degradation. Nuclease activity has been reported for many bacteria (Park *et al.*, 1980; Flint & Thomson, 1990; Gibson & McKee, 1993; Nakajima, *et al.*, 1994; Ruiz *et al.*, 2000), including those found in the oral cavity such as fusobacteria, porphyromonads, prevotellae, bacteroides, peptostreptococci (Porschen and Sonntag, 1974; Rudek and Haque, 1976), lactobacilli (Smith and Bodily, 1967) and streptococci (Miller, *et al.*, 1971). The presence of nuclease activity in *P. gingivalis* was demonstrated by its ability to degrade *P. gingivalis* DNA as well as the internal positive control (IPC-BT) DNA. Therefore, all DNA extraction procedures tested in this study required consideration of not only the type of cell wall treatment, but also the possibility of nuclease activity of the organisms.

Despite the investigation of different techniques using an extensive series of experiments, it was not possible to efficiently extract DNA from both Gram-negative and Gram-positive bacteria, while also preventing nuclease degradation. Problems were noted with a number of the techniques which prevented efficient DNA extraction. In summary, DEPC, EDTA, EGTA and SDS showed some incompatibility with mutanolysin, thereby preventing lysis of Gram-positive bacteria. However, the Gram-negative microbes required DEPC, EGTA and/or EDTA for nuclease control and SDS for cell lysis, and attempts to remove SDS, by washing or Triton X-100 treatment, to facilitate the action of mutanolysin were ineffective.

Boiling the sample was shown to release and protect the DNA of *P. gingivalis* to a limited extent and has been used in a number of studies as the DNA extraction method (Söder *et al.*, 1993; Slots *et al.*, 1995; Ashimoto, *et al.*, 1996; García, *et al.*, 1998; Conrads, *et al.*, 1999;

Corless, *et al.*, 2000; Siqueira, *et al.*, 2001). However, Bickler *et al.* (1992) reported that nuclease activity could be restored following heat treatment or by changes in Mg^{2+} concentration and cautioned that heat treatment alone was inadequate for DNase inactivation.

Other published extraction methods use a lysis buffer (usually 10mM tris hydrochloride or 10mM sodium phosphate) generally containing 1-10mg ml^{-1} lysozyme (Parrish and Greenberg, 1995; Dymock, *et al.*, 1996) and/or 0.1-1mg ml^{-1} proteinase K (Smith *et al.*, 1989; Conrads *et al.*, 1997). Other additions include 1% SDS, 1-20mM EDTA or 10mM EGTA (Conrads, *et al.*, 1997; Jung *et al.*, 2000; Odell *et al.*, 1999). Lysozyme is used to weaken the cell wall of Gram-negative organisms which subsequently lyse on the addition of a detergent (Chassy & Giuffrida, 1980). Proteinase K is also used for cell lysis and has been demonstrated to be effective in lysing some strains of *P. intermedia* and Gram-positive species such as *Actinomyces odontolyticus, A. naeslundii* and *Eubacteria saburreum* (Grenier, 1994). Other Gram-negative species lysed to a lesser extent by proteinase K included *Capnocytophaga ochracea, F. nucleatum, Treponema denticola* and *H. actinomycetemcomitans.* Overall the Gram-positive species are resistant to the action of lysozyme and proteinase K, with the exception of those previously mentioned, and all strains of *S. mutans* have been found to be quite resistant (Grenier, 1994). Digestion of the Gram-positive cell wall is an essential pre-requisite prior to lysis with a detergent or the DNA yield from a sample will be substantially reduced (Dymock, *et al.*, 1996; Harper-Owen, *et al.*, 1999). Therefore, other methods were tested for their ability to lyse these bacteria including lysozyme at higher concentrations and for longer incubation periods (Roussel *et al.*, 1993), lysozyme incubation with a protease in an acidic buffer followed by incubation with bicarbonate anions at physiologic concentrations (Pollock *et al.*, 1987) and the use of 2mg ml^{-1} mutanolysin, which hydrolyses the peptidoglycan of the Gram-positive cell wall (Simpson *et al.*, 1993). In the present study 2mg ml^{-1} mutanolysin was used in a phosphate buffer in place of a Tris-EDTA buffer for all experiments to optimize the activity of the mutanolysin (Simpson, *et al.*, 1993). Those experiments that incorporated bicarbonate in the presence of lysozyme and proteinase K with an extended incubation period showed good recovery of DNA from only *S.*

mutans. The gel images of DNA from *L. acidophilus* appeared smeared and only minimal amounts of DNA were recovered for the remaining organisms.

Some bacterial nucleases were inactivated and DNA degradation inhibited by boiling, or the addition of 5mM $ZnCl_2$, 100mM EDTA, 100mM EGTA or 20mM DEPC during DNA isolation. This is supported by the findings of Leduc *et al.* (1994) who demonstrated nuclease inhibition of *P. gingivalis* by heating above 70°C or treatment with 5mM $ZnCl_2$ or 100mM EDTA, and Bendjennat *et al.* (1997) who showed the effectiveness of 5mM EDTA, 5mM EGTA and 0.1%DEPC on *Mycoplasma pneumoniae* nuclease inhibition. In the present study 5mM $ZnCl_2$ was unable to prevent the degradation of *L. acidophilus* and although 100mM EDTA was useful in minimising *L. acidophilus* degradation it was observed to inhibit the action of mutanolysin on *S. mutans*. The presence of 20mM DEPC was an effective nuclease inhibitor for the DNA isolation of *F. nucleatum, P. melaninogenica* and *P. gingivalis,* moderately effective against *A. israelii* and *S. mutans,* and least effective with *L. acidophilus*. Other chemicals assessed as possible nuclease inhibitors included aurin tricaboxylic acid, a topoisomerase inhibitor (Hallick *et al.,* 1977), trisodium citrate (Ruiz, *et al.,* 2000) and ascorbic acid. The hydroxyl radical scavengers were only minimally effective in inhibiting nuclease activity as evidenced by smearing of the DNA bands in the agarose gel images. Additional experiments were then conducted to explore the effect of DEPC under different conditions in an attempt to find a general nuclease inhibitor.

Successful extraction of DNA from bacterial cultures in the presence of DEPC with an increased lysozyme concentration was noted in agarose gel images for all the Gram-negative bacteria and *S. mutans*. To increase the recovery of DNA from the remaining Gram-positive microbes and to dissociate aggregations or "corn-cob" arrangements of bacteria, additional mechanical treatments were included. However, only *A. israelii, P. melaninogenica* and *S. mutans* showed increased DNA recovery following sonication.

As DNA could only be reliably extracted from the Gram-negative bacteria, a comparison was made between DNA recovery following the DEPC procedures and the ATL buffer from the QIAamp DNA Mini Kit. Data from this experiment demonstrated that for each of the

Gram-negative cultures the DNA recovered using ATL was significantly greater than that following the DEPC procedures, with less deterioration of the DNA as shown by the clear bands in the ATL gel images. Although the ATL buffer was ineffective in lysing Gram-positive bacteria, *M. micros* showed increased DNA release following isolation in ATL compared with DEPC. This behaviour might be explained by disruption of the cell wall integrity that has been reported when anaerobic bacteria are exposed to oxygen (Johnson *et al.*, 1995a).

Clinical samples are known to contain PCR inhibitors such as potassium ions, blood, urine, vitreous humor, sputum, saliva (Mättö, *et al.*, 1998; Fredricks and Relman, 1999). Inhibitors from other sources include sodium phosphate from transport media (Johnson *et al.*, 1995b), calcium alginate and aluminium swab shafts (Wadowsky *et al.*, 1994). These inhibitors must be diluted, removed or inactivated to allow DNA amplification to proceed. The inhibitory effect of $ZnCl_2$ has been demonstrated and adequate dilution and/or purification allowed the sample to be quantified. Therefore, purification was considered to be an essential component of DNA preparation of the clinical samples prior to PCR. Of those studies which incorporated DNA purification in their extraction process, the QIAamp Tissue Kit Protocol (Mini Kit) has been most commonly reported (Oberst *et al.*, 1998; Conrads, *et al.*, 1999; Odell, *et al.*, 1999; Tran and Rudney, 1999). However, studies directly comparing the efficiency of purification kits provide conflicting reports (de Kok *et al.*, 1998; Dixon *et al.*, 1998). To ensure that PCR had successfully been carried out and that inhibition had not created false negative results, monitoring the amplification of a second target nucleic acid, the IPC-BT, during the reaction served as a useful internal control.

In summary, some success was found in extracting DNA from the anaerobic Gram-negative bacteria and the Gram-positive *M. micros* in ATL, and *S. mutans* in DEPC. However, reasonable amounts of DNA could not be reliably recovered by any technique from either *A. israelii* or *L. acidophilus*, the latter almost always seen on gel images with marked smearing indicating considerable deterioration of the DNA product. As the culture data of Gram-positive microaerophilic bacteria from the dentine samples (Chapter 4) provided information comparable with the findings of other authors, PCR quantitation of these microbes from the

carious samples was not attempted. However, as a result of limitations regarding the efficient culture of anaerobic microorganisms, PCR quantitation of the anaerobic microbes from the clinical samples was carried out using the proprietary buffer ATL, followed by purification with the QIAamp DNA Mini Kit. The results of this study are reported in the next chapter.

CHAPTER 6

QUANTITATION OF ANAEROBIC BACTERIA FROM HUMAN CARIOUS DENTINE USING REAL-TIME PCR

6.1 Introduction

While the cultivation of Gram-positive microaerophilic bacteria yields reliable results, attempts to culture anaerobic bacteria from carious dentine result in a significant underestimation of the numbers present (Nadkarni *et al.*, 2002). Anaerobic bacteria are difficult to grow, require special techniques, may take days to weeks to be identified and may be lost during processing (Rosenblatt, 1997). In contrast, molecular techniques have the potential to produce a reliable means of quantifying bacterial DNA and therefore bacterial numbers. One method of achieving this is real-time PCR, a cyclical enzymatic reaction in which two synthetic oligonucleotide primers and a fluorogenic probe hybridise to the nucleotide base sequences specific for the target organism within a sample. By monitoring the release of fluorescence with each PCR cycle, the progress of the reaction can be recorded in real-time and the amount of DNA in the sample quantified, thus allowing a given bacterial species to be enumerated (Heid *et al.*, 1996). Final quantitation is, however, dependent upon an accurate knowledge of the number of copies of rDNA operons in any given species at the time of sampling; information which is unknown for most dental pathogens. Another factor that affects final quantitation involves the choice of standard DNA used during amplification of sample DNA from complex communities.

Few studies have analysed the microflora from the deep carious lesion using culture techniques and there are no reports of PCR investigations of this tissue. In the previous chapter different methods of extracting and isolating DNA for PCR quantitation were assessed. The

final choice of the proprietary buffer ATL (Qiagen) was deemed to provide the most efficient method of DNA extraction from the anaerobic bacteria while protecting against nuclease activity. In this study real-time PCR was used to enumerate a group of anaerobic bacterial species from human carious dentine samples.

As stated previously, much of the PCR method development was a conjoint effort with Dr M Nadkarni. Where she had the prime role or completed the final design of an amplicon this has been indicated in the text.

6.2 Materials and methods

6.2.1 Effect of the source of standard DNA on the measurement of DNA concentration (Dr M Nadkarni)

E. coli DNA was primarily used as the standard for determining bacterial number by real-time PCR in the method development (Chapter 5). In order to determine the effect of variations in rDNA copy number as well as the multiplying effect of the generation time (t_d) on the calculation of bacterial number, DNA standards were prepared from two rapidly growing aerobic bacteria, *S. aureus* and *P. aeruginosa*, with t_d *in vitro* in the order of 20–50min and two slow growing obligate oral anaerobes, *P. melaninogenica* and *P. endodontalis*, with t_d *in vitro* in the order of 5–15h.

Porphyromonas endodontalis, P. melaninogenica, E. coli, P. aeruginosa and *S. aureus* were cultivated as described in section 5.2.1.1. Once grown, ~10^9 cells of each bacterial species were harvested by centrifugation (14,000*g*, 2min, 18 – 20^{0}C) then resuspended in 200µl of 10mM sodium phosphate buffer pH 6.7 containing 2mg lysozyme ml^{-1} and 2mg mutanolysin ml^{-1}, and for *P. endodontalis* and *P. melaninogenica* 5mM $ZnCl_2$ was included. The suspensions were incubated for 30min at 60^{0}C, the bacteria lysed in the presence of 1% SDS and following the addition of 2mg proteinase K ml^{-1} were incubated at 55^{0}C for 10min. All samples were then incubated for a further 10min after the addition of 1mg RNase ml^{-1} prior to purification using the QIAamp DNA Mini Kit following the manufacturer's directions. After elution into AE buffer (Qiagen), the DNA concentration (A_{260}) and purity (A_{260}/ A_{280}) were measured

spectrophotometrically (Beckman DU640, Beckman-Coulter Australia Pty Ltd, Gladesville, NSW, Australia).

The species-specific standard DNA graphs were generated from *E. coli* DNA within the range 238fg–2.38ng, from *P. aeruginosa* DNA within the range 25fg–2.5ng, from *S. aureus* DNA within the range 27.5fg–2.75ng, from *P. melaninogenica* DNA within the range 1.12pg–112ng and from *P. endodontalis* DNA within the range 240fg–24ng. PCR was carried out in duplicate reactions, in a 25µl reaction volume using 2.5µl of 100-fold diluted DNA with 100nM each of the universal primers and fluorogenic probe using the TaqMan PCR Core Reagents Kit. The reaction conditions for amplification of DNA were 95^{0}C for 10min and 40cycles of 95^{0}C for 15s and 60^{0}C for 1min. The standard graphs were prepared from data accumulated at the same time as the test samples in order to act as internal controls. Data were analysed using the Sequence Detection Software Version 1.6.3 supplied by Applied Biosystems.

6.2.2 Design of probes and primers for real-time PCR

A series of amplicons were designed to detect anaerobic bacteria reported, in the literature, in association with carious dentine and root canal pathology. The species-specific probes and primers were designed from the variable region, and the *Prevotella* probe and primers from the conserved region of the 16S rDNA sequences reported in GenBank and accessed through ANGIS, as described previously in section 5.2.1.3 and listed in Table 6.1.

6.2.2.1 Fusobacterium

The *Fusobacterium* primers-probe set generated a 108bp amplicon spanning nucleotides 457 to 564 (inclusive) in the *F. nucleatum* 16S rDNA sequence (GenBank accession no. X55401).

6.2.2.2 *M. micros*

The *M. micros* primers-probe set generated a 100bp amplicon spanning nucleotides 148 to 247 (inclusive) in the *M. micros* 16S rDNA sequence (GenBank accession no. U60326).

6.2.2.3 P. endodontalis

The *P. endodontalis* primers-probe set generated a 149bp amplicon spanning nucleotides 614 to 762 (inclusive) in the *P. endodontalis* 16S rDNA sequence (GenBank accession no. L16491).

6.2.2.4 P. melaninogenica

The *P. melaninogenica* primers-probe set generated a 91bp amplicon spanning nucleotides 150 to 240 (inclusive) in the *P. melaninogenica* 16S rDNA sequence (GenBank accession no. L16469).

6.2.2.5 *Prevotella*

The *Prevotella* primers-probe set generated a 152bp amplicon spanning nucleotides 363 to 514 (inclusive) in the *Prevotella intermedia* 16S rDNA sequence (GenBank accession no. X73965).

6.2.2.6 S. mutans

The *S. mutans* primers-probe set generated a 208bp amplicon spanning nucleotides 427bp to 634bp (inclusive) in the *S. mutans* 16S rDNA sequence (GenBank accession no. AJ243965). Dr M Nadkarni was responsible for the final design of the *S. mutans* amplicon. The author's role involved both optimising and specificity testing of the *S. mutans* probe and primers set.

All primers-probe sets fulfilled the recommended guidelines set by Applied Biosystems except the *Prevotella* and *S. mutans* amplicons which were longer than the recommended 150bp and the *Prevotella* forward primer and *Fusobacterium* reverse primer which contained 3 instead of 2 GCs at the 3′ end. GenBank data-base searches showed that each set was specific for the target bacterium. The designed primers and probe set for *Fusobacterium* spp. would allow the detection of *F. nucleatum* along with *Fusobacterium periodonticum, Fusobacterium alocis* and *Fusobacterium simiae.* Once designed, all probes and primers were synthesised by Applied Biosystems. The oligonucleotide probes were labelled with the fluorescent dyes FAM at the 5′-end and TAMRA at the 3′ end.

To assess the size of the designed amplicons, 5µl of 1:100 diluted primers from each of the primer/probe sets, including universal and *P. gingivalis,* together with 5µl of 1:100 diluted appropriate DNA and 35µl of TaqMan Universal Master Mix were amplified by PCR (CR FTS-30 Thermal Cycler, Corbett Research, Mortlake, NSW, Australia) using conditions of 95°C for 10min and 42 cycles of 95°C for 15s, 60°C for 1min and 25°C for 1min. Aliquots were loaded in

Table 6.1. Sequences of oligonucleotide primers and probes.

Bacterium detected	Primer/ Probe	Sequence (5′ → 3′)[a]	T_m (°C)[b]
Fusobacterium[c]	Forward	AAGCGCGTCTAGGTGGTTATGT	58.8
	Reverse	TGTAGTTCCGCTTACCTCTCCAG	58.6
	Probe	CAACGCAATACAGAGTTGAGCCCTGCATT	69.9
Prevotella	Forward	CCAGCCAAGTAGCGTGCA	58.1
	Reverse	TGGACCTTCCGTATTACCGC	58.5
	Probe	AATAAGGACCGGCTAATTCCGTGCCAG	68.8
P. endodontalis	Forward	GCTGCAGCTCAACTGTAGTCTTG	58.1
	Reverse	TCAGTGTCAGACGGAGCCTAGTAC	58.6
	Probe	CATTCCGCATACCTTCGGTCTCCTCTAGC	69.6
P. melaninogenica	Forward	GTGGGATAACCTGCCGAAAG	58.1
	Reverse	CCCATCCATTACCGATAAATCTTTA	58.3
	Probe	CAAATCTGATGCCGTCATCGAAGACTATGC	69.4
M. micros	Forward	AGTGGGATAGCCGTTGGAAA	58.1
	Reverse	GACGCGAGCCCTTCTTACAC	58.5
	Probe	ACCGCATGAGACCACAGAATCGCA	68.6
S. mutans	Forward	TGTAAGTCAAGAACGTGTGTGAGAGT	58.1
	Reverse	GACAGTTTCCAGAGCACACTATGG	58.8
	Probe	TGAGCCATAGCCTTTTACTCCAGACTTTCCTG	68.9

[a] All probes were labelled at the 5′ end with 6-FAM and at the 3′ end with TAMRA

[b] The melting temperature of DNA (T_m) was determined using Primer Express Version 1.0 (Applied Biosystems)

[c] The *Fusobacterium* primers and probe set designed for the detection of *F. nucleatum* would also detect *Fusobacterium periodonticum, Fusobacterium alocis* and *Fusobacterium simiae* if present in a sample.

an agarose gel and run against a 100bp ladder (New England Biolabs, Genesearch Pty Ltd, Queensland, Australia) to visualise the amplicons.

6.2.3 Specificity of the primers and probes for real-time PCR

The bacteria used to test the specificity of the probes and primers included *S. mutans* LT11, *S. gordonii, S. salivarius, S. sanguinis, F. nucleatum, F. necrophorum, A, israelii, A. naeslundii, P. gingivalis, P. endodontalis, P. melaninogenica, P. loescheii, M. micros, P. anaerobius, L. acidophilus* and *L. rhamnosus.* The source and culture conditions for these bacteria have been described previously (section 5.2.1.1). Reference bacteria were cultured to late exponential phase, harvested by centrifugation (14,000*g*, 18-20°C, 2min) and re-suspended in 10mM phosphate buffer pH 6.7 containing 2mg lysozyme ml^{-1} and 2mg mutanolysin ml^{-1} with 5mM $ZnCl_2$ then processed and purified using the QIAamp DNA Mini Kit as described earlier (5.2.1.5.3.2). DNA concentration (A_{260}) and purity (A_{260}/ A_{280}) were recorded.

Specificity of the probe and primers sets for the target DNA was tested in duplicate using the TaqMan Universal PCR Master Mix in the ABI PRISM 7700 Sequence Detection System. For *P. endodontalis,* the specificity of the primers and probe set was checked using the TaqMan PCR Core Reagent Kit since no PCR product was detected using TaqMan Universal PCR Master Mix. Each real-time PCR reaction was carried out in a 25µl volume containing 100nM each of the forward primer, reverse primer and probe and 2.5µl of 100-fold diluted template DNA (between 10-100pg μl^{-1}). The real-time PCR conditions used have been described previously (5.2.1.4).

6.2.4. Detection of individual and mixed bacterial DNAs using real-time PCR

Once the specificity of the primers and probe sets was established, optimisation of these sets was undertaken to select the concentrations that provided the most efficient amplification of the target DNAs. The optimised conditions were verified in triplicate, both individually, and by mixing approximately equal amounts of DNA extracted from *F. nucleatum, P. melaninogenica, P. endodontalis, P. gingivalis* and *M. micros*. Samples were prepared to ensure that 3-4pg μl^{-1} DNA from a particular species was present in both the individual and mixed samples. For PCR, the optimised conditions for the forward primer, reverse primer and

fluorogenic probe were 300nM, 300nM, 200nM respectively for *Fusobacterium*, 100nM, 300nM, 200nM respectively for *P. melaninogenica*; 100nM, 200nM, 175nM respectively for *P. endodontalis*; 100nM, 100nM, 150nM respectively for *P. gingivalis* and 200nM, 200nM, 100nM respectively for *M. micros*. The genus-specific *Prevotella* and the universal primers and probe sets were also optimised such that the forward primer, reverse primers and probe were 300nM, 300nM, 175nM respectively for the universal set and 300nM, 600nM and 200nM respectively for the *Prevotella* set. The reaction-volume of 25µl included the optimised probe and primers, 2.5µl of 100-fold diluted DNA and the TaqMan PCR Core Reagent Kit. A standard curve based on *P. melaninogenica* DNA (829fg–8.29ng) was used to determine bacterial numbers.

6.2.5 Sensitivity of detection of bacterial DNA by real-time PCR

The sensitivity of real-time PCR in detecting DNA was determined in triplicate with DNA extracted from *F. nucleatum, P. melaninogenica, P. gingivalis, P. endodontalis* and *M. micros* using the appropriate homologous DNA as standard (ranges 2.0fg–2.0ng, 82.9fg–8.29ng, 3.6fg–3.6ng, 24.1fg–2.4ng and 77.2fg–7.72ng respectively), as well as from the genus *Prevotella* and total bacteria using *P. melaninogenica* DNA as the standard (range 82.9fg–8.29ng), with PCR conditions previously described.

6.2.6 Isolation of bacterial DNA using ATL buffer with and without six minutes pulse sonication

To assess the requirement for mechanical disruption of bacterial clumps, "corn-cob" formations or bacterial cells trapped within the dentinal matrix, one clinical carious dentine sample was processed in ATL with and without pulsed sonication. An 80µl aliquot of thawed homogenised carious dentine was combined with 100µl ATL, 2 mg ml^{-1} proteinase K and 1µl (620pg DNA/µl) of IPC-BT. A second 80µl aliquot was combined with 100µl ATL and subjected to 6 minutes sonication in pulse mode prior to the addition of 2mg ml^{-1} proteinase K and 1µl of IPC-BT. Both samples were then incubated for 40min at 56°C with intermittent vortexing for 10sec every 10min. Ribonuclease (1mg ml^{-1}) was added, followed by a further incubation at 37°C for 10min before purification using the QIAamp DNA Mini Kit. Quantification of DNA used the optimised probe/primer sets for the universal, *Fusobacterium*,

P. endodontalis, P. gingivalis, P. melaninogenica and *M. micros* primers and probe sets, and 100nM each for the primers and probe of the IPC-BT with the TaqMan Core Reagent Kit. The reactions were carried out in triplicate using a 25µl reaction volume containing 2.5µl aliquots of a 1:10 dilution of purified DNA based on a standard graph generated from known amounts of *P. melaninogenica* DNA.

6.2.7 Comparison of the viable cell count with the theoretical number of cells from A_{260} DNA reading and real-time PCR

Bacterial cultures of *F. nucleatum, M. micros, P. endodontalis, P. gingivalis, P. melaninogenica* and *S. mutans* were grown as described previously until mid-exponential phase, when serial dilutions of 100µl of each culture was plated, in duplicate, onto appropriate agar in the range of 10^{-4} to 10^{-6}. After incubation under suitable conditions for 2d for *S. mutans* and 7-10d for the remaining bacteria, the number of CFU were counted. The bacterial cultures of *F. nucleatum* (~5 x 10^8), *M. micros* (~9 x 10^6), *P. endodontalis* (~5 x 10^8) and *P. melaninogenica* (~2 x 10^8) were pelleted by centrifugation (14,000*g*, 2min for each 2ml of culture, 18–20^{0}C) and resuspended in buffer containing 10mM sodium phosphate pH 6.7 containing 2mg lysozyme ml^{-1}, 2mg mutanolysin ml^{-1} and 5mM $ZnCl_2$. Following incubation for 30min at 60^{0}C, the cells were lysed with 1% SDS, then incubated with 2mg proteinase K ml^{-1} for 10min at 56^{0}C and further incubated with 1mg RNase ml^{-1} for 10min at 37^{0}C. *P. gingivalis* culture (~5 x 10^8) was resuspended in 400µl of AE buffer using the ATL method (5.2.1.5.3.10). *S. mutans* (~2 x 10^8) was resuspended in 200µl of buffer using the DEPC method (5.2.1.5.3.8). Thereafter, all suspensions were purified using the QIAamp DNA Mini Kit and the A_{260} recorded. Samples of purified DNA were quantified in triplicate by real-time PCR using optimised, species-specific primers and probes with the TaqMan Core Reagent Kit. The 25µl reaction volume contained 1µl of 1:100 diluted sample DNA and was run against a standard graph generated using homologous DNA, with PCR reaction conditions described previously.

6.2.8 Enumeration of anaerobic bacteria in carious dentine by real-time PCR

The homogenised carious dentine samples were thawed on ice, 80µl aliquots removed and combined with 100µl ATL buffer, 2mg proteinase K ml^{-1} and 1µl IPC-BT. The samples were

incubated for 40min at 56^0C with intermittent vortexing for 10sec every 10min. Ribonuclease (1mg ml^{-1}) was added, followed by a further incubation at 37^oC for 10min before purification using the QIAamp DNA Mini Kit and elution into AE buffer. The DNA was used with optimised concentrations of each probe and primers set, and an appropriate homologous standard to separately enumerate the total anaerobic bacterial load and specific individual anaerobic species present in the carious dentine. The reaction volume of 25µl included 2.5µl of 10-fold diluted sample DNA with the TaqMan PCR Core Reagents Kit using reaction conditions previously described. Amplification for each probe and primer set was run separately and not multiplexed. All analyses were performed in triplicate and the mean ±SEM calculated. Positive controls consisted of approximately 4pg homologous bacterial DNA μl^{-1} and negative controls of sterile H_20. The C_T values for the IPC-BT from each of the samples were also measured.

6.2.9 Calculation of bacterial cell numbers by real-time PCR

The amount of anaerobic bacterial DNA measured by real-time PCR was converted to theoretical cell numbers to allow comparison with the culture data. In order to achieve this, the real-time PCR data was optimised using standard curves derived from DNA extracted from the anaerobic species being enumerated, except in the case of the genus-specific *Prevotella* and the universal primers and probe sets, where *P. melaninogenica* DNA was selected as the standard on the basis of its reported prevalence in carious dentine (Massey *et al.*, 1993). However, as accurate quantification using real-time PCR also requires knowledge of the size of the genome and copy number of 16S rDNA within a cell (Farrelly *et al.*, 1995; Nadkarni, *et al.*, 2002) and since this information is unknown for most oral anaerobes, it was necessary to assume that the genome size of all anaerobes was similar to *P. gingivalis* (2.2Mb; TIGR Microbial Database [http://www.tigr.org]) and that each cell therefore contained 2.37fg DNA. One copy of the 16S rDNA was also assumed based on the findings of Klappenbach *et al.* (2000) who reported that slow growing bacteria contained on average 1.4 copies of the gene per genome.

6.2.10 Statistical analyses

Mean and standard error of the mean were calculated from the duplicate or triplicate PCR results following DNA quantitation. Where appropriate, significance was determined using the *t*-test and Wilcoxon signed-rank statistic.

6.3 Results

6.3.1 Effect of the source of standard DNA on the measurement of DNA concentration

In order to confirm that a DNA standard other than that of *E. coli* should result in a difference in the relative amount of DNA detected due to variations in rDNA copy number and the effect of the t_d on this number, the relative amount of DNA from the rapidly growing aerobic bacteria *S. aureus, E. coli* and *P. aeruginosa* were compared with the slow growing obligate oral anaerobes, *P. melaninogenica* and *P. endodontalis*. In each instance the relative amount of DNA was estimated by real-time PCR using each of the 5 DNAs as standards and compared with the amount of DNA determined at A_{260} nm (set at 100%). It would be expected that comparison of like DNA by real-time PCR with the known amount of added DNA would be approximately 100%. In two instances this was not the case. For both *P. aeruginosa* and *P. melaninogenica* approximately twice the amount of DNA was detected. This was due in part to the fact that the relative amounts of DNA were calculated by the Sequence Detection System Version 1.6.3 software supplied by Applied Biosystems based upon the arbitrary placement of the horizontal threshold line used to determine the C_T (cf Figure 5.1a). The horizontal threshold line was therefore adjusted to bring these two values as close to 100% as possible and the relative amount of DNA recalculated (Table 6.2).

As expected, variation in the relative amount of DNA was observed when the standard DNA differed from that of the species being evaluated (Table 6.2). However, significant error (> 3-fold) was only observed when the fast growing aerobic bacteria were compared with the DNA standards of the slow growing obligate anaerobes (over-estimation) or conversely, when the obligate anaerobes were compared to the DNA of the fast growing aerobes (under-estimation) (Table 6.2). The data in Table 6.2 allowed an estimation of the ratio of the number of copies of the 16S rDNA operons in different species. An average ratio of 20:10:9:1:1 (to the

Table 6.2. Effect of species-specific DNA standards on the relative estimation of DNA using the universal primers-probe set for real-time PCR.

Bacterium	Relative Amount of DNA (%)[a]					
	A_{260} nm[b]	*S. aureus* DNA St'd	*E. coli* DNA St'd	*P. aeruginosa* DNA St'd	*P. endodontalis* DNA St'd	*P. melaninogenica* DNA St'd
S. aureus	100	106	145	294	1231	2278
E. coli	100	46	96	139	550	1304
P. aeruginosa	100	48	96	139	456	669
P. endodontalis	100	8	17	9	108	201
P. melaninogenica	100	5	11	10	68	122

[a] The species-specific standard DNA graphs (C_T vs [DNA]) were generated from *E. coli* DNA within the range 238fg–2.38ng, from *P. aeruginosa* DNA within the range 25fg–2.5ng, from *S. aureus* DNA within the range 27.5fg–2.75ng, from *P. melaninogenica* DNA within the range 1.12pg–112ng and from *P. endodontalis* DNA within the range 240fg–24ng. The means of duplicate determinations are shown. Variation between duplicates was ≤2.7 % except where underlined where the values for the *E. coli*, *P. melaninogenica* and *P. aeruginosa* DNA standard varied by 4.8%, 10.5% and 15.9% respectively.

[b] The concentration of DNA was determined spectrophotometrically and normalised to 100% prior to diluting in the range of 100- to 1000-fold for determination by real-time PCR.

Culture and collation of data for the aerobic organisms was carried out by Dr M Nadkarni and by the author for the anaerobes, *P. endodontalis* and *P. melaninogenica*.

nearest integer) for the copy numbers in *S. aureus, E. coli, P. aeruginosa, P. endodontalis* and *P. melaninogenica* respectively fitted the data. This implied that the fast growing aerobes, *S. aureus, E. coli* and *P. aeruginosa* possessed approximately twice the known chromosomal complement of 16S rDNA operons. The data also predicted that the obligate anaerobes possess only one or two 16S rDNA operons *per* chromosome. The exact copy numbers are currently unknown.

6.3.2 Specificity and sensitivity of the primers and probes in detecting bacterial DNA using real-time PCR

The agarose gel in Figure 6.1 shows bands representing the amplicons of the universal, *Fusobacterium, P. endodontalis, P. gingivalis, Prevotella, P. melaninogenica* and *M. micros* primers following PCR. DNA from the individual bacteria was used with the species-specific amplicons and *P. melaninogenica* DNA was used with the universal and *Prevotella* amplicons. The positions of the amplicons in the gel image equate with their respective sizes.

The *Prevotella, Fusobacterium* and species-specific probes and primers sets for *P. melaninogenica, P. endodontalis, P. gingivalis, M. micros* and *S. mutans* were shown to be specific for the target organism with no cross-reactivity evident. Each of the probe and primers sets was also checked for recognition of the human DNA supplied in the Beta-actin Detection Kit and found to be negative.

Given amounts of DNA from *Prevotella* or the five different selected species could be accurately quantified by real-time PCR whether they were present as the sole DNA in a sample or a mixture of all five (Table 6.3). This indicated that quantification of DNA from a particular species or genus was not affected despite the presence of DNA from a variety of other species. Sensitivity testing showed that the estimated detection limit for the universal probe and primers set was 43 bacteria, while that for *Prevotella, Fusobacterium, P. melaninogenica, P. endodontalis, P. gingivalis* and *M. micros* was 173, 1, 39, 8, 3 and 16 bacteria respectively.

6.3.3 Isolation of bacterial DNA using ATL buffer with and without six minutes pulse sonication

PCR data showed little difference in DNA quantitation between the two methods as detected by the primers/probe sets, except the *P. gingivalis* set which recorded a greater

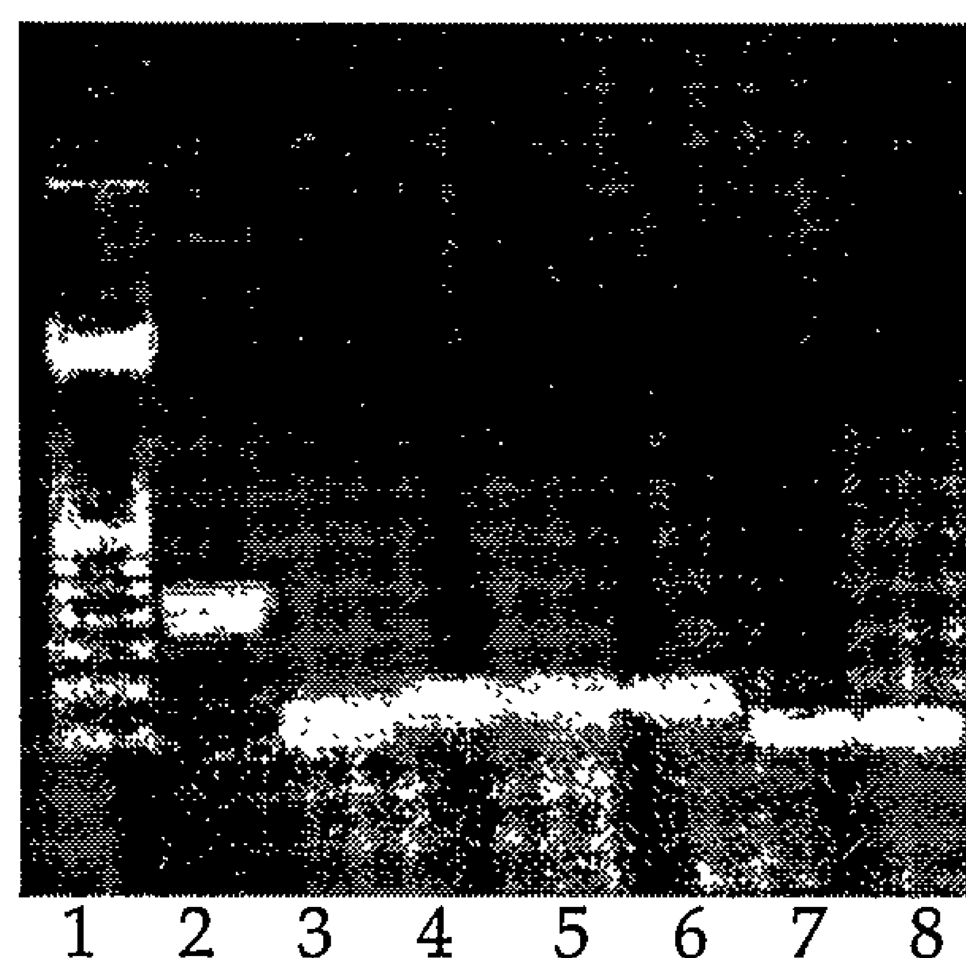


Figure 6.1. Agarose gel electrophoresis of the designed amplicons. Lane 1: 110-bp ladder; Lane 2: universal amplicon (466bp); Lane 3: *Fusobacterium* amplicon (108bp); Lane 4: *P. endodontalis* amplicon (149bp); Lane 5: *P. gingivalis* amplicon (150bp); Lane 6: *Prevotella* amplicon (152bp); Lane 7: *P. melaninogenica* amplicon (91bp) and Lane 8: *M. micros* amplicon (100bp). DNA from individual bacteria was used for the species-specific amplicons and *P. melaninogenica* DNA was used for the universal and *Prevotella* amplicons.

Table 6.3. Specificity of primers and probes for detecting 16S rDNA by real-time PCR in an homologous sample or as part of a mixture of DNAs.

Bacterium	Amount of DNA Detected ± SEM (%) [a]	
	Homologous DNA	Mixed DNA
Prevotella	100.0 ± 5.8	98.1 ± 10.7
P. melaninogenica	100.0 ± 13.8	105.2 ± 19.0
Fusobacterium[b]	100.0 ± 3.5	100.6 ± 4.3
M. micros	100.0 ± 2.5	103.6 ± 1.8
P. endodontalis	100.0 ± 8.0	92.4 ± 8.9
P. gingivalis	100.0 ± 0.5	100.6 ± 2.7

[a] Mean ± SEM were determined from triplicates using *P. melaninogenica* DNA in the range 829fg to 8.29ng as the standards. No significant differences were found by *t*-test between the estimation of DNA as sole source or as part of a mixture of the five DNAs.

[b] The *Fusobacterium* primers and probe set designed for the detection of *Fusobacterium nucleatum* would also detect *F. periodonticum, F. alocis* and *F. simiae* if present in a sample.

amount of DNA from the procedure without 6min pulse sonication. *Fusobacterium* was the only probe/primer set to detect slightly more DNA following 6min pulse sonication (Figure 6.2).

6.3.4 Comparison of viable cell count with the theoretical number of cells from A_{260} DNA reading and real-time PCR

The theoretical bacterial cell number estimated by A_{260} and PCR were comparable with the viable count only for the microaerophilic bacterium, *S. mutans* (Table 6.4). For the anaerobic organisms the theoretical bacterial cell counts from A_{260} and PCR were similar, and between 4 and 15-fold greater than the viable counts. The discrepancy range for *P. endodontalis*, *P. gingivalis* and *M. micros* showed a 10 to 15-fold increase in cell detection following PCR. The discrepancy for *F. nucleatum* and *P. melaninogenica* was 4 and 5-fold respectively.

6.3.5 Enumeration of anaerobic bacteria in carious dentine by real-time PCR

Initial experiments using undiluted purified sample DNA produced unpredictable results. In some samples no signal was produced despite successful amplification of the internal positive control and previous culture data which showed obvious growth of anaerobes. This problem raised questions regarding the possible interference by PCR inhibitors introduced during sample purification. Further experiments (data not shown) indicated that a 1:10 dilution of the sample DNA prior to PCR produced reliable, reproducible data. A lack of reliable data was also noted when carrying out PCR with the universal and IPC-BT probes and primers multiplexed. For subsequent experiments this problem was overcome by amplifying the probes and primers in separate tubes.

The theoretical number of anaerobic bacteria per mg wet wt of dentine was estimated by real-time PCR from bacterial DNA extracted from the 65 samples (Table 6.5). Considerable variability in the number and nature of the microflora was apparent, with up to a 52-fold difference in total anaerobic microbial load between samples. In those samples where they were detected, the number of *Prevotella* and *Fusobacterium* spp. varied by up to four orders of magnitude, *M. micros* and *P. endodontalis* by at least three and *P. gingivalis* by two. The data showed 100% of teeth were positive for *Fusobacterium* spp. (65/65), 97% for *Prevotella* spp. (63/65), 88% for *P. melaninogenica* (57/65), 71% for *M. micros* (46/65), 60% for *P. endodontalis*

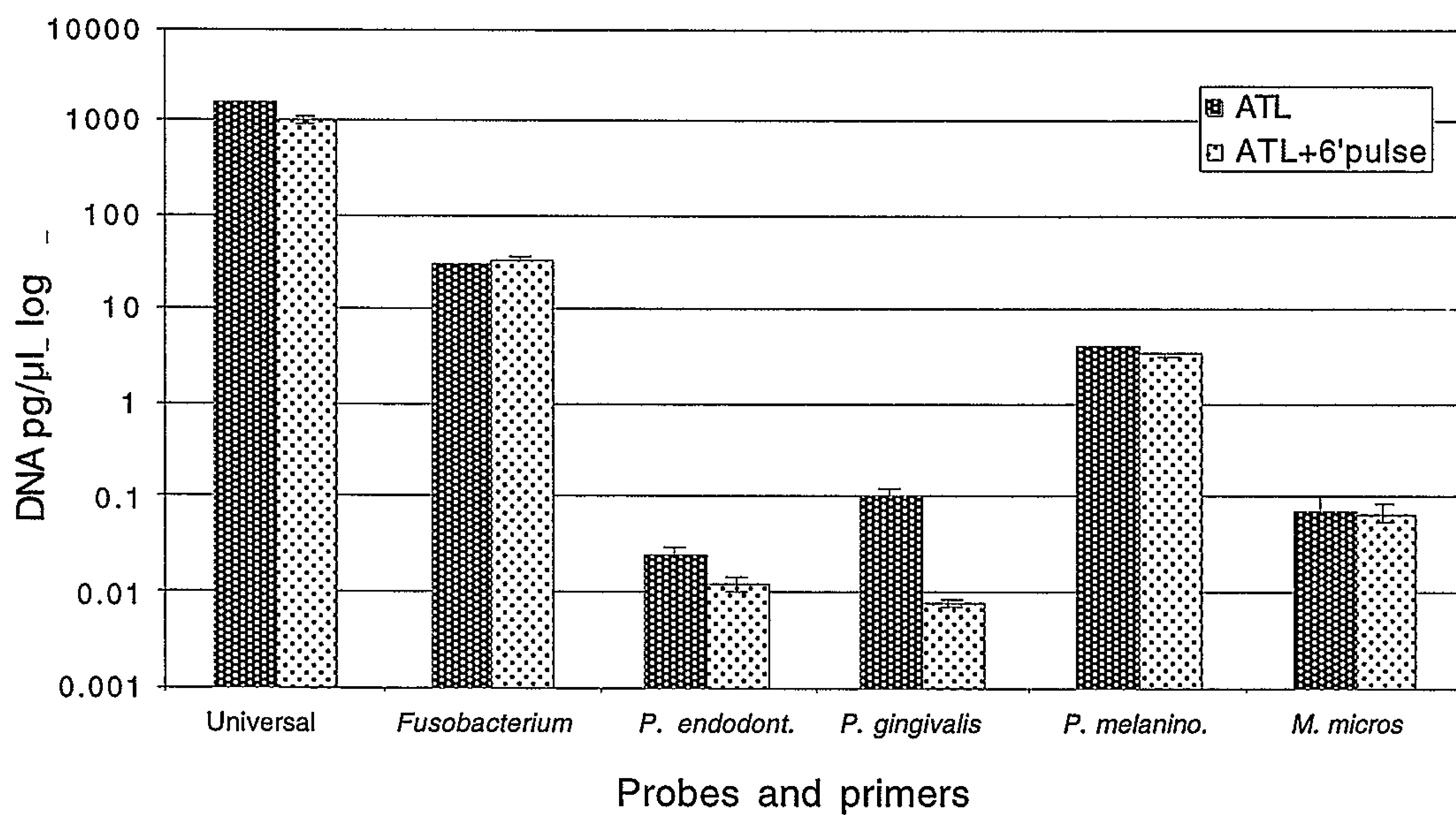


Figure 6.2. PCR quantitation of a carious dentine sample comparing the effect of DNA isolation in ATL with and without 6min pulse sonication using the universal, *Fusobacterium, Prevotella* and species-specific *P. endodontalis, P. gingivalis, P. melaninogenica* and *M. micros* primers and probes against a *P. melaninogenica* standard curve. Data shows one representative experiment of two, expressed as mean values from triplicate readings ±SEM.

Table 6.4. Comparison of the viable count with the theoretical number of cells estimated from A_{260} DNA reading and from real-time PCR.

Bacterium	Viable CFU per ml [a]	A_{260} nm per ml [b]	PCR per ml [c] mean (±SEM)
F. nucleatum	1.1×10^9	4.2×10^9	$4.2 \pm 1.1 \times 10^9$
M. micros	8.5×10^6	7.2×10^8	$3.2 \pm 0.3 \times 10^8$
P. endodontalis	3.3×10^8	4.1×10^9	$3.3 \pm 1.3 \times 10^9$
P. gingivalis	1.8×10^8	4.8×10^9	$4.3 \pm 1.1 \times 10^9$
P. melaninogenica	9.0×10^8	3.5×10^9	$3.9 \pm 0.3 \times 10^9$
S. mutans	5.4×10^9	9.3×10^9	$6.4 \pm 1.0 \times 10^9$

[a] Data represents the means of duplicate determinations. Variation between duplicates ≤10.0%.
[b] Data represents the means of triplicate determinations. Variation between triplicates ≤8.0%.
[c] PCR values obtained using species-specific primers and probes from standard curves based on homologous DNA. Dr M Nadkarni provided the data for *P. gingivalis* and *S. mutans*.

Table 6.5. Bacteria detected in carious dentine by real-time PCR.

Bacterium	Cell Number (mg dentine)$^{-1}$ [a]		
	Range	Median	Mean ± SEM [c]
Total anaerobic [b]	$2.1 \times 10^7 - 1.1 \times 10^9$	2.6×10^8	$3.0 \pm 0.3 \times 10^8$
Prevotella	$0.0 - 2.0 \times 10^8$	16×10^6	$39 \pm 7.0 \times 10^6$
P. melaninogenica	$0.0 - 3.5 \times 10^6$	0.1×10^6	$0.3 \pm 0.1 \times 10^6$
Fusobacterium	$4.8 \times 10^2 - 5.7 \times 10^6$	0.1×10^6	$0.4 \pm 0.1 \times 10^6$
M. micros	$0.0 - 1.6 \times 10^7$	0.4×10^6	$1.5 \pm 0.5 \times 10^6$
P. endodontalis	$0.0 - 1.3 \times 10^6$	0.01×10^6	$0.1 \pm 0.04 \times 10^6$
P. gingivalis	$0.0 - 2.5 \times 10^4$	0.001×10^6	$0.004 \pm 0.001 \times 10^6$

[a] Data collected from 65 samples.
[b] Detected by the universal primers and probe set (Nadkarni *et al.*, 2002).
[c] Determined from triplicate measurements.

(37/65) and 52% for *P. gingivalis.* Data representing the amplification of the internal control (IPC-BT), following its processing and purification with the carious dentine samples, showed no deterioration of the signal with C_T values ranging between 24.6 to 28.1.

Where comparative culture data were available, the number of samples positive for a specific bacterium increased and the number of bacteria estimated by real-time PCR were greater compared with the number determined by CFU (Table 4.1). The total number of anaerobic bacteria estimated by employing the universal amplicon and real-time PCR was 41-fold greater than that measured by anaerobic culture. For *Prevotella,* using the genus-specific amplicon, the estimation was 82-fold greater than for culture, whereas for *Fusobacterium* spp. the estimate was 2.4-fold greater. The number of samples positive for *Fusobacterium* increased from a detection level of 97% by colony counting, to 100% by real-time PCR. Similarly, the number of samples positive for *Prevotella* increased from 91% to 97%. In contrast, greater numbers of *P. melaninogenica* were observed following culture of the samples than were detected by real-time PCR. In all cases except five, however, the inability to detect the presence of a given species by real-time PCR was confirmed by colony counting. In the five exceptions, CFUs identified in the samples as *P. melaninogenica* by biochemical tests could not be detected by real-time PCR. One further anomaly showed *P. melaninogenica* detected in a sample not identified by the *Prevotella* genus-specific probe and primers set.

A Wilcoxon signed–rank statistic was used to compare the number of bacteria determined by colony counting with that obtained by real-time PCR. The results showed a significant difference ($p<0.0001$) between the two methods for the total anaerobic and *Prevotella* counts, with no difference found between the two for *Fusobacterium* spp. ($p = 0.109$) and *P. melaninogenica* ($p = 0.238$).

6.4 Discussion

The data demonstrate that failure to compare DNA from Groups of bacteria possessing similar growth rates readily leads to an under or over estimation of the amount of DNA by one order of magnitude. The analyses, however, show that if the ratio of the estimated amount of DNA measured against a rapidly growing bacterium, such as *S. aureus,* to that measured

against a slow growing bacterium, such as *P. melaninogenica*, is < 1.0, then enumeration of the number of bacteria in the sample should be estimated using DNA extracted from the fast growing bacterium. If the ratio > 1.0, the alternative standard DNA from the slow growing bacterium should be used. In practice, this may simply require reference to a standard curve where the DNA is derived from a bacterium considered to represent the predominant species in the sample. Others, however, have come to different conclusions. For instance, Lyons *et al.* (2000) found no difference in the number of rDNAs per bacterial cell for *H. actinomycetemcomitans*, *P. gingivalis*, *E. coli* and group G streptococci and therefore assumed that the average number of 16S rDNA operons in each bacterial cell was similar in all dental plaque samples. Thus, they made no attempt to compensate for differences in 16S rDNA copy number.

Despite inclusion of the QIAamp DNA Minikit purification step to remove potential PCR inhibitors from the DNA samples, some degree of inhibition remained in the undiluted samples. This inhibition was subsequently eliminated by a 10-fold dilution of the sample prior to PCR, a finding in agreement with others (Mahony *et al.*, 1998; Carroll *et al.*, 2000), although Holland *et al.* (2000) noted that faecal samples purified by the QIAamp DNA Minikit required no further dilution for efficient PCR. The interference with PCR quantitation by multiplexing might have been due to an inadequate supply of reaction components and the problem could not be overcome except by amplifying the universal and IPC-BT probes and primers in separate tubes. This, in effect, became a more efficient and cost effective method of processing the samples as the additional positive and negative controls could be eliminated from each PCR experiment.

As anticipated, results from the viable culture count, A_{260} reading and PCR quantitation for the microaerophilic bacterium, *S. mutans*, were comparable (Table 6.4), indicating that culture of these organisms provides an accurate representation of their numbers, assuming care is taken to adequately disperse the cells prior to culture. However, PCR data of the anaerobic organisms indicated a considerable underestimation by culture (Nadkarni *et al.*, 2002). It was noted that this discrepancy increased for the more fastidious species (Table 6.4).

A number of studies in endodontic and periodontal microbiology have compared culture methodologies with PCR for the detection of specific bacteria, with a consensus that bacterial detection by PCR is more sensitive, more reliable and shows enhanced species recognition compared with culture data from the same samples (Ali *et al.*, 1994; Moncla *et al.*, 1994; Tran *et al.*, 1997; Mättö *et al.*, 1998; Rolph *et al.*, 2001). The finding in the present study, of an increased incidence of fusobacteria and prevotellae detection and a significant increase in *Prevotella* spp. and total anaerobic load in carious dentine following bacterial enumeration by real-time PCR, is in agreement with these observations. Limitations of the culture techniques used with these samples could be attributed to an inability of the anaerobes to be grown under the culture conditions provided, because they had entered a non-culturable state or were present in low numbers (Leys *et al.*, 1994; Amann *et al.*, 1995). The reported inability to cultivate *P. gingivalis* or *P. endodontalis* from carious dentine (Massey, *et al.*, 1993) is pertinent to this argument despite the latter species being identified in dentine caries by immuno-histological staining using species-specific antisera (Ozaki *et al.*, 1994). In this study *P. gingivalis* and *P. endodontalis* were readily detected by real-time PCR in 52% and 60% of samples respectively. *M. micros* were also detected by real-time PCR in 71% of carious dentine samples. Both *M. micros* and *P. endodontalis* have been associated with endodontic pathology following pulpal necrosis related to advanced coronal caries (Sundqvist *et al.*, 1989; Hashioka *et al.*, 1992), and *M. micros* and *P. gingivalis* have been implicated in periodontal disease (Rams *et al.*, 1992; Travis *et al.*, 1997).

The theoretical quantitation of *F. nucleatum* pure cultures by PCR was found to be 4-fold greater than that recorded by culture, which was comparable with the 2.4-fold discrepancy between fusobacteria values from the clinical samples. This similarity of the PCR and culture results is not surprising as *F. nucleatum* is a robust anaerobe capable of growing in 6% oxygen and surviving exposure to air for 100 minutes (Moore *et al.*, 1984). However, the similarity of PCR and culture data in this study is in contrast to the findings of others who reported a significantly greater recovery of *F. nucleatum* using molecular techniques (PCR and cloning) (Maiden *et al.*, 1991; Dymock *et al.*, 1996; Wade *et al.*, 1997). It is possible that use of the CVE

agar in this study supported the growth of strains of *Fusobacteria* spp. not detected by the probe and primers utilised in PCR. Despite the description of two distinct colony forms of *F. nucleatum* on CVE agar (Walker, *et al.*, 1979), it has been reported that colony morphology of fusobacteria does not provide a consistent parameter for species identification (Tunér *et al.*, 1992). Further, Morris *et al.* (1997) found that currently assigned type strains did not represent any of the distinct genetic clusters of isolates identified in their study.

A discrepancy in the current study was the finding that the number of CFU of *P. melaninogenica* exceeded the number enumerated by real-time PCR. This was in contrast to the data shown in Table 6.4 where bacterial cell numbers determined by PCR were greater than those found by CFU. This anomaly could be accounted for by incorrect biochemical identification of the colonies. Difficulty in identifying *P. melaninogenica* in clinical samples using commercial biochemical test kits has been reported (Haraldsson and Holbrook, 1998). It is possible that some of the colonies identified as *P. melaninogenica* could have been *Prevotella tannerae,* a recently described black pigmented prevotellae previously identified incorrectly as *Prevotella intermedia* and *Prevotella nigrescens* (Xia *et al.*, 1999). The discrepancy in one sample where *P. melaninogenica* was detected but not identified by the *Prevotella* probe was due to different sensitivities of the probe/primers sets. The detection limit for *P. melaninogenica* was 39 cells, whereas the detection limit for the *Prevotella* set was 173 cells.

Studies of selected microorganisms quantified from the 65 carious dentine samples have provided data for the primarily Gram-negative anaerobic bacteria using real-time PCR and the Gram-positive microaerophilic bacteria using culture techniques (Chapter 4). The following chapter examines the histopathology of the pulp tissue from each of the samples and correlates the histopathological findings with the previously quantified bacterial species.

CHAPTER 7

THE HISTOPATHOLOGY OF CHRONIC PULPITIS

7.1 Introduction

The microbial populations involved in dental caries are known to be highly complex and variable and have not yet been fully identified, although key organisms are generally recognized as being associated with disease progression. The bacteria involved in caries initiation and early caries development, particularly the mutans streptococci and lactobacilli, have been well documented (van Houte and Kent, 1994). As the lesion progresses, there is a transition from predominantly facultative Gram-positive bacteria in early caries to anaerobic Gram-positive rods and cocci, and Gram-negative rods in deep carious lesions (Hoshino, 1985). Previous research has associated the presence of anaerobic Gram-negative rods, such as fusobacteria, prevotellae and porphyromonads with symptomatic teeth (Hahn *et al.*, 1991; Massey *et al.*, 1993), infected pulps (Zavistoski *et al.*, 1980) and periapical abscesses (van Winkelhoff *et al.*, 1985; Sundqvist *et al.*, 1989), whereas anaerobic Gram-positive cocci such as peptostreptococci are associated with pain and swelling related to apical infections (Brook *et al.*, 1991).

While it is recognized that bacteria and their products play a major role in dental caries and associated pulpal inflammation (Brännströmm, 1981), attempts to correlate clinical signs and symptoms with pulpal histopathology or to relate the presence of specific bacteria in root canal infections with clinical symptoms have been unsuccessful (Dummer *et al.*, 1980; Siqueira *et al.*, 2000). These limitations may be a natural characteristic of complex polymicrobial infections (caries, apical periodontitis) or the result of an incomplete microbial profile of the environment. However, few studies have analysed the microbiology of deep carious dentine or examined the relationship between the microflora of dentine caries and the histopathology of chronic pulpitis. Particular difficulties arise when trying to analyse data from previous studies in this area. For example, protocols were not standardised between studies and the sampling methods used may have been compromised by contamination with supra-gingival dental

plaque. In addition, pulpitis is chronic and episodic, and the active stage of tissue destruction is difficult to identify (Massler, 1967).

This study examined the histopathology of the pulp tissue taken from the 65 clinical samples. Correlations were made between the type and number of the microorganisms identified in the carious dentine samples, analysed in the previous chapters, and the pulpal cellular responses in order to investigate pathogenic mechanisms in carious pulpitis which may contribute to pulpal destruction.

7.2 Material and methods

Immediately following dentine processing (4.2.2) the extracted teeth were removed from the anaerobic chamber and the exposed pulp tissue or overlying thin dentine was point-stained at the deepest site with Toluidine Blue prior to removal of the pulp. This allowed orientation of the pulp tissue relative to the carious lesion. The teeth were then incompletely sectioned through the enamel and dentine using a water-cooled tungsten carbide bur in a high-speed dental handpiece. The remaining layer of dentine was split using hand instruments and the pulp tissue retrieved. Any pulp samples that appeared torn or necrotic were rejected from the study along with the associated dentine samples. The remaining 65 paired carious dentine samples and pulp specimens were subsequently assessed.

The pulp tissue samples were processed by fixation for 24 hours at 4°C with 4% paraformaldehyde in phosphate buffered saline. The pulp tissue was dehydrated in graded acetones, infiltrated and embedded at 4°C in JB4 glycolmethacrylate embedding resin (Polysciences Inc., Warrington, PA, USA). Three serial sequences comprising ten sections of 2μm thickness each, 100μm apart were cut from each processed tissue block using glass knives and mounted on glass slides. Sectioning commenced in the region of the pulpal tissue closest to the carious lesion, as defined by the Toluidine Blue point staining. Representative sections were stained for assessment using 1% Toluidine Blue.

All tissue sections were coded to avoid examiner bias. The pulp tissue sections were examined initially at low magnification (X50) and from these, areas were selected randomly to be examined at a higher magnification (X312). The tissue samples were then viewed through a

graticule eyepiece that divided the section into a series of fields. With frequent use of reference slides, the tissue fields were assigned to one of four categories with a particular tissue appearance:

(i) Minimal inflammatory change which consisted of an essentially normal tissue pattern with minimal inflammatory infiltrate and soft tissue disturbance.

(ii) Soft tissue degeneration which consisted of some abnormal connective tissue architecture, changes in thickening of the basement membrane through to replacement of tissue by hyaline-affected material, often interspersed with diffuse calcification.

(iii) Hard tissue degenerative change which consisted of evidence of dystrophic or diffuse calcification.

(iv) Inflammatory degenerative change which consisted of widespread infiltration of acute and/or chronic inflammatory cells, abscess formation and extensive necrotic changes.

Depending on the cross-sectional area of the pulp tissue, between 11 and 261 fields were examined for each slide. Thirty sections were examined for each pulp specimen and although most sections showed evidence of a number of different types of pathology, an index representing the dominant pathological category was derived for each pulp by determining the category most frequently identified within the graticule fields. In addition, the extent of different categories of pathological change throughout the specimens was also determined for each category of pathology from the pooled data. This was determined by dividing the number of graticule fields showing a particular pathological change by the total number of available fields from all sections and expressed as a percentage of total pulp volume.

7.2.1 Statistical analyses.

Basic descriptive statistics were applied to the microbial counts from the culture data including mean, standard error, median, minimum and maximum counts. Wilcoxon signed-rank statistics, Kruskal-Wallis, analysis of variance, t-tests and correlation matrices were applied to test differences, including those between real-time PCR and the CFU methods of enumerating bacteria, various specificity tests, and interspecies relationships between pathological category and bacterial load.

7.3 Results

7.3.1 Histopathology

For descriptive purposes, histopathological sections of the 65 pulps were divided into four groups based on the dominant pathology, although the majority of sections showed more than one type. To assess the extent of the different categories of pathological change throughout the specimens, the percentage of total pulp volume contributed by each presentation was also determined (Figure 7.1). Based on these assignments, the dominant histology in 43% of pulps and 39% of total pulp volume (determined as a percentage for the 65 pulps) was a category (i) pattern with essentially normal histology (Figure 7.2). Those pulps with a category (i) dominant presentation also exhibited focal areas showing inflammatory infiltration or soft tissue disturbances.

Soft tissue degeneration (category ii) was noted as the dominant pathology in 29% of the samples and 25% of total pulp volume. These pulps showed evidence of some abnormal connective tissue architecture including pulpal fibrosis with accompanying loss in cellularity and a granular appearance of the matrix (Figures 7.3 and 7.4). Alterations to the microvasculature appeared as thickening of the basement membrane of small vessels with narrowing of the vessel lumen (Figures 7.5 and 7.6). These samples were also characterised by irregular calcific deposits. In some cases pulpal tissue was replaced by hyaline-affected material interspersed with embedded necrotic cells (Figure 7.7).

Hard tissue degenerative change or category (iii) change characterised by calcification as the dominant pathology was evident for 11% of the pulp specimens and 14% of the total pulp volume. These were noted as discrete calcific deposits often occupying a substantial amount of the pulp section. These deposits were invariably surrounded by pulpal tissue showing amorphous hyaline changes and fibrosis, with some inflammatory cell infiltrate. Other presentations of calcific change were seen as large collections of small discrete calcifications or areas of diffuse calcification within hyaline-affected tissue (Figures 7.8 and 7.9).

The final category of inflammatory degenerative change (category iv) was evident in 17% of pulp specimens as the dominant pathology and 22% of total pulp volume. This category was

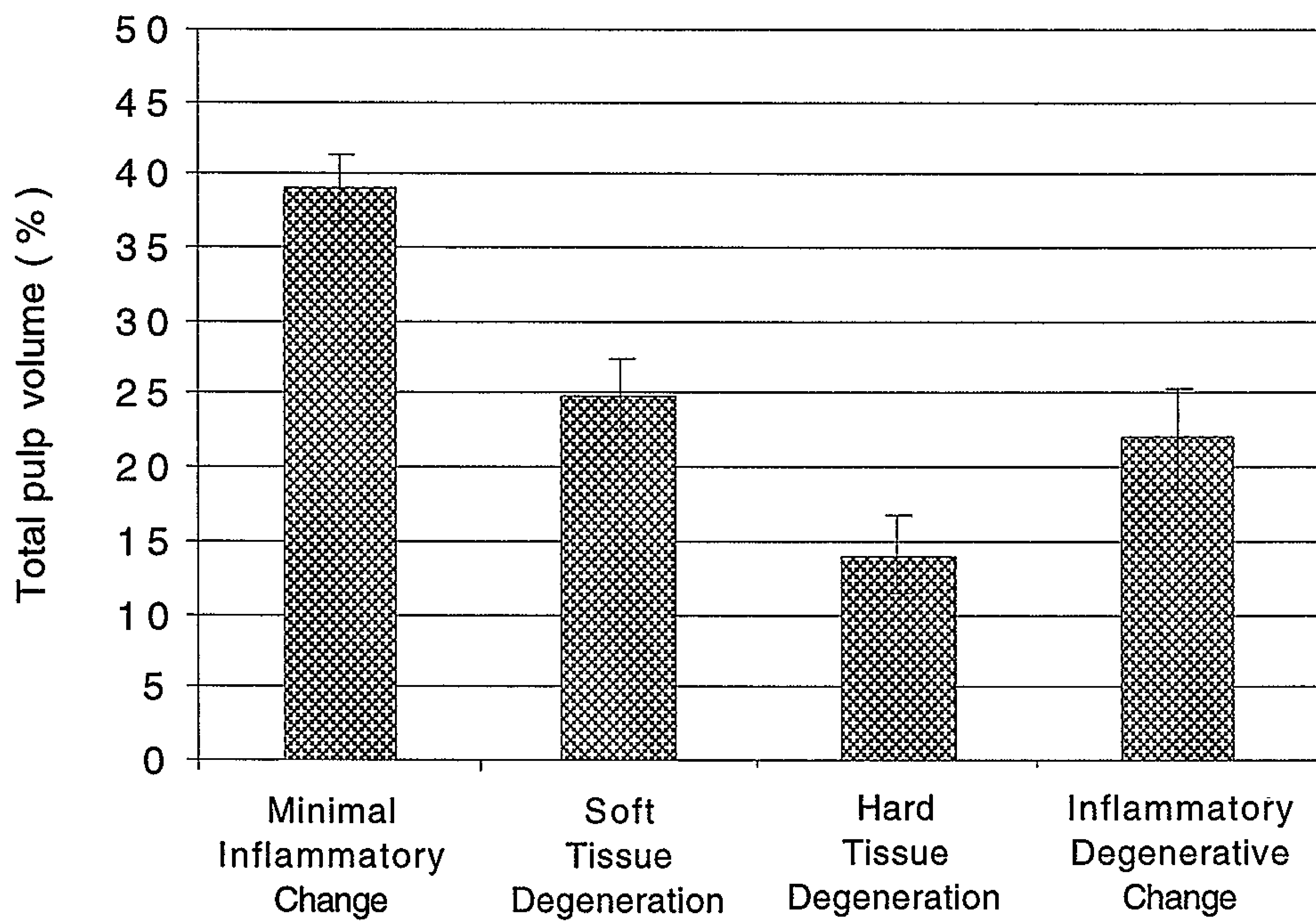


Figure 7.1. Histogram showing the distribution of the four main categories of pulp pathology from the pooled results of all pulps. Total pulp volume for each category was calculated by dividing the number of graticule fields showing a particular histopathological change by the total number of fields from all sections of all pulps. Data are expressed as means ± SEM for the 65 pulps studied.

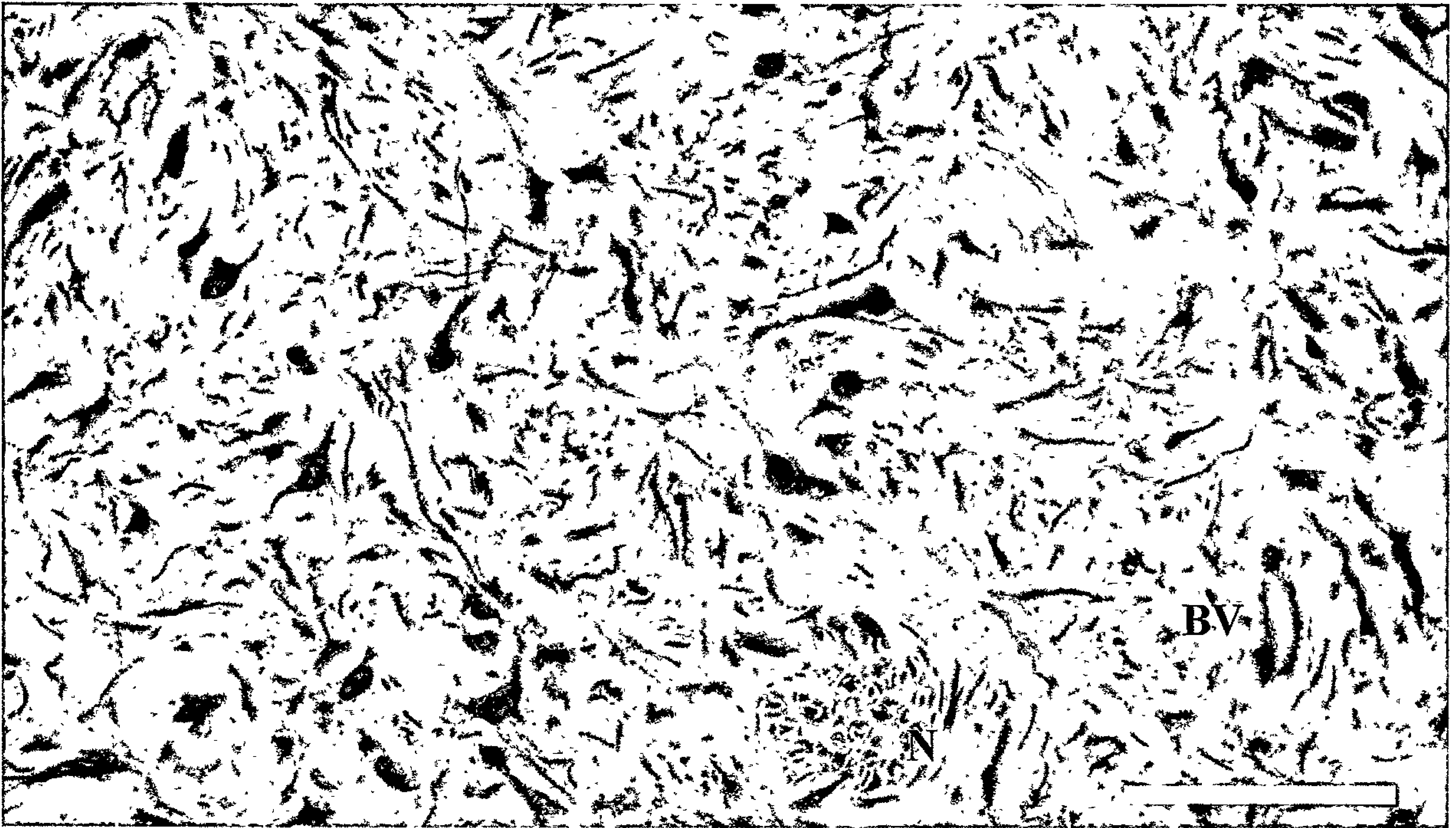


Figure 7.2. Normal pulp histology showing the typical loose connective tissue containing fibroblasts, putative mesenchymal cells and collagen fibres with nerves (N) and blood vessels (BV) present. Scale bar represents 50μm.

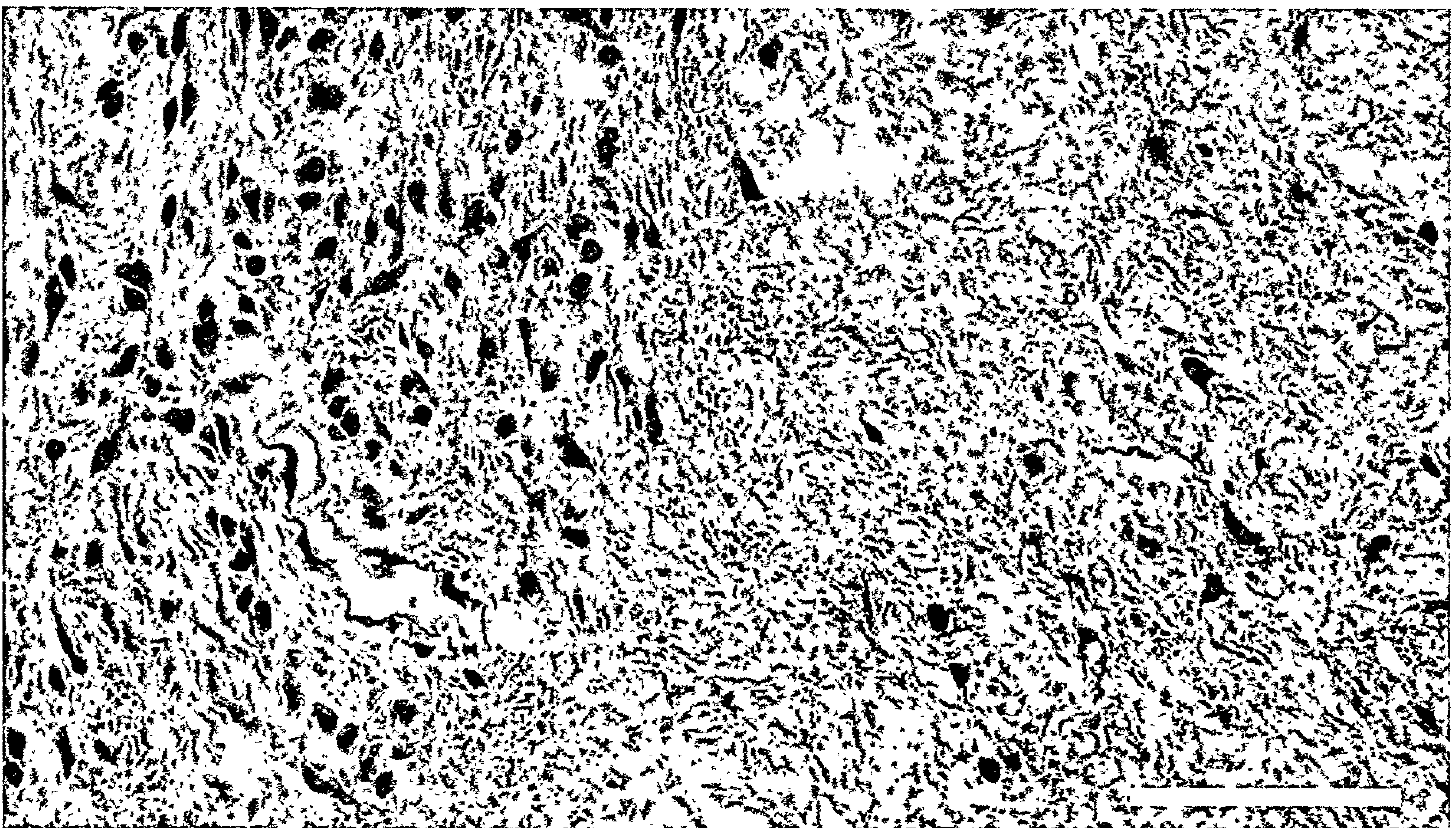


Figure 7.3. Pulp tissue showing hypocellular regions with disordered collagen arrangements on the right side of the image suggestive of a disintegrating matrix. Scale bar represents 50μm.

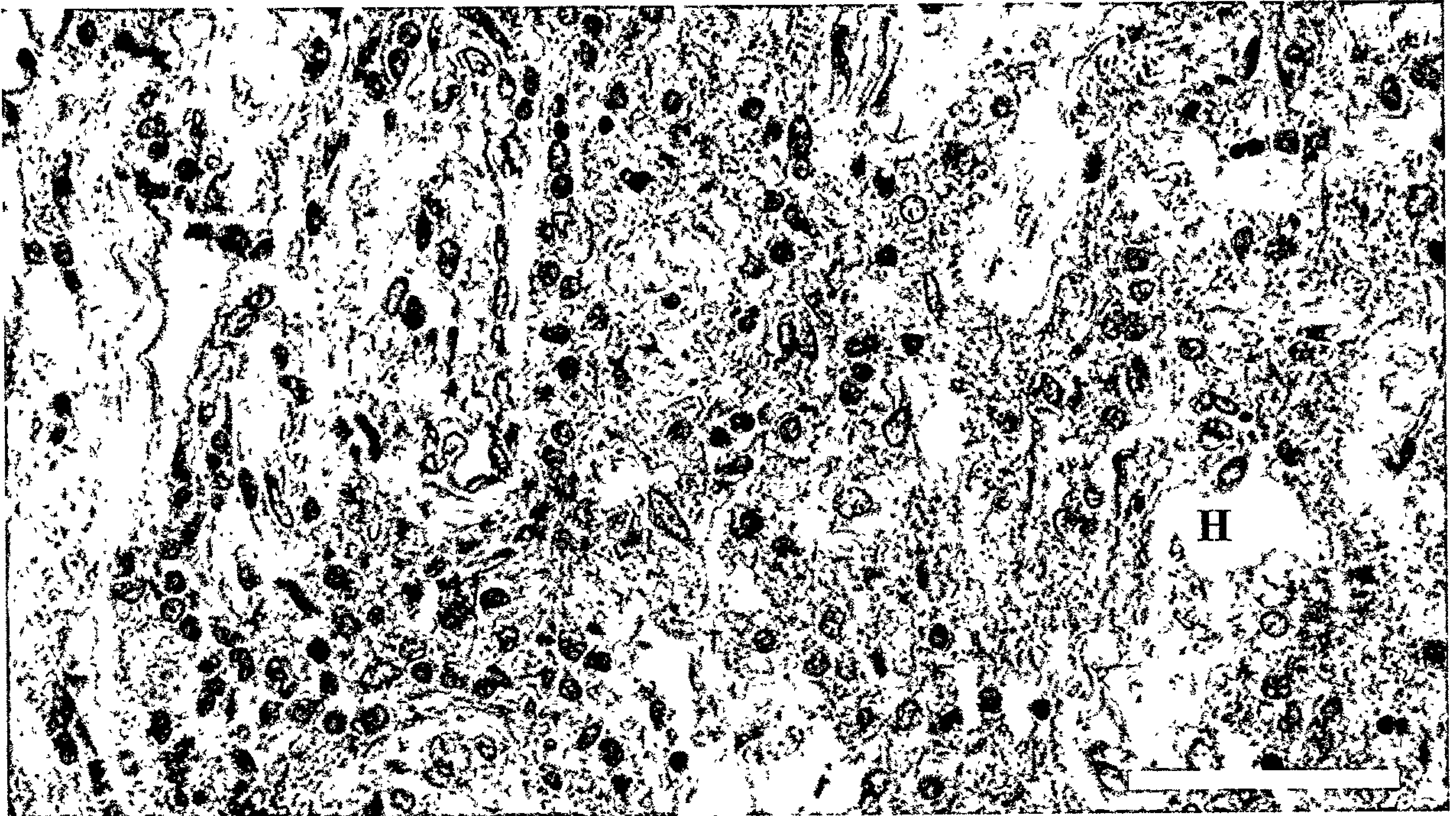


Figure 7.4. Soft tissue alterations including hyaline changes (H) and small collagen fragments interspersed with active fibroblasts. Scale bar represents 50μm.

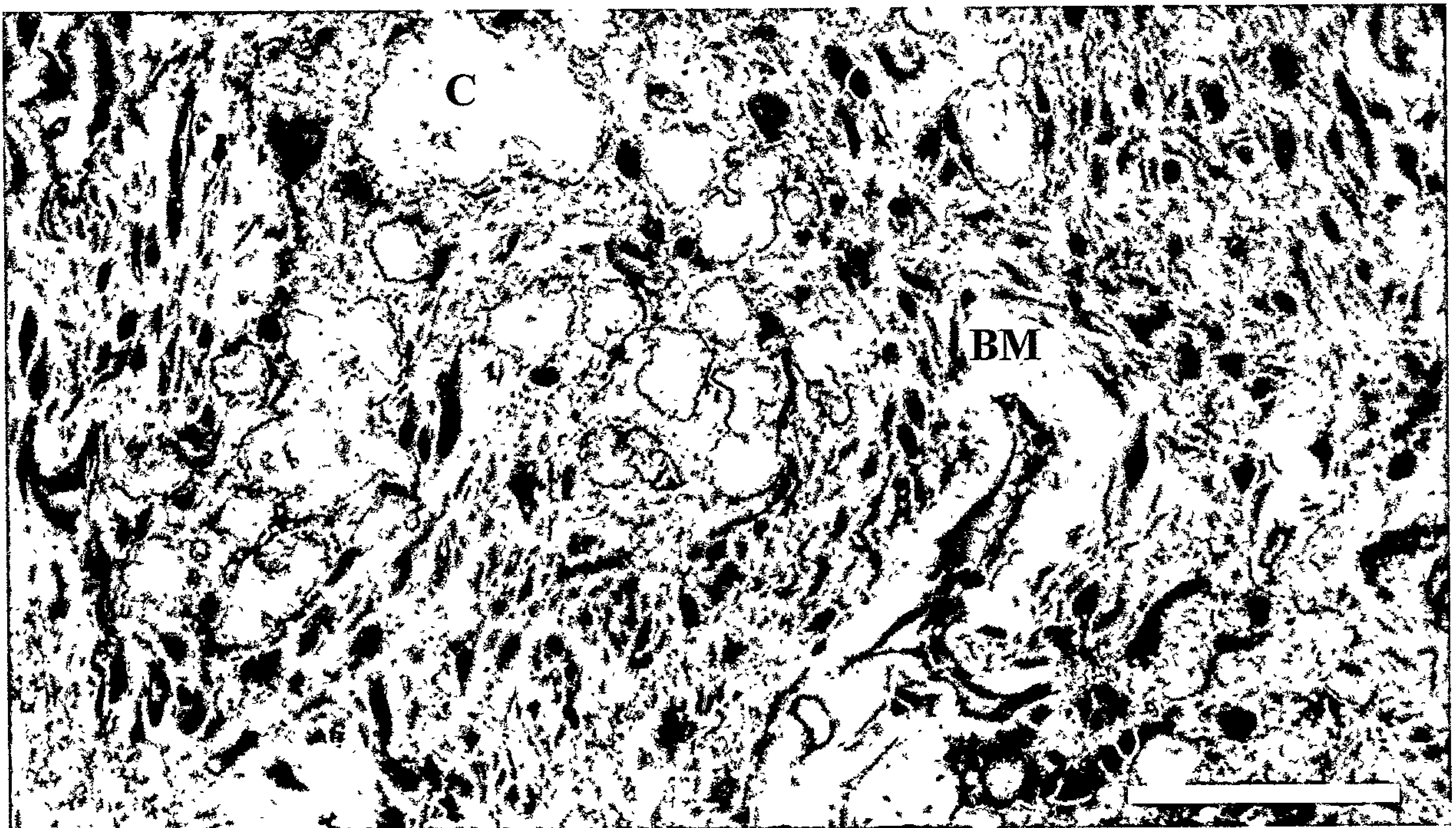


Figure 7.5. Numerous calcific foci (C) and hyaline changes affecting the small vessels (BM) throughout the connective tissue matrix. Scale bar represents 50μm.

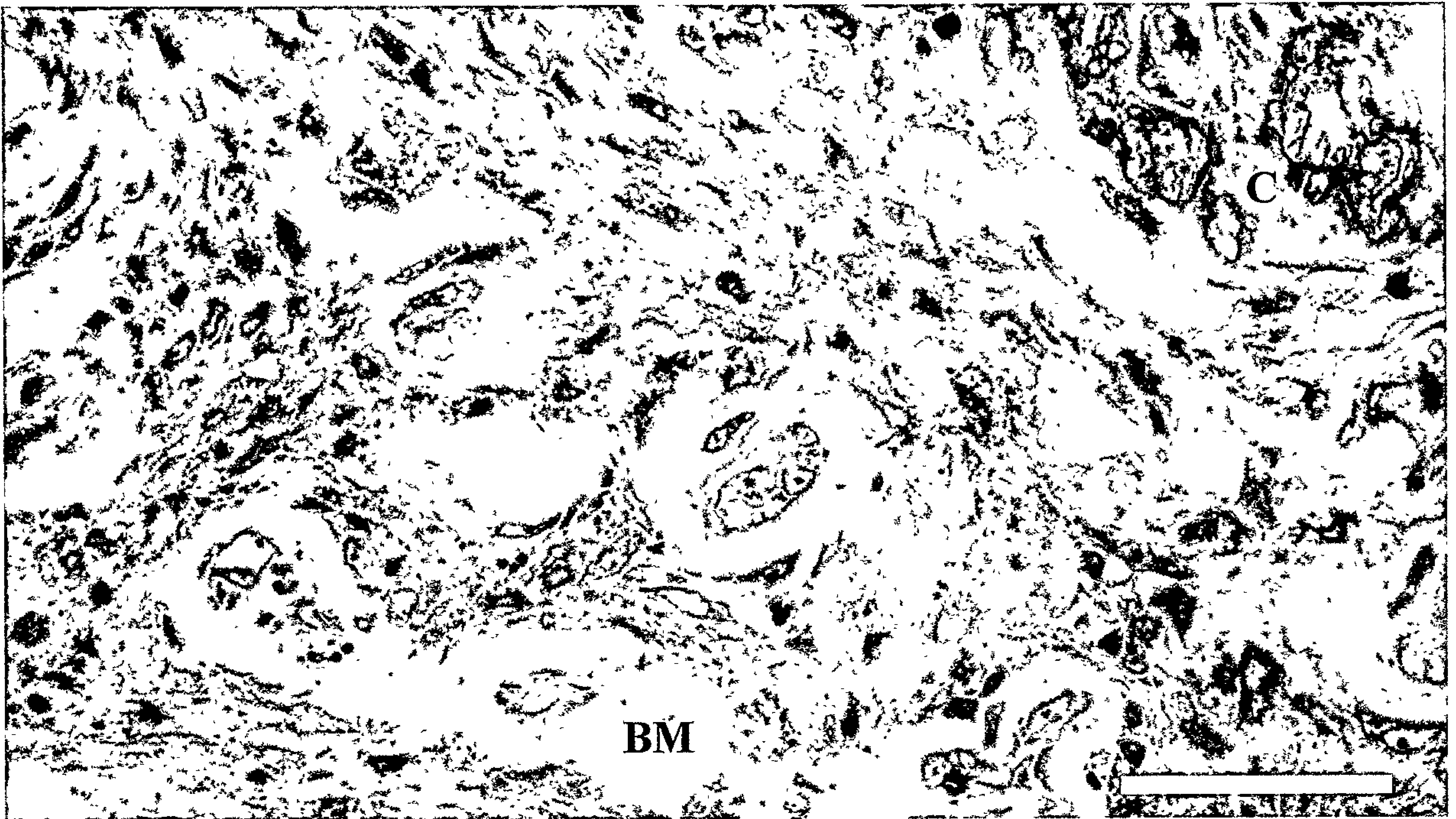


Figure 7.6. Hyaline changes resulting in basement membrane thickening of small vessels (BM) containing entrapped cells. Note calcific changes(C). Scale bar represents 50μm.

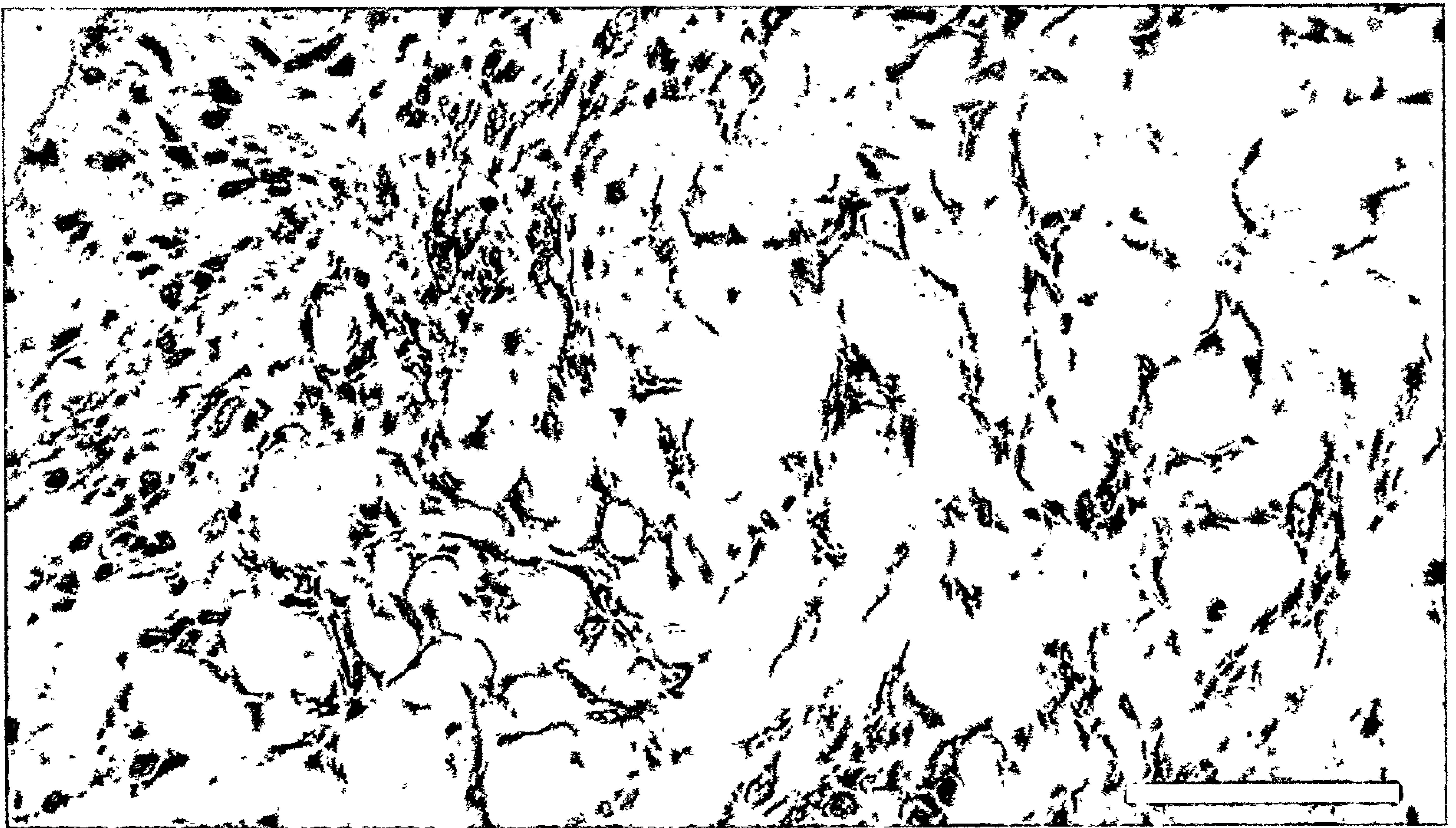


Figure 7.7. Most of the pulp tissue replaced by hyaline degeneration containing entrapped necrotic cells. Scale bar represents 50μm.

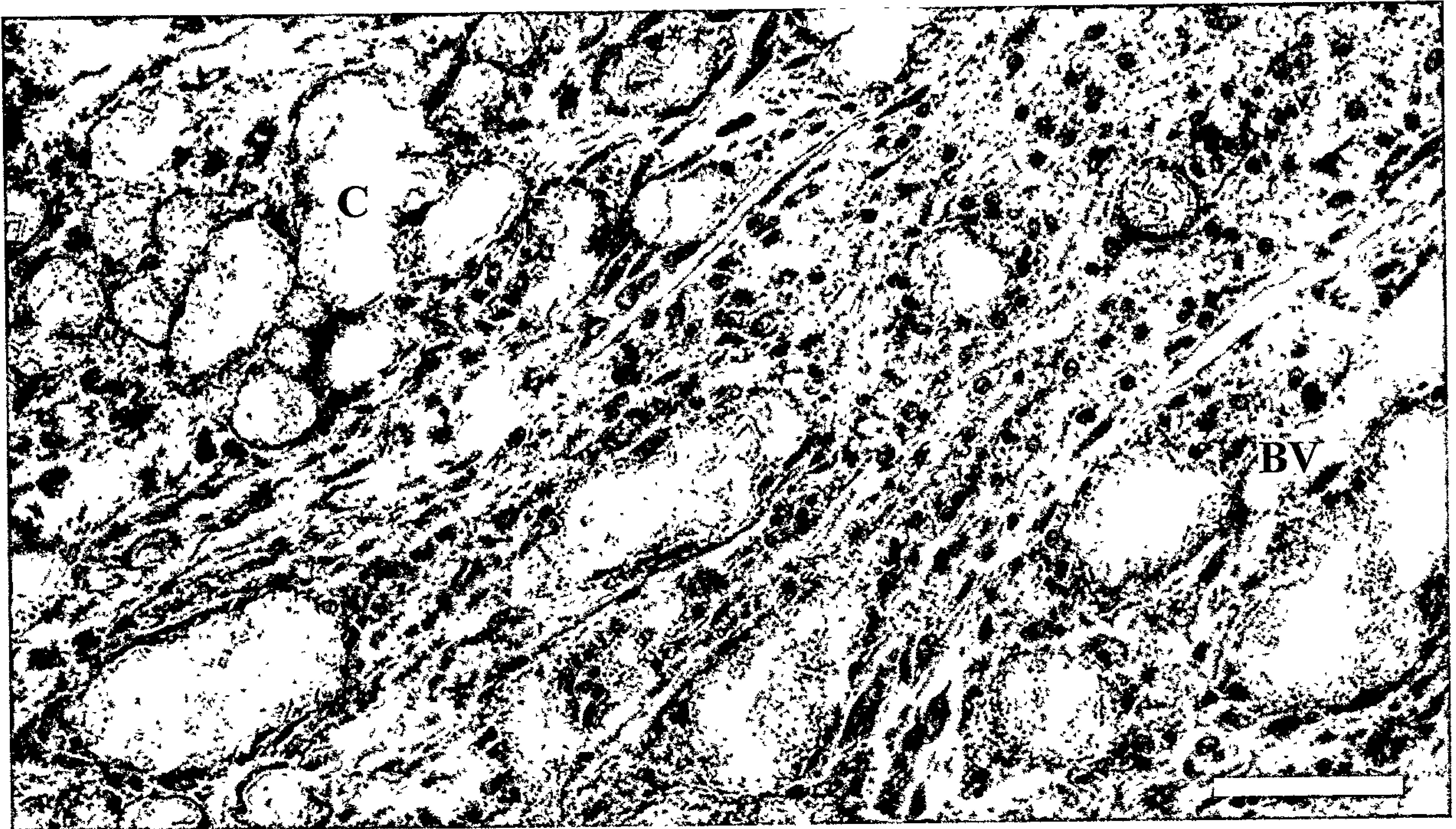


Figure 7.8. Numerous calcific foci (C) dispersed throughout a degenerate matrix. Note the engorged blood vessels (BV). Scale bar represents 50μm.

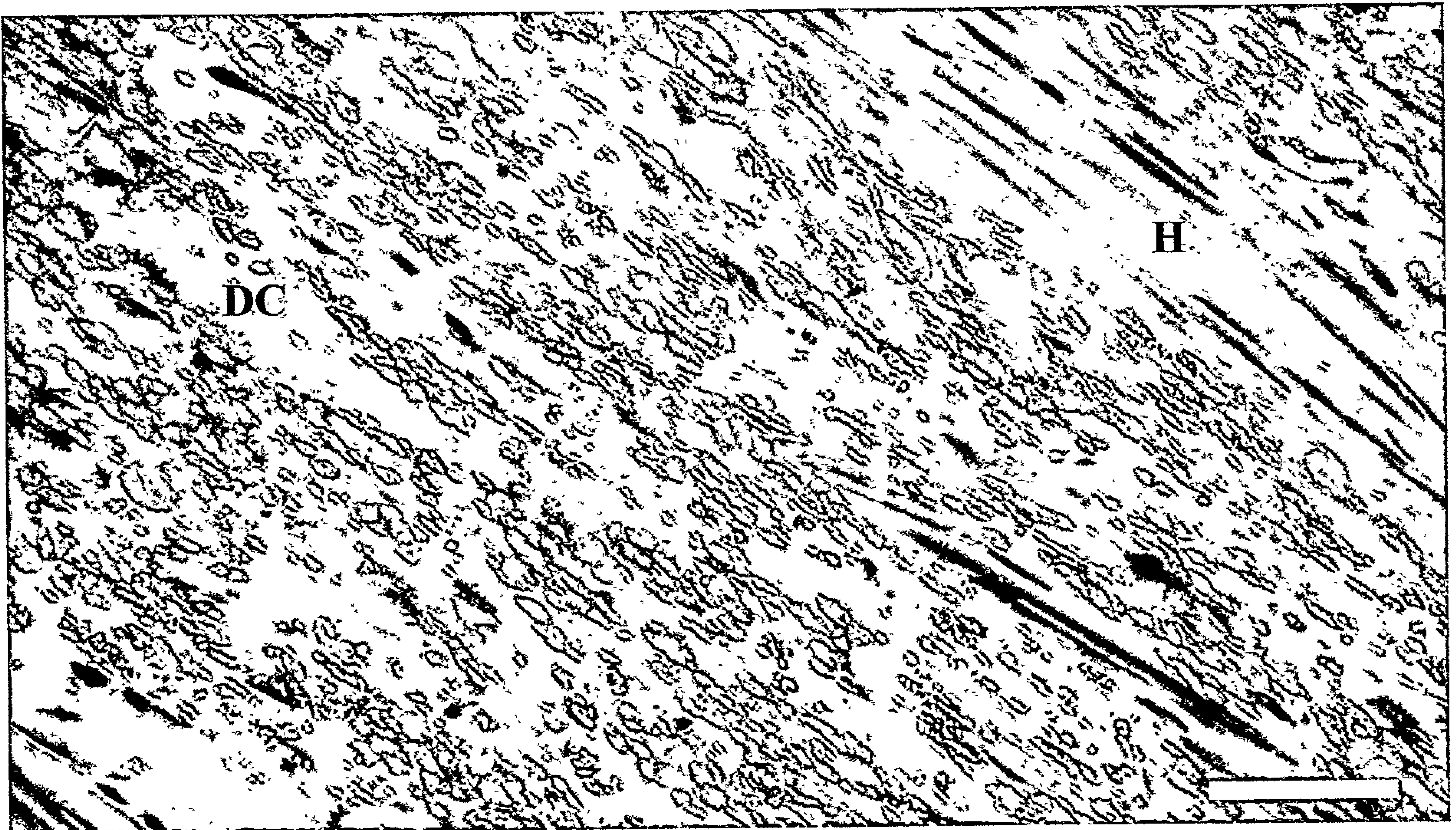


Figure 7.9. Diffuse calcification (DC) seen affecting most of the pulp tissue interspersed with areas of hyaline change (H). Scale bar represents 50μm.

characterised by a mononuclear infiltrate of primarily plasma cells, often seen in various stages of maturation, with smaller numbers of macrophages and lymphocytes (Figures 7.10 and 7.11). Some sections showed evidence of necrotic tissue encircled by accumulations of polymorphs and foamy macrophages (Figures 7.12 and 7.13) separating the chronic inflammatory cells and fibrous tissue surrounding these foci (Figure 7.14). The presence of bacteria (cocci, and short and long rod forms) was noted under high power magnification either extra or intra-cellularly within foamy macrophages in 14% of samples (Figure 7.15). In the majority of cases the presence of bacteria was associated with a marked inflammatory infiltrate, however, in two instances there was minimal inflammatory reaction.

Where considerable inflammation of the tissue was evident, responses in blood vessels ranged from subtle changes in cell morphology to lysis of endothelial cells, and included vessel wall disruption, vessel thrombosis and extravascular haemorrhage. Also noted were phagocytic cells in various stages of lysis. Areas of necrosis (Figure 7.16) were seen associated with marked inflammatory infiltrate in 12% of samples. Five percent of samples showed almost total tissue destruction with few cellular, blood vessel or fibrous elements recognisable within a disintegrated amorphous matrix containing numerous bacteria (Figure 7.17).

7.3.2 Inter-bacterial associations within carious dentine and associations with the histopathology of pulpitis

Multivariate analyses were performed on the number of bacteria determined by colony counting and by real-time PCR, and the histopathological data (Tables 7.1 to 7.8). Irrespective of the method used to enumerate the bacteria in carious dentine, no significant relationship was apparent between the total number of anaerobic or microaerophilic bacteria and the histopathological category (p values for anaerobic CFU 0.92, microaerophilic CFU 0.152 and anaerobic PCR 0.65 using the Kruskal-Wallis test). Similarly, no significant relationship was found between histopathological category and the selective microbial load for the genera *Actinomyces, Fusobacterium, Lactobacillus, Prevotella* and *Streptococcus* as determined by colony counts (p values 0.29, 0.84, 0.64, 0.44 and 0.19 respectively, Kruskal-Wallis) or for *Fusobacterium, Prevotella,* or the species *P. melaninogenica, M. micros, P. endodontalis* and

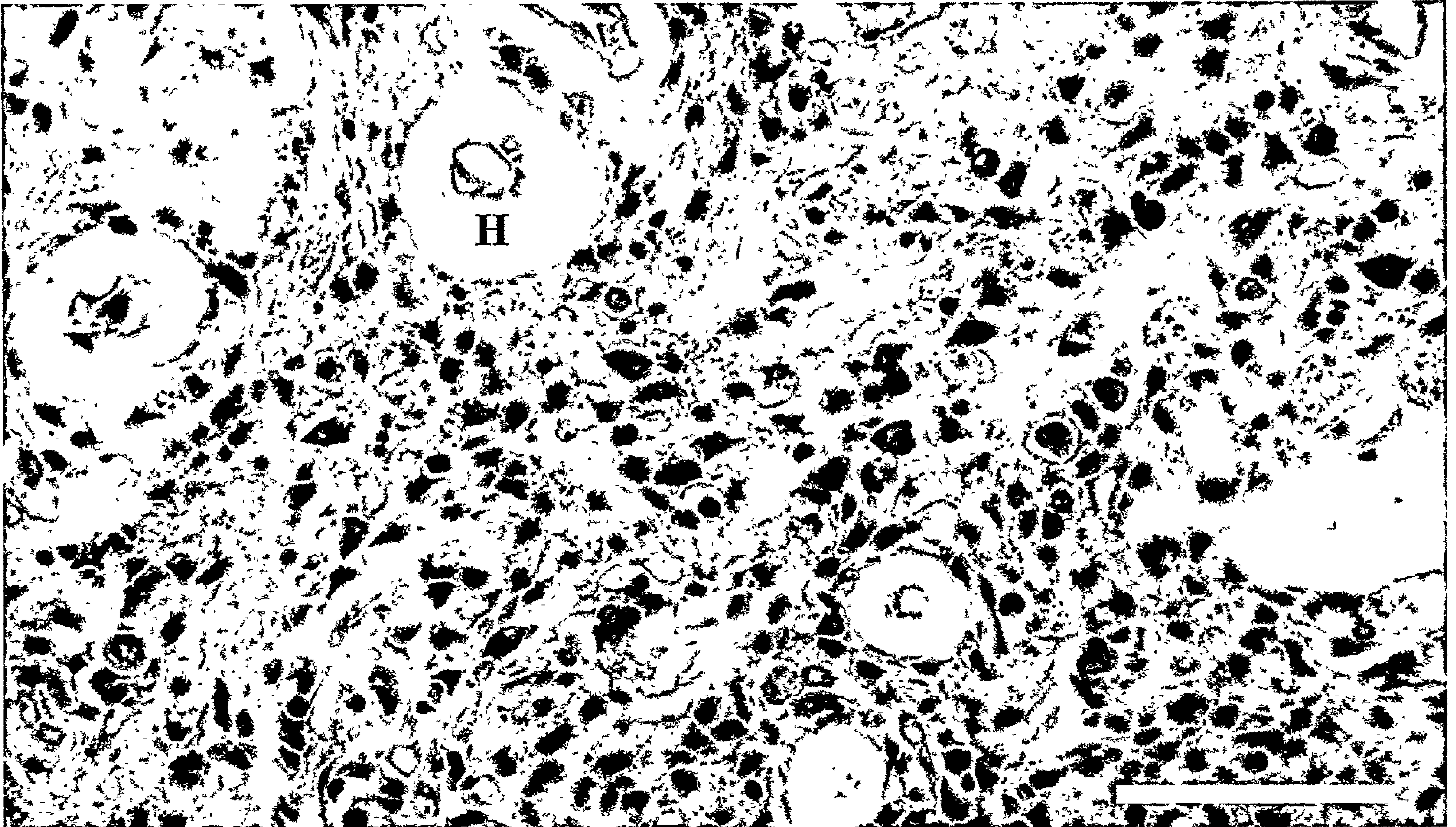


Figure 7.10. Inflammatory infiltrate with varying numbers of lymphocytes and monocytes. Hyaline basement membrane changes (H) noted around small vessels. Scale bar represents 50μm.

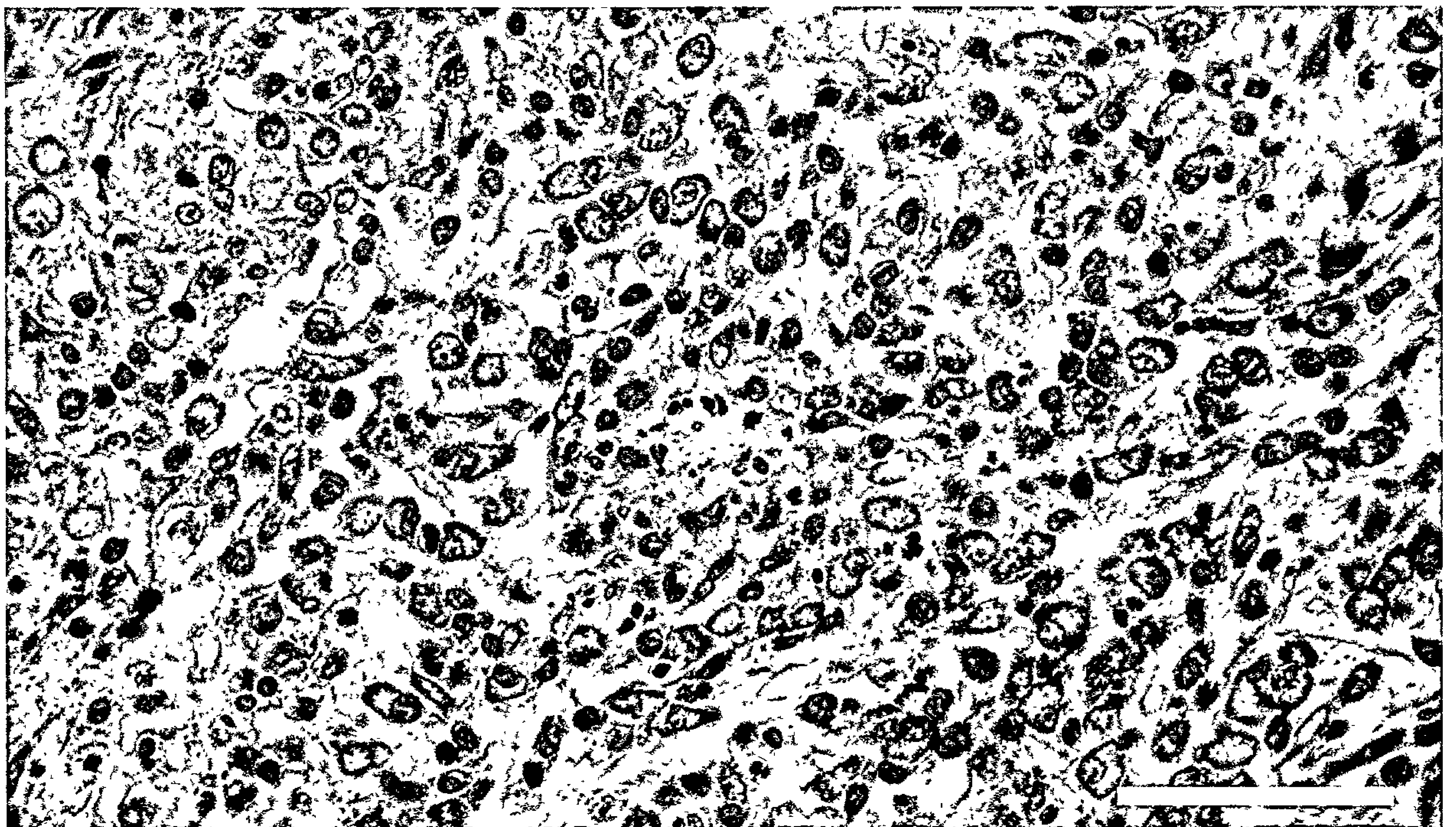


Figure 7.11. Plasma cell infiltration of the pulp tissue surrounding a small number of degenerating polymorphs. Scale bar represents 50μm.

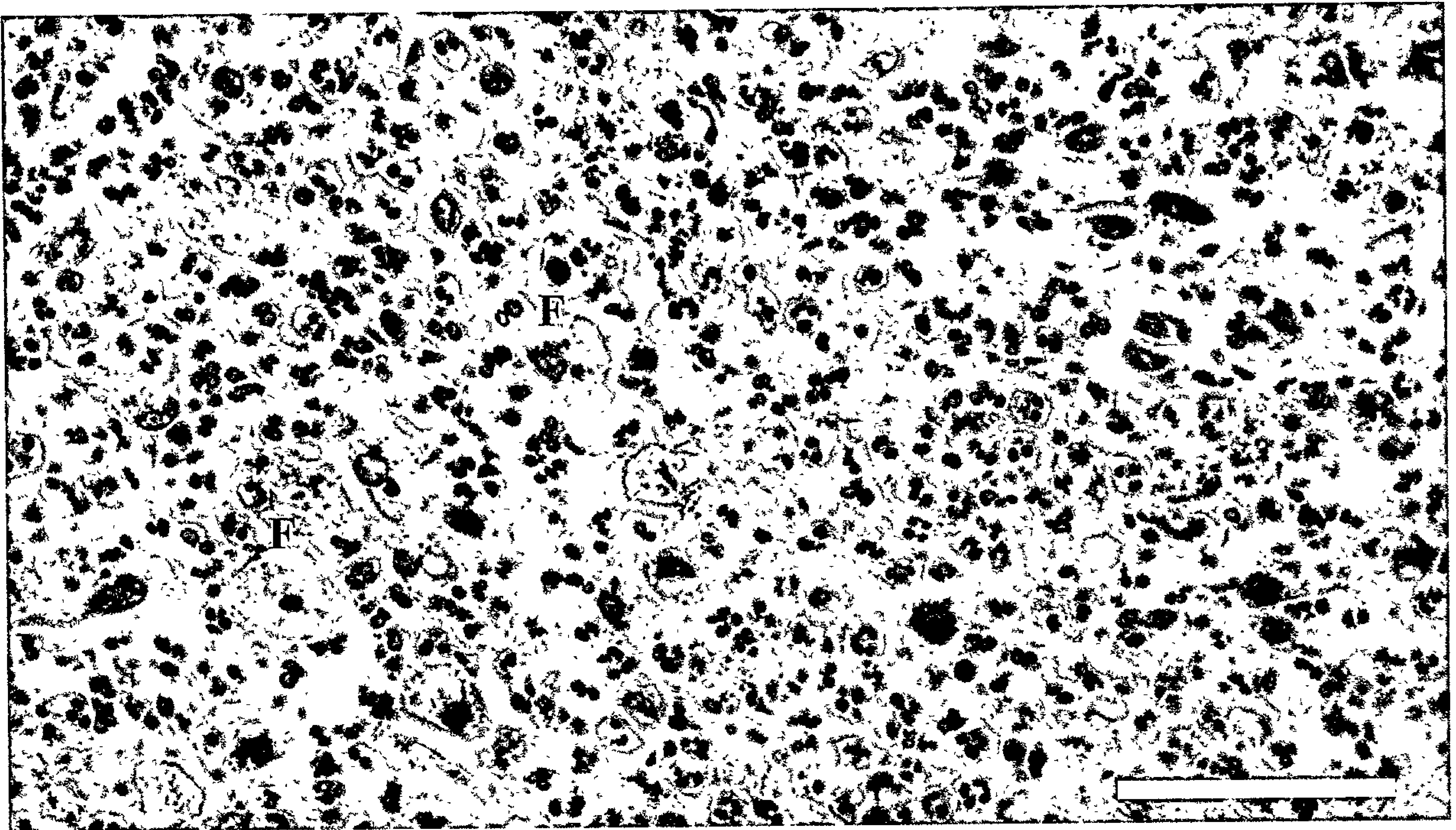


Figure 7.12. Areas of acute inflammation characterised by infiltration of polymorphonuclear leukocytes and "foamy macrophages" (F). Scale bar represents 50μm.

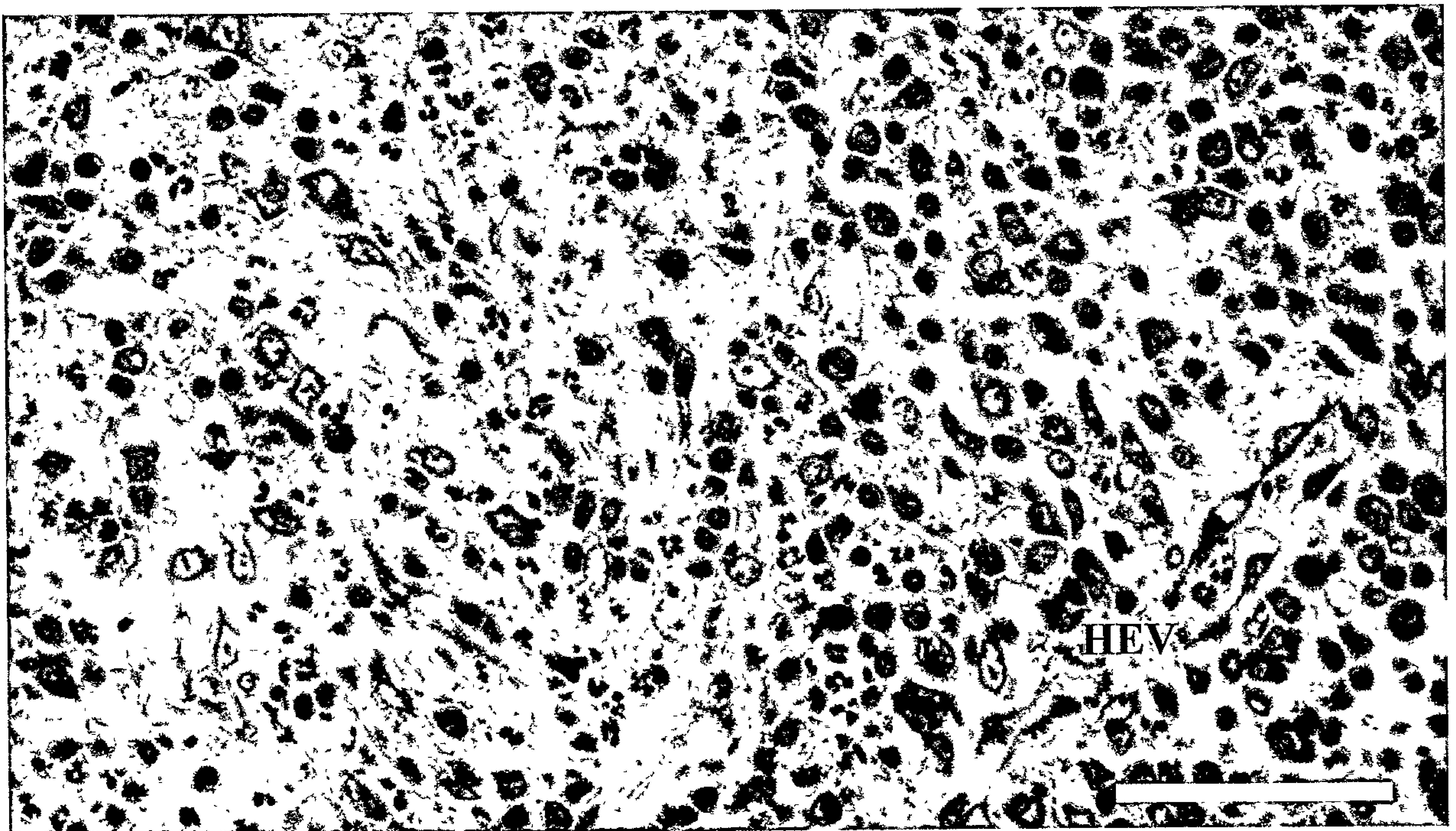


Figure 7.13. Inflammatory infiltrate of polymorphs, plasma cells, macrophages and monocytes within the connective tissue. Small vessels in the area of infiltration show features resembling high endothelial venules (HEV). Scale bar represents 50μm.

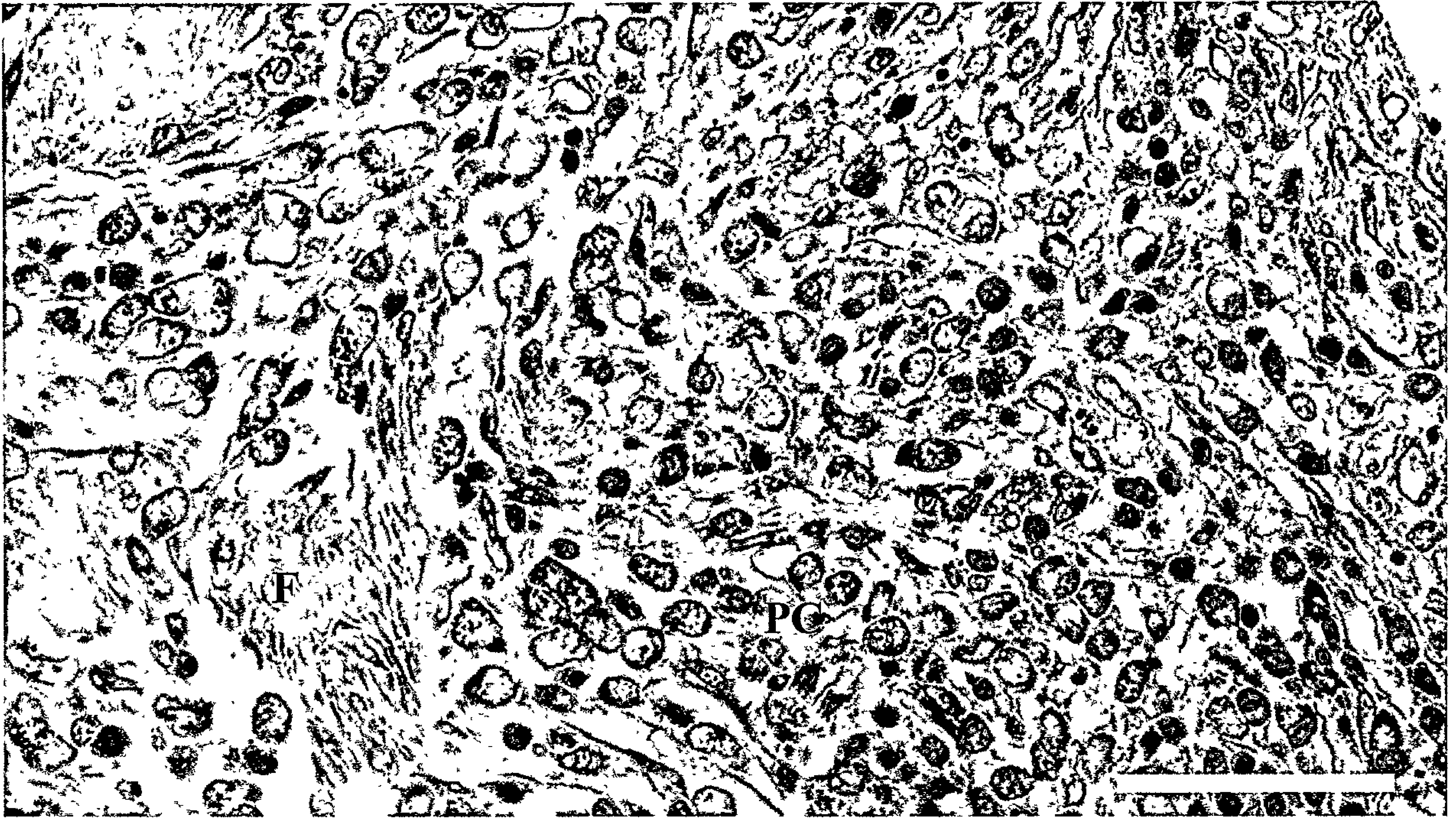


Figure 7.14. Infiltration of plasma cells (PC) with smaller numbers of lymphocytes and macrophages into a connective tissue matrix showing fibrous tissue changes (F). Scale bar represents 50μm.

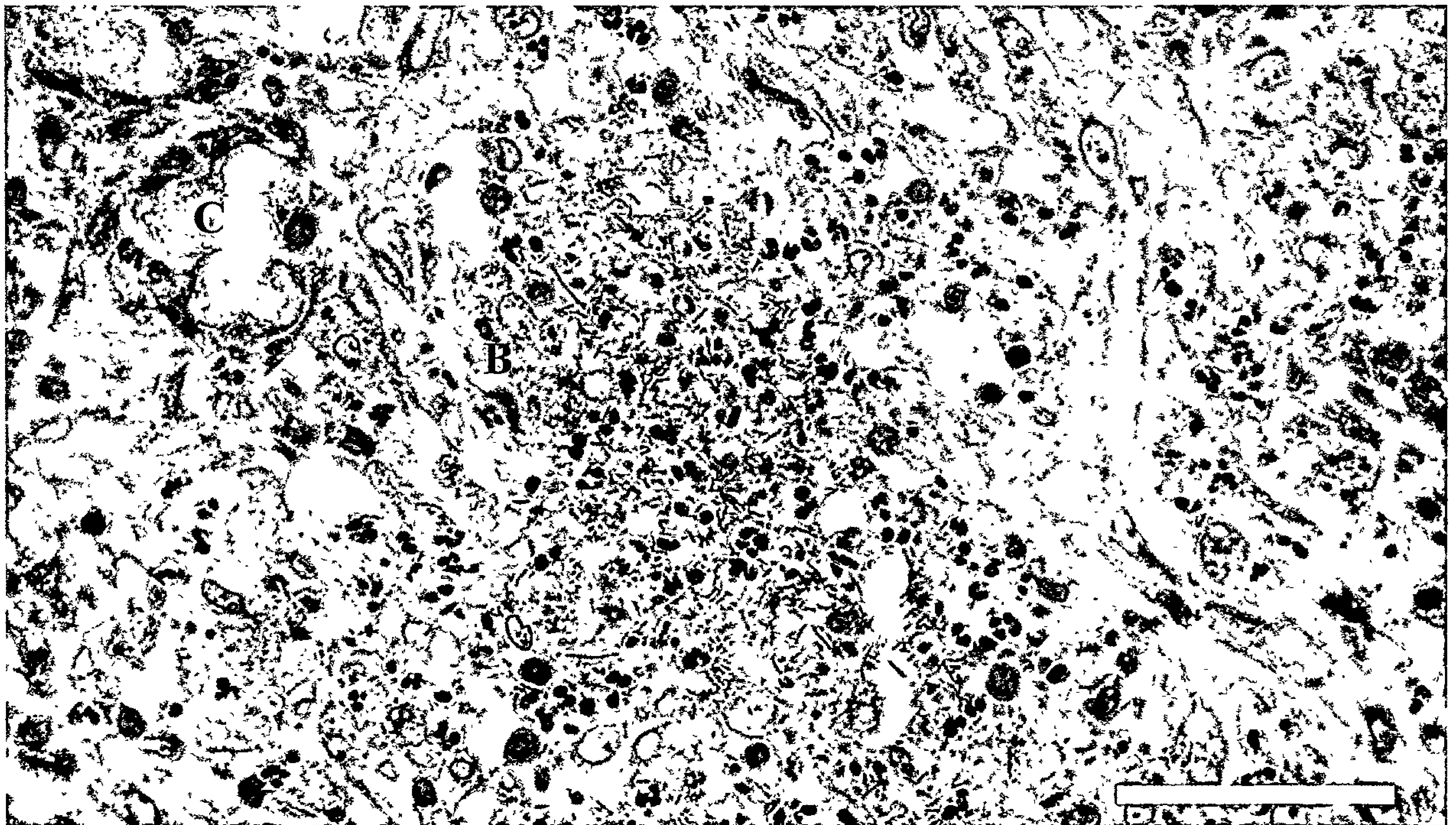


Figure 7.15. Inflammatory reaction of polymorphs to the presence of bacteria (B), seen as long and short rods, and cocci. Note calcific foci (C). Scale bar represents 50μm.

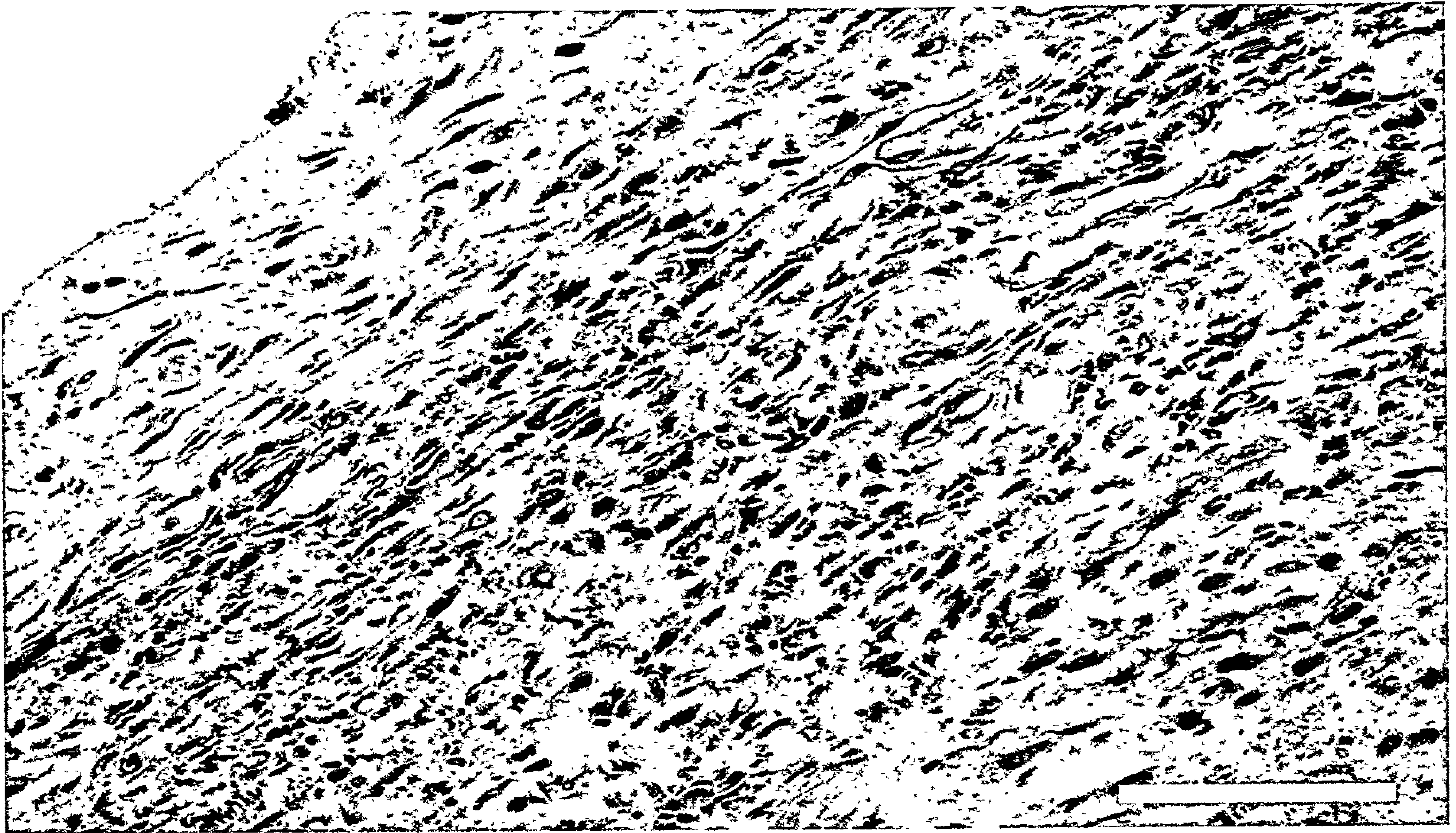


Figure 7.16. Pulp tissue containing a large number of degenerating and necrotic cells. Scale bar represents 50μm.

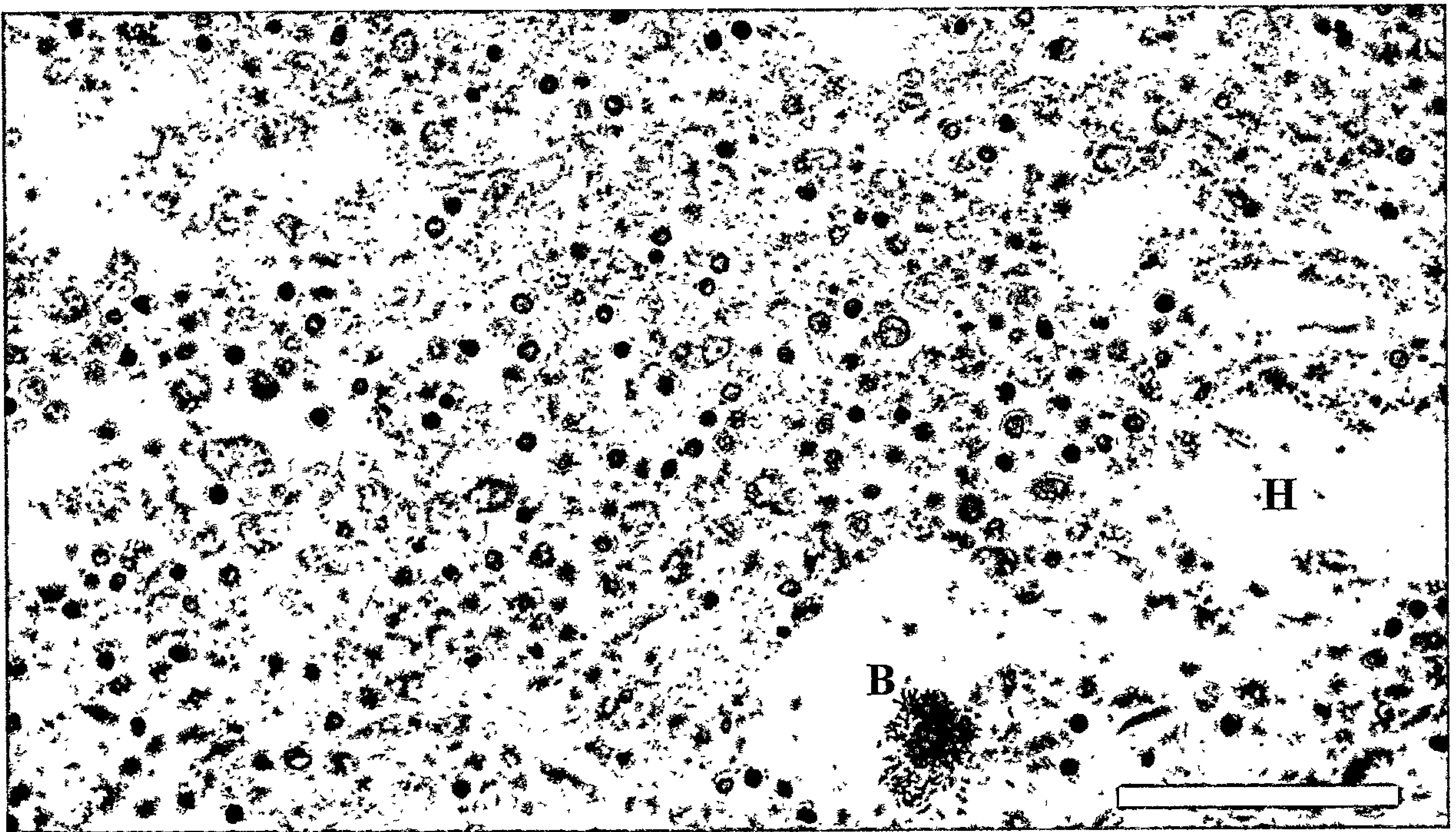


Figure 7.17. Necrotic pulp tissue containing degenerating cells, areas of hyaline change (H) and a clump of bacteria (B). Scale bar represents 50μm.

Table 7.1. Culture data CFU per mg wet weight dentine. Bacteria detected in carious dentine by colony counting from category (i), minimal inflammatory change.

Bacterium	CFU (mg dentine) $^{-1\,a}$		
	Range	Median	Mean ± SEM
Anaerobic	$1.4 \times 10^5 - 2.1 \times 10^7$	57×10^5	$74 \pm 12 \times 10^5$
Microaerophilic	$2.5 \times 10^4 - 3.3 \times 10^6$	1.2×10^5	$13 \pm 2.0 \times 10^5$
Prevotella	$0.0 - 4.4 \times 10^6$	1.0×10^5	$5.5 \pm 2.2 \times 10^5$
P. melaninogenica	$0.0 - 3.3 \times 10^6$	12×10^5	$13 \pm 2.0 \times 10^5$
F. nucleatum	$0.0 - 7.4 \times 10^5$	0.8×10^5	$1.7 \pm 0.4 \times 10^5$
Actinomycetes	$0.0 - 5.3 \times 10^6$	0.6×10^5	$3.5 \pm 2.0 \times 10^5$
Lactobacilli	$0.0 - 1.9 \times 10^7$	5.2×10^5	$14 \pm 7.0 \times 10^5$
Streptococci	$0.0 - 3.1 \times 10^6$	2.0×10^5	$5.1 \pm 1.5 \times 10^5$

[a] Data collected from 28 samples.

Table 7.2. Culture data CFU per mg wet weight dentine. Bacteria detected in carious dentine by colony counting from category (ii), soft tissue degenerative change.

Bacterium	CFU (mg dentine) $^{-1\,a}$		
	Range	Median	Mean ± SEM
Anaerobic	$3.3 \times 10^5 - 3.7 \times 10^7$	58×10^5	$80 \pm 22 \times 10^5$
Microaerophilic	$3.5 \times 10^4 - 4.7 \times 10^6$	4.9×10^5	$8.4 \pm 2.6 \times 10^5$
Prevotella	$0.0 - 5.1 \times 10^6$	2.1×10^5	$5.0 \pm 3.0 \times 10^5$
P. melaninogenica	$0.0 - 5.1 \times 10^6$	1.6×10^5	$4.9 \pm 3.1 \times 10^5$
F. nucleatum	$0.0 - 9.1 \times 10^5$	0.5×10^5	$1.7 \pm 0.6 \times 10^5$
Actinomycetes	$0.0 - 2.6 \times 10^5$	0.7×10^5	$1.1 \pm 0.2 \times 10^5$
Lactobacilli	$0.0 - 5.2 \times 10^6$	3.6×10^5	$7.3 \pm 2.8 \times 10^5$
Streptococci	$0.0 - 1.1 \times 10^6$	1.1×10^5	$1.9 \pm 0.6 \times 10^5$

[a] Data collected from 19 samples.

Table 7.3. Culture data CFU per mg wet weight dentine. Bacteria detected in carious dentine by colony counting from category (iii), hard tissue degenerative change.

Bacterium	CFU (mg dentine)$^{-1 a}$		
	Range	Median	Mean ± SEM
Anaerobic	$1.9 \times 10^6 - 1.0 \times 10^7$	45×10^5	$58 \pm 12 \times 10^5$
Microaerophilic	$2.4 \times 10^5 - 3.7 \times 10^6$	15×10^5	$17 \pm 5.0 \times 10^5$
Prevotella	$0.0 - 2.8 \times 10^5$	0.4×10^5	$1.1 \pm 0.5 \times 10^5$
P. melaninogenica	$0.0 - 2.1 \times 10^5$	0.3×10^5	$0.9 \pm 0.4 \times 10^5$
F. nucleatum	$5.0 \times 10^3 - 1.0 \times 10^6$	0.4×10^5	$2.1 \pm 1.4 \times 10^5$
Actinomycetes	$0.0 - 1.8 \times 10^5$	0.3×10^5	$0.6 \pm 0.3 \times 10^5$
Lactobacilli	$1.3 \times 10^5 - 2.4 \times 10^6$	3.3×10^5	$9.1 \pm 4.0 \times 10^5$
Streptococci	$1.0 \times 10^4 - 9.9 \times 10^5$	2.5×10^5	$3.2 \pm 1.3 \times 10^5$

[a] Data collected from 7 samples.

Table 7.4. Culture data CFU per mg wet weight dentine. Bacteria detected in carious dentine by colony counting from category (iv), inflammatory degenerative change.

Bacterium	CFU (mg dentine)$^{-1 a}$		
	Range	Median	Mean ± SEM
Anaerobic	$4.4 \times 10^5 - 2.2 \times 10^7$	38×10^5	$68 \pm 22 \times 10^5$
Microaerophilic	$1.1 \times 10^5 - 2.5 \times 10^6$	8.7×10^5	$11 \pm 2.0 \times 10^5$
Prevotella	$0.0 - 1.4 \times 10^6$	3.5×10^5	$4.7 \pm 1.5 \times 10^5$
P. melaninogenica	$0.0 - 1.1 \times 10^6$	3.5×10^5	$4.1 \pm 1.3 \times 10^5$
F. nucleatum	$5.0 \times 10^2 - 5.6 \times 10^5$	1.5×10^5	$1.9 \pm 0.6 \times 10^5$
Actinomycetes	$0.0 - 6.5 \times 10^5$	1.3×10^5	$2.0 \pm 0.6 \times 10^5$
Lactobacilli	$1.5 \times 10^4 - 1.4 \times 10^6$	7.7×10^5	$6.7 \pm 1.4 \times 10^5$
Streptococci	$2.5 \times 10^4 - 8.8 \times 10^5$	2.1×10^5	$3.5 \pm 0.9 \times 10^5$

[a] Data collected from 11 samples.

Table 7.5. Bacteria detected in carious dentine by real-time PCR per mg wet weight dentine from category (i), minimal inflammatory change.

Bacterium	Cell Number (mg dentine)$^{-1\,a}$		
	Range	Median	Mean ± SEM
Anaerobic [b]	$2.7 \times 10^7 - 1.1 \times 10^9$	2.4×10^8	$3.1 \pm 0.5 \times 10^8$
Prevotella	$0.0 - 1.7 \times 10^8$	18×10^6	$39 \pm 10 \times 10^6$
P. melaninogenica	$0.0 - 3.5 \times 10^6$	$.08 \times 10^6$	$0.3 \pm 0.2 \times 10^6$
Fusobacterium	$4.8 \times 10^2 - 5.7 \times 10^6$	0.1×10^6	$0.5 \pm 0.2 \times 10^6$
M. micros	$0.0 - 4.8 \times 10^6$	0.4×10^6	$0.9 \pm 0.3 \times 10^6$
P. endodontalis	$0.0 - 1.3 \times 10^6$	0.01×10^6	$0.1 \pm 0.1 \times 10^6$
P. gingivalis	$0.0 - 2.5 \times 10^4$	0.001×10^6	$0.005 \pm 0.002 \times 10^6$

[a] Data collected from 28 samples and determined from triplicate measurements when detected.
[b] Detected with the universal probe and primers set.

Table 7.6. Bacteria detected in carious dentine by real-time PCR per mg wet weight dentine from category (ii), soft tissue degenerative change.

Bacterium	Cell Number (mg dentine)$^{-1\,a}$		
	Range	Median	Mean ± SEM
Total anaerobic [b]	$2.1 \times 10^7 - 9.1 \times 10^8$	2.4×10^8	$2.8 \pm 0.5 \times 10^8$
Prevotella	$1.1 \times 10^4 - 2.0 \times 10^8$	21×10^6	$45 \pm 14 \times 10^6$
P. melaninogenica	$0.0 - 1.1 \times 10^6$	0.04×10^6	$0.1 \pm 0.1 \times 10^6$
Fusobacterium	$5.8 \times 10^2 - 3.1 \times 10^6$	0.05×10^6	$0.3 \pm 0.2 \times 10^6$
M. micros	$0.0 - 1.2 \times 10^7$	0.1×10^6	$1.1 \pm 0.9 \times 10^6$
P. endodontalis	$0.0 - 4.3 \times 10^5$	0.007×10^6	$0.05 \pm 0.04 \times 10^6$
P. gingivalis	$0.0 - 8.8 \times 10^3$	0.001×10^6	$0.003 \pm 0.001 \times 10^6$

[a] Data collected from 19 samples and determined from triplicate measurements when detected.
[b] Detected with the universal probe and primers set.

Table 7.7. Bacteria detected in carious dentine by real-time PCR per mg wet weight dentine from category (iii) hard tissue degenerative change.

Bacterium	Cell Number (mg dentine)$^{-1}$ [a]		
	Range	Median	Mean ± SEM
Total anaerobic [b]	1.2 – 5.3 x 10^8	2.1 x 10^8	2.6 ± 0.6 x 10^8
Prevotella	1.2 x 10^5 – 8.0 x 10^7	7.3 x 10^6	16 ± 11 x 10^6
P. melaninogenica	0.0 – 7.6 x 10^5	0.07 x 10^6	0.2 ± 0.1 x 10^6
Fusobacterium	1.4 x 10^3 – 1.3 x 10^6	0.07 x 10^6	0.3 ± 0.2 x 10^6
M. micros	0.0 – 1.5 x 10^6	0.2 x 10^6	0.4 ± 0.3 x 10^6
P. endodontalis	0.0 – 1.8 x 10^4	0.006 x 10^6	0.01 ± 0.004 x 10^6
P. gingivalis	0.0 – 1.6 x 10^4	0.0007 x 10^6	0.004 ± 0.003 x 10^6

[a] Data collected from 7 samples and determined from triplicate measurements when detected.
[b] Detected with the universal probe and primers set.

Table 7.8. Bacteria detected in carious dentine by real-time PCR per mg wet weight dentine from category (iv) inflammatory degenerative change.

Bacterium	Cell Number (mg dentine)$^{-1}$ [a]		
	Range	Median	Mean ± SEM
Total anaerobic [b]	1.0 – 4.9 x 10^8	3.4 x 10^8	3.3 ± 0.4 x 10^8
Prevotella	7.0 x 10^4 – 1.7 x 10^7	19 x 10^6	40 ± 17 x 10^6
P. melaninogenica	2.4 x 10^3 – 1.7 x 10^6	0.2 x 10^6	0.4 ± 0.2 x 10^6
Fusobacterium	2.5 x 10^3 – 2.4 x 10^6	0.1 x 10^6	0.4 ± 0.2 x 10^6
M. micros	0.0 – 1.6 x 10^7	0.7 x 10^6	4.9 ± 2.9 x 10^6
P. endodontalis	0.0 – 2.5 x 10^5	0.005 x 10^6	0.07 ± 0.05 x 10^6
P. gingivalis	0.0 – 7.0 x 10^3	0.004 x 10^6	0.004 ± 0.001 x 10^6

[a] Data collected from 11 samples and determined from triplicate measurements when detected.
[b] Detected with the universal probe and primers set.

P. gingivalis as determined by PCR (*p* values 0.90, 0.72, 0.14, 0.63, 0.82 and 0.57 respectively, Kruskal-Wallis).

The relation between pairs of microbial species present in carious dentine was determined using Pearson's correlation, while any association between multiple species was determined using the Bonferroni adjustment preset at a significance level of 0.05. Correlations were determined for all the bacterial data and for individual histopathological categories. Although the bacterial counts presented in the tables gives an appearance of uniformity, significant multiple associations were noted for all the bacterial data between the number of CFUs of *Prevotella* spp. and *F. nucleatum* and between those of lactobacilli and streptococci in carious dentine (Table 7.9). Analyses of the different anaerobic bacteria (determined by real-time PCR) indicated further associations between the *Prevotella* spp., *P. melaninogenica* and *Fusobacterium* spp., and between *Fusobacterium* spp., *M. micros* and *P. endodontalis* (Table 7.10).

Using correlation matrices, relationships between microbial species were examined and calculated for individual pathological categories. For culture data (Tables 7.11 to 7.14), there was no significant relationship between the specific genera and/or species of bacteria and the histopathological category except in the case of minimal inflammatory change (category i), for which analyses indicated an association between *F. nucleatum* and *Prevotella* ($r>0.532$), as well as between streptococci and lactobacilli ($r>0.532$). However, analyses of anaerobic bacteria determined by real-time PCR indicated significant multiple associations of *Fusobacterium* spp. in combination with *P. endodontalis, M. micros* and/or *Prevotella* with the first three histopathological categories (summary in Table 7.15, Tables 7.16 to 7.19). In the category of inflammatory degenerative change (category iv), only one significant microbial association was observed; that between *P. endodontalis* and *M. micros* (summary in Table 7.15, and Table 7.19), although a high, but non-significant correlation, also existed between the anaerobes, *Fusobacterium* spp. and *P. endodontalis* ($r = 0.839$).

Table 7.9. CFU correlations: all data.

	Prevotella	*F. nucleatum*	Lactobacilli	Streptococci	Actinomycetes
Prevotella	1	0.578*	0.031	0.314	0.249
F. nucleatum	0.578*	1	0.091	0.237	0.268
Lactobacilli	0.031	0.091	1	0.627*	0.190
Streptococci	0.314	0.237	0.627*	1	0.326
Actinomycetes	0.249	0.268	0.190	0.326	1

Data collected from 65 samples. Correlation coefficients for multiple comparisons were obtained using the Bonferroni adjustment preset at a significance level of 0.05.

Table 7.10. PCR correlations: all data.

	Prevotella	*P. melaninogen.*	*Fusobacterium*	*M. micros*	*P. endodontalis*	*P. gingivalis*
Prevotella	1	0.465*	0.693*	0.343	0.357	0.326
P. melaninogen	0.465*	1	0.464*	0.122	0 154	0.215
Fusobacterium	0.693*	0.464*	1	0.591*	0.687*	'0.222
M. micros	0.343	0.122	0.591*	1	0.574*	0.300
P. endodontalis	0.357	0 154	0.687*	0.574*	1	0.012
P. gingivalis	0.326	0.215	0.222	0.300	0.012	1

Data collected from 65 samples.

Table 7.11. CFU correlations: Category (i), minimal inflammatory change.

	Prevotella	*F. nucleatum*	Lactobacilli	Streptococci	Actinomycetes
Prevotella	1	0.701*	0.028	0.272	0.435
F. nucleatum	0.701*	1	0.177	0.190	0.480
Lactobacilli	0.028	0.177	1	0.635*	0.055
Streptococci	0.272	0.190	0.635*	1	0.188
Actinomycetes	0.435	0.480	0.055	0.188	1

Data collected from 28 samples.

Table 7.12. CFU correlations: Category (ii), soft tissue degenerative change.

	Prevotella	*F. nucleatum*	Lactobacilli	Streptococci	Actinomycetes
Prevotella	1	0.559	0.083	0.300	0.093
F. nucleatum	0.559	1	0.040	0.104	- 0.159
Lactobacilli	0.083	0.040	1	0.571	0.433
Streptococci	0.300	0.104	0.571	1	0.567
Actinomycetes	0.093	- 0.159	0.433	0.567	1

Data collected from 19 samples.

Table 7.13. CFU correlations: Category (iii), hard tissue degenerative change.

	Prevotella	*F. nucleatum*	Lactobacilli	Streptococci	Actinomycetes
Prevotella	1	0.510	0.289	0.336	0.311
F. nucleatum	0.510	1	0.557	0.709	0.204
Lactobacilli	0.289	0.557	1	0.864	0.230
Streptococci	0.336	0.709	0.864	1	0.536
Actinomycetes	0.311	0.204	0.230	0.536	1

Data collected from 7 samples.

Table 7.14. CFU correlations: Category (iv), inflammatory degenerative change.

	Prevotella	*F. nucleatum*	Lactobacilli	Streptococci	Actinomycetes
Prevotella	1	0.400	- 0.333	0.286	- 0.251
F. nucleatum	0.400	1	- 0.423	0.301	0.253
Lactobacilli	- 0.333	- 0.423	1	0.580	0.016
Streptococci	0.286	0.301	0.580	1	- 0.102
Actinomycetes	- 0.251	0.253	0.016	- 0.102	1

Data collected from 11 samples.

Table 7.15. Summary of correlations between anaerobic bacteria determined by real-time PCR and histopathological categories of carious dentine.

Histopathological Category	Microbial Associations	r [a]
Minimal inflammatory change	*Fusobacterium*[b], *P. endodontalis*	> 0.518
	Fusobacterium, Prevotella	> 0.518
Soft tissue degenerative change	*P. endodontalis, M. micros*	> 0.618
	Fusobacterium, M. micros	> 0.618
	Fusobacterium, Prevotella	> 0.618
Hard tissue degenerative change	*Fusobacterium, Prevotella*	> 0.907
Inflammatory degenerative change	*P. endodontalis, M. micros*	> 0.779

[a] Correlation coefficients (*r*) for multiple comparisons were obtained using the Bonferroni adjustment preset at a significance level of 0.05.

[b] The *Fusobacterium* primers and probe set designed for the detection of *Fusobacterium nucleatum* would also detect *Fusobacterium periodonticum, Fusobacterium alocis* and *Fusobacterium simiae,* if present in a sample

Table 7.16. PCR correlations: Category (i), minimal inflammatory change.

	Prevotella	*P. melaninogen.*	*Fusobacterium*	*M. micros*	*P. endodontalis*	*P. gingivalis*
Prevotella	1	0.473	0.611*	0.057	0.221	0.410
P. melaninogen	0.473	1	0.521	- 0.342	- 0 148	0.353
Fusobacterium	0.611*	0.521	1	0.397	0.731*	0.119
M. micros	0.057	- 0.342	0.397	1	0.468	- 0.176
P. endodontalis	0.221	- 0.148	0.731*	0.468	1	- 0.217
P. gingivalis	0.410	0.353	0.119	- 0.176	- 0.217	1

Data collected from 28 samples.

Table 7.17. PCR correlations: Category (ii), soft tissue degenerative change.

	Prevotella	*P. melaninogen.*	*Fusobacterium*	*M. micros*	*P. endodontalis*	*P. gingivalis*
Prevotella	1	0.693*	0.719*	0.607	0.494	0.473
P. melaninogen	0.693*	1	0.467	0.113	- 0 038	0.093
Fusobacterium	0.719*	0.467	1	0.772*	0.577	0.767
M. micros	0.607	0.113	0.772*	1	0.899*	0.828
P. endodontalis	0.494	- 0.038	0.577	0.899*	1	0.784
P. gingivalis	0.473	0.093	0.767	0.828	0.784	1

Data collected from 19 samples.

Table 7.18. PCR correlations: Category (iii), hard tissue degenerative change.

	Prevotella	*P. melaninogen.*	*Fusobacterium*	*M. micros*	*P. endodontalis*	*P. gingivalis*
Prevotella	1	0.270	0.923*	0.570	- 0.344	0.325
P. melaninogen	0.270	1	- 0.067	0.280	NA	0.582
Fusobacterium	0.923*	- 0.067	1	0.585	- 0.230	0.175
M. micros	0.570	0.280	0.585	1	0.242	- 0.651
P. endodontalis	- 0.344	NA	- 0.230	0.242	1	- 0.739
P. gingivalis	0.325	0.582	0.175	- 0.651	- 0.739	1

Data collected from 7 samples.
NA The p value could not be calculated, as only 2 observations were available.

Table 7.19. PCR correlations: Category (iv), inflammatory degenerative change.

	Prevotella	*P. melaninogen.*	*Fusobacterium*	*M. micros*	*P. endodontalis*	*P. gingivalis*
Prevotella	1	0.185	0.729	0.486	0.633	- 0.181
P. melaninogen	0.185	1	0.631	0.585	0.672	- 0.036
Fusobacterium	0.729	0.631	1	0.666	0.839	- 0.074
M. micros	0.486	0.585	0.666	1	0.985*	- 0.079
P. endodontalis	0.633	0.672	0.839	0.985*	1	- 0.045
	- 0.181	- 0.036	- 0.074	- 0.079	- 0.045	1

Data collected from 11 samples.

7.4 Discussion

Histopathology data collected from the 65 pulps were compared with the findings of Massey *et al.* (1993) using the same categories for evaluation and showed a similar range of variability in the tissue responses. In the present study, a higher number of pulps showing soft tissue degeneration (category ii) as the dominant pathology (29% vs 21%) were found when compared with results from the study of Massey *et al.* (1993). In addition, a lower number of pulps demonstrating minimal inflammatory change (category i) (43% vs 47%), and inflammatory degenerative change (category iv) (17% vs 21%), were noted. A similar number of pulps demonstrating hard tissue degeneration (category iii) as the dominant pathology was seen in both studies. As all teeth were selected using essentially the same clinical criteria, it is assumed these differences represent the variability in pulp responses to deep caries. This is in agreement with the findings of others (Dummer, *et al.*, 1980; Langeland, 1981) where attempts to correlate the clinical signs and symptoms of pulpitis with pulpal histopathology have been unsuccessful.

The hyaline thickening of micro-vessels noted in many of the pulp sections has been previously reported in association with chronic periodontitis (Pinchback *et al.*, 1996; Zoellner and Hunter, 1989a). The appearance of this amorphous hyaline material as viewed by the light microscope has been described, following electron microscopic examination, to consist of multiple layers of basement membrane impregnated with type IV collagen fibrils and cellular debris (Zoellner and Hunter, 1989a). It is considered to result from the combined effects of angiogenic stimuli and irritants from bacterial products and toxins over an extended period causing a progressive and irreversible response by the endothelial cells (Pinchback, *et al.*, 1996). In dental pulps, the presence of these perivascular hyaline deposits could interfere with the diffusion of chemotactic bacterial products from the carious dentine into the pulp tissue with ensuing loss of stimuli to attract PMNL into the area and also prevent PMNL movement through the thick hyaline sheets; in effect, compromising the local host defence response to bacteria and their products (Zoellner and Hunter, 1991). An additional feature seen in chronic pulpitis and also reported in chronic periodontitis was the presence of high endothelial-like

venules, which facilitate the migration of PMNL into the surrounding tissues (Zoellner and Hunter, 1989b).

In many of the pulp specimens hyaline deposits were not only confined to the vessels but also appeared to replace the extracellular matrix of the pulp tissue. A similar finding was also reported by Hebling *et al.* (1999) in areas of persistent inflammation and oedema in a study involving the capping of exposed human pulp tissue with a bonding resin (All Bond 2, Bisco Inc, Itasca, IL, USA). Some pulp sections in the present study demonstrated hyaline-affected tissue accompanied by diffuse calcification. These features have also been described by Martinez *et al.* (2000) and attributed to degeneration of the connective tissue matrix as a result of the ageing process. Two types of pathologic mineralisation found in inflamed dental pulps have been described (Seltzer *et al.*, 1977). The first involved beading of fibres and cells following degenerative change, with coalescence of the mineralising fibres into dystrophic mineralised masses. The second type involved the deposition of prismatic crystals unrelated to any cells, often obliterating much of the pulp and interspersed with islands of connective tissue. The description of beading has been more recently described as matrix vesicles of mineralising tissues which contain alkaline phosphatase (ALP) and play an important role in the extracellular mineralisation of tissues (Dean *et al.*, 1994). High levels of ALP activity have been demonstrated in pulp cells, particularly pulp fibroblasts (Tsukamoto *et al.*, 1992) and ALP function has been postulated to be involved in repair and healing following pulpal injury (Hill *et al.*, 1993). This is supported by a report of increased ALP levels in reversibly inflamed pulps following dental caries. In the reversible pulpitis specimens, diffuse ALP activity was noted in the stroma, whereas the irreversible pulp specimens showed reduced overall ALP activity that was confined to macrophages and neutrophils (Spoto *et al.*, 2001).

Dystrophic calcifications were noted in many pulp specimens and were classified as the dominant pathology in 11% of samples. Although their presence has been reported in sound and even unerupted teeth (Langeland and Langeland, 1965), calcifications commonly occur following calcium precipitation of dead or dying cells and are therefore classified as pathologic entities with the potential to cause organ dysfunction (Langeland, 1987; Cotran *et al.*, 1999). In

support of this finding, a study of the prevalence of pulp stones found a higher incidence in carious and restored teeth compared with sound teeth (Ranjitkar *et al.*, 2002).

Of those samples displaying inflammatory degeneration of the pulp tissue, all exhibited a plasma cell infiltrate, with less than one third of the sections showing evidence of an acute inflammatory reaction. The plasma cell infiltrates were generally accompanied by degenerating collagen and fibroblasts, a pattern previously reported in association with caries pulpitis (Massler, 1967; Torneck, 1974; Torneck, 1977; Torneck, 1981; Langeland, 1987; Massey, *et al.*, 1993) and also found in chronic periodontitis (Page and Schroeder, 1976). Other similarities between the two diseases include evidence of progression from an initial acute inflammatory T lymphocyte lesion, in the respective tissues, to a lesion dominated by B lymphocytes and plasma cells in more advanced or established pulpitis and periodontitis (Izumi *et al.*, 1995; Kinane and Lappin, 2001). This has been further developed in a study by Hahn *et al.* (1989) who demonstrated increased numbers of T helper and B cells in irreversible compared with reversible pulpitis. They postulated that under the continuous influx of antigens from an untreated carious lesion, the destructive or protective response of the pulp might be dependent upon the regulatory functions of the CD8+ T lymphocytes in controlling B cell activities. Thus the ratios of immunocompetent cells may be important in the pathogenesis of pulpitis.

The histopathological categories used in this study were based on those described by Massey *et al.* (1993) with a minor modification to the category of soft tissue degeneration to include some diffuse calcification of the hyaline-affected tissue. Microbial correlations of the CFU data within the histopathological categories in the present study revealed positive associations between *F. nucleatum* and prevotellae, and between streptococci and lactobacilli with minimal inflammatory change. No other associations were evident for the remaining categories. This was in contrast to the results of Massey *et al.* (1993) where a significant association between the number of *Prevotella* and *Actinomyces* spp. was reported with advanced pulpal pathology. The use of real-time PCR, therefore, facilitated the investigation of additional microorganisms and their possible association with pathological change.

Correlations made between the histopathological changes in the pulp tissue and the anaerobic bacteria detected by real-time PCR clearly demonstrated that similar groups of bacteria were positively associated with most categories of pulpal pathology, including those pulps in category (i) that were minimally inflamed. Despite the presence of predominantly normal pulp tissue in this group, there was frequent evidence of other forms of pathology in the sections. The variations noted in pulpal pathology at the time of extraction might represent differences in timing of the disease progression, acute or chronic phases of development, host responses or virulence of bacteria at specific sites.

Using the previously described categories of pathology, it was noted that no association could be found between pathological category and PCR bacterial cell numbers of specific organisms. With additional analyses of the numeric relationship between pairs or groups of microorganisms and the limited number of samples available within each category it was observed that *Fusobacterium* spp. was associated with one or more of *P. endodontalis, Prevotella* spp. or *M. micros* in all but the most severe category of pulpal inflammation. Similar associations have been reported in infected root canals (Lana *et al.*, 2001; Sundqvist, 1994) and species of *Bacteroides* (*Prevotella* and *Porphyromonas*), *Fusobacterium* and *Peptostreptococcus* (including *Micromonas*) have been strongly linked to periapical tissue destruction (Sundqvist, *et al.*, 1989). Synergistic anaerobic infections in animal models have also demonstrated the pathogenic potential of both *Prevotella* and *Porphyromonas* spp. (MacDonald *et al.*, 1963; Siqueira *et al.*, 1998), particularly when associated with *F. nucleatum* (Baumgartner *et al.*, 1992) and *M. micros* (van Dalen *et al.*, 1998). These findings are supported by literature that identifies *F. nucleatum* as one of few microorganisms capable of coaggregating with a wide variety of organisms, including oxygen-tolerant and obligately anaerobic species (Kolenbrander *et al.*, 1993), and which also facilitates the survival of obligate anaerobes in an aerated environment (Bradshaw *et al.*, 1998). It could be assumed that these properties would be beneficial in the progression of dental caries. In addition, *F. nucleatum* has been shown to be metabolically versatile in its ability to obtain energy by the fermentation of simple sugars and some amino acids as it has been detected in periodontally diseased sites together with organisms such as *P.*

gingivalis which possess endopeptidase activities (Rogers, 1998). Despite a lack of proteolytic activity, the main pathogenic potential of *F. nucleatum* is found in its synergism with other bacteria and its production of toxic metabolites including acetate, butyrate, propionate and formate which have been shown to inhibit the proliferation of gingival fibroblasts (Bolstad *et al.*, 1996; Rogers, 1998).

Although the significance of *Prevotella* and *Porphyromonas* spp. in carious dentine is not fully understood, correlations between the *Prevotella* spp. present in dentine caries and thermal sensitivity (Hahn *et al.*, 1993), advanced inflammatory pulpal change (Massey, *et al.*, 1993), and pulpal necrosis (Griffee *et al.*, 1980) have been reported. In this regard *Porphyromonas* and *Prevotella* spp. both demonstrate a large number of virulence factors that support their pathogenic potential. Included in these are proteolytic activities directed at degrading human plasma proteins, complement components C3 and C5, immunoglobulins IgG and IgM, connective tissue components and bioactive peptides controlling inflammatory processes (Sundqvist *et al.*, 1985; Nakata *et al.*, 2000; Rosen *et al.*, 2001), and interfering with the functions of PMNL through limiting phagocytosis, chemotaxis and bacteriocidal activity, thereby altering the inflammatory process. In addition, these organisms are capable of damaging host cells and tissues through the release of toxic metabolic products including butyrate, propionate, indole, ammonia and volatile sulphur compounds (Sundqvist, 1993). *M. micros* has also demonstrated pathogenic properties including proteolytic and hyaluronidase activity, inhibition of cell growth, release of volatile sulphur compounds and the ability to enhance the pathogenicity of *Prevotella* and *Porphyromonas* spp. (Tam and Chan, 1985; Rams *et al.*, 1992). Murdoch (1998) has suggested that the strong proteolytic activity of *M. micros* may be significant in the development of mixed anaerobic abscesses

The positive microbial association noted between *P. endodontalis* and *M. micros* with inflammatory degenerative change might be explained by a synergistic relationship based on the nutritional requirements of *P. endodontalis*. Although this organism has limited protease and peptidase activities (Rosen, *et al.*, 2001) it could benefit from the wide range of proteolytic enzymes exhibited by *M. micros* in degrading proteins and amino acids (ter Steeg and van der

Hoeven, 1989; Rams, *et al.*, 1992). However, this is in contrast to a negative association found by Zerr *et al.* (1998) between *P. endodontalis* and *M. micros* in supplemented mixed culture experiments *in vitro*.

Results from this study have demonstrated the presence of a diverse group of anaerobes including representatives of the genera *Fusobacterium, Micromonas, Porphyromonas* and *Prevotella* in carious dentine associated with pathologic changes of the dental pulp. In addition, this group of anaerobes has been consistently associated with both acute and chronic anaerobic wound infections (Bowler *et al.*, 2001), and pulmonary infections in humans (Bartlett *et al.*, 1974a and 1974b). It would therefore appear that these organisms play a significant role in anaerobic infections in varied locations.

The following chapter examines the achievements, limitations and future direction for investigation of research into carious pulpitis.

CHAPTER 8

GENERAL DISCUSSION

Perusal of the literature reveals a limited number of studies reporting the microbiology of deep caries, with only one reporting an association between microbial populations and pulpal histopathology (Massey *et al.*, 1993). Results from these studies indicated a predominance of anaerobic organisms in this environment. With regard to specific anaerobic species, prevotellae have been reported in dentine caries associated with thermal sensitivity (Hahn *et al.*, 1993), advanced inflammatory change (Massey, *et al.*, 1993) and pulpal necrosis (Griffee *et al.*, 1980). Despite this knowledge, it is still not known which microorganisms drive the carious process culminating in pulpal infection or what key interactions exist between microbes to assist this process.

The objectives of the work described in this thesis were to examine the microbial populations of extracted carious teeth with symptoms of pulpitis, using both culture and molecular techniques, and to study the histopathology of the associated pulpal tissue in an attempt to identify microbial predictors associated with carious pulpitis.

8.1 Achievements

Information from the culture studies of carious dentine revealed a complex and diverse group of bacteria dominated by anaerobes, particularly Gram-negative organisms. Also present were large numbers of microaerophilic Gram-positive bacteria. Further studies of this anaerobic group using PCR indicated previous under-reporting of these microbes by culture and the existence of species, such as *P. gingivalis* and *M. micros*, not previously reported in dentine caries.

Histopathological analysis of the pulpal tissues correlated with the culture and PCR data indicated no significant relationship between the total number of anaerobic or microaerophilic bacteria and histopathological category. However, significant multiple associations of *Fusobacterium* spp. in combination with *P. endodontalis, M. micros* and/or *Prevotella* spp. were found with different

categories of pulpal histopathology including minimal inflammatory change, soft tissue and hard tissue degenerative change of the pulpal tissues. These findings, therefore, suggest a potential for the association of groups of bacteria with pathological change. The identification of a number of significant microbial associations from the PCR studies and the diversity of species found by culture are suggestive of widespread cooperation and interdependence between the different species inhabiting dentine caries.

The significance of the Gram-negative anaerobic bacteria identified in carious dentine is not fully understood, although the association of fusobacteria with other anaerobes in all but the most severe category of pulpal inflammation indicates its synergistic role in disease development (Kolenbrander *et al.*, 1993a). In addition, the prevotellae and porphyromonads produce a range of virulence factors (Sundqvist, 1994), which are reportedly enhanced in the presence of *M. micros* (Rams *et al.*, 1992). The toxic effects of these bacteria on the pulp matrix and endothelial cells probably contribute to the pathological changes seen in the pulp sections. Of significance are the soft tissue changes seen as pulpal fibrosis, perivascular deposition of hyaline material and replacement of pulpal stroma by hyaline-affected material, possibly as a result of injury from bacterial irritants and toxins over an extended period. In some sections large areas of pulpal tissue showed hyaline material with superimposed calcific changes. These effects create further hypoxia within the pulpal tissues encouraging the proliferation of anaerobes, and leaving the pulp ill-equipped to mount effective defence or repair mechanisms.

Bacterial assault of the pulpal tissues has been proposed to evoke a primarily mononuclear infiltration of lymphocytes, plasma cells and macrophages, with acute reactions involving the mobilisation of polymorphonuclear leukocytes only when the pulp is in imminent danger of bacterial invasion. Whether the pulp succumbs to the insult or can mount an effective defence and recover depends on the health of the pulpal tissue and the virulence of the microorganisms. It is unlikely that a single microbial species is responsible for pulpitis and pulp death. It is more likely that the disease represents the effects of a combination of microorganisms, although, the pathogenic species will not

be readily discerned unless they can be repeatedly detected (Socransky *et al.*, 1987), which is problematic using traditional culture techniques.

The initial stage of PCR quantitation required the development of a universal amplicon to detect total bacterial load in the samples and to provide a reference for the quantitation of other bacterial species within the samples. A 466bp amplicon was designed and successfully detected 49 strains representing 34 different groups from the major Groups of bacteria described by Holt *et al.* (1994). When compared with other published broad-spectrum amplicons, the amplicon designed in this study was found to be more efficient in detecting a wide range of bacterial species, and in a shorter time (Nadkarni *et al.*, 2002). Other "universal" amplicons reported for use with real-time PCR showed numerous mismatches with the sequences of many of the dental pathogens (Corless *et al.*, 2000) or required additional steps, and hence an increased reaction time because of the longer amplicon (Lyons *et al.*, 2000).

The enumeration of different groups of bacteria from the samples involved the development of a series of genus-specific and species-specific primers and probes to identify and quantitate bacteria reported from the literature to be found in dentine caries and root canal infections including fusobacteria, prevotellae, *M. micros*, *P. endodontalis*, *P. gingivalis*, *P. melaninogenica* and *S. mutans*. Only one report exists of a *P. gingivalis* primers/probe set for use with real-time PCR (Lyons, *et al.*, 2000), which at 900bp is considerably longer than the 150 bp amplicon designed in this study. The additional length may lead to lower efficiency of the reaction.

8.2 Limitations

A number of concerns relate to the study of advanced caries. The caries site can be potentially contaminated by plaque bacteria, while liberation and dispersion of microorganisms from necrotic dentine fragments is required without damaging less robust cells and the cultivation of fastidious anaerobes is necessary. Additional problems include the presence of unknown species in a sample and the knowledge that no single medium can support the growth of all species. The situation is made more complex by the succession of microbial populations with changes in environmental conditions and nutritional requirements within the carious lesion. Microbial sampling at an inadequate depth or

at an inactive stage could distort the bacterial profile. Mixed infections also create problems in terms of the confounding presence of opportunistic species. Further, the pulpal tissue response is likely to be chronic and episodic.

Following bacterial culture of the carious dentine samples some anomalies were noted. These were identified as different growth intensities found on non-selective and selective agar plates for the same samples, a distribution of Gram-negative anaerobes observed with Gram-staining but not supported by growth on agar and variability of colony morphology and associated biochemical test results. Concerns with the reliability of the culture data therefore necessitated the inclusion of PCR techniques as an additional method to enumerate bacteria from the samples.

Quantitation of bacteria from clinical samples by PCR is also associated with limitations. Some of these relate to the sensitivity of the reaction itself, while others involve the paucity of information associated with genome size and the number of rDNA operons for many bacterial species. Further, there is no standard method for extracting and protecting DNA and PCR inhibition from components of clinical samples must be removed or diluted for PCR to occur. In this study, the development of a single method to efficiently extract DNA from a range of Gram-positive and Gram-negative bacteria, whilst also preventing nuclease degradation, was not achieved despite an extensive series of experiments using different techniques. As it was not possible to enumerate all bacteria using real-time PCR, a compromise enabled the correlation of histopathology results with data for the microaerophilic bacterial growth from the culture study and data for anaerobic bacteria from the PCR study.

Due to a lack of information regarding the genome length and copy number of 16S rDNA of dental microbes at the time of quantitation, the calculation of bacterial numbers for all species following PCR was based on the genome size of *P. gingivalis* (2.2Mb) and one copy of the 16S rDNA per genome. More recent information reports a genome length of 2.4Mb for *F. nucleatum* ssp. *polymorphum* and 3.8Mb for *Prevotella intermedia* (TIGR Microbial Database [http://www.tigr.org) which would slightly alter the bacterial cell calculations. Despite these differences, other known

genome lengths for microaerophilic Gram-positive bacteria from this environment include *S. mutans* at 2.2Mb and *L. acidophilus* at 1.9Mb.

Quantitation of the total anaerobic load from the 65 samples using the universal amplicon with real-time PCR was compared with data following quantitation with the genus-specific and species-specific probes and primers. The results indicated that, on average, only 12% of the total anaerobic microflora present in the carious dentine (range <1% to 71%) was detected by the specific probes and primers, despite using amplicons designed to detect the most frequently reported anaerobic organisms from deep caries. It is therefore necessary to exercise some caution in assigning important positive or negative correlations using only a minor percentage of the species present in the carious dentine samples. This problem also highlights the difficulty of identifying the bacterial profile of a complex environment that contains many previously uncultured species.

8.3 Directions for future research

The quantitation of DNA and therefore bacterial numbers using real-time PCR offers great potential in studying the pathogenesis of many diseases, including caries pulpitis, and could provide information for the design of useful diagnostic tools.

One area where PCR could provide more focused information is in the identification of microbes *in situ*. In the present study the carious samples incorporated both superficial and deep dentine from individual teeth. Additionally, the pulps were removed from the tooth for separate processing and fixation. The advantage in identifying spatial relationships between those microorganisms forming the carious front of the lesion and the adjacent pulpal response would provide more accurate assessment of those microbes responsible for pathology. Published methods utilising this approach include micro-dissection using either laser capture or scalpel excision of relevant areas of material from clinical sections mounted on histological slides. Use of this technology has been previously hampered by the traditional preparation of archival tissues using 10% formalin fixation over long periods, followed by high temperature paraffin embedding. These processes inevitably cause cross-linking of the tissue components with resultant fragmentation of the nucleic acids, estimated between 200-300bp in length for DNA (Lehmann and Kreipe, 2001). Newer techniques resulting in less DNA fragmentation

recommend fixation in 2% paraformaldehyde solution for 2h followed by cold temperature resin embedding using a water-miscible methyl methacrylate polymer (Finkelstein *et al.*, 1999).

The ability of the universal amplicon to detect previously uncultured species by PCR of the dentine samples has been discussed previously. Techniques for DNA cloning and sequencing can be used to isolate and identify uncultured bacteria by determining the phylogenetic relatedness through comparison with other 16S rDNA sequences from ribosomal databases (Wilson *et al.*, 1997). Using these techniques, one of the carious dentine samples from the group with advanced inflammatory degenerative pulpal change was selected for further investigation (CE Caldon, unpublished data). Utilising the previously extracted DNA, the 16S rDNA fragments were amplified with the universal primers. The PCR products were cloned and 47 fragments were sequenced (data not shown). The largest group, with 43% of the clones, showed some identity with the genus *Atopobium*, a newly described group with unknown pathogenic potential (Collins and Wallbanks, 1992). A further 21% were identified as prevotellae, 6% as *P. tannerae*; the remainder may represent new *Prevotella* species. The other 36% were identified as representatives from the genera *Pseudoramibacter, Lactobacillus, Peptostreptococcus, Dialister, Capnocytophaga, Fusobacterium* and *Catonella.*

As a result of the type of samples collected for this study, the histopathology of the pulpal tissues illustrated a late stage in the inflammatory process. Additional studies are therefore necessary, using these new techniques, to correlate the microbiology of dentine at the carious front with the histopathology of adjacent pulpal tissues in a sequential manner, using teeth with different stages of caries progression and varied clinical symptoms in an attempt to build an accurate profile of the microbiology of the advancing carious front and resultant inflammatory responses of the pulpal tissues.

Use of an *in situ,* sequential approach to sample collection combined with cloning and sequencing to identify unknown bacteria, together with quantitation by real-time PCR, could provide more focused information to answer significant questions regarding microbial species that drive the carious process, bacterial interactions critical to this process and interactions associated with pulpitis. Answers to these questions should allow the development of diagnostic tools and new methods of

treatment, including the use of appropriate therapeutic agents. The treatment of carious pulpitis has evolved little over the last five decades. In addition to the removal of carious dentine, pulpal therapy uses medicaments that mummify pulpal tissues (formocresol), allay symptoms in the short term (combination of tetracycline and corticosteroid) or require removal of the pulp tissue for symptom relief. More appropriate treatment options will only occur with knowledge of the causes and mechanisms driving carious pulpitis.

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APPENDIX

REAGENTS

Reduced Transport Fluid

Reference: Syed and Loesche (1972)

To make 1 litre place the following in a 2 litre flask:

Stock solution No. 1	75ml
Stock solution No. 2	75ml
sodium carbonate stock solution	5ml
EDTA 0.1M	10ml
distilled water	815ml

Autoclave, allow to cool and add the following after filter sterilisation:

dithiothreitol (DTT) (Clelands reagent)	0.2gm
distilled water	20ml

Stock solution No. 1

dibasic potassium phosphate (K_2HPO_4)	6.0gm
distilled water	1000ml

Stock solution No. 2

sodium chloride	12gm
ammonium sulphate	12gm
monobasic potassium phosphate (KH_2PO_4)	6gm
magnesium sulphate stock solution	10ml
distilled water	990ml

Magnesium sulphate stock solution

$MgSO_4.7H_2O$	2.5% solution

Sodium carbonate stock solution

Na_2CO_3	8% solution

MEDIA

Cadmium fluoride acriflavin tellurite medium

Reference: Zylber and Jordan (1982)

To make 1 litre place the following in a 2 litre flask:

trypticase soy broth	30gm
glucose	5gm
agar	15gm
distilled water	950ml

Autoclave for 15 minutes at 15psi and cool to 50°C in a water bath. Then mix:

cadmium sulphate	13mg
sodium fluoride	85mg
neutral acriflavine	1.2mg
potassium tellurite	2.5mg
basic fuchsin	1.25mg
distilled water	20ml

filter sterilise and add to media with 30ml horse blood.

Crystal violet erythromycin medium

Reference: Walker *et al.* (1979)

To make 1 litre place the following in a 2 litre flask:

agar	15gm
tryptone	10gm
yeast extract	5gm
sodium chloride	5gm
glucose	2gm
L-tryptophan	0.2gm
distilled water	950ml

Autoclave for 15 minutes at 15psi and cool to 50°C in a water bath. Then mix:

crystal violet (50mg in 50ml)	1ml
erythromycin	4mg
distilled water	19ml

filter sterilise and add to media with 30ml horse blood.

Enriched trypticase soy agar

Reference: Loesche *et al.* (1972)

To make 1 litre place the following in a 2 litre flask:

potassium nitrate	0.5gm
trypticase soy agar	40gm
yeast extract	1gm
glucose	1gm
sodium lactate	1ml
sodium formate	0.5gm
sodium succinate	0.5gm
agar	4gm
distilled water	950ml

Autoclave for 15 minutes at 15psi and cool to 50°C in a water bath. Then mix:

sodium carbonate	0.4gm
cysteine hydrochloride	0.4gm
dithiothreitol	0.1gm
haemin stock	1ml
menadione stock	2ml
sodium fumarate stock	10ml
distilled water	7ml

filter sterilise and add to media with 30ml horse blood.

Haemin stock

Dissolve 50mg of haemin chloride in 1ml 1N NaOH and make up to 100ml with distilled water.

Menadione stock

Dissolve 50mg of menadione in 50ml ethanol and make up to 100ml with distilled water. Store away from light.

Sodium fumarate stock

10% solution of sodium fumarate in distilled water.

Kanamycin Vancamycin agar

Reference: Dowell (1982)

To make 1 litre place the following in a 2 litre flask:

trypticase soy agar	40g
agar	5g
yeast extract	5g
L-cysteine	0.4g
(dissolve the L-cysteine in 5ml 1N NaOH before adding to other ingredients)	
distilled water	967ml

Autoclave for 15 minutes at 15psi and cool to 50°C in a water bath. Then mix:

kanamycin	100mg
vancamycin	7.5mg
hemin stock	1ml
menadione stock	2ml

filter sterilise and add to media with 30ml horse blood.

Mitis-salivarius agar

Reference: Chapman (1944)

Formula provided by the manufacturer (grams/litre):

peptone P	10gm
tryptone	10gm
dextrose	1gm
sucrose	50gm
dipotassium phosphate	4gm
tryptan blue	0.075gm
crystal violet	0.0008gm
agar	12gm

To rehydrate the medium, suspend 90gm in 1.0 litre of distilled water. Autoclave for 15 minutes at 15psi. Cool to 50°C then add 1.0ml of 1% tellurite solution.

Rogosa agar

Reference: Rogosa *et al.* (1951)

Formula provided by the manufacturer (grams/litre):

tryptone	10gm
yeast extract	5gm
potassium dihydrogen phosphate	6gm
dextrose	20gm
Tween 80	1gm
ammonium citrate	2gm
sodium acetate	25.gm
glacial acetic acid	1.32ml
$MgSO_4.7H_2O$	0.575gm
$Mn.2H_2O$	0.12gm
$FeSO_4. 7H_2O$	0.034gm
agar	20gm

To rehydrate the medium, suspend 62gm in 1.0 litre of distilled water. Autoclave for 15 minutes at 15psi. Cool to 50°C then pour.

All solutions and agars stored at 4°C.

FIXATION AND EMBEDDING SOLUTIONS

Paraformaldehyde

Specimens were stored in 4% paraformaldehyde prepared in phosphate buffered saline for 24 hours at 4°C. .

JB4 glycolmethacrylate embedding resin

Prepared following the manufacturer's directions (Polysciences Inc., Warrington, PA, USA)
Solution A preparation:
To 100ml of solution A (glycol methacrylate liquid) add 0.9gm of benz-pyrine catalyst powder. Stir with magnetic flea to ensure complete mixing. Soak tissue samples overnight prior to embedding at 4°C in the prepared solution A plus catalyst.
Embedding procedure:
Mix solution A containing the catalyst with solution B in the ratio 20:1 at 4°C.

Pour into plastic wells, position the tissue samples and stubs.
Use paraffin wax to seal around each stub to exclude any air from the setting resin.
Allow to set overnight at 4°C before removing the blocks, then dry to harden them prior to sectioning.