CAPNOCYTOPHAGA SPP. AND

DIABETES MELLITUS-PERIODONTITIS

Thesis submitted by

Marilou Ciantar BChD(Hons), MSc(Lond)

for the degree of

DOCTOR OF PHILOSOPHY

in the Faculty of Medicine,

University of London

Department of Microbiology,

Eastman Dental Institute for Oral Health Care Sciences,

University College London, 256, Gray's Inn Road

London WC1X 8LD

2002

ProQuest Number: U642951

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.
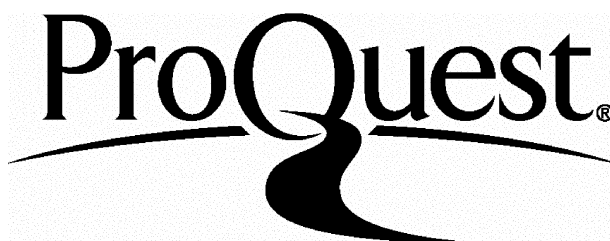
ABSTRACT

Diabetes Mellitus (DM) renders the individual more susceptible to infection and chronic periodontitis (Periodontitis) seems to be no exception. Indeed, DM-Periodontitis subjects manifest increased prevalence and severity of periodontitis than their non-DM-Periodontitis counterparts. The oral bacterial flora associated with DM-Periodontitis has not been thoroughly investigated. It is hypothesised that potentially elevated glucose levels in gingival crevicular fluid in DM-Periodontitis subjects could confer an ecological advantage for saccharolytic organisms such as *Capnocytophaga* spp. Members of this genus have been recovered from a range of periodontal diseases including DM-Periodontitis. *In vitro* studies have shown that *Capnocytophaga* spp. grow better and produce more enzymes relevant to periodontal destruction when grown in glucose-enriched conditions that simulate the human diabetic situation.
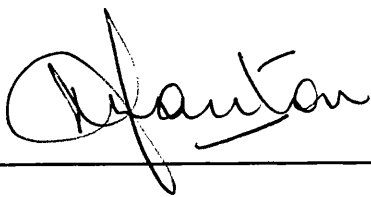
The main aim of this study was to investigate whether *Capnocytophaga* spp. were more prevalent in DM-Periodontitis. Prior to commencement of the clinical study, fastidious anaerobe agar (FAA) was identified as the best culture medium for the recovery of these species from clinical samples. Identification of *Capnocytophaga* clinical isolates to species level was based on 16S rRNA PCR-RFLP with *Cfo* I as the restriction enzyme, developed specifically as part of this study. The clinical study comprised 21 DM-Periodontitis (median HbA_{1c} = 9.3%) & 25 non-DM-Periodontitis (median HbA_{1c} = 5.3%) subjects. Subgingival plaque samples were collected from 3 healthy (PPD $\leq$ 3 mm) and 3 diseased sites (PPD $\geq$ 5 mm) per subject. Total counts for *Capnocytophaga* spp. and (facultative and obligate) anaerobes from each sample were determined. A total of 848 *Capnocytophaga* clinical isolates were isolated and identified using 16S rRNA PCR-RFLP. Statistical analyses were performed using multilevel modelling. The results showed significantly higher numbers of *Capnocytophaga* spp. (P < 0.001) and facultative and obligate anaerobes (P < 0.001) in diseased sites in DM-Periodontitis subjects when compared to healthy sites in non-DM-Periodontitis and DM-Periodontitis subjects. A proportion (39%) of *Capnocytophaga* clinical isolates were subjected to antimicrobial sensitivity testing. The majority were sensitive to most of the antibacterial agents used in clinical practice, with the exception of metronidazole. A micro-assay for the quantification of GCF-glucose was developed and used to quantify the GCF-glucose concentration in healthy, DM-Periodontitis and non-DM-Periodontitis subjects. The results showed that GCF-glucose concentration was higher in diseased & healthy sites in the DM-Periodontitis group.

2

In conclusion, the results of this investigation have shown that *Capnocytophaga* spp. are recovered in higher numbers in DM-Periodontitis patients, and more so in the diseased sites in these patients. This could be due to the different subgingival ecological environment resulting from the elevated levels of GCF-glucose at these sites.

DECLARATION

I hereby certify that the work embodied in this thesis is entirely the result of my own investigations except for the statistical analysis involving Multivariate Multilevel Modelling. This part of the analysis was performed in close collaboration with Dr. M. S. Gilthorpe.

This research project has not been submitted either in part or in full for a degree or diploma to this or any other University or examination board elsewhere.

Marilou Ciantar

May 2002.

To my Mama & Daddy, Sister, Brothers

& their respective families

ACKNOWLEDGMENTS

CONTENTS

Chapter 3: Selection of a culture medium for the isolation

of *Capnocytophaga* spp.

Chapter 4: Strategy for the identification of *Capnocytophaga* spp.

List of Figures

List of Figures (cont'd)

List of Figures (cont'd)

List of Tables

List of Tables (cont'd)

List of Tables (cont'd)

List of Appendices

List of Abbreviations

16S rRNA gene	16S ribosomal Ribonucleic Acid gene
AAP	American Academy of Periodontology
ADA	American Diabetes Association
AGE	Advanced Glycation End products
ATCC	American Type Culture Collection
BI	Bleeding Index
bp	basepair
C. cn	*C. canimorsus*
C. cy	*C. cynodegmi*
C. g	*C. gingivalis*
C. gr	*C. granulosa*
C. h	*C. haemolytica*
C. o	*C. ochracea*
C. s	*C. sputigena*
Cap Medium	*Capnocytophaga* culture medium (Rummens *et al.* 1985)
CI	Confidence Intervals
CIPD	Chronic Inflammatory Periodontal Diseases
CO_2	Carbon dioxide
DM	Diabetes Mellitus
DM-Periodontitis	Periodontitis in patients with Diabetes Mellitus
DNA	Deoxyribonucleic acid
FAA	Fastidious Anaerobe Agar
GCF	Gingival crevicular fluid
GT	Glucose transport proteins
H_2O_2	Hydrogen peroxide
HbA_{1c}	Glycated haemoglobin
Ig	Immunoglobulins
IL-1	Interleukin-1
IL-6	Interleukin-6
INF-γ	Interferon-γ
kb	kilobasepair
LB Medium	Leadbetter culture medium (Leadbetter *et al.* 1979)
LCAL	Lifetime cumulative attachment loss
LPS	Lipopolysaccharide

24

List of abbreviations (cont'd)

Mac	Macrophage
Mash	Mashimo medium (Mashimo *et al.* 1983b)
mg dL^{-1}	Milligrams per decilitre
MIC$_{90}$	Minimum inhibitory concentration for 90% of strains (μg ml^{-1})
MLM	Multilevel modelling
mmoll^{-1}	Millimoles per litre (mM)
MMPs	Matrix Metalloproteinases
Mo	Monocyte
MV MLM	Multivariate Multilevel modelling
NCTC	National Culture Type Collection
NF-κB	Nuclear Factor- κB
Non-DM-Periodontitis	Periodontitis in non-Diabetes Mellitus patients
O$_2$	Oxygen
OGTT	Oral Glucose Tolerance test
OR	Odds ratio
PCR	Polymerase Chain Reaction
PDL	Periodontal Ligament
PI	Plaque Index
PMNL(s)	Polymorphonuclear Leucocyte(s)
PPD	Pocket probing depth
RAGE	Receptor for Advanced Glycation End products
RE	Restriction enzyme
RFLP	Restriction Fragment Length Polymorphism
RI	Random Intercepts
RIGLS	Restricted Iterative Generalised Least Squares
RTF	Reduced Transport Fluid
rcf	Rotational centrifugal force
Slots Medium	Slots medium for *A. a* (Slots *et al.* 1982)
TAC	Total Anaerobic Count (obligate & facultative, cfu ml^{-1})
T*Capno*	Total *Capnocytophaga* counts (cfu ml^{-1})
TIMPs	Tissue Inhibitors of Matrix Metalloproteinases
TNF-α	Tumor Necrosis Factor-α
VC	Variance Components
μg	Micrograms

List of grants awarded in support of this project

1999   Shirley Glasstone Hughes Memorial Prize, British Dental Association. Isolation, identification and biodiversity assessment of *Capnocytophaga* spp. recovered from Diabetes Mellitus-associated periodontitis (£24,102). **Dr. Marilou Ciantar,** Dr. David Spratt, Prof. H. Newman and Prof. M. Wilson

1999   Oral & Dental Research Trust, SmithKline Beecham Award. Comparison of gingival crevicular glucose content recovered from Diabetes Mellitus and non-Diabetes patients (£825). Dr. David Spratt, **Dr. Marilou Ciantar.**

1999   Isolation, identification and biodiversity of *Capnocytophaga* species recovered from Diabetes Mellitus-associated periodontitis. Eli Lilly Diabetes Research (equipment grant). (£8000) Dr. David Spratt, **Dr. Mariiou Ciantar**, Prof. H. N. Newman, Prof. M. Wilson.

1998   Oral & Dental Trust, Procter & Gamble Research Award 1998. An investigation into the role of virulence in *Capnocytophaga* species in Diabetes Mellitus-associated periodontitis. Dr. David Spratt, **Dr. Marilou Clantar** (£637).

1997   Commonwealth Fellowship Award (one year; Research Fellow).

Grants awarded in support of additional research

2001   Oral & Dental Research Trust, Procter & Gamble Research Award. Bone resorbing potential of lipopolysaccharide extracted from *Capnocytophaga spp.* (£905). **Dr. Marilou Ciantar**, Dr. Sajeda Meghji, Dr. David Spratt.

2001   Oral & Dental Research Trust, Smithkline Beecham Award. Black pigmented anaerobes: enhanced growth & diversity in Diabetes Mellitus-periodontitis? (£900). **Dr. Marilou Ciantar**, Dr. Jonathan Pratten and Dr. David Spratt.

List of publications arising from this thesis

1. Assessment of five culture media for the growth and isolation of *Capnocytophaga* species.

   Ciantar M, Spratt DA, Newman HN, Wilson M (2001). *Clin Microbiol Infect* 7: 158 - 160.

2. *Capnocytophaga granulosa* and *Capnocytophaga haemolytica*: novel species in subgingival plaque.

   Ciantar M, Spratt DA, Newman HN, Wilson M (2001). *J Clin Periodontol* 28: 701 - 705.

3. Development of an *in-vitro* micro-assay for glucose quantification in submicrolitre volumes of biological fluid.

   Ciantar M, Spratt DA, Newman HN, Wilson M (2002). *J Periodont Res* 37: 79 - 85.

4. GCF-glucose: potential differences between DM- and non-DM-Periodontitis subjects?

   Ciantar M, Spratt DA, Gilthorpe MS, Hurel S, Newman HN, Wilson M. *J Dent Res* (submitted)

List of presentations pertaining to this research

Oct 2001   ESE Conference: poster presentation: Molecular identification of *Capnocytophaga* spp. isolated from root canals

May 2001   BSP Conference: poster presentation: GCF-glucose: potential differences between DM and non-DM periodontitis patients?

May 2001   BSP Conference: poster presentation: Glucose utilisation by *Capnocytophaga* species isolated from periodontitis patients with & without diabetes mellitus: a preliminary study

July 2000   International Association of Dental Students & Young Dentists Worldwide, Malta Dental Congress 2000. (Guest speaker): *Capnocytophaga* spp. & DM-Periodontitis: research trends for the new millennium

April 2000   IADR: poster presentation: *In-vitro* micro-assay for quantification of GCF-glucose

Nov 1999   Royal Society of Medicine, London (Oral & Dental Research Trust Award, Smithkline Beecham 1999). Comparison of Gingival Crevicular Fluid glucose content recovered from Diabetes Mellitus and non-Diabetes-Mellitus patients

June 1999   7th International Academy of Periodontology Meeting, Lubljana, Slovenia. Poster presentation: Assessment of five culture media for the isolation of *Capnocytophaga* spp.

Oct 1998   Royal Society of Medicine, London (Oral & Dental Research Trust Award, Procter & Gamble 1998). An investigation into the role of virulence of *Capnocytophaga* species in Diabetes Mellitus- associated periodontitis

Chapter 1

Introduction

1.1 Diabetes Mellitus

1.1.1 Definition

The term Diabetes Mellitus (DM) describes a metabolic disorder of multiple aetiology characterised by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both (Alberti & Zimmet 1998, WHO 1999). Chronic hyperglycaemia is responsible for the pathological features characteristic of DM, namely ophthalmic, neurological, cardiovascular, renal and vascular dysfunction and damage (WHO 1999).

Insulin is a major anabolic hormone produced by the β cells (contained within the islets of Langerhans) in the pancreas (DeFronzo *et al.* 1992). It is responsible for lowering blood glucose by promoting uptake of glucose from blood and formation of glycogen for storage in liver and skeletal muscle cells (Fig.1.1). It is also responsible for the conversion of glucose to triglycerides and for nucleic acid and protein synthesis (Crawford & Cotran 1994). Insulin also prevents the breakdown of stored glycogen other than in periods of fasting or increased glucose demand, when a decrease in insulin production and increased glucagon levels lead to release of glucose from glycogen stores, thus re-establishing euglycaemia (Mealey 2000). Insulin is therefore vital to maintain homeostasis.
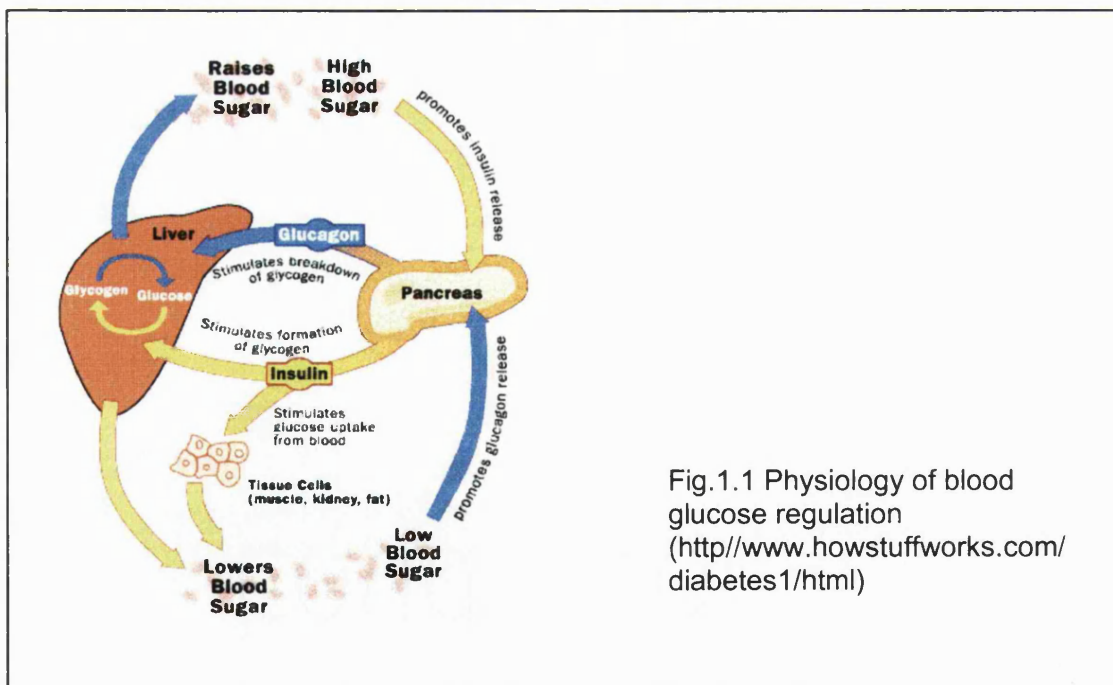
Fig.1.1 Physiology of blood glucose regulation (http//www.howstuffworks.com/diabetes1/html)

1.1.2 Epidemiology

Diabetes mellitus is a major chronic health problem and ranks among the top 10 causes of death in the western world (Crawford & Cotran 1994). Its prevalence varies according to country, ethnic origin and age (King & Rewers 1993).

In the United States of America, DM affects approximately 16 million people (7%), with a further estimated 8 million suffering from undiagnosed diabetes (National Diabetes Data Group 1995). Its incidence amounts to 600,000 cases per year, and its treatment consumes about 12% of the US annual health budget (Edelman 1998).

In the UK, DM complications are the seventh leading cause of death. Approximately 2 - 3% of the UK adult population suffer from DM although it is estimated that a similar proportion remains undiagnosed. Treatment of DM accounts for 5 -10% of the UK NHS annual expenditure.

In Europe the prevalence of DM is of the order of 2-5%, with the exception of Malta and Sweden where its prevalence reaches the 5-10% level (WHO 1985).

Racial differences can account, in part, for some of the global differences observed. Caucasians are 1.5 times more likely to develop Type 1 DM than Afro-Caribbeans. In Japan, the incidence is <0.8/100,000, while in Finland it is >27.9/100,000. The prevalence rates for Type 2 DM in the US are highest among Afro-Caribbeans, Hispanics and American Indians.

Prevalence figures are also dependent on age. Type 1 DM observed mainly among the younger population (<40 years) accounts for 5-10% of all diabetic patients, while Type 2 DM (>40 years) constitutes circa 90% of the diabetic population (National Diabetes Data Group, NDDG, 1985). Within this subgroup, the prevalence increases to 10% above the age of 70 (Crawford & Cotran 1994).

1.1.3 Classification

It was not until the late 1970s that the first classification of DM was published by the NDDG (1979). This classification established clear guidelines for the various categories of glucose tolerance based chiefly on standardising fasting blood glucose and glucose tolerance testing. Its main disadvantage was that it was primarily based on the pharmacological treatment used to manage the disease. In addition, classification of DM was based on age of onset (i.e. juvenile versus adult), which is now known to be incorrect, thus leading to inappropriate patient categorisation. In view of this, the American Diabetes Association (ADA 1997) proposed a new classification based on aetiology (Table 1.1).

Table 1.1 ADA Classification of DM

Type 1 (formerly insulin dependent DM, IDDM or juvenile DM):
autoimmune
idiopathic

Type 2 (formerly non-insulin dependent DM, NIDDM or maturity onset DM):
Obese Type II DM
Non obese Type II DM
Maturity Onset Diabetes of the Young (MODY)

Gestational DM

DM secondary to systemic disease:
genetic defects in β cell function
genetic defects in insulin action
pancreatic diseases or injuries
infections
drug / chemical induced DM
endocrinopathies
other genetic syndromes with associated DM

1.1.4 Diagnostic Criteria

The primary methods used to diagnose and monitor blood glucose levels have traditionally been fasting blood glucose, a combination of fasting blood glucose and a 2 hour post-prandial glucose loading and oral glucose tolerance tests (ADA 1997, Table 1.2).

Impaired fasting glucose and impaired glucose tolerance signify a metabolic state intermediate between euglycaemia and hyperglycaemia and present an increased risk for future development of DM and a higher risk of cardiovascular disease (Fuller *et al.* 1980).

Table 1.2 ADA Diagnostic Criteria for DM (1997)

DM may be diagnosed by any one of the three methods. Whichever method is used, it must be confirmed on a subsequent day using any 1 of the 3 methods.

1. Symptoms of DM plus casual (non-fasting) plasma glucose $\geq$ 11.1 mmoll^{-1}. Casual is defined as any time of day without regard to time since last meal. Classic symptoms of DM include polyuria, polydipsia, polyphagia and unexplained weight loss.

2. Fasting plasma glucose $\geq$ 7 mmoll^{-1}. Fasting is defined as no caloric intake for at least 8 hours.

3. 2-hr post-prandial glucose $\geq$ 11.1 mmoll^{-1} during an oral glucose tolerance test. The test should be performed as described by WHO (Appendix 1) using a glucose load containing the equivalent of 75 g of anhydrous glucose dissolved in water.

Categories of fasting plasma glucose (FPG) include:

1. FPG < 6.1 mmoll^{-1} = normal fasting glucose.

2. FPG $\geq$ 6.1 mmoll^{-1} and $\leq$ 7 mmoll^{-1} = impaired fasting glucose.

3. FPG $\geq$ 7 mmoll^{-1} = provisional diagnosis of DM (must be confirmed on a subsequent day).

Categories of 2 hr post-prandial glucose (2 hr PG) include:

1. 2 hr PG < 7.8 mmoll^{-1} = normal glucose tolerance.

2. 2 hr PG $\geq$ 7.8 mmoll^{-1} and < 11.1 mmoll^{-1} = impaired glucose tolerance.

3. 2 hr PG > 11.1 mmoll^{-1} = provisional diagnosis of DM (must be confirmed on a subsequent day).

The data quoted in this table refer to glucose concentrations in venous plasma.

Glycaemic control is monitored using the glycated haemoglobin test (HbA$_{1c}$), formerly called glycosylated haemoglobin (Tsuji *et al.* 1991). It has also been used as a screening test (Tsuji *et al.* 1991). The HbA$_{1c}$ assay measures the amount of glucose that is irreversibly bound to haemoglobin. Its value is proportional to the glucose level present in the blood and is dependent on the half-life of red blood cells (30 - 90 days) (Cooper *et al.* 2000). It also relays information about the level of glucose control in

diagnosed DM subjects during the previous three months (Table 1.3). In this respect it is a more accurate monitoring test compared to fasting or casual plasma glucose which gives the value of blood glucose at a specified point in time.

Table 1.3 Laboratory evaluation of diabetes control - Glycated haemoglobin assay (HbA1c) (Adapted from Mealey 2000)

4 to 6%	Normal
< 7%	Good diabetes control
7 to 8%	Moderate Diabetes control
> 8%	Poor control

1.1.5 Pathogenesis

1.1.5.1 Pathogenesis of Type 1 DM

Type 1 DM is caused by cell-mediated autoimmune destruction of the insulin-producing pancreatic β cells (Atkinson & Maclaren 1994). The rate of destruction varies between individuals, resulting in differing rates of decline in insulin production (ADA 1997). In all cases it will invariably lead to complete destruction of these cells and subsequently an absolute deficiency of insulin. This explains why all patients with Type 1 DM need insulin treatment for survival (Alberti & Zimmet 1998).

Recent evidence for the aetiology of Type 1 DM indicates an interaction between genetic makeup, immune reaction and environmental factors (Atkinson & Eisenbarth 2001).

The most important genes (responsible for both susceptibility towards, and protection from, the disease) are located within the major histocompatibility complex (MHC) HLA class II region on chromosome 6p21, namely the HLA-DR(3 & 4) and HLA-DQ(α & β) haplotypes (Tait & Harrisson 1991, Buzzetti *et al.* 1998). Alterations in these MHC antigens on cell surfaces may explain why individuals become intolerant to self-antigens, resulting in T-cell-mediated destruction of pancreatic β cells (Atkinson & Maclaren 1994). Although the concordance rate for Type 1 DM between monozygous twins is about 30 - 50%, the complex polygenic susceptibility of Type 1 DM is not fully understood (Crawford & Cotran 1994).

The postulated role of autoimmunity in Type 1 DM is supported by the results of several studies (Eisenbarth 1986). Lymphocytic infiltration of the islet cells is intense causing the characteristic insulitis. Islet cells from these patients aberrantly express a

cell surface MHC antigen (Laufer *et al.* 1993). In addition, patients with impending Type 1 DM also have elevated levels of islet cell antibodies (Atkinson & Maclaren 1993).

Although various environmental agents have been suggested as important in the aetiology of DM, no one factor has been established. To date the most extensively investigated environmental agents have been: viral infections, early infant diet and chemical toxins.

The implicated viral infections are caused by β-cell tropic viruses, namely coxsackie virus and cytomegalovirus (Dahlquist 1997). Although direct viral injury is not observed, it is postulated that viruses cause mild β cell injury, which, in turn, may initiate an autoimmune response directed towards the damaged cells in individuals with HLA-linked susceptibility (Yoon 1991).

Early ingestion of cow's milk may elicit an antibody response to bovine serum albumin (BSA) (Kostraba *et al.* 1993). This antibody may cross react with a β cell surface protein (*p69*), expression of which is induced only after the immature gut shows signs of infection (Karjalainen *et al.* 1992). In a genetically susceptible child, this may lead to a progressive destruction of β cells and hence DM.

Some chemical toxins, e.g. streptozotocin, alloxan and pentamidine, are known to precipitate DM (Knip & Akerblom 1999). They act either directly on the β cells or by eliciting an immune response.

Other non-genetic disease-modifying factors include vaccine administration, psychological stress and climatic influences (Knip & Akerblom 1999).

A postulated model for the development of Type 1 DM suggests that a certain degree of immune dysregulation present in a genetically susceptible individual and in combination with inherent target organ defects are modulated by life-long exposure to multiple environmental factors such as infectious agents, diet and toxins (Atkinsen & Eisenbarth 2001). The subsequent onset of acute insulitis leads to autoimmune β cell injury, which manifests clinically as DM.

However, some forms of Type 1 DM do not show any evidence of autoimmunity and are hence classified as idiopathic (MacLarty *et al.* 1990). The clinical features are the same as those manifested by autoimmune Type 1 DM.

1.1.5.2 Pathogenesis of Type 2 DM

While the specific aetiologies of Type 2 DM are not known, autoimmune destruction of the β cells does not occur (ADA 1997). It results from the interplay between genetic factors, obesity, increasing age and lack of physical exercise (Yki-Järvinen 1993).

Type 2 DM has a stronger genetic component than Type 1 DM with a concordance rate of 90% among Type 2 DM identical twins (Newman et al. 1987). In the majority of Type 2 DM, no specific gene abnormality has been identified. In a few subgroups, however, genetic abnormalities have been defined e.g. MODY (Maturity Onset Diabetes of the Young) patients may manifest well-characterised chromosomal defects for glucokinase (Frogel et al. 1992), hepatocyte nuclear factor-α (Yamagata et al. 1996) and insulin promoter factor-1 (Stoffers et al. 1997). Point mutations in mitochondrial DNA have also been found to be associated with DM and deafness (Kadowaki et al. 1994).

The pathophysiology of Type 2 DM is characterised by 3 major abnormalities:

i) impaired insulin secretion,

ii) peripheral resistance to insulin,

iii) hepatic insulin insensitivity.

Impaired insulin secretion is due to reduced cell mass and altered insulin release (Crawford & Cotran 1994). It has been postulated that the modest reduction in β cell mass is due to the fact that these cells are genetically susceptible to injury, which leads to accelerated cell turnover, premature ageing and reduction in cell mass (Crawford & Cotran 1994). The remaining islet cells manifest altered cell function. The pulsating release of insulin in response to a high glucose load seen in non-DM subjects is lost in Type 2 DM cases. This may be due to reduced transmembrane glucose transporters. Paradoxically, some Type 2 DM patients manifest hyperinsulinaemia resulting from a compensatory increase in insulin production (Crawford & Cotran 1994).

Resistance to insulin, which is demonstrable in first-degree relatives of patients with DM and in the prediabetic state, is a major factor in Type II DM (Kolterman et al. 1981). The exact mechanism of such resistance is as yet unknown but it is possibly due to:

i) a decrease in number of insulin receptors

ii) post-receptor defects, and

iii) impaired post-receptor signalling (Crawford & Cotran 1994).

1.1.6 Cellular metabolic changes

Hyperglycaemia induces cellular pathological changes along two fronts:

i) extracellularly via nonenzymatic protein glycosylation (Brownlee *et al.* 1988) and

ii) intracellularly via disruption of polyol pathways (Greene *et al.* 1987).

1.1.6.1. Extracellular pathologic changes

Non-enzymatic protein glycosylation (Fig.1.2) results from the irreversible chemical binding of glucose to proteins/lipids in the absence of enzymes (Brownlee *et al.* 1988, Bucala *et al.* 1993). The degree of glycosylation is directly proportional to concentrations of reactants i.e. glucose and protein (as described for HbA1c above) (Cooper *et al.* 2000). The products of this first step are unstable in their initial stages and may revert to the original components (i.e. glucose and protein). However, they eventually undergo slow chemical changes, leading to cross-linking thus forming irreversible advanced glycosylation end products (AGEs) (Brownlee *et al.* 1988). AGEs form on protein, lipoproteins and nucleic acids.

Glucose + Protein / lipids ⟷ Schiff Base (Glucose + Protein/ lipid)
reversible (hours)

⟷ reversible (days)

AGE (protein-protein cross link) ← Amadori product (AGE)
irreversible (weeks)

Fig.1.2. Schematic diagram showing formation of AGEs
(Adapted from Brownlee *et al.* 1988, Bucala *et al.* 1993).
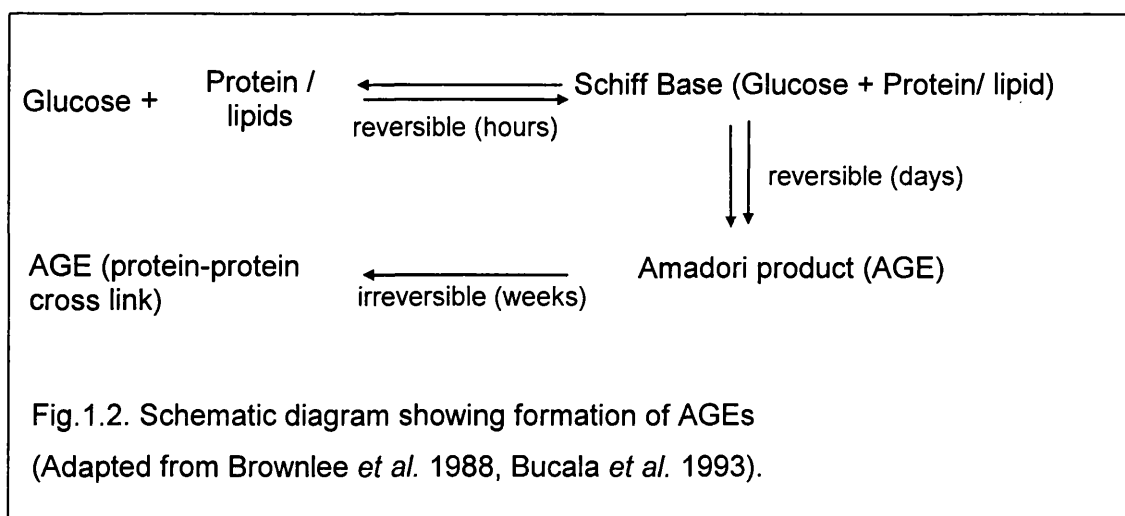
The formation of AGEs is a function of glucose concentration and time. AGEs accumulate both inside insulin-independent cells and outside on cell membrane proteins, structural proteins and circulating proteins (Brownlee *et al.* 1984). AGEs lead to quantitative and qualitative changes in a wide range of biologically relevant proteins and thus are accountable for the pathology associated with DM (Table 1.4). AGE formation is 2 - 3 fold higher in DM than in non-DM subjects (Vlassara 1991).

Table.1.4 AGE-induced pathology

System affected	Pathology	Result
Red cell membrane	Cell membrane rigidity	Interference with perfusion
Haemoglobin	Oxygen exchange decreased	Insufficient oxygenation
Leucocytes	Defective adhesion, chemotaxis, phagocytosis & bactericidal activity	Increased risk of infection
Lymphocytes	Decreased mitogen proliferative response, reduction in T and B cell surface receptors	Increased risk of infection
Fibrinogen	Increased plasma viscosity	Increased risk of thrombus formation
Antithrombin III	Decreased levels	Increased risk of thrombus formation
Basement membrane	Thickening	Microangiopathy
Plasma protein abnormalities	Increased blood viscosity	Decreased blood flow
Macrophages	Increased production of growth factors & cytokines	Proliferation of cells and matrix
Endothelial cells	Increased production of vasoconstrictors	Vasoconstriction and focal thrombosis
DNA (prokaryotic)	Mutations and altered gene expression	Inactivation of (plasmid) tetracycline resistance
Lens crystallins	Increased protein cross-links	Visual impairment
Lipoprotein	Trapped by AGEs	atheroma formation
NF-κB	Activation of response-to-injury genes	Excessive cytokine release

Compiled from Brownlee & Cerami 1981, Brownlee *et al.* 1984, Vlassara 1991, Brownlee 1992, 1994.

Besides the pathology associated with the formation of AGEs, they bind to specific receptors for AGEs (known as RAGEs). RAGEs are located on the cell surface and are the best characterised AGE receptors to date (Schmidt & Stern 2000). They consist of an extracellular domain, a transmembrane domain and a cytosolic tail, which mediates the interaction with cytosolic signal transduction molecules (Fig.1.3).

Fig.1.3 AGE-RAGE interaction
(Adapted from Schmidt & Stern 2000)
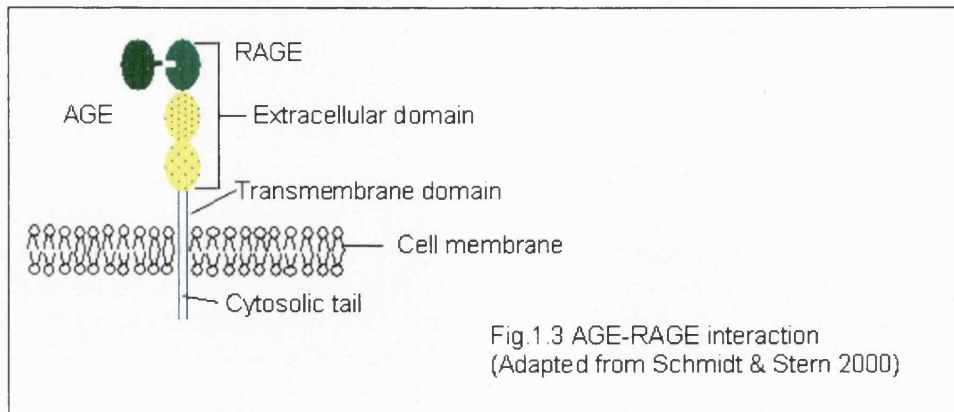
RAGEs are expressed in a range of cell types but at low levels in homeostasis. However, their expression is greatly enhanced in conditions of stress e.g. diabetes and inflammation (Schmidt & Stern 2000).

AGE-RAGE interaction results in enhanced (Schmidt & Stern 2000):

i) expression of adhesion molecules (vascular cell adhesion molecules, VCAM-1, intercellular adhesion molecules, ICAM-1)

ii) vascular permeability

iii) generation of procoagulant tissue factors

iv) cellular activation and hence increased cytokines and growth factors

v) modulation of gene expression

vi) oxidant stress and generation of reactive oxygen intermediates (ROIs).

Whilst they are resistant to proteolytic degradation, they are capable of accumulating non-glycosylated proteins (Vlassara *et al.* 1994). AGEs bind to receptors on many cell types including endothelium, monocytes, macrophages, lymphocytes and mesangial cell types. Binding elicits a range of biological activities including monocyte migration, cytokine and growth factor release, increased vascular permeability, procoagulant activity, enhanced cellular proliferation and increased extracellular matrix production (Vlassara *et al.* 1994). All of these effects can potentially contribute to DM complications.

1.1.6.2 Intracellular pathologic changes

Some cells e.g. lens, neurones and pericytes, do not require insulin for transmembrane transport of glucose. Thus hyperglycaemia leads to high levels of intracellular glucose (Greene *et al.* 1987). The excess glucose is metabolised to sorbitol (a polyol) and

eventually fructose. Accumulation of sorbitol and fructose lead to increased intracellular osmolarity and influx of water, which eventually leads to osmotic cell injury. Clinically this manifests, for example, as lens opacity (cataracts), neurological damage and retinal aneurysms (Greene *et al.* 1987).

1.1.7 Histopathology

Hyperglycaemia accounts for the histopathological changes that characterise DM (Nathan 1993). Whilst these features are not exclusively pathognomonic of DM, they are greatly exacerbated in this endocrine disease.

The microangiopathy observed in DM is due to:

i) basement membrane (BM) thickening

ii) periendothelial thickening

iii) narrowing of vessel lumen

iv) stasis in micro-circulation.

BM thickening is a common feature in all tissues that manifest DM-associated pathologic changes. It is due to sustained hyperglycaemia that results in the formation of AGEs. The increased thickness alters normal homeostatic transport across the membrane. The chemical and biological properties associated with AGEs lead to periendothelial thickening and hence micro- and macroangiopathy. Cytokine-induced proliferation of endothelial cells leads to narrowing of vascular lumen and vascular stasis (Vlassara *et al.* 1994).

1.1.8 Clinical symptoms

The classic triad of symptoms of DM are polyuria, polydipsia and polyphagia. Additional features include lethargy, weight loss, candidal infection and recurrent infections. The pathophysiology underlying these clinical features can be explained in terms of the elevated blood glucose content subsequent to an inadequate supply of insulin required to meet the physiological needs of the body at a cellular level.

Hyperglycaemia may reach levels, which exceed the renal threshold resulting in glycosuria (Fig.1.4). This induces an osmotic diuresis resulting in polyuria. The subsequent volume depletion leads to intracellular dehydration, which manifests as polydipsia. Increased lipolysis, seen in Type 1 DM, leads to a rapid build up of ketone bodies which, if not removed, results in an increase in hydrogen ion concentration and systemic metabolic ketoacidosis which can lead to diabetic coma.

Insulin deficiency

Hyperglycaemia   Increased lipolysis

Polyphagia

Glycosuria   Increased free fatty acids (FFA)

Volume depletion   Increased FFA oxidation

Polydipsia

Diabetic coma   ◄—   Ketoacidosis
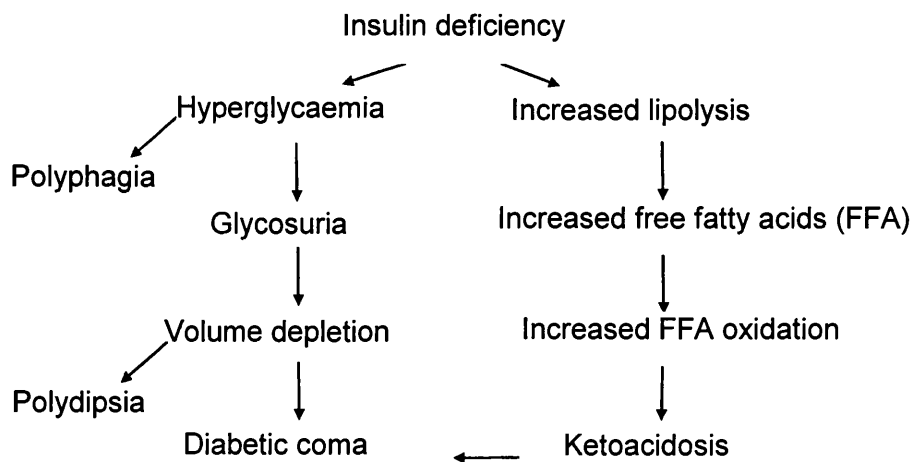
Fig.1.4 Metabolic Derangement observed in Diabetes Mellitus

Type 1 DM has an acute onset and most often, though not exclusively, presents in young individuals. Due to the acute nature of the condition, rapid deterioration to diabetic ketoacidosis is likely. This is followed by severe vomiting and hence electrolyte imbalance with ensuing diabetic coma. The condition may be fatal unless the condition is dealt with effectively and immediately by insulin administration.

Type 2 DM assumes a more gradual onset. Polyphagia, polydipsia and polyuria may accompany the fasting hyperglycaemia but ketoacidosis is rare. Due to its insidious nature, it may remain undiagnosed and may be detected as part of routine investigations or due to its complications. The clinical characteristics of Type 1 and 2 DM are summarised in Table 1.5. Other non-specific clinical features of DM include lethargy, fainting episodes, blurring of vision, emaciation and increased susceptibility to infection.

Table 1.5 Typical clinical features of Type 1 and Type 2 DM

Clinical feature	Type 1	Type 2
Age of onset (commonest)	<20 years	> 40 years
Body type (stature)	Normal / thin	Obese
Race	White	African-American, Hispanics, American-Indian, Pacific Islanders
Family History	Minimal	Very common
Clinical onset	Acute	Insidious
Endogenous insulin	None / reduced	Decreased, normal, elevated
Susceptibility to ketoacidosis	High	None
Treatment options	Insulin, diet, exercise,	Diet, exercise, oral agents, insulin.

1.1.9 Inflammation and glucose control

Inflammation is known to alter the endocrinologic-metabolic status of the host thus leading to difficulty in controlling blood sugar levels (Rayfield et al. 1982). This seems to be mediated through the interaction of various cytokines with the insulin receptor (Ling et al. 1994, Kanety et al. 1995). The proinflammatory cytokine Tumour Necrosis Factor-α (TNF-α) interacts with the insulin receptor inducing a serine- rather than a tyrosine-phosphorylation, thus impairing insulin action (Kanety et al. 1995).

The insulin receptor (Fig.1.5) is found on the surface of many different cell types e.g. monocyte and macrophage. It is a tetramer consisting of 2α and 2β glycoprotein subunits (Gannong 1983, Lönnroth 1991). The α subunits are directed extracellularly and bear the hormone-coupling domain.

Fig.1.5 Schematic diagram of the insulin receptor. (Adapted from http://arbl.cvmbs.colostate.edu/hbooks/pathophys/endocrine/pancreas/insulin_phys.html)
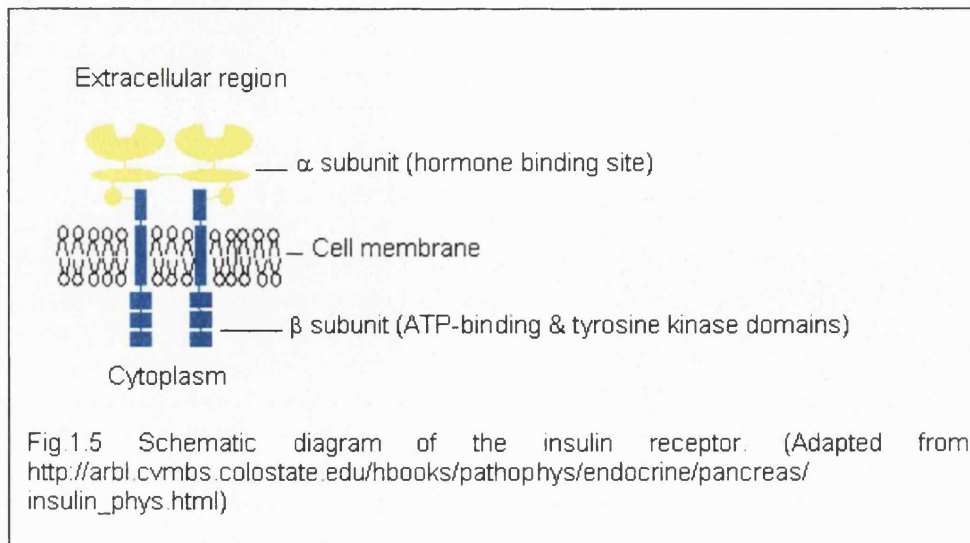
The β subunits, which have a transmembrane component, are coupled to the α subunits by sulphide bridges. Binding of insulin to the α subunits leads to conformational changes in the receptor initiating a series of intracellular insulin signals. The β subunits undergo autophosphorylation leading to the formation of tyrosine kinase as well as phosphorylation of other intracellular proteins. Most importantly, glucose transport proteins appear on the cell surface (Fig.1.6), thus facilitating cellular uptake of glucose (Gannong 1983, Crawford & Cotran 1994, Lönnroth 1991).

Fig.1.6 Cascade of events leading to intracellular glucose uptake. (Adapted from http://arbl.cvmbs.colostate.edu/hbooks/pathophys/endocrine/pancreas/insulin_phys.html).
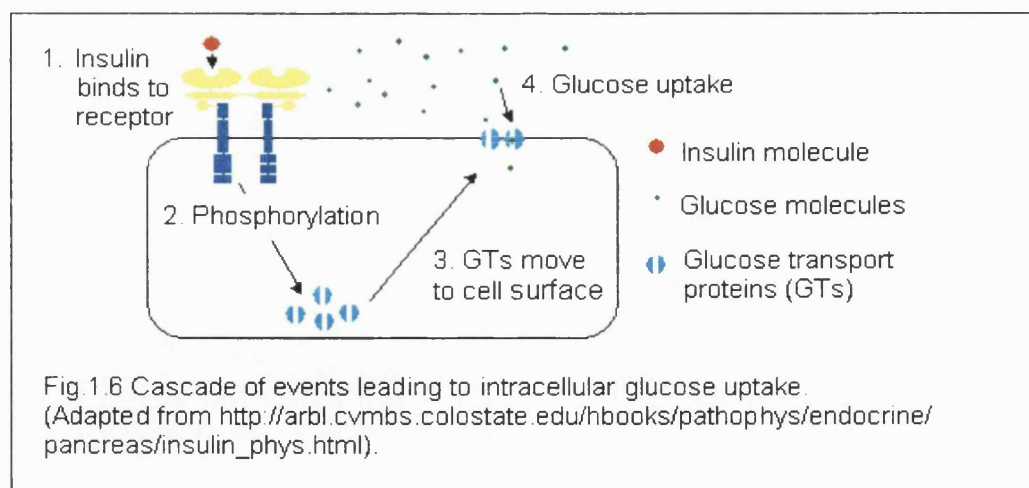
1.1.10 Susceptibility to infection

By virtue of their hyperglycaemic state, DM subjects are more prone to infections (Rayfield *et al.* 1982). Whereas previously this was based on anecdotal evidence, recent clinical and laboratory investigations have supported this notion (Tierney *et al.* 1995, Muchova *et al.* 1999). DM subjects also tend to suffer from an increased frequency and severity of *unusual* infections when compared to their non-diabetic counterparts. These can be of bacterial or fungal origin e.g. malignant external otitis and rhinocerebral mucormycosis (Pozzilli *et al.* 1994, Tiereny *et al.* 1995). The nature and severity of infection is related the level of metabolic control (Rayfield *et al.* 1982).

In vitro investigations have consistently indicated that hyperglycaemia impairs the physiology of polymorphonuclear leucocytes (PMNLs) (Bagdade *et al.* 1978, Delamaire *et al.* 1997). Since these cells are the first line of cellular immune defence, their impairment will severely undermine the host's response to infection. Abnormalities in PMNL chemotaxis (Moutschen *et al.* 1992), phagocytosis (Wilson *et al.* 1986) and intracellular killing (Gin *et al.* 1984) have been observed. Altered cellular and humoral responses in DM subjects have also been detected (Horita *et al.* 1982, Andersen *et al.* 1988). These dysfunctions, in conjunction with the reduced vascular supply and hence anaerobiosis, may explain the increased susceptibility to infections observed in DM subjects.

1.1.11 Complications of DM

1.1.11.1 Systemic Complications

The acute complications of DM, namely hyper- or hypoglycaemia, are most often due to improper glucose control by the patient. Hyperglycaemia may arise from excessive carbohydrate intake or inadequate therapy. It can also be precipitated in cases of severe stress (i.e. that associated with critical illness) due to the production of circulating counter-regulatory hormones and cytokines (McCowen *et al.* 2001). Hypoglycaemia is due to missed meals, over-treatment or excessive exercise. The clinical features of such acute complications are featured in Table 1.6. Optimal glycaemic control involves a delicate balance between medication and exercise.

Table 1.6 Acute complications of DM

Hyperglycaemia	Hypoglycaemia
Slow onset	Sudden onset
Disorientation	Mental confusion
Rapid and deep breathing	Sudden mood change
Hot, dry skin	Tachycardia
Loss of consciousness	Nausea
	Hunger
	Increased gastric motility
	Cold clammy skin
	Hypothermia
	Loss of consciousness

The chronic complications associated with DM arise due to the effects of hyperglycaemia and may be listed as (Crawford & Cotran 1994):

i) macroangiopathy: responsible for cardiovascular, cerebrovascular and peripheral vascular disease

ii) microangiopathy which manifests as:

    a) nephropathy: which leads to end stage renal failure

    b) retinopathy: which is the leading cause of blindness in developed countries

    c) neuropathy: which is responsible for sensory loss and subsequent undetected trauma, the most common site being the feet, and

    d) delayed wound healing: which leads to increased risk of infection and diabetic gangrene.

1.1.11.2 Oral Complications of DM

Although no oral condition is pathognomonic of DM, certain conditions manifest more frequently and are exacerbated in DM subjects. These include:

i) chronic inflammatory periodontal disease, especially if this is out of proportion to the patient's age (Shlossman *et al.* 1990, Emrich *et al.* 1991, Seppälä & Ainamo 1992, Löe 1993). Multiple periodontal abscesses presenting in young subjects may be the first sign of Type 1 DM. Indeed Periodontitis has been listed as the sixth complication of DM (Löe 1993; Refer to section 1.3).

ii) oral candidiasis, which is reported to be more prevalent in DM subjects (Guggenheimer *et al.* 2000)

iii) xerostomia (which arises as a complication of the disease and / or secondary to medication) and high salivary glucose concentrations, which may predispose the individual to candidal infections (Borg Andersson *et al.* 1998, Guggenheimer *et al.* 2000)

iv) burning mouth, glossopyrosis and altered taste sensation which are common manifestations secondary to xerostomia, candidiasis and possibly diabetic neuropathy (Rees *et al.* 1994)

v) decreased salivary flow rate occasionally accompanied by parotid enlargement (Rees 2000)

vi) increased caries incidence in some DM patients. This seems to be related more to the degree of self-awareness and therefore control of DM and level of oral hygiene.

1.2 Periodontitis

1.2.1 Definition

Periodontitis is a family of related diseases that differ in aetiology, natural history, disease progression and response to therapy, but which share a common underlying chain of events i.e. the histopathological and ultrastructural features of tissue destruction as well as healing and regeneration are very similar, if not identical, for all forms of periodontitis (Page *et al.* 1997).

Adult Periodontitis is a form of periodontitis defined as a chronic plaque-induced inflammation of the periodontium which manifests clinically as destruction of the periodontal ligament, loss of crestal alveolar bone and apical migration of the junctional epithelium, collectively known as loss of clinical attachment (Williams *et al.* 1992; Fig.1.7). It is a multifactorial, infectious disease in adults caused mainly by Gram-negative anaerobic, capnophilic and microaerophilic micro-organisms (Page 1998, Kornman & Newman 1999). It is viewed as an opportunistic infection caused by micro-organisms residing within the oral cavity (Newman 1990, Page *et al.* 1997).

46
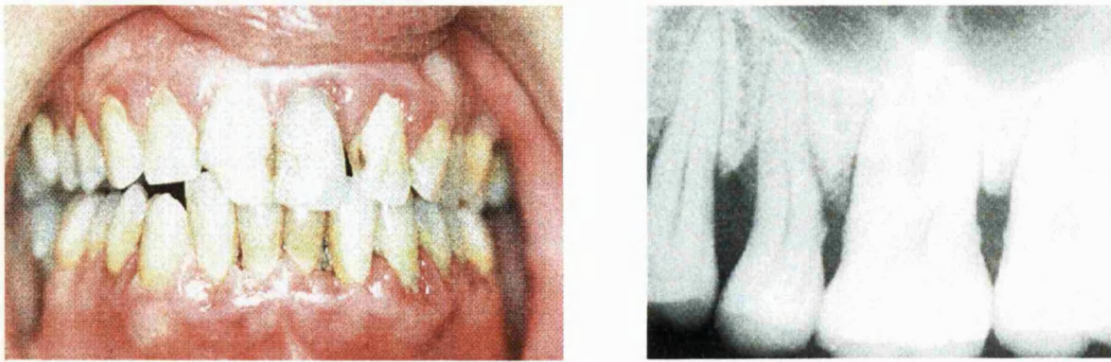
Fig.1.7 Clinical and radiographic features of periodontitis

1.2.2 Epidemiology

Chronic Inflammatory Periodontal Diseases (CIPDs) constitute a major chronic oral health problem as they are among the most common infections which afflict mankind. Acute exacerbation may lead to discomfort or pain, and untreated disease may eventually lead to tooth loss. A number of studies from different countries have shown that on average 8% of the population manifest severe periodontal destruction leading to tooth loss by early adulthood, 81% of subjects experience moderate destruction while the remaining 11% are resistant to the disease (Löe *et al.* 1978, 1986, Papapanou *et al.* 1989).

1.2.3 Classification

The rapid increase in knowledge and hence recognition of the different types of CIPD, and of periodontitis itself, has seen the classification revised thrice over a ten year period: The American Academy of Periodontology (AAP) World Workshop in 1989; Attström *et al.* 1993 and the 1999 International Workshop for a classification of periodontal conditions and diseases (Armitage *et al.* 1999). The classification used in this study was the 1989 AAP classification (Table 1.7). This was chosen for the following reasons:

i) the 1993 classification contained insufficient information for proper patient categorisation into different clinical entities

ii) this study commenced in 1997 i.e. prior to the 1999 International Workshop classification. The patient selection criteria used were therefore based on the 1989 system

iii) the 1999 International Workshop classification admits to an "apparent inconsistency" with respect to the periodontal conditions associated with systemic

47

disease. Whilst this classification recognises "diabetes-mellitus gingivitis", it excludes diabetes as one of the systemic factors in which periodontitis is a frequent manifestation. Whilst it is in no way being implied in this thesis that Diabetes Mellitus (DM) causes periodontitis (refer to section 1.2.5 for the aetiology of periodontitis), there seems to be sufficient evidence to implicate DM as a modifying or risk factor in the aetiology and progression of periodontitis (refer to section 1.3).

Table 1.7 1989 AAP Classification for Periodontitis

I Adult Periodontitis

II Early-Onset Periodontitis

A. Prepubertal Periodontitis	B. Juvenile Periodontitis
1. Generalised	1. Generalised
2. Localised	2. Localised

C. Rapidly Progressive Periodontitis

III Periodontitis Associated with Systemic Disease

A. Primary neutrophil disorder	B. Secondary neutrophil impairment
Agranulocytosis	DM Type I
Cyclic neutropenia	DM Type II
Chediak Higashi Syndrome	Papillon Lefèvre Syndrome
Neutrophil Adhesion abnormalities	Down's Syndrome
Job's Syndrome	Crohn's Disease
Lazy leukocyte Syndrome	Preleukaemic Syndrome
Neutrophil functional abnormalities	Addison's Disease
	AIDS

C. Systemic disease associated with changes in structure of the periodontal attachment apparatus (not truly infectious).
Ehler Danlos Syndrome
Histiocytosis
Sarcoidosis
Scleroderma
Hypophosphatasia
Hypoadrenocorticism
Hypothyroidism

IV Necrotising Ulcerative Periodontitis

V Refractory Periodontitis

VI Miscellaneous conditions

1.2.4 Diagnostic criteria

By definition, the main criterion for the diagnosis of periodontitis is loss of clinical attachment (Williams et al. 1992). The clinical diagnostic methods as set out by the 1989 AAP are the following:

i) visual signs of inflammation (redness, swelling)

ii) probing for estimation of pocket depths / loss of clinical attachment

iii) presence of suppuration

iv) radiographs & other imaging techniques to detect and assess the severity of bone loss

1.2.5 Aetiology

The seminal work by Löe et al. (1965) was the first to scientifically implicate bacteria as aetiological agents of CIPDs. Since then, the periodontal literature has become replete with further supporting evidence (Theilade et al. 1966, van Palenstein Helderman 1981, Kornman & Löe 1993). Although it could be implied that bacteria associated with periodontitis are the result rather than the cause of the disease, evidence from various therapeutic modalities and animal studies shows that bacteria are an essential prerequisite for periodontal destruction (Table 1.8). Perhaps the most convincing evidence emerges from studies involving germ-free animals, as these do not develop periodontal destruction (Socransky & Haffajee 1994).

Table 1.8 Evidence for a bacterial aetiology in periodontitis

Human studies	Reference
Mechanical suppression of bacteria	Lindhe & Nyman 1975, 1984, Rosling et al. 1976, Oosterwaal et al. 1987
Chemical suppression of bacteria	Soh et al. 1982, Haskel et al. 1986
Antibiotic treatment of periodontitis	Christersson et al. 1993, van Winkelhoff et al. 1996
Immune response to periodontal bacteria	Ebersole et al. 1982, 1987
Correlation of certain bacteria with disease	Dzink et al. 1985, 1988

Table 1.8 Evidence for a bacterial aetiology in periodontitis (cont'd)

Animal studies	
Gnotobiotic animals	Irving *et al.* 1975, Heijl *et al.* 1980
Ligature induced periodontitis	Sallay *et al.* 1982
Indigenous plaque	Isogai *et al.* 1985

Microbial Pathogenicity	
Bacterial virulence factors	Slots & Genco 1984, Fives-Taylor *et al.* 1999
Bacterial invasion of tissues	Chistersson *et al.* 1987, Lamont *et al.* 1992, Sandros *et al.* 1993, Rudney *et al.* 2001

Thus the two most important contending factors in the aetiology of periodontitis are the bacteria and the host. During the early stages of supragingival plaque formation, bacterial cells accumulate on enamel and are, initially, outside the body's main defence system although subject to those components of the host defence that are present in saliva and gingival crevicular fluid (Socransky & Haffajee 1998). These pioneer bacteria increase and eventually build a coherent climax bacterial community (Marsh & Martin 1999). The significance of the resultant quantitative and qualitative changes in dental plaque formed the basis of the Specific (Loesche 1976) and Non Specific (Theilade 1986) Plaque Hypotheses.

The Specific Plaque Hypothesis (SPH) proposes that specific pathogens in plaque are responsible for the observed destruction, whilst the Non Specific Plaque Hypothesis (NPH) implies that it is the sheer bulk of plaque that initiates CIPD. Neither of these theories can be wholly accepted or refuted outright. The increase in plaque bulk favours colonisation by specific putative periodontal pathogens such as *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* (thus supporting the SPH), however these species are not exclusive to disease and are in fact associated with healthy sites though in fewer numbers (Dahlen *et al.* 1992, Wolff *et al.* 1993). However, periodontal therapy is applied in a non-specific way (thus supporting the NPH). A more plausible and unifying theory is the Ecological Plaque Hypothesis (Marsh 1991), which states that a change in key environmental factor/s will trigger a shift in the balance of the resident plaque microflora and this might predispose a site to disease. This latter hypothesis recognises that biofilms formed at the tooth-gingiva interface may be in a state of dynamic equilibrium with the host until such a time when imposed ecological stress disrupts this balance.

Prior to discussing the pathobiology of periodontitis, it is relevant to briefly review the histology of the dento-gingival interface and the development and physiology of biofilms with particular reference to plaque at such an interface.

1.2.5.1 Dento-gingival junction

It is the uniqueness of this interface i.e. where a hard, non-shedding mineralised surface (tooth) pierces the integument, which distinguishes CIPD from the many other diseases that afflict mankind (Fig.1.8). In health, the components constituting this interface (enamel and gingiva) are both of neuroectodermal origin, thereby maintaining the embryological integrity of the integument and forming a continuum in the epithelial defence barrier. In disease, this integrity is breached as the gingiva migrates apically on to cementum (derived from mesoderm). It has been postulated that apical migration is the host's method of defending itself against the advancing bacterial front.

Enamel (ectodermal origin)

Junctional epithelium

Gingiva (ectodermal origin)

Cementum (mesodermal origin)

Periodontal ligament

Alveolar Bone

Pristine periodontium

Periodontium affected by periodontitis
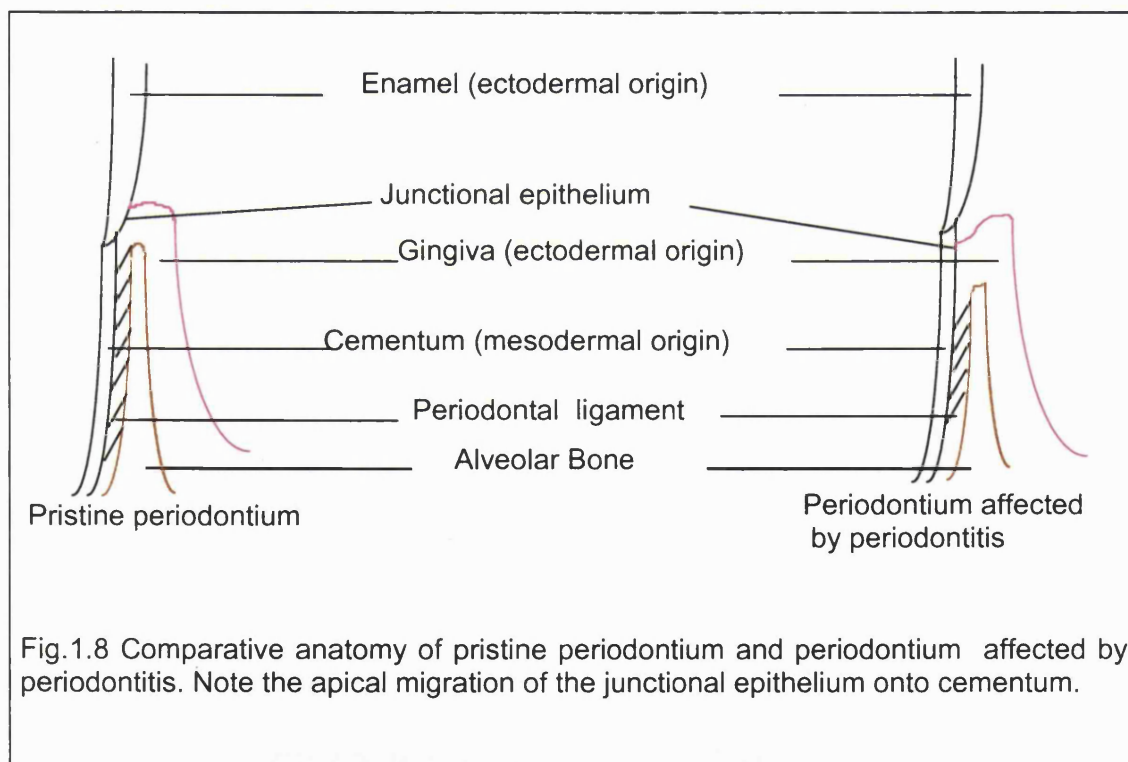
Fig.1.8 Comparative anatomy of pristine periodontium and periodontium affected by periodontitis. Note the apical migration of the junctional epithelium onto cementum.

At its most apical part, the JE is about 3 cells wide whilst towards the gingival crevice, it is about 12 - 18 cells wide (Scott & Symons 1982, Williams *et al.* 1992). The key characteristic features of the JE are:

i)   it has the highest cell turnover in the body (4 - 11 days)

ii) it has wider intercellular spaces relative to tissue volume when compared to the oral epithelium

iii) the size of JE cells is large relative to tissue volume when compared to oral epithelium

iv) it contains fewer desmosomes relative to the oral epithelium

v) it does not contain any membrane bound granules implying that it is maintained in an early state of differentiation.

These features signify that the JE is highly adapted to its role of ensuring a firm and effective attachment between the tooth and oral mucosa. Its early state of differentiation, in addition to the large intercellular spaces, makes the JE highly permeable allowing the bi-directional flow of fluid and / or cells from the crevice into tissues and vice versa. Thus bacterial cells (Lamont *et al.* 1992, Rudney *et al.* 2001), or their components e.g. lipopolysaccharide (Schwartz *et al.* 1972), situated in the crevice can readily enter the gingival tissues. Gingival tissue fluid can drain via the gingival sulcus, thus forming gingival crevicular fluid (Brill & Krasse 1958, Brill 1959). Neutrophils can also migrate through the JE and into the gingival crevice / periodontal pocket (Attström & Egelberg 1970, Attström 1975).

1.2.5.2 Biofilm formation and physiology

Biofilms are defined as "matrix-enclosed bacterial populations adherent to each other and / or to surfaces or interfaces" (Costerton *et al.* 1994). Bacteria readily attach to an organic layer present on a tooth surface, whether it is to the remnants of the enamel cuticle (Newman 1973) or of salivary origin (Ericson 1967). Although both the tooth surface and bacterial cell have a net negative charge, bacterial attachment to the tooth surface takes place through molecular mechanisms, namely hydrophobic interactions (Rosenberg *et al.* 1983), calcium bridging (Rose *et al.* 1993), extracellular polysaccharides (Germaine *et al.* 1977) and adhesin-receptor interactions (Ofek & Perry 1985).

Bacterial cells approaching the tooth surface may initially bind reversibly, however some do so irreversibly leading to single cell colonisation of the surface (adhesion) (Fig.1.9). Thereafter a gradual quantitative and qualitative increase in bacterial mass occurs both by internal multiplication and surface apposition leading to increased bacterial diversity (Costerton *et al.* 1987). Co-aggregation takes place between

genetically distinct initial colonisers on a tooth surface, which include members of the genera *Streptococcus, Actinomyces, Haemophilus, Neisseria* and *Veillonella* (Liljemark *et al.* 1986, Nyvad & Kilian 1987). Secondary colonisers e.g. *Fusobacterium nucleatum,* can form a bridge between early and late bacterial colonisers (Kolenbrander 1993).

Fig.1.9 Stages in Biofilm formation
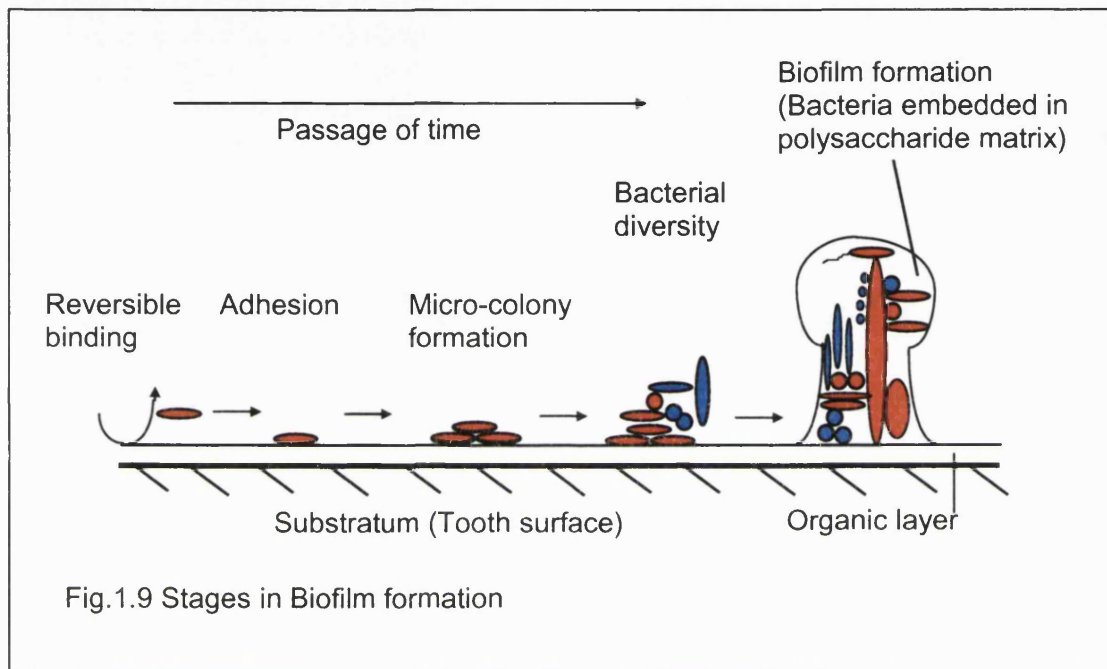
The bacterial diversity of plaque has been well documented microscopically (Newman 1975, Listgarten 1976, Vrahopoulos *et al.* 1992, Listgarten 1994). The characteristic corn-cob, toothbrush and rosette formation clearly demonstrate bacterial co-aggregation. However these images failed to portray the dynamic equilibrium that exists within these microbial communities. As plaque matures, it diversifies not only spatially (as seen above) but also temporally. The initial colonisers are usually aerobic organisms e.g. *Streptococcus sanguinis*, whilst late colonisers are anaerobic e.g. *Porphyromonas gingivalis.* Each microbial community develops into a functional unit with its own metabolic communication (Marsh 1994, Kolenbrander 1993, 2000). Bacterial interaction also takes place via quorum sensing (Frias *et al.* 2001). The bacterial cells in these micro-colonies are embedded in a gel-like matrix with differing gradients of oxygen, redox potential, pH and nutrients, and interlaced with water channels (Costerton *et al.* 1987, Marsh & Bradshaw 1997). Antagonistic conditions e.g. bacteriocin production by certain bacteria may prevent other bacteria from becoming established in the biofilm (van der Hoeven & Rogers 1979).

Microbial homeostasis results from physical and metabolic co-operation between cells within the biofilm (Sanders & Sanders 1984). It is also advantageous to the host as it prevents colonisation by exogenous micro-organisms (van der Waaij et al. 1971). In addition, the matrix embedding the bacterial cells protects the bacteria from white blood cells (Peterson et al. 1978) and antibiotics (Nickel et al. 1985).

1.2.5.3 Subgingival Biofilms

The range of bacteria that colonise a site is dictated by the environmental factors that operate at that site. Subgingival plaque forms from supragingival plaque. Individual anaerobic bacteria found in supragingival plaque may migrate apically as they are attracted by the lower redox potential in subgingival pockets (Kenny & Ash 1969), nutrients in gingival crevicular fluid (Shah & Gharbia 1995) or because they are able to migrate along the tooth surface (Poirier et al. 1979). The bacteria at the advancing plaque front release substances e.g. lipopolysaccharide and fermentation products, which stimulate an (subclinical) inflammatory response. This will lead to swelling of the gingiva resulting in more plaque becoming located subgingivally (Fig.1.10).
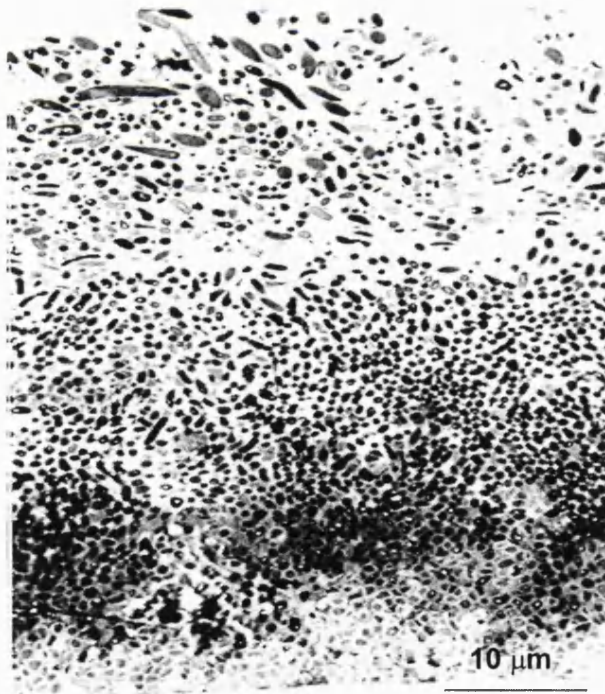
Fig.1.10 Transmission Electron microscopic section through plaque (Vrahopoulos et al. 1989; depth of plaque = 70 µm).

The subgingival bacterial biofilm is partly limited by the physical constraints of the gingival crevice / periodontal pocket. It is bathed in GCF so that bacterial cells may detach either singly or as aggregates and become suspended in it. The composition and physiology of the planktonic bacteria are different to those of the biofilm (Darveau *et al.* 1997). This might account for some of the differences observed in microbial composition between studies which used different subgingival plaque sampling methods e.g. paper points versus curettes (Tanner & Goodson 1986). Although GCF is rich in nutrients, it also contains antibacterial molecules (e.g. complement, immunoglobulins) (Cimasoni 1983b). However these antibacterial agents cannot easily penetrate the biofilm matrix (Baltimore & Mitchell 1980), although they may assist in controlling colonisation and plaque growth.

Putative periodontal pathogens are not exclusively present at diseased sites, nor does their presence or absence necessarily relate to disease (Dahlèn *et al.* 1992, Haffajee & Socransky 1994). Indeed CIPD results when the ecological conditions at the plaque-host interface disrupt the previous dynamic equilibrium between the bacteria and a susceptible host. Ecological stresses e.g. nutrients, pH, bacteriocins (Marsh 1994, 1997) may lead to changes in the quantity of bacteria, but more importantly they may encourage:

i) bacterial diversification and hence colonisation by putative periodontal pathogens e.g. *P. gingivalis, A. actinomycetemcomitans, F. nucleatum, Eikenella corrodens, Tannerella forsythensis, Prevotella intermedia, Campylobacter rectus* and *Peptostreptococcus micros* (Haffajee & Socransky 1994, Sakamoto *et al.* 2002)

ii) presence of virulent strains (Neiders *et al.* 1989)

iii) expression of bacterial virulence factors not previously expressed (Barua *et al.* 1990)

iv) dominance by certain clonal types (Loos *et al.* 1993).

Increased host susceptibility to periodontitis may be due to one or more risk factors:

i) systemic disease e.g. Diabetes Mellitus

ii) local susceptibility e.g. PMN dysfunction (Hart *et al.* 1994)

iii) genetic susceptibility (Michalowicz *et al.* 1991, Kornman *et al.* 1997)

iv) smoking (Bergström & Eliasson 1987).

1.2.6 Microbiology of Periodontitis

The microbiology of the oral cavity and therefore that associated with periodontitis is indeed one of the most complex microbial communities harboured by the human body. It is estimated that, on average 10^3 to 10^4 bacteria are cultured from a plaque sample taken from a healthy site, though this number increases ten thousand-fold if the sample is taken from a moderately deep (4-5 mm) periodontal pocket (Moore & Moore 1994). The basic principles of biofilm formation (as described above) hold true both for supragingival and for subgingival plaque formation. However, the different conditions which prevail supra- and subgingivally are reflected by the different organisms that colonise the respective sites.

Early studies on bacterial morphotypes contained in dental plaque showed a gradual transition from a predominantly coccoid microflora (associated with periodontal health), to one mainly composed of rods and spirochaetes (associated with periodontal inflammation; Löe et al. 1965, Newman 1975, Listgarten 1976, Listgarten & Helldén 1978). Indeed such morphological differences were supported by cultural microbiology (Theilade et al. 1966, Dzink et al. 1985, 1988, Moore & Moore 1994).

The predominant isolates of supragingival plaque are streptococci - mainly *Streptococcus sanguinis, but also S. mutans, S. mitis* and *S. salivarius. Actinomyces* spp. *(A. viscosus, A. naeslundii, A. israelii)* as well as *Lactobacillus* and *Veillonella* species are also frequently present in high numbers (Listgarten 1976, Loesche & Syed 1978, Moore & Moore 1994). As plaque matures and starts to assume a more subgingival location, these pioneer species are replaced by microbial complexes composed of mainly of Gram-negative, obligate and facultative anaerobic species. The predominant species cultured from periodontitis associated plaque include: *Por. gingivalis, A. actinomycetemcomitans, Prev. intermedia, T. forsythensis, E. corrodens* and *F. nucleatum* (Slots & Genco 1984, Dzink et al. 1985, 1988, Vrahopoulos et al. 1988, Listgarten 1994, Moore & Moore 1994). Although these organisms have been associated with diseased sites, as they have been recovered in higher numbers from such sites, it has still not been proven beyond reasonable doubt that they are the cause of the disease. Indeed, they might be found in higher numbers as they take advantage of the anaerobic environment within the pocket. Some authors have gone further to suggest that the different CIPDs harbour specific microbial complexes (Moore & Moore 1994, Petsios et al. 1995, Kamma et al. 1995, 1998, Ximenez-Fyvie et al. 2000, Socransky et al. 2002; Table 1.9).

Table 1.9 Representative putative periodontal pathogens implicated in the different CIPDs

Adult Periodontitis	Juvenile Periodontitis	Rapidly Progressive Periodontitis	Refractory Periodontitis	Prepubertal periodontitis	Necrotising Ulcerative Periodontitis
Por. gingivalis	*A. a*	*A. a*	*A. a*	*A. a*	*Treponema* spp.
A. a	*Prev. intermedia*	*Por. gingivalis*	*Por. gingivalis*	*Capno.* spp.	*Selenomonas* spp.
Prev. intermedia	*E. corrodens*	*T. forsythensis*	*Prev. intermedia*	*Prev. intermedia*	*Prev. intermedia*
E. corrodens	*Capno* spp.	*C. rectus*	*T. forsythensis*		*Fusobacterium* spp.
F. nucleatum	*C. rectus*	*E. corrodens*	*C. rectus*		
C. rectus		*Capno.* spp	*P. micros*		
T. forsythensis			*Candida* spp.		
P. micros			· *Capno.* spp.		
Treponema spp.					
Selenomonas spp.					
Eubacterium spp.					
Capno. spp (?)*					

*Capno. spp. = Capnocytophaga spp: C. rectus = Campylobacter rectus; Por. gingivalis = Porphyromonas gingivalis; Prev. intermedia = Prevotella intermedia; A. a = Actinobacillus actinomycetemcomitans; T. forsythensis = Tannerella forsythensis; E. corrodens = Eikenella corrodens; P. micros = Peptostreptococcus micros; F. nucleatum = Fusobacterium nucleatum.

Compiled from Slots 1976, Newman & Socransky 1977, Tanner *et al.* 1979, Slots *et al.* 1980, 1896, Loesche *et al.* 1982, Mandell 1984, Savitt & Socransky 1984, Dzink *et al.* 1985, 1988, Sweeney *et al.* 1987, Moore & Moore 1994, Petsios *et al.* 1995, Kamma *et al.* 1995, 1998.

1.2.7 Pathobiology of Periodontitis

In 1976, Page & Schroeder set the scene for the clinical and histological staging of the gradual transition that occurs between periodontal health and periodontitis. Twenty-five years later, their accurate description has been supported by an abundance of information regarding the bacteria-host interactions involved in periodontal destruction. Certainly a wealth of information is now available with respect to cytokine networks in periodontal health and disease (Okuda & Murakami 1998). A brief explanation of cytokine physiology is essential for a better understanding of the interactions that take place in periodontal pathobiology.

1.2.7.1 Cytokines

Cytokines are defined as soluble proteins produced by cells that serve as mediators of cell function, thus altering cellular physiology either locally or systemically. In health, cytokines are constitutively secreted by resident cells and play an important role in tissue homeostasis, maintaining the delicate dynamic balance between anabolic and catabolic reactions thereby regulating cellular proliferation, differentiation and migration of resident cells and turnover of tissue matrix. A typical example would be the production and breakdown of collagen by fibroblasts (Fig.1.11).

Fig.1.11 Collagen production and degradation by fibroblasts. PDGF: platelet derived growth factor; FGF: fibroblast derived growth factor; TGF-β: transforming growth factor β; IFN-γ: interferon γ; IL-1; interleukin-1.
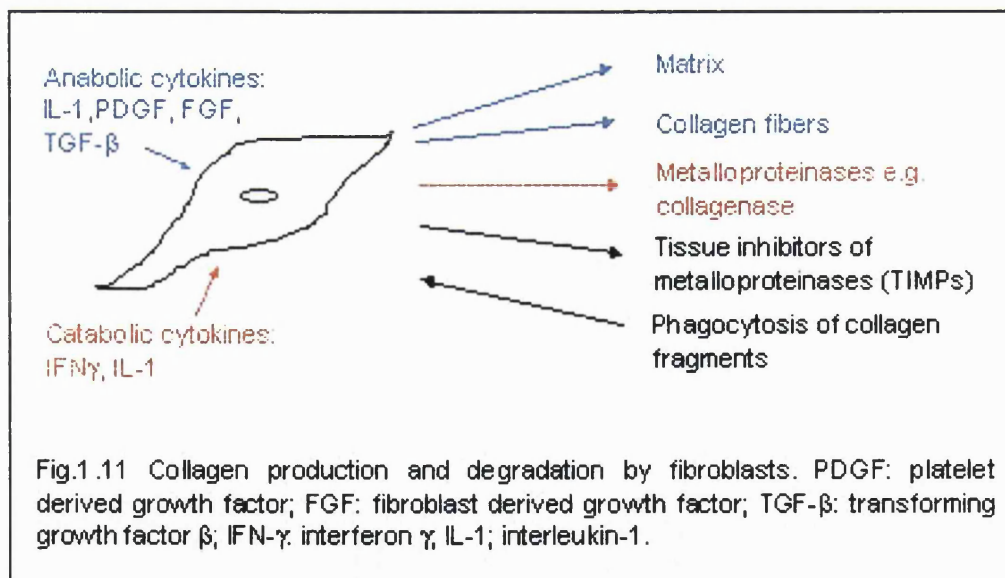
In disease, this delicate balance in cytokine networks which maintains tissue homeostasis is disrupted. Catabolic cytokines are induced in response to bacteria-host interactions taking place at the dento-gingival junction giving rise to an immuno-inflammatory response which may account for most of the destruction seen in

periodontitis (Wilson 1995, Page *et al.* 1997). Bacterial products e.g. LPS are known to induce cytokine production by monocytes and macrophages with wide ranging effects (Wilson 1995). Bacteria with tissue-invading properties (e.g. *Por. gingivalis*) are known to down-regulate cytokines e.g. interleukin-8 (IL-8) located in the JE and which act as chemoattractants for PMNLs, i.e. the first line of cellular defence in the body (Sandros *et al.* 2000). The disruption of the gradient will result in PMNL accumulation and degranulation in the tissues rather than in the crevice, with detrimental consequences (Kornman *et al.* 1997). Thus, in a genetically susceptible individual, the net increase in catabolic cytokine production both by resident (e.g. epithelial cells, fibroblasts) and immigrant (PMNLs, macrophages, monocytes) host cells in response to the bacterial challenge will result in periodontal breakdown (Wilson 1995, Kornman *et al.* 1997, Page 1998). Indeed genetically susceptible individuals manifest a hyper-responsive monocyte phenotype leading to upregulated cytokine production by monocytes (Molvig *et al.* 1988, Garrison *et al.* 1988, Offenbacher 1996).

1.2.7.2 Periodontitis: initial stages in bacteria-host interactions

Once the bulk of plaque reaches a critical mass and contains virulent clones of periodontal organisms, the host's immune response can never efficiently eliminate it. This results in a chronic inflammatory response that may bring about tissue destruction.

The subgingival plaque biofilm is bathed in GCF. PMNLs are constantly migrating outwards through the JE and are seen on the plaque surface (Theilade & Attström 1985), while monocytes are usually observed within the JE (Schroeder *et al.* 1975). Thus PMNL dysfunction leads to severe periodontal destruction (Barr-Agholme *et al.* 1992). Host antibacterial agents may remove bacterial cells singly suspended in GCF, however certain species e.g. *Capnocytophaga* spp. can evade this response for example by degrading immunoglobulins (Kilian *et al.* 1981, Grenier *et al.* 1989). The bacteria enclosed within the biofilm matrix are protected from the host defence mechanisms (Fig.1.2.2; Costerton 1987, Marsh & Martin 1999). Bacteria are a constant source of vesicles and LPS, which, due to the porous nature of the JE (Section 1.2.5.1), are capable of diffusing into and through the JE thereby eliciting periodontal destruction either directly e.g. via enzyme production, or indirectly by eliciting an inflammatory host response. Bacterial enzymes may include fibronectin-

degrading enzymes (Larjava *et al.* 1987, Smalley *et al.* 1988) which could potentially facilitate the dissipation of bacteria or their products into the tissues.

Bacterial products (proteins & LPS) from the biofilm are known to enhance the migration of leucocytes from blood vessels into the tissues and thence into the crevice and to activate the release of pro-inflammatory cytokines e.g. IL-8, prostaglandins (PG) and matrix metalloproteinases (MMPs) by various component cells of the gingiva (Page 1998). MMPs degrade collagen (e.g. perivascular collagen) and the extracellular matrix (Meikle *et al.* 1989). The cytokines diffuse towards the connective tissue and enhance the inflammatory response (Abe *et al.* 1991) e.g. IL-8 acts as a chemoattractant for neutrophils leading to an increased number of PMNLs exuding through the JE and into the crevice (Schroeder & Attström 1980). The effect of IL-8 on PMNLs seems to be dose dependent: lower concentrations attract PMNLs whilst higher doses induce antibacterial activity (Baggiolini *et al.* 1989). PMNL antibacterial activity can be elicited either through oxygen-dependent or oxygen independent mechanisms (Kinane & Lindhe 1997). Some PMNLs become entrapped within the JE and connective tissue and may thus contribute to tissue destruction. Other inflammatory cytokines include IL-1, IL-6 and TNF-α, all of which propagate the inflammatory response (Okada & Murakami 1998).

The JE provides a physical barrier to bacterial penetration though some bacteria, namely *Por. gingivalis*, *A. actinomycetemcomitans* and *C. gingivalis,* can penetrate the oral epithelium (Lamont *et al.* 1992, Sandros *et al.* 1993, Rudney *et al.* 2001). Protection is also provided by its high rate of cellular turnover (section 1.2.5.1). Keratinocytes within the JE are able to sense, and react to, bacterial stimuli by producing relevant cytokines (Fiero *et al.* 1995), e.g. IL-8, a chemoattractant to PMNLs (Baggiolini *et al.* 1989). Thus the JE plays an important role in the immuno-inflammatory response.

The gradual accumulation of proinflammatory cytokines, LPS and bacterial proteins in the tissues induces the endothelial cells in the subjacent capillary beds to express endothelial adhesion molecules (E-selectins), which bind to leukocytes thereby enhancing diapedesis (Darveau *et al.* 1997). LPS contributes towards this cellular migration as it activates the alternative complement pathway that results in the production of vasoactive amines and which, in turn, leads to increased permeability and vasodilatation of the capillary plexus beneath the JE. The blood vessels become

engorged and blood flow through these vessels decreases. The intercellular junctions between the endothelial cells open up leading to leakage of plasma proteins into the tissues (Adonogianaki *et al.* 1994). The increased plasma-derived enzymes in the tissues will lead to amplification of the inflammatory response (Kornman *et al.* 1997). LPS and bacterial proteins have an effect on cells of myeloid lineage i.e. lymphocytes and monocytes. T lymphocytes are recruited to the area and activated through their interactions with the various cytokines, the net result being that they produce a wide range of cytokines (Fig.1.12) (MacDonald 1999).

Th1 ⟶ IL-2, -12, IFN-γ, TGFβ ⟶ Cell mediated immunity

T-cells ⟶ Th2 ⟶ IL-4,-5,-6,-10,-13 ⟶ Antibody production

Th3 ⟶ TGF-α

Fig.1.12 Differentiation of T lymphocytes
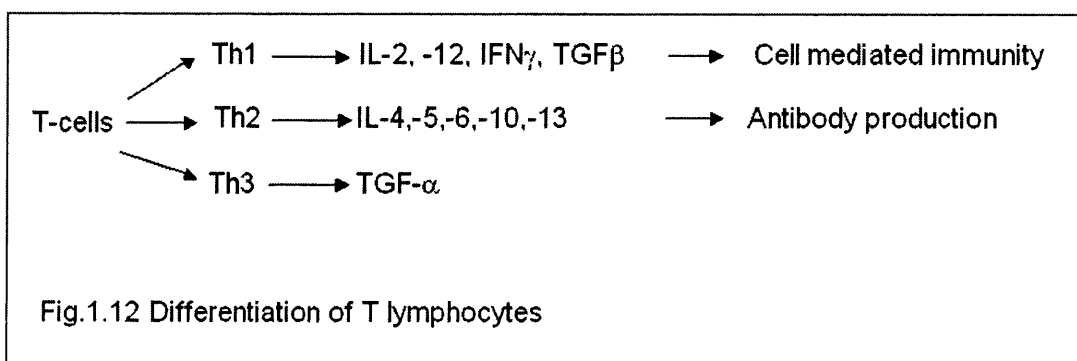
In the presence of antigen and various cytokines, these cells secrete a variety of cytokines namely IL-2, -3, -4, -5, -6, -10 and interferon gamma (INFγ) (Gemmel *et al.* 1997). These cytokines induce responding T-cells to differentiate into clones of $CD4^+$ and $CD8^+$, and B cells to differentiate into clones of antibody producing plasma cells.

Monocytes respond by releasing IL-8, PGE_2 and MMPs, which collectively enhance leukocyte recruitment, increase the chemotactic gradient and contribute to tissue destruction (Darveau *et al.* 1997). MMPs lead to degradation of the perivascular collagen and extracellular matrix. The clinical signs and symptoms at this stage are those of gingival redness, swelling and bleeding. Breakdown of the epithelial attachment and connective tissue results in loss of clinical attachment. The JE migrates apically and becomes ulcerated as it forms the pocket epithelium. These lesions may progress to the advanced stages of periodontitis.

1.2.7.3 Periodontitis: advanced stages in host-bacteria interactions

A persistent bacterial load at the dento-gingival interface leads to a sustained build-up in the immuno-inflammatory response by the host.

61

In the advanced stages of inflammation, the resident cells of the periodontium (e.g. fibroblasts, endothelial cells and epithelial cells) change phenotype and become active secretors of inflammatory cytokines (Kornman et al. 1997, Page 1998). The fibroblast phenotype changes to one that produces high levels of MMPs and low levels of tissue inhibitors of matrixmetalloproteinases (TIMPs: Fig.1.2.4; Reynolds & Meikle 1997), thus aiding periodontal tissue degradation. Periodontally diseased tissue is characterised by high levels of proinflammatory cytokines e.g. IL-1β, TNF-α & IFNγ (Page et al. 1997). The high number of macrophages in the area leads to the production of reactive oxygen metabolites (H_2O_2, O_2^-) as well as MMPs and PGE$_2$ and Leukotriene B$_4$. PGE$_2$ has been implicated in adult periodontitis and may mediate much of the tissue destruction that occurs in periodontal disease (Offenbacher et al. 1993, Offenbacher 1996).

The sustained perfusion of LPS diffusing through the tissues stimulates gingival macrophages to secrete a wide array of cytokines, the presence of which alters the local environment considerably by:

i)   recruiting more monocytes and lymphocytes to the area (Yu & Graves 1995)

ii)   releasing more MMPs which enhance collagen degradation

iii) activating CD4$^+$ T cells to release cytokines which induce B-cell differentiation and production of antibodies (IgG) (Ebersole et al. 1993).

The clinical features at this advanced stage include true loss of clinical attachment and radiographic evidence of bone loss superimposed on the preceding inflammatory signs.

In conclusion, adult periodontitis is the clinical manifestation of the advanced stages of the host-bacteria interaction at the dento-gingival junction. Periodontal breakdown occurs when the ecological factors at a site favour the accumulation of a concoction of virulent periodontal pathogens in a genetically susceptible individual. Risk factors that are definitely known to affect disease initiation and progression include smoking (Bergström & Eliasson 1987), neutrophil dysfunction (Hart et al. 1994), and systemic diseases such as diabetes mellitus.

1.3 Diabetes Mellitus-Periodontitis (DM-Periodontitis)

The first reported observation of a possible relationship between DM and periodontitis seems to be that by Seiffert (1862). During the 1920s - 1940s the acceptable profile for a definite clinical entity known as "diabetic periodontoclasia" was established (Williams

1926). Notable clinical changes in the periodontium included severe periodontal inflammation, increased tendencies towards periodontal abscesses and rapid alveolar bone loss (Hirshfield 1934, Cohen 1947).

1.3.1 Definition

The more recent nomenclature 'DM-associated Periodontitis', is a term which has been coined to imply the concurrent presence of both diseases in the same individual (AAP 1989, Lalla *et al.* 2000). In this thesis the more concise term DM-Periodontitis will be used. It is not intended to imply that DM-Periodontitis is a specific clinical entity but rather that the pathobiology underlying both diseases may interact at the cellular and molecular level thus leading to a two-way relationship between the two conditions (Grossi & Genco 1998). DM-Periodontitis is clinically indistinguishable from adult periodontitis (Fig.1.13).

Fig.1.13 Clinical appearance of DM-Periodontitis

1.3.2 Epidemiology

There are several reports in the periodontal literature pertaining to the epidemiology of periodontitis in DM subjects. The results are somewhat conflicting.

Most of the evidence supports the view that periodontitis is more prevalent and severe in DM subjects (Glavind *et al.* 1968, Sznajder *et al.* 1978, Hugoson *et al.* 1989, Shlossman 1990, Emrich *et al.* 1991, Cianciola *et al.* 1992, Löe 1993, Oliver & Tervonen 1993, Collin *et al.* 1998) especially those in whom DM is poorly controlled (Tervonen & Knuttila 1986, Ainamo *et al.* 1990, Safkan-Seppälä & Ainamo 1992). Additionally, the severity of periodontal destruction seems to be related to the duration of DM (Thorstensson & Hugoson 1993, Cerda *et al.* 1994, Firatli *et al.* 1997). There

are, however, other studies that do not support such a relationship (Hove & Stallard 1970, Nichols *et al.* 1978, Barnett *et al.* 1984, Rylander *et al.* 1986, Pinson *et al.* 1995). The confusion and disagreement which prevails in the periodontal literature and which prohibits any direct comparison between studies is not surprising considering that:

i)   the two different types of DM were frequently mixed in the groups being studied. Most studies (Barnett *et al.* 1984, Safkan-Seppälä & Ainamo 1992, Collin *et al.* 1998) still use the terminology "insulin-dependent" and "non insulin-dependent" DM in spite of the American Diabetes Association recommendations to avoid this terminology (section 1.1.3). This might lead to patient categorisation based on treatment rather than aetiology. Although the basic pathology of the two types of DM does not differ, the fact that Type I DM has a stronger genetic component than Type II and is of earlier onset (section 1.1.5.1) might affect the severity of periodontitis

ii)   the change in criteria used to define both DM (pre- and post-1979) and periodontitis (pre- and post-1989) have made direct comparisons practically impossible

iii) the cut-off point for the categorisation of DM subjects into well- and poorly-controlled DM based on glycated haemoglobin results was not consistent (Tervonen & Knuttila 1986, Pinson *et al.* 1995)

iv)   controls have often been presumed to be non-diabetic without documentation (Thorstensson & Hugoson 1993)

v)   different measures and indices of periodontal destruction were used. Some studies used partial screening indices (Nichols *et al.* 1978, Emrich *et al.* 1991), whilst others used full-mouth periodontal charting (Rylander *et al.* 1986)

vi)   variable and non-comparable data sets have been collected and analysed by widely varying statistical methods

vii) the relatively high prevalence of both diseases makes it difficult to conduct studies with statistical power sufficient to provide reliable data (Soskolne 1998)

viii) the racial mix in the groups under investigation has not been well documented. The prevalence of DM is known to vary between populations (King & Rewers 1993). Thus if DM is considered to be a risk factor for Periodontitis (Oliver & Tervonen 1994, Yalda *et al.* 1994), the genetic make-up of the subjects recruited in such studies must not be overlooked.

Perhaps the most reliable study to date is that by Emrich *et al.* (1991) who showed that DM increases the risk of developing periodontitis three-fold "in a manner which cannot be explained on the basis of age, sex or oral hygiene measures alone". The only drawback of this study is that it used only six (Ramfjord) index teeth. Whilst this made such a large study practicable, it might have included subjects manifesting Localised Juvenile Periodontitis, rather than Adult Periodontitis (AP), which could therefore have contributed to an apparent increased overall incidence of AP. In an effort to clarify the epidemiology of periodontal diseases in diabetics, and taking some of the above points into consideration, Soskolne (1998) reviewed the literature between 1989 and 1998 and concluded that DM subjects have an increased prevalence and severity of periodontitis. This may be explained by the common pathological processes that underlie the two diseases.

1.3.3 Pathobiology

Recent research into the pathobiology of DM-Periodontitis has shed light on different mechanisms, which may explain the exacerbated periodontal destruction observed in these subjects. These may be divided into:

i) AGE-induced cellular and molecular mechanisms, and

ii) the effect of elevated glucose on component cells of the periodontium.

1.3.3.1 AGE-induced cellular & molecular pathobiology

Although DM and periodontitis are two separate and distinct disease entities, AGE-induced cellular and molecular pathologies may help to explain the several potential interactions between them (Fig.1.14).

The pathology of periodontitis has already been explained previously (Section 1.2) and will not be elaborated upon here, other than to comment on the total surface area of the periodontal lesion. It has been estimated that in moderate or severe periodontitis, the total surface area of the pocket epithelium coming into contact with the subgingival bacterial biofilm is equivalent to the surface area of the palm of one's hand or forearm respectively (Page 1998, Seymour & Steele 1998). Thus at the dento-gingival interface, the host is continuously challenged by bacteria or their products.

Fig.1.14 Pathobiology of DM-Periodontitis

DM pathology

Hyperglycaemia & Hyperlipidaemia

DM-Periodontal pathology

Periodontitis

In cell membrane

AGE formation

outside cell
basement membrane thickening
collagen breakdown
↓ endothelial proliferation
↓ haematological abnormalities

Host - bacteria interaction

Perturbs cell
membrane lipid
composition

Inside cell

AGE-RAGE
interaction

Immuno-inflammatory
response

Altered cell biology

DNA damage
? mutation

altered cellular
phenotype

↓ tissue perfusion & ↓O$_2$
↓ Mo & Mac migration

PMNL, Mo, Mac
accumulation

inflammatory
phenotype

↑ risk of infection with Gram-
negative anaerobes (LPS)

oxidant stress

NF-κB

H$_2$O$_2$ + IL-1

Cytokine release

↓ Growth factors

Reactive oxygen
intermediates

↑ TNFα, IL-1, IL-6

Interferes with lipid and
glucose metabolism

Exacerbates periodontal
tissue destruction

Mac = Macrophages, Mo = Monocytes, PMNL = Polymorphonuclear Leucocytes, LPS = Lipopolysaccharide, NF-κB = Nuclear Factor-κB.
Compiled from: Brownlee *et al.* 1984, Vlassara *et al.* 1991, Brownlee 1992, Bucala *et al.* 1993, 1994, Brownlee 1993, Yan *et al.* 1994, Iacopino 1995, Kanety *et al.* 1995, Schmidt *et al.* 1996, Grossi & Genco 1998, Nishimura *et al.* 1998, Iacopino & Cutler 2000, Lalla *et al* 2000.

AGE formation and the sequelae of AGE-RAGE interaction have already been discussed (Section 1.1.6). In this section these will be reviewed vis-à-vis the pathobiology of DM-Periodontitis.

AGE formation is responsible for changes that take place:

i) outside cell membranes

ii) in cell membranes

iii) inside cells, whilst

iv) AGE-RAGE interactions lead to cellular perturbation and hence phenotypic changes in component cells of the periodontium.

i) AGE formation outside cells

The microangiopathy characteristic of DM complications, e.g. basement membrane thickening, is observed in gingival capillaries (Campbell 1971, Frantzis *et al.* 1971, Seppälä *et al.* 1997). AGE cross-links between protein molecules (e.g. type IV collagen) are resistant to protein degradation yet enhance protein deposition, thus partly accounting for basement membrane thickening (Crawford & Cotran 1994). The increased vascular permeability subsequent to AGE-RAGE interaction (Schmidt & Stern 2000) may encourage the accumulation of proteins e.g. immunoglobulin G in basement membranes which may be responsible for the activated complement membrane attack and hence tissue destruction seen in some tissues (Falk *et al.* 1983). In addition, AGE-induced haematological cellular malfunction (Table 1.4, Bagdade *et al.* 1972, 1978) may undermine host defences. Collectively, these changes may impede oxygen diffusion, metabolic waste elimination, leukocyte migration and diffusion of immune products and thus contribute to periodontitis in DM patients (Oliver & Tervonen 1994). In addition, these conditions may favour the growth of an anaerobic subgingival microflora, which in a susceptible individual, may elicit host-bacteria interactions leading to cytokine upregulation (e.g. TNFα, IL-1 & IL-6) which results in tissue destruction. Infections are known to alter the endocrinologic-metabolic status of the host (Rayfield *et al.* 1982) as cytokines such as TNFα interfere with insulin action at the cellular level (Kanety *et al.* 1995). This may potentially explain why some periodontal intervention studies have shown a slight improvement in HbA$_{1c}$ levels after periodontal treatment (Miller *et al.* 1992, Grossi *et al.* 1996, 1997, Taylor *et al.* 1996, Iwamoto *et al.* 2001).

ii) AGE formation in cell membranes

Insulin is required for the enzymatic metabolism of dietary essential fatty acids that subsequently form structural components of membrane phospholipids (Dutta-Roy 1994). The physicochemical properties of membranes e.g. membrane fluidity, insulin receptor expression and insulin action are largely determined by the nature of the fatty acids within the phospholipid bilayer (Borkman et al. 1993) and which in turn may influence diverse cellular functions including the activity of membrane bound enzymes and transporters (including glucose transporters) and the control of nuclear events operating to govern gene transcription (Clarke & Jump 1993, Dutta-Roy 1994). This could explain the increased IL-1β and reduced platelet derived growth factor levels in hyperlipidaemic DM-periodontitis patients (Cutler et al. 1999).

iii) AGE formation inside cells

Intracellular AGEs bind with nucleic acids or their associated proteins and induce DNA damage (Bucala et al. 1993, Mullokandov et al. 1994). AGE modification of DNA has been associated with in vivo mutations and altered gene expression in both prokaryotic (Bucala et al. 1985) and eukaryotic cells (Bucala et al. 1993). Such altered gene expression in diabetic cells may contribute to cellular pathologic dysfunction associated with DM including increased periodontal destruction observed in DM-Periodontitis subjects.

iv) AGE-RAGE Interaction (section 1.1.6, Fig.1.3)

The expression of RAGEs is enhanced in conditions of stress e.g. inflammation and diabetes (Schmidt & Stern 2000). This increases AGE-RAGE interaction, which leads to intracellular oxidant stress and formation of reactive oxygen intermediates (ROI) (Nishimura et al. 1998, Lalla et al. 1998, 2000), which in turn activate Nuclear Factor-κB (NF-κB), a pleiotropic nuclear transcription factor and important regulator of the immuno-inflammatory response through cytokine expression and adhesion molecules (Yan et al. 1994).

Another mechanism by which AGE-RAGE interaction may contribute to periodontal destruction is by alteration of cellular phenotypic expression e.g. altered macrophage gene expression. After binding of the ligand (AGE) with the cellular receptor, the cytosolic end of RAGE induces the macrophage to undergo phenotypic changes and thus express increased levels of cytokines namely TNF-α, IL-1 and IL-6 via activation of NF-κB (Iacopino 1995, Salvi et al. 1998). The increased generation of

proinflammatory cytokines such as TNF-α in inflammatory foci has been linked to the regulation and activation of matrix metalloproteinases (Birkedal-Hansen 1993). It has also been proposed that the elevated serum lipids will maintain the macrophage in an inflammatory state and prevent its maturation to a reparative phenotype (which would be associated with the production of essential growth factors e.g. platelet derived growth factor-β, Transforming growth factor β1, basic fibroblast growth factor) thus interfering with periodontal repair and potentially contributing to periodontal destruction (Iacopino 1995).

Other cells which manifest altered cellular phenotypes include endothelial cells and fibroblasts. AGE interactions with endothelial RAGE lead to increased vascular permeability (Wautier *et al.* 1996) and increased expression of vascular cell adhesion molecule-1 (Schmidt *et al.* 1995). AGE and fibroblast RAGE lead to increased production of matrix metalloproteinases and decreased collagen production (Lalla *et al.* 2000). Thus it has been hypothesised that increased accumulation of AGEs and their interaction with RAGEs in diabetic gingiva leads to vascular hyperpermeability, loss of effective tissue integrity and barrier function. This in turn leads to an exaggerated response to periodontal pathogens resulting in accelerated destruction of non-mineralised connective tissue and bone (Schmidt *et al.* 1996, Lalla *et al.* 2000).

Thus, at a cellular and molecular level various interactions could lead to increased production of proinflammatory cytokines, exacerbating periodontitis in DM subjects. In addition, hyperglycaemia may affect periodontal ligament (PDL) cells including osteoclasts.

1.3.3.2 Elevated blood glucose and cells of the periodontium

Diabetic complications, e.g. periodontitis, retinopathy, are directly related to the degree of blood glucose control (Ainamo *et al.* 1990, Löe 1993, Karjalainen *et al.* 1994). Thus the cells of the periodontium, which in poorly controlled DM subjects would be bathed in tissue fluid high in glucose (Schmidt *et al.* 1993), may function differently and partly explain why poorly controlled DM-subjects manifest severe periodontitis.

1.3.3.2.1 Periodontal Ligament Cells

The role of PDL cells in periodontal tissue regeneration is well characterised (Nyman *et al.* 1982a, b). Thus factors that alter the normal physiology of these cells would be expected to have a detrimental effect on periodontal healing. *In vitro* studies have shown that hyperglycaemia affects the phenotypic characteristics of PDL cells (Sasaki

et al. 1992) and PDL features such as adhesion, motility and protein synthesis (Sasaki *et al.* 1992, Nishimura *et al.* 1996, 1998), with the net result of severe periodontal destruction especially in poorly controlled DM-subjects.

1.3.3.2.2 Bone cells

A characteristic feature of periodontitis is loss of alveolar bone, both vertically & horizontally. The quality of bone in general is adversely affected by diabetes leading to the condition known as diabetic osteopenia (McNair *et al.* 1979). Thus DM may exacerbate bone loss in periodontitis. Indeed, this has been supported not only by several clinical studies which demonstrated an increased incidence of bone loss in DM subjects (Shlossman *et al.* 1990, Emrich *et al.* 1991), but also by recent studies which suggest that DM increases the risk for more severe progression of alveolar bone loss (Seppälä & Ainamo 1994, Taylor *et al.* 1998). Such bone loss may also be due partly to altered collagen metabolism in DM (Ramamurthy & Golub 1983). An *in vitro* investigation has demonstrated that sustained high glucose concentrations are capable of inhibiting osteoblast proliferation (Terada *et al.* 1998).

1.3.4 Gingival crevicular fluid (GCF)

Since the composition of GCF reflects the physiology of the surrounding tissues, a number of inflammatory markers found in GCF have been proposed as markers for periodontitis (Offenbacher *et al.* 1986, Lamster *et al.* 1988, Salvi *et al.* 1997, 1998). Statistically significantly higher levels of proinflammatory cytokines (IL-1β, PGE$_2$, & TNF-α) have been detected in DM subjects, possibly due to a genetically determined enhanced monocyte response (Salvi *et al.* 1998). Indeed these cytokines were higher in DM-subjects manifesting advanced periodontitis.

Since GCF originates from plasma it has been postulated that it may reflect blood glucose composition (Ficara *et al.* 1975). However, other reports do not support such a relationship (Hara & Löe 1969). Very limited research (using rather crude methods) has been performed in this field which makes it difficult to draw any major conclusions. Suffice to say that if higher GCF-glucose levels were to be found in DM-Periodontitis subjects, this could have profound implications on the diversity (Mashimo *et al.* 1983a) and on the phenotypic (Spratt *et al.* 1996) expression of some of the component species of the subgingival microflora.

1.3.5 Subgingival microflora

The microflora of DM-Periodontitis is poorly understood. Induction of experimental DM in an animal model is known to cause a shift in the subgingival microflora from one that consists of a nearly equal mixture of Gram-positive and Gram-negative cocci and short rods to one predominated by Gram-negative rods and cocci and which precedes deepening of periodontal pockets (McNamara *et al.* 1982). Infection with such Gram-negative organisms resulted in increased production of gingival collagenase (Chang *et al.* 1988). Various attempts have been made to characterise the microflora associated with DM-Periodontitis in humans, however the results are inconsistent (Sanchez-Cordero *et al.* 1979, Mandell *et al.* 1992, Mashimo *et al.* 1983a, Zambon *et al.* 1988, Sastrowijoto *et al.* 1989, Yuan *et al.* 2001). Some studies suggest that some bacterial species e.g. *Capnocytophaga* spp. (Mashimo *et al.* 1983), *Prevotella intermedia*, *Campylobacter rectus*, and *Porphyromonas gingivalis* (Zambon *et al.* 1988, Mandell *et al.* 1992) are recovered in higher proportions in DM-Periodontitis. However, other studies disagree with such findings and suggest that the subgingival microflora in DM-Periodontitis is, in fact, no different from that in chronic adult periodontitis (Sastrowijoto *et al.* 1989, Tervonen *et al.* 1994, Thorstensson *et al.* 1995, Sbordone *et al.* 1995, 1998). Direct comparison between studies is precluded due to the different clinical and laboratory procedures involved (explained further in Section 1.5). Most of the studies have looked at the relative proportions of one or more of the five putative periodontal pathogens commonly implicated in periodontitis, i.e. *A. actinomycetemcomitans, Por. gingivalis, Prev. intermedia, E. corrodens* and *F. nucleatum* (Zambon *et al.* 1988, Mandell *et al.* 1992, Yuan *et al.* 2001). Whilst there is sufficient evidence to infer these organisms as putative periodontal pathogens in periodontitis, they constitute only 1.6% of the total oral cultivable microflora (i.e. 5 from 300 cultivable species). Furthermore, the microbiological changes that take place in DM-Periodontitis might be more of a qualitative than a quantitative nature considering the subgingival ecological pressures (e.g. potentially elevated glucose in GCF), which might arise as a result of DM. Indeed, there might be other organisms, which could be equally or more pathogenic than the traditional putative periodontal pathogens and which have not, as yet, been investigated. One such group of organisms, which has been associated with severe and advanced periodontal destruction, and might therefore be involved in DM-Periodontitis, is *Capnocytophaga* spp.

1.4 *Capnocytophaga* spp.

1.4.1 Genus *Capnocytophaga*

The genus *Capnocytophaga*, first proposed by Leadbetter *et al.* (1979), consisted of three species: *Capnocytophaga gingivalis, C. ochracea, C. sputigena* all isolated from the human oral cavity (Leadbetter *et al.* 1979, Holt *et al.* 1979, Socransky *et al.* 1979, Williams & Hammond 1979). It comprises a group of capnophilic / facultatively anaerobic Gram-negative, fusiform, slender rods (Fig.1.15), which perform fermentative metabolism and exhibit gliding motility (Fig.1.16) when grown on solid culture media.
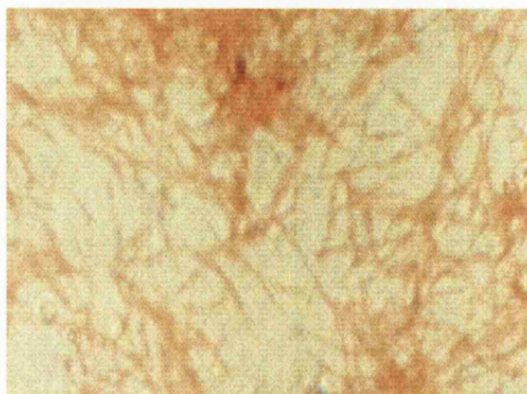
Fig.1.15 Gram stain reaction of *Capnocytophaga* spp.
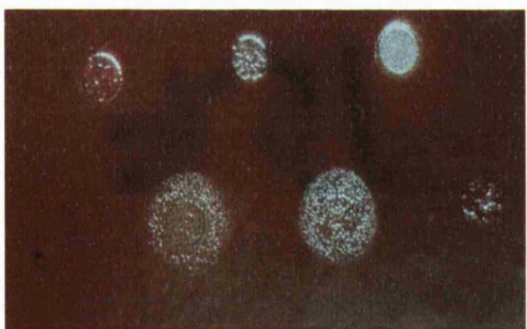
Fig.1.16 Spreading colonies of *Capnocytophaga* spp.

In 1989 another two species, *C. canimorsus* (previously known as Centre of Disease Control, CDC, group DF-2) and *C. cynodegmi* (previously known as CDC group DF-2 like), were added to this genus (Brenner *et al.* 1989). Although these two species share several similarities (e.g. gliding motility, growth in CO_2-enriched environment) with members of the genus *Capnocytophaga,* they are phenotypically and genetically distinct from Leadbetter's original species (Brenner *et al.* 1989). Their main differentiating features from the original *Capnocytophaga* species are that they are oxidase- and catalase-positive (Brenner *et al.* 1989). However, the authors preferred

72

"to place them in *Capnocytophaga* on the basis of their overall phenotypic similarity to this genus". *C. canimorsus* and *C. cynodegmi* form part of the oral flora in dogs and cats (Brenner *et al.* 1989). *C. canimorsus* can cause human systemic infections (Maher & Raik 1992) whilst *C. cynodegmi* may lead to localised infections (Brenner *et al.* 1989), both infections arising by way of dog or cat scratches or bites.

Another two species, *C. granulosa* and *C. haemolytica*, also isolated from the human mouth, were added to this genus by Yamamoto *et al.* (1994).

Thus, on the basis of phenotypic and genotypic characteristics, a total of seven species can be differentiated within this genus.

Prior to the Leadbetter papers, *C. ochracea* was originally known as *Bacteroides elongatus* var. *ochraceus* (Leadbetter *et al.* 1979). This species and *Ristella ochracea* were considered synonymous and the designation *B. ochraceus* was proposed (Newman *et al.* 1979, Williams & Hammond 1979). The latter name was eventually changed to *C. ochracea* (Leadbetter *et al.* 1979, Williams *et al.* 1979).

1.4.2 Habitat of *Capnocytophaga* spp.

The habitat of *C. gingivalis*, *C. ochracea*, *C. sputigena*, *C. granulosa* and *C. haemolytica* is the human oral cavity, more specifically supra- and / or subgingival plaque (Holt *et al.* 1979, Yamamoto *et al.* 1994, Ciantar *et al.* 2001). *Capnocytophaga gingivalis*, *C. ochracea*, and *C. sputigena* were originally designated as commensal organisms associated with periodontal health (Moore *et al.* 1982). However, some studies have implicated them as being opportunistic pathogens. Several studies report them as ranking among the more predominant cultivable microbiota isolated from patients with rapidly destructive periodontitis lesions (Tanner *et al.* 1979, Kamma *et al.* 1995), periodontal abscesses (Newman & Sims 1979), adult periodontitis (Rawlinson *et al.* 1993, Petsios *et al.* 1995), periodontitis in insulin dependent diabetes mellitus subjects (Mashimo *et al.* 1983a) and juvenile periodontitis (Nonnenmacher 2001). These species have also been identified in large numbers in the apical portion of subgingival plaque (Socransky 1977, Mordan *et al.* 1999). To date only one study has reported the isolation of *C. granulosa* and *C. haemolytica* from subgingival plaque from chronic periodontitis subjects (Ciantar *et al.* 2001).

1.4.3 Pathogenicity of *Capnocytophaga* spp.

Capnocytophaga spp. manifest various pathogenic features particularly their ability to adhere and to produce various tissue degrading enzymes. They are also capable of evading the host response.

1.4.3.1 *Capnocytophaga* spp. and adhesion

One of the most important features of a pathogenic organism is its ability to adhere to and colonise a site, since failure to colonise means failure to initiate disease (Socransky & Haffajee 1991). *C. sputigena* is capable of producing large amounts of surface-associated material (exopolysaccharide) and has long fibrils on its surface (Holt *et al.* 1979, Poirier *et al.* 1979). The cell surfaces of *C. ochracea* and *C. gingivalis*, however, appear smooth and free of any apparent extracellular material (Holt *et al.* 1979, Poirier *et al.* 1979). *Capnocytophaga* spp. manifest an affinity for cementum (Somerman *et al.* 1985) and can co-aggregate with other bacteria via lectin-carbohydrate interactions (Cisar *et al.* 1982, Weiss *et al.* 1987, Kolenbrander & London 1993), an important feature in biofilm formation. These organisms demonstrate gliding motility (Leadbetter *et al.* 1979) and, due to cell-to-cell interactions with other bacteria, they could potentially transport other bacteria with them from a supra- to a subgingival location (Poirier *et al.* 1979). An added advantage is that co-aggregated bacterial cells may exhibit resistance to phagocytosis and to the bactericidal action of PMNLs. Thus *Capnocytophaga* spp. may play a central role in establishing and maintaining the dental plaque biofilm. Furthermore, several *in vitro* studies involving *Capnocytophaga* spp. have shown that these organisms may play a role in periodontitis both directly via tissue destruction and indirectly via evasion of the host response (Genco & Slots 1984).

1.4.3.2 *Capnocytophaga* spp. and tissue destruction

Capnocytophaga spp. elaborate an epitheliotoxin (Birkedal-Hansen *et al.* 1982) which may damage crevicular epithelium. *C. ochracea* is also capable of degrading collagen types I & IV and elastin (Uitto *et al.* 1988, Söderling *et al.* 1991). Type I collagen is the predominant collagen type in gingiva and in the periodontal ligament (Bartold and Narayanan 1996). Type IV is the main collagen that constitutes the basement membrane beneath the junctional epithelium, whilst elastin is a minor component of the gingival connective tissue (Bartold 1991).

Capnocytophaga spp. elaborate various proteolytic enzymes, all of which are relevant to periodontal destruction (Laughon *et al.* 1982, Grenier & Turgeon 1994, Gazi *et al.* 1997) (Table 1.10). *Capnocytophaga* spp. exhibit potent aminopeptidase activity (Laughon *et al.* 1982, Nakamura & Slots 1982, Spratt *et al.* 1994, 1995). Such enzyme activity is of particular pathological significance for three reasons:

i) the organism producing it creates an ecological advantage for itself by providing a steady supply of proteins (Nakamura & Slots 1982)

ii) the resulting proteolysis could provide nutrients to a consortium of organisms present within the periodontal pocket (Spratt *et al.* 1995) where competition for nutrients is high

iii) aminopeptidase may contribute to the formation of bradykinin (a vasodilator peptide) and thus contribute to inflammation (Gannong 1983).

Table 1.10 Virulence factors produced by *Capnocytophaga* spp.

Enzyme	Reference
Aminopeptidases	Laughon *et al.* 1982, Nakamura & Slots 1982, Spratt *et al.* 1994,1995,
Arylaminopeptidase	Suido *et al.* 1986
Acid phosphatase	Nakamura & Slots 1982, Poirier & Holt 1983, Spratt *et al.* 1994
Alkaline phosphatase	Nakamura & Slots 1982, Poirier & Holt 1983, Spratt *et al.* 1994
Collagenase	Söderling *et al.* 1991
Trypsin-like protease	Slots 1981, Laughon *et al.* 1982, Uitto *et al.* 1988, Shibata *et al.* 1992, Grenier & Turgeon 1994, Spratt *et al.* 1995, Gazi *et al.* 1997
α-glucosidase	Slots 1981, Laughon *et al.* 1982
β-glucosidase	Slots 1981, Laughon *et al.* 1982
Chymotrypsin-like protease	Shibata *et al.* 1992, Grenier & Turgeon 1994, Gazi *et al.* 1997
Phospholipase A$_2$	Sandholm *et al.* 1988
Dextranase	Igarashi *et al.* 1998
Elastase	Uitto *et al.* 1988, Grenier & Turgeon 1994, Gazi *et al.* 1997
Superoxide dismutase	Gregory *et al.* 1978
N-acetyl-β-glucosaminidase	Slots 1981, Laughon *et al.* 1982.
Sialidase	Moncla *et al.* 1990
Immunoglobulin G protease	Kilian *et al.* 1981,Gregory *et al.* 1992, Jansen *et al.* 1994
Immunoglobulin A1 protease	Kilian *et al.* 1981, Frandsen *et al.* 1991,

Trypsin-like protease activity by *Capnocytophaga* spp. (Slots 1981, Laughon *et al.* 1982, Uitto *et al.* 1988, Shibata *et al.* 1992, Grenier & Turgeon 1994, Spratt *et al.* 1995, Gazi *et al.* 1997) may be particularly destructive in the periodontal pocket as trypsin can:

i) disrupt cell-cell or cell-substratum adhesion (Britch & Allen 1980)

ii) activate latent collagenase (Birkedal-Hansen *et al.* 1975)

iii) activate the alternative complement pathway (Ward 1973).

Capnocytophaga spp. also produce chymotrypsin (Shibata *et al.* 1992, Grenier & Turgeon 1994, Gazi *et al.* 1997). Bacterial chymotrypsin is known to degrade Type IV collagen, laminin and fibronectin (Grenier *et al.* 1990), all of which are basement membrane components. Thus the presence of these enzymes could encourage periodontal tissue destruction.

Furthermore, *Capnocytophaga* spp. possess significant acid- and alkaline-phosphatases which are located both within the cell (periplasmic space) and on its surface (Nakamura & Slots 1982, Poirer & Holt 1983, Spratt *et al.* 1994). These enzymes catalyse the hydrolysis of esterified phosphoric acid, releasing inorganic phosphates. In addition they could dephosphorylate phosphoproteins, the phosphate groups of which are essential for the structural maintenance of mineralised and connective tissue (Poirier & Holt 1983). Several bacterial substances have been shown to diffuse through the connective tissue of the periodontium (Schwartz *et al.* 1972). Whilst it is not known how much these enzymes penetrate these tissues, it is possible that *in vivo* they could partly contribute to destruction of periodontal tissues, as *Capnocytophaga* spp. have been shown to cause widespread periodontal destruction in gnotobiotic rats (Irving *et al.* 1978).

Capnocytophaga spp. produce α- and β-glucosidase (Nakamura & Slots 1982, Spratt *et al.* 1994). These saccharolytic enzymes, and the proteolytic enzymes referred to above, can generate nutrients for these species. A quantitative and qualitative (specific enzyme activity) increase in hydrolytic enzymes produced by *C. gingivalis* was observed when this organism was grown in chemostat culture containing increased glucose concentrations (Spratt *et al.* 1996), which led the authors to hypothesise that glucose could maximise the pathogenic potential of this organism. Hydrolytic enzyme production was upregulated when these species were grown in a protein-rich environment (Spratt *et al.* 1999) as would be the case subgingivally. Additional

bacterial nutrients may also be obtained through the production of dextranases, which supply oligosaccharides for utilisation by bacteria in dental plaque (Igarashi *et al.* 1998).

C. ochracea produces phospholipase A_2 (Sandholm *et al.* 1988), an enzyme which perturbs cell membrane stability by degrading structural phospholipids in cell membranes e.g. of mast cells, PMNLs, with inflammatory consequences. *C. sputigena* produces a fibroblast inhibitory factor (Stevens *et al.* 1980, Letzelter *et al.* 1998) which could interfere with healing.

C. ochracea and *C. sputigena* exhibit sialidase activity (Moncla *et al.* 1990). Bacterial sialidases have been considered as virulence factors in many pathogenic species that colonise mucosal surfaces (Moncla *et al.* 1990). They may cause tissue damage by indirectly activating the alternative complement pathway (Pangburn *et al.* 1978).

Lipopolysaccharide (LPS) from *Capnocytophaga* spp. possesses moderate (Poirier *et al.* 1983) to strong (Iino & Hopps 1984, Kim *et al.* 1994) endotoxic activity. These differences could be due either to different LPS extraction methods or to actual differences between species / strains. Although in the *in vivo* situation LPS is a structural component of the Gram-negative cell wall, it is released as a function of bacterial growth / lysis and can penetrate intact gingival epithelium (Schwartz *et al.* 1972). LPS extracted from *Capnocytophaga* spp. has a pleiotropic effect on various immune cells resulting in the production of a wide range of cytokines e.g. TNF-α, IL-1β, IL-8 (Kim *et al.* 1994, Lindemann *et al.* 1995, Yoshimura *et al.* 1997), IL-2 (Lindemann *et al.* 1995) and IL-6 (Lindemann *et al.* 1996, Frieling *et al.* 1997). These cytokines may act either singly or in concert to accentuate periodontal tissue destruction. Another pathway through which LPS from *Capnocytophaga* spp. may modulate the local immune network is through autoantibody production (Hara *et al.* 1996, Kaneko *et al.* 1999).

1.4.3.3 *Capnocytophaga* spp. and evasion of the host response

Another aspect of bacterial virulence is the ability of bacteria to evade or perturb the protective host response. *Capnocytophaga* spp. are among very few oral isolates capable of degrading immunoglobulins (Ig) A and G (Frandsen *et al.* 1991, 1997, Gregory *et al.* 1992, Jansen *et al.* 1994). Such degradation not only provides the bacterial consortium with a nutrient source but it may also debilitate the local immune response.

In vitro and *in vivo* studies have shown that these organisms are not only weakly chemotactic to PMNLs (Lindhe & Socransky 1979, Wennström *et al.* 1980) but may inhibit PMNL migration altogether (van Dyke *et al.* 1982). In addition, they are also capable of altering PMNL function (Shurin *et al.* 1979) and exhibit a poor capacity to induce vascular exudation (Lindhe & Socransky 1979). Since PMNL are the first line of cellular defence in the periodontium, such perturbation undermines the host's defence against bacterial infection of the periodontium.

Capnocytophaga spp. produce an exopolysaccharide, which not only suppresses the immune response (Bolton *et al.* 1983, Ochiai *et al.* 1998) but can also induce polyclonal B-cell activation (Bick *et al.* 1981, Kim *et al.* 1994). *Capnocytophaga* spp. are also capable of counteracting the host response by producing bacterial proteases capable of digesting host protease inhibitors (Grenier 1996, Frandsen *et al.* 1997).

1.4.4 *Capnocytophaga* spp. as putative periodontal pathogens

None of the oral bacteria associated with periodontitis can be designated as strict pathogens as none fulfil Koch's postulates (Newman 1990). All of the putative periodontal pathogens, including *Capnocytophaga* spp., can be found in various stages of periodontal health and disease (Dzink *et al.* 1985, 1988, Rawlinson *et al.* 1993). However in recent years, the association of a putative pathogen with a disease has been adapted to the periodontal context to include criteria of bacterial association, elimination, animal pathogenicity, virulence factors and modulation of host response. The organisms which to date have been implicated as putative periodontal pathogens e.g. *A. actinomycetemcomitans* and *Por. gingivalis* have fulfilled these criteria. Based on these same criteria, an increasing body of evidence has accumulated which similarly implicates *Capnocytophaga* spp. as putative periodontal pathogens (Table 1.11).

Table 1.11 Evidence implicating *Capnocytophaga* spp. as putative periodontal pathogens

Criterion	Findings
Association	Found on crevicular epithelia Detected at apical plaque border Detected / elevated in various types of periodontitis
Host response	Host unable to mount an adequate immunoglobulin response Ability of species to suppress and degrade immunoglobulins
Virulence factors	Various hydrolytic enzymes capable of degrading periodontal tissue components Adverse affect on PMNLs Can induce bone resorption
Animal studies	Induce periodontitis in gnotobiotic rats.

Studies investigating the association of *Capnocytophaga* spp. with the different types of chronic inflammatory periodontal conditions revealed that these species could be recovered from a wide range of such conditions (Table 1.12).

Table 1.12 *Capnocytophaga* spp. and chronic inflammatory periodontal conditions

Periodontal Diagnosis	Reference
Adult Periodontitis	Rawlinson *et al.* 1993, Könönen *et al.* 1994, Petsios *et al.* 1995, Nonnemacher *et al.* 2001
Pubertal gingivitis	Gusberti *et al.* 1983, Mombelli *et al.* 1990
Rapidly progressive periodontitis	Kamma *et al.* 1995
Juvenile periodontitis	Page *et al.* 1985, Kim *et al.* 1992
Prepubertal periodontitis	Sweeney *et al.* 1987, Watanabe *et al.* 1990, Delaney *et al.* 1997, Kamma *et al.* 1998
Refractory periodontitis	Listgarten *et al.* 1993
Peri-implantitis	Augthun *et al.* 1997, Fardal *et al.* 1999
Pericoronitis	Leung *et al.* 1993

In some of these conditions there prevails an underlying immune defect e.g. decreased PMNL chemotaxis, which may potentially lead to *Capnocytophaga* spp. acting as opportunistic periopathogens. Such immune defects may be locally (i.e. periodontally)

induced (e.g. due to the organisms' ability to undermine the host response) or of a systemic nature. In the latter instance, the opportunistic nature of *Capnocytophaga* spp. may be supported by their increased prevalence in a range of systemic diseases which are characterised by defects in PMNLs or lymphocytes and which manifest severe periodontal destruction (Table 1.13).

Table 1.13 *Capnocytophaga* spp. associated with systemic diseases that manifest severe periodontal destruction

Systemic condition	Reference
Papillon-Lefèvre Syndrome	Tinanoff *et al.* 1986, Robertson *et al.* 2001
Down's syndrome	Barr-Agholme *et al.* 1992, Amano *et al.* 2000
Hypophosphatasia	Baab *et al.* 1986,
Neutropenia	Kamma *et al.* 1998
HIV-seropositive	Okuda *et al.* 1994
Diabetes Mellitus	Mashimo *et al.* 1983a

A number of case-reports have documented the recovery of *Capnocytophaga* spp. from infections at sites distant from the oral cavity (Table.1.14). Only some of these cases provided conclusive evidence that oral ulceration or severe periodontal disease was concurrently present at the time of systemic infection. However, since the oral cavity is the only site where *Capnocytophaga* spp. are found, it is most probable that this could have been the original site for systemic seeding of bacteria.

Table 1.14 Systemic infections caused by *Capnocytophaga* spp.

Infection	Oral disease	Name of species	Reference
Bacteraemia	Yes	Species not identified[§]	Montejo Baranda *et al.* 1984
Bacteraemia	Yes	*C. ochracea, C. sputigena*	Martino *et al.* 2001
Bacteraemia - NIC*	Yes	Species not identified	Parenti & Snydman 1985
Bacteraemia - IC**	Yes	Species not identified	Parenti & Snydman 1985
Peritonitis	NA[†]	*C. sputigena*	Testilliano Tarrero *et al.* 1988, Esteban *et al.* 1995
Peritonitis	NA	*C. ochracea*	Mortensen *et al.* 1985
Endophthalmitis	NA	*C. ochracea*	Rubsamen *et al.* 1993
Keratitis	NA	Species not identified	Alexandrakis *et al.* 2000
Liver abscess	NA	Species not identified	Weber *et al.* 1997
Septic arthritis	No[‡]	*C. ochracea*	Winn *et al.* 1984
Empyema	Yes	Species not identified	Parenti & Snydman 1985
Lung abscess	No	Species not identified	Parenti & Snydman 1985
Sinusitis	No	Species not identified	Parenti & Snydman 1985
Conjunctivitis	No	Species not identified	Parenti & Snydman 1985
Subphrenic abscess	No	Species not identified	Parenti & Snydman 1985
Wound abscess	No	Species not identified	Parenti & Snydman 1985
Osteomyelitis	No	Species not identified	Parenti & Snydman 1985
Amniotic fluid infection	NA	Species not identified	McDonald & Gordon 1988
Chorioamnionitis	NA	Species not identified	Mercer 1985
Endocarditis	NA	Species not identified	Montejo Baranda *et al.* 1984
Endocarditis	Dental treatment	*C. ochracea*	Buu-Hoi *et al.* 1988,
Endocarditis	Dog bite	*C. canimorsus*	Ngaage *et al.* 1999
Lumbosacral abscess	Possibly oral source	*C. granulosa*	Ebinger *et al.* 2000
Portal-systemic shunt	Dental treatment	Species not identified	Mello *et al.* 1990
Septicaemia	Yes	Species not identified	Warren & Allen 1986

Table 1.14 Systemic infections caused by *Capnocytophaga* spp. (cont'd)

Infection	Oral disease	Name of species	Reference
Septicaemia	Cat scratch	*C. canimorsus*	Mahrer & Raik 1992
Septicaemia	Periodontal abscess	Species not identified	Alberio & Lammle 1998
Septicaemia	Dog bite	*C. canimorsus*	Hantson *et al.* 1991, Cheng *et al.* 1999
Acute renal failure	NA	*C. canimorsus*	Bateman *et al.* 1992

*NIC   = Non immunocompromised

**IC   = Immunocompromised

†NA   = Not available (i.e. information re presence / absence of oral disease)

‡NO   = Oral disease not present

§Species not identified   = Individual species within genus *Capnocytophaga* were not identified

Thus, *Capnocytophaga* spp., which reside in the human oral cavity, are known to cause severe systemic infections. In addition, the various virulence factors, which they possess, could implicate them as putative periodontal pathogens, especially in conditions where the host's response is jeopardised as in DM.

1.5 *Capnocytophaga* spp. & DM-periodontitis

In spite of the *in vitro* and *in vivo* evidence supporting the pathogenicity of *Capnocytophaga* spp., and the multitude of studies investigating the microbiology of DM-periodontitis, relatively few studies have included this genus amongst the putative periodontal pathogens in DM-periodontitis (Mashimo *et al.* 1983a, Zambon *et al.* 1988, Sastrowijoto *et al.* 1989, Mandell *et al.* 1992, Sbordone *et al.* 1995, 1998, Thorstensson *et al.* 1995). The data emanating from these studies are conflicting. Direct comparisons between the studies are precluded primarily due to the different clinical parameters (Table 1.15) and laboratory procedures (Table 1.16) employed.

1.5.1 Clinical parameters (Table 1.15)

Subject categorisation: The clinical classification of DM based on pharmacological treatment (rather than according to disease aetiology) led to patient categorisation based on insulin requirements i.e. IDDM or NIDDM. This proved to be an inappropriate method of classification as the IDDM group could have included subjects who were originally NIDDM, but whose poor glycaemic control eventually warranted insulin therapy. Although the histopathology of the two disease categories is the same, the complications in Type 1 DM (previously IDDM) may be more severe due to its early onset, and hence increased duration, compared with Type 2 DM (previously NIDDM). It is rather unfortunate that all the subjects in the studies pertaining to *Capnocytophaga* spp. in DM-Periodontitis are classified as either IDDM or NIDDM. In addition, whilst some studies did not include control subjects (Mashimo *et al.* 1983a, Zambon *et al.* 1988, Sastrowijoto *et al.* 1989, Mandell *et al.* 1992), the ones that did (Sbordone *et al.* 1995, 1998, Thorstensson *et al.* 1995) presumed them to be non-diabetics without documentation.

Table 1.15 *Capnocytophaga* spp. & DM-periodontitis studies (Clinical Data)

Reference	Subjects	DM Control (HbA1c)	Mean Age (yrs)	No of sites	AL (mean, mm)	PPD (mean, mm)	Data analysis
Mashimo *et al.* 1983	9 IDDM	NA	17.4	3 - 8 D sites / patient (n = 38)	NA	7.05	% Microbial counts
Zambon *et al.* 1988	8 NIDDM	OGTT	NA	2 D sites / patient	NA	$\geq$ 5	Coverage analysis of Good
Sastrowijoto *et al.* 1989	22 IDDM	Good (7.7%,n = 12)	37.2	1H & 1D site / patient	NA	2.8	*Mann-Whitney U* test
		Poor (9.9%,n = 10	31. 8	1H & 1D site / patient	NA	3	
Sastrowijoto *et al.* 1990	6 IDDM	Poor (12.9%)	32.3	1H & ID site / patient	NA	H = 1.8 D = 5.5	Friedman 2-way ANOVA
Mandell *et al.* 1992	15 IDDM	Poor (10.4%)	34.6	1H & 1D site / patient	H = 0.6 D = 2.8	H = 2.8 D = 5.8	Mean data, *Wilcoxon* signed rank test
Sbordone *et al.* 1995	16 IDDM 16 nonDM sibs	Moderate (8.76%)	Non-DM: 13.2 IDDM: 11.3	1H & 1D site / patient	H = 2.19* DM = 1.8*	H = 2.2* DM= 2.29*	Mean data, *Wilcoxon* signed rank test
Thorstensson *et al.* 1995	28 IDDM 34 NonDM	NA	IDDM: 40-59 Non-DM: 40-59	4 pooled samples /patient	NA	$\geq$4 - $\geq$6	*Student t*-test; χ^2 test; *Mann-Whitney U* test
Sbordone *et al.* 1998	16 IDDM 16 nonDM sibs	Moderate (value NA)	NA	1H & 1D site / patient	H = 2.2* DM = 2.3*	H = 2.2* DM = 2.3*	Mean data for DM /nonDM groups; % of total anaerobe counts

NA: not available; H: Healthy; D: diseased; IDDM: Insulin Dependent Diabetes Mellitus; NIDDM: Non Insulin Dependent Diabetes Mellitus; non-DM: not suffering from DM; OGTT: oral glucose tolerance test; * mean data refer to non-DM and IDDM subjects rather than sites.

Some studies (Sbordone *et al.* 1995, 1998) included non-DM siblings as controls. In these cases, all diabetic subjects were matched as to periodontal sites with their non-diabetic siblings in terms of age and sex. This was done in order to reduce possible environmental and familial factors. However, since the test and control subjects were of similar genetic profiles, they were most probably equally susceptible to developing periodontitis and / or DM. Thus the negative findings in these studies are not surprising.

The majority of the studies were based on a small number of subjects, and indeed only 6 patients in one study (Sastrowijoto *et al.* 1990). It is a basic principle in statistics that the larger the sample size, the more representative this would be of the population being investigated. The areas of periodontology and microbiology manifest large inherent variations, which seem to be additive when undertaking studies of periodontal microbiology. Thus, it would seem more important in such studies to recruit as large a sample population as possible, keeping within the remit of cost-effectiveness and practicality of each study. As a rule, a minimum number of 20 subjects are required for basic statistical analysis (Gilthorpe MS, *pers. comm.*). This number is derived on the basis of comparing a single factor across two groups, which in the above studies, was *Capnocytophaga* spp. in DM and non-DM subjects. The minimum number of subjects required is generated from a 2 x 2 contingency table; the sample number arises from the smallest "expected" number in each cell, which should be >5, hence the total expected should be > 20 (as there are four cells in the table). If differences between groups are being compared e.g. using *t*-tests, then even more observations are required, since it is not feasible to accurately assess the distribution properties of so few observations, i.e. 10 per group. Thus, a minimum of 20 observations for very basic non-parametric tests, and at least 20 observations per group for parametric tests are required. In the above studies only Sastrowijoto *et al.* 1989 and Thorstensson *et al.* 1995 had more than 20 subjects; all the other studies (Mashimo *et al.* 1983a, Zambon *et al.* 1988, Sastrowijoto *et al.* 1989, 1990, Mandell *et al.* 1992, Sbordone *et al.* 1995, 1998) collected samples from less than 20 subjects (Table 1.15).

HbA$_{1c}$: The level of glucose control and the duration of diabetes are of crucial importance in DM studies as they have a direct effect on the ensuing periodontal complications (Glavind *et al.* 1968, Emrich *et al.* 1991).

The level of glucose control in the studies shown in Table 1.15 varies from good to moderate to poor. Thus even fewer studies within each group were available for comparison. Surprisingly three of the studies (Mashimo et al. 1983a, Thorstensson et al. 1996, Sbordone et al. 1998) did not reveal the level of glycated haemoglobin. Zambon et al. (1988) utilised the Oral Glucose Tolerance Test (refer to Appendix 1), i.e. a test which determines the level of blood glucose on the day of testing and is subject to previous food intake by the patient. It is, therefore, unreliable as a test for monitoring the control of DM.

The HbA$_{1c}$ (the result being quoted as a percentage) is the most accurate method for determining the control of blood glucose during the preceding 8 - 12 weeks. There are a number of ways in which glycated haemoglobin can be measured: methods of separating the haemoglobin molecules are based on differences in their electronic charge, molecular structure or immunological reactivity (Cooper et al. 2000). Unfortunately, the different methods measure slightly different things and there is no standard method, and therefore no standard reference range. This lack of standardisation means that the HbA$_{1c}$ measurements made by different laboratories cannot be easily compared, unless the laboratory performing the test provides the reference range. No such information was revealed in the studies quoted above. In addition it is important not to confuse the target range with the normal range. A target range may indicate acceptable values for a diabetic person and this may differ slightly from the normal range seen in the non DM population.

Only three studies (Mandell et al. 1992, Sastrowijoto et al. 1989, Thorstensson et al. 1995) revealed information regarding the duration of DM for their subjects. The mean DM duration in the Mandell et al. (1992) study was 20.6 years; in the Sastrowijoto et al. 1989 study 13.2 years, whilst in the Thorstensson et al. (1995) the mean DM duration was 21 years in the 40 - 49 age category and 25.6 years in the 50 - 59 year age group.

Age: The mean age of the patients in these studies varies extensively from a minimum of 9 years (Sbordone et al. 1995) to a maximum of 60 years (Sastrowijoto et al. 1989). Since the cumulative effects of periodontal destruction are age-dependent, the broad age categories might have obscured any potential differences due to DM. In addition, the mean age of the subjects in some of the studies (Mashimo et al. 1983a, Zambon et al. 1988, Sastrowijoto et al. 1990, Mandell et al. 1992, Sbordone et al. 1995) and the poorly controlled diabetic subjects in the Sastrowijoto et al. (1989) study were all below 35 years, i.e. the age limit which was then used to classify subjects as manifesting

either adult onset periodontitis (if > 35 years of age) or early onset periodontitis (if < 35 years of age). Thus it is not clear whether the clinical manifestation of periodontitis was because of the DM or in spite of it. The Mashimo *et al.* (1983a) study included subjects whose age ranged between 13 - 27 years. The authors described their patients as suffering from "bone loss which was severe and often localised to the first molars and incisors", and which therefore might be suggestive of juvenile periodontitis. However, this is not made clear in the paper. Their conclusion is that "*Capnocytophaga* spp. were the most numerous isolates in periodontitis lesions" from IDDM-periodontitis subjects. Only Thorstensson *et al.* (1995) recruited subjects within the "adult" age range for their test and control groups; therefore this precludes comparisons with the other studies listed in Table 1.15 and makes it the only study from which some inferences regarding the potential association of *Capnocytophaga* spp. and DM-Periodontitis may be drawn.

Site Data: The majority of studies were cross-sectional (Zambon *et al.* 1988, Sastrowijoto *et al.* 1989, Mandell *et al.* 1992, Sbordone *et al.* 1995, Thorstensson *et al.* 1995) with only 1 or 2 sites per subject sampled for microbiological investigation. Site selection was based on probing depth rather than attachment loss. In fact, in some studies no details of attachment loss were given (Mashimo *et al.* 1983a, Zambon *et al.* 1988, Sastrowijoto *et al.* 1989, 1990, Thorstensson *et al.* 1995). None of the papers revealed details of the general periodontal status, thus it is not known whether periodontal breakdown was generalised or localised or indeed whether true loss of attachment was manifest. Whilst it is difficult to determine whether disease was active or inactive on a site basis, the lack of data pertaining to loss of attachment prevented patient categorisation based on disease severity. Three papers (Mashimo *et al.* 1983a, Zambon *et al.* 1988, Thorstensson *et al.* 1995), report radiographic evidence of bone loss, though again these papers do not indicate the extent or severity of alveolar bone resorption.

Statistical Analysis: Data analysis of clinical variables and *Capnocytophaga* spp. associated with healthy or diseased sites were analysed by widely varying statistical methods. The data sets were subjected to parametric (e.g. *t*-tests, ANOVA) or non - parametric tests (e.g. *Mann Whitney-U* test, *Wilcoxon* signed rank test). The counts of *Capnocytophaga* species were reported as either mean data or percentage microbial counts. Most data analyses were performed using site data rather than subjects as the

unit of analysis. This important statistical issue in periodontal research is discussed in detail in section 1.6.

1.5.2 Laboratory parameters (Table 1.16)

Sampling methods: Inconsistencies between the studies are also evident with respect to the methods used to collect plaque samples. In some studies, subgingival plaque was collected with paper points (Mashimo *et al.* 1983a, Zambon *et al.* 1988, Thorstensson *et al.* 1995) whilst in others curettes (Sbordone *et al.* 1995, 1998) or broaches were used (Sastrowijoto *et al.* 1989; Table 1.16). Of the three methods of sampling, curettes seemed to remove the largest bulk of subgingival plaque (Kiel & Lang 1983, Moore *et al.* 1985). Kiel & Lang (1983) found that curette samples yielded 10 - 1000 fold higher colony forming units than paper point samples. They estimated that scalers removed 61 - 91% of the pocket microflora, while paper points removed only 7 - 41%. Paper points would tend to collect more of the bacteria suspended in the gingival crevicular fluid rather than the bacteria forming part of the plaque biofilm on the root surface (Tanner & Goodson 1986). This could potentially explain qualitative and quantitative differences in bacterial counts between studies.

The studies also used different transport media for the plaque samples namely Ringer's solution (Mashimo *et al.* 1983a, Mandell *et al.* 1992, Sbordone *et al.* 1995, 1998), BM broth (Sastrowijoto *et al.* 1989, 1990) and VGMA III (Thorstensson *et al.* 1995). Very limited information is available as to which is the best transport medium.

Table 1.16 *Capnocytophaga* spp. & DM-Periodontitis studies (Microbiological Data)

Reference	Plaque Samples	Culture Media	ID tests	Microflora
Mashimo *et al.* 1983	Subgingival plaque collected with paper points; Ringer's; vortexed x 20 sec	TSBA	Colony morphology & biochemical tests	*Capnocytophaga* spp. = 24% of microflora
Zambon *et al.* 1988	Subgingival plaque collected with paper points; Ringer's; vortexed x 60 sec	ETSA	Colony morphology, Gram reaction & biochemical tests.	43.8% sites positive for *Capnocytophaga* spp.
Sastrowijoto *et al.* 1989	Subgingival plaque collected with cotton wool on broach; BM broth; vortexed x 10 sec	Mashimo & Cap; BA for total counts	Colony morphology & API ZYM	Low levels of *Capnocytophaga* spp. in H & D sites
Sastrowijoto *et al.* 1990	Subgingival plaque collected with cotton wool on broach; BM broth; vortexed x 10 sec; homogenised x 3 times	TSBV, Mashimo, Cap, BA for total counts	Colony morphology & API ZYM	Low levels of *Capnocytophaga* spp. in H & D sites
Mandell *et al.* 1992	Subgingival plaque collected with scaler; Ringer's solution; sonicated x 5 sec	Mashimo for *Capno.* spp.; TSBA for total counts	Based on growth on selective media.	*Capnocytophaga* spp not significantly different between H & D
Sbordone *et al.* 1995	Subgingival plaque collected with curette; Ringer's solution; vortexed x 10 sec; sonicated x 20 sec	TSA for total counts; TSBA for *Capno.* spp	Colony morphology, Gram reaction & biochemical tests.	*Capnocytophaga* spp.similar in both groups
Thorstenssson *et al.* 1995	Subgingival plaque from all teeth pooled; few kept separate; paper points; VGMA III and mailed to lab	BBA	Colony morphology & Gram reaction	*Capnocytophaga* spp. not different between groups
Sbordone *et al.* 1998	Subgingival plaque collected with curet; Ringer's; vortexed x 10 sec; sonicated x 20 sec.	TSBA	Colony morphology, Gram reaction & biochemical tests	*Capnocytophaga* spp. not a risk factor for periodontal breakdown

ETSA = Enriched Trypticase Soy Agar; TSBA = Trypticase Soy Agar + 5% blood; BA = Blood Agar; BBA = Brucella Blood Agar; BM = prereduced anaerobic transport medium; Mashimo & Cap = selective media for *Capnocytophaga* spp.

H = Healthy; D = Diseased; DM = Diabetes Mellitus.

All plaque samples were dispersed by vigorous agitation (vortexed) for varying time periods. The samples in the Mandell *et al.* 1992, Sbordone *et al.* 1989 and 1990 were also sonicated. Whilst both are established methods for dispersing plaque, sonication may selectively disrupt the cell membranes of certain bacteria (Robrish *et al.* 1976, Moore *et al.* 1985). The plaque samples in the Sastrowijoto *et al.* (1990) study were vortexed and homogenised with glass beads, which could have ruptured bacterial cell membranes and hence affected the outcome of the cultured bacteria. An interesting point to note is that the subgingival plaque samples in the Thorstensson *et al.* (1995) study were mailed to the microbiology laboratory and processed the next day. Even though the samples were contained in a prereduced anaerobic transport medium (VGMA III), the results of this study must be interpreted with caution, as a 24-hour delay in processing the samples may have jeopardised the growth of anaerobic bacteria. Furthermore the plaque samples from different sites were pooled. Pooling of plaque samples before analysis could lead to dilution to undetectable levels of pathogenic organisms characteristic of individual sites.

Culture Media: The culture medium used for growing the bacteria in a plaque sample is perhaps the most crucial factor in determining the recovery of the component organisms.

The studies in question used a diverse range of culture media some of which were selective for *Capnocytophaga* spp. (Sastrowijoto *et al.* 1989, Mandell *et al.* 1992), while others were not (Mashimo *et al.* 1983a, Zambon *et al.* 1988). Differences in the nutritional composition of the culture media (refer to Chapter 3, Table 3.2), some of which contained antibiotics (Mashimo, Cap and TSBV) could have altered the recovery of *Capnocytophaga* spp. (For a detailed review on the recovery of *Capnocytophaga* spp. on the culture media most often used in studies, see Chapter 3 of this thesis).

Species identification: The identification methods used in all studies were rather crude, employing colony morphology and biochemical tests as the means for identification to genus level. These methods are only capable of identifying the isolates to genus level (Kristiansen *et al.* 1984, Speck *et al.* 1987). Identification to species level would have been more informative as different *Capnocytophaga* spp. have been shown to manifest different pathogenic potential (Irving *et al.* 1978). (For a more detailed review on identification of *Capnocytophaga* spp., see Chapter 4 of this thesis).

In conclusion therefore, although the pathogenic role of *Capnocytophaga* spp. is supported by several studies (Tables 1.11 - 1.14), concrete evidence for the role of *Capnocytophaga* spp. in DM-periodontitis is lacking. Besides the factors described above, another important determinant which could have had a major impact and therefore obscured any potential association between *Capnocytophaga* spp. and DM-Periodontitis in studies performed to date, are the statistical methods employed when analysing such data.

1.6 Statistical Issues in Periodontal Research

1.6.1 Introduction

The significance and relevance of research is based not only on the quality of research itself, but equally so on the method of data analysis used to derive and then interpret the results obtained. When performing research it would be ideal to have all the conditions constant other than the one being investigated. Whilst this might be possible in laboratory experiments, this is far from practical when performing many clinical investigations. Such studies involve biological systems, which are inherently variable. Although patient inclusion and exclusion criteria aim to recruit a cognate sample population, the random inherent variation present between different subjects must be accounted for during subsequent data analysis if the correct data interpretation is to be obtained.

1.6.2 Historical aspects of periodontal disease progression: site data

Initial studies on the natural history of CIPDs gave the impression that the condition progressed slowly and relentlessly and that tooth exfoliation was the inevitable outcome (Löe *et al.* 1978 a, b, c). These conclusions were based on mean (i.e. pooled) values of longitudinal site data. The results of subsequent studies, which monitored disease progression using independent site data, showed that the extent and severity of disease varied between sites in the same individual (Goodson *et al.* 1982, Lindhe *et al.* 1983, Socransky *et al.* 1984). In addition, new concepts of CIPD progression emerged which demonstrated the random and episodic nature of periodontal breakdown (Socransky *et al.* 1984). In fact it is the number, size and location of bursts, which account for the pattern of periodontal destruction observed. These studies also showed that acute bursts of activity were limited to a few "active" (as compared to the non progressing or "inactive") sites, thus confirming that the different forms of chronic periodontitis were site-specific diseases (Socransky *et al.* 1984, Haffajee & Socransky

1986). Thus, this fundamental biological maxim of differences between sites must be taken into consideration when analysing data emanating from clinical studies of destructive periodontitis.

1.6.3 Multifactorial nature of CIPDs: subject data

As discussed earlier, CIPDs are multifactorial in origin (Page 1998). Thus the many factors operating at the subject-level e.g. diabetes mellitus, smoking habits, genetic composition, may confer an increased risk for disease. Lack of such knowledge in earlier studies might have led to incongruent patient groups and could have also confounded data analysis.

Disease severity, e.g. pocket probing depth or loss of clinical attachment, recorded at the subject-level represents the clinical manifestation of biochemical processes occurring at the cellular (site) level. It is now well established that CIPDs are site-specific (Socransky & Haffajee 1997) and therefore a certain degree of variation may occur at the site-level. Such biological variation may also be expected between subjects. Thus, there exists a "biological concern about combining unlike sites together in a single descriptive number and a statistical concern about regarding individual sites as independent units" (Socransky & Haffajee 1997). These different strata i.e. sites nested within teeth, which are in turn nested within subjects, establish a hierarchy (Fig.1.17). Thus, in the periodontal context, the data collected are inevitably of a hierarchical nature, with variations occurring at the site, tooth and subject-level. Any subsequent data interpretation should take this hierarchy into account if any reliable results are to be obtained.

Fig.1.17 A 3-level multilevel model

Subjects	Level 3
Teeth	Level 2
Sites	Level 1
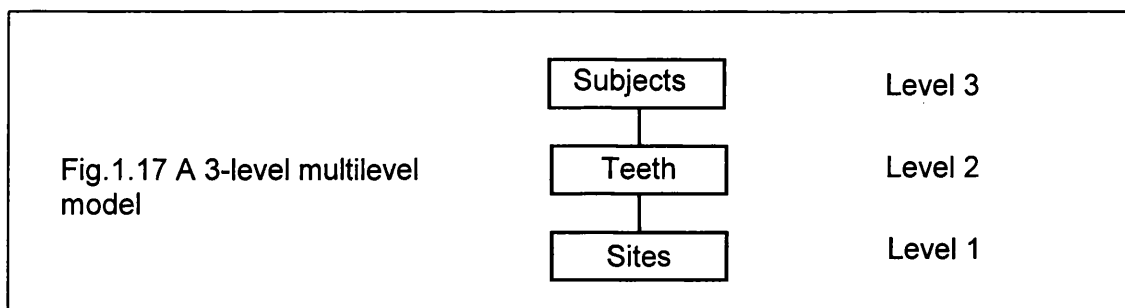
Previously, because it was impossible to perform complex computational analyses thus allowing exploration of such data structures statistically, this hierarchy was thought of as being a nuisance and therefore often ignored or worked around. Any such data

were erroneously interpreted by considering site data to be independent and therefore analysed using invalid single-level analyses e.g. t-tests (Wikström et al. 1993, Seppälä & Ainamo 1994, Noack et al. 2000, Yuan et al. 2001) or non-parametric Mann-Whitney tests (Salvi et al. 1997, Collin et al. 1998, Sbordone et al. 1998). Alternatively, the approach of aggregating the site data to the subject-level was more often used (Papapanou et al. 1993, Liljenberg et al. 1994, Salvi et al. 1997, Rocha et al. 2001). Whilst statistically valid, this led to loss of valuable information. In addition, there exists the added risk of higher-level associations (i.e. subject) being wrongly inferred to exist at the site-level, i.e. the ecological fallacy, the corollary to this being the atomistic fallacy (Jones et al. 1992).

1.6.4 Site versus Tooth versus Subject as the unit of analysis

Data analysis pertaining to the site or subject as the fundamental unit of analysis has been a long-established contentious issue in dental research in general, and in periodontology in particular. This probably stems from the fact that most clinicians are not mathematically minded, and therefore do not (through no fault of their own) understand the basic mathematical concepts implicit in evaluating the clinical data which they so meticulously collect. Most studies involve small data sets (samples drawn from a larger population). The conclusions drawn from such studies can only be of a descriptive nature. It is not unknown, however, for inferences about the population at large to be made from such data sets (Sastrowijoto et al. 1990, Tervonen et al. 1994). The sample used in a study comprises only a small subset of the population, thus the conclusions drawn about the population are subject to uncertainty, which is expressed as a probability of any finding occurring by chance alone (e.g. the P-value).

In Periodontology, measurements are often taken from several sites within the same mouth. Due to the common oral environment, the data will be correlated to some degree. Overlooking such inherent data correlations and hence ignoring the inherent hierarchical structure described above (Fig.1.17) is highly inappropriate (DeRouen et al. 1995). The problem may be further compounded by improper utilisation of statistical analysis based on data being independent (Fleiss et al. 1988, Emrich 1990). The studies which are most likely to have a long-lasting impact on the research community are those which aim to achieve statistical as well as clinical / biological excellence. Thus the characteristics of the data set must be taken into account during subsequent statistical analysis if pertinent conclusions are to be established from the study in question.

93

The debate regarding the basic computational unit (i.e. site or subject) in statistical analysis of periodontal data dates back almost 20 years (Haffajee *et al.* 1983a, Fidler 1984, Blomqvist 1985). In addition, it has been shown that ignoring the anatomical structure imposed by teeth can also lead to erroneous analysis if not taken into consideration within the natural hierarchy of the site-teeth-subjects (Gunsolley *et al.* 1994). The crucial factor favouring the site as the basic unit of analysis is the site-specific nature of periodontitis (Lindhe *et al.* 1983, Socransky *et al.* 1984, Haffajee & Socransky 1986). Examination of any periodontal patient reveals that not all sites within the same mouth are affected to the same degree. Indeed some sites may be healthy. It is widely agreed that the number of progressing (active) sites tends to be rather small compared to the number of non-progressing (inactive) sites (Haffajee *et al.* 1983b, Badersten *et al.* 1985, Lang *et al.* 1986). In addition, the microbial composition of subgingival plaque differs between sites in the same mouth and may also differ at that same site over time (Moore *et al.* 1984, Dzink *et al.* 1985, 1988). However in spite of all this evidence, some studies still pool samples from different sites and site data are averaged across sites and across subjects (Blomqvist 1985, Papapanou *et al.* 1993, Salvi *et al.* 1997).

1.6.5 Alternative statistical methods

It is evident from the foregoing that, in a given study the chosen analytical strategy will have profound implications on the results of that study. Thus, differences in outcome between studies may be accounted for not only by the clinical / biological methods employed but also by the method of analysis.

Some analytical methods have tried to address the issue of sites clustered within subjects. Indeed, one of these methods is analysis of variance (ANOVA). The principle underlying ANOVA is that the total variability in a data set is partitioned into its component parts. However in spite of its name, ANOVA is based on comparison of mean values for two or more independent groups of numerical observations where the (n) factors of interest are those that define the groups. In addition, one of the assumptions underlying ANOVA is that the observed variances are equal between groups, which might not always be the case in periodontal studies.

Another analytical method that takes into account the problem of clustered data is the generalised estimating equations (GEE) method (DeRouen *et al.* 1991). GEE employs the use of a broad range of regression models, taking into account and adjusting for

dependence between observations. The GEE method focuses on modelling the relationship between the response mean (e.g. disease progression) and various covariates (e.g. treatment), whereas the correlation between responses, within the same patient, is considered a nuisance (DeRouen *et al.* 1995). Thus, the GEE method focuses on data analysis at the lowest levels of the hierarchy and in fact perceives the hierarchy as a nuisance, rather than as a statistical model worth exploring (Gilthorpe *et al.* 2000c).

The importance of accounting for both site and subject factors when performing data analysis seems to have been first highlighted by Imrey (1986). Thus in an effort to address such issues, whilst respecting the clustered and hierarchical nature of periodontal data, Empirical Bayesian statistics (Gilthorpe *et al.* 2000a) and Multilevel Modelling (MLM) (Sterne *et al.* 1988, 1990, Albander & Goldstein 1992, Gilthorpe *et al.* 2000b,c) have been introduced into dental research (Refer to appendix 9 for a glossary of MLM terminology).

1.6.6 Multilevel Modelling

1.6.6.1 Concepts of MLM

MLM makes the assumption that differences between subjects are the consequence of random (or biological) variation, or effects that are systemically related to some measurable factor, or both. MLM accounts for the clustered effects of the data - i.e. multiple <u>sites</u> clustered within <u>teeth</u> within <u>subjects</u>. The advantages of MLM are that it is not necessary to aggregate information to the subject-level (which is the correct level of independence), whilst lower level information (sites and teeth) may be analysed separately, though simultaneously, thus accounting for the hierarchy whilst retaining maximum use of all the available data.

Consequently, MLM has implications on the statistical power of the analyses, since there are many more observations (N) at the lowest level of a hierarchical data structure (e.g. sites) than there are truly independent observations at the top of the hierarchy (e.g. subjects). For example, in this study with 50 subjects and six sites per subject, there are a total of 300 sites (one per tooth for six teeth, hence the tooth-level becomes synonymous with the site-level resulting in a 2-level hierarchy). If the site data were considered independently (hence N = 300), the standard error (SE) would be inversely proportional to the square-root of the number of observations, in this case $1/\sqrt{N}$ or $1/\sqrt{300} \approx 1/17.3$. If N were taken to be equal to 50 (i.e. as within the single-level aggregate approach), then the SE = 1/7.1. Thus, ignoring hierarchy would

erroneously yield smaller SEs (which is a measure of the precision of the estimate and it therefore gives an indication of how "good" the sample estimate is of the population value; the smaller the SE the more precise the estimate). Similarly, ignoring hierarchy would erroneously yield smaller Confidence Intervals (CI, which are approximately equal to ± 2SE) by around 40% in this example, leading to potential Type I errors (i.e. where a factor is incorrectly identified as influencing the outcome of interest). If the correct single-level approach were adopted (i.e. aggregation and thus N = 50), the SE would be valid but much larger. This would lead to the potential of Type II errors (i.e. where a factor is incorrectly observed to have no significant impact upon the outcome of interest). Using MLM, the number of "equivalent" independent observations lies somewhere between 50 and 300. If the upper-level variance (i.e. those at the subject-level) is small, this number will be closer to 300. If the lower level variance is small (i.e. there is little variation between sites within subjects) then this number of "equivalent" independent observations would lie closer to 50. Thus, by using MLM, although the number of truly independent units (subjects) remains only 50, the multilevel structure and the multilevel analysis have increased the statistical power over that of a subject-based analysis, due to the clustered nature of the collected data. For this reason, it is advantageous to exploit the use of MLM, especially where it becomes easier to take multiple sites per subject than to recruit more subjects, each contributing only one site, providing there is sufficient between-site variation within subjects. As previous studies using MLM in periodontal research have shown there to be greater variation, in general, between teeth than between sites within the same tooth (Gilthorpe *et al.* 2000c), it is for this reason that this study selected multiple sites per subject, but chose separate teeth for each site. The level of teeth thereby becomes redundant if only one site per tooth is selected. Nevertheless, this restriction only applied to this study; the general scenario is that illustrated in Fig 1.17.

1.6.6.2 Development of MLM

MLM (Goldstein 1995), also known as hierarchical linear modelling (HLM, Bryk & Raudenbush 1992), is the technique of analysing clustered or hierarchical data using generalised linear models (McCullagh & Nelder 1989). HLM was originally introduced by Lindley & Smith (1972), who elaborated a general framework for clustered data with complex error structures. However, due to the fact that their method could not deal with unbalanced data (i.e. unequal numbers within each cluster), and because at that time there was a lack of adequate computer software, their concepts could not find

96

practical application. The progressive development of statistical modelling (Dempster *et al.* 1981, Mason *et al.* 1983, Goldstein 1986, Longford 1987) and the recent advances in desktop computers have led to the development of a number of sophisticated statistical computing programmes which can extend hierarchical data analysis to the nth level, where n is often limited only by computer memory (Rashbash *et al.* 2000).

MLM is the modified version of statistical methods available for the analysis of single-level data structures (e.g. multiple regression, logistic regression). The standard regression equation for a straight line is given by:

$$y = \beta_0 + \beta_1 x$$

where y = dependent (response) variable

β_0 = intercept

β_1 = estimate for slope of the explanatory variable x (differential, dy / dx).

The simplest MLM for a 2-level hierarchy (the site-level denoted by i, and the subject-level denoted by j), and can be specified by the equation:

$$y_{ij} = \beta_0 + u_{0j} + e_{0ij}$$

where y_{ij} is the response variable, β_0 is the overall mean of all site values across all sites and subjects; u_{0j} is the residual difference between the overall mean (β_0) and the mean for subject j, and e_{0ij} is the additional residual difference for site i (within subject j) between the subject mean and the site value. Thus, the response y_{ij} is a function of the mean across all sites (β_{0j}) for each subject plus the residual difference between sites (e_{0ij}) within each subject.

When no explanatory variables are present, the dependent (response) variable y_{ij} is equal to β_0. This is known as the Null Model or Variance Components (VC) model, since MLM partitions the total variance across all modelled levels (e.g. the site- and subject-levels). The model can then be extended to include explanatory variables (covariates), i.e. factors which might explain the variation in the response variable (y_{ij}), leading to the *Random Intercept* model, given by the equation (for a single covariate, x_1):

$$y_{ij} = \beta_{0ij} + \beta_1 x_1$$

where $\beta_{0ij} = \beta_0 + e_{0ij} + u_{0j}$, with again β_0 = the overall mean intercept; e_{0ij} = the random variation across sites (within subjects); u_{0j} = the random variation across subjects, and β_1 = the estimated change in outcome (y_{ij}) per unit change in the covariate (x_1).

1.6.6.3 Multivariate Multilevel Modelling (MV MLM)

This brief review of MLM has, so far, described the model based on one outcome variable. However most experimental studies might want to investigate two or more outcome measures (i.e. multiple variables or *variates*, hence multi*variate*) (Cunningham 2000, Cunningham *et al.* 2000, Gilthorpe & Cunningham 2000). Prior to MLM, such investigations could be undertaken by using multiple regression and analysing each outcome independently. This has two disadvantages:

i) whilst this method might facilitate the interpretation of the results in terms of each outcome, not all the data would be used in the analysis, thus reducing the statistical power of the analytical process;

ii) an underlying assumption of multiple regression is that the data are independent. For the reasons discussed above, such an assumption is incompatible with the nature of data (e.g. dental data) which are inherently correlated and which constitute a hierarchy.

Multivariate multilevel analysis overcomes this by analysing the entire outcome measures simultaneously structured in a multilevel model. It is therefore:

i) more effective because it optimises statistical power,

ii) more efficient (statistically), because each independent outcome regression "borrows information" from the parallel processes of other simultaneous outcome regressions thus making use of similarities in how covariates operate across several outcomes.

1.6.6.4 MLM in dental research

Some studies have embraced the MLM concept and have highlighted the importance of addressing the data hierarchy. The first such study is apparently that by Sterne *et al.* (1988) which evaluated the results of a clinical trial investigating the use of metronidazole in CIPD. Thereafter, MLM has been increasingly used in periodontal research (Albander & Goldstein 1992, Kallestal and Uhlin 1992, Albander 1993, Gunsolley *et al.* 1994, Axtelius *et al.* 1999, Gilthorpe *et al.* 2000c, 2001). MLM has also been used in other areas of dentistry: orthodontics (Buschang *et al.* 1989, Hoeksma and van der Beek 1991, Cunningham 2000, Gilthorpe and Cunningham 2000), prosthodontics (Söderfeldt and Palmqvist 1998) and conservation (Albander *et al.* 1995a, Lewsey *et al.* 2001, Gilthorpe 2002).

Thus, considering the hierarchical nature of periodontal data, it may be considered logical that a statistical model which encompasses data hierarchy be used when analysing such data.

The advantages of MLM may be summarised as:

i)   improved estimation of coefficients (and therefore reduced Type I errors)

ii) increased statistical power (and therefore reduced Type II errors)

iii) the ability to focus on the units of interest

iv) the ability to focus on all levels of a complex system simultaneously

v)   greater insight into real biological systems

vi) the ability to focus on variation as an aspect of interest

vii) the emergence of several new statistical tools that have no single-level analogy

viii) the opportunity to develop multiple models from different perspectives and the ability to review and reanalyse old data sets.

1.6.6.5 Some issues regarding MLM

Like other techniques that claim generality and superiority over established methods, MLM raises some concerns about potential limitations relative to ordinary linear regression models.

MLM is a relatively novel concept in dentistry and therefore clinicians might be challenged by this established (in other fields) and as yet unfamiliar concept of mathematical modelling. Indeed extra effort is required to understand and build the models. MLM need only be applied when the data are hierarchical and indeed there may be situations when the full complexity of the model is not required (hence the important use of variance components models, Chapter 5, section 5.3.6). Like any other statistical model, the assumptions underlying MLM must be satisfied and statistically significant inferences, if any, must be interpreted in the light of clinical significance.

1.7 Conclusions from the literature

The periodontal microflora of DM-Periodontitis has not been well characterised. Some studies suggest that it is no different to that found in adult periodontitis and that the traditional putative periodontal pathogens implicated in the aetiology of adult periodontitis are the main culprits responsible for the extensive periodontal destruction characteristic of poorly-controlled DM subjects. Other periopathogens might contribute to the generalised destruction, typical of DM-Periodontitis. The literature indicates that

one such group of organisms might be *Capnocytophaga* spp. It has also been suggested that GCF from DM-Periodontitis patients might contain higher levels of glucose, which could support the growth of saccharolytic organisms such as *Capnocytophaga* spp.

The human isolates of *Capnocytophaga* spp. are found only in the oral cavity. Several *in vitro* studies have shown that *Capnocytophaga* spp. produce a range of virulence factors relevant to periodontal breakdown. *In vivo* studies have recovered these species not only from periodontally healthy sites, but also from a wide range of periodontitis lesions, and especially those conditions that manifest an underlying immune defect (periodontally and systemically). In addition, *Capnocytophaga* spp. are known to be capable of causing severe systemic infections. The lack of a rapid and accurate method for the identification of these organisms to species level has hampered the potential association of each species with the pathological condition from which they are recovered. In addition, from a therapeutic standpoint, limited information is available regarding the antimicrobial sensitivity of each of the *Capnocytophaga* species.

Only a handful of microbiological studies have indicated a potential association between *Capnocytophaga* spp. and DM-Periodontitis. Then again, none of these studies set out to exclusively investigate the potential association of *Capnocytophaga* spp. and DM-Periodontitis *per se*; rather these species were amongst several others, so that the conditions for their in depth study may not have been optimal. The results of such studies are conflicting and, in some cases, contradictory. These discrepancies could be due to:

i) a true lack of association between *Capnocytophaga* spp. and DM-Periodontitis

ii) inconsistencies in study design (as described in section 1.5)

iii) inappropriate statistical analysis.

There is much to be said about the statistical analysis and interpretation of data. This has been a cause of concern for both statisticians and periodontists alike. However, the larger bulk of periodontal data sets are still being analysed using single-level (parametric or non parametric) statistical methods. These methods are based on the assumption that the data are independent. Since periodontal data are inherently correlated and hierarchical in nature, such statistical tests are highly inappropriate for analysing periodontal data.

Thus, from the available evidence, there seems to be sufficient justification to investigate the potential association between *Capnocytophaga* spp. and DM-Periodontitis.

1.8 Aims of study

The aims of this investigation were to:

i) isolate and enumerate *Capnocytophaga* spp. from subgingival plaque collected from DM- and non-DM-Periodontitis human subjects

ii) identify all isolates to species level

iii) isolate these organisms from the patients' blood

iv) subject the isolates to antimicrobial sensitivity testing

v) quantify and compare GCF-glucose content between DM- and non-DM-Periodontitis subjects.

1.9 Null Hypotheses

The null hypotheses proposed for this study were:

i) there is no difference in the number of *Capnocytophaga* spp. (cfu ml^{-1}) in subgingival plaque between DM- and non-DM-periodontitis subjects

ii) there is no difference in the GCF-glucose concentration between DM- and non-DM-periodontitis subjects.

1.10 Schematic plan of study

This aim of this section is to give a brief outline of this multifaceted study. The principal aim of this investigation was to research the potential association of *Capnocytophaga* spp. and DM-Periodontitis on the basis of a clinical study, which compared the DM-Periodontitis and non-DM-Periodontitis subjects (Chapter 2). Initial experimental work identified a solid culture medium that best supported the growth of *Capnocytophaga* spp. (Chapter 3); the formulation of molecular methods for the identification of *Capnocytophaga* spp. (Chapter 4) was performed prior to commencement of the clinical study (Chapter 5). *Capnocytophaga* clinical isolates were subjected to antimicrobial sensitivity testing (Chapter 6). One of the study hypotheses proposed that the GCF-glucose concentration collected from DM-Periodontitis patients could be higher than non-DM-Periodontitis subjects thereby supporting the growth of saccharolytic micro-organisms such as *Capnocytophaga* spp. Development of a micro-

assay for quantification of GCF-glucose in healthy subjects as well as DM- and non-DM-Periodontitis subjects formed the final part of this investigation (Chapter 7).

1. Selection of DM- and non-DM-Periodontitis subjects　　　　(Chapter 2)

2. *Capnocytophaga* spp.
Identification of a culture medium　　　　(Chapter 3)

Strategy for the molecular identification of
Capnocytophaga spp.　　　　(Chapter 4)

Isolation & identification of *Capnocytophaga* spp.
from subginginval plaque & blood from DM- &
non-DM-Periodontitis subjects　　　　(Chapter 5)

Antimicrobial sensitivity testing of clinical isolates　　　　(Chapter 6)

Tooth surface

Periodontal
pocket

3. GCF-glucose
Quantification of GCF-glucose in DM- &
non-DM-Periodontitis subjects　　　　(Chapter 7)

Blood
vessel
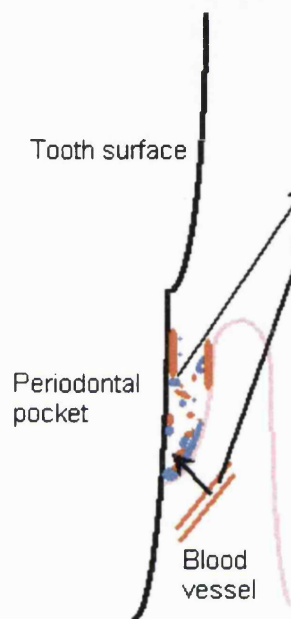
Fig.1.18 Schematic plan of study

Chapter 2

General materials & methods

2.1 Patient Recruitment

2.1.1 Ethical Approval

Prior to commencement of the study, ethical approval was obtained from the Joint Research and Ethics Committees (JREC) of the Eastman Dental Institute and Hospital, and the Joint University College London (UCL) / UCL Hospital (UCLH) Committees on the Ethics of Human Research, Middlesex Hospital, London (JREC Ref.: 99/E008; 26/4/99 and 7/10/99 respectively).

2.1.2 Criteria for patient recruitment

Patient recruitment for this study was undertaken from the Periodontology Unit, Eastman Dental Hospital (UCLH, NHS Trust) and from the Diabetes Unit, Middlesex Hospital (UCLH, NHS Trust), London, UK. Patients had already completed a standardised health questionnaire, as per UCLH Trust regulations. The patients' informed, voluntary written consent for this project (Appendices 2 & 3) was obtained prior to clinical examination. Patient details were recorded (see section 2.2.1). A detailed (extra-oral & intra-oral) clinical examination was undertaken. Inclusion and exclusion criteria for patient recruitment are summarised in Tables 2.1 & 2.2. Test patients were those concurrently suffering from DM and chronic adult periodontitis (henceforth referred to as DM-Periodontitis subjects), while the controls were non-diabetic subjects suffering from chronic adult periodontitis (non-DM-Periodontitis).

Table 2.1 Patient inclusion criteria

Patient's age	$\geq$35, $\leq$65 years
Clinical evidence of CAP:	
Probing pocket depth (true pockets, generalised)	$\geq$ 5 mm
Radiographic evidence of bone loss*	yes
Minimum number of teeth (excluding third molars)	20
Diabetes Mellitus-Periodontitis	test group
Non-Diabetes Mellitus-Periodontitis	control group

* Standardised full-mouth long cone periapical radiographs

Table 2.2 Patient exclusion criteria

Patients who had received antibiotics ≤ 3 months previously
Patients who had received periodontal therapy ≤ 3 months previously
Patients with systemic conditions requiring antibiotic cover
Patients with systemic conditions manifesting periodontal complications
Patients suffering from other infectious diseases e.g. HIV, hepatitis etc.
Patients manifesting signs of oral pathology other than periodontitis
Lactating or pregnant females
Patients with dental restorations at or near gingival margins
Patients with long-span dental bridges
Patients with endocrine disorders other than DM

2.2 Clinical materials & methods

2.2.1 Patient data

Patient details regarding name, contact information, age, smoking habits, gender, ethnic group, date of previous periodontal treatment and date of last course of antibiotic were recorded (Appendix 4). The medical questionnaire was checked prior to recording the clinical periodontal data.

2.2.2 Periodontal data

After recording the presence or absence of teeth, the following clinical periodontal variables (full mouth excluding third molars) were recorded for each patient:

i)   Pocket probing depth (PPD) using an EN 15 probe (Dentsply, Surrey, UK)

ii) Lifetime cumulative attachment loss (LCAL) using an EN 15 probe (Dentsply)

iii) Plaque Index (Silness & Löe 1964)

iv) Bleeding Index (Saxton & van der Ouderaa 1989)

2.2.3 Clinical Samples

Subgingival plaque was collected from three healthy and three diseased sites per patient according to the criteria in Table 2.1. In the case of the diseased sites, the deepest sites were selected. The sites were isolated with cotton wool rolls and clinically-evident supragingival plaque was removed with a periodontal probe. This was done by holding the graduated end of the periodontal probe horizontally at the level of the gingival margin and scraping the side of the tooth towards the incisal edge /

occlusal surface. Subgingival plaque was then obtained by inserting a sterile Gracey curette (Hu Friedy, Dorset, UK) to the full depth of the periodontal pocket and subsequently moving it vertically along the side of the root to the gingival margin (Socransky *et al.* 1998, Darby *et al.* 2000) The plaque sample was then placed in a labelled vial containing pre-reduced trypticase soy broth and immediately transferred to the laboratory for processing. Each plaque sample was vigorously agitated for 20 sec and serially diluted (10^{-2} to 10^{-5}) in Reduced Transport Fluid (RTF, Syed & Loesche 1972, Appendix 5). A known volume of each dilution was then inoculated onto a previously labelled fastidious anaerobic agar (FAA, Lab M, Lancashire, UK) plate containing 5% (v/v) defibrinated horse blood (E&O Labs, Stirlingshire, UK) and incubated anaerobically in 80% N_2, 10% H_2, 10% CO_2 (MACS-MG-100 Anaerobic Workstation, Don Whitely Scientific, Yorkshire, UK) for five days at 37°C. *Capnocytophaga* species, selected on the basis of their distinctive colonial morphology and Gram staining reaction, were subcultured to purity on FAA incubated in 5% (v/v) CO_2 (Leec Lab. & Elec. Eng. Co., Nottinghamshire, UK) at 37°C for 3 days. When not in use, the strains were aseptically transferred to a sterile vial containing Brain Heart Infusion (BHI, Lab M) containing 10% glycerol (BDH, Dorset, UK) and stored at -70°C.

2.2.4 DM data

For each patient, the following blood samples were withdrawn by venepuncture from the antecubital fossa:

i)   4 ml were collected in a K_2 EDTA Vacutainer tube (Beckton Dickinson, Oxfordshire, UK) for quantification of glycated haemoglobin (HbA_{1c}). This analysis was performed at the Chemical Pathology Laboratory, Middlesex Hospital.

ii)   10 ml were collected in a K_3 EDTA Vacutainer tube and subjected to lysis filtration (Chapter 5, section 5.2.3).

2.3 Laboratory materials & methods

2.3.1 Bacterial strains

The *Capnocytophaga* Type strains used in this study were obtained from the American Type Culture Collection (ATCC), Manassas, VA, USA and consisted of:

C. gingivalis ATCC 33624, *C. ochracea* ATCC 27872, *C. sputigena* ATCC 33612,

C. granulosa ATCC 51502, *C. haemolytica* ATCC 51501, *C. canimorsus* ATCC 35979 and *C. cynodegmi* ATCC 49044.

The strains were inoculated and maintained by subculture every 3 days on FAA and incubated in 5% (v/v) CO_2 at 37°C. When not in use, the strains were stored frozen as described for clinical isolates (2.2.3). Purity of cultures was established by examination of a fixed Gram-stained slide preparation (Lillie's modification 1928, as per Cowan & Steel 1993) as follows:

i) Ammonium oxalate-crystal violet stain for 30 sec

ii) Slide rinsed with tap water

iii) Slide flooded with Lugol's iodine (30 sec).

iv) Lugol's iodine drained

v) Slide decolourised with acetone for 2 - 3 sec.

vi) Slide counterstained with 0.5% safranin for 30 sec.

vii) Slide rinsed, dried and examined under oil immersion by transmitted light microscopy (microscope: Zeiss, Jena, Germany) at a magnification x 1000.

2.4 Statistical Analysis

2.4.1 Patient allocation to test / control groups

Patients who fulfilled the inclusion criteria were assigned to test (DM-Periodontitis) or control (non-DM-Periodontitis) groups

2.4.2 Determination of sample size

The number of test (25) and control (25) subjects was determined by standard power calculations using Statistical Product and Service Solutions computer software programme (SPSS Manual 1990; see Chapter 5, section 5.3.1).

2.4.3 Determination of number of sites per subject

The number of test and control sites per subject was determined using SPSS based on information available in the periodontal literature (See Chapter 5, section 5.3.2).

2.4.4 Data analysis

2.4.4.1 Group data analysis

Comparison of the clinical variables age, HbA_{1c}, PPD, LCAL (Chapter 5, Tables 5.1 and 5.2) and GCF volume (Chapter 7, Tables 7.2 & 7.5) within and between groups were performed using Student's t-tests. PI and BI (Chapter 5, Table 5.2, and Chapter 7, Tables 7.2 & 7.5) were compared using the *Mann-Whitney U* test.

2.4.4.2 Site data analysis

Analyses of site data for total *Capnocytophaga* counts and total anaerobic counts (Chapter 5, section 5.4.2) and GCF-glucose (Chapter 7, section 7.4.2) were based on multilevel modelling (MLM) and performed using the MLM computer software programme *MLwiN* (Rasbash *et al.* 2000).

Chapter 3

Selection of a culture medium for the isolation of
Capnocytophaga spp.

3.1 Introduction

Isolation of an organism/s from clinical samples is crucial for accurate diagnosis and treatment. In microbial ecology studies, inappropriate recovery of the species in question may lead to its / their pathologic features being assigned to other, perhaps less important, organisms. Several factors influence bacterial recovery from clinical specimens, one of which is the provision of the appropriate culture medium. Different microbial studies involving *Capnocytophaga* spp. have reported varied and even conflicting data regarding the isolation of these organisms from samples of dental plaque. Some studies have recovered these species in high numbers from plaque samples and therefore intimate a pathogenic role for this species in various periodontal conditions (Chapter 1, section 1.4.2). However, other studies do not support such an association (Moore *et al.* 1985). Some authors have even stated that the significance of these organisms has been overestimated (Holdeman *et al.* 1985). Most of the studies that have isolated *Capnocytophaga* spp. lack identification to species level. Furthermore, investigations in which identification has been performed relate to *C. gingivalis, C. ochracea* and *C. sputigena* i.e. the first three identified species (*C. granulosa* and *C. haemolytica* had not yet been formally named). Thus conclusive evidence for a pathogenic role for any of the five human oral *Capnocytophaga* species is lacking. In addition, it is only more recently that *C. canimorsus* and *C. cynodegmi* have been considered as being important pathogens in human subjects (Hantson *et al.* 1991, Mahrer & Raik 1992, Ngaage *et al.* 1999).

Comparison between periodontal studies is difficult, if not impossible, primarily due to the different technical procedures involved e.g. method of plaque collection, transport medium used and laboratory processing of the samples in question. Moreover, different studies have used a diverse range of microbial culture media and this could potentially explain the qualitative and quantitative differences observed between the respective clinical samples. The infrequent recovery of *Capnocytophaga* spp. may thus be more apparent than real.

Selective culture media for *Capnocytophaga* spp. were formulated by Mashimo *et al.* (1983b) and by Rummens *et al.* (1985), henceforth referred to as Mashimo and Cap media respectively. However, in spite of their selectivity for this genus, they have seldom been used in studies aiming to recover *Capnocytophaga* species from plaque specimens. Two other frequently used media were that used by Leadbetter *et al.* (1979; referred to as LB) and that proposed by Slots (1982; referred to as Slots). The latter however is primarily selective for *A. actinomycetemcomitans*.

The aim of this investigation, therefore, was to perform a quantitative and qualitative comparison of these culture media for optimal recovery of *Capnocytophaga* species. A fifth medium, FAA, was also included as it has been "shown to be superior to other formulations as a primary isolation medium for fastidious organisms" (Griffiths & Wade 1987).

3.2 Materials and methods

3.2.1 Media

The constituents for one litre of each medium are listed below.

Mashimo Medium: (Mashimo *et al.* 1983b) Tryptone soy agar (Oxoid, Hampshire, UK; 40 g), yeast extract (Lab M; 1 g), glucose (BDH; 2 g), defibrinated sheep blood (Oxoid, 50 ml), distilled water (960 ml), bacitracin (Sigma, Dorset, UK; 50 mg), polymyxin B (Sigma; 100 mg).

Cap Medium: (Rummens *et al.* 1985) Columbia blood agar (Difco, Oxford, UK; 44 g), haemoglobin (Sigma; 100 ml), PolyViteX (BioMérieux, Marcy-l'Etoile, France; 10 ml), polymyxin B (Sigma; 15 mg), vancomycin (Sigma; 5 mg), trimethoprim (Sigma; 2.5 mg), amphotericin B (Sigma; 2.5 mg).

LB Medium: (Leadbetter *et al.* 1979): Trypticase soy broth (Difco; 30 g), Bacto-Agar (Difco, 30 g).

Slots Medium: (Slots 1982) Trypticase soy agar (Oxoid; 40 g), yeast extract (Lab M; 1 g), horse serum (Oxoid; 100 ml), bacitracin (Sigma; 75 mg), vancomycin (Sigma; 5 mg).

Fastidious Anaerobe Agar: Fastidious anaerobe agar (Lab M; 46 g), defibrinated horse blood (50 ml).

The media were prepared as specified in the respective references, stored at 2 - 4°C and used within 7 days of preparation.

3.2.2 Bacterial strains

The *Capnocytophaga* strains used consisted of:

i) the 7 ATCC Type strains (refer to Chapter 2, section 2.3.1)

ii) 7 clinical isolates (previously obtained from patients with non-DM-Periodontitis as per the criteria in Table 2.1)

In addition, seven subgingival plaque specimens (from patients with non-DM-Periodontitis as per the criteria in Table 2.1) were collected and processed as per the protocol in section 2.2.3.

The 7 Clinical isolates (strains MLC 74, 92, 87, 75, 62, 61 and 97) had been previously recovered from human dental plaque (collected as per the protocol in section 2.2.3) from non-DM-Periodontitis patients based on the selection criteria stated in Tables 2.1 & 2. These were stored as frozen stocks until this investigation. All strains (type strains and clinical isolates) were maintained as specified in 2.3.1. Preliminary identification of these organisms as *Capnocytophaga* spp. was performed by colonial morphology and Gram-staining reaction. Definitive identification of each isolate to species level was performed using DNA fingerprinting, more specifically 16S rRNA PCR restriction fragment length polymorphism (RFLP) analysis employing the restriction enzyme *Cfo* 1 (Wilson *et al.* 1995; validated with 16S rRNA gene sequencing; refer to Chapter 4).

Three-day-old cultures of the 7 ATCC Type strains and the 7 clinical isolates were used in all experiments. A small quantity of each culture was suspended in separate, labelled vials containing freshly prepared RTF (Appendix 5). Each neat suspension was vortexed for 20 seconds, and its optical density adjusted to 0.2 (Ultrospec, Cambridge, UK) at a wavelength of 590 nm. Serial dilutions (10^{-2} - 10^{-5}) were prepared using RTF. Twenty µl aliquots of each serial dilution were dispensed onto previously labelled plates containing the respective media. Each aliquot was plated out using a sterile spreader. The inoculated plates were incubated at 37°C in 10% CO_2 and examined after 3 days and again after 7 days of incubation. For each strain grown on each of the different media, the mean number of colony forming units per ml (cfu ml^{-1}) was calculated from four replicate plates.

3.2.3 Statistical analysis

The raw data (cfu ml^{-1}) for each type strain and clinical isolate were tested for variance using the *F*-test. Since variation was equal between the different strains, analysis of variance (ANOVA) was used to compare recovery of the different strains on the five media ($P < 0.05$).

3.3 Results

3.3.1 ATCC *Capnocytophaga* Type strains (Fig.3.1)

FAA was the only medium capable of supporting growth of all the seven different *Capnocytophaga* ATCC Type strains. FAA yielded the maximum number of colony forming units per ml (cfu ml^{-1}) for all Type strains except for *C. sputigena,* which showed a marginally better recovery on Mashimo medium. FAA showed statistically significantly better recovery of isolates than Mashimo medium for ATCC strains *C. ochracea, C. granulosa, C. canimorsus* and *C. cynodegmi* ($P < 0.05$). It was also statistically significantly better than the Leadbetter medium for *C. ochracea, C. granulosa, C. canimorsus* and *C. cynodegmi.*
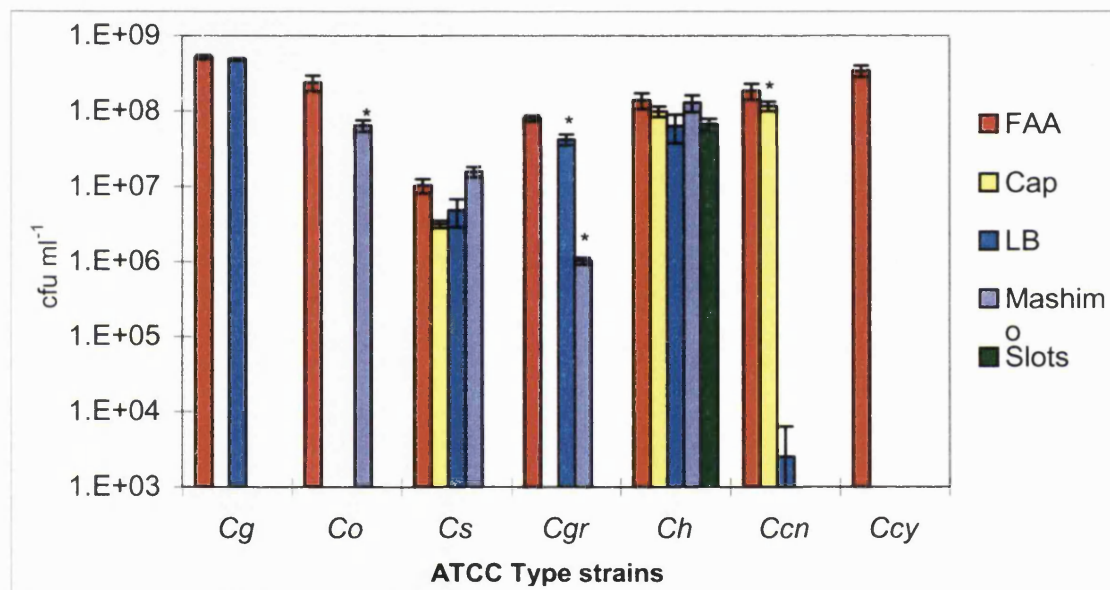
Fig.3.1 Growth of *Capnocytophaga* ATCC strains on various media. *Cg* = *C. gingivalis, Co* = *C. ochracea, Cs* = *C. sputigena, Cgr* = *C. granulosa, Ch* = *C. haemolytica, Ccn* = *C. canimorsus, Ccy* = *C. cynodegmi.* Bars represent mean values (n = 4); error bars represent standard deviations. *Significantly different from FAA.

3.3.2 *Capnocytophaga* clinical isolates (Fig.3.2)

The seven clinical isolates were identified as three *Capnocytophaga* strains; MLC 74 as *C. gingivalis,* MLC 92, 87, 75, 61 and 62 as *C. ochracea* and MLC 97 as *C. granulosa.*
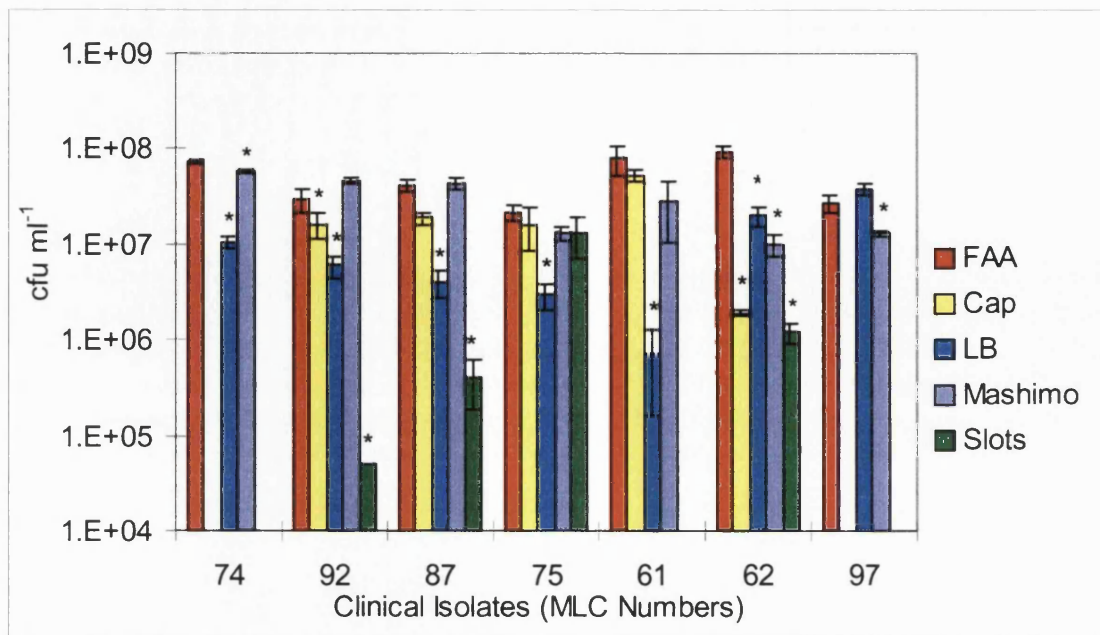
Fig.3.2 Growth of *Capnocytophaga* clinical isolates on various media. Bars represent mean values (n = 4); error bars represent standard deviations. * Significantly different from FAA

FAA, LB and Mashimo supported growth of all clinical isolates i.e. *C. gingivalis*, *C. ochracea* and *C. granulosa*. Cap and Slots' media supported the growth of all the *C. ochracea* clinical isolates, with the exception of MLC 61 (*C. ochracea* strain), which failed to grow on Slots' medium.

In the majority of cases, growth of all isolates on FAA was statistically significantly better (*P* < 0.05) than on the other media.

3.3.3 Clinical samples (Fig.3.3)

Data are represented for 5 plaque samples. No *Capnocytophaga* spp. were isolated from patient 2. In the case of patient 5, two samples were collected and these were considered as one during subsequent data analysis. One plaque sample (patient 6) was very scanty and did not show any growth at all. In all cases, the media that supported the growth of *Capnocytophaga* spp. were FAA, Cap, LB and Mashimo media.
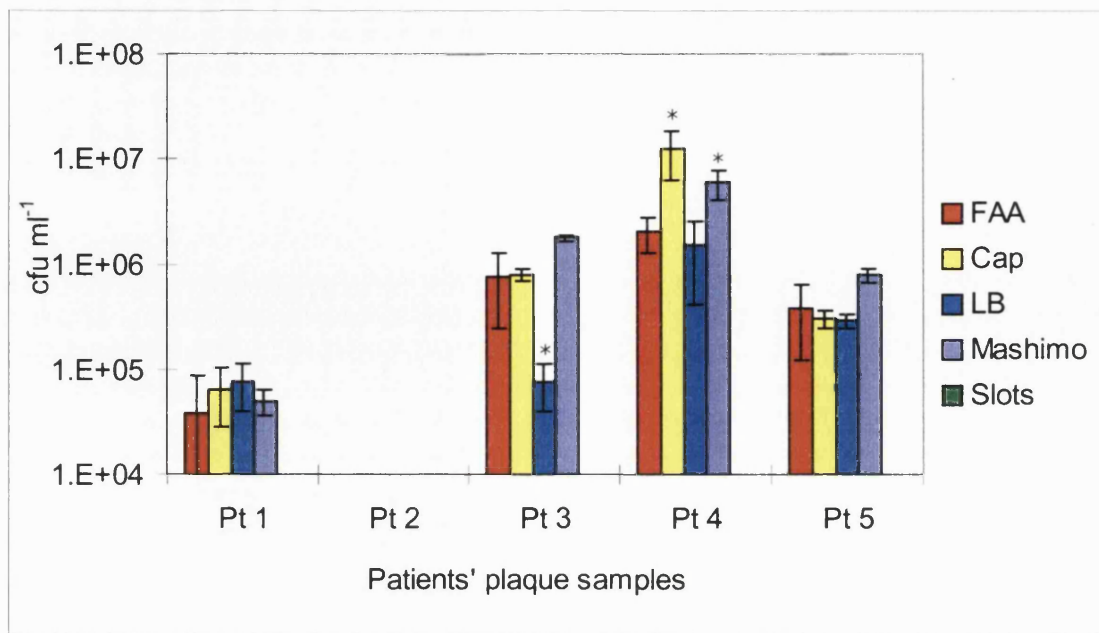
Fig.3.3 Growth of *Capnocytophaga* isolated from patients' plaque samples on various media. Bars represent mean values (n = 4); error bars represent standard deviations. *Significantly different from FAA

Slots' medium was the only medium that did not support the growth of clinical isolates of *Capnocytophaga*. Within each plaque sample, the recovery of the isolates on the different media was comparable. Exceptions to this were noted in patient 3 from whom the recovery of *Capnocytophaga* strains was statistically significantly ($P < 0.05$) greater on FAA than on LB medium and in patient 4 from whom the recovery of these species was statistically significantly greater on Cap and Mashimo media ($P < 0.05$) than on FAA. A representative selection of isolates (45) were purified and identified by 16S rRNA PCR-RFLP analysis (subsequently validated using 16S rRNA gene sequencing; see chapter 4). The isolates were identified as *C. gingivalis* (7), *C. ochracea* (18), *C. sputigena* (1), *C. granulosa* (15) and *C. haemolytica* (4). Information generated from this part of the investigation showed that Mashimo medium was not entirely selective for *Capnocytophaga* strains.

3.4 DISCUSSION

One of several laboratory factors which may affect recovery of bacterial isolates, from a clinical sample is the culture medium used for isolation and growth of the organisms in question. Furthermore, the isolates recovered from clinical samples need to be

accurately identified to species level in order to obtain precise and reliable information as to which isolates may be expected to be consistently recovered on the medium under investigation. Despite increasing interest in the genus *Capnocytophaga*, infections caused by these organisms have been infrequently recognised in clinical practice, possibly due to the lack of reliable data regarding the most appropriate isolation medium.

The media compared in this study were: Mashimo, Cap, LB, Slots and FAA, selected on the basis of their stated selectivity for *Capnocytophaga* spp. (Mashimo and Cap), their frequent use in previous studies (LB and Slots), and, in the case of FAA, as it has been shown to be "superior to other formulations as a primary isolation medium for fastidious organisms" (Griffiths & Wade 1987).

The Mashimo and Cap media were specifically formulated to select for *Capnocytophaga* species (Mashimo *et al.* 1983b, Rummmens *et al.* 1985); their selectivity was attributed to the addition of antibiotics (bacitracin, polymyxin B, vancomycin and trimethoprim). However, information regarding the antimicrobial sensitivity profile of each *Capnocytophaga* spp. to these agents was not available as the studies which set out to test the antimicrobial susceptibility of *Capnocytophaga* spp., did not identify the organisms to species level (Forlenza *et al.* 1981, Sutter *et al.* 1981). Mashimo medium (which contains bacitracin and polymyxin B), yielded a high recovery of *Capnocytophaga* species (80%) however it was not totally selective for this genus (Mashimo *et al.* 1983b), a finding corroborated in this study.

Cap medium (Rummens *et al.* 1985) was formulated to be an improvement on Mashimo medium. Its nutritional characteristics are enhanced by the addition of PolyViteX, a chemically defined enrichment supplement that is added to media to isolate and grow fastidious micro-organisms. Cap medium also contains a mixture of antibiotics (polymyxin B, vancomycin, trimethoprim) and an antifungal agent (amphotericin B). This medium has been claimed to be an "excellent medium for the recovery of *Capnocytophaga* species from contaminated clinical specimens" (Rummens *et al.* 1985). However, Hawkey *et al.* (1987) reported that 18% of *Capnocytophaga* strains used in their study were sensitive to trimethoprim. The major shortcoming of the investigations evaluating these selective media is that both studies failed to identify the *Capnocytophaga* isolates to species level. Sutter *et al.* (1981) found that bacitracin and vancomycin could inhibit the growth of some *Capnocytophaga* strains. This may explain the reduced growth of some

Capnocytophaga strains on media (Cap, Mashimo and Slots) containing either or both of these antibiotics.

Leadbetter *et al.* (1979) suggested using either Trypticase soy broth (TSB) solidified with agar (3% w/v) or Trypticase soy agar containing sheep's blood (5% v/v). In this investigation TSB solidified with agar was used. The enhanced nutritional characteristics of the TSB medium containing blood could potentially support the growth of a different range or quantity of *Capnocytophaga* species.

In this study, LB medium performed nearly as well as FAA.

Slots' medium, a selective medium for *A. actinomycetemcomitans*, has been used in several investigations in which *Capnocytophaga* species were concurrently isolated with *A. actinomycetemcomitans*. Its selectivity is imparted by the antibiotics bacitracin and vancomycin.

The differing growth patterns (Table 3.1) could be explained by the diverse nutritional components of the different media (Table 3.2). FAA is claimed to be superior to other formulations as a primary isolation medium for fastidious organisms. Mashimo, LB and Slots are all composed of Tryptone Soy Agar, a nutritious medium capable of supporting the growth of a wide variety of organisms. Mashimo added yeast extract and glucose while Slots added yeast extract only. Cap medium consists of Columbia blood agar, enriched with haemoglobin (a source of protein) and PolyViteX.

The literature regarding the sensitivity of *Capnocytophaga* spp. to antibiotics used in Cap medium is confusing with some authors suggesting growth inhibition (Sutter *et al.* 1981, Hawkey *et al.* 1987) while others implying intrinsic resistance (Rummens *et al.* 1986). Since these studies did not identify the isolates used, accurate information regarding which species are sensitive or resistant to the antibiotics used cannot be deduced. Although it might be assumed that organisms within the same genus would tend to have the same antimicrobial susceptibility pattern, definitive information can only be obtained if the tested isolates are accurately identified. This is of particular clinical significance considering the systemic infections caused by these organisms (Parenti & Snydman 1985, Warren & Allen 1986, Esteban 1985, Martino *et al.* 2001) especially in immunocompromised patients (Parenti & Snydman 1985).

Table 3.1 Growth pattern of *Capnocytophaga* spp. on the isolation media

Media	ATCC strains (n = 7) 7 species							Clinical isolates (n = 7) 3 species			Plaque isolates (n = 5) 5 species				
	Cg	*Co*	*Cs*	*Cgr*	*Ch*	*Ccn*	*Ccy*	*Cg*	*Co*	*Cgr*	*Cg*	*Co*	*Cs*	*Cgr*	*Ch*
FAA	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓		✓	
Cap			✓		✓	✓			✓			✓		✓	✓
LB	✓		✓	✓	✓	✓		✓	✓	✓	✓		✓	✓	
Mashimo			✓	✓	✓			✓	✓	✓		✓		✓	✓
Slots					✓				✓						

✓Indicates growth (not absolute number)

Table 3.2 Comparison of media constituents

FAA (gL⁻¹)		Mashimo (gL⁻¹)		Cap (gL⁻¹)		LB (gL⁻¹)		Slots (gL⁻¹)	
Peptone mix	23.0	Tryptone Soy Agar (Oxoid)	40.0	Columbia Blood Agar: (Difco)	44.0	Trypticase Soy Broth (Difco)	30.0	Trypticase Soy Agar (Difco)	40.0
Sodium chloride	5.0	Tryptone	15.0	Bacto Pantone	10.0	Bacto Tryptone	15.0	Bacto Tryptone	17.0
Soluble starch	1.0	Soya peptone	5.0	Bacto Bitone	10.0	Bacto Soytone	5.0	Bacto Soytone	3.0
Agar No.2	12.0	Na Cl	5.0	Tryptic digest of beef heart	3.0	Na Cl	5.0	Bacto dextrose	2.5
Sodium bicarbonate	0.4	Agar	15.0	Corn starch	1.0	agar	15.0	NaCl	5.0
Glucose	1.0			Na Cl	5.0	Bacto Agar	30.0	K₂PO₄	2.5
Sodium pyruvate	1.0	Yeast extract	1.0	Bacto Agar	15				
Cysteine HCl. H₂0	0.5	Glucose	2.0	Haemoglobin (ml)	100.0			Yeast extract	1.0
haemin	0.01	bacitracin	0.05	Polyvitex (ml)	10.0			Bacitracin	0.075
Vitamin K	0.001	polymyxin B	0.1	Polymyxin B	0.15			Vancomycin	0.005
L-arginine	1.0	Sheep Blood (ml)	50.0	Vancomycin	0.005			Horse serum (ml)	100.0
Soluble pyrophosphate	0.25			Trimethoprim	0.0025				
Sodium succinate	0.5			Amphotericin B	0.0025				
Horse blood (ml)	50.0								

Prior to plate inoculation, the optical densities of the bacterial suspensions of the *Capnocytophaga* ATCC strains and 7 clinical isolates were adjusted to an absorbance of 0.2 (λ = 590 nm). This OD value adjustment was based on a previous calibration graph (OD versus cfu ml^{-1}; data not shown) and was performed to ensure that the bacterial concentration inoculated onto the plates could be easily counted.

The third part of this investigation was undertaken to exclude the possibility that maintaining subcultures of *Capnocytophaga* spp. on FAA could potentially bias the results in favour of FAA. It is evident, with the exception of patient 4 (Fig.3.3), that strains of *Capnocytophaga* from the plaque samples grew equally well on all media. Isolates recognised as being *Capnocytophaga* spp. were selected at random and identified via 16S rRNA RFLP analysis. This part of the investigation was not intended to identify all the *Capnocytophaga* isolates recovered from the plaque samples, but rather to identify a representative sample of clinical isolates. Thus, absolute comparison of the identified isolates from the plaque samples with the respective Type strains and clinical isolates was precluded. The trend in the growth pattern of *Capnocytophaga* ATCC strains, clinical isolates and plaque isolates on the different media is summarised in Table 3.1. It was observed that clinical isolates of *C. gingivalis*, *C. ochracea* and *C. granulosa* grew on a wider range of media than did the corresponding ATCC strains. The predominant species isolated from plaque were: *C. gingivalis* (7), *C. ochracea* (18) and *C. granulosa* (15), *C. sputigena* (1) and *C. haemolytica* (4).

In conclusion, the results of this investigation have shown that the culture medium is a determining factor in isolating the full range of *Capnocytophaga* spp. from clinical samples. FAA was the only medium that supported the growth of all *Capnocytophaga* ATCC Type strains and clinical isolates.

Thus based on the results of this investigation, and the fact that FAA was relatively easier to prepare when compared to some of the suggested media (in particular Mashimo and Cap media), FAA was chosen as the medium for use throughout this study for the isolation of *Capnocytophaga* spp. Ease of media preparation was an asset due to the large number of samples (an estimated 300 plaque samples and 50 blood samples) which were to be processed during this study. Furthermore, since FAA does not contain any antibiotics and is capable of supporting the growth of several fastidious organisms found in plaque e.g. *Eubacterium* spp., *F. nucleatum, Por. gingivalis, Prev. asaccharolytica, Prev. melaninogenica* and *Veillonella* spp., (Lab M

Culture Media Manual), more information could be derived from each sample when using FAA e.g. besides the total *Capnocytophaga* counts, the total counts for bacteria which grew anaerobically (i.e. both facultative and obligate anaerobes) were also quantified (refer to Chapter 5).

This investigative work has been published as:

Assessment of five culture media for the growth and isolation of *Capnocytophaga* species. Ciantar M, Spratt DA, Newman HN & Wilson M (2001). *Clin Microbiol Infect* 7: 158 - 160.

Chapter 4

Strategy for the identification of *Capnocytophaga* spp.

4.1 Introduction

Traditionally bacterial identification was established using a broad range of phenotypic characteristics. Organisms were assigned to groups based on morphological features (macro- and microscopic, including staining characteristics) and physiological attributes such as nutritional requirements, fermentation products, growth conditions (oxygen, temperature and inhibitory products) and the ability to form spores. This formed the basis of classification for bacterial genera (Cowan & Steel 1993). These phenotypic features were shared by many species and were therefore non-specific. They varied depending on the cultural conditions and / or age of colonial growth. The number of tests, duration and labour involved and their costs most often limited the range of chosen tests. The choice of tests undertaken and their standardisation varied between laboratories, which in turn was a potential source of misclassification (Goodfellow and Dickinson 1985, Totten *et al.* 1987). In addition, strain-specific tests which claimed to differentiate between species within a genus failed to do so conclusively (Kristiansen *et al.* 1984, Speck *et al.* 1987).

The accessibility and ease of use of bacterial genetic information heralded a new era in bacterial systematics. The major breakthrough in determining the evolution and phylogeny of prokaryotes came with the introduction of ribosomal ribonucleic acid (rRNA) sequencing techniques (Woese *et al.* 1975). The 16S rRNA gene is universally distributed and highly conserved (Woese *et al.* 1987). Due to its conserved nature and ease of manipulation, it has been extensively used to establish accurate identification of clinical bacterial isolates. Amplification of the 16S rRNA gene using the polymerase chain reaction (PCR, Mullis & Faloona 1987) allows generation of high copy numbers of this gene, which may subsequently be used for bacterial identification. Cleavage of PCR-generated 16S rRNA gene amplicons by a restriction enzyme(s) results in restriction fragment length polymorphisms (RFLP). The 16S rRNA PCR-RFLP may be separated by electrophoresis and visualised on an agarose gel. This has been used extensively as a method for bacterial species identification (Wilson *et al.* 1995, Marshall *et al.* 1999, Conville *et al.* 2000, Steinhauserova *et al.* 2001).

Identification of *Capnocytophaga* spp. from clinical isolates is crucial in epidemiological typing and clinical diagnosis. Investigations into possible associations of species within this genus with various pathological conditions have been hampered by the lack of a reliable scheme for species identification. Previous studies have attempted to distinguish between these species by biochemical tests (Socransky *et al.* 1979,

Laughon *et al.* 1982), protein profiles (Khwaja *et al.* 1990, Vandamme *et al.* 1996), multilocus enzyme electrophoresis and serotyping of immunolgobulin A1 protease (Frandsen & Wade 1996), DNA probes (Conrads *et al.* 1997), 16S rRNA PCR-RFLP (Wilson *et al.* 1995) and 16S rRNA sequence analysis (Conrads *et al.* 1992, Vandamme *et al.* 1996). Most of these methods (other than 16S rRNA PCR-RFLP) are labour intensive, costly and time consuming and therefore not amenable to most microbiology laboratories, especially when several hundred clinical isolates are to be identified.

The aim of this study therefore was to develop a strategy for the molecular identification of *Capnocytophaga* spp. using 16S rRNA PCR-RFLP.

The study was performed in 2 parts:

i) the first part entailed screening different endonuclease restriction enzymes (RE) in order to select one which yielded a different restriction pattern for each of the *Capnocytophaga* Type strains. The identity of the Type strains obtained via 16S rRNA PCR-RFLP was confirmed via 16S rRNA sequencing

ii) the second part involved identification of 187 clinical isolates of *Capnocytophaga* spp. via 16S rRNA PCR-RFLP with subsequent verification via 16S rRNA gene sequencing.

4.2 Materials and methods

4.2.1 Bacterial strains

The 7 *Capnocytophaga* ATCC Type strains used were those referred to in Chapter 2, section 2.3.1. Clinical isolates of *Capnocytophaga* spp. (187) were obtained as described in Chapter 2 section 2.2.3.

4.2.2 DNA extraction

Extraction of bacterial DNA was performed using a 2-minute boil preparation. Briefly, a few bacterial colonies were aseptically suspended in sterile nuclease-free water (Anachem, Bedfordshire, UK) contained in sterile, labelled 0.5 ml Eppendorf tubes (Sarsted, Leicestershire, UK). The tubes were then sealed, vigorously agitated and boiled for 2 minutes. Immediately after boiling, the tubes were placed on ice. A 1:10 dilution of this bacterial suspension in sterile nuclease-free water was subsequently prepared and kept on ice. It was later used as the nucleic acid template for the PCR amplification.

4.2.3 16S rRNA PCR-RFLP analysis

The PCR master mix (2 x 100 µl) contained: 5 mM Taq NH₄ Buffer, 200 µM each of dNTPs (dATP, dCTP, dGTP and dTTP; Promega, Madison, USA), 2.5 mM MgCl₂, 25 pmol µl⁻¹ of each PCR primer 27f (Genosys, Cambridge, UK, Table 4.1) and 1492r (Genosys, Table 4.1) and 1 U Taq Polymerase (Bioline, London, UK). Fifty µl each of bacterial DNA template and master mix were placed in PCR tubes (ABgene, Surrey, UK). Two reactions per strain were performed, one for 16S rRNA PCR-RFLP analysis and the other for 16S rRNA gene sequencing.

Table 4.1 Primers used for 16S rRNA PCR amplification and sequencing

Primer	Sequence
27f	5' AGAGTTTGATCMTGGCTCAG
1492r	5' TACGGYTACCTTGTTACGACTT
357f	5' CTCCTACGGGAGGCAGCAG

M = C:A; Y = C:T

PCR amplification was performed in a Biometra Uno II Thermal Cycler (Anachem) according to the following conditions: 95°C for 5 min, then 29 cycles at 94°C for 1 min, 54°C for 1 min, 72°C for 1 min followed by an extension period of 72°C for 5 min. Negative and positive controls using sterile, nuclease-free water and *Escherichia coli* template respectively were also prepared. Assessment of PCR amplicons (circa 1500 bp) was performed by loading 10 µl of the PCR product into separate wells of a 0.8% w/v agarose gel (Agarose I, Anachem) containing ethidium bromide (0.5 µg / ml). A molecular weight marker (2000 - 50 bp; Anachem) was loaded into the end well. The gel was immersed in Tris-acetate EDTA buffer (BDH, Dorset, UK) and subjected to a voltage difference of 70 volts that led to separation of the fragments. The gel was visualised after excitation under UV transillumination by placing it in a MultiImage Light Cabinet (AlphaInnotech Corp., Staffordshire, UK) and the resulting image captured by a computer software programme (AlphaEase™, AlphaInnotech).

The PCR-RFLP reaction mixture for the *Capnocytophaga* ATCC strains (25 µl) each contained: 21.5 µl PCR amplicon, 1 U of RE i.e. either *Cfo* I or *Hae* III or *Rsa* I (Promega, Wisconsin, USA) and 2.5 µl of corresponding RE buffer. The RE digests were performed in sterile, labelled Eppendorf tubes. The RE mixture was briefly mixed, pulse spun and incubated overnight at 37°C. The resulting 16S rRNA PCR-RFLP digests (25 µl) were transferred into separate wells of a 2% w/v superfine resolution

agarose gel (Anachem) containing ethidium bromide (0.5 μg ml^{-1}). The same molecular weight marker (10μl) was loaded into each of the end wells of the gel. The fragments were allowed to separate electrophoretically and visualised as described above.

4.2.4 16S rRNA gene sequencing

One of the PCR tubes containing 100 μl of PCR product was purified using the Qiagen purification kit (Qiagen, West Sussex, UK) and used for DNA sequencing. The purified DNA was stored at -20°C until sequencing. The sequencing PCR was performed as follows (7 μl reaction): 2 μl of 1:4 dilution in 5x sequencing buffer (400 mM TRIS, 10 mM MgCl$_2$) of ABI Prism BigDye Terminator Cycle Sequencing Kit (AB Applied Biosystems, Warrington, UK), 1 μl of 357f sequencing primer (5 pmol μl^{-1}; Genosys; Table 4.1), 1 μl DNA template and 3 μl sterile nuclease-free water. The sequencing reaction was performed in a thermal cycler (Biometra Uno II) programmed as follows: 95°C for 10 sec, 50°C for 5 sec and 60°C for 4 min for 99 cycles. At the end of the sequencing reaction, each 7 μl PCR was increased to 20 μl by aseptically adding sterile nuclease-free water and transferred to a sterile, labelled 1.5 ml Eppendorf tube containing 2 μl sodium acetate (3M, pH 5.2) and 50 μl of 95% cold (-20°C) ethanol (Anachem). This mixture was incubated on ice for 10 min and subsequently centrifuged at 15,800 rcf for 20 min at 4°C. The fluid in the tube was gently aspirated, care being taken not to dislodge the DNA pellet, which was then washed with 250 μl of 70% cold ethanol. The tubes were centrifuged at 15,800 rcf for 15 min. The fluid was again aspirated and the tubes dried on a heating block at 95°C for a few seconds. The purified DNA was then re-suspended in 20 μl template suppression reagent (Applied Biosystems), incubated at 95°C for 2-3 min, briefly mixed, pulse spun and put on ice for 2-3 min. The tube contents were then transferred to correspondingly labelled sequencing tubes. Sequence separation took place in an automated DNA sequencer (ABI Prism 310 Genetic Analyzer, Applied Biosystems). The resulting electrophoretogramme were analysed via the computer software programme Chromas (version 1.43, Queensland, Australia). Only sequences that were 300 bases or longer were analysed by comparison with the 16S rRNA gene databases located at the Ribosomal Database Project (RDP-II, Michigan, USA; Maidek *et al.* 2001) and also subjected to Basic Logic Alignment Search Tool 2.2.1 analysis (BLAST; Altschul *et al.* 1990, 1997). Stringent criteria (tall, distinct peaks, minimal number of "Ns" and very

low background noise) were set for the electrophoretogram prior to submission of each sequence to both databases. A target cut-off $\geq$ 0.8 was set for the RDP-II data.

The 187 *Capnocytophaga* clinical isolates were processed exactly as described above for the Type strains. Since the analysis of the 16S rRNA PCR-RFLP patterns of the *Capnocytophaga* ATCC strains with *Cfo* I (Promega, WI, USA) revealed seven different patterns (See results section 4.3.1), the RE digests of the clinical isolates were performed using only this enzyme (*Cfo* I).

4.2.5 *In silico* PCR-RFLP analysis of the 16S rRNA *Capnocytophaga* genes available on gene databases

The 16S rRNA gene sequences (n = 37) of all the *Capnocytophaga* spp. available on the RDP-II and BLAST databases were downloaded and saved as Microsoft Word documents. *In silico* 16S rRNA PCR-RFLP analysis of these sequences was performed based on the restriction site for *Cfo* I (GCG▼C). The sequences available on the RDP-II and BLAST databases are listed in Table 4.2.

4.2.6 Phylogenetic analysis of *Capnocytophaga* spp.

The 16S rRNA gene sequences for *Capnocytophaga* spp (except for *Capnocytophaga* spp. oral clone BM058) located on the RDP-II and BLAST databases were used to construct a phylogenetic tree for these species. The oral clone BM058 was omitted from the analysis as the sequence available on the database was a very short partial sequence. This would have severely distorted the dendrogram if included in the analysis. The sequences were aligned using ClustalX (Thompson *et al.* 1997). The resulting sequence alignment was edited using BioEdit v5.0.9 (Hall 1999) and subsequently used to produce a phylogenetic tree by the neighbour-joining method using ClustalX. Tree rooting was established by including the 16S rRNA gene for *P. gingivalis*.

Capnocytophaga species	16S rRNA gene sequences at:		Accession #
	RDP	BLAST	
C. gingivalis ATCC 33624T*	✓	✓	X67608
C. gingivalis 16S rRNA gene	✓	✓	L14639
C. gingivalis LMG 12118	✓	✓	U41346
Capnocytophaga spp. str S3	✓	✓	AY005073
Capnocytophaga spp. str S1	✓	✓	U42008
Capnocytophaga spp. LMG 12116	✓	✓	U41352
C. ochracea ATCC 27872T*	✓	✓	U41350
C. ochracea ATCC str. 25	✓	✓	X67610
C. ochracea ATCC 33596T	✓	✓	L14635
C. ochracea LMG 12117	✓	✓	U41351
C. ochracea LMG 12115 (=FDC 7)	✓	✓	U41353
C. ochracea FDC 7b	✓	✓	U41354
Capnocytophaga spp. str S1	✓	✓	U42008
Capnocytophaga spp. str. S1b	✓	✓	U42009
C. sputigena ATCC 33612T*	✓	✓	L14636
C. sputigena 16S rRNA gene	✓	✓	X67609
C. sputigena str 897 CIP 100	✓	✓	AF133536
C. granulosa ATCC 51502T*	✓	✓	X97248
C. granulosa LMG 16022T	✓	✓	U41347
C. granulosa LMG 12119	✓	✓	U41348
C. haemolytica ATCC 51501T*	✓	✓	X97247
C. haemolytica LMG 16021T	✓	✓	U41349
C. canimorsus ATCC 35979T*	✓	✓	L14637
C. canimorsus 16S rRNA gene	✓	✓	X97246
C. cynodegmi ATCC 49044T*	✓	✓	L14638
C. cynodegmi 16S rRNA gene	✓	✓	X97245
Capnocytophaga spp. oral clone X089		✓	AY05080
Capnocytophaga spp. oral clone EL043		✓	AY008312
Capnocytophaga spp. oral clone DS022		✓	AF366270
Capnocytophaga spp. oral clone AA032		✓	AY005079
Capnocytophaga spp. oral clone AH015		✓	AY005074
Capnocytophaga spp. oral clone X066		✓	AY005078
Capnocytophaga spp. oral clone A47ROY		✓	AY005077
Capnocytophaga spp. oral clone BB167		✓	AY005076
Capnocytophaga spp. oral clone DZ074		✓	AF385494
Capnocytophaga spp. oral clone BM058		✓	AY005075
Capnocytophaga spp. oral clone BU084		✓	AF385569

Table 4.2 16S rRNA gene sequences located at the RDP-II and BLAST databases (as per May 2002). *Capnocytophaga* ATCC strains used in this study to produce the 16S rRNA PCR-RFLP shown in Fig 4.1.

4.3 RESULTS

4.3.1 Selection of RE for 16S rRNA PCR-RFLP

Of the 3 RE (*Cfo* I, *Hae* III and *Rsa* I) used for RFLP analysis of the 7 ATCC *Capnocytophaga* strains, *Cfo* I was the only RE which yielded 7 different 16S rRNA PCR-RFLP, with each RFLP characterising a particular ATCC species (Fig.4.1). Thus *Cfo* I was the RE chosen for RFLP analysis of the clinical isolates. Gram staining of the ATCC strains confirmed their cellular morphology as Gram-negative, slender, fusiform rods.
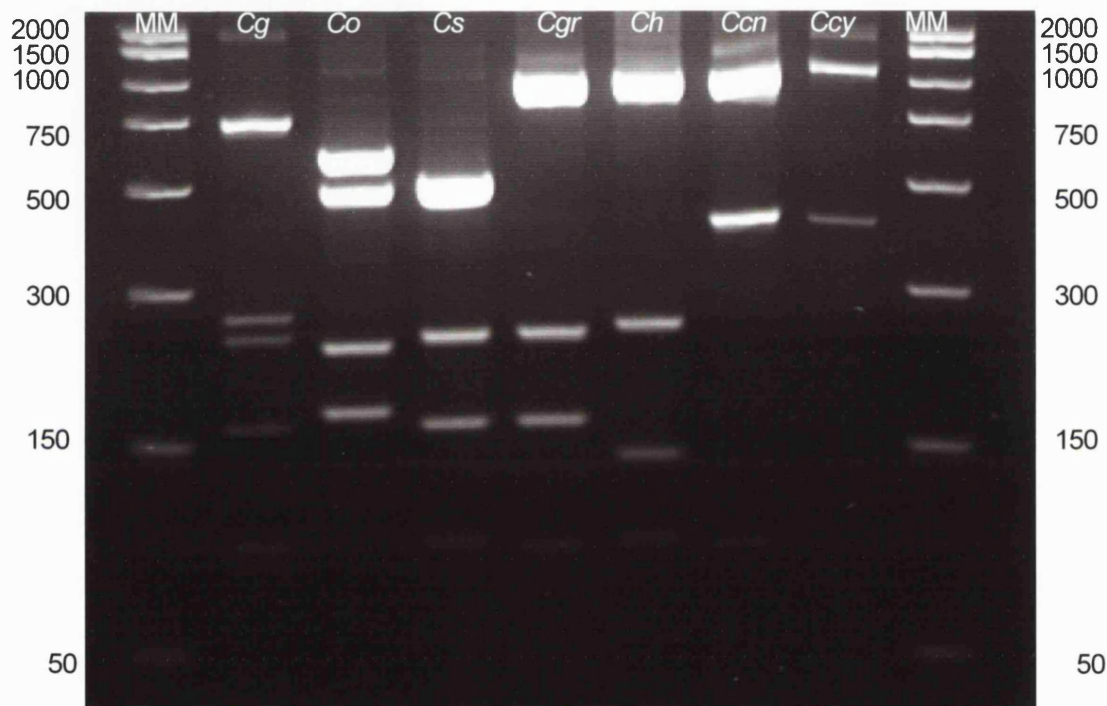
Fig.4.1 16S rRNA PCR-RFLP of ATCC *Capnocytophaga* spp. with *Cfo* I

4.3.2 *Capnocytophaga* ATCC strains: comparison of identification via 16S rRNA PCR-RFLP and 16S rRNA gene sequencing

The identity of the *Capnocytophaga* Type strains was confirmed by submitting the respective sequenced 16S rRNA genes to the RDP-II and BLAST databases.

4.3.3 *Capnocytophaga* clinical isolates: identification via 16S rRNA PCR-RFLP

Preliminary identification of all clinical isolates of *Capnocytophaga* was performed by examination of their colonial morphology and Gram-stain reaction. Identification of all clinical isolates was initially accomplished by comparing their 16S rRNA PCR-RFLP

with those of the *Capnocytophaga* ATCC strains used in this study (Fig.4.1). The 16S rRNA PCR-RFLP of a representative group of *Capnocytophaga* clinical isolates are depicted in Figs.4.2, 4.3 and 4.4. The larger majority of the clinical isolates' 16S rRNA PCR-RFLP conformed exactly to those of the ATCC strains e.g. the *C. ochracea* (typical) and *C. granulosa* in Fig.4.2; *C. gingivalis* in Fig.4.3 lanes 2, 3 & 4; *C. sputigena* in Fig.4.3 lane 18. An advantage of this method was that strain contamination was easily detected (Fig.4.3 lane 17).
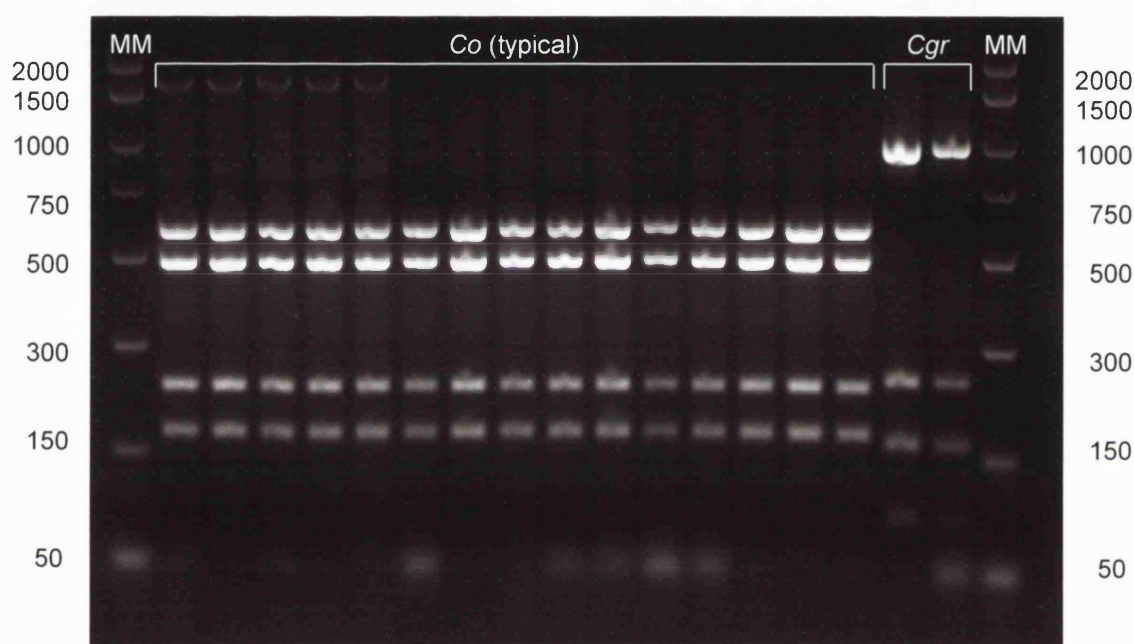
Fig. 4.2 16S rRNA PCR-RFLP of clinical isolates of *Capnocytophaga* species with *Cfo* I. *Co* = *C. ochracea*; *Cgr* = *C. granulosa*; MM = Molecular weight Marker

Some strains showed subtle differences in their RFLPs; these strains were hence labelled as "variants" e.g. *C. ochracea* variant (Fig.4.3 lane 6, Fig.4.4 lane 8). Isolates conforming to this pattern were identified as oral clones of *Capnocytophaga* spp. via 16S gene sequencing. An RFLP pattern similar to that of *C. canimorsus* was observed (Fig.4.3 lane 14), however identification via 16S rRNA gene sequencing revealed this to be a variant of *C. gingivalis*.
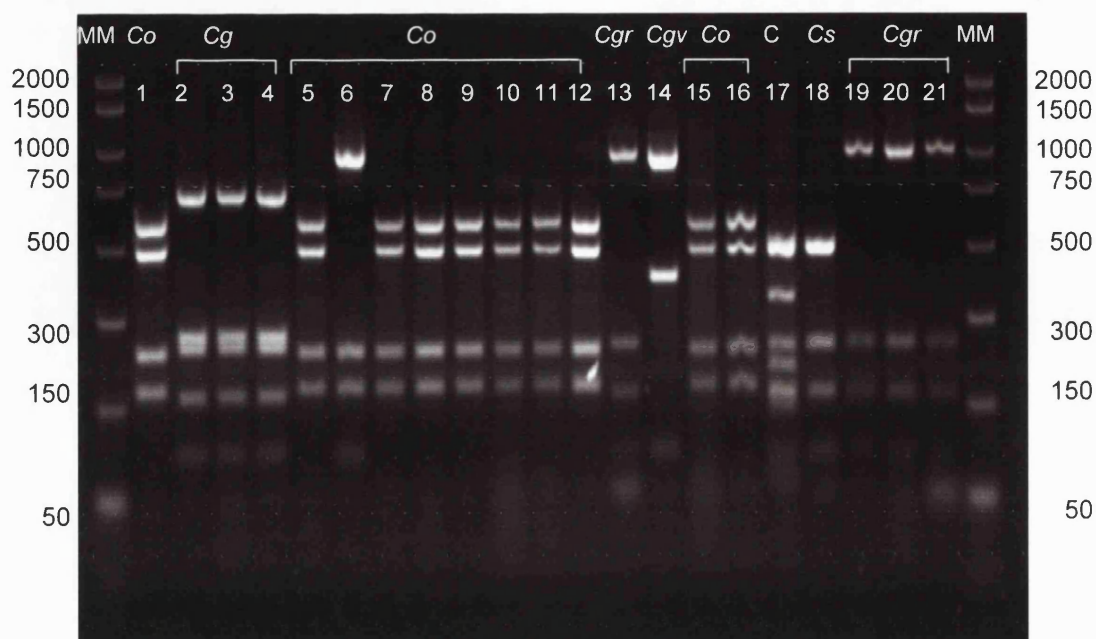
Fig.4.3 16S rRNA PCR-RFLP of clinical isolates of *Capnocytophaga* species with *Cfo* I. *Co* = *C. ochracea*; *Cg* = *C. gingivalis*; *Cgr* = *C. granulosa*; *Cs* = *C. sputigena*; *Cgv* = *C. gingivalis* variant; C = Contaminant; MM = Molecular weight Marker.
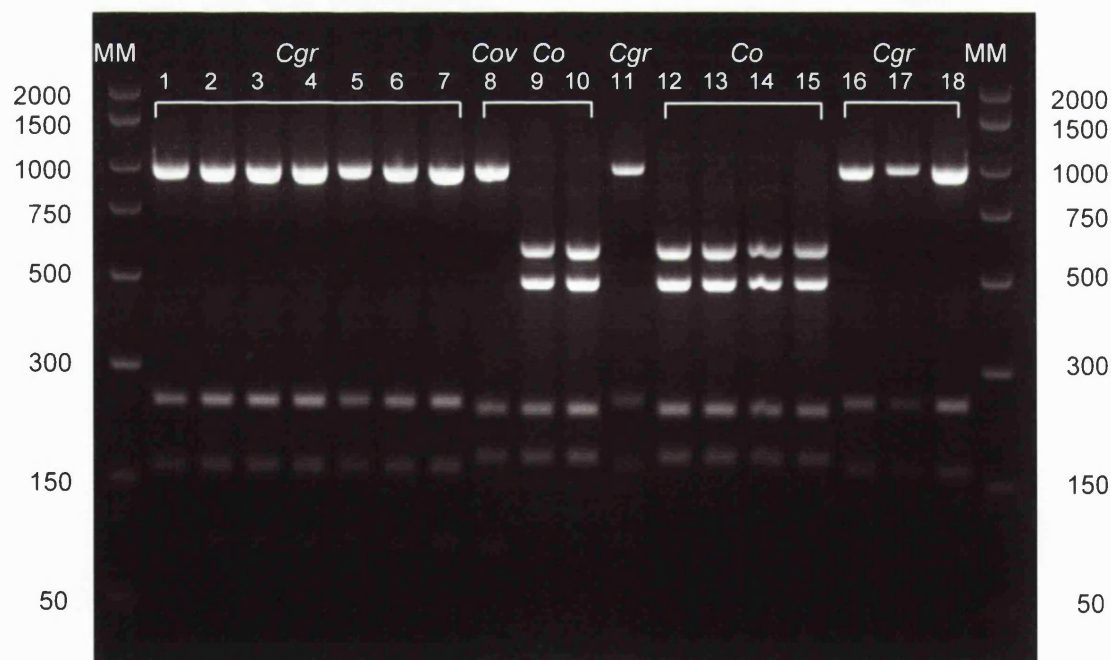
Fig.4.4 16S rRNA PCR-RFLP of clinical isolates of *Capnocytophaga* species with *Cfo* I. *Cov* = *C. ochracea* variant; *Co* = *C. ochracea*; *Cgr* = *C. granulosa*; MM = Molecular weight Marker.

4.3.4 *Capnocytophaga* clinical isolates: identification via 16S rRNA gene sequencing

Definitive identification of *Capnocytophaga* clinical isolates was achieved by comparing the sequenced 16S rRNA genes with those available on the RDP-II and BLAST databases. In the case of the RDP-II data, although a similarity value cut-off ≥ 0.8 had been set, some data points with a similarity value ≤ 0.8 had to be accepted as reprocessing of these strains e.g. *C. ochracea* variant strains, failed to yield better similarity values in spite of the electrophoretogram fulfilling the predetermined criteria. The *Capnocytophaga* 16S rRNA gene sequences contained on each of the two databases are listed in Table 4.2.

4.3.5 *Capnocytophaga* clinical isolates: comparison of identification obtained via 16S rRNA PCR-RFLP versus 16S rRNA gene sequencing

Validation of identification of clinical isolates via 16S rRNA PCR-RFLP versus 16S rRNA gene sequencing is presented in Table 4.3. The results showed that the sequencing data confirmed the initial identification of clinical isolates obtained via 16S rRNA PCR-RFLP only when the RFLP exactly matched that of the ATCC patterns used in this study. The identification results obtained via BLAST demonstrated better corroboration with the RFLP results than did the RDP-II data. The BLAST sequence results were more accurate and, unlike the RDP-II data, included all the *Capnocytophaga* oral clones deposited by Paster *et al.* (2001). This explained why some RDP-II sequence results, e.g. *C. ochracea* variant, gave low similarity values and were incorrectly identified as *C. sputigena* in spite of a good quality electrophoretogram. The lower number of sequences on the RDP-II databases accounted for the low level of correlation in identification between RFLP and RDP for the *C. ochracea* variant strains (41.8%). The corresponding comparison between RFLP and BLAST gave 100% correlation.

One *C. ochracea* (typical) strain (processed twice, hence n = 2) was incorrectly identified as *C. sputigena* via RDP in spite of the similarity values being 0.858 and 0.827. This same strain was identified as *Capnocytophaga* spp. oral clone X089 via BLAST.

Species identified via sequencing

Species identified via RFLP	No. of strains	ID correlation: RFLP versus RDP No. of strains (%)	ID correlation: RFLP versus BLAST No. of strains (%)
C. gingivalis (typical)	8	8 (100.0)	8 (100.0)
C. gingivalis (variant)	3	3 (100.0)	3 (100.0)
C. ochracea (typical)	46	44 (95.0)	46 (100.0)
C. ochracea (variant)	43	18 (41.8)	43 (100.0)
C. sputigena	7	7 (100.0)	7 (100.0)
C. granulosa	67	62 (93.9)	67 (100.0)
C. haemolytica	8	8 (100.0)	8 (100.0)
C. canimorsus	2	2 (100.0)	2 (100.0)
C. cynodegmi	3	2 (66.0)	3 (100.0)

Table 4.3 Validation of identification of *Capnocytophaga* spp. via 16S rRNA PCR-RFLP versus 16S rRNA sequencing

The strains initially identified as *C. canimorsus* via RFLP were identified as *C. gingivalis* 33624T / *Capnocytophaga* sp. str. S3 / *C. gingivalis* LMG 12118 when submitted to both databases, hence the corresponding RFLP pattern was labelled as *C. gingivalis* variant. As can be seen from Fig.4.5 (*in silico* analysis), the RFLP pattern of these strains simulated the RFLP for either *C. gingivalis* LMG 12118 or *C. canimorsus* ATCC 35979 more closely than it did the *C. gingivalis* ATCC 33624. Sequence alignment via ClustalW (version 1.8, 1999; Thompson *et al.* 1994) of *C. gingivalis* LMG 12118 with *C. canimorsus* yielded 91.2% similarity, demonstrating species difference rather than strain variation. *C. gingivalis* variant (*C g* LMG 12118) could be differentiated from *C. canimorsus* by performing a second, separate digest of the 16S rRNA gene using the RE *Hae* III. Alignment of *C. gingivalis* ATCC 33624 versus *C. gingivalis* LMG 12118 revealed 97.8% similarity (implying strain variation).

4.3.6 *In silico* PCR-RFLP analysis of 16S rRNA *Capnocytophaga* spp. genes available on database sequences

The results of the *in silico* analysis of representative strains of 16S rRNA *Capnocytophaga* spp. genes available on the RDP-II and BLAST databases are shown

in Fig.4.5. Strains of *C. gingivalis* and *C. ochracea* conformed to two patterns, whilst *C. sputigena*, *C. granulosa*, *C. haemolytica*, *C. canimorsus* and *C. cynodegmi* conformed to a single RFLP fingerprint. Strains with RFLP similar to the representative strains shown in Fig.4.5 are listed beneath the figure. Oral clones of *Capnocytophaga* spp. were also analysed *in silico* (Fig.4.6). Sequence alignment of *C. gingivalis, C. ochracea* and *Capnocytophaga* spp. oral clones showed some degree of intraspecies diversity.
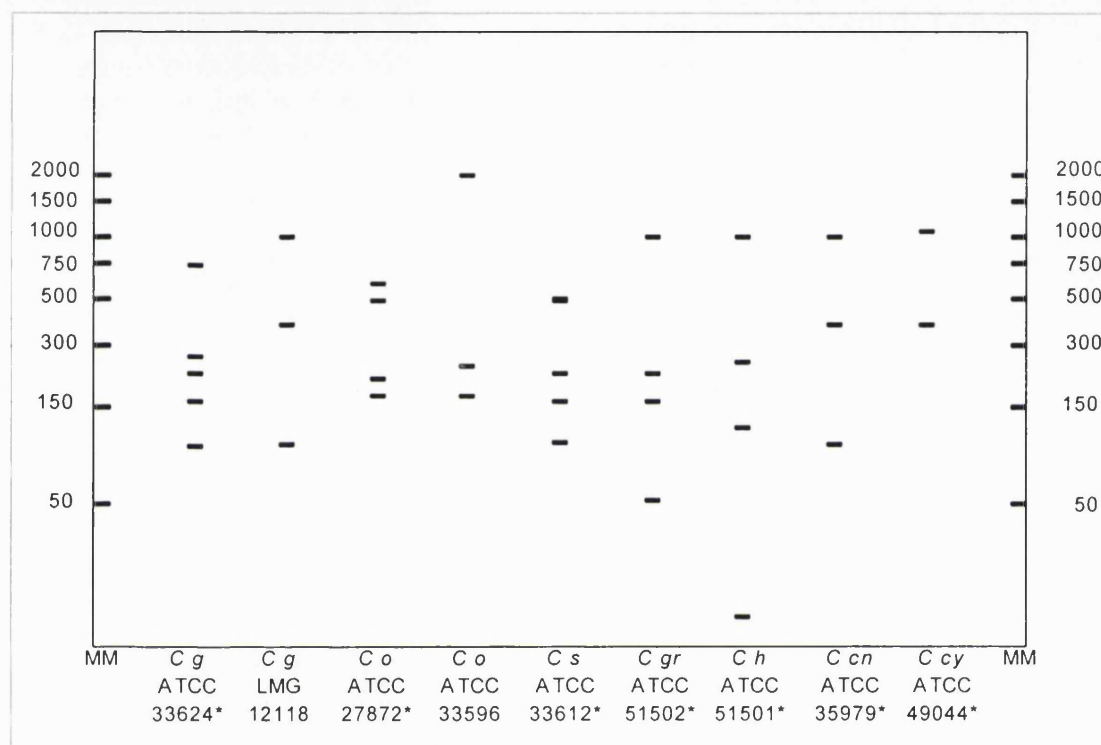
Fig.4.5 *In silico* 16S rRNA PCR-RFLP of representative *Capnocytophaga* spp. obtained from the RDP-II and BLAST databases

C g = *C. gingivalis; C o* = *C. ochracea; C s* = *C. sputigena; C gr* = *C. granulosa; C h* = *C. haemolytica; C cn* = *C. canimorsus; C cy* = *C. cynodegmi.* *Type strains used in this study; MM = Molecular weight marker

Strains with similar fingerprints to:

C g LMG 12118	= *Capnocytophaga* spp. str. S3
C o ATCC 27872T	= *Co* FDC 7b, LMG 12115, LMG 12117, *Capnocytophaga* spp. str. S1
C s 33612T	= *Cs* str. 897 CIP 100, *C s* 16S rRNA gene
C gr ATCC 51502	= LMG 16022, LMG 12119
C h ATCC 51502	= LMG 16021
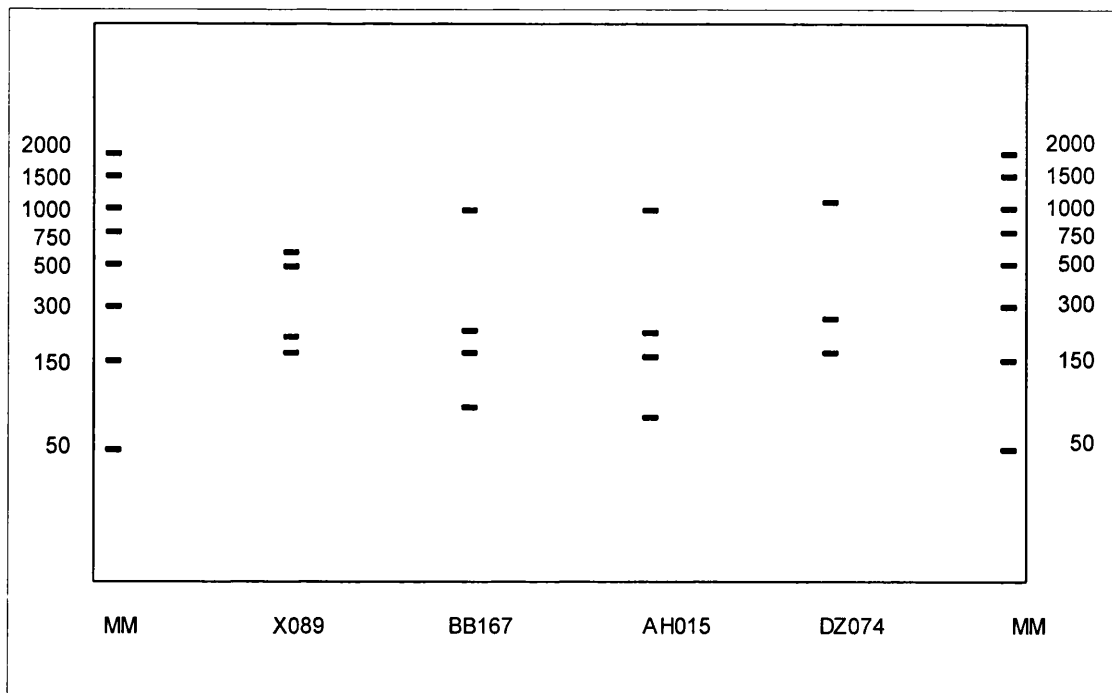
Fig.4.6 *In silico* 16S rRNA PCR-RFLP of representative oral *Capnocytophaga* clones obtained from the BLAST database (Paster *et al.* 2001)

Clones with similar fingerprints to:

X089 = EL043, DS022, AA032, X066, A47ROY, BU084

4.3.7 Phylogenetic analysis of *Capnocytophaga* spp. 16S rRNA genes located at RDP-II & BLAST databases

The results of the phylogenetic analysis are presented in Fig. 4.7. The dendrogram showed that the strains were very closely related, which was to be expected since these species all form members of the same genus. However, subdivisions within the genus can be observed resulting in the formation of three large clusters: *C. gingivalis* / *C. granulosa* group, the *C. canimorsus* / *C. cynodegmi* group and the *C. ochracea* / *C. sputigena* group. *C. haemolytica* spp. formed a separate, small group on its own.

The *C. ochracea* / *C. sputigena* formed the largest group and most of the oral clones fell into this group. The oral *Capnocytophaga* clone BU084 and the uncultured *Capnocytophaga* oral clone DS022, although closely related to the *C. ochracea* / *C. sputigena* group, formed a separate distinct branch in the dendrogram. This suggested that these could form a separate species. The results also showed that with the exception of 7 strains, all of these 16S rRNA gene sequences were isolated during the course of this study.

135
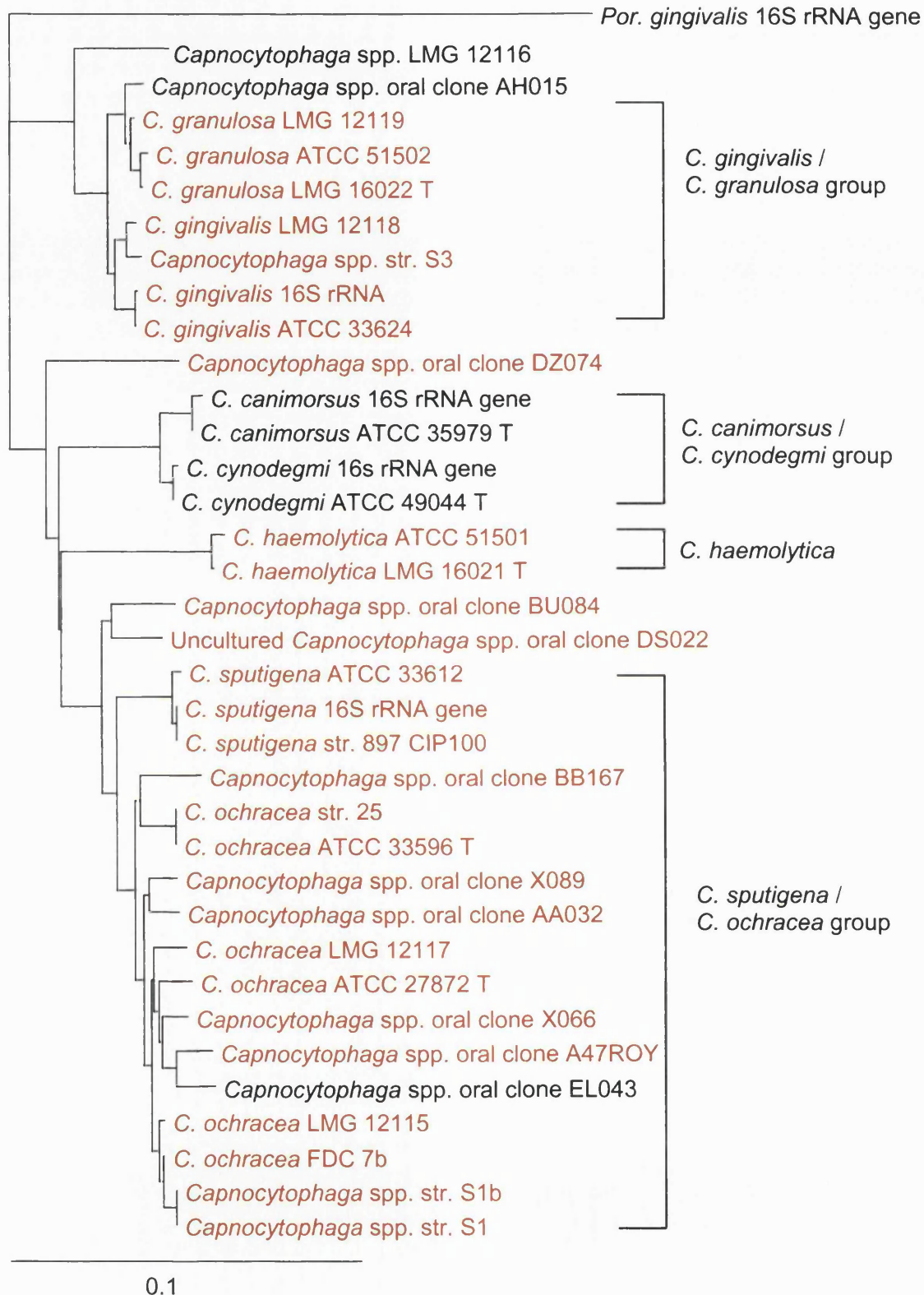
Fig.4.7 Phylogenetic tree for 16S rRNA genes of *Capnocytophaga* spp. & *Por. gingivalis* (reference strain) located on the RDP-II & BLAST databases based on 16S rRNA sequence similarity. Red coloured strains were isolated during this study. (*Capnocytophaga* spp. oral clone BM058 omitted – see text section 4.2.6).

4.4 DISCUSSION

Traditionally, identification of *Capnocytophaga* spp. has been performed using conventional biochemical methods (Socransky *et al.* 1979, Rummens *et al.* 1986, Forlenza *et al.* 1981, Jolivet-Gougeon *et al.* 2000). However such methods failed to distinguish between the more closely phylogenetically related species within this genus, e.g. *C. ochracea* and *C. sputigena* (Kristiansen *et al.* 1984, Speck *et al.* 1987). Such findings were corroborated by preliminary experiments in this investigation (data not shown), the results of which led to the exclusion of biochemical testing as a method of strain identification.

Several molecular methods (e.g. full gene sequencing, genomic restriction analysis, conventional ribotyping, 16S-23S intergenic spacer gene sequencing) are currently available for intrageneric differentiation of bacterial species. Indeed 16S rRNA sequencing is regarded as the gold standard in bacterial identification. Most of these procedures however are lengthy, time-consuming and expensive and therefore not accessible to most microbiology laboratories. Conventional ribotyping methods usually involve the hybridisation of restriction fragments of chromosomal DNA to respective probes via Southern blotting. Modification of the conventional ribotyping obviates the use of Southern blotting which is a labour intensive procedure. The main advantages of identification via 16S rRNA PCR-RFLP using this modified ribotyping method are that:

i)   it can be performed by the majority of laboratories

ii) it allows a high throughput of strains

iii) it is less expensive, and

iv) results can be obtained within 24 hr (maximum).

The known human oral strains (to date) of *Capnocytophaga* are *C. gingivalis*, *C. ochracea*, *C. sputigena*, *C. granulosa* and *C. haemolytica*. In this study the *C. canimorsus* and *C. cynodegmi* Type strains were included as, although they are inhabitants of the canine and feline oral flora (Brenner *et al.* 1989), they are capable of causing severe and sometimes fatal infections in humans (Griego *et al.* 1995). Thus a method for their rapid identification would be clinically important.

A large number of strains (187) were identified by two methods:

i) 16S rRNA PCR-RFLP analysis

ii) 16S rRNA gene sequencing and submission to RDP-II and BLAST databases.

Bacterial DNA was easily extracted by a 2 minute boil preparation. The DNA served adequately as a template for PCR thus precluding the need for lengthy DNA extraction protocols, further reducing procedure time.

The *Capnocytophaga* ATCC strains were sequenced to authenticate the identity of the Type strains. This was also performed to judge the quality of the sequences deposited on the 16S rRNA gene databases and which were to be subsequently used to identify the clinical isolates. These ATCC strains were concurrently subjected to 16S rRNA PCR-RFLP analysis and the results obtained from the two processes were compared. This was done to verify the suitability of RFLP as a method for their identification. The primers used for the PCR were 27f & 1492r. These were chosen as they are ideal primers for PCR amplification of 16S rRNA genes (Medlin *et al.* 1988).

The number of clinical isolates (187) identified using this strategy was deemed to comprise a representative sample of the collection of *Capnocytophaga* isolates (n = 848) obtained as a result of this investigation. The two parts of the study, i.e. 16S rRNA PCR-RFLP and 16S rRNA gene sequencing, were performed blindly to avoid any bias in interpreting the results. The sequence obtained for each strain was submitted to both RDP-II and BLAST databases. This eliminated the possibility that any differences in results from the database could have been due to differences in DNA sequencing results rather than actual differences between strains. Some strains were processed twice. This was done for 2 reasons:

i) as self quality control during 16S rRNA gene sequencing, and

ii) due to low RDP-II similarity values in spite of good quality electrophoretograms.

The 16S rRNA gene sequences for *Capnocytophaga* spp. available on the RDP-II and BLAST databases are summarised in Table 4.2. The number of 16S rRNA gene sequences on the BLAST database totals to 37 as compared to the 26 sequences on the RDP-II database. None of the oral clones of *Capnocytophaga* spp. located on the BLAST database were available on the RDP-II database. This explained why some strains identified as *C. ochracea* (typical) via RFLP and as oral clones of *Capnocytophaga* spp. via BLAST (one of which also gave a *C. ochracea* typical RFLP following *in silico* analysis, Fig.4.6) were identified as *C. sputigena* via RDP. These strains were reprocessed on different occasions using different sequencing primers (27f and 1492r were used as sequencing primers), however the same results were obtained. This could not have been the result of amplicon contamination as the negative control (water) was in fact negative. Rather they were identified as *C.*

138

sputigena by the RDP-database as this strain is closely related phylogenetically to *C. ochracea*.

This study was based on an initial 16S PCR-RFLP analysis by Wilson *et al.* (1995), in which they described 8 RFLP patterns for *Capnocytophaga* spp. Although some RFLP patterns (including variants) simulated the ones found in this study, comparison of RFLP fingerprints between studies was precluded as the authors used a 2kb DNA ladder, the lower fragment size of which was 154. This could have easily obscured fragments of smaller size found in most strains e.g. *C. gingivalis* ATCC 33624, *C. gingivalis* LMG 12118, *C. sputigena*, *C. granulosa*, *C. haemolytica* and *C. canimorsus* and therefore gave a distorted RFLP fingerprint. In addition, no sequencing data was available. Another factor that precluded direct comparison between this and the Wilson *et al.* (1995) study was that Wilson *et al.* modified the 27f and 1492r primers which they used for 16S rRNA gene amplification. Their 27f primer was longer than (though it included) the sequence used in this study. The 1492r primer was however completely different; the authors gave no reason why they modified the primers. A drawback of that study was that it supported the division of the genus *Capnocytophaga* into 3 species (*C. gingivalis*, *C. ochracea* and *C. sputigena*) but it did not reveal any information with respect to the other 4 remaining species (*C. granulosa*, *C. haemolytica*, *C. canimorsus* and *C. cynodegmi*) all of which are clinically relevant. In their phylogenetic analysis, the authors report the finding of 8 strains of *C. gingivalis*. Comparison of their results with the phylogenetic tree in Fig.4.7 suggested that some of their isolates could have been *C. granulosa*.

The division of the genus *Capnocytophaga* into 7 species was confirmed by Vandamme *et al.* (1996) using protein profile electrophoresis. They too reported on the division of *C. ochracea* into two groups. In that study, as in this one, considerable genotypic heterogeneity was noted within the genus *Capnocytophaga* in spite of minimal phenotypic differences. Whilst this might be due to previously unidentified species within this genus, percentage 16S rRNA similarity values greater than 97% are insufficient to guarantee a new species. It is not known whether these genetically different species are associated with specific pathological conditions.

The differences between the 16S rRNA PCR-RFLPs "typical" and "variant" strains could be explained following close examination of the *in silico* sequencing analysis. The latter showed that the variant RFLP fingerprints resulted either from a different

base or the presence of an insertion / deletion (indel) sequence at the restriction site. These nucleotide differences prevented restriction by the enzyme thus resulting in a different RFLP pattern.

The oral clones of *Capnocytophaga* spp. deposited by Paster *et al.* (2001) on the BLAST database seemed to be most closely related to *C. ochracea* when sequence alignment was performed using ClustalW. Those clones were obtained from subgingival plaque retrieved from different periodontal conditions, which did not include adult periodontitis, as distinct from this study, which seems to be the first to report the isolation of these species from subgingival plaque.

Comparison of the 16S rRNA sequencing results obtained from RDP-II and BLAST emphasised the importance of using a database, which is more up-to-date and contains a more diverse range of sequences. The main drawback is that although 16S rRNA gene sequencing is considered to be the gold standard for bacterial identification, sequences submitted for bacterial identification are compared with those available on the database, which, in some cases, might be partial sequences. Furthermore, the quality of the deposited sequences is not vetted in any way and the query sequence might not be available on the database thus giving a poor correlation because of its absences rather than because of it being of poor quality.

The phylogenetic relatedness of the *C. ochracea* & *C. sputigena* species explained the similar biochemical features shared between these two species. This similarity made biochemical differentiation between them impossible. This finding, experienced during the early course of this study (unpublished data), has been corroborated by other investigators (Kristiansen *et al.* 1984, Speck *et al.* 1987). The majority of the clones deposited on the BLAST database by Paster *et al.* (2001) have been cultured and identified during this study. These included the uncultured *Capnocytophaga* oral clone DS022. The phylogenetic relationship of the species explained the discrepancies in bacterial identification when the same sequence was submitted to the RDP-II and BLAST databases. Since the RDP-II database contained a smaller range of *Capnocytophaga* 16S rRNA gene sequences than did the BLAST database (Table 4.1) and did not contain any of the cloned sequences, all the sequences which were identified as oral *Capnocytophaga* clones by the BLAST database were identified as a closely related species within that cluster of organisms. Thus for example, strains identified as *C. ochracea* str. 25 on the RDP-II database were identified as

Capnocytophaga spp. oral clones BB167, X066 or A47ROY on the BLAST database. The absence of oral *Capnocytophaga* clones from the RDP-II database helped to explain the poor identification correlation (41.8%) between 16S rRNA PCR-RFLP and RDP (Table 4.3).

C. gingivalis variant (*Cg* LMG 12118) and *C. canimorsus* gave similar 16S rRNA PCR-RFLPs when *Cfo* I was used as the restriction digest. These strains could be easily differentiated with *Hae* III as the RE. In addition, they showed only 91.2% similarity when aligned using ClustalW, indicating that they were completely different species.

This study might be criticised on the basis that microbial culture could have biased the *Capnocytophaga* strains isolated during this project. This is highly unlikely as the culture medium used (i.e. FAA, Chapter 3) was shown to support the growth of all the *Capnocytophaga* Type strains and of the majority of identified strains located on the 16S rRNA gene databases. In addition, FAA supported the growth of an isolate which when identified was shown to be as yet uncultured (*Capnocytophaga* spp. oral clone DS022).

In conclusion, the results of this investigation have shown that 16S rRNA PCR-RFLP using *Cfo* I as the restriction enzyme is a reliable, rapid and accurate method for the identification of clinical isolates of *Capnocytophaga* spp. especially when large numbers of clinical isolates need to be identified. More importantly such methods are cost-effective and available to most routine microbiology laboratories thus allowing for more accurate identification of clinical isolates.

The isolation of *C. granulosa* and *C. haemolytica* from subgingival plaque and the identification of *Capnocytophaga* spp. via 16S rRNA PCR-RFLP, has been published as:

Capnocytophaga granulosa and *Capnocytophaga haemolytica*: novel species in subgingival plaque. Ciantar M, Spratt DA, Newman HN & Wilson M (2001). *J Clin Periodontol* 28: 701 – 705.

Chapter 5

Capnocytophaga spp. isolated from subgingival plaque & blood from DM- & non-DM-Periodontitis subjects

5.1 Introduction

Historically, an organism is held accountable for a particular disease if it fulfils Koch's postulates. Whilst this holds for many infections occurring at previously sterile sites in the human body (e.g. tuberculosis, tetanus), the situation is somewhat different when commensal organisms are involved. By definition, commensal organisms are those which routinely colonise the body surfaces without doing harm, a typical example being those found in dental plaque. However, infections at sites colonised by commensal organisms still arise, chronic gingivitis and periodontitis probably being typical examples. Shifts in the bacterial composition of dental plaque have been related to different periodontal conditions (Slots & Rams 1991). In DM-periodontitis, some studies have suggested that the microflora does not differ from that found in adult periodontitis (Zambon *et al.* 1988, Mandell *et al.* 1992), whilst others (Mashimo *et al.* 1983a), report that certain organisms e.g. *Capnocytophaga* spp., are recovered in higher numbers.

These conflicting reports may have arisen not only due to differences in basic scientific methods used to generate and collect data, but also because of the statistical methods employed during subsequent analysis. Chronic Periodontitis may be regarded as a heterogeneous disease process with different risk factors operating at both site- and subject-levels (Socransky & Haffajee 1997, Page 1998). Differences in its rate of progression are evident between subjects, between different sites within the same subject and at the same site at different time points (Löe *et al.* 1978, Goodson *et al* 1982, Socransky *et al.* 1984). There has been much debate in the periodontal literature as to whether site or subject should constitute the fundamental unit of analysis (see Chapter 1, section 1.6). This is where most studies differ in their statistical analysis. Indeed, the appropriate unit of analysis should be based on the medical / biological question being asked (Emrich 1990). In studies investigating microbial differences between sites, it is axiomatic to consider the site as the unit of statistical analysis. Since sites within a subject are nested within teeth, which are in turn nested within subjects, a hierarchy is established (Chapter 1, section 1.6.3). Any subsequent data interpretation must take this hierarchy into account if any reliable results are to be obtained. In addition, biological variation is to be expected at site-, tooth- and subject-levels. Such stratification (sites / teeth / subjects) which encompasses variation at each level, warrants the use of multilevel modelling (MLM, Gilthorpe *et al.* 2000b, c).

143

The recent resurgence of interest in the role of oral microflora in systemic disease, i.e. focal infection (Debelian *et al.* 1994, Newman 1996, Gendron *et al.* 2000), has been based on the notion that the ulcerated periodontal pocket surfaces may be a potential site of entry for oral bacteria (Page 1998, Seymour & Steele 1998). The focal infection theory (Miller 1890) proposes that seeding of pathogenic micro-organisms and / or their virulent components / metabolites present in local foci of infection (e.g. the oral cavity) may spread from such sites via the blood to distant body sites (Belizzi 1980, Newman 1996). Indeed, *Capnocytophaga* spp. have been known to cause such infections and the only potential site for systemic access seems to have been the oral cavity (Parenti & Snydman 1985, Warren & Allen 1986, Martino *et al.* 2001). However, to date no studies have attempted to recover *Capnocytophaga* spp. from blood taken from DM-Periodontitis patients.

Thus the aim of this study was three-fold:

i)   to isolate *Capnocytophaga* spp. from subgingival plaque taken from DM- and non-DM-Periodontitis patients

ii) to identify all the isolates to species level using 16S rRNA PCR-RFLP (Chapter 4), and

iii) to isolate *Capnocytophaga* spp. from blood taken from this cohort of patients using lysis filtration.

5.2 Materials & methods

5.2.1 Patient recruitment

Subjects recruited for this study were based on the inclusion and exclusion criteria outlined in Chapter 2, Tables 2.1 and 2.2. A detailed patient history and periodontal examination were performed (Chapter 2).

5.2.2. Subgingival plaque samples

Subgingival plaque samples were collected and processed as described in Chapter 2, section 2.2.3. After each plaque sample had been incubated for 5 days, the total *Capnocytophaga* counts (T*Capno*) per plate were recorded and expressed as colony forming units per ml (cfu ml^{-1}). Similarly, the total anaerobic counts (TAC) per plate were calculated and expressed as cfu ml^{-1}.

5.2.3. Blood samples for lysis filtration

In addition to the blood sample drawn for quantification of glycated haemoglobin (Fig.5.1a), a second blood sample (10 ml) was drawn from the same site using an EDTA K_3 Vacutainer tube (Beckton Dickinson). This sample was subjected to lysis filtration (Heimdahl *et al.* 1985). Immediately after collection, each sample was taken to the laboratory and divided into 2 aliquots:

i) 6 ml were immediately processed by lysis filtration;

ii) 4 ml were stored at -70°C for future use.

The 6 ml blood sample was aseptically transferred to 180 ml of lysis solution (Appendix 6) that had been previously warmed to 37°C (Fig.5.1b). After incubating the blood-lysis solution mixture for 10 min at 37°C, vacuum filtration was performed using a 47 mm diameter, 0.45 μm pore size filter (Millipore, Sartorius AG, Goettingen, Germany; Fig.5.1c). The filters were aseptically cut in half; one half of the filter was placed on a previously labelled FAA plate supplemented with 5% horse blood (E&O Labs) and incubated anaerobically at 37°C (80% N_2, 10% H_2, 10% CO_2; MACS-MG-100 Anaerobic Workstation) for five days. The other half of the filter was placed on a blood agar (Lab M, Lancashire, UK) plate also supplemented with 5% horse blood (E&O Labs) and incubated aerobically at 37°C for 5 days. If any colonial growth was observed on the filter after the stipulated period of incubation (Fig.5.1d), the colonies were subcultured to purity on the respective plates and grown under the same conditions. Preliminary identification was performed via phenotypic features, Gram-stain reaction (Chapter 2, section 2.3.1) and biochemical tests (catalase & oxidase, Appendix 7).
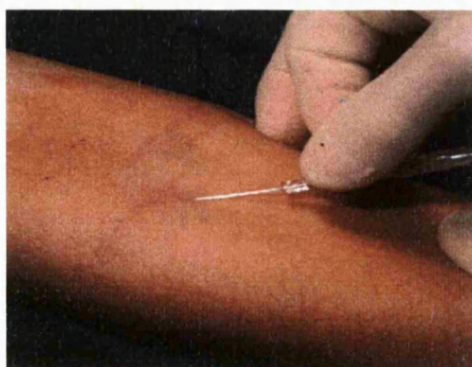
Fig.5.1(a) Venepuncture for blood sample   (b) Blood incubated in lysis solution
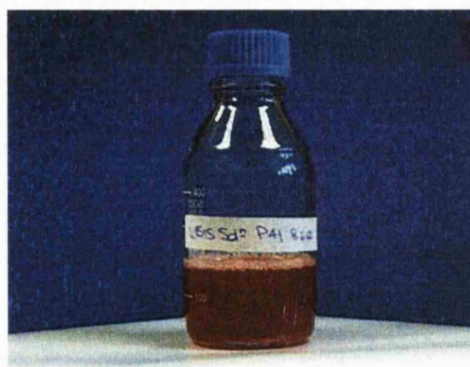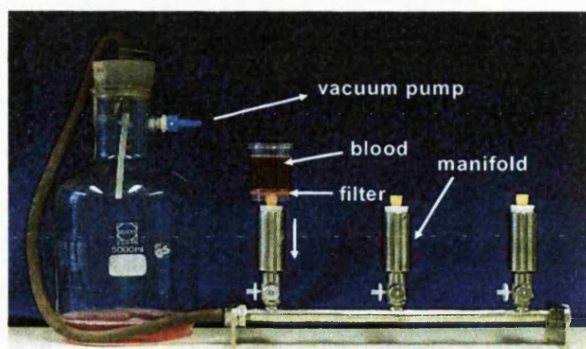
Fig.5.1(c) Blood lysis filtration kit   (d) Bacterial growth on filter

5.3 Statistical analysis

5.3.1 Determination of sample size

Standard sample power calculations were performed using SPSS statistical package (SPSS Manual, 1990) undertaken for a two-sided proportions test and *t*-test for two independent samples with common variance on several estimates of results from the literature, where the outcomes were either mean percentage of *Capnocytophaga* spp. (Mashimo *et al.* 1983a, Zambon *et al.* 1988, Sastrowijoto *et al.* 1989, Sbordone *et al.* 1995) or number of colony forming units per ml (Müller *et al.* 1997). Based on this limited information, this study aimed to collect a sample size of 25 patients for each of the test and control groups i.e. total of 50 patients.

5.3.2 Determination of number of sites per subject

Based on the above calculation with 25 subjects in each group, it was anticipated that one test and one control sample per patient would suffice to detect any significant differences in outcome measures (bacterial counts and GCF glucose). However, a total of 6 samples were collected from each patient i.e. 3 samples from periodontally diseased sites and 3 samples from periodontally healthy sites for the following reasons:

i)   in order to overcome any potential loss of sample (and hence loss of patient)

ii)   since it is easier to collect more samples per patient rather than recruit more patients, and

iii) to further increase the statistical power of the analysis.

5.3.3 Raw data

5.3.3.1 Measures of normality: skewness and kurtosis

The distribution properties of the raw data counts (cfu ml^{-1}) for TAC and T*Capno* were checked for skewness (i.e. the degree of asymmetry of a distribution around the mean) and kurtosis (i.e. the degree of smoothness of the distribution curve) prior to analysis. Since each data set was not normally distributed, it was transformed in order to achieve normal distribution. Logarithmic transformations resulted in zero skewness and minimal kurtosis. Data were initially entered on an Excel spreadsheet (Microsoft Excel for Windows 95 version 7) and subsequently converted for use by *MLwiN* (Rasbash *et al.* 2000).

5.3.3.2 Coding prior to modelling

Site status and DM status were binary variables and were therefore coded as 0 or 1 for: healthy versus diseased sites, and non-DM versus DM subjects.

5.3.4 Within- and between-patient group comparisons

Statistical comparisons for patients' clinical data within (i.e. healthy versus diseased sites in the same group) and between patient groups (i.e. DM-Periodontitis versus non-DM-Periodontitis) were performed using single-level analyses in SPSS version 10. Parametric tests were used for quantitative data, i.e. HbA$_{1c,}$ age, PPD, LCAL and GCF volume. Two-sample independent *t*-tests were used for comparing patients' age and HbA$_{1c,}$ as well as for comparing PPD and LCAL between groups. Within group comparisons for PPD, LCAL and GCF were performed using paired *t*-tests. The Plaque Indices and Bleeding Indices (ranked data) within and between groups were compared using the non-parametric *Mann-Whitney U* test. The assumptions underpinning the use of these tests were satisfied.

It is imperative to point out at this stage that significant differences between groups for HbA$_{1c}$ between DM-Periodontitis and non-DM-Periodontitis subjects, and PPD and LCAL between healthy and diseased sites were expected as these were the clinical features which defined the categories being compared in this study. Single-level analyses were thus used to justify retrospectively the differences between the categories.

5.3.5 Data hierarchy and content

A 2-level hierarchy was used when modelling this data set as follows (Fig.5.2):

Covariates:

Level 2   [Subject]   ◄────── DM vs non DM

                                ▲
                                │ Interaction (DM-status)
                                ▼

Level 1   [Site]   ◄────── Site status: healthy vs diseased

Fig.5.2 A (2-level) multilevel model
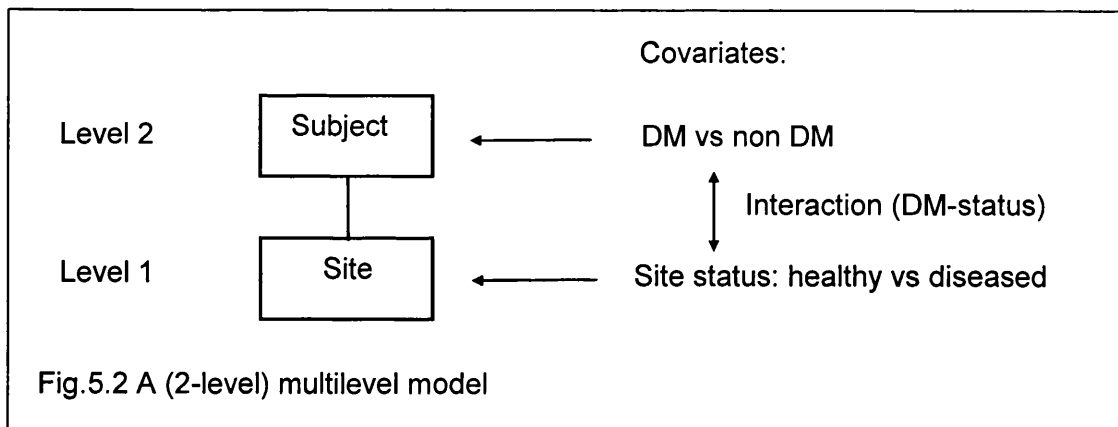
Subjects were at the higher level of the hierarchy (level-2) whilst sites occupied the lower level of the hierarchy (level-1; Fig.5.2).

The subject-level covariate (explanatory variable) was DM status whilst that site-level covariates were site status (healthy versus diseased sites).

The outcome variables were:

i)   total *Capnocytophaga* counts

ii)   total anaerobic counts

Data analysis was performed using the multilevel modelling (MLM) statistical package *MLwiN* (Rasbash *et al.* 2000). For all models, significance was assumed at the 5% level. Model assumptions for the variance components models and the covariate (Random Intercept, see below) models were confirmed through analysis of model residuals generated by the software (Fig.5.4, 5.5, 5.8, 5.9). The distribution of residuals (performed by plotting the standardised residuals versus their normal scores) was used to assess the normality assumptions underpinning the modelling process. Linearity assumptions were also checked by plotting residuals against their covariates.

5.3.6 Variance Components (VC) models

In order to determine the mean value for each outcome variable, as well as the degree of variation (variance) at each level of the data hierarchy (i.e. at subject- and site-levels), variance components models were developed for the transformed T*Capno* counts and TAC. In each variance components model the mean (intercept) and the variance at each level were estimated using the *MLwiN* Restricted Iterative Generalised Least Squares (RIGLS) algorithm provided within the software package. Instead of having the usual (single-level) construct of a mean and standard deviation,

148

the multilevel variance components model yields the overall mean and the degree of variation that occurs across each level of the hierarchy. The formulaic expression for the VC model for the 2-level data structure used in this study was:

$$y_{ij} = \beta_0 + e_{0ij} + u_{0j}$$

where β_0 = mean value of the outcome variable y_{ij}. The total variance is partitioned across each level:

$e_{0ij} \sim N(0, \sigma^2_{0e})$ where zero is the mean value and σ^2_{0e} is the variance across sites;

$u_{0j} \sim N(0, \sigma^2_{0u})$ where zero is the mean value and σ^2_{0u} is the variance across subjects.

If the variances within the components models are significantly different from zero, then this justifies the use of MLM for data analysis.

5.3.7 Random Intercepts (RI) models

RI models were developed by incorporating explanatory variables in the variance components models, in the same way that single-level multiple regression analysis incorporates explanatory variables. However, in a multilevel framework, these covariates may operate at a specific level (i.e. the subject- or site-level) of the hierarchy. For each subject-level covariate such as DM, all sites within each subject have the same mean relationship between the outcome variable and covariate(s). For site-level covariates such as site-status, every site within each subject may have a different relationship between the covariate and outcome, dependent on the covariate value.

Model improvements were assessed using the -2 loglikelihood (-2LL) statistic which is automatically outputted by the software. The change in the -2LL between successive models follows a χ^2 distribution, with the degrees of freedom equal to the number of covariates either added or subtracted (but not both) to the model.

5.3.8 Multivariate Multilevel Modelling (MV MLM)

ML MVM was performed to investigate the role of several explanatory variables (DM and site-status) on *each* of the *Capnocytophaga* spp. (which had been previously identified via 16S rRNA PCR-RFLP; Chapter 4). The 7 different species were *C. gingivalis*, *C. ochracea*, *C. sputigena*, *C. granulosa*, *C. haemolytica*, *C. gingivalis* variant, *C. ochracea* variant.

The MV MLM is given by the simultaneous regression equations:

$$y_{ij}^{(k)} = \beta_{0ij}^{(k)}$$

where y_{ij} = logit $(p_{ij}^{(k)})$ = ln $(p_{ij}^{(k)}/ 1- p_{ij}^{(k)})$ where $p_{ij}^{(k)}$ expresses the probability (between 0 & 1) of the outcome (e.g. *C. gingivalis*) being present across both sites and subjects. (The *logit* transformation is used because the outcomes are binary; hence *logistic regression* was used).

k = 1, ...7 for all seven outcomes; $\beta_{0ij}^{(k)}$ = intercept = $\beta_0^{(k)} + u_{0j}^{(k)} + e_{0ij}^{(k)}$; where $e_{0ij}^{(k)}$ = site-level residuals for each outcome, which are normally distributed and have a mean of zero; u_{0j} = subject-level residuals for each outcome, which are normally distributed and have zero mean, and $\beta_0^{(k)}$= mean value of the outcome variable $y^{(k)}$.

The absence of an organism at a site was coded as 0, whilst its presence was coded as 1. Since the multiple responses were binary (and hence binomially distributed), logistic regression analysis was performed. Missing data were coded as -99, which informed the software not to include these data records. Sensitivity tests were performed to evaluate the effect of different ways of coding missing data. A multivariate VC model using the RIGLS algorithm was set up to establish the mean (intercept) and the variance / covariance structure for all 7 organisms simultaneously. The explanatory variables included in this model were DM, site-status and their interaction.

As in the VC models described for MLM, in MV MLM there is a measure of variance associated with each outcome. Furthermore, there are additional covariance terms derived: one for each pairwise combination of outcomes. This more sophisticated variance structure yields an array of outcome correlations, derived by the following formula:

$$Corr\,(O_k, O_l) = \frac{Cov\,(O_k, O_l)}{\sqrt{var\,(O_k)\,var(O_l)}} \quad \text{................Equation (1)}$$

where O_k = outcome variable k (1.........7) and O_l = outcome variable l (1......7).

It is important to note that the overall (total) correlation between any two outcomes is partitioned by the hierarchy. In other words, the correlation between sites (within subjects) is separately determined from the correlation between subjects. Once covariates are included and if these covariates "explain" the outcome variation, then the overall (total) variance / covariance will reduce.

5.4 Results

5.4.1 Patient data

The final number of patients recruited for this study totalled 46; their clinical details are summarised in Tables 5.1 and 5.2.

Subject status No. of sites	DM-Periodontitis (n = 21)	Non-DM-Periodontitis (n = 25)
Female	12	14
Male	9	11
Age (yrs)	52 (46.5, 57.0)	45.0 (43.0, 55.0)
HbA_{1c} (%)[†]	9.3 (8.0, 10.0)	5.3 (4.97, 5.72)
Duration (yrs)	8.0 (5.0, 13.5)	0
Ethnic group: Caucasian	6	19
Asian	10	5
Other	5	1

Table 5.1 Summary of patients' clinical details.

[†] = DM greater than non-DM ($P < 0.001$). All data are presented as the median (interquartile range). Independent t-tests were used for HbA_{1c} and age.

The level of HbA_{1c} was significantly greater in DM-Periodontitis subjects ($P < 0.001$). No significant difference was observed for age.

Subject status	DM-Periodontitis		Non-DM-Periodontitis	
Site-status	Healthy [†#]	Diseased[¶]	Healthy [†#]	Diseased[¶]
No. of sites	63	63	75	75
PPD[†#¶] (mm)	3.0 (2.0, 3.0)	7.0 (6.0, 8.0)	2.0 (2.0, 3.0)	7.0 (6.0, 8.0)
LCAL[†#¶](mm)	0.0 (0.0, 3.0)	7.0 (6.0, 10.0)	0.0 (0.0, 3.0)	7.0 (6.0, 8.0)
PI*	1.0 (1.0, 1.0)	1.0 (1.0, 2.0)	0.0 (0.0, 1.0)	1.0 (1.0, 1.0)
BI[†#¶]	0.0 (0.0, 0.0)	2.0 (1.0, 2.0)	0.0 (0.0, 0.0)	2.0 (0.0, 2.0)

Table 5.2 Summary of periodontal clinical details. All data are presented as the median (interquartile range). Paired t-tests were used for PPD and LCAL; *Mann-Whitney U* test was used for PI & BI.

[†] Significant difference between healthy & diseased sites within groups ($P < 0.001$)

[#] No significant difference between healthy sites between groups ($P = 0.280$)

[¶] No significant difference between diseased sites between groups ($P = 0.280$)

* PI significantly different between healthy sites between groups ($P < 0.003$)

151

5.4.2 Subgingival plaque samples

The total number of plaque samples processed (Chapter 2, section 2.2.3) was 276 (46 patients x 6 sites per patient). The total number of *Capnocytophaga* spp. isolated and identified during this study amounted to 848. These were identified to species level via 16S rRNA PCR-RFLP with *Cfo* I as restriction enzyme (Chapter 4). The actual numbers and percentage proportions of each of the *Capnocytophaga* spp. are presented in Table 5.3.

Species	Actual Number (n = 848)	Percentage of total isolates
C. gingivalis	44	5.2
C. gingivalis variant	34	4.0
C. ochracea	366	43.8
C. ochracea variant	136	15.4
C. sputigena	52	6.1
C. granulosa	210	24.8
C. haemolytica	6	0.7

Table 5.3 Numbers of *Capnocytophaga* spp. isolated from subgingival plaque from all subjects.
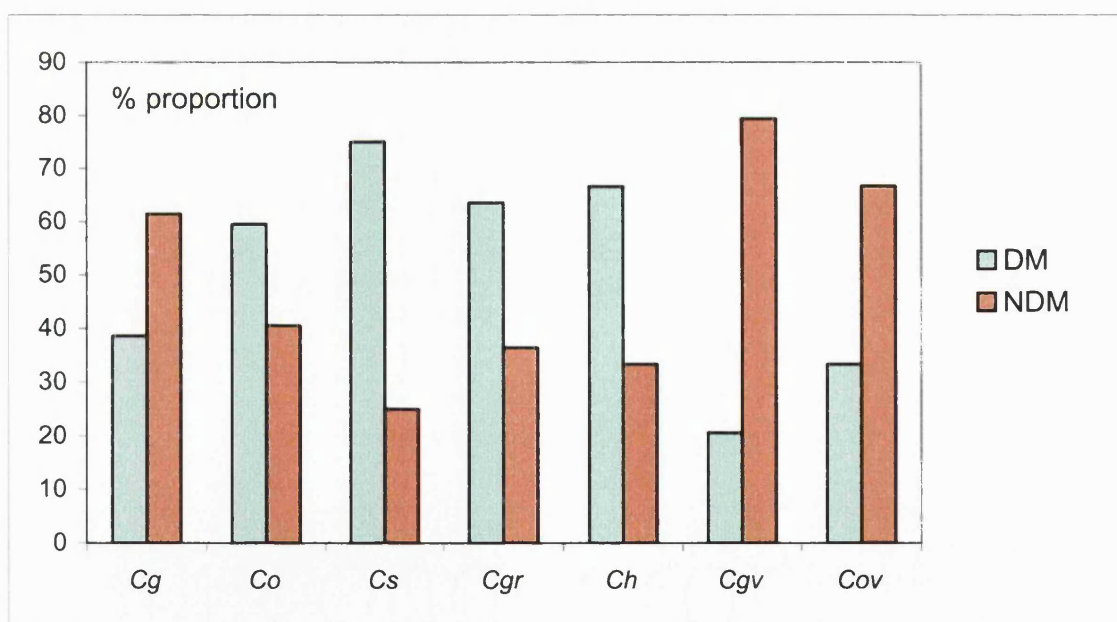
Fig.5.3 Percentage proportion of *Capnocytophaga* spp. in DM- and non-DM-Periodontitis subjects. *Cg* = *C. gingivalis*, *Co* = *C. ochracea*, *Cs* = *C. sputigena*, *Cgr* = *C. granulosa*, *Ch* = *C. haemolytica*, *Cgv* = *C. gingivalis variant*, *Cov* =*C. ochracea variant*.

Data analysis for T*Capno* and TAC was performed on 21 DM-Periodontitis subjects (Test) and 25 non DM-Periodontitis subjects using MLM and MV MLM.

5.4.2.1 VC Models for T*Capno* counts and TAC

The results from the VC model (i.e. explanatory variables not included; Table 5.4) for the outcome variable T*Capno* counts (or rather their *log* transformations) showed that the estimate for the intercept (mean) was significantly greater than zero at 8.47 ($P <$ 0.001) with a standard error of 0.32. This finding is expected, as the mean would inevitably be non-zero. It is more important that the variance terms are significantly different from zero, which they were.

	Estimate (95%CI)	*P*-value	
Fixed structure: Intercept (mean)	8.47 (7.83, 9.10)	<0.001	
Random structure	Variance (SE)		Proportion of total
Level-2 (subject)	3.45 (1.00)	<0.001	31%
Level-1 (site)	7.65 (0.72)	<0.001	69%
Total	11.10		100.0%

Table 5.4 VC model for T*Capno* counts (*log* transformed); N = 270. SE = standard error.

The level-2 (i.e. between subject) variance accounted for 31% of the total variance, while level-1 (i.e. between sites within subjects) variance accounted for 69% of the variance. These significant variances at each level (i.e. the coefficient being at least twice the SE), along with their sizeable proportion of the total variation, indicated that there was sufficient variation at each level to require the use of a hierarchical modelling approach. Ignoring this hierarchy would be erroneous. In addition, the large variance at site-level further justified the use of multiple site data per subject analysed using MLM rather than as pooled samples aggregated to subject level.

The results from the variance components model for the TAC (Table 5.5) showed that the estimate for the intercept (mean) was equal to 13.31 ($P < 0.001$). The estimate of the variances at the subject-level (1.83) and the site-level (5.23) were significantly different from zero ($P = 0.002$ and $P < 0.001$ respectively).

	Estimate (95%CI)	*P*-value	
Fixed structure Intercept (mean)	13.31(12.83, 13.79)	<0.001	
Random structure	Variance (SE)		Proportion of total
Level-2 (subject)	1.83 (0.58)	0.002	26%
Level-1 (site)	5.23 (0.50)	<0.001	74%
Total	7.06		100%

Table 5.5 Variance components model for TAC (log transformed); N = 263. SE = standard error

The subject-level variance accounted for 26% of the total variance whilst the site-level variance accounted for 74%. This demonstrated the presence of sufficient variance at each level to require the use of MLM for statistical analysis. The large variance at site level justified the use of multiple site data per subject.

The model assumptions underlying MLM were checked by plotting the ranked-standardised residuals (standardised residuals) against the standard normal cumulative distribution (normal score) for both T*Capno* and TAC counts (Fig. 5.4 and Fig. 5.5). These showed that the normality assumptions were satisfied since the plots demonstrated a near-linear relationship.
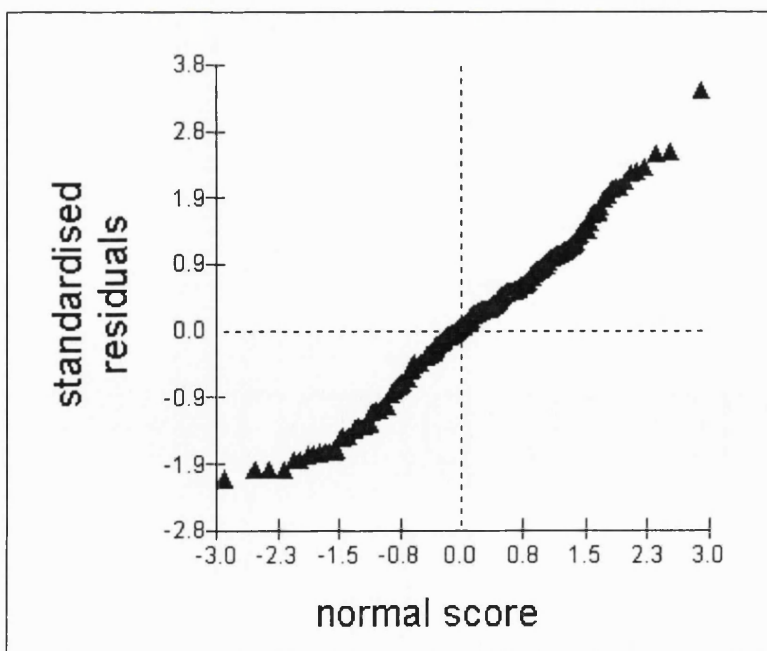
Fig.5.4 Residuals from Variance Components model for T*Capno*.
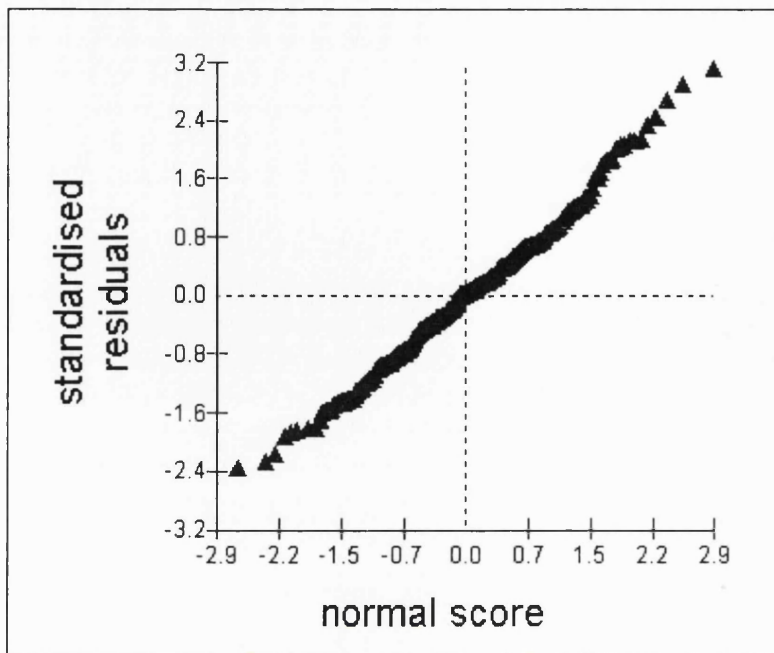
Fig.5.5 Residuals from Variance Components Models for TAC

5.4.2.2 RI models T*Capno* counts and TAC

The RI models for T*Capno* (Table 5.6) and TAC (Table 5.7) were generated after introducing DM (NDM = 0; DM =1) and site-status (healthy = 0; diseased = 1) as explanatory variables in the VC models. This was done initially using each explanatory variable (DM and site-status) separately.

Fixed effects (covariates)	Estimate (95%CI)	*P*-value
Intercept	7.83 (7.01, 8.65)	< 0.001
DM	1.39 (0.17, 2.61)	0.025
Random Effect	Variance (SE)	
Subject (level-2 σ^2_{ou})	3.06 (0.92)	<0.001
Site (level-1 σ^2_{oe})	7.65 (0.72)	<0.001
Total	10.71	

Table 5.6 RI Model for the effect of DM on the outcome variable T*Capno* counts (*log* transformed)

The RI model for the effect of DM on the T*Capno* counts (Table 5.6) showed that there was an 11% reduction in variance at the subject-level from 3.45 (Table 5.4) to 3.06 (Table 5.6). This meant that the presence of DM explained some of the variation at the

155

subject-level. The level of variance at site-level remained unchanged, which was to be expected as the DM covariate operates only at the subject level. In addition, the data showed that the T*Capno* counts in the DM-Periodontitis subjects were significantly higher than the T*Capno* counts in the non-DM-Periodontitis subjects ($P < 0.025$; Fig. 5.6).
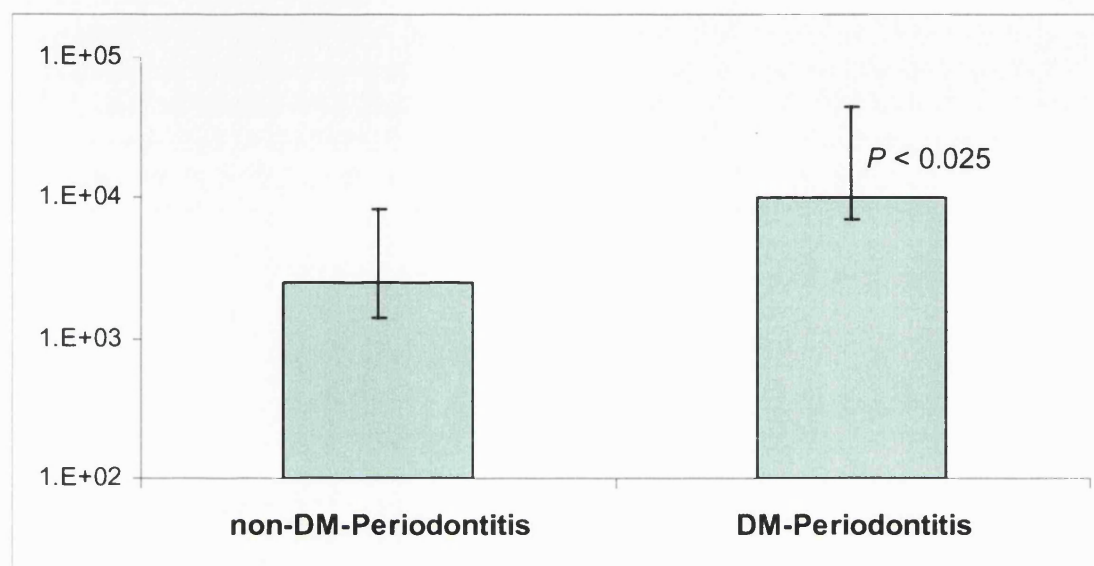
Fig.5.6 Mean counts for T*Capno* (cfu ml^{-1}) in non-DM-Periodontitis & DM-Periodontitis subjects. Bars represent 95% CI.

The RI model for the effect of site-status on the T*Capno* counts is shown in Table 5.7

Fixed effects (covariates)	Estimate (95%CI)	P-value
Intercept	7.80 (7.09, 8.52)	< 0.001
Site-status	1.31 (0.67, 1.96)	< 0.001
Random Effect	Variance (SE)	
Subject (level-2 σ^2_{0u})	3.59 (1.01)	<0.001
Site (level-1 σ^2_{0e})	7.16 (0.68)	<0.001
Total	10.75	

Table 5.7 RI model for the effect of site-status in the outcome variable T*Capno* counts (log transformed)

The RI model showed that the variation at subject level increased from 3.45 (Table 5.4) to 3.59 (Table 5.7). The site level variation decreased by 6.4% from 7.65 (Table 5.4) to 7.16 (Table 5.7). As the status covariate operated at the site level, it was expected that the variance at this level decreased; that fact that variance increased slightly at the

subject level was surprising. However, the subject level changes were well within the standard errors. Furthermore, it is likely that there exists some interaction between the DM and site-status covariates, which might also give rise to this unexpected change in subject level variance from the site-level covariate. The results of this analysis showed that the T*Capno* counts in diseased sites were significantly higher than in the healthy sites ($P < 0.001$; Fig.5.7).
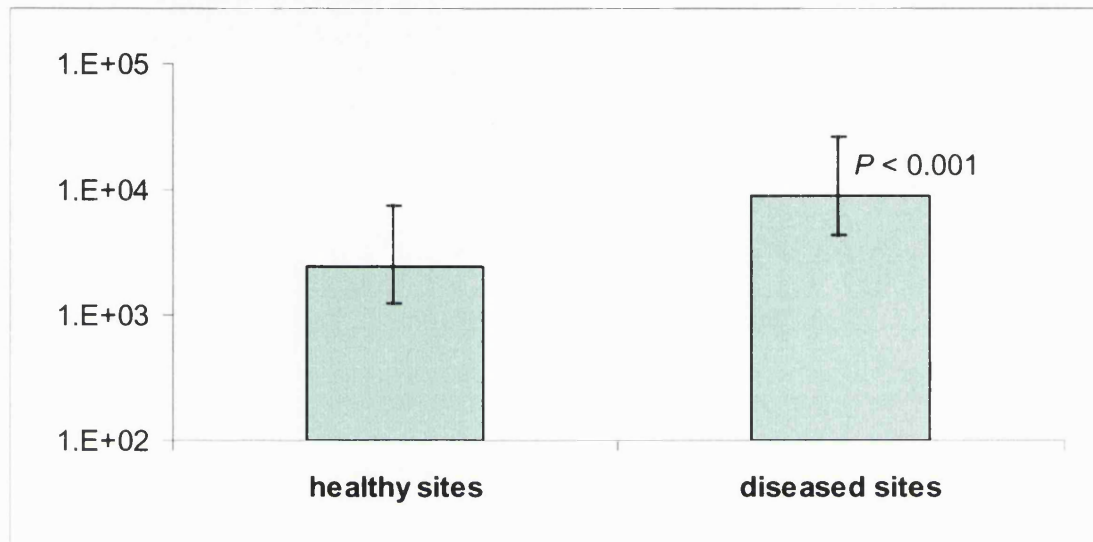
Fig.5.7 Mean counts for T*Capno* (cfu ml^{-1}) in healthy and diseased sites. Bars represent 95% CI.

Model assumptions were checked for both models by plotting the standardised residuals versus normal plots (Fig.5.8 & 5.9)
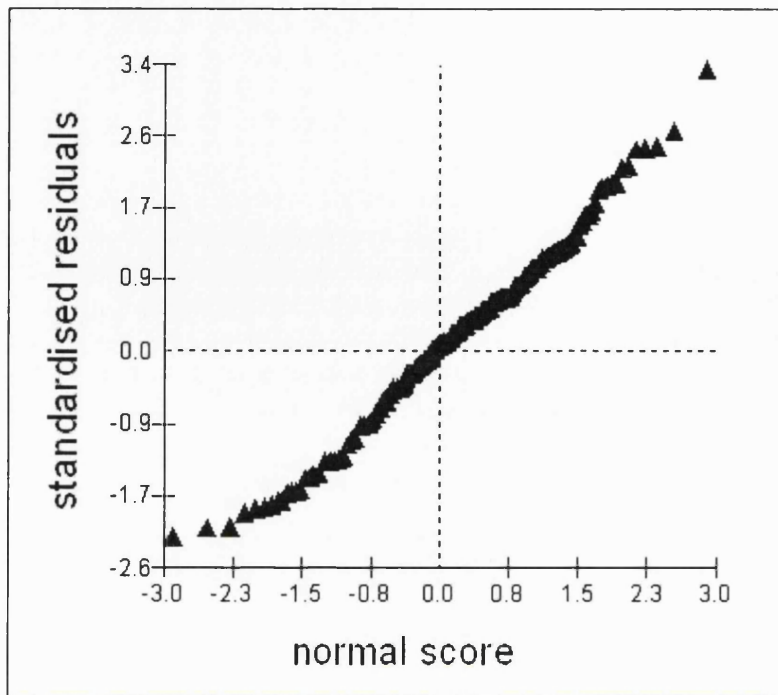
Fig.5.8 Residuals from DM model for T*Capno* counts
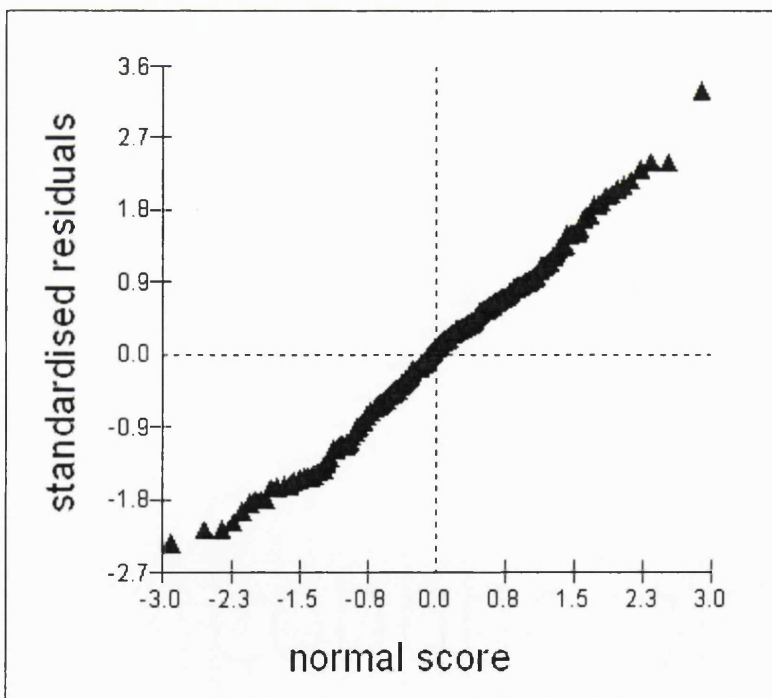
Fig.5.9 Residuals from site-status model for T*Capno* counts

The graphs demonstrated near-linear relationship, thereby showing that the model assumptions were satisfied.

These results show that when DM and site-status were analysed independently as explanatory variables, the T*Capno* counts were significantly higher in the DM-Periodontitis subjects than in non-DM-Periodontitis subjects ($P < 0.025$); these counts were also significantly higher ($P < 0.001$) in the diseased sites when compared to the healthy sites. However since this is a biological model and the two explanatory variables are not anticipated to operate independently *in vivo*, an interaction between DM and site-status was also investigated.

Fixed effects (covariates)	Estimate (95%CI)	*P*-value
Intercept	6.98 (6.04, 7.92)	<0.001
Subject-level (level-2) DM	1.78 (0.39, 3.16)	0.012
Site-level (level-1) Site-status	1.64 (0.76, 2.52)	<0.001
Interaction DM & site-status	-0.68 (-1.98, 0.62)	0.302
Random Effect	Variance (SE)	
Subject (level-2 σ^2_{ou})	3.16 (0.92)	<0.001
Site (level-1 σ^2_{oe})	7.16 (0.68)	<0.001
Total	10.32	

Table 5.8 RI model for the outcome variable T*Capno* (DM, Site-status & Interaction).

The results of the RI model for T*Capno* showed an 8.4% reduction in variances from 3.45 in the VC model (Table 5.4) to 3.16 (Table 5.8) at the subject-level, and a 6.4% reduction in variance from 7.65 (Table 5.4) to 7.16 (Table 5.8) at the site-level. This meant that the covariates explained some of the variation at both the subject- and site-levels. The results indicated that both DM and site-status were significant positive risk factors for the presence for *Capnocytophaga* spp. at these sites.

The interaction between the presence of DM and site-status had a sizeable effect on the outcome (coefficient of -0.68) which was too large to be ignored even though it was not formally significant at the 5%-level. However, this term needs to be interpreted in conjunction with each coefficient for both DM and site-status covariates as illustrated in Table 5.8 for this model. To correctly evaluate the significance of the interaction coefficient, it is necessary to evaluate the overall model improvement between the

models with and without the interaction term included. This was done using the model fit parameter -2 LogLikelihood (-2LL; see section 5.3.7 RI Models, p. 149). In this model the interaction was not significant (P = 0.302), but this could be well due to insufficient statistical power. For this reason, given that the interaction was both plausible (even likely) and quite sizeable, the preferred and final model was that which included the interaction.

Fixed effects	Estimate (95%CI)	P-value
Intercept	11.62 (10.92, 12.32)	<0.001
Subject-level (level-2)		
DM	1.76 (0.72, 2.81)	0.001
Site-level (level-1)		
Site-status	2.15 (1.47, 2.83)	<0.001
Interaction: DM & site-status	-0.81 (-1.83, 0.21)	0.118
Random Effects		
Subject-level (level-2 σ^2_{ou})	1.64 (0.50)	0.001
Site-level (level-1 σ^2_{oe})	4.26 (0.41)	<0.001
Total	5.90	

Table 5.9 RI model for the outcome variable TAC (DM, Site-status & Interaction).

There was a 10.4% decrease (from 1.83 to 1.64) in subject-level variance and an 18.5% decrease (from 5.23 to 4.26) at the site-level (Tables 5.5 and 5.9) when covariates were added to the VC model for TAC. This showed that these explanatory variables contributed to some of the variation in the outcome across both levels. The results showed that both DM and site-status acted as highly significant positive risk factors for TAC. When correctly evaluating the significance of the interaction coefficient using the model fit parameter -2LL, this was not significant (P = 0.120). However, this could again be due to insufficient statistical power. For this reason, given that the interaction was both plausible and sizeable, the preferred and final model was that which included the interaction.

Model assumptions for the combined and interaction models for T*Capno* and TAC were checked by plotting the standardised residuals versus normal plots (Figs. 5.10 & 5.11). The graphs demonstrate a near-linear relationship, thereby showing that the model assumptions were satisfied.
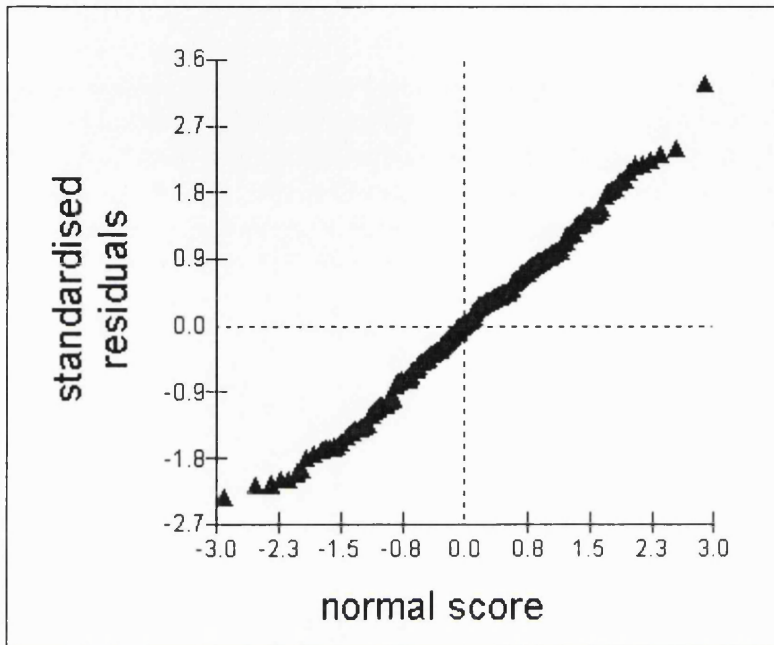
Fig.5.10 Residuals from Combined & Interaction model for T*Capno*.
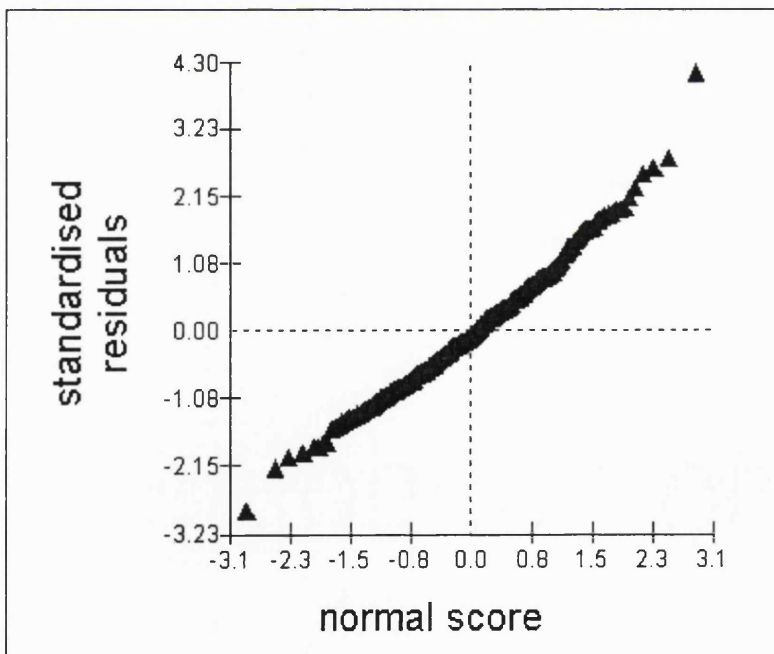
Fig.5.11 Residuals from Combined & Interaction model for TAC.

The T*Capno* counts (cfu ml^{-1}) and TAC (cfu ml^{-1}) for non-DM-Periodontitis (healthy and diseased sites) as well as for the DM-Periodontitis (healthy and diseased sites) are presented in Figs. 5.12 & 5.13 respectively.
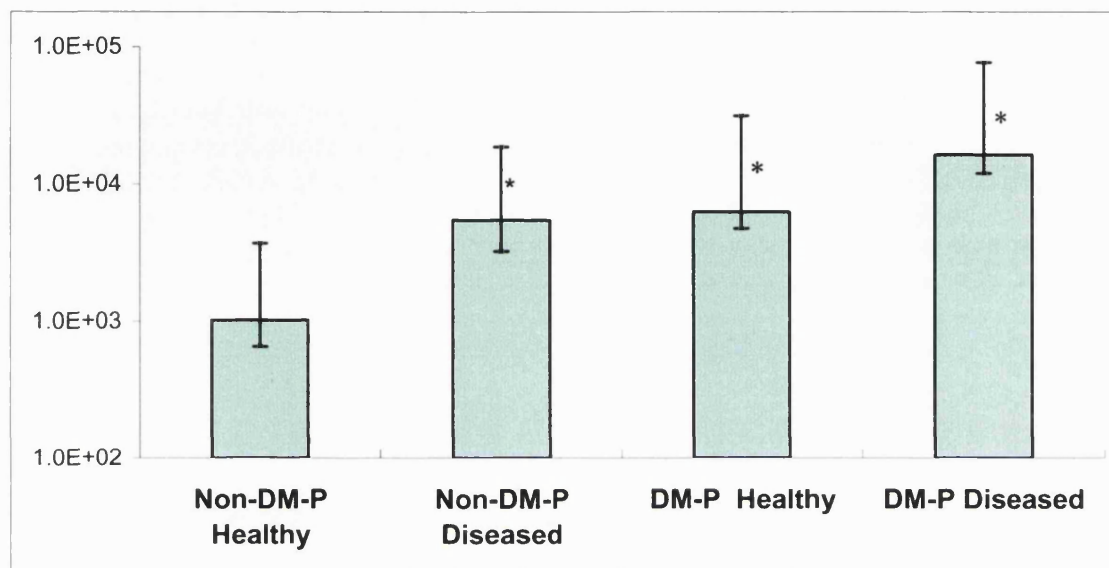
Fig.5.12 Total *Capnocytophaga* Counts (mean values, cfu ml^{-1}): effect of site health and DM. Bars represent 95% CI. *Significantly different from non-DM-Periodontitis healthy sites (For pair-wise contrasts see Table 5.10)
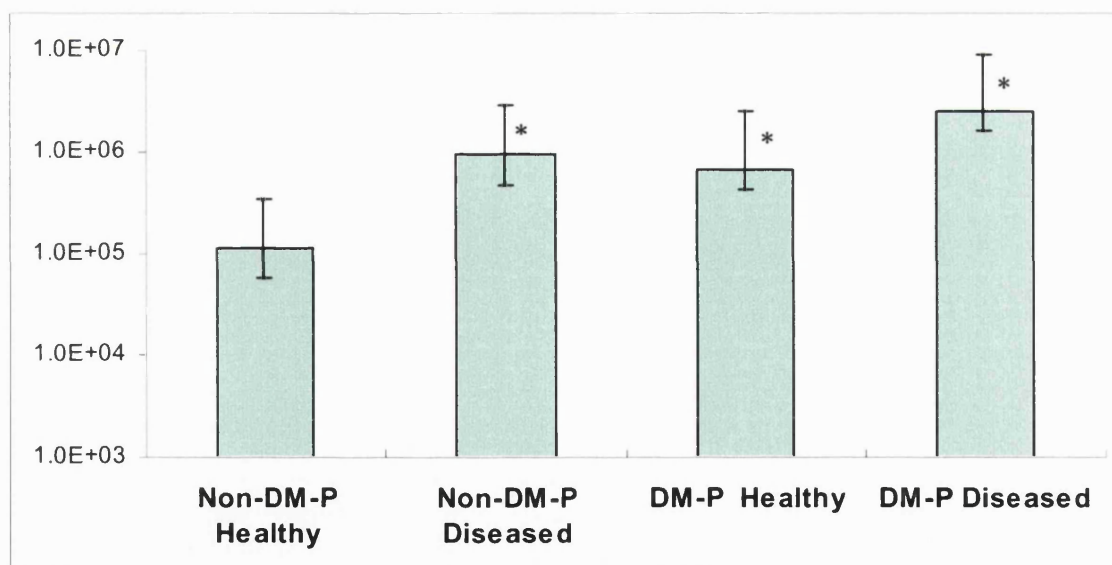
Fig.5.13 Total Anaerobic Counts (mean values, cfu ml^{-1}): effect of site health and DM. Bars represent 95% CI. *Significantly different from non-DM-Periodontitis healthy (For pair-wise contrasts see Table 5.11)

Total *Capnocytophaga* counts: Pair-wise contrasts for T*Capno* counts between groups for significance values (generated through Main Effects & Interactions model in *MlwiN*) are presented in Table 5.10.

Pair-wise contrasts

	Non-DM-P H	Non-DM-P D	DM-P H
Non-DM-P D	< 0.001		
DM-P H	0.013	0.888	
DM-P D	<0.001	0.135	0.050

Table 5.10 *P*-values resulting from pair-wise contrasts for total *Capnocytophaga* counts between groups: H = Healthy sites; D = Diseased sites; Non-DM-P H = healthy sites in the non-DM-Periodontitis group; Non-DM-P D = diseased sites in the non-DM-Periodontitis group; DM-P H = healthy sites in the DM-Periodontitis group; DM-P D = diseased sites in the DM-Periodontitis group.

The results show that the healthy sites in the non-DM-Periodontitis group (denoted by non-DM-P healthy in Fig.5.12) had the lowest total *Capnocytophaga* counts (cfu ml^{-1}) when compared with all the other groups. In this group (i.e. non-DM-Periodontitis group), the total *Capnocytophaga* count was significantly lower (P < 0.001) in the healthy sites when compared to the diseased sites (Table 5.10). The total *Capnocytophaga* count in the diseased sites in the non-DM-Periodontitis group (non-DM diseased) was almost equal to that in the healthy sites in the DM-Periodontitis group. In the DM-Periodontitis group, the diseased sites harboured the highest number of total *Capnocytophaga* counts (DM-P diseased) when compared to all the other groups. Within the DM-Periodontitis group, the diseased sites showed the highest count, this being significantly greater than that at the healthy sites in the non-DM Periodontitis group (P < 0.001); the difference between the diseased and healthy sites in the DM-Periodontitis group reached borderline significance (P = 0.050). Comparison between the DM- and non-DM-Periodontitis groups showed that there was a greater difference amongst the healthy sites (P = 0.013) than amongst the diseased sites (P = 0.135). That the latter was not significant at the 5%-level was most likely due to insufficient power, as it might be anticipated that the combined effect of DM and site-

status would be less than that of the two independent effects combined (hence the sizeable interaction term; Table 5.8).

Total Anaerobic Counts: Pair-wise comparisons between groups were generated (through the Main Effects and Interactions model in *MIwiN*) and the data presented in Table 5.11.

Pair-wise contrasts

	Non-DM-P H	Non-DM-P D	DM-P H
Non-DM-P D	< 0.001		
DM-P H	0.001	0.421	
DM-P D	<0.001	0.084	<0.001

Table 5.11 *P*-values resulting from pair-wise contrasts for TAC (facultative & obligate) between groups: H =Healthy sites; D = Diseased sites; Non-DM-P H = healthy sites in the non-DM-Periodontitis group; Non-DM-P D = diseased sites in the non-DM-Periodontitis group; DM-P H = healthy sites in the DM-Periodontitis group; DM-P D = diseased sites in the DM-Periodontitis group.

The healthy sites in the non-DM-Periodontitis group (denoted by Non-DM-P Healthy in Fig.5.13) harboured the lowest numbers of bacteria capable of growing anaerobically (i.e. obligate and facultative anaerobes). Within the non-DM-Periodontitis group, the diseased sites contained significantly higher numbers of organisms capable of growing anaerobically ($P < 0.001$) when compared to the healthy sites. The healthy sites in the DM-Periodontitis group had a slightly lower count than that in the diseased sites in the non-DM-Periodontitis group. Within the DM-Periodontitis group, the diseased sites contained significantly higher numbers of bacteria capable of growing anaerobically (i.e. obligate and facultative anaerobes) both when compared to non-DM healthy sites ($P < 0.001$) and when compared to the healthy sites in the DM-Periodontitis group ($P < 0.001$). Comparison between the DM- and non-DM-Periodontitis group showed that there was a greater difference amongst the healthy sites ($P < 0.001$) than amongst the diseased sites ($P = 0.084$). That the latter was not significant at the 5%-level was most likely due to insufficient power, as it might be anticipated that the combined effect of

DM and site-status would be less than that of the two independent effects combined (hence the sizeable interaction term; Table 5.9).

5.4.2.3 MV MLM for each of the *Capnocytophaga* spp. as outcome variables

In the multivariate analysis, the output of the *MLwiN* software determined a covariance matrix at each level of the hierarchy. This was then used to derive the multilevel outcome correlation structure, which is presented in Tables 5.12 (subject-level) and 5.13 (site-level) for each of the *Capnocytophaga* spp.

There were instances when counts for the site data were available, but the identity of species at these sites was not known. Thus, when the MV MLM models were specified, these data points were coded as -99. In order to eliminate any possibility that coding the data as -99 might affect the outcome, the models were rerun with these data points coded as zero. These sensitivity tests showed that results were consistent for both approaches and the former model was used as this made use of less data, thus erring on the more conservative side.

Subjects	C. g	C. o	C. s	C. gr	C. h *	C. gv	C. ov
C. g	1.000						
C. o	0.440	1.000					
C. s	0.323	0.447	1.000				
C. gr	0.080	0.692	0.407	1.000			
C. h *	-	-	-	-	1.000		
C. gv	-0.092	0.133	-0.354	0.092	-	1.000	
C. ov	-0.349	0.537	0.608	0.531	-	0.346	1.000

Table 5.12 MV MLM correlation matrix for *Capnocytophaga* spp. at subject-level

C. g = C. gingivalis; C. o = C. ochracea; C. s = C. sputigena; C. gr = C. granulosa;

C. h = C. haemolytica; C. gv = C. gingivalis variant; C. ov = C. ochracea variant.

*MLwiN could not determine any covariance structure for C. haemolytica across subjects, hence no correlation could be ascertained for this outcome.

Sites	C. g	C. o	C. s	C. gr	C. h *	C. gv	C. ov
C. g	1.000						
C. o	-0.106	1.000					
C. s	0.091	-0.134	1.000				
C. gr	0.141	0.041	0.221	1.000			
C. h *	0.074	0.077	0.083	0.213	1.000		
C. gv	-0.137	-0.116	0.115	0.016	0.082	1.000	
C. ov	0.031	-0.082	0.263	0.287	0.268	0.074	1.000

Table 5.13 MV MLM correlation matrix for *Capnocytophaga* spp. at site-level

C. g = *C. gingivalis*; *C. o* = *C. ochracea*; *C. s* = *C. sputigena*; *C. gr* = *C. granulosa*;

C. h = *C. haemolytica*; *C. gv* = *C. gingivalis* variant; *C. ov* = *C. ochracea* variant.

At the subject-level, *C. gingivalis* and *C. ochracea* showed a correlation of 0.440 (Table 5.12). However, these species manifested a negative correlation (-0.106) at the site level (Table 5.13). Similarly, *C. granulosa* and *C. ochracea* showed a correlation of 0.692 (Table 5.12) at the subject-level, but were very poorly correlated (0.041) at the site-level (Table 5.13). In contrast, *C. ochracea* variant and *C. granulosa* were highly correlated (0.531) at the subject-level (Table 5.12) but less well correlated (0.287) at the site level (Table 5.13). These results showed that some species might coexist in the same subject, though not necessarily at the same sites within that subject.

Three explanatory variables i.e. DM, site-status and the interaction site-status*DM, were introduced into the MV MLM for the seven *Capnocytophaga* spp., the results of which are shown in Table 5.14. The effects of the explanatory variables are best explained by the coefficients in section (b) of Table 5.14, i.e. the covariate model. Both site-status and DM are shown to have an effect on the seven species as shown by the coefficients. However, the significance of the interactions (denoted by site-status*DM) for each of the species in the MV MLM could not be determined due to software limitations, because the -2 LogLikelihood statistic was not provided when logistic regression was employed in the *MLwiN* software.

	Multivariate Multilevel Model Coefficients (95% Confidence Interval)			
	(a) VC Model	(b) Random Intercept Model (i.e. Covariate Model)		
	Intercept	Site-status	DM	Site-status x DM Interaction
C. gingivalis	-2.22 (-2.90, -1.54) **	-1.22 (-2.37, -0.07)*	-1.56 (-3.16, 0.04)	0.09 (-2.56, 2.73)
C. ochracea	0.28 (-0.13, 0.68)	0.59 (-0.20, 1.38)	0.00 (-1.02, 1.02)	0.13 (-1.03, 1.30)
C. sputigena	-2.26 (-2.94, -1.58)**	0.59 (-1.05, 2.22)	1.34 (-0.46, 3.14)	-1.31 (-3.42, 0.80)
C. granulosa	-0.78 (-1.20, -0.35)**	0.08 (-0.81, 0.97)	0.76 (-0.31, 1.83)	-0.21 (-1.42, 1.00)
C. haemolytica	-3.68 (-4.57, -2.79)**	-0.50 (-2.32, 1.32)	-0.33 (-2.15, 1.49)	1.88 (0.71, 4.47)
C. gingivalis variant	-2.20 (-2.80, -1.60)**	-0.38 (-1.47, 0.70)	-2.44 (-4.76, -0.12)*	-†
C. ochracea variant	-1.40 (-1.80,-0.99)**	-0.13 (-1.08,0.82)	0.20 (-0.85, 1.26)	-0.48 (-1.87, 0.90)

Table 5.14 Results of MV MLM analysis for *Capnocytophaga* spp. (a) Variance Components Model which, when reverse transformed, gives the overall prevalence of each species (see Fig. 5.14); (b) Random Intercept Model for the covariates: versus site-status, subject DM status, and the cross-level interaction of site-status with DM.

* *P* < 0.05; ** *P* < 0.001.

† Interaction term omitted because model convergence could not be achieved

The exponentiation of the covariates coefficient gives the odds ratio (OR) for the covariates for each organism (Fig.5.14). The healthy non-DM-Periodontitis (HnonDM) sites are taken as the baseline reference (OR = 1.0) against which are compared the healthy sites in DM-Periodontitis subjects (HDM), diseased sites in non-DM-Periodontitis subjects (DnonDM) and the diseased sites in DM-Periodontitis subjects (DDM).
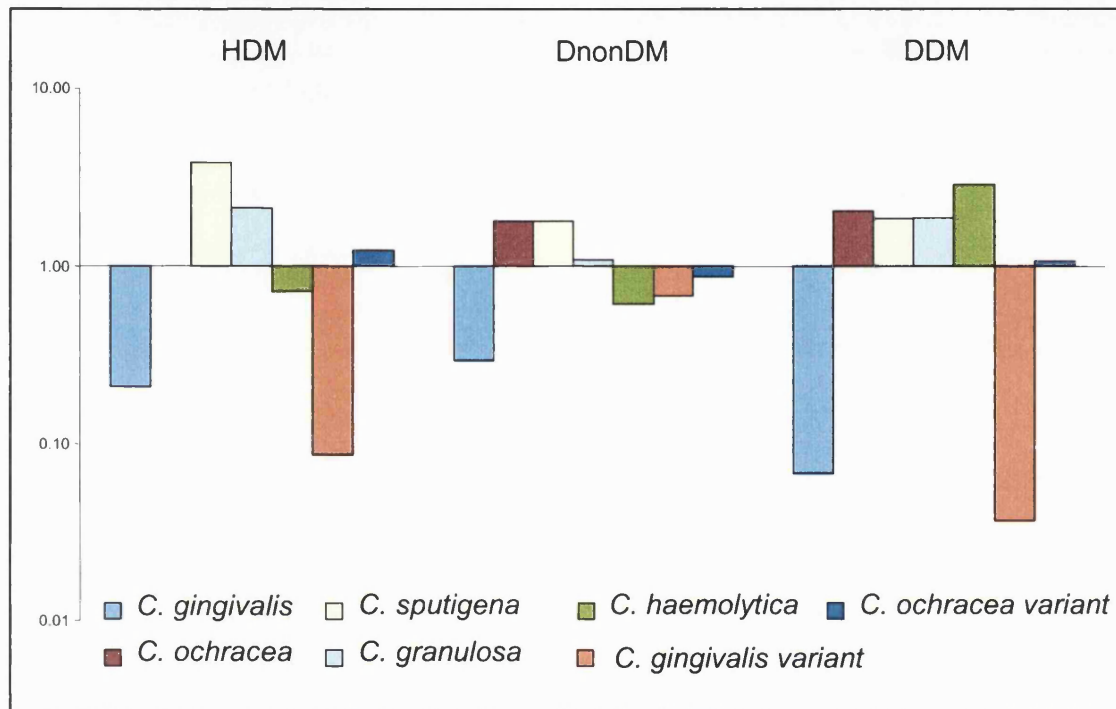
Fig.5.14 Odds ratio of finding each of the seven *Capnocytophaga* spp. in healthy sites in DM-Periodontitis patients (HDM), diseased sites in non-DM-Periodontitis patients (DnonDM) and in diseased sites in DM-Periodontitis patients (DDM). The baseline reference was healthy sites in non-DM-Periodontitis subjects.

The graph shows that the odds ratio of finding *C. gingivalis* and its variant in all 3 groups was decreased with respect to finding the organism in HnonDM sites. The odds ratio of finding *C. ochracea* in HDM sites were the same as that for HnonDM, whilst it increased in the DnonDM and DDM sites. The odds ratio for the presence of *C. sputigena* and *C. granulosa* in HDM, DnonDM and DDM sites were increased. *C. haemolytica* showed a decreased odds ratio in HDM and DnonDM. However, the odds ratio of finding this organism at DDM sites was increased. The odds ratio for *C.*

ochracea variant were increased at HDM and DDM sites and decreased at DnonDM sites.
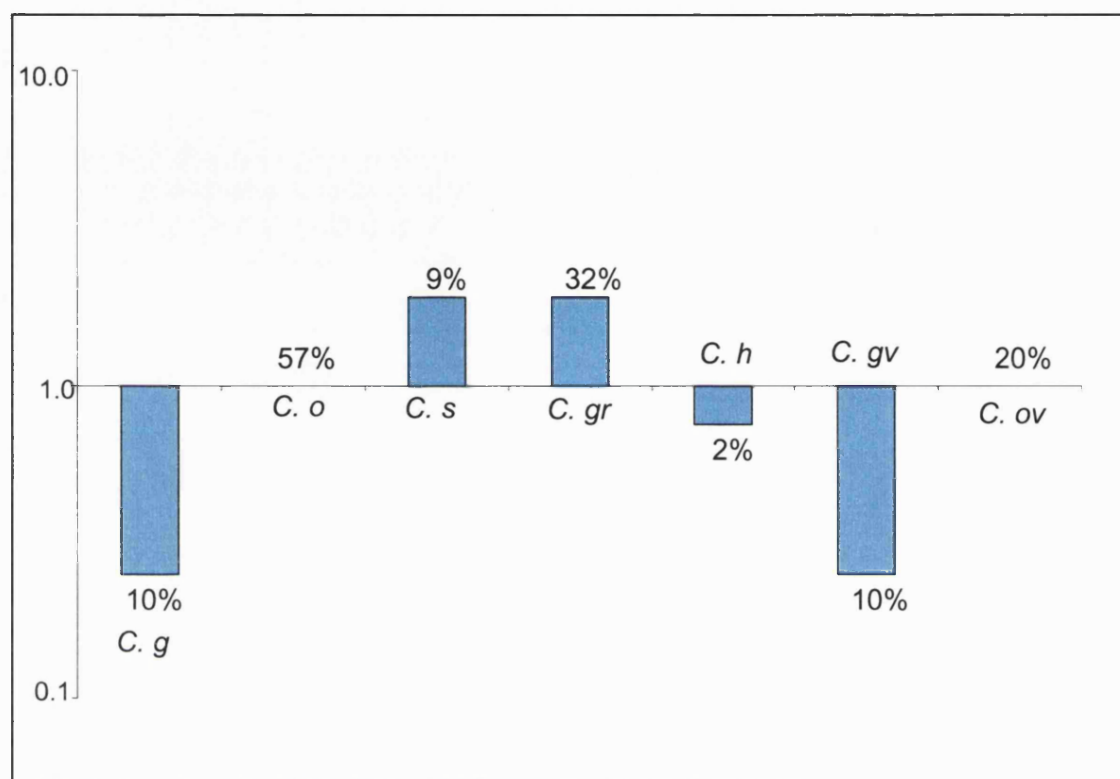
Fig.5.15 Prevalence of the seven *Capnocytophaga* spp. in DM-Periodontitis subjects
C. g = *C. gingivalis*, *C. o* = *C. ochracea*, *C. s* = *C. sputigena*, *C. gr* = *C. granulosa*,
C.h = *C. haemolytica*, *C. gv*= *C. gingivalis variant*, *C. ov* = *C. ochracea variant*.

The effect of DM (only) on the site prevalence of these organisms is depicted in Fig. 5.15. The graph shows that the overall site prevalence of *C. gingivalis* and its variant decreased by 10% for each organism; the prevalence of *C. haemolytica* decreased by 2%. The site prevalence for *C. ochracea* and its variant was unchanged, whilst that for *C. sputigena* and *C. granulosa* increased by 9% and 32% respectively. Thus, the results demonstrated that, whilst the net prevalence of *Capnocytophaga* species in DM subjects increased, the different species showed increasing or decreasing trends.

5.4.3 Blood Samples

The total number of blood samples processed via lysis filtration was 46. Only 7 samples showed bacterial growth on aerobic plates; however no growth was observed on the corresponding anaerobic plates. The phenotypic morphology of these colonies

(circular, pulvinate colonies, off-white in colour, not spreading) was not suggestive of *Capnocytophaga* species. Microscopic examination showed small, Gram-positive cocci, all of which were catalase-positive, oxidase-negative (Appendix 7) and coagulase-negative (Oxoid Staphytect Plus, Oxoid) suggestive of coagulase-negative *Staphylococcus* spp.

5.5 Discussion

Whilst it is difficult to determine (especially in a cross-sectional study such as this one) whether the bacterial populations at a site are the cause or the result of the pathology occurring at that particular site, it is still beneficial to be cognisant of the bacterial populations at the site-level, as this might lead towards an improved understanding of the microbial aetiology of the various periodontal diseases. In the case of *Capnocytophaga* spp. and DM-periodontitis, no study to date appears to have specifically investigated the potential association of this species with DM-Periodontitis. Therefore, this was the specific aim of this study.

Patients for this study were selected at random as they attended for treatment at either the Periodontology Unit or the Endocrine Unit in the respective Hospitals. Sample size was determined using SPSS as described in Chapter 2. It could be argued that patient selection was not random, as the fact that the patients were selected from referral clinics (Eastman Dental and Middlesex Hospitals), could have led to bias. This could have therefore resulted in DM subjects who were poorly controlled (as was indeed the case in this study, Table 5.1) and patients with more advanced periodontitis. However in the light of the aim of this study, which was to try to detect whether there were any differences between *Capnocytophaga* spp. between DM- and non DM-periodontitis subjects, and which had to be performed within the time constraints allowed for this study, selection of such patients can be justified.

A detailed patient personal and clinical history was undertaken, even though some of that information (e.g. genetic background, smoking habits as well as type, duration and level of control of DM, and PPD) was not included in the data analysis. Although these details were not crucial in testing the hypothesis for this study, it was imperative that such details were collected during the course of this study to be available for analysis at a later date. Blood samples were collected prior to periodontal probing and subgingival plaque sampling in order to avoid inducing a bacteraemia.

All patients had their HbA_{1c} estimated. This was done for 2 reasons:

i) to exclude any undiagnosed DM subjects in the control group, and

ii) to accurately determine the level of glucose control in the DM subjects.

The HbA_{1c} test was chosen in preference to random serum glucose, as it provides a more accurate indicator of blood glucose control (Cooper *et al.* 2000). HbA_{1c} determines the level of blood glucose over the preceding three months rather than during the hours prior to testing, as would be the case with a random blood test. The HBA_{1c} test also established the level of blood glucose control, i.e. good control <8%, moderate control 8-10%, poor control >10%. Since the degree of glucose control has been reported to affect the degree of periodontitis (Ainamo *et al.* 1990), this information would prove relevant during further data analysis.

The majority of DM subjects (20 / 21) were Type 2. This provided a more homogenous DM group and minimised the possibility that any clinical periodontal differences could be attributed to the differences in the aetiology of Type 1 and Type 2 DM.

Sample size determination: To determine a significant difference in outcomes with 95% confidence and 80% power, the number of patients required per group (DM- and non-DM both suffering from periodontitis) ranged from 3, for the most optimistic, to 85 for the most pessimistic. Sample sizes less than 20 yield unreliable data so the lower limit of 3 patients was excluded. The upper limit of 85 patients was unrealistic for the amount of clinical and microbiological work scheduled for this project to be completed within the specified time span. In addition these two figures were more extreme estimates with the majority of the estimated group sizes being between 16 and 38. The evidence used to make these estimates was sketchy with information extracted from only a few studies available that were related to this proposed study (Mashimo *et al.* 1983a, Sastrowijoto *et al.* 1989, Müller *et al.* 1997) but none of which set out to investigate *Capnocytophaga* species in DM-Periodontitis subjects as the focus of their research. Nor was it possible to contrast test (DM-Periodontitis) and controls (non DM-Periodontitis) from the same study data. Thus, with this limited information to estimate the sample size required, along with the practical limitations of collecting the data and processing of samples, this study aimed to collect a sample size of 25 patients for each of the test and control groups. Thus with a total sample-size of 50 subjects, it was anticipated that this would provide sufficient statistical power to detect any significant differences in outcome measures. A total of 46 patients were recruited for this study;

four subjects from the DM-periodontitis group withdrew from the study for personal reasons (work / family commitments).

Statistical analysis: MLM was used for statistical analysis as it takes into account:

i) the hierarchical nature of the data, and

ii) the inherent correlation between the data points,

This is not to say that MLM *must* be used in *all* studies. Indeed in some cases it might not be necessary to use such complex models for data analysis. However in this case, the clustered and hierarchical nature of the data warranted the use of MLM, which takes these features into account when modelling the data. It is important to know the degree of variation at each level as this is important when considering sample size, as this will in turn affect statistical power. Thus, VC models may be used as a guide to sampling decisions, which will have a direct bearing on the method of sampling i.e. one site / multiple sites per tooth, and which will in turn affect the number of patients recruited and study costs.

The use of single-level analyses for DM-Periodontitis and non-DM-Periodontitis groups and for PPD, LCAL, BI and PI between & within groups, for healthy and diseased sites, could be justified in this instance as the data sets were being analysed to ensure homogeneity of the groups being compared, i.e. DM versus non DM and healthy versus diseased sites. These analyses provided sufficient evidence that the groups being compared were in fact homogenous for severity of periodontitis and age. The high plaque index at healthy sites in DM-Periodontitis subjects reflected the poorer oral hygiene levels which prevail in most DM subjects. This is most likely due to the lack of self health awareness most notable in poorly controlled DM patients. The significant difference for HbA_{1c} values was expected, as this was the main discriminating feature between the two groups. Thus, on this basis, any differences between the groups being compared would be likely to be due to actual differences at site- and subject-level and not due to gross differences between the data sets. The data from these conventional single-level analyses were <u>not</u> used to test the hypotheses formulated for this investigation, since single-level analyses are based on the assumption that the data are independent, which was not the case with the hierarchical nature of the periodontal data set collected for this study (Chapter 1, section 1.6). It was realised that the *P*-values obtained via single-level analyses would be over optimistic. Nevertheless, the results obtained via this method would have been corroborated if the

more statistically powerful MLM were used; it was however unnecessary to obtain highly accurate *P*-values for this part of the data evaluation.

Examiner reproducibility via the traditional Cohen's *kappa* statistic (Cohen 1960) was not performed in this study. The value of the *kappa* statistic is somewhat arbitrary as the number of categories used to determine the statistic alters it final value (Gilthorpe *et al.* 2000b). Furthermore MLM can identify and correct for inter-examiner variability when several examiners have been involved in a study. In this study, where only one examiner was involved, the small standard errors obtained when MLM was used signified that the intra-examiner reproducibility was good (Gilthorpe *et al.* 2000b; Gilthorpe MS, *pers comm*).

Microbiological results: The results of this study have rejected the null hypothesis and show that the total *Capnocytophaga* count in DM-Periodontitis subjects was significantly higher than that in the non-DM-Periodontitis subjects ($P < 0.025$; Fig.5.6). This result was obtained when DM was the only explanatory variable in the RI model (Table 5.6). A significantly higher T*Capno* count was also observed in the diseased sites when compared the healthy sites ($P < 0.001$). However, since these factors (DM and site-status) were operating in a biological system, it was most unlikely that the two explanatory variables operated independently. This justified reanalysing the data set using the combined and interaction model. These results showed that the difference between DM- and non-DM-Periodontitis groups was greater among the healthy sites than amongst the diseased sites. Furthermore, the periodontally diseased sites in DM-Periodontitis subjects harboured significantly higher mean counts of *Capnocytophaga* spp. when compared to the healthy sites in this group ($P = 0.05$) and more so when compared to healthy sites in the non-DM-Periodontitis group ($P < 0.001$). This result is in agreement with Mashimo *et al.* (1983a), although direct comparison between the two studies is precluded, as the methodologies involved are different. Although no significant difference was observed between the T*Capno* counts in the diseased sites between the DM- and non-DM-Periodontitis groups ($P = 0.135$), this could have been due either to a true lack of significance or, more plausibly, due to the fact that the subdivision of the data set used in this study into four subgroups was underpowered and therefore failed to achieve a level of significance.

A greater degree of variance was observed at site-level than at subject-level. This implied greater heterogeneity within subjects than between subjects. What was more

important was not the value of the co-efficient at either site- or subject-level, but rather that both of these were significantly different from zero ($P < 0.001$) thereby warranting the use of sophisticated statistical methods such as multilevel modelling to appropriately partition the total variance across the natural hierarchy. This is a strong aspect of the multilevel methodology that the significant detection of small covariate effects can be achieved amidst only small variations between subjects despite large within-subject variation.

The data were also analysed on the basis of the odds ratio of finding each of the identified *Capnocytophaga* species with respect to site-status and presence or absence of DM (Fig.5.14). The results revealed that whilst there was a net increase in *Capnocytophaga* spp. at diseased sites, and in DM subjects (Fig.5.12), the individual species manifested different trends (Fig.5.14). These results were corroborated by prevalence analysis for each species (Fig.5.15). The trends shown were:

i)   *C. gingivalis* and its variant showed an overall decrease in OR

ii) *C. ochracea* showed no change in healthy DM-Periodontitis sites, but the OR of finding it in diseased non-DM- and DM-Periodontitis sites increased

iii) *C. sputigena* and *C. granulosa* showed an increased trend in all sites, whilst

iv) *C. haemolytica* showed a decreased OR in healthy DM-Periodontitis and diseased non-DM-Periodontitis sites, whilst its OR increased in diseased DM-Periodontitis sites

v)   *C. ochracea* variant showed an increased OR in healthy and diseased DM-Periodontitis sites, however it decreased in diseased non-DM-Periodontitis sites

These results must be interpreted within the context of the complexity of the oral micro flora. The decreased prevalence of *C. gingivalis* in DM subjects was unexpected. Investigations by Spratt *et al.* (1996) showed that the biomass of this organism increased five-fold when grown in batch culture at increasing glucose concentrations, which simulated the glucose levels in DM subjects. In this study, even though gingival crevicular fluid in DM subjects showed higher levels of glucose (Chapter 7), the prevalence of *C. gingivalis* decreased. Direct comparison between these two studies is precluded as the experimental conditions of the *in vitro* (Spratt *et al.* 1996) and *in vivo* (this) studies were completely different. The subgingival microbial biofilm present in the *in vivo* situation is known to exhibit different physiological properties to bacteria grown in batch culture (Marsh & Martin 1999). The findings for *C. granulosa* and *C. haemolytica* are indeed novel, as these organisms have not been isolated previously from subgingival plaque in DM-Periodontitis subjects and there is only one other report of the recovery of these organisms from subgingival plaque taken from non-DM-Periodontitis subjects (Ciantar *et al.* 2001). Furthermore, comparison of the prevalence of *C. gingivalis* and *C. ochracea C. sputigena*

is also precluded as previous studies on *Capnocytophaga* spp. in DM periodontitis failed to identify the organisms to species level. The large proportion of *C. ochracea* is noteworthy; these isolates were found to be of intermediate virulence when compared to *C. gingivalis* and *C. sputigena*. However all three isolates produced significant bone destruction in animal models (Irving *et al.* 1978, Socransky *et al.* 1979)

The results of this investigation also showed that of all the groups, the diseased sites in DM-Periodontitis group harboured the highest number of bacteria capable of growing anaerobically (i.e. facultative and obligate anaerobes), this being significantly greater than in the non-DM-Periodontitis group ($P < 0.001$; Fig.5.13, Table 5.11). Whilst the higher counts for facultative and obligate anaerobes at diseased sites when compared to the healthy sites was to be expected, and is in agreement with other studies (Dzink *et al.* 1985, 1988), the higher counts for total anaerobes (facultative and obligate) in DM- versus non-DM-Periodontitis group is a novel finding. The higher numbers in the DM-Periodontitis group (especially at the diseased sites) may be attributed to the more reduced (anaerobic) conditions present in the subgingival pockets resulting from the higher GCF-glucose levels (Chapter 7). The presence of a significantly higher count of facultative and obligate anaerobes at diseased sites in the DM-Periodontitis subjects might contribute to the more severe periodontitis often observed in these patients. An interesting observation is that the healthy sites in the DM-Periodontitis group harboured almost the same number of facultative and obligate anaerobes as the diseased sites in the non-DM-Periodontitis group (Fig.5.13).

Five hundred black-pigmented anaerobes were isolated from the subgingival plaque samples collected throughout the course of this study. However, since this was not within the remit of this study, these bacteria have been frozen (-70°C in 10% BHI) and will be analysed at a later date.

Blood samples

Blood samples were taken from each patient for the following reasons:

i) to establish the level of DM control (via HbA$_{1c}$ levels) of the DM subjects

ii) to eliminate any undiagnosed DM subjects within the control group

iii) to attempt to isolate bacteria from the blood samples.

Most of the DM-Periodontitis subjects were found to be poorly-controlled (Table 5.1); all controls subjects were non-DM.

Much has been said about the possibility of periodontitis related bacteria gaining systemic access via the ulcerated periodontal pocket hence resulting in a bacteraemia (Page 1998,

Seymour & Steele 1998). This notion has been based on the large numbers of periodontal bacteria which are in contact with the large surface area of moderate / severe (ulcerated) periodontitis lesions. However, to date this premise has been neither scientifically proved nor disproved. In addition, blood-borne bacterial dissemination forms one part of the focal infection concept (Newman 1996). Thus, one of the aims of this investigation was to culture periodontal bacteria from blood taken from periodontitis subjects.

The blood samples for this part of the study were collected in separate containers to those for quantification of HbA_{1c}. The samples were processed via lysis filtration (Heimdahl *et al.* 1985). The technique of lysis filtration was used as it was shown to be superior to conventional bottle broth technique for the detection of bacteraemia (Gill *et al.* 1984, Heimdahl *et al.* 1985). One advantage of this technique is that lysis of phagocytes releases any viable intracellular organisms, which would not otherwise be detected.

The results of this part of the study were negative except for 7 samples. Preliminary identification of these colonies was suggestive of *Staphylococcus* spp. These results may be interpreted in one of two ways:

i) either periodontal bacteria do not enter the systemic circulation as previously suggested, or

ii) the host is capable of mounting a potent phagocytic response which eradicates any bacteria which would have entered the circulation.

However, further investigation is warranted. Indeed, blood samples (4 ml) from these patients have been stored at -70°C and will be tested for detection of bacterial DNA via 16S rRNA PCR analysis (Ley *et al.* 1998).

In conclusion, the results of this study have shown that the total mean count of *Capnocytophaga* spp. were significantly higher in DM-Periodontitis subjects when compared to non-DM-Periodontitis ($P < 0.025$) and at diseased sites when compared to healthy sites ($P < 0.001$). Significant differences were also observed between healthy and diseased sites within groups. The mean total *Capnocytophaga* count at the DM-Periodontitis diseased sites was higher than at the diseased sites in non-DM-Periodontitis subjects, though at these sites statistical significance was not established. Data analysis for each individual species revealed that the outcome of each *Capnocytophaga* species varied with site-status and DM-status. *C. ochracea* and *C. granulosa* were the most common isolates (43.8% and 24.8% of all the *Capnocytophaga* species isolated). All the blood samples were negative for *Capnocytophaga* spp. via the lysis filtration method.

Chapter 6

Antimicrobial sensitivity testing of *Capnocytophaga* spp.

6.1 Introduction

Mechanical instrumentation is the primary therapeutic modality for the treatment of periodontitis (Badersten *et al.* 1981, 1984, 1985). This involves the disruption of supragingival plaque biofilms via the mechanical action of the toothbrush and the removal of subgingival biofilms via professional instrumentation (Axelsson & Lindhe 1981, Lindhe & Nyman 1984, Ramfjord *et al.* 1982, Cercek *et al.* 1983, Loos *et al.* 1987, Kieser 1990, Palmer 2001). In the majority of cases this would lead to resolution of periodontal inflammation; however there are some cases, e.g. early-onset periodontitis (Slots *et al.* 1983, Christersson *et al.* 1993) and refractory periodontitis (Mombelli *et al.* 1989), where the adjunctive use of antimicrobials could prove beneficial. Prophylactic antibiotic therapy is vitally important in subjects at risk from bacterial endocarditis (BSAC 1992). Thus knowledge of the antimicrobial sensitivity of oral bacteria is of clinical importance.

The recognition for a potential role for *Capnocytophaga* spp. in CIPDs (Tables 1.11 - 1.13) and as causative agents in localised or systemic infections (Table 1.14) including endocarditis (Montejo Baranda 1984, Buu-Hoi *et al.* 1988, Ngagge 1999), underlies the need for data regarding their antimicrobial susceptibility. As highlighted previously (Chapter 3), the studies that investigated the antimicrobial sensitivity of *Capnocytophaga* spp. (Forlenza *et al.* 1981, Sutter *et al.* 1981, Mashimo *et al.* 1983, Rummens *et al.* 1986, Hawkey *et al.* 1987, Roscoe *et al.* 1992, Jolivet-Gougeon *et al.* 2000), failed to identify the strains to species level. In addition, the studies used different procedures for antimicrobial sensitivity testing, none of which fully conformed to the guidelines prescribed by the National Committee for Clinical Laboratory standards (NCCLS 1993), thus precluding comparisons between studies and limiting the value of the results. In addition, culture media were made selective for *Capnocytophaga* spp. by the addition of antimicrobial / antifungal agents for which no previous record of sensitivity data was available.

Thus, the aim of this investigation was to determine the antimicrobial sensitivity of the 7 *Capnocytophaga* Type strains and of 398 identified *Capnocytophaga* clinical isolates, by the agar dilution method. The study was performed in two parts using:

i) a range of antimicrobial agents commonly used in clinical periodontal practice - penicillin V, amoxicillin, metronidazole, tetracycline, clindamycin and erythromycin;

ii) antimicrobial agents and an antifungal compound which were incorporated in media devised to be selective for *Capnocytophaga* spp. (Mash and Cap media, Chapter 3) and for which no previous susceptibility information was available.

178

6.2 Materials & methods

6.2.1. Bacterial strains

The 7 *Capnocytophaga* ATCC strains used were those referred to in Chapter 2 section 2.3.1. Additionally, three hundred and ninety eight (398) clinical isolates of *Capnocytophaga* species which had been previously isolated from subgingival plaque (Chapter 5), and which had been identified using 16S rRNA PCR-RFLP (Chapter 4), were also tested. Both the ATCC strains and clinical isolates were grown on FAA for 3 days at 37°C in 5% CO_2.

Plate inoculation was performed using a multi-point inoculator (Mast Group, Merseyside, UK). Each run consisted of 30 *Capnocytophaga* clinical isolates, 4 control strains (β-haemolytic *Streptococcus* spp., Oxford *Staphylococcus aureus* NCTC 6571, *E. coli* NCTC 10418, Vancomycin B resistant *Entercoccus faecalis* (clinical isolate) and 2 uninoculated broths as controls.

6.2.2 Antimicrobial stock solutions

The antimicrobials and antifungal agent were all supplied by Sigma. Stock solutions of these reagents were prepared at a concentration of 5120 μg/ml in the respective solvent (Table 6.1) according to the manufacturer's instructions. Each stock solution was aseptically filter-sterilised using 0.2 μm syringe filters (Nalgene Europe Ltd., Herefordshire, UK) into sterile containers (Bibby Sterilin Ltd., Staffordshire, UK). Aliquots (1 ml) of these stocks were subsequently dispensed into sterile, previously labelled Eppendorf tubes and stored at -20°C for a maximum period of fourteen days. One Eppendorf tube containing the respective antimicrobial was used to prepare a series of two-fold (log_2) dilutions of antimicrobial / antifungal agent using the corresponding diluent as shown in Table 6.2, and then discarded.

Table 6.1 Antimicrobial Agents and corresponding solvents & diluents

i) Agents used in clinical practice:		
Antimicrobial Agent	Solvent	Diluent
Penicillin V	97% ethanol	Deionised water
Ampicillin	Deionised water	Deionised water
Amoxicillin	Phosphate buffers*	Phosphate buffers*
Metronidazole	Deionised water	Deionised water
Tetracycline	Deionised water	Deionised water
Clindamycin	Deionised water	Deionised water
Erythromycin	97% ethanol + water (1:1)	Deionised water
ii) Agents incorporated in selective media[t]		
Antimicrobial Agent	Solvent	Diluent
Amphotericin B	Dimethylsulphoxide	Deionised water
Polymyxin B sulphate	Deionised water	Deionised water
Bacitracin	Deionised water	Deionised water
Trimethoprim	Propylene glycol	Deionised water
Vancomycin	Deionised water	Deionised water

[t] Used to prepare Mashimo (Mashimo *et al.* 1983) and Cap (Rummens *et al.* 1985) media referred to in Chapter 3.

*Refer to appendix 8

Table 6.2 Antimicrobial / antifungal dilution schedule for agar susceptibility testing (Modified from Woods & Washington 1995)

Conc.* of stock solution of antimicrobial agent (μg/ml)	Proportions of stock @ 5120 μg/ml + diluent	Actual volumes of stock (ml) + diluent (ml)	Final conc.* (μg/ml) after 1:10 dilution in agar
5120	1 + 0	6 + 0	512
2560	1 + 1	5 + 5	256
1280	1 + 3	2 + 6	128
640	1 + 7	1 + 7	64
320	1 + 15	0.5 + 7.5	32
160	1 + 31	0.5 + 15.5	16
80	1 + 63	0.1 + 6.3	8
40	1 + 127	0.1 + 12.7	4
20	1 + 255	0.1 + 25.5	2
10	1 + 511	0.01 + 5.11	1
5	1 + 1023	0.01 + 10.23	0.5
2.5	1 + 2047	0.01 + 20.47	0.25
1.25	1 + 4095	0.01 + 40.95	0.125

*Conc. = Concentration

6.2.3. Preparation of agar plates

Mueller Hinton Agar (MHA, Oxoid) was the culture medium used as recommended by NCCLS (1993). Several medical flat bottles containing 42.5 ml of the dissolved agar in water were prepared and sterilised at 121°C for 15 min. After autoclaving, the agar was allowed to cool and maintained at 47°C in a thermostatically controlled water bath.

Sterile Petri dishes (Bibby Sterilin, 100 mm, circular) were previously labelled with the name and concentration of the antimicrobial / antifungal agar to be poured into the respective plate. The range (5120 - 1.25 μg/ml) of concentrations of antimicrobial / antifungal agents were allowed to thaw and equilibrate to room temperature, ensuring that none of the agents precipitated during storage at -20°C.

Plate pouring was performed in a laminar flow cabinet (Medical Air Technology, Manchester, UK). The MHA (42.5 ml) was supplemented with 2.5 ml of defibrinated lysed horse blood (equivalent to 5% v/v) giving a total volume of 45 ml. This volume had been previously calculated to ensure 10-fold dilution of the stock solutions of

antimicrobial / antifungal agents in agar (Table 6.2). Thus for each agar bottle (sufficient for two plates) the final composition was:

42.5 ml agar + 2.5 ml lysed blood + 5 ml antimicrobial agent

The ingredients were added in this order. The agar was mixed to ensure uniform distribution of the antimicrobial agent and blood throughout the agar and the medium was then poured into the respective Petri dishes to a depth of 3-4 mm (20 -25 ml of agar per plate) care being taken to avoid bubble formation on the agar surface. Control plates containing drug-free MHA were also prepared. The pH of each batch was checked to confirm the appropriate pH range of 7.2 - 7.4. Once the agar had solidified, the plates were stored at 4 to 8°C and used within 7 days of preparation. Prior to inoculation, the agar plates were allowed to dry and to equilibrate with room temperature. The plates were inoculated with bacterial suspensions using a multipoint inoculator as explained below.

6.2.4 Inoculation procedure

The agar plates with the lowest concentration of antimicrobial agent were inoculated first. Bacterial broth cultures were prepared by aseptically suspending colonies (from three day old bacterial cultures) in sterile Muller Hinton Broth (Oxoid). The turbidity of the broth was adjusted to match that of 0.5 McFarland standard (BioMérieux, SA, Marcy-l'Etoile, France) equivalent to ca 10^8 cfu ml^{-1}. This suspension was diluted 10-fold (1:10) with MHB giving a final adjusted concentration of 10^7 cfu ml^{-1}.

Once the adjusted inoculum was prepared, plate inoculation was performed within 30 min using a multipoint inoculator (Fig.6.1). Five hundred µl of each bacterial suspension was aseptically transferred into a well of the seeding plate. The prongs of the multipoint inoculator transferred a standardised inoculum (approximately 0.001 ml) of bacterial suspension from the seeding plate to the agar surface, yielding the desired final inoculum of approximately 10^4 cfu ml^{-1} per inoculum spot. Control plates i.e. two drug-free MHA plates, 2 FAA plates and 2 Blood agar (BA) per stack of antimicrobial agent, were inoculated at the start and end of each batch of plates to serve as controls. The inoculated plates were allowed to stand until all the inocula were completely absorbed by the medium, then inverted and incubated.
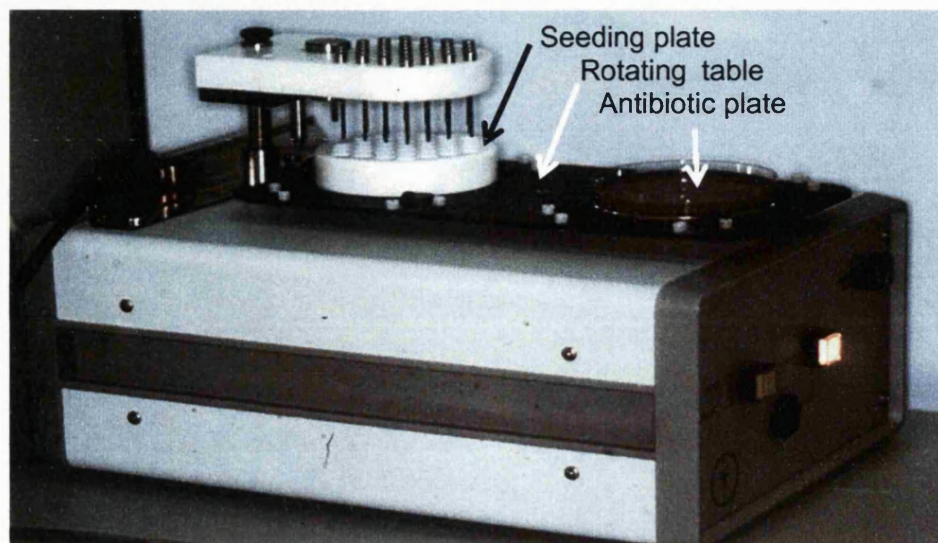
Fig.6.1 Multipoint inoculator

6.2.5 Incubation

The plates were incubated anaerobically (80% N_2: 10% H_2: 10% O_2) in an anaerobic cabinet (MACS Anaerobic Workstation). The MHA and FAA control plates were incubated anaerobically. One BA plate was incubated at 37°C in 5% CO_2. One BA plate was placed in the refrigerator. After a 48 hr incubation period, the plates were examined and the Minimum Inhibitory Concentration (MIC, defined as the lowest concentration of antimicrobial at which no growth was visible after a 48 hr incubation period) recorded.

A representative antibiotic plate showing growth after 48 hr incubation is shown in Fig. 6.2.
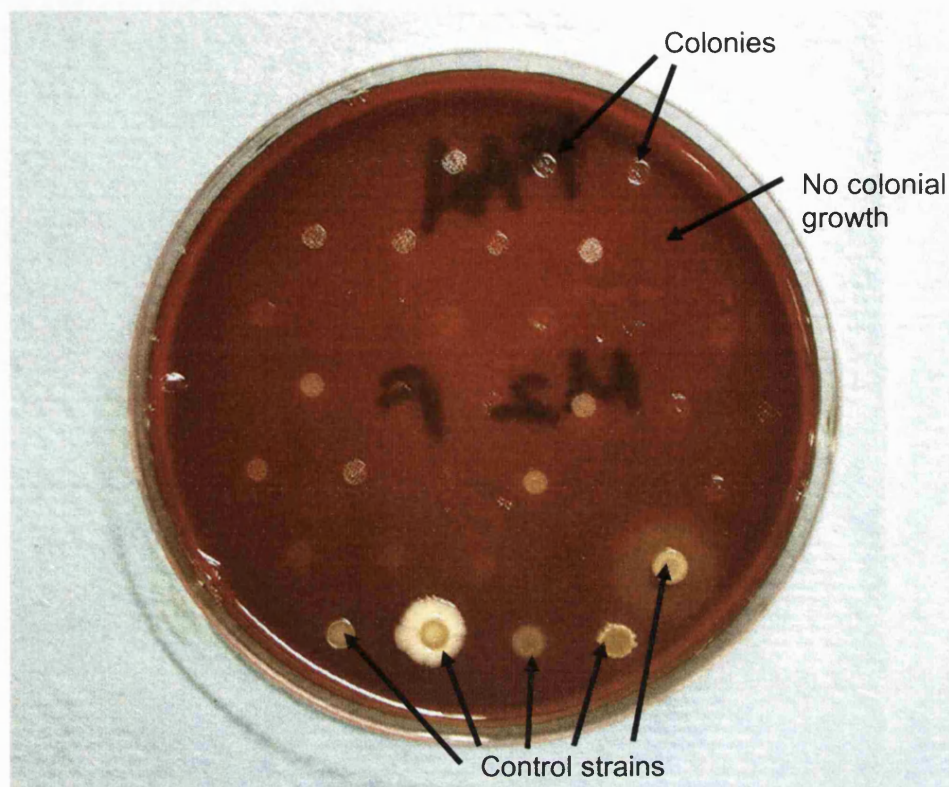
Fig.6.2 Colonial growth of *Capnocytophaga* spp. and control strains on culture medium containing antibiotic.

6.3 Results

The MIC values for 90% (MIC$_{90}$) for the 7 ATCC *Capnocytophaga* strains (10 replicates) are presented in Table 6.3. The corresponding data for the *Capnocytophaga* clinical isolates are presented in Table 6.4. Comparison of the MIC$_{90}$ values showed that the values for the ATCC *Capnocytophaga* strains were lower than the corresponding values for the *Capnocytophaga* clinical isolates, except for the penicillin V values for *C. granulosa* and *C. haemolytica* and erythromycin values for *C. ochracea*, *C. granulosa* and *C. haemolytica*.

A summary of the data for the antimicrobial sensitivity of the 398 clinical isolates of *Capnocytophaga* spp., is shown in Table 6.5. A more detailed presentation of the number of each species tested against each of the antimicrobial / antifungal agents is shown in Table 6.6.

184

The distribution of *Capnocytophaga* clinical isolates versus the MIC ranging from 0.125 to 512 μg ml^{-1} for the antimicrobial agents used most commonly in clinical practice is shown in Table 6.7. These results showed a unimodal distribution for clindamycin, amoxicillin and tetracycline with peak MIC at 0.125 and 0.25 μg ml^{-1}. Pencillin V and erythromycin manifested a bimodal distribution with peak values at 0.5 and at 0.125 μg ml^{-1}, whilst metronidazole showed a uniform distribution across the range of MIC used.

Table 6.3 MIC$_{90}$ (μg ml^{-1}) values for ATCC strains of *Capnocytophaga* spp.

	Antimicrobials used in clinical practice							Agents incorporated into selective media				
	Clind	Amox	Amp	Te	PenV	Eryth	Mz	Vanc	PolyB	Bacit	AmphB	Trimeth
C. gingivalis	≤0.125	≤0.125	≤0.125	≤0.125	0.5	≤0.125	16	≤0.125	8	4	8	≤0.125
C. ochracea	≤0.125	≤0.125	≤0.125	≤0.125	0.25	128*	1	4	256	256	64	≤0.125
C. sputigena	≤0.125	≤0.125	≤0.125	0.5	0.125	128	8	4	256	32	64	≤0.125
C. granulosa	≤0.125	≤0.125	≤0.125	0.5	4*	128*	32	4	128	256	256	0.25
C. haemolytica	≤0.125	0.5	1	1	4*	128*	2	32	≥512	≥512	≥512	≤0.125
C. canimorsus	≤0.125	NG	≤0.125	≤0.125	0.5	≤0.125	32	NG	NG	NG	NG	≤0.125
C. cynodegmi	≤0.125	≤0.125	≤0.125	≤0.125	0.5	128	64	4	1	1	64	≤0.125

Clind = clindamycin; Amox = amoxicillin; Amp = ampicillin; Te = tetracycline; PenV = penicillin V; Eryth = erythromycin; Mz = metronidazole; Vanc = vancomycin; PolyB = polymyxin B; Bacit = bacitracin; AmphB = amphotericin B; Trimeth = trimethoprim.

* MIC$_{90}$ for ATCC *Capnocytophaga* strains were greater than the corresponding MIC$_{90}$ values for *Capnocytophaga* clinical isolates (see Table 6. 4)

Table 6.4 MIC$_{90}$ (μg ml^{-1}) values for clinical isolates of *Capnocytophaga* spp.

	Antimicrobials used in clinical practice							Agents incorporated into selective media				
.	Clind	Amox	Amp	Te	PenV	Eryth	Mz	Vanc	PolyB	Bacit	AmphB	Trimeth
C. gingivalis	≤0.125	≤0.125	-	0.5	0.5	0.5	64	-	-	-	-	-
C. gingivalis v	≤0.125	≤0.125	-	0.5	0.25	0.25	64	-	-	-	-	-
C. ochracea	≤0.125	1	0.5	0.5	0.5	0.5	128	16	≥512	≥512	128	≤0.125
C. ochracea v	≤0.125	≤0.125	-	0.5	0.5	1	256	-	-	-	-	0
C. sputigena	≤0.125	≤0.125	≤0.125	2	1	128	128	4	256	64	256	≤0.125
C. granulosa	≤0.125	≤0.125	-	0.5	2	1	128	-	-	-	-	-
C. haemolytica	≤0.125	≤0.125	-	0.25	0.5	0.5	16	-	-	-	-	-

Clind = clindamycin; Amox = amoxicillin; Amp = ampicillin; Te = tetracycline; PenV = penicillin V; Eryth = erythromycin; Mz = metronidazole; Vanc = vancomycin; PolyB = polymyxin B; Bacit = bacitracin; AmphB = amphotericin B; Trimeth = trimethoprim.

C. gingivalis v = *C. gingivalis* variant

C. ochracea v = *C. ochracea* variant

Table 6.5 Summary of the *Capnocytophaga* clinical isolates tested for antimicrobial sensitivity.

Species	Number	%
C. gingivalis	19	4.8
C. gingivalis variant	11	2.8
C. ochracea	180	45.2
C. ochracea variant	72	18.0
C. sputigena	42	10.6
C. granulosa	70	17.6
C. haemolytica	4	1.0
Total	398	100

Table 6.6 Numbers of clinical isolates of *Capnocytophaga* spp. tested for antimicrobial / antifungal sensitivity.

	Antimicrobials used in clinical practice							Agents incorporated into selective media				
	Clind	Amox	Amp	Te	PenV	Eryth	Mz	Vanc	PolyB	Bacit	AmphB	Trimeth
C. gingivalis	17	14	-	17	17	17	17	-	-	-	-	-
C. gingivalis v	11	11	-	11	11	11	11	-	-	-	-	-
C. ochracea	144	142	5	143	142	144	139	5	5	5	5	5
C. ochracea v	67	68	-	68	68	68	68	-	-	-	-	-
C. sputigena	43	43	17	43	43	43	43	17	17	17	17	17
C. granulosa	59	59	-	59	59	59	55	-	-	-	-	-
C. haemolytica	3	3	-	3	3	3	3	-	-	-	-	-
	344	340	22	341	343	345	336	22	22	22	22	22

Clind = clindamycin; Amox = amoxicillin; Amp = ampicillin; Te = tetracycline; PenV = penicillin V; Eryth = erythromycin; Mz = metronidazole; Vanc = vancomycin; PolyB = polymyxin B; Bacit = bacitracin; AmphB = amphotericin B; Trimeth = trimethoprim.

C. gingivalis v = *C. gingivalis* variant

C. ochracea v = *C. ochracea* variant

Table 6.7 Distribution of antimicrobial sensitivity.

	Number of *Capnocytophaga* clinical isolates for which the MIC (μg ml^{-1}) was:													
	$\geq$512	256	128	64	32	16	8	4	2	1	0.5	0.25	$\leq$0.125	Total
Clind	-	-	-	-	-	-	2	-	1	2	-	1	338	344
Amox	-	-	-	-	-	-	1	1	1	34	14	12	277	340
Te	-	-	-	-	-	-	-	-	6	2	89	154	90	341
PenV	-	-	-	-	-	2	1	6	7	35	116	59	114	343
Mz	24	27	14	30	15	17	64	78	23	17	9	3	15	336
Eryth	-	-	23	-	-	-	1	3	7	33	84	44	150	345

Clind = clindamycin; Amox = amoxicillin; Te = tetracycline; PenV = penicillin V; Mz = metronidazole; Eryth = erythromycin.

6.4 Discussion

Previous studies have reported on the antimicrobial sensitivity of *Capnocytophaga* spp. (Forlenza *et al.* 1981, Sutter *et al.* 1981, Rummens *et al.* 1986, Bremmelgard *et al.* 1989, Jolivet-Gougeon *et al.* 2000), however to date none have reported on the antimicrobial sensitivity of each species within the genus. Although similar antimicrobial sensitivity values for bacterial species within a genus might be expected, it would be beneficial to have data for each species as this would:

i) detect potential differences in antibiotic profiles between the species

ii) enable more accurate treatment especially when infections are due to a single species (Ngaage *et al.* 1999, Ebinger *et al.* 2000).

In this study, the ATCC strains of the non-oral human species *C. canimorsus* and *C. cynodegmi*, were included as they are known to cause severe septicaemia and wound infections respectively in humans, after a dog or cat scratch or bite (Griego *et al.* 1995) and no antimicrobial sensitivity data on these species have been reported to date. The 398 clinical isolates tested (Table 6.5) were randomly selected from the 848 isolates obtained during the course of this study and which were identified to species level using 16S rRNA PCR-RFLP (Chapter 4). Some of the species failed to grow on the control plates or were contaminated and were therefore excluded from analysis. This accounts for the differing total numbers of isolates in Table 6.6. Antimicrobial sensitivity testing of the clinical isolates was performed blindly i.e. the identity of the species was not known at the time of testing. This was done in order to avoid bias when examining the plates and reading the results. However this resulted in only 5 *C. ochracea* and 17 *C. sputigena* clinical isolates having been tested (in parallel with the ATCC strains, Table 6.6) for their sensitivity against the antimicrobial agents used in selective media. Thus, with hindsight, it would have been better to have been aware of the identity of the isolates and to have selected a more representative proportion of each isolate for antimicrobial sensitivity testing against the antimicrobial agents used in the selective media.

The agar dilution method was chosen as it is recognised as the best method for evaluation of the susceptibility to antimicrobial agents of *Capnocytophaga* spp. and other fastidious anaerobic bacteria (Rummens *et al.* 1986, NCCLS 1993). In addition, it generates quantitative results (i.e. an MIC) rather than a category result, as would be the case with breakpoint testing. The procedures used throughout this study were those recommended by the NCCLS (Woods & Washington 1995). Lysed horse blood was used in preference to sheep blood, as it is the blood recommended for use when

191

testing fastidious anaerobes (Wexler & Doern 1995). Furthermore, sheep blood might antagonise the activity of certain antimicrobial agents e.g. trimethoprim (Bauer *et al.* 1964).

The antimicrobial agents were prepared and dispensed into separate sterile Eppendorf tubes in order to prevent several freeze-thawing cycles which would jeopardise the performance of the antimicrobial agents (manufacturer's recommendations). The stock solutions were stored for a maximum period of 14 days then discarded and new stock solutions prepared. In order to obtain the best results, the agar dilution plates were prepared 24 - 48 hours prior to inoculation.

The maximum number of inocula transferred to the plates at any one time was 36. This was determined by the (36) prongs on the multipoint inoculator. After inoculation the plates were allowed to dry before being inverted and incubated, otherwise the broths would run into each other leading to erroneous results.

One of the notable findings in this study was that the MIC_{90} for the ATCC *Capnocytophaga* strains were either equal to, or less than, the respective values for the *Capnocytophaga* clinical isolates, with the exception of penicillin V for *C. granulosa* and *C. haemolytica*, and erythromycin for *C. ochracea*, *C. granulosa* and *C. haemolytica*. This variation might be due to laboratory attenuation of the ATCC strains. Thus when comparing MIC values, it would be better to compare results between clinical isolates rather than between clinical isolates and ATCC strains. In addition, any data generated for these 2 categories of bacterial strains should be reported separately.

From the data in Table 6.7 it is evident that some of the *Capnocytophaga* clinical isolates grew above the MIC_{90} values of the antimicrobial agents used in clinical practice (Table 6.4). This could indicate the emergence of resistant strains of *Capnocytophaga* spp. This study however does not provide sufficient evidence to conclusively support such conclusions and indeed further testing of these isolates is warranted.

One of the aims of this study was to determine whether the concentrations of the antimicrobial agents incorporated in selective media (previously used for isolation of *Capnocytophaga* spp.) could have affected recovery of *Capnocytophaga* species from clinical samples. A comparison of the concentrations of the agents used in the

selective media versus the MIC_{90} for the *Capnocytophaga* ATCC strains and clinical isolates is shown in Table 6.8.

Medium	Antimicrobial agent used in selective medium	Conc. of antimicrobial agent (μg ml^{-1})	Range of MIC_{90} (μg ml^{-1}) for ATCC strains[t]	Range of MIC_{90} (μg ml^{-1}) for clinical isolates*
Mashimo	Bacitracin	50.0	1 - $\geq$512	64 - $\geq$512
Cap	Polymyxin B	1.96	1 - $\geq$512	256 - $\geq$512
	Vancomycin	5.0	$\leq$0.125 - 32	4 - 16
	Trimethoprim	2.5	$\leq$0.125 - 0.25	$\leq$0.125
	Amphotericin B	2.5	8 - $\geq$512	128 - 256
Slots	Bacitracin	75.0	1 - $\geq$512	64 - $\geq$512
	Vancomycin	5.0	$\leq$0.125 - 32	4 - 16

Table 6.8 Comparison of antimicrobial concentrations used in selective media and antimicrobial sensitivity results obtained as part of this study

[t] from Table 6.3

* from Table 6.4 (data refer to *C. ochracea* and *C. sputigena* - see discussion section).

The comparisons show that in the case of the ATCC strains the concentrations of bacitracin, polymyxin B, vancomycin and trimethoprim used in the selective media would affect the recovery of *Capnocytophaga* strains. In the case of the *Capnocytophaga* clinical isolates, the concentrations of bacitracin, vancomycin and trimethoprim would affect recovery of *C. ochracea* and *C. sputigena*. The results for bacitracin and vancomycin are in agreement with those of Sutter *et al.* (1981).

The high susceptibility of *Capnocytophaga* clinical isolates to amoxicillin and clindamycin is reassuring since these antimicrobial agents are used for endocarditis prophylaxis (BSAC 1992), which can be caused by *Capnocytophaga* spp. (Montejo Baranda *et al.* 1984, Buu-Hoi *et al.* 1988, Ngaage *et al.* 1999). Pencillin V and erythromycin (also used for endocarditis prophylaxis) showed slightly higher MIC_{90} values. The exception in the case of erythromycin was *C. sputigena* which yielded an MIC_{90} of 128 μg ml^{-1} (Table 6.4).

Direct comparison of these results with data from other studies (Forlenza *et al.* 1981, Sutter *et al.* 1981, Rummens *et al.* 1986, Hawkey *et al.* 1987, Bremmelgard *et al.* 1989, Jolivet-Gougeon *et al.* 2000) is difficult primarily because some studies used methods other than the agar dilution technique (Forlenza *et al.* 1981, Verghese *et al.* 1988) and those which did either used a small number of isolates or else did not conform to the NCCLS method of agar dilution (Bremmelgard *et al.* 1989, Kolokotronis 1995, Jolivet-Gougeon *et al.* 2000). Furthermore the strains being tested were not identified to species level as was the case in this study, thus precluding comparison at species level.

The clinical application of these findings must be considered with respect to the levels of these antimicrobial agents that can be obtained in gingival crevicular fluid (GCF; Table 6.9).

	Dose* (mg)	GCF conc. (μg ml^{-1})	MIC$_{90}$ (μg ml^{-1}) for *Capnocytophaga* clinical isolates[#]
Penicillin	500	ND	$\leq$0.25 – 2
Amoxicillin	500	3 - 4	$\leq$0.125 - 1
Tetracycline	500	5 - 12	$\leq$0.25 - 2
Clindamycin	150	1 - 2	$\leq$0.125
Metronidazole	500	8 - 10	64 - 256
Erythromycin	250	ND	0.25 - 128

Table 6.9 Comparison of GCF concentration of antimicrobial agent versus MIC$_{90}$ values (adapted from Lorian 1986, Slots & Rams 1990).

* systemic dose prescribed to patient

[#]from this study (Table 6.4)

conc. = concentration; ND = not detected

Thus, it is evident that at the stipulated dose prescribed to patients, the GCF levels of the above antimicrobial agents, except for metronidazole, seem to be sufficient for clinical efficacy as regards the inhibition of *Capnocytophaga* clinical isolates. A note of caution is warranted: these results pertain only to pure cultures of the isolates grown on solid culture media and it would not be surprising to find some differences if the study were to be repeated with the isolates grown ideally as mixed biofilms thus simulating subgingival plaque.

In conclusion therefore, this part of the study has established the antibiotic profiles of more than 336 *Capnocytophaga* human clinical isolates which had been identified to species level using molecular techniques. More importantly, these results have shown that the clinically administered doses of the antimicrobial agents used in clinical practice are sufficient to treat periodontal infections associated with *Capnocytophaga* spp. In addition, clinical isolates of *Capnocytophaga* spp. were highly sensitive to amoxicillin and clindamycin, i.e. antibiotics used for the prophylaxis of bacterial endocarditis.

196

Chapter 7

Comparison of GCF-glucose between DM and

Non-DM-Periodontitis subjects

7.1 INTRODUCTION

The nature of a particular microflora at a specific site is determined by the various factors that predominate at that site. The physical and chemical characteristics of a site determine the pioneer microbes, whilst local nutrients and those generated through the metabolic activities of the pioneer species, have an effect on successive colonisers (Loesche 1968), which eventually collectively constitute the climax community of the biofilm formed at that site (Marsh & Martin 1999). This holds true for any biofilm including dental plaque.

The diverse habitats present within the oral cavity dictate the consortia of micro organisms which establish themselves at a particular location at a specific point in time (Listgarten 1994). One of the most contrasting ecological differences in the oral cavity is that between supra- and subgingival niches. Although the physical and chemical features of these sites differ, the most striking is the nutritional difference observed between the two sites. Apart from microbial products produced by other species, the major source of nutrients for supragingival dental plaque are the glycoproteins of salivary origin (Morhart & Fitzgerald 1976) and food residues. In subgingival plaque, where saliva and host diet have little effect, it is the macromolecules from host tissues and gingival crevicular fluid (GCF), which sustain the growth of subgingival organisms (Loesche 1968). GCF is a tissue fluid that percolates through the junctional epithelium into the gingival crevice and thence into the oral cavity. Since it originates from plasma, its chemical composition is thought to mirror that of blood (Ficara *et al.* 1975). It has been suggested that the hyperglycaemia pathognomonic of DM will be reflected in gingival crevicular fluid. This may in turn select for, and encourage the growth of, a more saccharolytic flora e.g. *A. actinomycetemcomitans*, *Capnocytophaga* spp., and *Prev. intermedia* all of which have been recovered from subgingival plaque in DM-periodontitis patients (Mashimo *et al.* 1983). Furthermore, such selective pressure in the local subgingival microenvironment may induce expression of virulence factors, e.g. increased enzyme production, relevant to periodontal tissue degradation (Spratt *et al.* 1996). Moreover, glucose is a potent reducing agent, so that it could lower the redox potential of the subgingival environment even further, thus ecologically favouring the growth of putatively pathogenic anaerobic organisms. In addition, in the clinical scenario this might alter the physiology of the surrounding periodontal tissues (Nishimura *et al.* 1996, Terada *et al.* 1998) leading to exacerbated periodontal destruction observed in DM-Periodontitis subjects.

A few studies in the 1970s compared the levels of GCF-glucose to that in blood with disparate results (Hara *et al.* 1969, Kjellman *et al.* 1970, Ficara *et al* 1975). Different GCF sampling procedures were used and the analytical methods employed to quantify GCF-glucose were rather crude. Collectively these could explain the inconsistent data obtained. Further work on GCF-glucose quantification might have been hampered by the lack of a sensitive method for glucose quantification in submicrolitre volumes of the fluid sample.

The aims of this investigation therefore were to:

i)   develop a method for quantification of GCF-glucose

ii) compare GCF-glucose levels between DM-and non-DM-Periodontitis subjects.

The null hypothesis formulated for this investigation was that there was no difference in GCF-glucose between DM- and non-DM-Periodontitis patients.

The study was conducted in three parts:

i)   *in vitro* development of a micro-assay for GCF-glucose quantification

ii) *in vivo* quantification of GCF-glucose concentration in periodontally healthy and periodontitis subjects (validation study)

iii) *in vivo* quantification of GCF-glucose concentration in DM- and non-DM-Periodontitis subjects.

7.2 Materials and methods

7.2.1 Reagents

A commercially-available assay kit indicated for serum estimation (Glucose Trinder, procedure number 315, Sigma Diagnostics) was used in this micro-assay. A series of glucose (Analar, Dorset, UK) solutions were prepared over a concentration range of 50 - 400 mg dl^{-1}. A glucose / urea nitrogen standard containing a glucose concentration of 100 mg dl^{-1} (Sigma Diagnostics) was used as a control. Human serum (male, type AB, Sigma) was used as a substitute for GCF.

7.2.2. *In vitro* development of GCF-glucose micro-assay

Trinder's reagent was reconstituted according to the manufacturer's instructions. The absorbance of the freshly reconstituted solution was checked prior to each assay. The solution was discarded if the absorbance, when measured spectrophotometrically (Ultrospec 2000, Pharmacia Biotech. Ltd., Cambridgeshire, UK) in a 1 cm light path at 492 nm versus water as reference, exceeded 0.2. Trinder's reagent was pipetted into the wells of a 96-well round-bottomed micro-titre plate. A 1 µl Hamilton syringe (700

series, Bonaduz, Switzerland) with attached Cheney adaptor was used to dispense the samples (i.e. either glucose solutions or human serum spiked with glucose) into the respective wells. The volume of glucose sample was 0.5 µl. The volume of human serum spiked with glucose ranged between 0.2 µl and 0.8 µl. All reactions took place under standard conditions of temperature (20°C) and time (18 min). The colour change of the micro-assay was assessed spectrophotometrically at 492 nm using a microtitre plate reader (MRX Microplate Reader, Dynex Technologies (UK) Ltd, West Sussex, UK).

The manufacturer's recommendations for this particular assay were to use:

i) a minimum sample volume of 5 µl, and

ii) sample to reagent ratio of 5 µl to 1 ml.

Since this assay was aimed at determining the volume of glucose in submicrolitre volumes of fluid (GCF), this ratio was proportionally reduced to 0.5 µl sample to 100 µl reagent. The effect of alteration in sample and reagent volumes on the biochemical characteristics, and hence linearity, of the assay, was tested using a range of glucose solutions with a sample:reagent ratio of 0.5 µl:100 µl. The concentration of the glucose solutions used ranged from 50 mg dl^{-1} to 400 mg dl^{-1}.

The micro-assay was subsequently repeated using human serum spiked with glucose (concentration range 50 - 200 mg dl^{-1}). The sample (serum / glucose mixture) to reagent ratio was maintained at 0.5 µl:100 µl.

The assay was also performed using human serum spiked with glucose as the sample, the volume of which ranged from 0.2 µl to 0.8 µl while the reagent volume was increased to 200 µl. In order to simulate clinical sampling of GCF, the latter part of the study was performed by applying known volumes of the serum / glucose sample to Periopaper strips (Ora-flow Inc., Amityville, New York, USA). These commercially available filter paper strips are specifically intended for GCF collection.

7.2.3 *In vivo* study involving use of GCF-glucose micro-assay

Ethical approval and criteria for patient recruitment have been described in Chapter 2 (section 2.1). Patients' personal information and periodontal data were recorded as described in sections 2.2.1.and 2.2.2. Two groups were used: the first group consisted of (periodontally and systemically) healthy and non-DM-Periodontitis subjects section 7.2.3.1); the validation study of the *in vitro* micro-assay was performed using samples from these patients. The second group consisted of DM- and non-DM-Periodontitis subjects (7.2.3.2).

199

7.2.3.1 *In vivo* quantification of GCF-glucose: periodontally healthy and periodontitis subjects

Nineteen systemically healthy subjects accepted to participate in the study. The patients were divided into 2 groups: periodontally healthy (n = 11) with PPD $\leq$ 3 mm and periodontitis (n = 8) with PPD $\geq$ 5 mm. GCF was collected from 3 sites in periodontally healthy subjects and from 6 sites in periodontitis patients. In the latter case the samples were collected as follows: 3 samples from healthy sites (PPD $\leq$ 3 mm) and 3 from diseased sites (PPD $\geq$ 5 mm).

7.2.3.2 *In vivo* quantification of GCF-glucose: DM-Periodontitis and non-DM-Periodontitis subjects

Fifteen DM- and 17 non-DM-Periodontitis subjects were recruited for this part of the investigation. Patients were confirmed as DM or non-DM subjects via estimation of their glycated haemolgobin levels (HbA_{1c}). For each patient, 3 healthy sites (PPD $\leq$ 3 mm) and 3 diseased sites (PPD $\geq$ 5 mm and with radiographic evidence of bone loss) were selected for GCF collection.

7.2.4 GCF collection & processing

Sites selected for GCF collection were isolated with cotton wool rolls. A saliva ejector was used to avoid salivary contamination of the samples. Any clinically evident supragingival plaque was removed with a periodontal probe, care being taken not to elicit gingival bleeding and the area briefly and gently dried with air. Periopaper strips used for GCF collection were inserted into the crevice or pocket until mild resistance was felt and left *in situ* for 2 minutes (Fig.7.1a). Thereafter the volume of fluid collected was determined using the Periotron 8000 (Ora Flow Inc.) which was located at the chair-side. Once the sample had been collected, the filter paper strip was immediately placed in a dry, sterile, pre-labelled Eppendorf tube. The tubes were placed in liquid nitrogen and transported to the laboratory for glucose analysis. Quantification of glucose in GCF was performed using the modified micro-assay (Fig.7.1b). Any blood-stained GCF strips were excluded. Briefly, each strip was placed in a separate well of the microtitre plate with each well containing 100 μl Trinder's reagent. The reaction was allowed to proceed at room temperature for 18 minutes. A blank assay, a standard reagent and the patient's plasma sample were run in parallel with the GCF samples. The optical density (OD) of the samples was noted. The background OD was then subtracted from the actual OD

value and the quantity of glucose per 2-minute sample calculated from a previously established calibration graph.
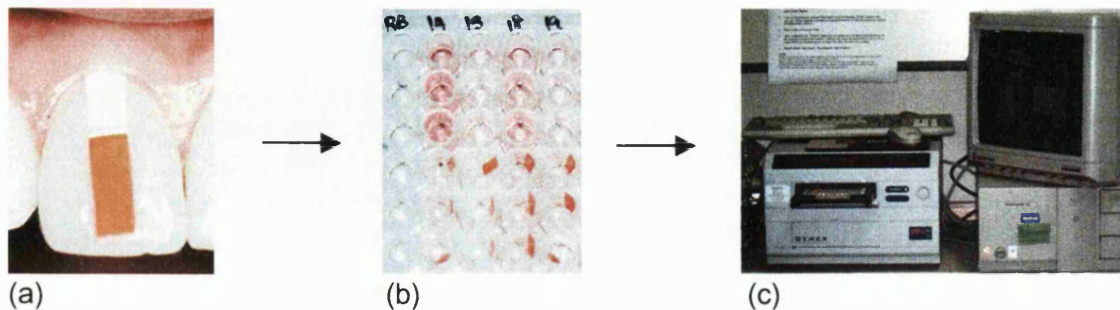
Fig.7.1 GCF collection (a); sample reaction was performed in a microtitre plate (b) and colour change quantified in a microtitre plate reader (c).

7.2.5 Blood samples

Blood samples were withdrawn from each patient as described in Chapter 2. One ml blood (taken from the 10 ml blood sample contained in the K_3 EDTA Vacutainer tube) was centrifuged (15,800 rcf) for 4 min at 4°C. Using a Hamilton syringe, 0.5 µl of plasma supernatant was added to the 100 µl of Trinder's reagent in the respective wells of the microtitre plate. For each patient, the assays for the blood samples were run in parallel with the GCF samples.

7.3 Statistical Analysis

Statistical comparisons for PPD, LCAL & GCF volume between and within groups were performed using the Student *t*-test; PI & BI were compared using *Mann-Whitney U* test, for the reasons given in Chapter 5. Statistical analysis of GCF-glucose concentration (µg µl^{-1}) was performed using MLM.

7.3.1 Measures of normality: skewness and kurtosis

The distribution properties of the raw data for GCF-glucose concentration (µg µl^{-1}) in both studies were checked for skewness and kurtosis prior to their respective analysis. Since each data set was not normally distributed, it was transformed in order to achieve normal distribution. Logarithmic transformations resulted in zero skewness and minimal kurtosis. Data were initially entered on an Excel spreadsheet (Microsoft Excel for Windows 95 version 7) and subsequently converted for use by *MLwiN* (Rasbash *et*

al. 2000). The logarithmic values for GCF-glucose concentration were then reverse transformed to obtain the actual GCF-concentration values (μg μl^{-1}).

7.3.2 Coding prior to modelling

Site status and DM status were binary variables and were therefore coded as 0 or 1 for healthy versus diseased sites, and non-DM versus DM subjects.

7.4 Results

7.4.1 *In vitro* GCF-glucose micro-assay

The results of the *in vitro* experiments investigating performance of the micro-assay (Fig.7.2) showed that the linearity of the assay was not affected when the sample (glucose solution) to reagent volume was lowered from 5 μl per ml (as instructed by the manufacturer) to 0.5 μl per 100 μl respectively.
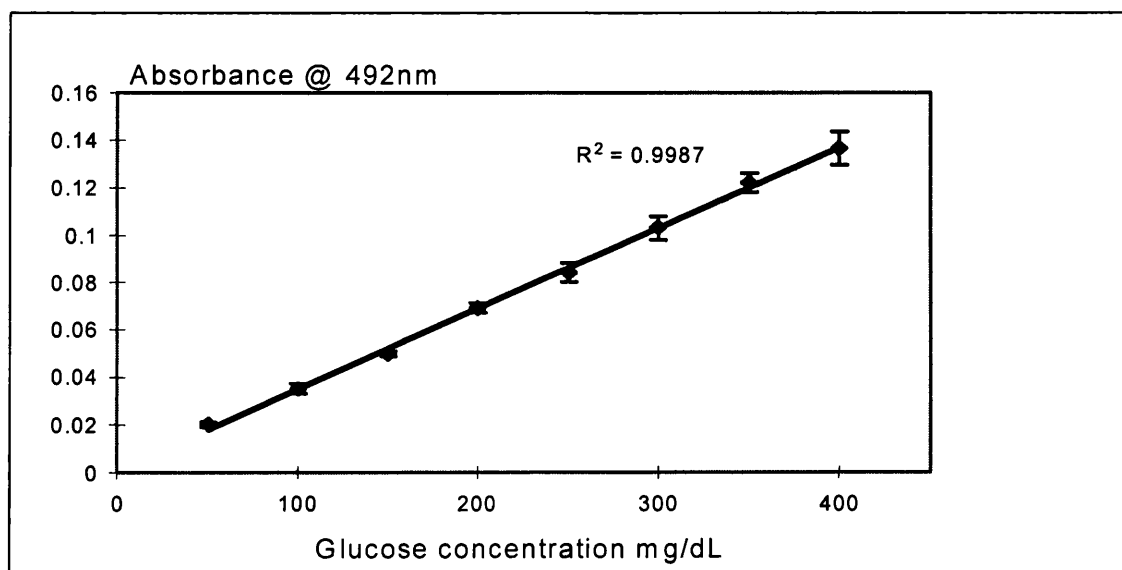
Fig.7.2 Absorbance of reaction mixture (glucose solution and Trinder's reagent) measured at 492 nm vs increasing glucose concentration. Glucose sample = 0.5 μl; Trinder's reagent = 100 μl; reaction time = 18 min. Each datum point represents mean values (n = 4); error bars represent standard deviations.

Furthermore, the linearity of the assay was also maintained when human serum spiked with glucose (over a concentration range of 50 to 200 mg / dL) was used as the sample inoculum (Fig.7.3). This part of the investigation was repeated twice. The linear equations, which best described the results for the different sample volumes,

consistently showed that the intercept was equivalent to the inherent serum glucose (i.e. human serum without added glucose).
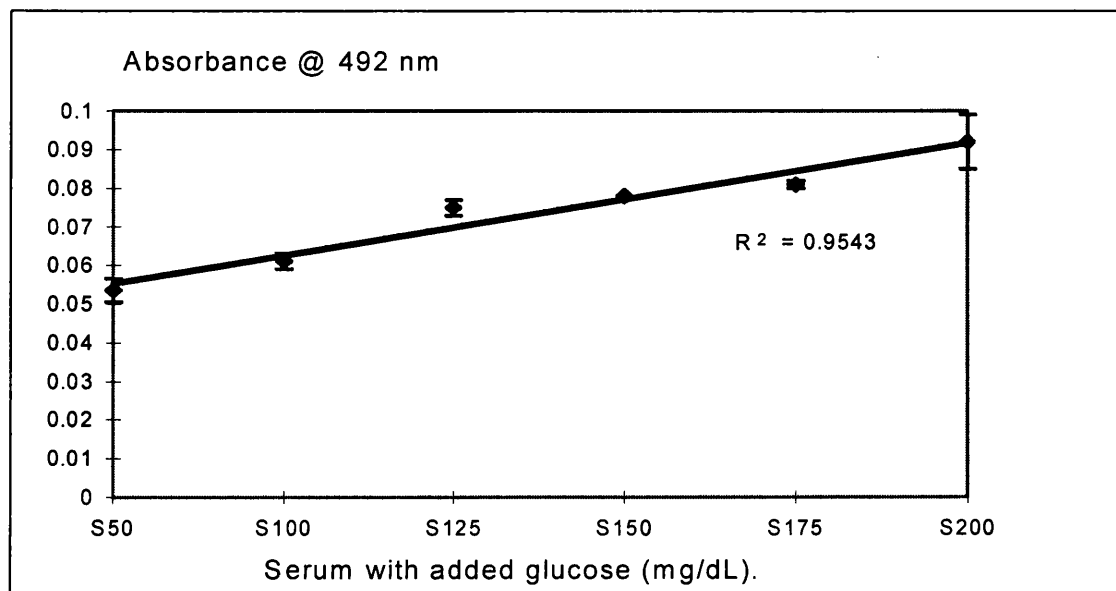
Fig.7.3. Absorbance of reaction mixture (serum with added glucose and Trinder's reagent) measured at 492 nm vs increasing glucose concentration. Sample volume = 0.5 µl; Trinder's reagent = 100 µl; reaction time = 18 min. Each datum point represents mean values (n = 4); error bars represent standard deviations. Intercept = inherent serum glucose.

The results of the micro-assay, when performed with human serum spiked with glucose (concentration range 50 to 200 mg dl^{-1}) over a volume range of 0.2 µl to 0.8 µl, are shown in Fig. 7.4. The reagent volume was equal to 200 µl. The results of these assays showed that the linearity of each assay was maintained even though the sample to reagent volume was altered. Furthermore the intercept given by each of the linear equations (Fig. 7.4) was consistently equal to inherent serum glucose values for the specified assay as shown in Table 7.1.
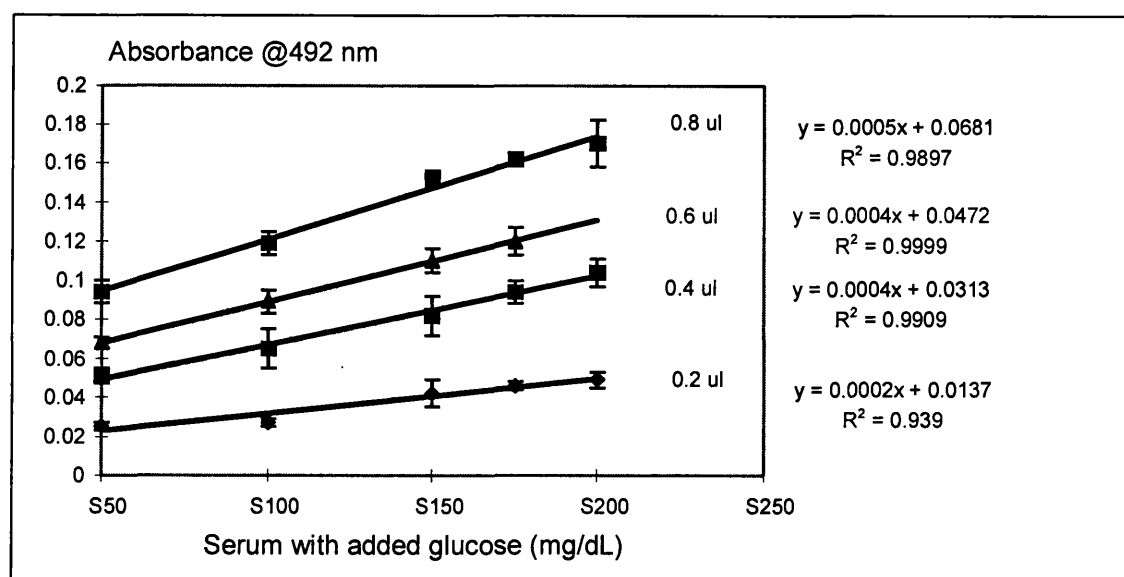
Fig.7.4. Absorbance of reaction mixture (serum + added glucose and Trinder's reagent) measured at 492 nm versus increasing glucose concentration. The sample volume varied between 0.2 μl and 0.8 μl; volume of Trinder's reagent used for all assays was equal to 200 μl; reaction time = 18 min. Each datum point represents mean values (n = 4); error bars represent standard deviations; intercept = inherent glucose in serum.

Sample volume (μl)	Absorbance at 492 nm of inherent serum glucose (serum without added glucose)	Intercept as calculated from equation in Fig.7.4
0.2	0.021	0.014
0.4	0.038	0.031
0.6	0.048	0.047
0.8	0.071	0.068

Table 7.1 Comparison of absorbance at 492 nm of inherent serum glucose (as assayed spectrophotometrically) with the calculated value obtained from the linear equation in Fig.7.4.

7.4.2 Clinical samples

7.4.2.1 GCF-glucose: periodontally healthy and periodontitis subjects

A total of 81 GCF samples were collected from 19 subjects (mean age 35y ± 11.8; range 23 -61 y; 9 men and 10 women; Table 7.2). Thirty-three samples were collected from periodontally healthy patients (median probing depth = 2.0 mm, median GCF volume 0.4 μl). Two samples were blood-stained and were excluded from the analysis. A

204

total of 48 samples were collected from the periodontitis subjects as follows: 24 from healthy sites (median PPD = 2.0 mm, median GCF volume 0.4 µl); 24 from diseased sites (median probing depth = 6 mm, median GCF volume = 1.1 µl). The absolute amount of glucose per strip (i.e. per 2-minute sample) was calculated from a previously established calibration graph. Data used for plotting this graph were those obtained when performing the micro-assay *in vitro*. Since the volume of each GCF sample was known, the GCF concentration (µg µl^{-1}) was calculated.

Subjects	Healthy	Periodontitis	
No. of subjects	11	8	
site status	healthy	healthy[†]	diseased
No. of sites	31	24	24
PPD[†] (mm)	2.0 (1.8, 3.0)	2.0 (2.0, 3.0)	6.0 (5.0, 7.0)
LCAL[†#] (mm)	0.0 (0.0, 0.0)	0.0 (0.0, 4.0)	6.0 (5.0, 7.0)
GCF[†] (µl)	0.4 (0.3, 0.6)	0.4 (0.3, 0.9)	1.1 (0.6, 1.6)
Plaque[†] Index	0.0 (0.0, 1.0)	0.5 (0.0, 1.3)	1.0 (1.0, 2.0)
Bleeding Index	0.0 (0.0, 0.0)	0.0 (0.0, 1.0)	0.5 (0.0, 2.0)
Blood glucose (mg dl^{-1})[□]	72.7 (69.2, 81.8)	100.0 (88.0, 125.0)	

Table 7.2 Summary of clinical details of subjects in the validation study. Data are presented as medians (interquartile range). Comparisons were performed using *t*-tests for PPD, LCAL & GCF volume and blood-glucose, and *Mann-Whitney U* tests for PI & BI.

[†]Significant difference between healthy and diseased sites ($P < 0.05$) in periodontitis group, except for BI ($P = 0.28$)

[#]Significant difference in LCAL only between healthy sites between the two groups

[□]Significant difference between groups

7.4.2.2 VC model for GCF-glucose: periodontally healthy and periodontitis subjects (validation study)

The results for the VC model for the outcome variable GCF-glucose concentration (µg µl^{-1}), showed that the estimate for the intercept (mean) was significantly different from zero at 0.11 (µg µl^{-1}) ($P < 0.001$), which was to be expected (Table 7.3).

	Estimate (95%CI)	*P*-value	
Fixed structure: Intercept (mean)	0.11 (0.05, 0.21)	<0.001	
Random structure Level-2 (subject) Level-1 (site)	Variance (SE) 1.64 (0.68) 1.59 (0.29)	<0.001 <0.001	Proportion of total 51% 49%
Total	3.23		100%

Table 7.3 VC model for GCF-glucose concentration ($\mu g \ \mu l^{-1}$) in periodontally healthy and periodontitis subjects; N = 77; SE = Standard error; 95%CI = 95% Confidence Interval.

The level-2 (i.e. between subject) variance accounted for 51% of the total variance, while the level-1 (i.e. between site) variance accounted for 49% of the total variance. The significant variances at each level ($P < 0.001$) indicated that there was sufficient variation at each level to require the use of MLM. In addition, the large variance at the site level justified the use of multiple site data per subject as single data rather than as pooled samples aggregated to the subject level. The MLM assumptions were checked by plotting the standardised residuals against the standard normal cumulative distribution (normal score). These showed that the assumptions were satisfied since the plots demonstrated a near-linear relationship (Fig.7.5).
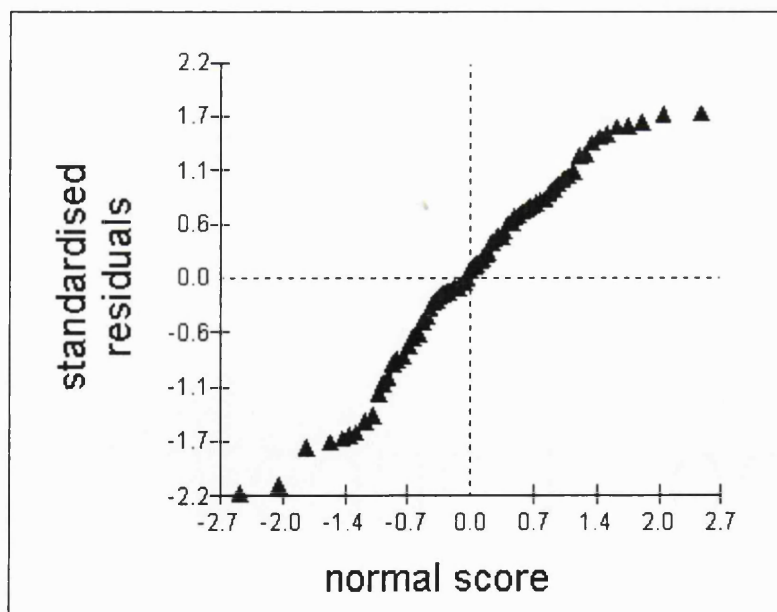
Fig.7.5 Residuals for the VC model for GCF-glucose concentration ($\mu g \ \mu l^{-1}$) in periodontally healthy and periodontitis groups.

7.4.2.3 RI Model for GCF-glucose: periodontally healthy and Periodontitis subjects

The RI model for the GCF-glucose for this group of patients was generated after introducing healthy non-Periodontitis sites and healthy and diseased sites from the Periodontitis group as explanatory variables (Table 7.4).

Fixed effects (covariates)	Estimate (95%CI)	P-value
Intercept (Healthy non-Periodontitis patients/ sites)	0.08 (0.03,0.19)	<0.001
Subject/site-level		
Healthy sites in Periodontitis group	0.14 (0.03,0.58)	0.389
Diseased sites in Periodontitis group	0.18 (0.04,0.75)	0.227
Random Effects	Variance (SE)	
Subject (level-2 σ^2_{ou})	1.64 (0.68)	0.018
Site (level-1 σ^2_{oe})	1.60 (0.30)	<0.001
Total	1.16	

Table 7.4 RI model for the outcome variable GCF-glucose concentration ($\mu g\ \mu l^{-1}$); N = 77; SE = Standard error; healthy (i.e. periodontally healthy subjects / sites) used as baseline.

The results of the RI model showed that there was no change in the subject-level variance (1.64 in Tables 7.3 & 7.4) after introducing explanatory variables to the model. The site-level variance increased negligibly (<0.01%) from the VC model to the RI model and covariates coefficients were far from significant (P = 0.389, P = 0.227). These results showed that the GCF-glucose concentration ($\mu g\ \mu l^{-1}$) was not affected by subject / status, which was to be expected since all the subjects in this part of the study were non-diabetic. The model assumptions underlying MLM were checked by plotting the standardised residuals against the standard normal cumulative distribution (normal score). These showed that the assumptions were satisfied since the plots demonstrated a near-linear relationship (Fig.7.6).
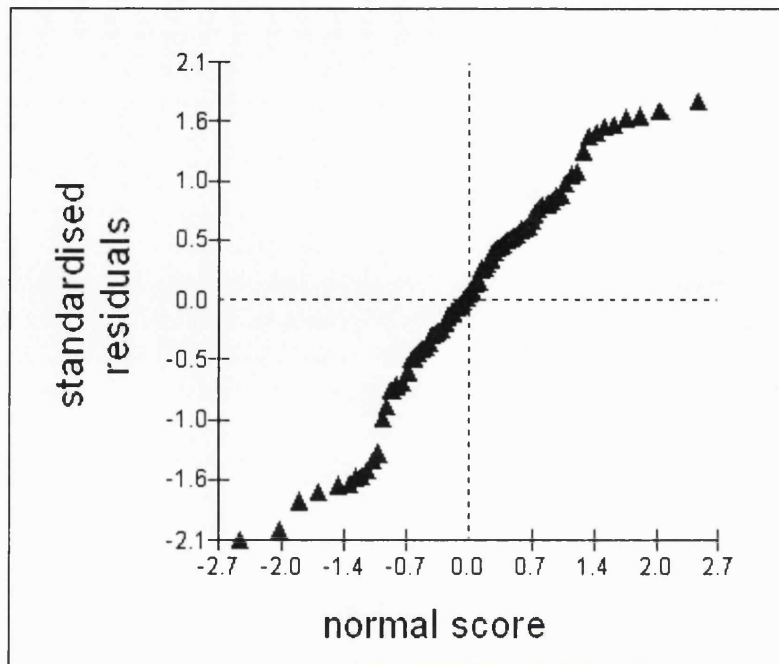
Fig.7.6 Residuals for RI Model for GCF-glucose concentration (μg μl^{-1}) in periodontally healthy and periodontitis subjects (validation study).

The levels of GCF-glucose concentration (μg μl^{-1}) for the healthy i.e. non-Periodontitis group (designated as the control group and thus used as baseline reference), healthy sites in the Periodontitis group (denoted by Healthy Perio) and diseased sites in the Periodontitis group (denoted by Diseased Perio) are shown in Fig.7.7. The results demonstrated a gradual increase in GCF-glucose concentration from the healthy subjects (controls) to the healthy sites in the Periodontitis group and the diseased sites in the Periodontitis group. The results showed that whilst there was no significant difference between the healthy sites in the Periodontitis group (Healthy Perio) and the controls (P = 0.126), the GCF-glucose concentration was significantly higher in the diseased sites in the Periodontitis group when compared to both the control group (P < 0.002) and the Healthy Perio group (P = 0.017).
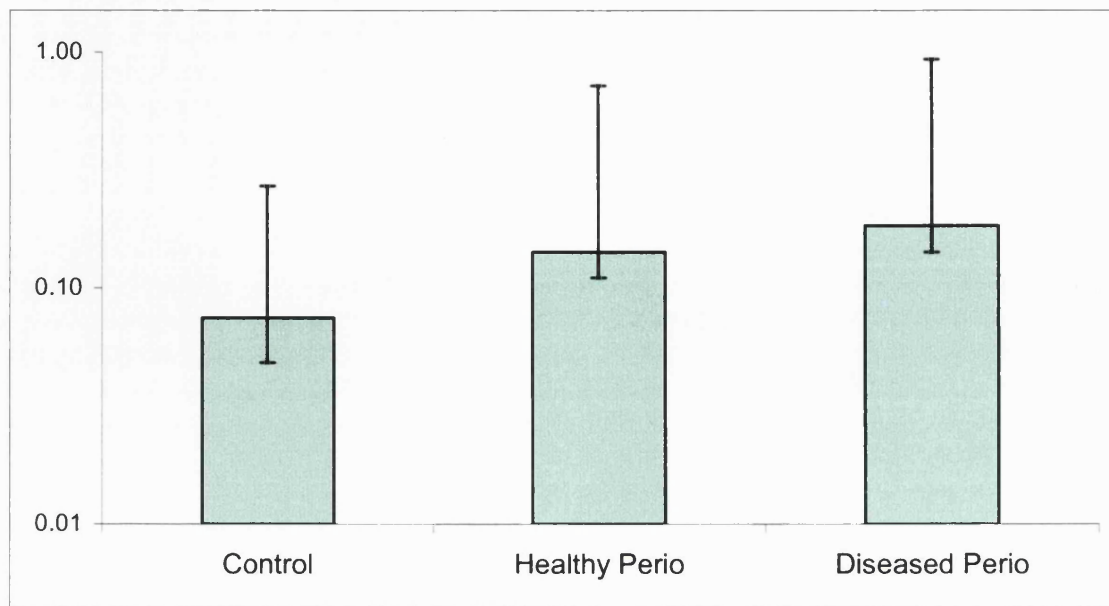
Fig.7.7 GCF-glucose concentration (mean values, µg µl^{-1}): effect of periodontitis, accounting for differences (amongst the periodontitis subjects) between healthy and diseased sites. N = 77. Bars represent 95% CI. Controls = healthy sites in Non-DM-Periodontitis subjects; Healthy Perio: healthy sites in periodontitis subjects; Diseased Perio: diseased sites in Periodontitis subjects.

7.4.2.5 GCF-glucose:Blood-glucose ratio in Healthy & non-DM-Periodontitis subjects

The ratios of GCF-glucose concentration (converted to mg dL^{-1}) to blood-glucose (mg dL^{-1}) are presented in Table 7.5. The results showed that the ratio decreased as periodontal disease status worsened.

Subject status	Healthy	Periodontitis	
Site status	Healthy	Healthy	Diseased
Blood-glucose (mg dL^{-1})	72.7	100.0	
GCF-glucose (mg dL^{-1})	8	14	18
GCF-glucose:Blood-glucose	1:9	1:7.1	1:5.5

Table 7.5 GCF-glucose:Blood-glucose ratios in Healthy & non-DM-Periodontitis subjects. N = 77.

7.4.2.6 GCF-glucose: DM- and non-DM-Periodontitis subjects

A summary of the clinical data collected from DM-Periodontitis and non-DM-Periodontitis groups is presented in Table 7.6. The data show that the patients had similar levels of periodontal disease, facilitating comparison between the groups. Comparisons of the clinical measurements (PPD and LCAL) for healthy and diseased sites within each group showed them to be distinctly different. Between groups comparisons (DM- versus non-DM-periodontitis) of PPD and LCAL for healthy sites were similar. Similarity in clinical measurements between groups was also observed at the diseased sites.

Subject status	DM-Periodontitis		Non-DM-Periodontitis	
No. of subjects	15		17	
Site status	Healthy[t]	Diseased[¶]	Healthy[t#¶]	Diseased[#¶]
No of sites	41	44	49	52
PPD[t¶] (mm)	3.0 (2.0, 3.0)	6.0 (0.5, 8.0)	2.0 (2.0, 3.0)	6.0 (5.0, 7.0)
LCAL[t¶] (mm)	0.0 (0.0, 3.0)	7.0 (5.3, 8.0)	0.0 (0.0, 4.0)	6.0 (5.0, 7.0)
GCF[¶] vol[#] (µl)	0.5 (0.3, 1.2)	1.3 (0.7, 1.6)	0.3 (0.3, 0.6)	1.1 (0.7, 1.6)
PI[#¶]	1.0 (1.0, 1.0)	1.0 (1.0, 2.0)	0.0 (0.0, 1.0)	1.0 (1.0, 2.0)
BI[t#¶*]	0.0 (0.0, 0.0)	2.0 (0.0, 2.0)	0.0 (0.0, 0.0)	2.0 (0.0, 2.0)
Blood-glucose (mg dl^{-1})[□]	205.4 (145.6, 257.8)		100.8 (86.0, 120.7)	

Table 7.6 Summary of patients' data, presented as medians (interquartile range). Comparisons were performed using t-tests for PPD, LCAL, GCF volume and blood-glucose, and *Mann-Whitney U* tests for PI & BI.

[t]Significant difference between healthy & diseased sites within groups ($P < 0.050$)

[#]Significant difference between healthy & diseased sites, non-DM-Periodontitis group only ($P = 0.001$)

[¶]No significant difference between diseased sites, between groups ($P = 0.200$)

*No significant difference between healthy sites, between groups

[□]Significantly different between DM & Non-DM groups ($P < 0.001$)

Glycosylated haemoglobin (HbA_{1c}) values were, as would be expected, significantly higher in DM-Periodontitis patients (mean 8.1% ± 1.61), compared to non-DM-Periodontitis subjects (mean 5.2% ± 0.5), ($P < 0.010$).

7.4.2.7 VC model for GCF-glucose: DM-Periodontitis and non-DM-Periodontitis subjects

The results of the VC model for GCF-glucose concentration (μg μl^{-1}) for the DM-Periodontitis and non-DM-periodontitis subjects are given in Table 7.7. The estimate for the intercept (mean) was significantly greater than zero at 0.18 (P < 0.001). Subject and site-level variances were significantly different from zero, which they were.

	Estimate (95%CI)	*P*-value	
Fixed structure: Intercept (mean)	0.18 (0.12, 0.25)	<0.001	
Random structure Level-2 (subject) Level-1 (site)	Variance (SE) 0.83 (0.23) 1.03 (0.11)	<0.001 <0.001	Proportion of total 45% 55%
Total	1.86		100%

Table 7.7 VC model for GCF-glucose concentration (μg μl^{-1}) in DM-Periodontitis & non-DM-Periodontitis subjects; N = 216. SE = standard error.

The level-2 (i.e. between subject) variance accounted for 45% of the total variance while the level-1 (i.e. between sites within subjects) variance accounted for 55% of the variance. These significant variances at each level along with their sizeable proportion of the total variation indicated that there was sufficient variation at each level to warrant the use of hierarchical modelling. These results confirmed that ignoring the inherent hierarchy would have been erroneous. In addition, the large variance at the site-level justified the use of multiple site data per subject.

The data set used to perform this analysis consisted of 216 data points i.e. the 186 data values from DM- and non-DM-Periodontitis subjects as well as the data points used in the validation study (a small subset of the Periodontitis group was common to both studies). The data sets were combined since the multilevel model could then maximize the statistical power from all available data when establishing the model. This had the added advantage of being able to include, and therefore compare, the healthy (i.e. non-Periodontitis) subjects used in the validation study in the final graphic presentation (Fig.7.10).

Model assumptions were verified by plotting the standardised residuals versus normal scores. The near-linearity of the graph (Fig.7.8) showed that the model assumptions were satisfied.
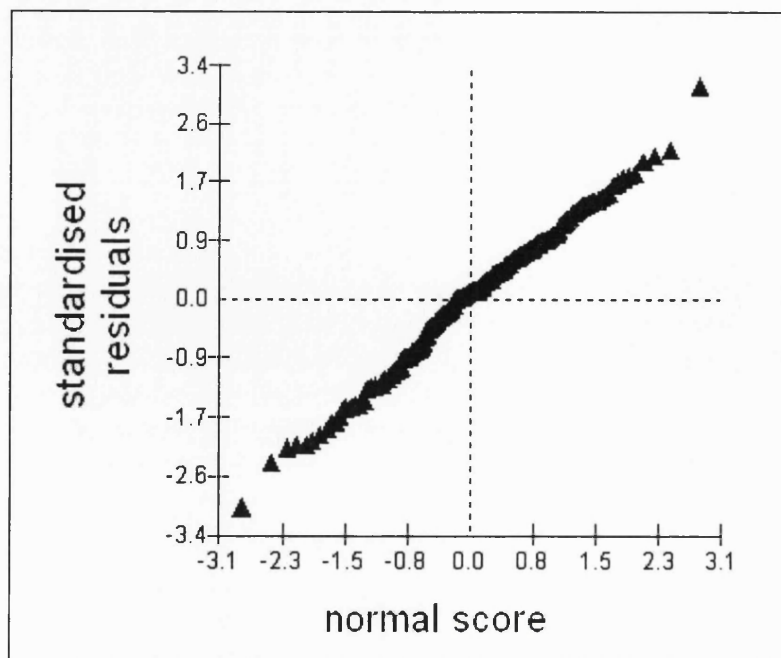
Fig.7.8 Residuals for VC model for GCF-glucose concentration (µg µl⁻¹) in the DM- and non-DM-Periodontitis subjects

7.4.2.8 RI model for GCF-glucose concentration: DM-Periodontitis and non-DM-Periodontitis subjects

The RI models for GCF-glucose concentration (µg µl⁻¹) for DM-Periodontitis & non-DM-Periodontitis subjects were generated after introducing DM and site-status as explanatory variables forming the following groups: healthy non-DM-non-Periodontitis (baseline), healthy DM-Periodontitis, diseased non-DM-Periodontitis and diseased DM-Periodontitis groups (Table 7.8).

Fixed effects (covariates)	Estimate (95%CI)	P-value
Intercept (Healthy non-DM, non-Periodontitis patients / sites)	0.09 (0.04, 0.19)	<0.001
Subject/site-level		
Healthy non-DM-Periodontitis	0.16 (0.06, 0.37)	0.249
Healthy DM-Periodontitis	0.41 (0.17, 0.96)	0.001
Diseased non-DM-Periodontitis	0.14(0.05, 0.34)	0.343
Diseased DM-Periodontitis	0.23 (0.09, 0.53)	0.057
Random Effects	Variance (SE)	
Subject (level-2 σ^2_{ou})	0.71 (0.20)	<0.001
Site (level-1 σ^2_{oe})	1.01 (0.11)	< 0.001
Total	1.72	

Table 7.8 RI model for GCF-glucose concentration ($\mu g\ \mu l^{-1}$) in DM-Periodontitis & non-DM-Periodontitis subjects; N = 216. SE = standard error (Healthy non-DM, non-Periodontitis subjects as controls).

The results of the RI model showed a 14% reduction in variance at the subject-level from 0.83 in the VC model (Table 7.7) to 0.71 in the RI model (Table 7.8). The variance at the site-level was reduced by 2% from 1.03 in the VC model (Table 7.7) to 1.01 in the RI model (Table 7.8). The results indicated that whilst both DM and site-status were positive risk factors for levels of GCF-glucose concentration as all were significantly different from the healthy non-Periodontitis group (baseline reference), the subject status (in this case the presence of DM) had a greater effect on the level of GCF-glucose as it accounted for more of the variation.

Model assumptions for the RI model were checked by plotting the standardised residuals versus the normal scores. The near-linearity of the graph (Fig.7.9) showed that the assumptions underlying MLM were well satisfied.
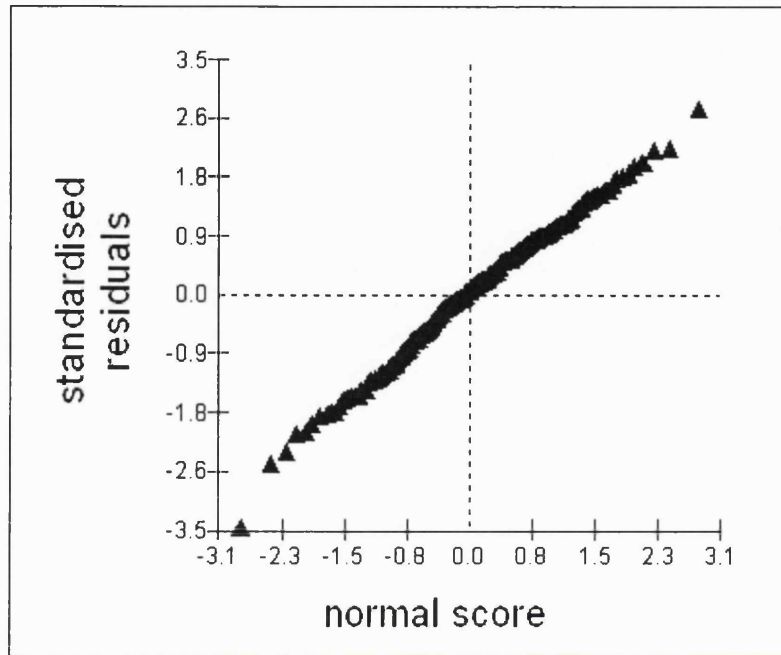
Fig.7.9 Residuals for the RI model for the GCF-glucose concentration (μg μl^{-1}) in DM-Periodontitis & non-DM-Periodontitis subjects

The GCF-glucose concentrations (μg μl^{-1}) at healthy and diseased sites in DM-Periodontitis and non-DM-Periodontitis subjects are shown graphically in Fig. 7.10. The GCF-glucose concentration (μg μl^{-1}) value obtained from the healthy non-Periodontitis group during the validation study (denoted as controls in Fig. 7.10) was used as the baseline reference.
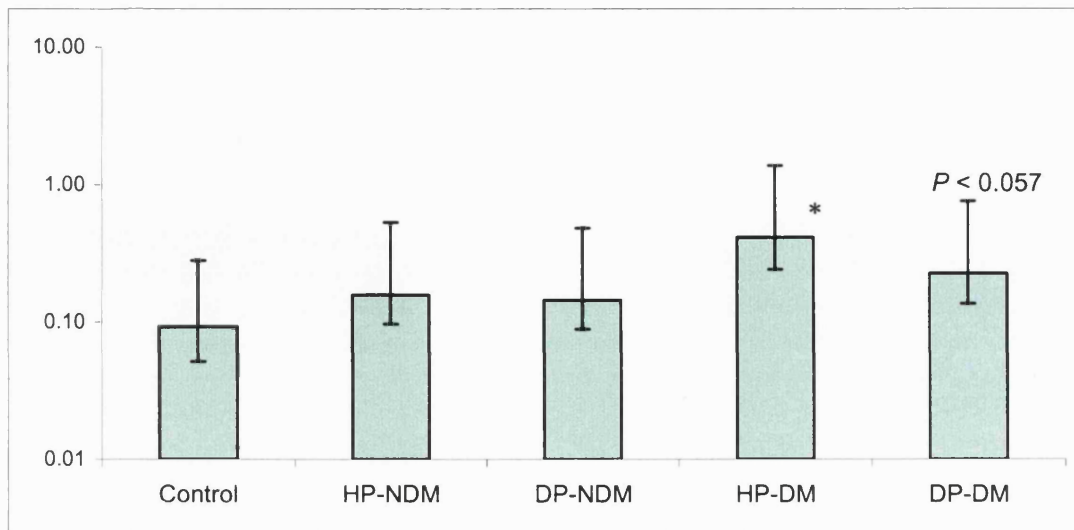
Fig.7.10 Comparisons of GCF-glucose concentrations (mean values, μg μl^{-1}) in controls and in healthy & diseased sites in DM-Periodontitis & non-DM-Periodontitis groups. N = 216; Bars represent 95% CI. Controls: healthy sites in non-Periodontitis group (from validation study); HP-NDM: healthy sites in non-DM-Periodontitis; HP-DM: healthy sites in DM-Periodontitis group; DP-NDM: diseased sites in the non-DM-Periodontitis group; DP-DM: diseased sites in DM-Periodontitis group. *Significantly different from control; $P < 0.057$ compared to control.

The graph (Fig. 7.10) showed that the GCF-glucose concentration (μg μl^{-1}) was least in the healthy sites in healthy subjects (taken from the validation study). In the Non-DM subjects, the GCF-glucose concentrations in the healthy and diseased sites were similar (0.16 & 0.14 respectively). Neither of these was significantly different from the GCF-glucose concentration in the control group. The healthy sites in the DM-Periodontitis group (HP-DM) showed the highest level of GCF-glucose concentration (0.41 μg μl^{-1}); this was highly significantly different from the control ($P < 0.001$) and from the healthy sites in non-DM-Periodontitis subjects ($P = 0.017$). The diseased sites in the DM-Periodontitis group (DP-DM) also showed elevated levels of GCF-glucose concentration compared to the controls, though this achieved only borderline significance ($P = 0.057$). Within the DM-Periodontitis group, GCF-glucose concentration was significantly greater in the healthy than in the diseased sites ($P = 0.012$).

The P-values resulting from the pair-wise contrasts of the GCF-glucose concentrations (μg μl^{-1}) between all the groups are presented in Table 7.9.

Pair-wise contrasts

	Controls	HP-NDM	DP-NDM	HP-DM
HP-NDM	0.249			
DP-NDM	0.343	0.681		
HP-DM	0.001	0.017	0.008	
DP-DM	0.057	0.378	0.262	0.012

Table 7.9 P-values resulting from pair wise contrasts between groups for GCF-glucose concentration ($\mu g\ \mu l^{-1}$): Controls = healthy sites in the non-Periodontitis group; HP-NDM = Healthy sites in non-DM-Periodontitis subjects; DP-NDM = Diseased sites in non-DM-Periodontitis subjects; HP-DM = Healthy sites in DM-Periodontitis subjects DP-DM = Diseased sites in DM-Periodontitis subjects.

7.4.2.9 GCF-glucose:Blood-glucose ratio in non-DM-Periodontitis & DM-Periodontitis subjects

The ratios of GCF-glucose concentration (converted to mg dL^{-1}) to blood-glucose (mg dL^{-1}) are presented in Table 7.10.In both the DM- and non-DM-Periodontitis groups, this ratio was slightly higher at the diseased sites than at the heatlhy sites.

Subject status	DM-Periodontitis		Non-DM-Periodontitis	
Site status	Healthy	Diseased	Healthy	Diseased
Blood glucose (mg dL^{-1})	205.4		100.8	
GCF-glucose (mg dL^{-1})	41	23	16	14
GCF-glucose:Blood-glucose (mg dL^{-1})	1:5.0	1:8.9	1:6.3	1:7.2

Table 7.10 GCF-glucose:Blood-glucose ratios in non-DM-Periodontitis and DM-Periodontitis subjects. N = 216.

7.5 Discussion

GCF originates from serum due to microvascular (postcapillary venule) leakage in the gingival vessels. It exudes through the junctional epithelium and into the gingival crevice in health and into the periodontal pocket in disease (Egelberg 1967, Cimasoni 1983a). The constituents of GCF are derived from a number of sources namely serum, connective tissue and epithelium, through which it passes on its way to the crevice. It also contains inflammatory cells and bacteria present within the crevice or pocket (Lamster 1997). Hence its biochemical composition reflects the physiological / pathological state prevailing within the crevice / pocket respectively at that point in time. Quantification of glucose in GCF would give further insight into the environmental conditions which prevail in the periodontal pockets of patients with, for example, diabetes mellitus (DM).

In vitro **GCF-glucose micro-assay.** Serum glucose determination is routinely undertaken when disorders in carbohydrate metabolism e.g. diabetes mellitus, are suspected. Biological fluids derived from serum e.g. GCF, are thought to reflect the glucose concentration in serum (Ficara *et al.* 1975). However, unlike a blood sample, which would consist of a few microlitres or millilitres of blood, a sample of GCF from any one site can only be collected in submicrolitre quantities.

A few studies around the 1970s investigated the levels of glucose in GCF with disparate results (Hara & Löe 1969, Kjellman 1970, Ficara *et al.* 1975). Direct comparison of these studies is impractical as the methods used for GCF sampling and volume determination varied between them. Two studies (Kjellman 1970, Ficara *et al.* 1975) used microlitre pipettes to collect GCF. No detail in these studies was given as to the amount of GCF collected or the unit time of the sampling process. Other investigators used calibrated microcapillary tubes to collect GCF during a 10 - 15 min interval (Kaslick *et al.* 1970). The drawback with this method was that the long duration of sampling time (10 - 15 min) increased the possibility of sampling error. Furthermore stroking the gingival margin as suggested by Ficara *et al.* (1975) would stimulate GCF flow leading to elevated volumes per unit time. Hara & Löe (1969) used filter paper strips, which were weighed pre- and post-sampling. In addition, the biochemical assays for glucose estimation have varied between studies, e.g. Ficara *et al.* (1975) used the hexokinase while Kjellman *et al.* 1970 used the glucose oxidase method.

The aim of the *in-vitro* investigation in this study was to address the above shortcomings by developing a standardised and sensitive micro-assay for glucose determination in

submicrolitre samples. A commercially available assay kit, which according to the manufacturer required a minimum sample volume of 5 μl, was modified to enable quantification of glucose in submicrolitre volumes of GCF. The sensitivity of the assay was such that a spectrophotometric change of 0.003 corresponded to 2 mg dL^{-1} (0.02 μg μl^{-1}) glucose when performed under the conditions stated by the manufacturer. The kit was found to be 96% reproducible within and between runs. The results showed that this modified assay was sensitive enough to quantify glucose in submicrolitre volumes of samples. The linear characteristics of the assay were not altered, even when the sample-to-reagent ratio was altered. Although variation in the sample-to-reagent ratio deviated from the manufacture's instructions, this was intentionally undertaken as the volume of GCF would vary *in-vivo*, thus resulting in a variation in the sample-to-reagent ratio. In addition, the sensitivity of the assay was such as to be able to detect the levels of glucose in the GCF samples.

It would have been ideal to use GCF for the *in vitro* evaluation of the micro-assay. However, this biological fluid cannot be collected in sufficiently large quantities to undertake such *in vitro* studies. The alternative was to use human serum since GCF originates from serum. Glucose was added to serum in order to attempt to simulate GCF collected from patients diagnosed with DM. Preliminary studies (data not shown) which involved performing the assay with and without the glucose solution applied to the filter paper strips, showed that the glucose did not irreversibly bind to the strips and was released unreservedly into the reagent solution.

The results obtained from this *in vitro* investigation show that this micro-assay is sensitive enough to quantify glucose over a concentration range of 50 - 200 mg dL^{-1} in submicrolitre volumes of biological fluids.

Quantification of GCF-glucose: Quantification of GCF components can be expressed in different ways (Lamster *et al.* 1985b, 1986, 1988). The most commonly used methods are either the total (absolute) amount per standardised collection time or concentration (amount / unit volume).

GCF volume quantification in the studies referred to above (Hara & Löe 1969, Kjellman 1970, Ficara *et al.* 1975) was performed rather inaccurately i.e. by weighing strips or volumetric quantification in capillary tubes. A recent and more accurate method for GCF quantification employs the use of a specific device, the Periotron 8000 (Ciantar & Caruana 1998). This device was designed to quantify submicrolitre volumes of fluid applied to a filter paper strip. The Periotron has been used in the majority of recent

studies pertaining to GCF volume quantification, especially to enable the calculation of the concentration of various GCF component/s. However, different investigators have proposed different mathematical models for converting the Periotron score to actual GCF volume (Bickel & Cimasoni 1984, Lamster *et al.* 1985b, van der Bijl *et al.* 1986, Griffiths *et al.* 1988, Preshaw *et al.* 1996, Ciantar & Caruana 1998). Thus, the same Periotron reading could potentially give a different value for GCF volume depending on which mathematical calculation has been used. In addition, minimal variation during GCF collection or its subsequent quantification via the Periotron could lead to disproportionately large errors in quantification of GCF components (Lamster *et al.* 1985b). Expressing GCF components as absolute amounts is an alternative option, as this would avoid having to quantify GCF volume precisely, thus eliminating the inherent errors associated with its quantification. However, one disadvantage would be that it would preclude direct comparison between different sites. In addition, attempts to accurately correlate a given GCF component with another clinical or microbiological variable would be impossible.

In the present study, the data were presented as GCF-glucose concentrations (μg μl^{-1}). The data were also analysed as absolute amounts (unpublished data). The data analysis for absolute amounts showed that, as expected, the levels of absolute GCF-glucose (per 2 min sample) were significantly higher at the diseased sites than at the healthy sites in both DM-Periodontitis and non-DM-Periodontitis subjects. The absolute amount of GCF-glucose in the DM-Periodontitis patients was significantly higher than that in the non-DM-Periodontitis subjects ($P < 0.010$).

An important criterion was that a standardised time was used for GCF collection. GCF-sampling times most often used have varied from 5 to 30 seconds. These short sampling periods might be too short for GCF collection especially at healthy sites where an extremely small GCF volume would be expected; this would compound the problems associated with GCF quantification. In this study a 2-minute GCF collection time (Curtis *et al.* 1990, Griffiths *et al.* 1997) was used to avoid the possibility of insufficient GCF volumes, particularly from healthy sites. Sufficient care (saliva ejector and site isolation with cotton wool rolls) was implemented to avoid contamination with saliva during the sampling procedure. In addition, the possibility of contamination of GCF with glucose from saliva was eliminated, as the subjects had not had anything by mouth for at least one hour prior to GCF collection.

Statistical analysis: Data analysis for GCF-glucose quantification was performed using MLM for the reasons elaborated upon in section 1.6 of the introduction and again in Chapter 5.

In vivo **results:** Most of the research concerning DM-Periodontitis pertains to clinical or microbiological variables (Mashimo *et al.* 1983, Shlossman *et al.* 1990). This study, however, attempted to investigate DM-periodontitis from a different perspective - an ecological perspective. Although GCF has been thoroughly investigated for a wide array of biochemical components, e.g. acute phase proteins (Adonogianaki *et al.* 1994), lactoferrin (Adonogianaki *et al.* 1993), alkaline phosphatase (Chapple *et al.*1993), lactate dehydrogenase, beta-glucuronidase and arylsulphatase (Lamster *et al.* 1985a), keratin (MacLaughlin *et al.* 1996), cytokines (Kurtis *et al.* 1999) and prostaglandin E (Offenbacher *et al.* 1984), limited research has been performed on GCF-glucose quantification (Hara & Löe 1969, Kjellman *et al.* 1970, Ficara *et al.* 1975). The earlier GCF-glucose studies used relatively crude and disparate methods for glucose quantification leading to inconclusive and conflicting results.

When analysing the GCF-glucose concentration for periodontally healthy and periodontitis subjects (i.e. the validation study), the level of variation was almost equal at subject- and site-level (Table 7.3). These variances did not change after introduction of the explanatory variables (Table 7.4). This could be explained as follows:

i) either there was a difference but this difference was too small to have been detected with the sample size used in the study, or

ii) there is no difference between the samples from each of the three groups.

The latter result would be more plausible in this case as all the subjects in this group were non-DM.

The ratio of GCF-glucose:Blood glucose showed a decreasing trend from healthy sites in healthy subjects to diseased sites in the Periodontitis group (Table 7.5). This could potentially be attributed to differences in periodontal microflora between healthy and diseased sites (Dzink *et al.* 1985, 1988). Cognisant of the fact that the eukaryotic cellular composition of healthy gingiva is different to that of inflamed gingiva (more inflammatory cells in the latter), it is not known whether cellular glucose utilisation changes with varying degrees of inflammation.

In the second part of the study, site-level variance (55%) was slightly greater than subject-level variation (45%; Table 7.7). Thus, data aggregation to subject-level would

220

certainly have been misleading. Introduction of explanatory variables in this model revealed that DM accounted for a 14% reduction in variance at the subject-level (Tables 7.7 & 7.8) clearly showing that DM had a significant impact on GCF-glucose concentration. These data supported the view that GCF-levels in DM subjects would be higher than in non-DM subjects as GCF originates from blood (blood glucose in the DM subjects in this study were significantly higher than in the non-DM subjects). These results are in agreement with those of other investigators (Kjellman 1970, Ficara *et al.* 1975). GCF-glucose concentrations for healthy subjects and healthy and diseased sites in DM-Periodontitis and non-DM-Periodontitis subjects are shown graphically in Fig 7.10, with the pair-wise comparison shown in Table 7.9. The GCF-glucose from diseased sites in DM-Periodontitis subjects was significantly lower (P = 0.012) than the corresponding value for healthy sites. As explained earlier this could be due to differences in the subgingival microflora, supported by the results obtained in Chapter 5 of this thesis which showed that the numbers of *Capnocytophaga* spp. and total cultivable anaerobes (facultative and obligate) at diseased sites in DM-Periodontitis subjects were higher when compared to their non-DM counterparts. It could be due also to more glucose utilisation by the surrounding tissue cells though no information seems to be available about this.

The ratio of GCF-glucose:blood-glucose (1:6.3) for the healthy sites in the non-DM-Periodontitis group (Table 7.10) was lower than that obtained during the first part of the study (1:1.9 & 1:7.1; Table 7.5). In the non-DM-Periodontitis group this value was reported to be 1:5 (Table 7.10). The ratio at the diseased sites increased to 1:7.2 (non-DM-Periodontitis) and 1:8.9 (DM-Periodontitis).

The GCF-glucose concentration was in the range of 11% - 20% that of blood. These results are at variance with those of Kjellman (1970) who reported GCF-glucose to be about 40% that of blood. These results differ from those of Hara & Löe (1969), who reported GCF-glucose as being six times higher that of serum. Ficara *et al.* (1975) found similar glucose concentrations in gingival fluid and serum, both in healthy and diabetic subjects. In the latter two studies, the authors did not explain the physiological mechanisms whereby the GCF-glucose could become more concentrated than in blood.

The results for the ratio of GCF-glucose:blood-glucose should be interpreted with caution. Since this was a random blood sample, the serum glucose level could have been affected by food intake in the hours preceding sample collection (as discussed

earlier a better way to monitor blood glucose levels is via glycated haemoglobin rather than random serum blood glucose).

Clinical implications: The higher GCF-glucose levels observed in this study could affect the physiology of the surrounding cells. *In vitro* experiments on periodontal ligament cells (PDL) revealed that these cells manifested a decreased chemotactic response when grown in culture media containing high glucose levels (Nishimura *et al.* 1996). In addition, under these conditions PDL cells and gingival fibroblasts manifested decreased proliferation and increased prostaglandin E_2 secretion, which could perpetuate a chronic inflammatory response (Sung-Jim 1998). In addition, high glucose levels have also been shown to impair proliferation of osteoblasts (Terada *et al.* 1998). Abnormalities in granulocyte adhesion (Bagdade *et al.* 1978), chemotaxis (Mowat & Baum 1971, Ramamurthy *et al.* 1979), phagocytosis (Bagdade *et al.* 1972) and microbicidal function (Nolan *et al.* 1978) were noted with impaired function increasing as the degree of hyperglycaemia increased. Collectively these could explain why DM-periodontitis subjects tend to manifest severe periodontal destruction and poorer healing capacity.

Microbiological implications: The higher GCF-glucose concentration observed in DM-Periodontitis subjects may act as a selective pressure on the subgingival microflora. It may lead not only to quantitative changes by encouraging the growth of subgingival species but also to qualitative changes by encouraging the growth of a more saccharolytic subgingival microflora (McNamara *et al.* 1982). The environmental pressure exerted by glucose may induce alterations in bacterial gene expression. For example *Candida albicans* possesses unique features that promote its virulence in a hyperglycaemic milieu (Hostetter 1990). In these circumstances it induces a surface protein, which enhances adhesion to mucosal surfaces and subverts phagocytosis by the host (Hostetter 1990). This could explain the higher prevalence of candidal infections in DM subjects (Guggenheimer *et al.* 2000). Ecological stress on bacteria could also manifest as increased virulence. For example when *Capnocytophaga gingivalis* was grown in chemostat culture at progressively increasing glucose concentrations, its growth rate doubled and its biomass increased five-fold (Spratt *et al.* 1996). Under such conditions the specific activity of enzymes relevant to periodontal breakdown was enhanced. Thus glucose could maximise the pathogenic potential of

Capnocytophaga spp., which have been implicated in DM-Periodontitis (Mashimo *et al.* 1983, Spratt *et al.* 1996).

In conclusion, therefore, a micro-assay for the quantification of GCF-glucose in submicrolitre volumes of fluid was developed during the course of this study. In addition quantification of GCF-glucose via this micro-assay confirmed that GCF-glucose concentration was higher in DM-Periodontitis than in non-DM-Periodontitis subjects. These elevated levels of glucose could result in a more reduced (anaerobic) environment, which would explain the increased levels of *Capnocytophaga* spp. and anaerobes in diseased sites in DM-Periodontitis subjects (Chapter 5, Figs.5.11 & 5.12). The saccharolytic nature of *Capnocytophaga* spp. and of some of the anaerobic organisms e.g. *P. intermedia,* could potentially explain the lower levels of GCF-glucose at diseased sites in the DM-Periodontitis subjects. Preliminary work pertaining to the proportions of black-pigmented anaerobes in DM-Periodontitis subjects revealed that 92% of these isolates were, in fact, saccharolytic (*Prev. intermedia* & *Prev. nigrescens*; D. Spratt, J. Pratten, *pers. comm.*).

Chapter 8

General discussion & conclusions

8.1 Introduction

During the past three decades, extensive clinical and laboratory research has been performed in the areas of periodontal microbiology and diabetes mellitus. This has resulted in a significantly better understanding of the aetiology and pathology of both topics and their potential interaction when periodontitis and DM co-exist in the same individual.

Although the association between bacteria and CIPDs was first proposed by Antonie van Leuwenhoek over 300 years ago, it was the seminal work of Löe *et al.* (1965) which led to a cascade of investigations which collectively formulated periodontal microbiology as we know it today. In the 1970s and '80s, periodontal microbiology was mostly concerned with the bulk of bacterial plaque at healthy and diseased sites. The majority of studies described the microflora in terms of bacterial morphotypes and shifts in proportions of Gram-positive and Gram-negative organisms (Listgarten 1976, Listgarten & Helldén 1978, Dzink *et al.* 1985, 1988, Moore & Moore 1994). This led to the establishment of the Non-Specific Plaque Hypothesis (Theilade 1986). To some extent the concept of plaque bulk still holds true in the present day. It is standard clinical practice that restitution of periodontal health is achieved partly by the implementation of oral hygiene procedures aimed to reduce the bulk of the plaque biofilm. However some studies of the periodontal microflora associated with various diseased states supported the Specific Plaque Hypothesis (Loesche 1976). The latter proposed that certain bacterial species are associated with disease and therefore act as putative periodontal pathogens, the six major organisms being *Por. gingivalis*, *A. actinomycetemcomitans*, *Prev. intermedia*, *T. forsythensis*, *E. corrodens* and *F. nucleatum*. Some investigators (Moore & Moore 1994, Petsios *et al.* 1995, Kamma *et al.* 1995, 1998) have proposed that certain bacterial clusters are associated with the different CIPDs (Table 1.9). It is important to note that none of these organisms are exclusively found at diseased periodontal sites; they *are* present in periodontal health, however their numbers increase significantly at diseased compared to healthy sites. Indeed one such group of organisms, which has been associated with various types of advanced periodontitis, is *Capnocytophaga* spp.

Capnocytophaga spp. form part of the commensal human oral microflora. However, they have been described as opportunistic pathogens as they have often been associated with localized (i.e. periodontal) and systemic infections. They possess

several characteristics (e.g. ability to adhere, to produce tissue destroying enzymes, to evade the host response; section 1.4.3) that enable them to act as opportunistic pathogens. They have been recovered from a wide range of advanced periodontal conditions (Table 1.12). Furthermore, they have been associated with systemic diseases that manifest severe periodontal destruction (Table 1.13). One such condition is periodontitis in subjects with DM (DM-Periodontitis).

DM is the most common of the endocrine diseases. Recent research has yielded tremendous insight into the molecular and cellular mechanisms that beget DM pathology. The widespread formation of AGEs throughout the body has enabled our understanding of the histopathological and pathophysiological changes which are consequent to the onset of DM. AGE formation has also been observed in periodontal tissues. A main characteristic feature of DM is that it predisposes the individual to infection/s. Indeed, DM subjects manifest an increased prevalence and severity of periodontitis (referred to as DM-Periodontitis). This could be due partly to the formation of AGEs in periodontal tissues. In addition, the elevated levels of certain cytokines (TNF-α, IL-1 & IL-6) that are a feature of inflammation including periodontitis are known to interfere with glucose uptake at a cellular level. The presence of infection in DM subjects is known to alter the endocrinologic status of the host leading to difficulty in controlling blood sugar levels. Thus, chronic infections, such as periodontitis, could potentially induce insulin resistance and lead to worsening of DM control. The increased severity of periodontitis could also be due to the presence of certain bacterial species in such individuals. One group of organisms that has the potential to take advantage of the immunocompromised state of DM subjects is *Capnocytophaga* spp.

The microflora associated with DM-Periodontitis has not been thoroughly investigated. *Capnocytophaga* spp. have been implicated as potential pathogens however no study to date has specifically investigated the potential association of *Capnocytophaga* spp. with DM-Periodontitis. This was the principal aim of this study.

The raison d'être underlying the hypothesis for this study was that the saccharolytic nature of *Capnocytophaga* spp. could potentially provide them an ecological advantage over other non-saccharolytic organisms, as it has been suggested that DM-Periodontitis subjects have higher GCF-glucose levels.

8.2 Cultural microbiology

An important aspect of any study involving cultural microbiology is the secure knowledge that the culture medium used supports the growth of the organism/s being investigated. At the outset of this study, a thorough literature search for a medium supporting the growth of all known *Capnocytophaga* isolates did not yield any conclusive results. Furthermore, since two *Capnocytophaga* species (*C. granulosa* & *C. haemolytica*) were recent additions to this genus, no conclusive evidence was available in the literature that suggested the use of one particular culture medium. Thus, it was imperative that a solid culture medium supporting the growth of all *Capnocytophaga* isolates was identified prior to the commencement of the clinical study. This investigation identified FAA as a medium that supported the growth of all *Capnocytophaga* clinical isolates (Chapter 3).

8.3 Bacterial identification

Bacterial enumeration alone provides insufficient evidence for the potential association between *Capnocytophaga* spp. and DM-Periodontitis. In an era when bacterial identification can be rapidly and accurately performed using molecular techniques, the isolates obtained during the course of this study were identified using 16S rRNA PCR-RFLP. This was based on previous work by Wilson *et al.* (1995). However, no conclusive results about the molecular identification of *Capnocytophaga* spp. via RFLP were obtained from that study primarily because the results were not supported by sequencing data (though it has to be admitted that 16S rRNA gene sequencing was not routinely available in microbiology laboratories at that time). In addition, a possible problem with that study was that the molecular weight marker (2 kb – 154 bp) used may have been inappropriate, as restriction enzyme analysis of the 16S rRNA gene of *Capnocytophaga* spp. with *Cfo* I in the present study, yielded fragments in the 50 bp range; it is likely therefore that these fragments were overlooked in the Wilson *et al.* (1995) study and therefore omitted during RFLP analysis.

The results of the 16S rRNA PCR-RFLP analysis with *Cfo* I obtained during this investigation yielded a distinctive pattern for each ATCC *Capnocytophaga* strain and isolate. However, prior to accepting RFLP analysis as a standard method for bacterial identification of the clinical isolates obtained during the clinical part of this investigation, a representative sample of isolates was concurrently (though blindly) identified via 16S rRNA gene sequencing. The 16S rRNA gene sequencing data obtained via comparison with those on the BLAST database supported the bacterial identification results

secured via 16S rRNA PCR-RFLP with *Cfo* I. Construction of a phylogenetic tree with sequences available on this database (all of which were cultured during this study except for 7 strains, see Fig. 4.7) demonstrated considerable genotypic heterogeneity within the genus *Capnocytophaga*. The genus can be divided into three large clusters consisting of: *C. gingivalis* & *C. granulosa* strains, *C. canimorsus* & *C. cynodegmi* strains and *C. ochracea* & *C. sputigena* strains. The *C. haemolytica* Type species stood on its own, as did the *Capnocytophaga* spp. oral clone DZ074. A considerable degree of genotypic heterogeneity was observed in the *C. ochracea* group. This was in agreement with Vandamme *et al.* (1996), who identified two clusters within the *C. ochracea* group. Two *Capnocytophaga* spp. oral clones (BU084 and DS022) were most closely related to, yet distinct from, the *C. ochracea* / *C. sputigena* cluster. This could potentially signify the presence of a new species, though full gene sequencing of these strains in addition to a range of morphological, biochemical and physiological tests would have to be performed, before such a conclusion can be made. Further investigation of the virulence properties of each of these strains is indicated, now that identification to species level of the *Capnocytophaga* species can be performed reliably and rapidly.

8.4 *Capnocytophaga* spp. isolated from subgingival plaque taken from DM- and non-DM-Periodontitis subjects

The presence of an organism at a diseased site does not automatically implicate it as a causative agent. Indeed its presence may be a consequence of disease as the organism might have proliferated as it takes advantage of clinical changes (e.g. increased GCF flow and composition, deeper pockets, lower Eh in periodontal pockets) associated with disease. However, once present at a site, it might contribute to the pathology at that site especially if it possesses the relevant armamentarium for enhancing periodontal breakdown.

The results of the clinical study (Chapter 5) showed that significantly higher counts of *Capnocytophaga* spp. (P < 0.025) were obtained from DM-Periodontitis compared to non-DM-Periodontitis patients. Significantly higher counts (P < 0.001) were recovered from diseased sites when compared to healthy sites. However, since the two explanatory variables were concurrently present in a biological system and were thus not anticipated to operate independently, subsequent data analysis investigated the interaction between DM and site-status. The diseased sites in the DM-Periodontitis

subjects showed the highest total *Capnocytophaga* count (Fig.5.12). Comparison of total *Capnocytophaga* spp. counts (mean values, cfu ml⁻¹) between healthy sites and diseased sites in DM-Periodontitis subjects showed borderline significance ($P = 0.050$) favoring higher a *Capnocytophaga* count at the diseased sites. In the non-DM-Periodontitis group, the total mean *Capnocytophaga* spp. count at diseased sites was significantly higher than that at corresponding healthy sites ($P < 0.001$). Between group comparisons of the total *Capnocytophaga* counts at the healthy sites showed a significantly higher mean *Capnocytophaga* count in the DM-Periodontitis group ($P = 0.013$). Comparison of the mean *Capnocytophaga* counts at diseased sites in the DM- and non-DM-Periodontitis groups revealed a higher count in the DM-Periodontitis group, though this was not statistically significant ($P = 0.135$). Lack of statistical significance could have been due either to a true lack of significance, or, more plausibly, due to the fact that the subdivision of the data set into four groups was underpowered and therefore failed to achieve a level of significance. The division of the data set into four groups, and therefore analysis of the interaction between DM and site-status was crucial as signified by the sizeable interaction term (Table 5.8).

It is in no way being implied that *Capnocytophaga* spp. are *the* causative agents of DM-Periodontitis. This conclusion cannot be derived from a cross-sectional study such as this one. Indeed, this study would have to be repeated and ideally performed on a longitudinal basis before such conclusions could be drawn. Another important aspect for further investigation would be a series of *in vitro* studies investigating virulence factors associated with this genus which would have to include the more recent species added to this genus (*C. granulosa* & *C. haemolytica*). However, from this study it can be concluded that *Capnocytophaga* spp. are present in higher numbers in DM-Periodontitis subjects.

The results of this study provide some novel findings. The results of the clinical study have rejected the null hypothesis formulated for this investigation i.e. that there is no difference in *Capnocytophaga* spp. counts (cfu ml⁻¹) isolated from subgingival plaque collected from DM and non-DM-Periodontitis subjects. This study seems to be the first to specifically investigate *Capnocytophaga* spp. and DM-Periodontitis. Thus, direct comparison with other studies is precluded (Mashimo *et al.* 1983, Zambon *et al.* 1988, Sastrowijoto *et al.* 1989, 1990, Sbordone *et al.* 1995, 1998, Thorstensson *et al.* 1995). Prior to commencement of the clinical study, it was imperative to address several

shortcomings pertaining to the isolation and identification *Capnocytophaga* spp. (Chapters 3 & 4), as not to do so could have jeopardized the results. Another reason why comparison with the above studies is impracticable is that none were consistent in either clinical or laboratory methodology (Tables 1.15 & 1.16). Furthermore, none of those studies identified the isolates to species level. Indeed none of them included the newer members of this genus i.e. *C. granulosa* & *C. haemolytica* (some studies were performed prior to the recognition of the newer species within the genus). There is only one paper in the literature that reports on the presence of *C. granulosa* and *C. haemolytica* in subgingival plaque (Ciantar *et al.* 2001). Indeed further studies on *C. granulosa* are warranted especially since this isolate was the second most common comprising 24.8% of the total *Capnocytophaga* clinical isolates in this study.

It was conclusively shown that the *Capnocytophaga* spp. counts (cfu ml⁻¹) were dependent on DM-status and site-status (i.e. healthy versus diseased). However, whilst there was a net increase in *Capnocytophaga* spp, data analysis of the odds ratio for each species (Fig.5.14) revealed that the different species showed varying trends. Furthermore the prevalence of these species varied based on site- and DM-status. These results must be interpreted in the light of the complexity of the oral microflora. *Capnocytophaga* spp. form only seven of some 350 cultivable species present in the oral cavity. Indeed there might well have been other saccharolytic organisms (e.g. *Prev. intermedia*, *A. actinomycetemcomitans*) that could have shown similar growth trends, however this was not within the remit of this study.

Five hundred black-pigmented anaerobes were isolated during the course of this investigation however these isolates are pending identification and data analysis as this was not one of the aims of this study. The results of this study revealed that within each group, the total counts (cfu ml⁻¹) of bacteria grown anaerobically (i.e. facultative and obligate anaerobes) were higher in the diseased sites when compared to the healthy sites, which was to be expected in view of the more reduced conditions prevailing within such periodontal pockets (Kenny & Ash 1969). Comparison of the diseased sites revealed that the mean count (cfu ml⁻¹) at diseased sites in the DM-Periodontitis group were in fact higher than those in the diseased sites in the non-DM-Periodontitis group (Fig.5.13).

8.5 Isolation of *Capnocytophaga* spp. from blood

Teeth have been considered as a focus of infection ever since the focal infection theory was first suggested by Hippocrates (Slots 1998). The major target sites of infection include the cardiovascular system, articular joints and the neurological system (Miller 1890, Newman 1996). It has been implied that mobility associated with periodontally involved teeth might exert a pumping action thereby forcing subginginval bacteria through the large ulcerated surface associated with moderate to severe periodontitis and thence into the blood stream (Page 1998, Seymour & Steele 1998). Anachoretic spread of oral bacteria might lead them to cause infections at sites distant from the oral cavity. However, to date no study appears to have attempted to detect oral bacteria in blood taken from periodontitis subjects.

Capnocytophaga spp., though located only in the oral cavity, have been documented to be the cause of infections at various sites in the human body (Table1.14), the most likely site of entry would be through ulcerated oral mucosa. The recognition of *Capnocytophaga* spp. as a cause of bacterial endocarditis and of septicaemia, amongst other infections, warranted an investigation into the detection of *Capnocytophaga* spp. from blood samples taken from periodontitis subjects. The results showed that all samples proved negative for *Capnocytophaga* spp. though seven samples grew colonies suggestive of coagulase-negative *Staphylococcus* spp., probably a skin contaminant. Blood samples from these patients are still available and will be analyzed for the presence of bacterial DNA (16S rRNA gene).

8.6 Antimicrobial sensitivity testing of *Capnocytophaga* isolates

Antimicrobial therapy proves a useful adjunct to mechanical periodontal therapy especially in cases of advanced and aggressive periodontal lesions. *Capnocytophaga* species have been associated with such periodontal conditions (Tables 1.12 & 1.13); therefore knowledge of antimicrobial sensitivity would be clinically relevant from a periodontal as well as a systemic perspective for the reasons described in the previous section.

Previous studies have reported on the antimicrobial sensitivity of *Capnocytophaga* spp., (Forlenza *et al.* 1981, Sutter *et al.* 1981, Rummens *et al.* 1986, Bremmelgard *et al.* 1989, Jolivet-Gougeon *et al.* 2000). However none had identified the clinical isolates to species level. The present study evaluated the antimicrobial sensitivity of each isolate identified using molecular techniques (Chapter 4); it also seems to be the largest study of its kind (336 clinical isolates). The agar dilution method was chosen as

it is a reliable and reproducible method recommended by the NCCLS for antimicrobial sensitivity testing. The agents used in this study were 6 antibiotics used often in clinical practice and 4 other agents (3 antibiotics and an antifungal agent) that have been incorporated in culture media selective for *Capnocytophaga* spp.

The results of this study showed that, with the exception of metronidazole (only active in strict anaerobes), the MIC_{90} (μg ml^{-1}) values for *Capnocytophaga* spp. were within the concentration range that these antibiotics attain in GCF following systemic antibiotic administration. The results also showed that the concentrations of antibiotics used in some of the selective media could affect the recovery of certain *Capnocytophaga* strains. Thus the results of studies using these selective media should be interpreted with caution.

8.7 GCF-glucose concentration

This part of the study was performed in order to investigate whether the glucose levels in GCF collected from DM-Periodontitis subjects differed from those in GCF taken from non-DM-Periodontitis subjects. The rationale behind this was that higher GCF-glucose levels, if present in DM-Periodontitis subjects, could affect the microflora growing subgingivally, in particular saccharolytic species such as *Capnocytophaga* spp.

A micro-assay was developed specifically for this purpose. The results for GCF-glucose concentration showed that the levels were marginally higher in DM-Periodontitis subjects. In the DM-Periodontitis group, the GCF-glucose concentration was slightly lower at diseased compared to healthy sites. This could be due to the higher total *Capnocytophaga* spp. counts and total facultative and obligate anaerobic counts (and other saccharolytic organisms found within subgingival plaque) recovered from the diseased DM-Periodontitis sites that could have been utilizing the freely available glucose in which they were bathed. Alternatively, host cells could have utilized the glucose.

8.8 Statistical analysis

The implementation of multilevel modeling for the statistical analysis of the data collated in Chapters 5 & 7 has attempted to overcome previous shortcomings when analyzing periodontal data. This method of analysis, though significantly more complex and time-consuming than single-level analysis, could be justified as it applied a mathematical model that addressed the hierarchical structure implicit when analyzing periodontal data. The main advantage of MLM is that it is not necessary to aggregate

data to the subject-level (which is the correct level of independence) whilst lower level information may be analyzed separately, though simultaneously, thus accounting for the hierarchy whilst retaining use of all the available data. In addition, MLM can investigate whether an interaction was operating between the explanatory variables (DM and site-status), which in this case there was. It would have been erroneous to overlook this interaction and any data analysis omitting this interaction would lead to incorrect conclusions. This method of analysis provided a wealth of invaluable data, the analysis of which had to be restricted within the confines of this thesis.

8.9 Future work

Consequent to the findings of this study, the following investigations are proposed:

i)   further clinical & microbiological investigations focusing on *Capnocytophaga* spp. in the various periodontal conditions, especially now that these species can be rapidly and reliably identified to species level

ii)   *in vitro* investigations of virulence factors produced by each species

iii)   an investigation of glucose utilization by each of these species

iv)   identification of the 500 black-pigmented anaerobes isolated from subginginval plaque during the course of this investigation

v)   detection of bacterial DNA (16S rRNA gene) from blood samples taken from Periodontitis subjects

Chapter 9

References

Abe T, Hara Y, Aono M (1991). Penetration, clearance and retention of antigen en route from the gingival sulcus to the draining lymph node of rats. *J Periodontal Res* 26: 429 - 439.

Adonogianaki E, Moughal NA, Kinane DF (1993). Lactoferrin in the gingival crevice as a marker of polymorphonuclear leucocytes in periodontal diseases. *J Clin Periodontol* 20: 26 - 31.

Adonogianaki E, Moughal NA, Mooney J, Stirrups D, Kinane DF (1994). Acute-phase proteins in gingival crevicular fluid during experimentally induced gingivitis. *J Periodont Res* 29: 196 - 202.

Ainamo J, Lahtinen A, Uitto V-J (1990). Rapid Periodontal destruction in adult humans with poorly controlled diabetes. *J Clin Periodontol* 17: 22 - 28.

Albander JM (1993). Juvenile periodontitis - pattern of progression and relationship to clinical periodontal parameters. *Comm Dent Oral Epidemiol* 21: 185 -189.

Albander JM, Buischi YAP, Axelsson P (1995a). Caries lesion and dental restorations as predisposing factors in the progression of periodontal diseases in adolescents. A 3-year longitudinal study. *J Periodontol* 66: 249 -254.

Albander JM, Goldstein H (1992). Multilevel statistical models in studies of periodontal disease. *J Periodontol* 63: 690 - 695.

Alberio M, Lämmle B (1998). *Capnocytophaga canimorsus* sepsis. *N Eng J Med* 339: 1827.

Alexandrakis A, Palma LA, Miller D, Alfonso EC (2000). *Capnocytophaga* keratitis. *Ophthalmol* 107: 1503 - 1506.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990). Basic local alignment search tool. *J Mol Biol* 215: 403 - 410.

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997). Gapped BLAST and PSI-Blast: a new generation of protein database search programs. *Nucleic Acids Res* 25: 3389 - 3402.

Amano A, Kishima T, Kimura S, Takiguchi M, Ooshima T, Hamada S, Morisaki I. Periodontopathic bacteria in children with Down's syndrome (2000). *J Periodontol* 71: 249 - 255.

American Academy of Periodontology (1989). Proceedings of the World Workshop in Clinical Periodontics, eds Nevins M, Becker W, Kornman K, Chicago, USA. I/23 - 24.

American Diabetes Association. Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (1997). *Diabetes Care* 20: 1183 - 1197.

Andersen B, Goldsmith GH, Spagnuolo PJ (1988). Neutrophil adhesive dysfunction in diabetes mellitus; the role of cellular and plasma factors. *J Lab Clin Med* 111: 275 - 285.

Armitage GC (1999). Development of a classification system for periodontal diseases and conditions. *Ann Periodontol* 4: 1 - 6.

Atkinson MA, Eisenbarth GS (2001). Type 1 diabetes: new perspective on disease pathogenesis and treatment. *Lancet* 358: 221 - 229.

Atkinson MA, Maclaren NK (1993). Islet cell autoantigens in insulin-dependent diabetes. *J Clin Invest* 92: 1608 - 1616

Atkinson MA, Maclaren NK (1994). The pathogenesis of insulin dependent diabetes. *N Engl J Med* 331: 1428 -1436.

Attström R (1975). The role of gingival epithelium and phagocytosing leukocytes in gingival defence. *J Clin Periodontol* 2: 25 - 32.

Attström R, Egelberg J (1970). Emigration of blood neutrophils and monocytes into the gingival crevices. *J Periodontal Res* 5: 48 - 55.

Attström R, van der Velden U. Consensus report (epidemiology) (1994). In: Proceedings of the 1st European workshop on Periodontics, Lang NP, Karring T, eds. Quintessence, pp. 120 - 126.

Augthun M, Conrads G (1997). Microbial Findings of Deep Peri-implant Bone defects. *Int J Oral Maxillofac Implants* 12: 106 - 112.

Axelsson P, Lindhe J (1981). The significance of maintenance care in the treatment of periodontal disease. *J Clin Periodontol* 8: 281 - 294.

Axtelius B, Söderfelt B, Attström R (1999). A multilevel analysis of factors affecting pocket probing depth in patients responding differently to periodontal treatment. *J Clin Periodontol* 26: 67 - 76.

Baab DA, Page RC, Ebersole JL, Williams BL, Scott CR (1986). Laboratory studies of a family manifesting premature exfoliation of deciduous teeth. *J Clin Periodontol* 13: 677 - 683.

Badersten A, Nilvéus R, Egelberg J (1981). Effect of nonsurgical periodontal therapy. I. Moderately advanced periodontitis. *J Clin Periodontol* 8: 57 - 72.

Badersten A, Nilvéus R, Egelberg J (1984a). Severely advanced periodontitis. II. *J Clin Periodontol* 11: 63 - 76.

Badersten A, Nilvéus R, Egelberg J (1985). Effect of nonsurgical therapy. VII. Bleeding, suppuration and probing depths in sites with probing attachment loss. *J Clin Periodontol* 12: 432 - 440.

Bagdade J, Nielson K, Bulger R (1972). Reversible abnormalities in phagocytic function in poorly controlled diabetic patients. *Amer J Med Sci* 263: 451 - 456.

Bagdade J, Stewart M, Waltera E (1978). Impaired granulocyte adherence: a reversible defect in host defence in patients with poorly controlled diabetes. *Diabetes* 27: 677 - 681.

Baggiolini M, Walz A, Kunkel SL (1989). Neutrophil-activating peptide-1/ interleukin 8, a novel cytokine that activates neutrophils. *J Clin Invest* 84: 1045 - 1049.

Baltimore RS, Mitchell M (1980). Immunologic investigations of mucoid strains of *Pseudomonas aeruginosa*: Comparison of susceptibility by opsonic antibody in mucoid and nonmucoid strains. *J Infect* 141: 238 - 247.

Barnett ML, Baker RL, Yancey JM, MacMillan DR, Kotoyan M (1984). Absence of periodontitis in a population of insulin-dependent diabetes mellitus (IDDM) patients. *J Periodontol* 55: 402 - 405.

Barr-Agholme M, Dahllöf G, Linder L, Modéer T (1992). *Actinobacillus actinomycetemcomitans, Capnocytophaga* and *Porphyromonas gingivalis* in subgingival plaque of adolescents with Down's Syndrome. *Oral Microbiol Immunol* 7: 244 -248.

Bartold MA (1991). Connective tissue of the periodontium. Research and clinical implications. *Aust Dent J* 36: 255 - 268.

Bartold MA, Narayanan AS (1996). The biochemistry and physiology of the periodontium. In: Fundamentals of Periodontics, ed. Wilson TG, Kornman KS, Quintessence; Chapter 6: 61 - 107.

Barua PK, Dyer DW, Nieders ME (1990). Effect of iron limitation on *Bacteroides ginigivalis. Oral Microbiol Immunol* 5: 263 - 268.

Bateman JM, Rainford DJ, Masterton RG (1992). *Capnocytophaga canimorsus* infection an acute renal failure. *J Infect* 25: 112 - 4.

Bauer AW, Sherris JC (1964). The determination of sulfonamide susceptibility of bacteria. *Chemotherapia* 9: 1 - 16.

Belizzi R, Cruse WP (1980). A historic review of endodontics, 1689 -1963. *J Endod* 6: 576 - 608.

Bergström J, Eliasson S (1987). Noxious effect of cigarette smoking on periodontal health. *J Periodontal Res* 22: 513 - 517.

Bick PH, Carpenter AB, Holdeman LV, Miller GA, Ranney RR, Palcanis KG, Tew JG (1981). Polyclonal-B cell activation induced by extracts of Gram-negative bacteria isolated from periodontally diseased sites. *Infect Immun* 34: 43 - 49.

Bickel M, Cimasoni G (1984). Reliability of volume measurements with the new Periotron 6000. *J Periodontal Res* 19: 313 - 316.

Birkedal-Hansen H (1993). Role of cytokines and inflammatory mediators in tissue destruction. *J Periodontal Res* 28: 500 - 510.

Birkedal-Hansen H, Caufield PW, Wane Muehler YM, Pierce R (1982). A sensitive screening assay for epitheliotoxin produced by oral micro-organisms. *J Dent Res* 61: 125

Birkedal-Hansen H, Cobb CM, Taylor RE, Fullmer HM (1975). Trypsin activation of latent collagenase from several mammalian sources. *Scand J Dent Res* 83: 302 - 305.

Blomqvist N (1985). On the choice of computational unit in statistical analysis. *J Clin Periodontol* 12: 873 - 876.

Bolton RW, Dyer DK, Reinhardt RA, Okano DK (1983). Modulation of *in vitro* human lymphocyte responses by an exopolysaccharide from *Capnocytophaga ochracea*. *J Dent Res* 62: 1186 - 1189.

Borg Andersson A, Birkhed D, Berntorp K, Lindgärde F, Matsson L (1998). Glucose concentration in parotid saliva after glucose / food intake in individuals with glucose intolerance and diabetes mellitus. *Eur J Oral Sci* 106: 931 - 937.

Borkman M, Storlien LH, Pan DA, Jenkins AB, Chisholm DJ, Campbell LV (1993). The relation between insulin sensitivity and the fatty acid composition of skeletal muscle phospholipids. *N Eng J Med* 328: 238 - 244.

Bremmelgard A, Pers C, Kristiansen JE, Korner B, Heltberg O, Frederiksen W (1989). Susceptibility testing of Danish isolates of *Capnocytophaga* and CDC group DF-2 bacteria. *APMIS* 97: 43 - 48.

Brenner DJ, Hollis DG, Fanning GR, Weaver RE (1989). *Capnocytophaga canimorsus* sp. nov. (formerly CDC group DF-2), a cause of septicaemia following a dog bite, and *C. cynodegmi* sp. nov, a cause of localised would infection following dog bite. *J Clin Microbiol* 27: 231 - 235.

Brill N (1959). Influence of capillary permeability on flow of tissue fluid into gingival pockets. *Acta Odont Scand* 17: 23 - 33.

Brill N, Krasse B (1958). The passage of tissue fluid into the clinically healthy gingival pocket. *Acta Odont Scand* 16: 233 - 245.

Britch M, Allen TD (1980). The modulation of cellular contractility and adhesion by trypsin and EGTA. *Exp Cell Res* 125: 221 - 223.

Brownlee M (1992). Glycation products and the pathogenesis of diabetic complications. *Diabetes Care* 15: 1835 - 1843.

Brownlee M (1994). Lilly Lecture 1993. Glycation and diabetic complications. *Diabetes* 43: 836 - 841.

Brownlee M, Cerami A (1981). The biochemistry of the complications of diabetes mellitus. *Ann Rev Biochem* 50: 385 - 342.

Brownlee M, Cerami A, Vlassara H (1988). Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications *N. Eng J Med* 318: 1315 - 1321.

Brownlee M, Cerami A, Vlassara H (1988). Advanced products of nonenzymatic glycosylation and the pathogenesis of diabetic vascular disease. *Diab Met Rev* 4: 437 - 451.

Brownlee M, Vlassara H, Cerami A (1984). Nonenzymatic glycosylation and the pathogenesis of diabetic complications. *Ann Intern Med* 101: 527 - 537.

Bryk AS, Raudenbush AW (1992). Hierarchical linear models: applications and data analysis methods. London: Sage.

BSAC (British Society for Antimicrobial Chemotherapy) - Working Party (1992). antibiotic prophylaxis for infective endocarditis. *Lancet* 1: 1292 - 1293.

Bucala R, Lee AT, Rouke L, Cerami A (1993). Transposition of an *Alu*-containing element induced by DNA-advanced glycosylation endproducts. *Proc Natl Acad Sci USA* 90: 2666 - 2670.

Bucala R, Makita Z, Koschinsky T, Cerami A, Vlassara H (1993). Lipid advanced glycosylation: pathway for lipid peroxidation. *Proc Natl Acad Sci USA* 90: 6434 - 6438.

Bucala R, Model P, Russel M, Cerami A (1985). Modification of DNA by glucose-6-phosphate induces DNA rearrangements in an *E. coli* plasmid. *Proc Natl Acad Sci USA* 82: 8439 - 8442.

Buschang PH, Tanguay R, Demirjan A, Lapalme L, Goldstein H (1989). Modelling longitudinal mandibular growth: Percentiles for gnathion from 6 to 15 years of age in girls. *Amer J Ortho Dentofac Orthopaed* 95: 60 - 66.

Buu-Hoi AY, Joundy S, Acar JF (1988). Endocarditis caused by *Capnocytophaga ochracea*. *J Clin Microbiol* 26: 1061 - 1062.

Buzzetti R, Quattrocchi CC, Nistico L (1998). Dissecting the genetics of type 1 diabetes: relevance for familial clustering and differences in incidence. *Diabetes Metab Rev* 14: 111 - 128.

Campbell MJA (1971). A light and electron microscope study of blood vessels from the gingival tissues of non-diabetic and diabetic patients. *Aust Dent J* 15: 235 - 239.

Cercek JF, Kiger RD, Garrett S, Egelberg J (1983). Relative effects of plaque control and instrumentation on the clinical parameters of human periodontal disease. *J Clin Periodontol* 10: 46 - 56.

Cerda J, Vázquez de la Torre C, Malacara JM, Nava LE (1994). Periodontal disease in non-Insulin dependent diabetes mellitus (NIDDM). The effect of age and time since diagnosis. *J Periodontol* 65: 991 - 995.

Chang KM, Ramamurthy NS, McNamara TF, Genco RJ, Golub LM (1988). Infection with a gram-negative organism stimulates gingival collagenase production in non diabetic and diabetic germfree rats. *J Periodontal Res* 23: 239 - 244.

Chapple ILC, Matthews JB, Thorpe GH, Smith JM, Saxby MS (1993). A new ultrasensitive chemiluminescent assay for the site-specific quantification of alkaline phosphatase in gingival crevicular fluid. *J Periodontal Res* 28: 266 - 273.

Cheng A, Nack Z, Graves S, McDonal M (1999). Knowing where to look: Meningococcal septicaemia following a dog bite. *Emerg Med* 11: 185 - 187.

Christersson LA, Albini B, Zambon JJ, Wikesjöt UME, Genco RJ (1987). Tissue localisation of *Actinobacillus actinomycetemcomitans* in Human Periodontitis. I. Light, Immunofluorescence and Electron Microscopic Studies. *J Periodontol* 58: 529 - 539.

Christersson LA, Zambon JJ (1993). Suppression of subgingival *Actinobacillus actinomycetemcomitans* in localised juvenile periodontitis by systemic tetracycline. *J Clin Periodontol* 20: 395 - 401.

Cianciola LJ, Park BH, Bruck E, Mosovich L, Genco RJ (1992). Prevalence of periodontal disease in insulin-dependent diabetes mellitus (juvenile diabetes). *J Am Dent Assoc* 104: 653 - 660.

Ciantar M, Caruana DJ (1998). Periotron 8000: calibration characteristics & reliability. *J Periodontal Res* 33: 259 - 264.

Ciantar M, Spratt DA, Newman HN, Wilson M (2001). *Capnocytophaga granulosa* and *Capnocytophaga haemolytica*: novel species in subgingival plaque. *J Clin Perio* 28: 701 - 705.

Ciantar M, Spratt DA, Newman HN, Wilson M (2002). Development of an *in vitro* microassay for glucose quantification in submicrolitre volumes of biological fluid. *J Periodontal Res* 37: 79 - 85

Cimasoni G (1983a). Crevicular fluid updated. In: Monographs in oral science 12, ed. HM Myers, Basle, Karger. Chapter I, pp. 1 - 52.

Cimasoni G (1983b). Crevicular fluid updated. In: Monographs in oral science 12, ed. HM Myers, Basle, Karger. Chapter VII, pp. 64 - 66.

Cisar JO (1982). Coaggregation reactions between oral bacteria: studies on specific cell-to-cell adherence mediated by microbial lectins. In: Host-parasite interactions in periodontal diseases, eds. Genco RJ, Mergenhagen SE, American Society of Microbiology, Washington DC, pp 121 -131.

Clarke SD, Jump DB (1993). Regulation of gene transcription by polyunsaturated fatty acids. *Prog Lipids Res* 32: 139 - 149.

Cohen J (1960). A coefficient of agreement for nominal scales. *Educ Pyschol Measurement* 20: 37 - 46.

Cohen MM (1947). Clinical studies of dental caries susceptibility in young diabetics. *J Am Dent Assoc* 34: 239 - 243.

Collin H-L, Uusitupa M, Niskanen L, Kontturi-Närhi V, Markkanen H, Koivisto A-M, Meurman JH (1998). Periodontal findings in elderly patients with non-insulin dependent diabetes mellitus. *J Periodontol* 69: 962 - 966.

Conrads G, Lutticken R (1992). Nucleotide sequences of 16S rRNA encoding genes from *Capnocytophaga ochracea* ATCC 33596, *Capnocytophaga sputigena* ATCC 33612 and *Capnocytophaga gingivalis* ATCC 33624. *Nucleic Acids Res* 20: 5847.

Conrads G, Mutters R, Seyfarth I, Pelz K (1997). DNA-probes for the differentiation of *Capnocytophaga* spp. *Mol Cell Probes* 11: 323 - 328.

Conville PS, Fischer SH, Cartwrigth CP, Witebsky FG (2000). Identification of *Nocardia* species by restriction endonuclease analysis of an amplified portion of the 16S rRNA Gene. *J Clin Microbiol* 38: 158 - 164.

Cooper GR, Mullins RE, Myers GL, Sampson EJ (2000). Methodology of measuring glucose, glycated serum protein and haemoglobin, and Lipids and Lipoprotein. In: Clinical Diabetes Mellitus: A Problem Oriented Approach, 3rd edition, ed. Davidson JK, Thieme, New York. Chapter 13, pp. 189 - 215.

Costerton JW, Cheng KJ, Geesey GG, Ladd TI, Nickel JC, Dasgupta M, Marrie TJ (1987). Bacterial biofilms in nature and disease. *Ann Rev Microbiol* 41: 435 - 464.

Costerton W, Lewandowski Z, DeBeer D, Caldwell D, Korber D, James G (1994). Biofilms, the customised microniche. *J Bacteriol* 176: 2137 - 2142.

Cowan & Steel (1993). Manual for the identification of medical bacteria. Cambridge University Press, 3rd edition, eds. Barrow GI, Feltham RKA.

Crawford JM, Cotran RS (1994). The Pancreas. In: Robbins Pathogenic Basis of Disease, eds. Schoen FJ, Cotran RS, Kumar V, Robbins SL. WB Saunders, Chapter 19, pp. 897 - 926.

Cunningham SJ (2000). Outcomes of orthognathic treatment. PhD Thesis, University of London.

Cunningham SJ, Gilthorpe MS, Hunt NP (2000). Are orthognathic patients different? *Eur J Orthodont* 22: 195 - 202.

Curtis MA, Sterne JAC, Price SJ, Griffiths GS (1990). The protein composition of gingival crevicular fluid sampled from male adolescents with no destructive periodontitis: baseline data of a longitudinal study. *J Periodont Res* 25: 6 – 16.

Cutler CW, Machen RL, Jotwani R, Iacopino AM (1999). Heightened gingival inflammation and attachment loss in Type 2 diabetics with hyperlipidaemia. *J Periodontol* 70: 1313 - 1321.

Dahlèn G, Manji G, Baelum V, Fejerskov O, (1992). Putative periodontopathogens in "diseased" and "non-diseased" persons exhibiting poor oral hygiene. *J Clin Periodontol* 19: 35 - 42.

Dahlquist GG (1997). Viruses and other perinatal exposures as initiating events or beta-cell destruction. *Ann Med* 29: 413 - 417.

Darby IB, Hodge PJ, Riggio MP, Kinane DF (2000). Microbial comparison of smoker and non-smoker adult and early-onset periodontitis patients by polymerase chain reaction.

Darveau RP, Tanner A, Page R (1997). The microbial challenge in periodontitis. *Periodontol 2000* 14: 12 - 32.

Debelian GJ, Olsen I, Tronstad L (1994). Systemic disease caused by oral microorganisms. *Endod Dent Traumatol* 10: 57 - 65.

DeFronzo RA, Bonadonna RC, Ferrannini E (1992). Pathogenesis of NIDDM: a balanced overview. *Diabetes Care* 15: 318 - 368.

Delamaire M, Maugendre D, Moreno M, Le Goff M-C, Allanic H, Genetet B (1996). Impaired leucocyte function in diabetic patients. *Diabet Med* 14: 29 - 34.

Delaney JE, Kornman KS (1987). Microbiology of subgingival plaque from children with localised prepubertal periodontitis. *Oral Microbiol Immunol* 2: 71 - 78.

Dempster AP, Rubin DB, Tsutakawa RK (1981). Estimation in covariance components models. *J Amer Stat Assoc* 76: 341 - 353.

DeRouen TA, Hujoel PP, Mancl LA (1995). Statistical analysis in periodontal research. *J Dent Res* 74: 171-173.

DeRouen TA, Mancl L, Hujoel P (1991). Measurement of associations in periodontal diseases using statistical methods for dependent data. *J Periodontal Res* 26: 218 - 229.

Dutta-Roy AK (1994). Insulin mediated processes in platelets, erythrocytes and monocytes / macrophages: effects of essential fatty acid metabolism. *Prostaglandins Leukot Essent Fatty Acids* 51: 385 - 399.

Dzink JL, Socransky SS, Haffajee AD (1988). The predominant cultivable microbiota of active and inactive lesions of destructive periodontal disease. *J Clin Periodontol* 15: 316 - 323.

Dzink JL, Tanner ACR, Haffajee AD, Socransky SS (1985). Gram-negative species associated with active destructive periodontal lesions. *J Clin Periodontol* 12: 648 - 659.

Ebersole JL, Singer RE, Steffensen B, Filloon T, Kornman KS (1993). Inflammatory mediators and immunoglobulins in GCF from healthy, gingivitis and periodontitis sites. *J Periodontal Res* 28: 543 - 546.

Ebersole JL, Taubman MA, Smith DJ, Frey DE, Haffajje AD, Socransky SS (1987). Human serum antibody responses to oral microorganisms. IV. Correlation with homologous infection. *Oral Microbiol Immunol* 2: 53 - 59.

Ebersole JL, Taubman MA, Smith DJ, Socransky SS (1982) Humoral Immune responses and diagnosis of human periodontal disease. *J Periodontal Res* 17: 478 - 480.

Ebinger M, Nichterlein T, Schumacher UK, Manncke B, Schmidt D, Böhn I (2000). Isolation of *Capnocytophaga granulosa* from an abscess in an immunocompromised adolescent. *Clin Infect Dis* 30: 606 - 607.

Edelman SV (1998). Type II diabetes mellitus. *Adv Internal Med* 43: 449 - 500.

Egelberg J (1967). The topography and permeability of vessels at the dento-gingival junction in dogs. *J Periodontal Res.* Suppl 1: 1 - 39.

Eisenbarth GS (1986). Type 1 diabetes mellitus. A chronic autoimmune disease. *N Eng J Med* 314: 1360 - 1368.

Emrich LJ (1990). Common problems with statistical aspects of periodontal research papers. *J Periodontol* 61: 206 - 208.

Emrich LJ, Shlossman M, Genco RJ (1991). Periodontal disease in non-insulin dependent diabetes mellitus. *J Periodontol* 62: 123 - 130.

Ericson T (1967). Adsorption to hydroxyapatite of proteins and conjugated protein from human saliva. *Caries Res* 1: 585 - 599.

Esteban J, Albalate M, Caramelo C, Reyero A, Carriazo MA, Hernandez J, Ortiz A, Soriano F (1995). Peritonitis involving a *Capnocytophaga* sp. in a patient undergoing continuous ambulatory peritoneal dialysis. *J Clin Microbiol* 33: 2471 - 2472.

Falk RJ, Dalmasso AP, Kim Y, Tsai CH, Scheinman JI, Gewurz H, Michael AF (1983) Neoantigen of the polymerized ninth component of complement. Characterization of a monoclonal antibody and immunohistochemical localization in renal disease. *J Clin Invest* 72: 560 - 573.

Fardal O, Johannessen AC, Olsen I (1999). Severe, rapidly progressing peri-implantitis. *J Clin Periodontol* 26: 313 - 317.

Ficara AJ, Levin MP, Grower MF, Kramer GD (1975). A comparison of the glucose and protein content of gingival fluid from diabetics and nondiabetics. *J Periodontal Res* 10: 171 - 175.

Fidler V (1984). Letter to the editor. *J Clin Periodontol* 11: 138 - 140.

Fiero D, Langkamp H, Piesco N, Kewitt G, Johns L, Agarwal S (1995). Characterisation of human oral epithelial cell immune response. *J Dent Res* 74: 540.

Firatli E (1997). The relationship between clinical periodontal status and insulin dependent diabetes mellitus. Results after 5 years. *J Periodontol* 68: 136 - 140.

Fives-Taylor PM, Hutchins Meyer D, Mintz KP, Brissette C (1999). Virulence factors of *Actinobacillus actinomycetemcomitans*. *Periodontol 2000* 20: 136 -167.

Fleiss JL, Wallenstein S, Chilton NW, Goodson JM (1988). A re-examination of within-mouth correlations of attachment level and of change in attachment level. *J Clin Periodontol* 15: 411- 414.

Forlenza SW, Newman MG, Horikoshi AL, Blachman U (1981). Antimicrobial susceptibility of *Capnocytophaga*. *Antimicrob Agents Chemother* 19: 144 - 146.

Frandsen EV, Kjeldsen M, Kilian M (1997). Inhibition of *Prevotella* and *Capnocytophaga* Immunoglobulin A1 proteases by human serum. *Clin Diag Lab Immunol* 4: 458 - 464.

Frandsen EVG, Reinholdt J, Kilian M (1991). Immunoglobulin A1(IgA1) proteases from *Prevotella (Bacteroides)* and *Capnocytophaga* species in relation to periodontal disease. *J Periodontal Res* 26: 297 - 299.

Frandsen EVG, Wade WG (1996). Differentiation of human *Capnocytophaga* species by multilocus enzyme electrophoretic analysis and serotyping of immunoglobulin A1 proteases. *Microbiol* 142: 441 - 448.

Frantzis TG, Reeve CM, Brown AL (1971). The ultrastructure of capillary basement membrane in the attached gingiva of diabetic and nondiabetic patients with periodontal disease. *J Periodontol* 42: 406 - 411.

Frias J, Olle E, Alsina M (2001). Periodontal pathogens produce quorum sensing signal molecules. *Infect Immun* 69: 3431 - 3434.

Frieling JTM, Mulder JA, Hendriks T, Curfs JHAJ, van der Linden CJ, Sauerwein RW (1997). Differential induction of pro- and anti-inflammatory cytokines in whole blood by bacteria: effects of antibiotic treatment. *Antimicrob Agents Chemother* 41: 1439 - 1443.

Froguel P, Vaxillaire M, Sun F, Velho G, Zouali H, Butel MO, Lesage S, Vionnet N, Clement K, Fougerousse F (1992). Close linkage of glucokinase locus on chromosome 7p to early-onset non-insulin dependent diabetes mellitus. *Nature* 356: 162 -164.

Fuller JH, Shipley MJ, Rose G, Jarrett RJ, Keen H (1980). Coronary-heart disease risk and impaired glucose tolerance: the Whitehall Study. *Lancet* 1: 1373 -1376.

Gannong WF (1983). Cardiovascular regulatory mechanisms. In: Review of Medical Physiology, 12th Edition, Lange Medical Publications, Los Altos, California, Chapter 31 pp. 485 - 496.

Garrison SW, Hilt SC, Nichols FC (1988). Lipopolysaccharide stimulated PGE_2 release from human monocytes. *J Periodontol* 59: 684 - 687.

Gazi MI, Cox SW, Clark DT, Eley BM (1997). Characteristics of protease activities in *Capnocytophaga* spp., *Porphyromonas gingivalis, Prevotella* spp., *Treponema denticola* and *Actinobacillus actinomycetemcomitans*. *Oral Microbiol Immunol* 12: 240 - 248.

Gemmel E, Marshall RI, Seymour GJ (1997). Cytokines and prostaglandins in immune homeostasis and tissue destruction in periodontal disease. *Periodontol 2000* 14: 112 - 143.

Genco RJ, Slots J (1984). Host responses in periodontal diseases. *J Dent Res* 63: 441 - 451.

Gendron R, Grenier D, Maheu-Robert L (2000). The oral cavity as a reservoir of bacterial pathogens for focal infections. *Microbes Infect* 2: 897 - 906.

Germaine GR, Harlander SK, Leung WL, Schachtele CF (1977) *Streptococcus mutans* dextran sucrase: functioning of primer dextran and endogenous dextransucrase in water soluble and water-insoluble glucan synthesis. *Infect Immun* 16: 637 - 648.

Gill VE, Zierdt CH, Wu TC, Stock F, Pizo PA, McLowry JD (1984). Comparison of lysis-centrifugation with lysis-filtration and a conventional unvented bottle for blood cultures. *J Clin Microbiol* 20: 927 - 932.

Gilthorpe MS (1995). The importance of normalisation in the construction of deprivation indices. *J Epidemiol Comm Health* 49: S45 - S50.

Gilthorpe MS, Cunningham SJ (2000). The application of multilevel, multivariate modelling to orthodontic research data. *Comm Dent Health* 17: 236 - 242.

Gilthorpe MS, Griffiths GS, Maddick IH, Zamzuri AT (2000c). The application of multilevel modelling to periodontal research data. *Comm Dent Health* 17: 227 -235.

Gilthorpe MS, Griffiths GS, Maddick IH, Zamzuri AT (2001). An application of multilevel modelling to longitudinal periodontal research data. *Comm Dent Health* 18: 79 - 86.

Gilthorpe MS, Maddick IH, Petrie A (2000a). Introduction to Bayesian modelling in dental research. *Comm Dent Health* 17: 218 - 221.

Gilthorpe MS, Maddick IH, Petrie A (2000b). Introduction to multilevel modelling in dental research. *Comm Dent Health* 17: 222 - 226.

Gilthorpe MS, Mayhew MT, Bulman JS (2002). Multilevel modelling the survival of amalgam restorations. *Comm Dent Health* 19: 3 -11.

Gin H, Brottier E, Aubertin J (1984). Influence of glycaemic normalisation by an artificial pancreas on phagocytic and bacterial functions of granulocytes in insulin-dependent diabetic patients. *J Clin Pathol* 37: 1029 - 1031.

Glavind L, Lund B, Löe H (1968). The relationship between periodontal state and diabetes duration, insulin dosage and retinal changes. *J Periodontol* 39: 341 - 347.

Goldstein H (1986). Multilevel mixed linear model analysis using iterative generalised least squares. *Biometrika* 73: 43 - 56.

Goldstein H (1995). Multilevel statistical models. Eds. Edward Arnold, London.

Goodfellow M, Dickinson CH (1985). Delineation and description of microbial populations using numerical methods. In: Computer-Assisted Bacterial Systematics, M. Goodfellow, D. Jones and FG, ed. Priest, Academic Press London. pp. 165 -225.

Goodson JM, Tanner ACR, Haffajee AD, Somberger GC, Socransky SS (1982). Patterns of progression and regression of advanced destructive periodontal disease. *J Clin Periodontol* 9: 472 - 481.

Greene DA, Lattimer SA, Sima AA (1987). Sorbitol, phosphoinositides and sodium potassium ATPase in the pathogenesis of diabetic complications. *N Eng J Med* 316: 599 - 606

Gregory EM, Moore WEC, Holdeman LV (1978). Superoxide dismutase in anaerobes: Survey. *Appl Environ Microbiol* 35: 988 - 991.

Gregory RL, Kim DE, Kindle JC, Hobbs LC, Lloyd DR (1992). Immunoglobulin-degrading enzymes in localised juvenile periodontitis. *J Periodontal Res* 27: 176 - 183.

Grenier D (1996). Degradation of host protease inhibitors and activation of plasminogen by proteolytic enzymes from *Porphyromonas gingivalis* and *Treponema denticola*. *Microbiol* 142: 955 - 961.

Grenier D and Turgeon J. (1994). Occurrence and identity of proteolytic bacteria in adult periodontitis. *J Peridontal Res* 29: 365 - 370.

Grenier D, Mayrand D, McBride BC (1989). Further studies on the degradation of immunoglobulins by black-pigmented *Bacteroides*. *Oral Microbiol Immunol* 4: 12 - 18.

Grenier D, Uitto V-J, McBride BC (1990). Cellular location of Treponema denticola chymotrypsin-like protease and importance of the protease migration through basement membrane. *Infect Immun* 58: 347 - 351.

Griego RD, RosenT, Orengo IF, Wolf JE (1995). Dog, cat and human bites: A review. *J Am Acad Dermatol* 33: 1019 - 1029.

Griffiths GS, Curtis MA, Wilton JMA (1988). Selection of a filter paper with optimum properties for the collection of gingival crevicular fluid. *J Periodontal Res* 23: 33 - 38.

Griffiths GS, Wilton JMA, Curtis MAC (1997). Permeability of the gingival tissues to IgM during an experimental gingivitis study in man. *Arch Oral Biol* 42: 129 – 136.

Griffiths M, Wade W (1987). Comparison of 6 media for cultivation of subgingival bacteria. *J Dent Res* 66: 334.

Grossi SG, Genco R (1998). Periodontal disease and diabetes mellitus: A two-way relationship. *Ann Periodontol* 3: 51 - 61.

Grossi SG, Skrepcinski FB, DeCaro T, Roberston DC, Ho AW, Dunford RG, Genco RJ (1997). Treatment of periodontal disease in diabetics reduces glycated haemoglobin. *J Periodontol* 68: 713 - 719.

Grossi SG, Skrepcinski FB, DeCaro T, Zambon JJ, Cummins D, Genco RJ (1996). Response to periodontal therapy in diabetics and smokers. *J Periodontol* 67: 1094 - 1102.

Guggenheimer J, Moore PA, Rossie K, Myers D, Mongelluzzo MB, Block HM, Weyant R, Orchard T (2000). Insulin-dependent diabetes mellitus and oral soft tissue

pathologies: II. Prevalence and characteristics of *Candida* and candidal lesions. *Oral Surg Oral Med Oral Path Oral Radiol Endod* 89: 570 – 576.

Gunsolley JC, Williams DA, Schenkein HA (1994). Variance component modelling of attachment level measurements. *J Clin Periodontol* 21: 289 - 295.

Haffajee AD, Socransky SS (1986). Attachment level changes in destructive periodontal diseases. *J Clin Periodontol* 13: 461 - 472.

Haffajee AD, Socransky SS (1994). Microbial etiological agents of destructive periodontal diseases. *Periodontol 2000* 5: 78 - 111.

Haffajee AD, Socransky SS, Goodson JM (1983b). Clinical parameters as predictors of destructive periodontal disease activity. *J Clin Periodontol* 10: 257 - 265.

Haffajee AD, Socransky SS, Lindhe J (1983a). Comparison of statistical methods of analysis of data from clinical periodontal trials. *J Clin Periodontol* 10: 247 - 256.

Hall TA (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp* 41: 95 - 98.

Hantson P, Gautier PE, Vekemans MC, Fievez P, Evrard P, Wauters G, Mahieu P (1991). Fatal *Capnocytophaga canimorsus* septicaemia in a previous healthy woman. *Ann Emerg Med* 20: 93 - 94.

Hara K and Loe H (1969). Carbohydrate components of the gingival exudate. *J Periodontal Res* 4: 202 – 207.

Hara Y, Kaneko T, Yoshimura A, Kato I. (1996). Serum rheumatoid factor induced by intraperitoneal administration of periodontopathic bacterial lipopolysaccharide in mice. *J Periodontal Res* 31: 502 - 507.

Hart TC, Shapira L, van Dyke TE (1994). Neutrophil defects are risk factors for periodontal disease. *J Periodontol* 65: 521- 529.

Haskel E, Esquenasi J, Yussin L (1986). Effects of subgingival chlorhexidine irrigation in chronic moderate periodontitis. *J Periodontol* 57: 305 - 310.

Hawkey PM, Smith S, Haynes J, Malnick H, Forlenza S (1987). *In vitro* susceptibility of *Capnocytophaga* species to antimicrobial agents. *Antimicrob Agents Chemother* 31: 331 - 332.

Heijl L, Wennström J, Lindhe J, Socransky SS (1980). Periodontal disease in gnotobiotic rats. *J Periodontal Res* 15: 405- 419

Heimdahl A, Josefsson K, von Konow L, Nord CE (1985). Detection of anaerobic bacteria in blood cultures by lysis filtration. *Eur J Clin Microbiol* 4: 404 - 407.

Higgins DG, Bleasby AJ, Fuchs R (1992). ClustalV: improved software for multiple sequence alignment. *CABIOS* 8: 189 - 191.

Hirshfield L (1934). Periodontal symptoms associated with diabetes. *J Periodontol* 5: 37 - 46.

Hoeksma JB, van der Beek MCJ (1991). Multilevel modelling of longitudinal cephalometric data explained for orthodontists. *Euro J Ortho* 13: 197 - 201.

Holdeman LV, Moore WEC, Cato EP, Burmeister JA, Palcanis KG, Ranney RR (1985). Distribution of *Capnocytophaga* in periodontal microfloras. *J Periodontal Res* 20: 475-483.

Holt SC, Leadbetter ER, Socransky SS (1979). *Capnocytophaga:* New genus of Gram-negative gliding bacteria. II. Morphology and ultrastructure. *Arch Microbiol* 122: 17-27.

Horita M, Suzuki H, Onodera T, Ginsberg-Fellner F, Fauci AS, Notkins AL (1982). Abnormalities of immunoregulatory T-cell subsets in patients with insulin dependent diabetes mellitus. *J Immunol* 129: 1426 -1429.

Hostetter MK (1990). Handicaps to host defense. Effects of hyperglycaemia on C3 and *Candida albicans*. *Diabetes* 39: 271 - 275.

Hostetter MK, Lorenz JS, Preus L, Kendrick KE (1990). The iC3b receptor on *Candida albicans*: subcellular localisation and modulation of receptor expression by glucose. *J Infect Dis* 161: 761 - 768.

Hove KA, Stallard RE (1970). Diabetes and the periodontal patient. *J Periodontol* 41: 713 - 718.

Hugoson A, Thorstensson H, Falk H, Kuylenstierna J (1989). Periodontal conditions in insulin-dependent diabetics. *J Clin Periodontol* 16: 215 - 223.

Iacopino AM (1995). Diabetes periodontitis: possible lipid induced defect in tissue repair through alteration of macrophage phenotype and function. *Oral Dis* 1: 214 - 229.

Igarashi T, Yamamoto A, Goto N. (1998). Detection of dextranase-producing gram-negative oral bacteria. *Oral Microbiol Immunol* 13: 382 - 386.

Iino Y, Hopps RM (1984). The bone-resorbing activities in tissue culture of lipopolysaccharide from the bacteria *Actinobacillus actinomycetemcomitans, Bacteroides gingivalis* and *Capnocytophaga ochracea* from human mouths. *Arch Oral Biol* 29: 59 - 63.

Imrey PB (1986). Considerations in the statistical analysis of clinical trials in periodontitis. *J Clin Periodontol* 13: 517 - 528.

Irving JT, Newman MG, Socransky SS, Heely JD (1975). Histological changes in experimental periodontal disease in rats mono-infected with a gram-negative organism. *Arch Oral Biol* 20: 219 - 220.

Irving JT, Socransky SS, Tanner AC (1978). Histological changes in experimental periodontal disease in rats monoinfected with gram-negative organisms. *J Periodontal Res* 13: 326 - 332.

Isenberg HD (1992). Preparation of routine media and reagents used in antimicrobial susceptibility testing. In: Clinical Microbiology Procedures Handbook, ASM Press, Washington DC, Vol 1, Chapter 5, pp 5.19.1 - 5. 19.28.

Isogai E, Isogai H Sawada H, Kaneko H, Ito N (1985). Microbial ecology of plaque in rats with naturally occurring gingivitis. *Infect Immun* 48: 520 - 527.

Iwamoto Y, Nishimura F, Nakagawa M, Sugimoto H, Shikata K, Makino H, Fukuda T, Iwamoto M, Murayama Y (2001). The effect of antimicrobial periodontal treatment on circulating tumor necrosis factor-alpha and glycated haemoglobin level in patients with Type 2 diabetes. *J Periodontol* 72: 774 - 778.

Jansen H-J, van der Hoeven JS, van der Kieboom CWA (1994). Degradation of immunoglobulin G by periodontal bacteria. *Oral Microbiol Immunol* 9: 345 - 351.

Jolivet-Gougeon A, Buffett A, Dupuy C, Sixou JL, Bonnaure-Mallet M, David S, Cormier M (2000). *In vitro* susceptibilities of *Capnocytophaga* isolates to β-lactam antibiotics and β-lactamase inhibitors. *Antimicrob Agents Chemother* 44: 3186 - 3188.

Jones K, Johnstone R, Pattie C (1992). People, places and regions: exploring the use of multilevel modelling in the analysis of electoral data. *Br J Political Science* 22: 343.

Kadowaki T, Kadowaki H, Mori Y, Tobe K, Sakuta R, Suzuki Y, Tanabe Y, Sakura H, Awata T, Goto Y, Takaki Hayakawa, Kenpei Matsuoka, Ryuzo Kawamori, Takenobu Kamada, Satoshi Horai, Ikuya Nonaka, Ryoko Hagura, Yasuo Akanuma, Yoshio Yazaki (1994). A subtype of diabetes mellitus associated with a mutation of mitochondrial DNA. *N Engl J Med* 330: 962 - 968.

Kallestal C, Uhlin S (1992). Buccal attachment loss in Swedish adolescents. *J Clin Periodontol* 19: 485 - 491.

Kamma JJ, Lygidakis NA, Nakou M (1998). Subgingival microflora and treatment in prepubertal periodontitis associated with chronic idiopathic neutropenia. *J Clin Periodontol* 25: 759 - 765.

Kamma JJ, Nakou M, Manti FA (1995). Predominant microflora of severe, moderate and minimal periodontal lesions in young adults with rapidly progressive periodontitis. *J Periodontal Res* 30: 66 - 72.

Kaneko T, Hara Y, Yoshimura A, Kato I (1999). Induction of anti-thymocyte/T lymphocyte antibodies in mice injected with lipopolysaccharides from periodontopathic bacteria. *J Periodontal Res* 34: 105 - 112.

Kanety H, Feistein R, Papa MZ, Hemi R, Karasiki A (1995). Tumor necrosis factor α-induced phosphorylation of insulin receptor substrate-1 (IRS-1). *J Biol Chem* 270: 23780 - 23784.

Karjalainen J, Martin JM, Knip M, Ilonen J, Robinson BH, Savilahti E, Akerblom HK, Dosch HM (1992). A bovine albumin peptide as a possible trigger of insulin-dependent diabetes mellitus. *N Eng J Med* 327: 302 – 307.

Karjalainen KM, Knuuttila ML, von Dickhoff KJ (1994). Association of the severity of periodontal disease with organ complications in Type 1 diabetic patients. *J Periodontol* 65: 1067 - 1072.

Kaslick RS, Chasens AJ, Weinstein D, Wakdman R, Pluhar T, Lazarra R (1970). Quantitative analysis of sodium, potassium and calcium in gingival fluid from gingiva with varying degrees in inflammation. *J Periodontol* 41: 93-97.

Kenny EB, Ash M (1969). Oxidation reduction potential of developing plaque, periodontal pockets and gingival sulci. *J Periodontol* 40: 630 - 633.

Khwaja KJ, Parish P, Aldred MJ, Wade WR (1990). Protein profiles of *Capnocytophaga* species. *J Appl Bacteriol* 68: 385 - 390.

Kiel RA, Lang NP (1983). Effect of subgingival sampling techniques on periodontal microbiological culturing. *J Dent Res* 62: 247.

Kieser JB (1990). The fundamental role of plaque control. In: Periodontics: a practical approach. Wright Publishers, Chapter 9, pp. 67 - 83.

Kilian M (1981). Degradation of immunoglobulins A2, A2, and G by suspected principal periodontal pathogens. *Infect Immun* 34: 757 - 765.

Kim KJ, Kim DK, Chung CP, Son S (1992). Longitudinal monitoring for disease progression of localised juvenile periodontitis. *J Periodontol* 63: 806 - 811.

Kim SJ, Kato T, Naito Y, Hirai K, Choi JI, Takazoe I, Okada K (1994). B-cell mitogenicity and IL-1 beta production of lipopolysaccharides from various *Capnocytophaga* strains. *Bull Tokyo Dent Coll* 35: 79 -83.

Kim SJ, Kato T, Naito Y, Hirai K, Choi JI, Takazoe I, Okuda K (1994). Lipopolysaccharide from various Capnocytophaga strains possess potent *Limulus* amoebocyte lysate clotting activity. *Bull Tokyo Dent Coll* 35: 17 - 21.

Kinane DF, Lindhe J (1997). Pathogenesis of Periodontitis. In: Clinical Implantology & Periodontology, eds. Lindhe J, Karring T, Lang NP, Chapter 5, pp. 189 - 225.

King H, Rewers M (1993). Global estimates for prevalence of diabetes mellitus and impaired glucose tolerance in adults. *Diabetes Care* 16: 157 - 177.

Kjellman O (1970). The presence of glucose in gingival exudate and resting saliva with insulin treated diabetes mellitus. *Swed Dent J* 63: 11 - 19.

Knip M, Akerblom HK (1999). Environmental factors in the pathogenesis of type 1 diabetes mellitus. *Exp Clin Endocrinol Diabetes* 107: S93 - 100.

Kolenbrander PE (1993). Co-aggregation of human oral bacteria: potential role in the accretion of dental plaque. *J Appl Bacteriol* 74: 79S - 86S.

Kolenbrander PE (2000). Oral Microbial Communities: Biofilms, Interactions and Genetic Systems. *Ann Rev Microbiol* 54: 413 - 437.

Kolenbrander PE, London J (1993), Adhere today, here tomorrow: oral bacterial adherence. *J Bacteriol* 175: 3247 - 3252.

Kolokotronis A (1995). Susceptibility of *Capnocytophaga* to antimicrobial agents. *J Chemother* 7: 414 - 416.

Kolterman OG, Gray RS, Griffin J, Burstein P, Insel J, Scarlett JA, Olefsky JM (1981). Receptor and postreceptor defects contribute to insulin resistance in noninsulin-dependent diabetes mellitus. *J Clin Invest* 68: 957 - 969.

Könönen E, Jousimies-Somer H, Asikainen S (1994). The most frequently isolated gram-negative anaerobes in saliva and subgingival samples taken from young women. *Oral Microbiol Immunol* 9: 126 - 128.

Kornman KS, Crane A, Wang HY, di Giovine FS, Newman MG, Pirk FW, Wilson TG, Higginbottom FL, Duff GW (1997). The interleukin-1 genotype as a severity factor in adult periodontal disease. *J Clin Periodontol* 24: 72 - 77.

Kornman KS, Löe H (1993). The role of local factors in the etiology of periodontal diseases. *Periodontol 2000* 2: 83 - 97.

Kornman KS, Newman MG (1999). Role of genetics in assessment, risk and management of adult periodontitis. In: Periodontal Medicine, eds. Rose LF, Genco RJ, Cohen DW, Mealey BL, BC Decker Inc., Ontario, Canada, Chapter 4, pp. 45 - 62.

Kornman KS, Page RC, Tonetti MS (1997). The host response to the microbial challenge in periodontitis: assembling the players. *Periodontol 2000* 14: 33 - 53.

Kostraba JN, Cruickshanks KJ, Lawler-Heavner J, Jobim LF, Rewers MJ, Gay EC, Chase HP, Klingensmith G, Hamman RF (1993). Early exposure to cow's milk and solid foods in infancy, genetic predisposition and risk of IDDM. *Diabetes* 42: 288 – 295.

Kristiansen JE, Bremmegard A, Busk HE, Heltberg O, Frederiksen W, Justersen T (1984). Rapid identification of *Capnocytophaga* isolated from septicaemic patients. *Eur J Clin Microbiol* 3: 236 - 240.

Kurtis B, Develioglu H, Taner IL, Balos K (1999). IL-6 levels in gingival crevicular fluid (GCF) from patients with non-insulin dependent diabetes mellitus (NIDDM), adult periodontitis and healthy subjects. *J Oral Sci* 41: 163 - 167.

Lab M Culture Media Manual (1997). Fastidious Anaerobe Agar (FAA, Lab 90) p 50.

Lalla E, Lamster IB, Drury S, Fu C, Schmidt AM (2000). Hyperglycaemia, glucoxidation and receptor for advanced glycation endproducts: potential mechanisms underlying diabetic complications, including diabetes-associated periodontitis. *Periodontol 2000* 23: 50 - 62.

Lalla E, Lamster IB, Schmidt AM (1998). Enhanced interaction of advanced glycation end products with their cellular receptor RAGE: implications for the pathogenesis of accelerated periodontal disease in diabetes. *Ann Periodontol* 3: 13 -19.

Lamont RJ, Oda D, Persson RE, Persson GR (1992). Interaction of *Porphyromonas gingivalis* with gingival epithelial cells maintained in culture. *Oral Microbiol Immunol* 7: 364 - 367.

Lamster IB (1997). Evaluation of components of gingival crevicular fluid as diagnostic tests. *Ann Periodontol* 2: 123-137.

Lamster IB, Hartley LJ, Oshrain RL (1985a). Evaluation and modification of spectrophotometric procedures for analysis of lactate dehydrogenase, beta-glucuronidase and arylsulphatase in human gingival crevicular fluid collected with filter-paper strips. *Arch Oral Biol* 30: 235 - 242.

Lamster IB, Hartley LJ, Vogel RI (1985b). Development of a biochemical profile for gingival crevicular fluid. *J Periodontol* 56: 13 - 21.

Lamster IB, Oshrain RL, Florello LA, Celenti RS, Gordon JM (1988). A comparison of 4 methods of data quantification for lysosomal enzyme activity in gingival crevicular fluid. *J Clin Periodontol* 15: 347 - 352.

Lamster IB, Oshrain RL, Gordon JM (1986). Enzyme activity in human gingival crevicular fluid: consideration in data reporting based on analysis of individual crevicular sites. *J Clin Periodontol* 13: 799 - 804.

Lang NP, Joss A, Orsanic T, Gusberti FA, Siegrist BE (1986). Bleeding on probing: a predictor for the progression of periodontal disease? *J Clin Periodontol* 13: 590 - 596.

Larjava H, Uitto VJ, Haapasalo M, Heino J, Vuento M (1987). Fibronectin fragmentation induced by dental plaque and *Bacteroides gingivalis. Scand J Dent Res* 95: 308 - 314.

Laufer TM, von Herrath MG, Grusby MJ, Oldstone MB, Glimcher LH (1993). Autoimmune diabetes can be induced in transgenic major histocompatibility complex class II-deficient mice. *J Exp Med* 178: 589 – 596.

Laughon BE, Syed SA, Loesche WJ (1982). API ZYM for the identification of *Bacteroides* spp., *Capnocytophaga* spp., and spirochetes of oral origin. *J Clin Microbiol* 15: 97 - 102.

Leadbetter ER, Holt SC, Socransky SS (1979). *Capnocytophaga:* New genus of Gram-negative gliding bacteria I. General characteristics, taxonomic considerations and significance. *Arch Microbiol* 122: 9 - 16.

Letzelter C, Croute F, Pianezzi B, Roques C, Soleilhavoup JP (1998). Supernatant cytotoxicity and proteolytic activity of selected oral bacteria against human gingival fibroblasts *in vitro. Arch Oral Biol* 43: 15 - 23.

Leung WK, Theilade E, Comfort MB, Lim PL (1993). Microbiology of the pericoronal pouch in mandibular third molar pericoronitis. *Oral Microbiol Immunol* 8: 306 - 312.

Lewsey JD, Gilthorpe MS, Gulabivala K (2001). An introduction to multilevel meta-analysis using an example for the probability of success of root canal treatment. *Comm Dent Health* 18: 131 - 137.

Ley BE, Linton CJ, Bennett DMC, Jalal H, Foot ABM, Millar MR (1998). Detection of bacteraemia in patients with fever and neutropenia using 16S rRNA gene amplification by polymerase chain reaction. *Eur J Clin Microbiol Infect Dis* 17: 247 - 253.

Liljemark WF, Fenner LJ, Bloomquist CG (1986). *In vivo* colonisation of salivary pellicle by *Haemophilus, Actinomyces* and *Streptococcus* spp. *Caries Res* 20: 481 - 497.

Liljenberg B, Lindhe J, Berglundh T Dahlén G (1994). Some microbiological, histopathological and immunohistochemical characteristics of progressive periodontal disease. *J Clin Periodontol* 21: 720 - 727.

Lillie RD (1928). The Gram stain. I. A quick method for staining Gram-positive organisms in the tissues. *Arch Path* 5: 828.

Lindemann RA, Kinder Haake SA, Kjeldsen M, Avanessian AB (1996). Effect of whole oral bacteria on peripheral leukocyte interleukin-6 and soluble interleukin-6 receptor production. *Oral Microbiol Immunol* 5: 332 - 336.

Lindemann RA, Kjeldsen M, Cabret M (1995). Effect of whole oral bacteria and extracted lipopolysaccharides on peripheral blood leucocyte interleukin-2 receptor expression. *J Periodontal Res* 30: 264 - 271.

Lindhe J, Haffajee AD, Socransky SS (1983). Progression of periodontal disease in adult subjects in the absence of periodontal therapy. *J Clin Periodontol* 10: 433 - 442.

Lindhe J, Nyman S (1975). The effect of plaque control and surgical pocket elimination on the establishment and maintenance of periodontal health. A longitudinal study of periodontal therapy in cases of advanced periodontitis. *J Clin Periodontol* 2: 65 - 79.

Lindhe J, Nyman S (1984). Long term maintenance of patients treated for advanced periodontal disease. *J Clin Periodontol* 11: 504 - 514.

Lindhe J, Socransky SS (1979). Chemotaxis and vascular permeability produced by human periodontopathic bacteria. *J Periodontal Res* 14: 138 -146.

Lindley DV, Smith AFM (1972). Bayes estimates for the linear model. *J Roy Stat Soc Series B* 34: 1 - 41.

Ling PR, Bistrian BR, Mendez B, Istfan NW (1994). Effects of systemic infusion of endotoxin, tumor necrosis factor and interlukin-1 on glucose metabolism in the rat: relationship to endogenous glucose production and peripheral tissue glucose uptake. *Metabolism* 3: 279 - 284.

Listgarten MA (1976). Structure of the microbial flora associated with periodontal health and disease in Man. A light and electron microscope study. *J Periodontol* 47: 1 - 18.

Listgarten MA (1994). The structure of dental plaque. *Periodontol 2000* 5: 52-65.

Listgarten MA, Helldén L (1978). Relative distribution of bacteria at clinically healthy and periodontally diseased sites in humans. *J Clin Periodontol* 5: 115 - 132.

Listgarten MA, Lai CH, Young V (1993). Microbial composition and pattern of antibiotic resistance in subgingival microbial samples from patients with refractory periodontitis. *J Periodontol* 64: 155 - 161.

Löe H (1993). Periodontal disease. The sixth complication of diabetes mellitus. *Diabetes Care* 16: 329 - 334.

Löe H, Anerud A, Boysen H, Smith MR (1978a). The natural history of periodontal disease in man. Study design and baseline data. *J Periodontal Res* 13: 550 - 562.

Löe H, Anerud A, Boysen H, Smith MR (1978b). The natural history of periodontal disease in man. Tooth mortality rate before 40 years of age. *J Periodontal Res* 13: 563 - 572.

Löe H, Anerud A, Boysen H, Smith MR (1978c). The natural history of periodontal disease in man. The rate of periodontal destruction before 40 years of age. *J Periodontol* 49: 607 - 620.

Löe H, Aneurd A, Boysen H, Morrison E (1986). Natural history of periodontal disease in man. Rapid, moderate and no loss of attachment in Sri Lankan labourers 14 to 46 years of age. *J Clin Periodontol* 13: 431 - 440.

Löe H, Theilade E, Jensen SB (1965). Experimental gingivitis in man. *J Periodontol* 36: 177 - 187.

Loesche WJ (1968). Importance of nutrition in gingival crevice microbial ecology. *Periodontics* 6: 245 -249.

Loesche WJ (1976). Chemotherapy of dental plaque infections. *Oral Sci Res* 9: 65 - 107.

Loesche WJ, Syed SA (1978). Bacteriology of human gingivitis: effect of plaque and gingivitis scores. *Infect Immun* 21: 830 - 839.

Loesche WJ, Syed SA, Laughon BE, Stoll J (1982). The bacteriology of acute necrotising ulcerative gingivitis. *J Periodontol* 53: 223 - 230.

Longford NT (1987). A fast scoring algorithm for maximum likelihood estimation in unbalanced mixed models with nested random effects. *Biometrika* 74: 817 - 828.

Lönnroth P (1991). Regulation of insulin action at the cellular level. *J Int Med* 735: 23 - 29.

Loos B, Kiger R, Egelberg J (1987). An evaluation of basic periodontal therapy using sonic and ultrasonic scalers. *J Clin Periodontol* 14: 29 - 33.

Loos BG, Dyer DW, Whittam TS, Selander RK (1993). Genetic structure of populations of *Porphyromonas gingivalis* associated with periodontitis and other oral infections. *Infect Immun* 61: 203 - 212.

Lorian V (1986). Antibiotics in laboratory medicine. 2nd edition, Williams & Wilkins, Baltimore, USA.

MacDonald H, Gordon DL (1988). *Capnocytophaga* species: a cause of amniotic fluid infection and preterm labour. *Pathol* 20: 74 - 76.

MacDonald TT (1999). Effector and regulatory lymphoid cells and cytokines in mucosal sites. *Curr Top Microbiol Immunol* 236: 113 - 135.

MacLarty DG, Arhaide I, Bottazzo GF, Swai ABM, Alberti KGMM (1990). Islet cell autoantibodies are not specifically associated with insulin-dependent diabetes in rural Tanzanian Africans. *Diabetes Res Clin Pract* 9: 219 - 224.

Mahrer S, Raik E (1992). *Capnocytophaga canimorsus* septicaemia associated with cat scratch. *Pathol* 24: 194 - 196.

Maidek BL, Cole JR, Lilburn TG, Parker CT Jr, Saxman PR, Farris RJ, Garrity GM, Olsen GJ, Schmidt TM, Tiedje JM (2001). The RDP-II (Ribosomal Database Project). *Nucleic Acids Res* 1: 173 - 174.

Mandell RL (1984). A longitudinal microbiological investigation of *Actinobacillus actinomycetemcomitans* and *Eikenella corrodens* in juvenile periodontitis. *Infect Immun* 45: 778 - 780.

Mandell RL, DiReinzo J, Kent R, Joshipura K, Haber J (1982). Microbiology of healthy and diseased periodontal sites in poorly controlled insulin dependent diabetics. *J Periodontol* 63: 274 - 279.

Marsh PD (1991). Sugar, fluoride, pH and microbial homeostasis in dental plaque. *Proc Finn Dent Soc* 87: 515 – 525.

Marsh PD (1994). Microbial ecology of dental plaque and its significance in health and disease. *Adv Dent Res* 8: 263 - 271.

Marsh PD, Bradshaw DJ (1997). Physiological approaches to the control of oral Biofilms. *Adv Dent Res* 11: 176 - 185.

Marsh PD, Martin MV (1999). Acquisition, adherence, distribution and metabolism of the oral microflora. In Oral Microbiology, 4th Edition, Wright Publishers, Chapter 4, pp. 34 - 57.

Marsh PD, Martin MV (1999). Dental Plaque. In: Oral Microbiology, Wright Publishers 4th edition, Chapter 5, pp. 58 - 81.

Marshall SM, Melito PI, Woodward DL, Johnson WM, Rodgers FG, Mulvey MR (1999). Rapid identification of *Campylobacter*, *Arcobacter* and *Helicobacter* isolates by PCR-Restriction Fragment Length Polymorphism analysis of the 16S rRNA Gene. *J Clin Microbiol* 37: 4158 - 4160.

Martino R, Ràmila E, Capdevila JA, Planes A, Rovira M, del Mar Ortego M, Plumé G, Gómez L, Sierra J (2001). Bacteraemia caused by *Capnocytophaga* species in patients with neutropenia and cancer: results of a multicentre study. *Clin Infect Dis* 33: 20 - 22.

Mashimo PA, Yamamoto Y, Nakamura M, Slots J (1983b). Selective recovery of oral *Capnocytophaga* spp. with sheep blood agar containing bacitracin and polymyxin B. *J Clin Microbiol* 17: 187 - 191.

Mashimo PA, Yamamoto Y, Slots J, Park BH, Genco RJ (1983a). The periodontal microflora of juvenile periodontitis. Culture, immunofluorescence and serum antibody studies. *J Periodontol* 54: 420 - 430.

Mason WM, Wong GM, Entwistle B (1983). Contextual analysis through the multilevel linear model. In: Sociological methodology, ed. Leinhardt S, San Francisco: Jossey-Bass, pp 72 - 103.

McCowen KC, Malhotra A, Bistrian BR (2001). Stress-induced hyperglycemia. *Crit Care Clin* 17: 107 - 124.

McCullagh P, Nelder JA (1989). Generalised linear models, 2nd edition, Chapman and Hall, London.

McDonald H, Gordon DL (1988). *Capnocytophaga* species: a cause of amniotic fluid infection and preterm labour. *Pathol* 20: 74 - 76.

McLaughlin WS, Kirkham J, Kowolik MJ, Robinson C (1996). Human gingival crevicular fluid keratin at healthy, chronic gingivitis and chronic adult periodontitis sites. *J Clin Periodontol* 23: 331 - 335.

McNair P, Madsbad S, Christiansen C, Christensen MS, Faber OK, Binder C, Transbol I (1979). Bone loss in diabetes: Effects of metabolic state. *Diabetologia* 17: 283 - 286.

McNamara TF, Ramamurthy NS, Mulvihill JE, Golub LM (1982). The development of an altered gingival crevicular microflora in the alloxan-diabetic rat. *Arch Oral Biol* 27: 217 - 223.

Mealey B (2000). Diabetes Mellitus. In: Periodontal Medicine, eds Rose LF, Genco RJ, Cohen DW, Mealey BL, BC Decker Inc, Ontario, Canada, Chapter 8, pp. 120 - 150.

Medlin L, Elwood HJ, Stickel S, Sogin ML (1988). The characterisation of enzymatically amplified eukaryotic 16S-like rRNA coding regions. *Gene* 71: 491 - 499.

Meikle M, Atkinson SJ, Ward RV, Murphy G, Reynolds JJ (1989). Gingival fibroblasts degrade Type I collagen films when stimulated with tumor necrosis factor and interleukin 1: Evidence that breakdown is mediated by metalloproteinases. *J Periodontal Res* 24: 207 - 213.

Mello KA, Syndman DR, Arora S (1990) *Capnocytophaga* infection involving a portal-systemic vascular shunt. *Digest Dis Sci* 35: 909 - 911.

Mercer LJ (1985). *Capnocytophaga* isolated from the endometrium as a cause of neonatal sepsis. *J Reprod Med* 30: 67 - 68.

Michalowicz BS, Aeppli D, Virag JG, Klump DG, Hinrichs JE, Segal NL, Bouchard TJ, Pihlstrom BL (1991). Periodontal findings in adult twins. *J Periodontol* 62: 293 - 299.

Miller LS, Manwell MA, Newbold D, Reding ME, Rasheed A, Blodgett J, Kornman KS (1992). The relationship between reduction in periodontal inflammation and diabetes control: A report of 9 cases. *J Periodontol* 63: 843 - 848.

Miller WD (1890). The microorgansims of the human mouth. The local and general diseases which are caused by them. SS White, Philadelphia pp 274 – 341.

Molvig J, Baek L, Christensen P, Manogue KR, Vlassara H, Platz P, Nielsen LS, Svejgaard A, Nerup J (1988). Endotoxin-stimulated human monocyte secretion of interleukin 1, tumour necrosis factor alpha, and prostaglandin E2 shows stable interindividual differences. *Scand J Immunol* 27: 705 - 16.

Mombelli A, Gusberti FA, Lang NP (1989). Treatment of recurrent periodontal disease by root planing and Ornidazole (Tiberal®). Clinical and microbiological findings. *J Clin Periodontol* 16: 38 - 45.

Mombelli A, Lang NP, Bürgin WB, Gusberti FA (1990). Microbial changes associated with the development of puberty gingivitis. *J Periodontal Res* 25: 331 - 338.

Moncla BJ, Braham P, Hillier SL (1990). Sialidase (neuraminidase) activity among gram-negative anaerobic and capnophilic bacteria. *J Clin Microbiol* 28: 422 - 425.

Montejo Baranda M, Acha Arrieta V, Hernandez Almaraz J, Perez Rodriguez M, Antoñana Oraeta M, Aguirre Errasti C (1984). Two cases of *Capnocytophaga* bacteraemia, one with endocarditis. *Can Med Assoc J* 130: 1420.

Moore WEC, Holdeman LV, Cato EP, Good IJ, Smith EP, Ranney EE, Palcanis KG (1984). Variation in periodontal floras. *Infect Immun* 46: 720 - 726

Moore WEC, Holdeman LV, Cato EP, Smibert RM, Burmeister JA, Palcanis KG, Ranney RR (1985). Comparative bacteriology of juvenile periodontitis. *Infect Immun* 48: 507 - 519.

Moore WEC, Moore LVH (1994). The bacteria of periodontal diseases. *Periodontol 2000* 5: 66 - 77.

Moore WEC, Ranney RR, Holdeman LV (1982). Supragingival microflora in periodontal disease: cultural studies. In: Host-Parasite Interactions in Periodontal Diseases, eds. Genco RJ, Mergenhagen SE, American Society for Microbiology, pp.13 - 26.

Mordan NJ, Newman HN, Slaney JM, Curits MA (1999). The apical plaque border in health and disease. In: Dental Plaque revisited - Oral Biofilms in health and disease, eds. HN Newman, M Wilson, Bioline, pp. 343 - 374.

Morhart RE, Fitzgerald RJ (1976). Nutritional determinants of the ecology of the oral flora. *Dent Clin N Amer* 20: 473 - 489.

Mortensen JE, LeMaistre A, Moore DG, Robinson A (1985). Peritonitis involving *Capnocytophaga ochracea*. *Diag Microbiol Infect* 3: 359 - 362.

Moutschen MP, Scheen AJ, Lefebvre P (1992). Impaired immune responses in diabetes mellitus: analysis of the factors involved. Relevance to the increased susceptibility of diabetic patients to specific infections. *Diabet Med* 18: 187 -201.

Mowat A, Baum J (1971). Chemotaxis of polymorphonuclear leukocyte from patients with diabetes mellitus. *N Eng J Med* 284: 621 - 627.

Muchová J, Liptáková A, Országhová Z, Garaiová I, Tisoñ P, Cársky J, Duracková Z (1999). Antioxidant systems in polymorphonuclear leucocytes of Type 2 diabetes mellitus. *Diabet Med* 16: 74 - 78.

Müller HP, Hiencke A, Borneff M, Knopf A, Kiencke C, Pohl S (1997). Microbial ecology of *Actinobacillus actinomycetemcomitans*, *Eikenella corrodens* and *Capnocytophaga* spp. in adult periodontitis. *J Periodontal Res* 32: 530 - 542.

Mullis KB, Faloona F (1987). Specific synthesis of DNA *in vitro* via a polymerase catalysed chain reaction. *Methods Enzymol* 155: 335 - 350.

Mullokandov EA, Franklin WAS, Brownlee M (1994). DNA damage by the glycation products of glyceraldehyde-3-phosphate and lysine. *Diabetologia* 37: 145 - 149.

Nakamura M, Slots J (1982). Aminopeptidase activity of *Capnocytophaga*. *J Periodontal Res* 17: 597 - 603.

Nathan DM (1993). Long-term complications of diabetes mellitus. *N Engl J Med* 328: 1676 -1685.

National Committee for Clinical Laboratory Standards (1993). Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria, Third Edition: Approved Standard. NCCLS document m11-A3 (ISBN 1-56238-210-1).

National Diabetes Data Group (1979). Classification and diagnosis of diabetes mellitus and other categories of glucose intolerance. *Diabetes* 28: 1039 - 1057.

National Diabetes Data Group (1985): Diabetes in America, National Institute of Health 2 ed. Bethesda, MD: Diabetes data compiled in 1984. NIH Publication No 85 -1268.

National Diabetes Data Group (1995): Diabetes in America, National Institute of Health, 2 ed. Bethesda, MD. NIH Publication no 95 - 1468.

Neiders ME, Chen PB, Suido H, Reynolds HS, Zambon JJ (1989). Heterogeneity of virulence among strains of *Bacteroides gingivalis*. *J Periodontal Res* 24: 192 - 198.

Newman B, Selby JV, King MC, Slemenda C, Fabsitz R, Friedman GD (1987). Concordance for type 2 (non-insulin-dependent) diabetes mellitus in male twins. *Diabetologia* 30: 763 - 768.

Newman HN (1973). The organic films on enamel surfaces. 1. The vestigial enamel organ. *Br Dent J* 135: 64 - 67.

Newman HN (1974). Microbial biofilms in nature. *Microbios* 9: 247 - 257.

Newman HN (1975). The gingival border of plaque. Morphological studies in 8 to 15-year-old children. *Br Dent J* 138: 335 - 345.

Newman HN (1990). Plaque and chronic inflammatory periodontal disease. A question of ecology. *J Clin Periodontol* 17: 533 - 541.

Newman HN (1996). Focal Infection. *J Dent Res* 75: 1912 - 1919.

Newman MG, Sims TN (1979). The predominant cultivable microbiota of the periodontal abscess. *J Periodontol* 50: 350 - 354.

Newman MG, Socransky SS (1977). Predominant cultivable microbiota in periodontosis. *J Periodontal Res* 12: 120 - 128.

Newman MG, Sutter VL, Pickett MJ, Blachman U, Greenwood JR, Grinenko V, Citron D (1979). Detection, identification and comparison of *Capnocytophaga, Bacteroides ochraceus*, and DF-1. *J Clin Microbiol* 10: 557 - 562.

Ngaage DL, Kotidis KN, Sandoe JAT, Nair U (1999). Do not snog the dog: infective endocarditis due to *Capnocytophaga canimorsus*. *Eur J Cardio Thor Surg* 16: 362 - 363.

Nichols C, Laster LL, Bodak-Gyovai LZ (1978). Diabetes mellitus and periodontal disease. *J Periodontol* 49: 85 - 88.

Nickel JC, Rueska I, Whitefield C, Marrie TJ, Costerton JW, (1985). Antibiotic resistance of *Pseudomonas aeruginosa* colonising a urinary catheter *in vivo*. *Eur J Clin Microbiol* 4: 213 - 218.

Nishimura F, Takahashi K, Kurihara M, Takashiba S, Murayama Y (1998). Periodontal Disease as a complication of diabetes mellitus. *Ann Periodontol* 3: 20 - 29.

Nishimura F, Terranova V, Foo H, Kurihara M, Kurihara H, Murayama Y (1996). Glucose-mediated alteration of cellular function in human periodontal ligament cells. *J Dent Res* 75: 1664 - 1671.

Noack B, Jachmann I, Roscher S, Sieber L, Kopprasch S, Lück C, Hanefeld M, Hoffmann T (2000). Metabolic diseases and their possible link to risk indicators of periodontitis. *J Periodontol* 71: 898 - 903.

Nolan C, Beaty H, Bagdade J (1978). Impaired granulocyte bactericidal function in patients with poorly controlled diabetes. *Diabetes* 127: 889 - 894.

Nonnenmacher C, Mutters R, Flores de Jacoby L (2001). Microbiological characteristics of subgingival microbiota in adult periodontitis localised juvenile periodontitis and rapidly progressive periodontitis subjects. *Clin Microbiol Infect* 7: 213 - 217.

Nyvad B, Kilian M (1987). Microbiology of early colonisation of human enamel and root surfaces. *Scand J Dent Res* 95: 369 - 380.

Ochiai K, Senpuku H, Kurita-Ochiai T (1998). Purification of immunosuppressive factor from *Capnocytophaga ochracea*. *J Med Microbiol* 47: 1087 - 1095.

Ofek I, Perry A (1985). Molecular basis of bacterial adherence to tissues. In: Molecular Basis of Oral Microbial Adhesion, eds. Mergenhagen SE, Rosen B, American Society of Microbiology, Washington DC, pp. 7 - 13.

Offenbacher S (1996). Periodontal diseases: pathogenesis. *Ann Periodontol* 1: 879 - 925.

Offenbacher S, Heasman PA, Collins JG, (1993). Modulation of host PGE_2 secretion as a determinant of periodontal disease expression. *J Periodontol* 64: 432 - 444.

Offenbacher S, Odel BM, Van Dyke T (1986). The use of crevicular fluid prostaglandin E_2 levels as a predictor of periodontal attachment loss. *J Periodontal Res* 21: 101 - 112.

Offenbacher S, Odle BM, Gray RC, Van Dyke TE (1984). Crevicular fluid prostaglandin E levels as a measure of the periodontal disease status and juvenile periodontitis patients. *J Periodontal Res* 19: 1 - 13.

Okuda H, Murakami S (1998). Cytokine expression in periodontal health and disease. *Crit Rev Oral Biol Med* 9: 248 - 266.

Okuda K, Yamanaka A, Kubodera T, Ikeda M, Takazoe I (1994). A study of the subgingival microflora of HIV-seropositive and CD4 cell depleted children with hemophilia. *Bull Tokyo Dent Coll* 35: 91 - 94.

Oliver R, Tervonen T (1993). Periodontitis and tooth-loss: comparing diabetics with the general population. *J Am Dent Assoc* 124: 71- 76.

Oliver RC, Tervonen T (1994). Diabetes – A risk factor for periodontitis in adults? *J Periodontol* 65: 530 - 538.

Oosterwaal PJM, Matee MI, Mikx FH, van't Hof MA, Renggli HH (1987). The effect of subgingival debridement with hand and ultrasonic instruments on the subgingival microflora. *J Clin Periodontol* 14: 528 - 533.

Page R (1998). The pathobiology of periodontal diseases may affect systemic diseases: inversion of a paradigm. *Ann Periodontol* 3: 108 - 120.

Page R, Offenbacher S, Schroeder HE, Seymour GJ, Kornman K (1997). Advances in the pathogenesis of periodontitis: summary of developments, clinical implications and future directions. *Periodontol* 2000 14: 216 - 248.

Page R, Schroeder HE (1976). Pathogenesis of inflammatory periodontal disease. A summary of current work. *Lab Invest* 33: 235 - 249.

Page RC, Vandesteen GE, Ebersole JL, Williams BL, Dixon IL, Altman LC (1985). Clinical and laboratory studies of a family with a high prevalence of juvenile periodontitis. *J Periodontol* 56: 602 - 610.

Palmer RA (2001). Long-term periodontal problems - the chemotherapeutic aspect. *Br Dent J* 190: 218.

Pangburn MK, Muller-Eberhard HJ (1978). Complement C3 convertase: cell surface restriction of B1H control and generation of restriction on neuraminidase-treated cells. *Immunol* 75: 2416 - 2420.

Papapanou PN, Sellén A, Wenström JL, Dahlén G (1993). An analysis of the subgingival microflora in randomly selected subjects. *Oral Microbiol Immunol* 8: 24 - 29.

Papapanou PN, Wennström JL, Gröndahl K (1989). A 10 year retrospective study of periodontal disease progression. *J Clin Periodontol* 16: 403 - 411.

Parenti DM, Snydman DR (1985). *Capnocytophaga* species: Infections in nonimmunocompromised and immunocompromised hosts. *J Infect Dis* 151: 140 - 147.

Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, Sahasrabudhe A, Dewhirst FE (2001). Bacterial diversity in human subgingival plaque. *J Bacteriol* 183: 3770 - 3783.

Peterson PK, Wilkinson BJ, Kom Y, Schmeling D, Quie PG (1978). Influence of encapsulation on staphylococcal opsonisation and phagocytosis by human polymorphonuclear leukocytes. *Infect Immun* 19: 943 - 949.

Petsios A, Nakou M, Manti F (1995). Microflora in adult periodontitis. *J Periodontal Res* 30: 325 - 331.

Pinson M, Hoffman WH, Garnick JJ, Litaker MS (1995). Periodontal disease and type 1 diabetes mellitus in children and adolescents. *J Clin Periodontol* 22: 118 - 123.

Poirer TP, Holt SC (1983). Acid and alkaline phosphatases of *Capnocytophaga* spp. I. Production and cytological localisation of the enzymes. *Can J Microbiol* 29: 1350 - 1360.

Poirer TP, Holt SC (1983). Acid and alkaline phosphatases of *Capnocytophaga* spp. II. Isolation, purification and characterisation of the enzymes from *Capnocytophaga ochracea*. *Can J Microbiol* 29: 1361 - 1368.

Poirier TL, Tonelli SJ, Holt SC (1979). Ultrastructure of gliding bacteria: scanning electron microscopy of *Capnocytophaga sputigena, Capnocytophaga gingivalis* and *Capnocytophaga ochracea*. *Infect Immun* 26: 1146 - 1158.

Poirier TP, Mishell R, Trummel CL, Holt SC (1983). Biological and chemical comparison of butanol- and phenol-water extracted lipopolysaccharide from *Capnocytophaga sputigena*. *J Periodontal Res* 18: 541 - 557.

Pozzilli P, Leslie RDG (1994). Infections and Diabetes: mechanisms and prospects for prevention. *Diabet Med* 11: 935 - 941.

Preshaw PM, Kelly PJ, Heasman PA (1996). Quadratic calibration curves for the Periotron 6000. *J Periodontal Res* 31: 441 - 443.

Ramamurthy NS, Siegel M, Iacono VJ, Golub LM (1979). Leucocyte response in the gingival crevice of the diabetic rat. *J Periodontal Res* 14: 289 - 296.

Ramfjord SP, Morrison EC, Burgett FG, Nissle RR, Shick RA, Zann GJ, Knowles JW. (1982). Oral hygiene and maintenance of periodontal support. *J Periodontol* 53: 26 - 30.

Rasbash J, Browne W, Cameron B, Charlton C (2000). *MLwiN* version 1.10.0006, Multilevel Models Project, Institute of Education, London.

Rawlinson A, Duerdin BI, Goodwin L (1993). New findings on the microflora associated with adult periodontitis. *J Dent* 21: 179 - 184.

Rayfield EJ, Ault MJ, Keush GT, Brothers MJ, Mechemias C, Smith H (1982). Infection and diabetes: the case for glucose control. *Am J Med* 72: 439 - 450.

Rees TD (1994). The diabetic dental patient. *Dent Clin N Amer* 38: 447 - 463.

Rees TD (2000). Periodontal management of the patient with diabetes mellitus. *Periodontol 2000* 23: 63 - 72.

Reynolds JJ, Meikle MC (1997). Mechanisms of connective tissue destruction. Importance of the balance of metalloproteinases and inhibitors in tissue destruction

and implications for human periodontitis and its treatment. *Periodontol 2000* 14: 144 - 157.

Robertson KL, Drucker DB, James J, Blinkhorn AS, Hamlet S, Bird PS (2001). A microbiological study of Papillon-Lefevre syndrome in two patients. *J Clin Pathol* 54: 371 - 376.

Robrish SA, Grove SB, Bernstein RS, Marucha PT, Socransky SS, Amdur B (1976). Effect of sonic treatment on pure cultures and aggregates of bacteria. *J Clin Microbiol* 3: 474 - 479.

Rocha M, Nava LE, Vázquez de la Torre C, Sánchez-Marin F, Garay-Sevilla MA, Malacara JM (2001). Clinical and radiological improvements of periodontal diseases in patients with Type 2 diabetes mellitus treated with alendronate: A randomised, placebo controlled trial. *J Periodontol* 72: 204 - 209.

Roscoe DL, Zemcov JV, Thornber D, Wise R, Clarke AM (1992). Antimicrobial susceptibilities and β-Lactamase characterisation of *Capnocytophaga* spp. *Antimicrob Agents Chemother* 36: 2197 - 2200.

Rose RK, Dibdin GH, Shellis RP (1993). A quantitative study of calcium binding and aggregation in selected oral bacteria. *J Dent Res* 72: 78 - 84.

Rosenberg M, Judes H, Weiss E (1983). Cell surface hydrophobicity of dental plaque microorgansims. *Infect Immun* 42: 831 - 834.

Rosling B, Nyman S, Lindhe J (1976). The effect of systematic plaque control on bone regeneration in infrabony pockets. *J Clin Periodontol* 3: 38 - 53.

Rubsamen PE, McLeish WM, Pflugfelder S, Miller D (1993). *Capnocytophaga* Endophthalmitis. *Ophthalmol* 100: 456 - 459.

Rudney JD, Chen R, Sedgewick GJ (2001). Intracellular *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in buccal epithelial cells collected from human subjects. *Infect Immun* 69: 2700 - 2707.

Rummens J L, Fossepre JM, De Gruyter M, Van de Vyver H, Neyt L, Vanuyt HW (1985). Isolation of *Capnocytophaga* species with a new selective medium. *J Clin Microbiol* 22: 375-378.

Rummens JL, Gordts B, van Landuyt HW (1986). *In vitro* susceptibility of *Capnocytophaga* species to 20 antimicrobial agents. *Antimicrob Agents Chemother* 30: 739 - 742.

Rylander H, Ramberg P, Blohmé G, Lindhe J (1986). Prevalence of periodontal disease in young diabetics. *J Clin Periodontol* 14: 38 - 43.

Safkan-Seppälä B, Ainamo J (1992). Periodontal conditions in insulin-dependent diabetes mellitus. *J Clin Periodontol* 19: 24 - 29.

Sakamoto M, Suzuki M, Umeda M, Ishikawa I, Benno Y (2002). Reclassification of *Bacteroides forsythus* (Tanner *et al.* 1986) as *Tannerella forsythensis* corrig., gen. nov. comb. nov. *Int J Sys Evol Microbiol* 52: 841 - 849.

Sallay K, Sanavi F, Ring I, Pham P Behling UH, Nowotny A (1982). Alveolar bone destruction in the immunocompromised rat. *J Periodontal Res* 17: 263 - 274.

Salvi GE, Beck JD, Offenbacher S (1998). PGE_2, IL-1β and TNF-α responses in diabetics as modifiers of periodontal disease expression. *Ann Periodontol* 3: 40 - 50.

Salvi GE, Yalda B, Collins JG, Jones BH, Smith FW, Arnold RR, Offenbacher S (1997). Inflammatory mediator response as a potential risk marker for periodontal diseases in insulin-dependent diabetes mellitus patients. *J Periodontol* 68: 127 - 135.

Sanchez-Cordero S, Hoffman H, Stahl SS (1979). Occurrence of *Staphylococcus* in periodontal pockets of diabetic and nondiabetic adults. *J Periodontol* 50: 109 - 113.

Sanders WE, Sanders CC (1984). Modification of normal flora by antibiotics: effects on individuals and the environment. In: New dimensions in antimicrobial chemotherapy, eds. Koot RK, Sande MA, New York, Churchill Livingstone, pp. 217 - 241.

Sandholm L, Mahlberg K, Jousimies-Somer H (1988). Phospholipase A_2: a possible virulence factor of *Capnocytophaga ochracea*. *Oral Microbiol Immunol* 3: 18 - 21.

Sandros J, Karlsson C, Lappin DF, Madianos PN, Kinane DF, Papapanou PN (2000). Cytokine responses of oral epithelial cell to *Porphyromonas gingivalis* infection. *J Dent Res* 79: 1808 - 1814.

Sandros J, Papapanou PN, Dahlen G (1993). *Porphyromonas gingivalis* invades oral epithelial cells *in vitro*. *J Periodontal Res* 28: 219 - 226.

Sasaki T, Ramamurthy NS, Yu Z, Golub LM (1992). Tetracycline administration increases protein (presumably procollagen) synthesis and secretion in periodontal ligament fibroblasts of streptozotocin-induced diabetic rats. *J Periodontal Res* 27: 631 - 639.

Sastrowijoto S, Hillemans P, van Steebergen TJM, Abraham-Inpijin L, de Graaff J (1989). Periodontal conditions and microbiology of healthy and diseased periodontal pockets in type 1 diabetes mellitus patients. *J Clin Periodontol* 16: 316 - 322.

Sastrowijoto SH, van der Velden U, van Steenbergen TJM, Hillemans P, Hart AAM, de Graaff J, Abraham-Inpijn J (1990). Improved metabolic control, clinical periodontal

status and subgingival microbiology in insulin-dependent diabetes mellitus. *J Clin Periodontol* 17: 233 - 242.

Savitt ED, Socransky SS (1984). Distribution of certain subginginval microbial species in selected periodontal conditions. *J Periodontal Res* 19: 111 - 123.

Saxton CS, van der Ouderaa FJ (1989). The effect of a dentifrice containing zinc citrate and Triclosan on developing gingivitis. *J Periodontal Res* 24: 75 - 80.

Sbordone L, Ramaglia L, Barone A, Ciaglia RN, Tenore A, Iacono VJ (1995). Periodontal status and selected cultivable anaerobic microflora of insulin-dependent juvenile diabetics. *J Periodontol* 66: 452 - 461.

Sbordone L, Ramaglia L, Barone A, Cigalia RN, Iacono VJ (1998). Periodontal status and subgingival microbiota of insulin-dependent juvenile diabetics: A 3-year longitudinal study. *J Periodontol* 69: 120 -128.

Schäffer AA, Aravind L, Madden TL, Shavirin S, Spouge JL, Wolf YI, Koonin EV, Altschul SF (2001). Improving the accuracy of PSI-BLAST protein database searches with composition-based statistics and other refinements. *Nucleic Acids Res* 15: 2994 - 3005.

Schmidt AM, Hori O, Chen J, Li JF, Crandal J, Zhang J, Cao R, Yan SD, Brett J, Stern D (1995). Advanced glycation endproducts interacting with their endothelial receptor induce expression of vascular cell adhesion molecule-1 (VCAM-1): a potential mechanism for the accelerated vasculopathy of diabetes. *J Clin Invest* 96: 1395 - 1403.

Schmidt AM, Stern DM (2000). RAGE: A new target for the prevention and treatment of the vascular and inflammatory complications of diabetes. *Trends Endocrinol Metabol* 11: 368 - 375.

Schmidt AM, Weidman E, Lalla E, Yan SD, Hori O, Cao R, Brett JG (1996). Advanced glycation endproducts (AGEs) induce oxidant stress in the gingiva: a potential mechanism underlying accelerated periodontal disease associated with diabetes. *J Periodontal Res* 31: 508 - 515.

Schmidt FJ, Sluiter WJ, Schoonen AJ (1993). Glucose concentration in subcutaneous extracellular space. *Diabetes Care* 16(5): 695 - 670.

Schroeder HE, Attström R (1980). Pocket formation: an hypothesis. In: Borderland between caries and periodontal disease, ed. Lehner T, Cimasoni G, Vol. II, Orlando, Grune & Stratton.

Schroeder HE, Graf-de Beer M, Attström R (1975). Initial gingivitis in dogs. *J Periodontal Res* 10: 128 - 142.

Schwartz J, Stinson FL, Parker RB (1972). The passage of tritiated bacterial endotoxin across intact gingival crevicular epithelium. *J Periodontol* 43: 270 - 275.

Scott JH, Symons NBB (1982). The mucous membrane of the mouth. In: Introduction to Dental Anatomy, Churchill Livingstone, chapter 17, pp. 295 - 319.

Seiffert A (1862). Der Zahnaszt Als Diagnostiker, Dtsch Wehn Zahnheil 3: 153.

Seppälä B, Ainamo J (1994). A site-by site follow-up study on the effect of controlled versus poorly controlled insulin-dependent diabetes mellitus. *J Clin Periodontol* 21: 161 - 165.

Seppälä B, Ainamo J (1994). A site-by-site follow-up study in the effect of controlled versus poorly controlled insulin-dependent diabetes mellitus. *J Clin Periodontol* 21: 161 - 165.

Seppälä B, Sorsa T, Ainamo J (1997). Morphometric analysis of cellular and vascular changes in gingival connective tissue in long-term insulin-dependent diabetes. *J Periodontol* 68: 1237 - 1245.

Seymour R, Steele JG (1998). Is there a link between periodontal disease & coronary heart disease? *Br Dent J* 184: 645 - 660.

Shah HN, Gharbia SE (1995). The biochemical milieu of the host in the selection of anaerobic species in the oral cavity. *Clin Infect Dis* 20: 291 - 300.

Shibata Y, Fujimura S, Nakamura T 1992. Isolation and characterization of enzymes hydrolyzing chymotrypsin synthetic substrate (Enzyme I) and trypsin synthetic substrate (Enzyme II) from the envelope of *Capnocytophaga gingivalis*. *Med Microbiol Immunol* 81: 107 - 115.

Shlossman M, Knowler WC, Pettitt DJ, Genco RJ (1990). Type 2 diabetes mellitus and periodontal diseases. *J Am Dent Assoc* 121: 532 - 536.

Shurin BS, Socransky SS, Sweeney E, Stossel TP (1979). A neutrophil disorder induced by *Capnocytophaga*, a dental micro-organism. *N Eng J Med* 16: 849 - 854.

Silness J, Löe H (1964). Periodontal disease in pregnancy. II. Correlation between oral hygiene and periodontal condition. *Acta Odont Scand* 22: 112 – 135.

Slots J (1976). The predominant cultivable organisms in juvenile periodontitis. *Scand J Dent Res* 84: 1 - 10.

Slots J (1981). Enzymatic characterisation of some oral and nonoral Gram-negative bacteria with the API ZYM System. *J Clin Microbiol* 14: 288 - 294.

Slots J (1982). Selective medium for isolation of *Actinobacillus actinomycetemcomitans*. *J Clin Microbiol* 15: 606 - 609.

Slots J, Genco R (1984). Black Pigmented *Bacteroides* species, *Capnocytophaga* species and *Actinobacillus actinomycetemcomitans* in human periodontal disease: virulence factors in colonisation, survival and tissue destruction. *J Dent Res* 63: 412 - 421.

Slots J, Rams TE (1990). Antibiotics in periodontal therapy: advantages and disadvantages. *J Clin Periodontol* 17: 479 - 493.

Slots J, Rams TE (1991). New views on periodontal microbiota in special patient categories. *J Clin Periodontol* 18: 411 - 420.

Slots J, Reynolds HS, Genco RJ (1980). *Actinobacillus actinomycetemcomitans* in human periodontal disease: a cross sectional microbiological investigation. *Infect Immun* 29: 1013 - 1020.

Slots J, Rosling BG (1983). Suppression of the periodontopathic microflora in localised juvenile periodontitis by systemic tetracycline. *J Clin Periodontol* 10: 465 - 476.

Slots S, Genco RJ (1984). Microbial pathogenicity. Black pigmented *Bacteroides species, Capnocytophaga species,* and *Actinobacillus actinomycetemcomitans* in human periodontal disease: virulence factors in colonisation, survival and tissue destruction. *J Dent Res* 63: 412 - 421.

Smalley JW, Birss AJ, Shuttleworth CA (1988). The degradation of type 1 collagen and human plasma fibronectin by the trypsin-like enzyme and extracellular membrane vesicles of *Bacteroides gingivalis* W50. *Arch Oral Biol* 33: 323 - 329.

Socransky SS (1977). Microbiology of periodontal disease: present status and future considerations. *J Periodontol* 48: 497 - 504.

Socransky SS, Gibbons RJ, Dale AC, Bortnik L, Rosenthal E, MacDonald JB (1963). The microbiota of the gingival crevice in man-I. Total microscopic and viable counts and counts of specific organisms. *Arch Oral Biol* 8: 275 -280.

Socransky SS, Haffajee A (1998). Microbiology of periodontal disease. In Clinical Periodontology and Implant Dentistry, eds Lindhe, Karring, Lang, Chapter 4, pp. 138 - 188.

Socransky SS, Haffajee AD (1991). Microbial mechanisms in the pathogenesis of destructive periodontal disease. *J Periodontal Res* 26: 195 - 212.

Socransky SS, Haffajee AD (1994). Evidence of bacterial etiology: a historical perspective. *Periodontol* 2000 5: 7 - 25.

Socransky SS, Haffajee AD (1997). The nature of periodontal diseases. *Ann Periodontol* 2: 3 -10.

Socransky SS, Haffajee AD, Cugini Ma, Smith C, Kent Jr RL, (1998). Microbial complexes in subgingival plaque. *J Clin Periodontol* 25: 134 -144.

Socransky SS, Haffajee AD, Goodson JM, Lindhe J (1984). New concepts of destructive periodontal disease. *J Clin Periodontol* 11: 21 - 32.

Socransky SS, Holt SC, Leadbetter ER, Tanner ACR, Savitt E, Hammond BF (1979). *Capnocytophaga:* new genus of Gram-negative gliding bacteria. III. Physiological characterisation. *Arch Microbiol* 122: 29 - 33.

Socransky SS, Smith C, Haffajee AD (2002). Subgingival profiles in refractory periodontal disease. *J Clin Periodontol* 29: 260 - 268.

Söderfelt B, Palmqvist S (1998). A multilevel analysis of factors affecting the longevity of fixed partial dentures, retainers and abutments. *J Oral Rehab* 25: 245 - 252.

Söderling E, Mäkinen PL, Syed S, Mäkinen KK (1991). Biochemical comparison of proteolytic enzymes present in rough- and smooth-surfaced *Capnocytophaga* isolated from the subgingival plaque of periodontitis patients. *J Periodontal Res* 26: 17 - 23.

Soh LL, Newman HN, Strahan JD (1982). Effects of subgingival chlorhexidine irrigation on periodontal inflammation. *J Clin Periodontol* 9: 66 - 74.

Somerman MJ, Kagermier AS, Bowers MR, Fox PC (1985). *In vitro* attachment of bacteria to extracts of cementum. *J Oral Path* 14: 793 - 799.

Soskolne WA (1998). Epidemiology and clinical aspects of periodontal disease in diabetics. *Ann Periodontol* 3: 3 - 12.

Speck H, Kroppenstedt RM, Mannheim W (1987). Genomic relationships and species differentiation in the genus *Capnocytophaga. Zbl Bakt Hyg* A 266: 390 - 402.

Spratt DA (1994). A study of aminopeptidase and other hydrolytic enzymes produced by *Capnocytophaga* gingivalis. PhD Thesis, University of the West of England, Bristol, UK.

Spratt DA, Greenman J, Schaffer AG (1995). *Capnocytophaga gingivalis* aminopeptidase: a potential virulence factor. *Microbiol* 141: 3087 - 3093.

Spratt DA, Greenman J, Schaffer AG (1996). *Capnocytophaga gingivalis*: effects of glucose concentration on growth and hydrolytic enzyme production. *Microbiol* 142: 2161 - 2164.

Spratt DA, Greenman, Schaffer AG (1999). Growth and hydrolytic enzyme production of *Capnocytophaga gingivalis* on different protein substrates. *Oral Microbiol Immunol* 14: 122 - 126.

SPSS Manual (1990). Statistical Data Analysis. Base System User's Guide. MJ Norusis / SPSS Inc. pp.113 - 4; 365.

SPSS Sample Power: http:/www.SPSS.com/software/Spower/

Steinhauserova I, Ceskova J, Fojtikova K, Obrovska I (2001). Identification of thermophilic *Campylobacter* spp. by phenotypic and molecular methods. *J Appl Microbiol* 90: 470 - 475.

Stern IB (1981). Current concepts of the dentogingival junction. The epithelium and connective tissue attachments to the tooth. *J Periodontol* 52: 465 - 476.

Sterne JAC, Curtis MA, Gillett IR, Griffiths GS, Maiden MFJ, Wilton JAM, Johnson NW (1990). Statistical models for data from periodontal research. *J Clin Periodontol* 17: 129 - 137.

Sterne JAC, Johnson NW, Wilton JMA, Joyston-Bechal S, Smales FC (1998). Variance components analysis of data from periodontal research. *J Periodontal Res* 23: 148 - 153.

Stevens RH, Sela MN, Shapira J, Hammond BF (1980b). Detection of a fibroblast proliferation inhibitory factor from *Capnocytophaga sputigena. Infect Immun* 27: 271 - 275.

Stoffers DA, Ferrer J, Clarke WL, Habener JF. Early-onset type-II diabetes mellitus (MODY 4) linked to 1PF1. *Nature Genetics* 117: 138 -139.

Suido H, Nakamura M, Mashimo PA, Zambon JJ, Genco RJ (1986). Arylaminopeptidase activities of oral bacteria. *J Dent Res* 65: 1335 -1340.

Sung-Jim K, Jong-Hyuk C, Joon-Bong P, Young-Hyuk K (1998). High glucose stimulates production of PGE_2. *J Dent Res* 77: 1873.

Sutter VL, Pyeatt D, Kwok KK (1981). *In vitro* susceptibility of *Capnocytophaga* strains to 18 antimicrobial agents. *Antimicrobial Agents Chemother* 20: 270-271.

Sweeney EA, Alcoforado GAP, Nyman S, Slots J (1987). Prevalence and microbiology of localised prepubertal periodontitis. *Oral Microbiol Immunol* 2: 65 - 70.

Syed SA, Loesche WJ (1972). Survival of human dental plaque flora in various transport media. *App Microbiol* 24: 638-644.

Sznajder N, Carraro JJ, Rugna S, Sereday M (1978). Periodontal findings in diabetic and non diabetic patients. *J Periodontol* 49: 445 - 448.

Tait BD, Harrison LC. The major histocompatibility complex and IDDM. *Ballieres Clin Endocrinol Metab* 5: 211 – 228.

Tanner ACR, Goodson JM (1986). Sampling of micro-organisms associated with periodontal disease. *Oral Microbiol Immunol* 1: 15 -20.

Tanner ACR, Hadfer C, Bratthal GT, Visconte RA, Socransky SS (1979). A study of the bacteria associated with the advancing periodontitis in man. *J. Clin Periodontol* 6: 278-307.

Taylor GW, Burt BA, Becker MP, Genco RJ, Shlossman M (1998). Glycemic control and alveolar bone loss progression in type 2 diabetes. *Ann Periodontol* 3: 30 - 39.

Taylor GW, Burt BA, Becker MP, Genco RJ, Shlossman M, Knowler WC, Pettitt DJ (1996). Severe periodontitis and risk for poor glycaemic control in patients with non-insulin dependent diabetes mellitus. *J Periodontol* 67: 1085 - 1093.

Terada A, Inaba M, Yano Y, Hasuma T, Nishizawa Y, Morii H, Otani S (1998). Growth-inhibitory effect of a high glucose concentration on osteoblast-like cells. *Bone* 22: 17 - 23.

Tervonen T, Knuttila M (1986). Relation of diabetes control to periodontal pocketing and alveolar bone level. *Oral Surg Oral Med Oral Pathol* 61: 346 - 349.

Tervonen T, Oliver RC, Wolff LF, Bereuter J, Anderson L, Aeppli DM (1994). Prevalence of periodontal pathogens with varying metabolic control of diabetes mellitus. *J Clin Periodontol* 21: 375 - 379.

Testillano Tarrero M, Montejo Baranda M, Arizaga JI, Canela MM (1989). Peritonitis involving *Capnocytophaga ochracea*. *Am J Gastroenterol* 84: 206 - 207.

Theilade E, Attström R (1985). Distribution and ultrastructure of subgingival plaque in beagle dogs with gingival inflammation. *J Periodontal Res* 10: 131- 145.

Theilade E, Wright WH, Jensen SB (1966). Experimental gingivitis in man II. *J Periodontal Res* 1: 1 -13.

Theilade E. (1986). The non-specific theory in microbial etiology of inflammatory periodontal disease. *J Clin Periodontol* 13: 905 - 911.

Thompson JD, GibsonTJ, Plewniak F, Jeanmougin F, Higgins DG (1997). The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 24: 4876 - 4882.

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22: 4673 - 4680.

Thorstensson H, Dahlèn G, Hugoson A (1995). Some suspected periodontopathogens and serum antibody response in adult long-duration insulin-dependent diabetics. *J Clin Periodontol* 22: 449 - 458.

Thorstensson H, Hugoson A (1993). Periodontal disease experience in adult long duration insulin-dependent diabetics. *J Clin Periodontol* 20: 352 - 358.

Tierney MR, Sullivan Baker A (1995). Infections of the head and neck in diabetes mellitus. *Infect Dis Clinics N Amer* 9: 195 - 216.

Totten PA, Patton CM, Tenover FC, Barrett TJ, Stamm WE, Steigerwalt AG, Lin JY, Holmes KK, Brenner DJ (1987). Prevalence of characterisation of hippurate-negative *Campylobacter jejuni* in King Country, Washington. *J Clin Microbiol* 25: 1747 - 1752.

Trinder P (1969). Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. *Ann Clin Biochem* 6: 24 - 27.

Tsuji I, Nakamoto K, Hasegawa T, Hisashige A, Inawashiro H, Fukao A, Hisamichi S (1991). Receiver operating characteristic analysis of fasting plasma glucose, HbA1c, and fructosamine on diabetes screening. *Diabetes Care* 14: 1075 - 1077.

van der Bijl, Dreyer WP, Grobler SR (1986). The Periotron gingival crevicular fluid meter. *J Periodontal Res* 21: 39 - 44.

van der Hoeven JS, Rogers AH (1979). Stability of the resident microflora and bacteriocinogeny of *Streptococcus mutans* as factors affecting its establishment in specific pathogen free rats. *Infect Immun* 23: 206 - 212.

van der Waaij D, Berghuis de Vries JM, Lekkerkerk van der Wees JEC (1971). Colonisation resistance of the digestive tract in conventional and antibiotic treated-mice. *J Hyg* 69: 405 - 411.

Van Dyke T, Bartholomew E, Genco RJ, Slots J, Levine MJ (1982). Inhibition of neutrophil chemotaxis by soluble bacterial products. *J Peridontol* 53: 502 - 508.

Van Palenstein Helderman (1981). Microbial etiology of periodontal disease. *J Clin Periodontol* 8: 261 - 280.

van Winkelhoff AJ, Rams TE, Slots J (1996). Systemic antibiotic therapy in periodontics. *Periodontol 2000* 10: 45 - 78.

Vandamme P, Vancanneyt M, Van Belkum A, Segers P, Quint WGV, Kersters K, Paster BJ, Dewhirst FE (1996). Polyphasic approach of strains of the genus *Capnocytophaga* and centres for disease control group DF-3. *Int J Sys Bacteriol* 46: 782 - 791.

Verghese A, Hamati F, Berk S, Franzus B, Berk S, Smith K (1988). Susceptibility of dysgonic fermentor 2 to antimicrobial agents *in vitro*. *Antimicrob Agents Chemother* 32: 78 - 80.

Vlassara H (1991). Non-enzymatic glycosylation. In: *Diab Annual/6*, eds KGMM Alberti and LP Krall, Elsevier Science Publishers, Chapter 18, pp. 371 - 389.

Vlassara H, Bucala R, Striker L (1994). Pathogenic effects of advanced glycosylation: biochemical, biological and clinical implications. *Lab Invest* 70: 138 – 151.

Vrahopoulos TP, Barber P, Liakoni H, Newman HN (1988). Ultrastructure of the periodontal lesion in a case of Papillon-Levfèvre syndrome (PLS). *J Clin Periodontol* 15: 17 - 26.

Vrahopoulos TP, Barber PM, Newman HN (1992). The apical border plaque in chronic adult periodontitis. An ultrastructural study. I. Morphology, structure and cell content. *J Periodontol* 63: 243 - 252.

Vrahopoulos TP. (1989) An ultrastructural study of dental plaque. PhD Thesis Vol.2: 28.

Ward PA, Chapitis J, Conroy MC, Lepow IH (1973). Generation by bacterial proteinases of leukotactic factors from human serum and human C3 and C5. *J Immunol* 110: 1003 - 1009.

Warren JS, Allen SD (1986). Clinical, pathogenetic and laboratory features of *Capnocytophaga* infections. *Am J Clin Pathol* 86: 513 - 518.

Watanabe K (1990). Prepubertal periodontitis: a review of diagnostic criteria, pathogenesis and differential diagnosis. *J Periodontal Res* 25: 31 - 48.

Wautier JL, Zoukourian C, Chappey O, Wautier MP, Guillausseau PJ, Cao R, Hori O Stern D, Schmidt AM (1996). Receptor mediated endothelial cell dysfunction in diabetic vasculopathy: soluble receptor for advanced glycation endproducts blocks hyperpermeability. *J Clin Invest* 97: 238 - 243.

Weber G, Abu-Shakra M, Hertzanu Y, Borer A, Sukenik S (1997). Liver abscess caused by *Capnocytophaga* species. *Clin Infect Dis* 25: 152 - 153.

Weiss EI, London J, Kolenbrander PE, Kagameier AS, Andersen RN, (1987). Characterisation of lectinlike surface components on *Capnocytophaga ochracea* ATCC 33596 that might mediate coaggregation with Gram-positive oral bacteria. *Infect Immun* 55: 1198 - 1202.

Wennström J, Heijl L, Lindhe J, Socransky S (1980). Migration of gingival leukocytes mediated by plaque bacteria. *J Periodontal Res* 15: 363 - 372.

Wexler HM, Doern GV (1995). Susceptibility testing of anaerobic bacteria. In: Manual of Clinical Microbiology, ASM Press, Ed. Murray PR, 6th edition, Chapter 115, pp. 1350 - 1355.

WHO (1985). Diabetes Mellitus. Report of WHO Study Group. WHO technical Report Series No. 727.

WHO (1999). Definition, Diagnosis and Classification of Diabetes Mellitus and its complications. Report of a WHO Consultation. Part 1: Diagnosis and Classification of Diabetes Mellitus. WHO/NCD/NCS/99.2

Wikström M, Renvert S, Johnsson T, Dahlén G (1993). Microbial associations in periodontitis sites before and after treatment. *Oral Microbiol Immunol* 8: 213 - 218.

Williams BL, Hammond BF (1979). *Capnocytophaga:* new genus of Gram-negative gliding bacteria. IV.DNA Base composition and sequence homology. *Arch Microbiol* 122: 35 - 39.

Williams BL, Hollis D, Holdeman LV (1979). Synonymy of strains of center for disease control group DF-1 species for *Capnocytophaga. J Clin Microbiol* 10: 550 - 555.

Williams DM, Hughes FJ, Odell EW, Farthing PA (1992). The nature of periodontal disease. In: Pathology of Periodontal Disease, Oxford Medical Publications, Chapter 1, pp. 1 -16.

Williams DM, Hughes FJ, Odell EW, Farthing PA (1992). The normal periodontium. In: Pathology of Periodontal Disease, Oxford Medical Publications, Chapter 2, pp. 16 – 31.

Williams JB (1926). Diabetic periodontoclasia. *J Am Dent Assoc* 15: 523 - 529.

Wilson M (1995). Biological activities of lipopolysaccharides from oral bacterial and their relevance to the pathogenesis of periodontitis. *Sci Prog* 78: 19 - 34.

Wilson MJ, Wade WG, Weightman AJ (1995). Restriction fragment length polymorphism analysis of PCR amplified 16S ribosomal DNA of human *Capnocytophaga. J Appl Bacteriol* 78: 394 - 401.

Wilson RM, Reeves WG (1986). Neutrophil function in diabetes. In: Recent advances in Type 2 DM, ed. Nattrass M, London Churchill Livingstone, pp. 127 -138.

Winn RE, Chase WF, Lauderdale PW, McClesky FK (1984). Septic arthritis involving *Capnocytophaga ochracea. J Clin Microbiol* 19: 538 - 540.

Woese CR (1987). Bacterial evolution. *Microbiol Rev* 51: 221- 271.

Woese CR, Fox GE, Zablen L, Uchida T, Bonen L, Pechman K, Lewis BJ (1975). Conservation of primary structure in 16S ribosomal RNA. *Nature* 254: 83 - 86.

Wolff LF, Aeppli DM, Pihlstrom BL, Anderson L, Stoltenberg J, Osborn J, Hardie N, Shelburne C, Fischer G. (1993). Natural distribution of five bacteria associated with periodontal disease. *J Clin Periodontol* 20: 699 - 706.

Woods GL, Washington JA (1995). Antibacterial susceptibility tests: dilution and disk diffusion methods. In: Manual of Clinical Microbiology, ASM Press, ed. Murray PR, 6th edition, Chapter 113, pp. 1327 - 1341.

Ximenez-Fyvie LA, Haffajee AD, Socransky SS (2000). Comparison of the microbiota of supra- and subgingival plaque in healthy and periodontitis. *J Clin Periodontol* 27: 648 - 657.

Yalda B, Offenbacher S, Collins JG (1994). Diabetes as a modifier of periodontal disease expression. *Periodontol 2000* 6: 37 - 49.

Yamagata K, Furuta H, Oda N, Kaisaki PJ, Menzel S, Cox NJ, Fajans SS, Signorini S, Stoffel M, Bell GI (1996). Mutations in the hepatocyte factor-4α gene in maturity-onset diabetes of the young (MODY 1). *Nature* 384: 458 - 460.

Yamamoto T, Kajiura S, Hirai Y, Watanabe T (1994). *Capnocytophaga haemolytica* sp. nov. and *Capnocytophaga granulosa* sp. nov., from human dental plaque. *Int J Sys Bacteriol* 44: 324-329.

Yan SD, Schmidt AM, Anderson GM, Zhang J, Brett J, Zou YS, Pinsky D, Stern D (1994). Enhanced cellular oxidant stress by the interaction of advanced glycation end products with their receptors/binding proteins. *J Biol Chem* 269: 9889 - 9897.

Yki-Järvinen H (1993). Pathogenesis of non-insulin-dependent diabetes mellitus. *Lancet* 343: 91 - 95.

Yoon JW (1991). Role of viruses in the pathogenesis of IDDM. *Ann Intern Med* 23: 437 - 445.

Yoshimura A, Hara Y, Kaneko T, Kato I (1997). Secretion of IL-1β, TNF-α, IL-8 and IL-1ra by human polymorphonuclear leukocytes in response to lipopolysaccharides from periodontopathic bacteria. *J Periodontal Res* 32: 279 - 286.

Yu X, Graves DT (1995). Fibroblasts, mononuclear phagocytes and endothelial cells express monocyte chemoattractant protein (MCP-1) in inflamed human gingiva. *J Periodontol* 66: 80 - 88.

Yuan K, Chang C-J, Hsu P-C, Sun HS, Tseng C-C, Wang J-R (2001). Detection of putative periodontal pathogens in non-insulin-dependent diabetes mellitus and non-diabetes mellitus by polymerase chain reaction. *J Periodontal Res* 36: 18 - 24.

Zambon JJ, Reynolds H, Fisher JG, Shlossman M, Dunford R, Genco R (1988). Microbiological and immunological studies of adult periodontitis in patients with non-insulin dependent diabetes mellitus. *J Periodontol* 59: 23 - 31.

APPENDIX 1: THE ORAL GLUCOSE TOLERANCE TEST (OGTT; WHO, 1999).

The OGTT is principally used for diagnosis when blood glucose levels are equivocal, during pregnancy or in epidemiological studies.

The OGTT should be administered in the morning after at least 3 days of unrestricted diet (greater than 150 g of carbohydrate daily) and usual physical activity. Recent evidence suggests that a reasonable (30 - 50 g) carbohydrate-containing meal should be consumed on the evening before the test. The test should be preceded by an overnight fast of 8 -14 hours, during which water may be drunk. Smoking is not permitted during the test. The presence of factors that influence interpretation of the results of the test must be recorded (e.g. medications, inactivity, infection etc.).

After collection of the fasting blood sample, the subject should drink a solution of 75 g of anhydrous glucose or 82.5 g of glucose monohydrate (or partial hydrolysates of starch of the equivalent carbohydrate content) in 250 - 300 ml of water over the course of 5 minutes. For children, the test load should be 1.75 g of glucose per kg body weight up to a total of 75 g glucose. Timing of the test is from the beginning of the drink. Blood samples must be collected 2 hours after the test load.

Unless the glucose concentration can be determined immediately the blood sample should be collected in a tube containing sodium fluoride (6 mg per ml whole blood) and immediately centrifuged to separate the plasma; the plasma should be frozen until the glucose concentration can be estimated.

(Refer to Table 1.2 for reference range of blood glucose values).

APPENDIX 2:   **EASTMAN DENTAL INSTITUTE AND HOSPITAL**
SUBJECT INFORMATION SHEET

DIABETES MELLITUS - GUM DISEASE RESEARCH PROJECT

PLEASE READ CAREFULLY. PLEASE ASK IF YOU DO NOT UNDERSTAND OR WOULD LIKE MORE INFORMATION.

PURPOSE:
The aim of this investigation is to study the bacteria associated with gum disease in diabetes patients. In order to do this, two groups of subjects are required - one group having diabetes mellitus and gum disease and the other group having gum disease only.

PROCEDURE:
As part of this study, you will be asked to attend once. However you may have more frequent appointments if you are attending for treatment in this or any other department. Participation in this study will not hinder you from receiving any other form of dental treatment. This visit should last under one hour.
During the visit, your mouth will be examined and six sites (three healthy and three diseased sites) will be selected. Clinical data collections will involve recording clinical measurements (using a probe) relevant to gum disease and taking plaque samples (i.e. bacteria which forms around your teeth, using a dental instrument). A blood sample will be taken from all patients. This is important so that we can establish the level of control of diabetes in the diabetes patients and to exclude any possibility of undiagnosed diabetes in our non Diabetes group. Your GP may need to be contacted during the course of the study.

CONFIDENTIALITY:
Data collected as part of this investigation will be used for research purposes only. You are free to withdraw from the study at any time without giving a reason. This **will not** in any way jeopardise any treatment you are receiving at this hospital. Data may be scrutinised by other persons within the Eastman Dental Institute but it will be treated as confidential and kept secure. Your personal details will not be disclosed. You will be kept informed of any relevant facts arising as the project progresses. Ethical approval of this study has been obtained.

DISCOMFORT:
You are not to experience any discomfort during this investigation other than a small prick when taking the blood sample. However in the event that you encounter problems, please contact the investigator: Dr Marilou Ciantar on telephone number 020 7915 1280 (Tues., Thurs. & Fri.) or on 020 7915 2309 /1016 (Mon. & Wed.) during office hours; alternatively on 020 7837 8888 ext. 2253 after hours.

PARTICIPATION:
Your participation in this study is completely voluntary. You are free to withdraw from the study at any time and without any prior notice. You may be withdrawn from the trial by the investigator if it is in your best interests to do so. You are allowed time to consider participation in the study.

APPENDIX 3:   SUBJECT'S CONSENT FORM FOR RESEARCH ON DIABETES & GUM DISEASE
Eastman Dental Institute and Hospital Joint Research and Ethics Committee

PLEASE READ THIS FORM CAREFULLY. PLEASE ASK IF YOU DO NOT UNDERSTAND OR WOULD LIKE MORE INFORMATION.

CONSENT BY THE PARTICIPANT:
Title of study: Isolation, identification of *Capnocytophaga* species associated with Diabetes Mellitus-periodontitis.
The purpose of this research is to study the possible association between Diabetes Mellitus and certain bacteria by the name of *Capnocytophaga,* which are recovered from inflamed gums.

Name of Investigator: Dr. Marilou Ciantar
This study will be performed at the Periodontology Department, Eastman Dental Hospital & Institute, 256, Gray's Inn Road, London WC1X 8LD.

Subject's Name:...
Date of Birth...   Gender: Female / Male.
I acknowledge that an information sheet has been provided. This I have read and understood and any questions I had have been answered to my satisfaction.
I understand that this study will contribute to scientific knowledge and that no personal benefit is obtained by participating in this study. I have been assured of confidentiality of any patient data collected. I agree that my general practitioner may be contacted and that he / she may release any relevant information on my past medical / dental history. I have informed the investigator of any medication which I am taking.
I am aware that I am under no obligation to participate in this study. Refusal to participate will not in any way jeopardise any treatment, which I may be receiving at this hospital. I understand that I may withdraw from the study at any time. I agree that the investigator responsible for this study may withdraw me from participating if it is in my best interest to do so. I am aware that blood and plaque (from around my teeth) samples will be collected in the course of the study.
The nature and purpose of this investigation have been detailed to me in an information sheet and have been explained to me by Dr. Marilou Ciantar.

I.. (Full name)

of...(Address)
hereby fully and freely consent to participate in the above study.

Signed...

Date......................................

DECLARATION BY THE INVESTIGATOR:
I confirm that I have provided an information sheet and that I have explained the nature and procedures involved in participating in this study. The subject participating in this trial has given his / her full consent freely and voluntarily.

Signed...
Date..........................Name ....

279

APPENDIX 4:   *CAPNOCYTOPHAGA* **Spp. - DM-PERIODONTITIS STUDY**

Clinical Data Information sheet

Patient Name: Lab No:

                                       Date:
                                       Ethnic origin
                                       Gender: M / F

Hospital No: MLC Nos.:

                                       BPA Nos.:

Address:

Tel No:

Patient Information sheet given	Yes	No
Consent form signed	Yes	No

DM	Yes	No	Blood glucose:	
Smoker	Yes	No	Quantity:	
Recent antibiotics	Yes	No	Name	
			Date taken	
Mouthwash	Yes	No	Name	
			Frequency	
Perio Rx	Yes	No	Date	
Blood sampled	Yes	No	LF	HbA$_{1c}$

Sites sampled for plaque						
PD (mm)						

Sites sampled for GCF						
PD (mm)						

NOTES:

280

APPENDIX 5: COMPOSITION OF REDUCED TRANSPORT FLUID (RTF)

Stock 1

Dibasic potassium phosphate	0.6 g
Distilled water	100 ml

Magnesium Sulphate stock solution

Magnesium sulphate	2.5 g
Distilled water	100 ml

Stock Solution 2

Potassium chloride	1.2 g
Ammonium sulphate	1.2 g
Monobasic potassium phosphate	0.6 g
Magnesium sulphate stock	1 ml
Distilled water	99 ml

Sodium Carbonate Solution

Sodium carbonate	0.8 g
Distilled water	10 ml

Preparation for 100 ml

Stock 1	7.5 ml
Stock 2	7.5 ml
Sodium carbonate	0.5 ml
Distilled water	80 ml

Autoclave solutions at 121°C for 15 min and allow to cool. Once cool, add a filter-sterilised solution of dithiothreitol (Sigma, 0.02 g in 5 ml distilled water).

APPENDIX 6: LYSIS SOLUTION

Recipe for 5 bottles, 190 ml each:

1. Weigh 0.76 g sodium carbonate

2. Dissolve in 950 ml distilled water

3. Adjust to pH 10 using 5M HCl (take care; wear gloves and use eye protection)

4. Dispense 190 ml into a clean 500 ml Duran bottle

5. Add approx. 100 μl of triton X-100 to each bottle (2 drops using a Pasteur pipette)

6. Label bottle with your name and lysis solution

7. Autoclave for 15 min at 121°C. Solution appears cloudy when just autoclaved

8. Store at room temperature until use

9. Warm solution to 37°C prior to use

APPENDIX 7: CATALASE & OXIDASE TESTS

Catalase test

Using a clean inoculating loop, growth from the centre of a colony was transferred to the surface of a clean glass slide. A loop full of 3% hydrogen peroxide (Sigma) was subsequently added to the bacteria. The smear was observed for bubble formation. The rapid and sustained appearance of bubbles / effervescence was reported as positive. The following strains were used as controls:

- positive control: *Staphylococcus aureus*
- negative control: *Streptococcus mutans.*

Oxidase Test (Cowan & Steel 1993)

A 1% solution of tetramethyl-*p*-phenylenediamine (Sigma) was prepared prior to each test. A drop of this solution was placed onto a filter paper contained in a Petri dish. Using a platinum or plastic loop or wooden applicator, some of the colonial growth was smeared onto the moistened paper. A positive reaction was recorded by the development of a purple colour within 10 seconds. The following strains were used as controls:

- positive: *Pseudomonas aeruginosa*
- negative: *E. coli*

APPENDIX 8: PHOSPHATE BUFFER (pH 6.0, 0.1 M) USED AS SOLVENT / DILUENT FOR AMOXICILLIN

(Isenberg 1992).

1. Prepare stock buffers (A & B):

    A. Stock buffer A: KH_2PO_4 (0.2 M)

        1. Add 13.6 g of KH_2PO_4 (0.2 mol, monobasic potassium phosphate, anhydrous) to a 500 ml volumetric flask).

        2. Add 100 ml of deionised water

        3. Shake to dissolve

        4. Bring to 500 ml with deionised water

        5. Dispense into 500 ml screw cap bottles

        6. Autoclave for 15 min at 121°C, alternatively filter through a 0.22 μm-pore-size membrane filter.

    B. Stock buffer B: K_2HPO_4 (0.2 M)

        1. Add 22.82 g of $K_2HPO_4.3H_2O$ (dibasic potassium phosphate) to a 500 ml volumetric flask

        2. Add 100 ml of deionised water

        3. Shake to dissolve

        4. Bring to 500 ml with deionised water

        5. Dispense into 500 ml screw cap bottles

        6. Autoclave for 15 min at 121°C, alternatively filter through a 0.22 μm-pore-size membrane filter.

Store sterile stock buffers at 2 - 8°C for up to 1 year.

2. Prepare 0.1 M, pH 6.0 phosphate working buffer by adding 43.85 ml of stock buffer A and 6.15 ml of stock buffer B.

3. Bring to 100 ml with deionised water

4. Check pH with pH meter. It should be close to the desired value. If necessary adjust with 1 N NaOH or 0.1 M phosphoric acid (if more than a few drops of base or acid are necessary to adjust pH, remake buffer).

5. Dispense approximately 150 ml into screw cap bottles.

6. Autoclave for 15 min at 121°C, alternatively filter through a 0.22 μm-pore-size membrane filter.

7. Store sterile working buffers at 25°C for up to 1 year.

APPENDIX 9: GLOSSARY OF MLM TERMINOLOGY

Cluster: a group sharing a common environment containing "lower level" elements e.g. cluster = subjects, lower level elements = teeth.

Explanatory variables (covariates, independent variables): factors which explain variation in the outcome. In the fixed part of the model, this is usually denoted by x.

Fixed effects: those depicted by a factor which has multiple (categorical or continuous), states where each state yields differences in the outcome(s). In MLM, the fixed effects represent the average model for the data set being analysed. The fixed effects are expressed by the coefficients for the intercept and all explanatory variables.

Interaction: occurs when the effect of one covariate is not constant for all values of another covariate, i.e. the effect differs in concordance with the values of the other covariate.

Kurtosis: of a data set measures a distribution's relative peakedness or flatness, indicating "heavy tails" or clumped distributions. A distribution with zero kurtosis is normally distributed.

Level: a component of the hierarchy. Level 1 is the lowest level, e.g. sites within teeth (level 2, the next level up), which in turn is nested within subjects = level 3. (Refer to Fig.1.17).

Multilevel modelling (hierarchical linear modelling): the technique of analysing clustered or hierarchical data using generalised linear modelling.

Nesting: the clustering of units into a hierarchy.

Random effects: those depicted by factors describing samples of states drawn from a larger population from all possible states. In MLM, these are given by the variances within- and between-groups. Note: "the intercept" is a fixed covariate used to derive the intercept but it is also a random covariate at each level, determining the variances at each level; the intercept and the variances are the "effects", the intercept covariate is neither a fixed or random "effect".

Residual (error term): the difference between the actual outcome value and the predicted outcome value. Residuals are used to check the assumptions of the regression model; in particular they should be approximately normally distributed (plot of ranked standardised residuals versus standard normal cumulative distribution should show the data points forming a straight line).

Response variable (outcome or dependent variable): often denoted by y in the linear regression model.

285

Skewness: characterise the degree of asymmetry of a distribution around its mean value. A distribution with zero skewness is symmetric about the mean.

Unit: an entity defined at a level of a data hierarchy e.g. an individual tooth would be (in a two-level tooth-subject structure) a level-1 unit (tooth) contained within a level-2 unit which is the subject.

Variance components models: those which split the total variance into separate components, one for each level of the natural hierarchy, and have no explanatory variables (i.e. only the intercept is present).

Variance: A measure of the random or systematic variation; it is the square of the degree of spread, known as the standard deviation.

(From Gilthorpe MS 1995, Gilthorpe MS *et al.* 2000c, d).

CONCISE COMMUNICATIONS

Assessment of five culture media for the growth and isolation of *Capnocytophaga* spp.

M. Ciantar[1], *D. A. Spratt*[2], *H. N. Newman*[3] and *M. Wilson*[2]

[1]Department of Microbiology and Periodontology, [2]Department of Microbiology, Eastman Dental Institute for Oral Health Care Sciences, London WC1X 8LD, UK; [3]Emeritus Professor of Periodontology and Preventive Dentistry, University of London, UK

*Tel: +44 171915 2308/1016   Fax: +44 171915 1127   E-mail: m.ciantar@eastman.ucl.ac.uk

The genus *Capnocytophaga* comprises a group of capnophilic/facultatively anaerobic Gram-negative, fusiform, slender rods which exhibit gliding motility [1–4]. The genus consists of seven species, namely *Capnocytophaga gingivalis*, *C. ochracea*, *C. sputigena*, *C. granulosa*, *C. hemolytica*, *C. canimorsus* and *C. cynodegmi*.

The ecologic niche for the first five species is the human oral cavity [1,5]. These species have been associated with varying degrees of periodontal health and disease [6,7]. They have been shown to gain systemic access, causing bacteremia [8] and endocarditis [9]. The natural habitat for *C. canimorsus* and *C. cynodegmi* is the oral cavity of dogs and cats [10]. *C. canimorsus* and *C. cynodegmi* can be pathogenic to humans following a dog/cat scratch or bite.

Data regarding the isolation frequency of *Capnocytophaga* spp. from clinical samples are conflicting, primarily due to the different microbial procedures employed. Isolation of these organisms is important for proper diagnosis and treatment of the systemic infections which they cause. Several factors influence bacterial recovery from clinical specimens, one of which is the provision of the appropriate culture medium. Two culture media selective for *Capnocytophaga* were formulated by Mashimo et al [11] and by Rummens et al [12]. However, in spite of their selectivity for this species, they have seldom been used in studies aiming to recover *Capnocytophaga* spp. from clinical samples. Two other media frequently used to culture *Capnocytophaga* spp. are that used by Leadbetter et al (LB) [1] and Slots' selective medium [13], primarily intended for *Actinobacillus actinomycetemcomitans*. The aim of this investigation, therefore, was to perform a quantitative and qualitative comparison of culture media used for recovery of *Capnocytophaga* spp.

Five culture media were used in the study, namely: Mashimo medium [11], Cap medium [12], LB medium [1], Slots' medium [13] and fastidious anaerobe agar (FAA) (Lab M) [14]. The media were prepared as specified in the respective references and used within 7 days of preparation.

The *Capnocytophaga* type strains (obtained from ATCC) used in this investigation were: *C. gingivalis* ATCC 33624, *C. ochracea* ATCC 27872, *C. sputigena* ATCC 33612, *C. granulosa* ATCC 51502, *C hemolytica* ATCC 51501, *C. canimorsus* ATCC 35979 and *C. cynodegmi* ATCC 49044.

Fresh clinical isolates MLC 74, 92, 87, 75, 62, 61 and 97 were recovered on FAA from human dental plaque and were stored as frozen stocks until this investigation. All strains (type strains and clinical isolates) were subcultured and maintained on FAA (Lab M) plus 5% (v/v) horse blood and incubated at 37 °C in 10% carbon dioxide. Preliminary identification was performed on the basis of colony morphology and Gram staining. Definitive identification was performed using 16S rRNA PCR RFLP analysis (previously validated with 16S rRNA gene sequencing; data not shown).

Three-day-old microbial cultures were suspended in Reduced Transport Fluid (RTF) [15]. The neat suspension was vortexed, serially diluted and inoculated on the five media. The plates were incubated at 37 °C in 10% carbon dioxide and examined after 3 days and 7 days of incubation. The numbers of CFU/mL for each strain on the different media were calculated from the average of four replicate plates.

Subgingival plaque samples were obtained from periodontitis patients attending the Periodontology Department, Eastman Dental Hospital, London. Plaque was collected from periodontal pockets ≥ 5 mm. At the time of sampling, the selected teeth were isolated with cotton wool rolls and dried. Supragingival plaque was removed with a periodontal probe. Subgingival plaque samples were obtained by using sterile Gracey curettes, with a single stroke from pocket base to orifice, and suspended in RTF. Bacterial identification was performed as for the clinical isolates.

The raw data (CFU/mL for each type strain and clinical isolate) were tested for variance using the F-test. Since variance was equal between the different strains, analysis of variance (ANOVA) was used to compare recovery of the different strains on the five media ($P < 0.05$).

FAA was the only medium capable of supporting growth of all the seven different *Capnocytophaga* ATCC type strains. FAA

yielded the maximum number of CFU/mL for all type strains except for *C. sputigena*, which showed marginally better recovery on Mashimo medium. FAA showed statistically significantly better recovery of isolates than Mashimo medium for ATCC strains *C. ochracea*, *C. granulosa*, *C. canimorsus and C. cynodegmi* (*P* < 0.05). It was also statistically significantly better than the Leadbetter medium for *C. ochracea*, *C. granulosa*, *C. canimorsus and C. cynodegmi*.

The seven clinical isolates were identified as three *Capnocytophaga* strains, namely: MLC 74 = *C. gingivalis*, MLC 92, 87, 75, 61 and 62 = *C. ochracea*, and MLC 97 = *C. granulosa*.

FAA, LB and Mashimo supported growth of all clinical isolates, i.e. *C. gingivalis*, *C. ochracea* and *C. granulosa*. Cap and Slots' media supported the growth of all the *C. ochracea* clinical isolates, with the exception of MLC 61 (*C. ochracea* strain), which failed to grow on Slots' medium.

In the majority of cases, growth of the three strains on FAA was statistically significantly better (*P* < 0.05) than that on the other media.

Only four of the five plaque samples showed growth of *Capnocytophaga* spp. on the media under investigation. In all four cases, the media which supported the growth of the organisms were FAA, Cap, LB and Mashimo media. Slots' medium was the only medium which did not isolate strains of *Capnocytophaga*. Within each plaque sample, the recovery of the isolates on the different media was comparable. Exceptions to this were noted in patient 3, where the recovery of

Capnocytophaga strains was statistically significantly (*P* < 0.05) better on FAA than on LB medium, and in patient 4, where the recovery of these organisms was statistically significantly better on Cap and Mashimo media (*P* < 0.05) than on FAA. A representative selection of isolates (45) was purified and identified by 16S rRNA RFLP analysis. The isolates were identified as *C. gingivalis* (7), *C. ochracea* (18), *C. sputigena* (1), *C. granulosa* (15) and *C. hemolytica* (4). Information generated from this part of the investigation showed that Mashimo medium was not entirely selective for *Capnocytophaga* strains.

Capnocytophaga spp. have been isolated from human dental plaque retrieved from both healthy and diseased sites. However, the reported relative isolation frequency between studies has varied considerably. Some studies have recovered these species in high numbers from periodontitis lesions [7], while others reported low numbers [6,16]. Discrepancies in data may be due to the culture media used for isolation and growth of these organisms. Few studies have used media selective for *Capnocytophaga* spp., namely Mashimo and Cap [11,12]. Furthermore, many of the investigations that have isolated *Capnocytophaga* spp. have failed to identify the isolates. Therefore, direct comparison between such studies is precluded.

Mashimo and Cap media were formulated to select for *Capnocytophaga* spp. from polymicrobial clinical specimens [11,12]. Their selectivity was imparted by the addition of antibiotics based on previous susceptibility testing of *Capnocytophaga* strains [17,18]. The major disadvantage of these

Table 1 Growth of the ATCC *Capnocytophaga* type strains, clinical isolates and plaque samples on five different culture media

	FAA	Cap	LB	Mashimo	Slots
ATCC type strains					
Cg	5.2×10^8 (3.6×10^7)	0	4.9×10^8 (1.8×10^7)	0	0
Co	2.4×10^8 (5.6×10^7)	0	0	6.5×10^7 (1.1×10^7)	0
Cs	1×10^7 (2×10^6)	3.1×10^8 (3.4×10^5)	4.9×10^8 (2×10^6)	1.6×10^7 (2.5×10^6)	0
Cgr	8.1×10^7 (7.2×10^6)	0	4.2×10^7 (6.9×10^6)	1×10^6 (1×10^5)	0
Ch	1.4×10^8 (3.3×10^7)	1×10^8 (1.6×10^7)	6.4×10^7 (2.6×10^7)	1.3×10^8 (3.3×10^7)	6.8×10^7 (1.2×10^7)
Ccn	1.9×10^8 (4.4×10^7)	1.2×10^8 (1.6×10^7)	2.5×10^3 (3.8×10^3)	0	0
Ccy	3.4×10^8 (5.7×10^7)	0	0	0	0
Clinical isolates					
74 (Cg)	7.3×10^7 (5×10^6)	0	1.1×10^7 (1.7×10^6)	5.8×10^7 (1.7×10^6)	0
92 (Co)	3×10^8 (8.2×10^6)	1.6×10^7 (4.6×10^6)ᵃ	6×10^6 (1.6×10^6)ᵃ	4.6×10^7 (2.8×10^6)	5×10^4 (0)ᵃ
87 (Co)	4.2×10^7 (5.1×10^6)	1.9×10^7 (2.7×10^6)	4.1×10^6 (1.3×10^6)ᵃ	4.4×10^7 (6.4×10^6)	4.1×10^5 (2.2×10^5)ᵃ
75 (Co)	2.1×10^7 (4.1×10^6)	1.6×10^7 (7.7×10^6)	3×10^6 (8.5×10^5)ᵃ	1.3×10^7 (2×10^6)	1.3×10^7 (6.1×10^6)
61 (Co)	7.9×10^7 (2.7×10^7)	5.3×10^7 (8.4×10^6)	7.1×10^5 (5.5×10^5)ᵃ	2.8×10^7 (1.8×10^7)	0
62 (Co)	9.3×10^7 (1.2×10^7)	1.9×10^6 (1.3×10^5)ᵃ	2×10^7 (4.8×10^6)ᵃ	1×10^7 (2.6×10^6)ᵃ	1.2×10^6 (4.4×10^5)ᵃ
97 (Cgr)	2.7×10^7 (5.5×10^6)	0	3.8×10^7 (5.1×10^6)	1.3×10^7 (1.2×10^6)ᵃ	0
Plaque samples					
1	3.8×10^4 (4.8×10^4)	6.5×10^4 (3.7×10^4)	7.5×10^4 (3.5×10^4)	5×10^4 (1.3×10^4)	0
2	0	0	0	0	0
3	7.5×10^5 (5×10^5)	8×10^5 (9.1×10^4)	7.5×10^4 (3.5×10^4)ᵃ	1.8×10^6 (1.3×10^5)	0
4	2×10^6 (7.1×10^5)	1.2×10^7 (6×10^6)ᵃ	1.5×10^6 (1.1×10^6)	6×10^6 (1.9×10^6)ᵃ	0
5	3.8×10^5 (2.5×10^5)	3.1×10^5 (6.4×10^4)	2.9×10^5 (4.8×10^4)	7.9×10^5 (1.1×10^5)	0

Bold numbers are mean values of CFU/mL (*n* = 4); numbers in parentheses indicate standard deviations. Cg, *C. gingivalis*; Co, *C. ochracea*; Cs, *C. sputigena*; Cgr, *C. granulosa*; Ch, *C. hemolytica*; Ccn, *C. canimorsus*; Ccy, *C. cynodegmi*. ᵃCFU/mL significantly different from FAA values at *P* < 0.05.

investigations which set out to test the antimicrobial suscept-ibility of *Capnocytophaga* spp. was that the bacterial strains used were not identified. This precludes accurate information being obtained regarding the antibiotic sensitivity of the different *Capnocytophaga* strains and which could explain the results obtained in this investigation. Although Mashimo medium was formulated to be specific for *Capnocytophaga* spp., Mashimo et al [11] found that only 80% of the recovered strains were *Capnocytophaga* spp., a finding corroborated in this study.

Cap medium is a nutritionally richer medium, formulated to be an improvement on Mashimo medium. It was claimed to be an 'excellent medium for the recovery of *Capnocytophaga* species from contaminated clinical specimens' [12].

The literature regarding the sensitivity of *Capnocytophaga* spp. to the antibiotics used in Cap medium is confusing, with some authors suggesting growth inhibition [18,19] and others imply-ing intrinsic resistance [20]. Since these studies did not identify the isolates, such conclusions cannot be made. Therefore, antibiotic susceptibility testing of the different *Capnocytophaga* spp. warrants further investigation.

LB medium was included in the study on the basis of re-commendations by Leadbetter et al [1], who used it during the original *Capnocytophaga* isolation and characterization studies. In our studies, this medium performed nearly as well as FAA.

Slots' medium [13], a selective medium for *Actinobacillus actinomycetemcomitans*, has been used in several investigations in which *Capnocytophaga* spp. were concurrently isolated with *A. actinomycetemcomitans*. Its selectivity is imparted by the anti-biotics bacitracin and vancomycin.

The third part of this investigation was undertaken to rule out the possibility that maintaining subcultures of *Capnocytophaga* spp. on FAA could potentially bias the results in favor of FAA. It is evident that, with the exception of patient 4, strains of *Capnocytophaga* from the plaque samples grew equally well on the media under investigation. Isolates recognized as being *Capnocytophaga* spp. were selected at random and identified via 16S rRNA RFLP analysis. It has to be emphasized that this part of the investigation was not intended to identify all the *Capno-cytophaga* isolates recovered from the plaque samples. Thus, direct comparison of the identified isolates from the plaque samples with the respective type strains and clinical isolates is precluded.

In conclusion, the results of this investigation have shown that the culture medium is a determining factor in isolating the full range of *Capnocytophaga* spp. from clinical isolates. FAA was the only medium which supported the growth of all *Capnocytophaga* ATCC type strains and clinical isolates.

REFERENCES

1. Leadbetter ER, Holt SC, Socransky SS. *Capnocytophaga:* new genus of Gram-negative gliding bacteria I. General characteristics,

taxonomic considerations and significance. *Arch Microbiol* 1979; 122: 9–16.

2. Holt SC, Leadbetter ER, Socransky SS. *Capnocytophaga:* new genus of Gram-negative gliding bacteria. II. morphology and ultrastructure. *Arch Microbiol* 1979; 122: 17–27.

3. Socransky SS, Holt SC, Leadbetter ER, Tanner ACR, Savitt E, Hammond BF. *Capnocytophaga:* new genus of Gram-negative gliding bacteria. III. Physiological characterisation. *Arch Microbiol* 1979; 122: 29–33.

4. Williams BL, Hammond BF. *Capnocytophaga:* new genus of Gram-negative gliding bacteria. IV. DNA base composition and sequence homology. *Arch Microbiol* 1979; 122: 35–9.

5. Yamamoto T, Kajiura S, Hirai Y, Watanabe T. *Capnocytophaga haemolytica* sp. nov. & *Capnocytophaga granulosa* sp. nov., from human dental plaque. *Int J Syst Bacteriol* 1994; 44: 324–9.

6. Moore WEC, Ranney RR, Holdeman LV. Supragingival microflora in periodontal disease: cultural studies. In: Genco RJ, Mergenhagen SE, eds. *Host–Parasite Interactions in Periodontal Diseases.* Washington: American Society for Microbiology, 1982: 13–26.

7. Tanner ACR, Hadfer C, Bratthal GT, Visconte RA, Socransky SS. A study of the bacteria associated with the advancing periodontitis in man. *J Clin Periodontol* 1979; 6: 278–307.

8. Montejo Baranda M, Acha Arrieta V, Hernandez Almarez J, Perez Rodriguez M, Antonana Oraeta M, Aguirre Erasti C. Two cases of *Capnocytophaga* bacteraemia, one with endocarditis. *J Can Med Assoc* 1984; 130: 1420.

9. Buu-Hoi AY, Souad J, Acar JF. Endocarditis caused by *Capnocytophaga ochracea. J Clin Microbiol* 1988; 23: 1061–2.

10. Brenner DJ, Hollis DG, Fanning GR, Weaver RE. *Capnocytophaga canimorsus* sp. nov. (formerly CDC group DF-2), a cause of septicaemia following a dog bite, and *C. cynodegmi* sp. nov, a cause of localised wound infection following dog bite. *J Clin Microbiol* 1989; 27: 231–5.

11. Mashimo PA, Yamamoto Y, Nakamura M, Slots J. Selective recovery of oral *Capnocytophaga* spp. with sheep blood agar containing bacitracin and polymyxin B. *J Clin Microbiol* 1983; 17: 187–91.

12. Rummens JL, Fossepre JM, De Gruyter M, Van de Vyver H, Neyt L, Vanuyt HW. Isolation of *Capnocytophaga* species with a new selective medium. *J Clin Microbiol* 1985; 22: 375–8.

13. Slots J. Selective medium for isolation of *Actinobacillus actinomy-cetemcomitans. J Clin Microbiol* 1982; 15: 606–9.

14. Griffiths M, Wade W. Comparison of 6 media for cultivation of subgingival bacteria. *J Dent Res* 1987; 66: Abstract 334.

15. Syed SA, Loesche WJ. Survival of human dental plaque flora in various transport media. *Appl Microbiol* 1972; 24: 638–44.

16. Holdeman LV, Moore WEC, Cato EP, Burmeister JA, Palcanis KG, Ranney RR. Distribution of *Capnocytophaga* in periodontal microfloras. *J Periodont Res* 1985; 20: 475–83.

17. Forlenza SW, Newman MG, Horikoshi AL, Blachman U. Antimicrobial susceptibility of *Capnocytophaga. Antimicrob Agents Chemother* 1981; 19: 144–6.

18. Sutter VL, Pyeatt D, Kwok KK. In vitro susceptibility of *Capnocytophaga* strains to 18 antimicrobial agents. *Antimicrob Agents Chemother* 1981; 20: 270–1.

19. Hawkey PM, Smith S, Haynes J, Malnick H, Forlenza S. In vitro susceptibility of *Capnocytophaga* species to antimicrobial agents. *Antimicrob Agents Chemother* 1987; 31: 331–2.

20. Rummens JL, Gordts B, van Landuyt HW. In vitro susceptibility of *Capnocytophaga* species to 20 antimicrobial agents. *Antimicrob Agents Chemother* 1986; 30: 739–42.

Journal of Clinical
Periodontology

Short Communication

Capnocytophaga granulosa and Capnocytophaga haemolytica: novel species in subgingival plaque

M. Ciantar[1], D. A. Spratt[2],
H. N. Newman[3] and M. Wilson[2]
Departments of [1]Microbiology &
Periodontology, [2]Microbiology, Eastman
Dental Institute for Oral Health Care
Sciences, University College London, 256,
Gray's Inn Road, London, WC1X 8LD, UK;
[3]Department of Periodontology & Preventive
Dentistry, University College London, London,
UK (Emeritus Professor)

Abstract
Background: The oral cavity accommodates one of the most diverse microfloras in the human body. Knowledge of this microflora, and of the periodontal microflora in particular, proves crucial towards an understanding of the bacterial-host interactions which lead to the development of infectious inflammatory periodontal diseases. *Capnocytophaga* species have been implicated as putative periodontal pathogens. To date, only 3 members of this genus (*C. gingivalis*, *C. ochracea* and *C. sputigena*) have been isolated from subgingival plaque.
Aim: This communication reports the isolation of 2 recently-speciated strains, namely *C. granulosa* and *C. haemolytica*, from subgingival plaque collected from adult periodontitis patients.
Material and Methods: Subgingival plaque was collected from 29 patients with chronic adult periodontitis. Plaque samples were inoculated onto fastidious anaerobe agar and incubated anaerobically for 5 days. Routine identification of clinical isolates was performed by 16S rRNA PCR-RFLP analysis, using *Cfo* I as restriction enzyme and corroborated by 16S rRNA gene sequencing.
Results: 16 of 29 patients (55%) tested positive for either *C. granulosa* and or *C. haemolytica*. A total of 70 isolates (63 *C. granulosa* and 7 *C. haemolytica*) were cultivated from subgingival plaque. 15 (51%) patients tested positive for *C. granulosa*, and 3 (10%) patients tested positive for *C. haemolytica*.
Conclusion: This is the 1st report which recounts the presence of *C. granulosa* and *C. haemolytica* in subgingival plaque. Further research is required to establish the relative proportions of these species subgingivally in health and disease.

The periodontal microflora is complex and poorly understood. Some 350 periodontal species have been cultivated and identified; however, it is estimated that this figure equates to only 50% of the total bacteria encountered in the subgingival region (Socransky 1963, Socransky & Haffajee 1994). The remaining 50% of the as-yet-uncultivable microflora have not been identified, as knowledge of their physiology is lacking. Thus, the requisite laboratory conditions necessary for their isolation from dental plaque are not yet known.

Capnocytophaga species form part of the normal human oral flora both in health and disease. The genus was established by Leadbetter et al. (1979) and then consisted of the 3 species *Capnocytophaga gingivalis*, *Capnocytophaga ochracea* and *Capnocytophaga sputigena*, isolated from dental plaque. The virulence factors manifested by these species, namely an epitheliotoxin (Birkedal-Hansen et al.1982), a fibroblast inhibitor factor (Stevens et al. 1980), and their ability to degrade immunoglobulins G and A (Killian et al. 1981) have implicated them as putative periodontal pathogens. These species are also capable of causing extensive alveolar bone destruction (Irving et al. 1978). All 3 species have been isolated from diseased periodontal sites in cases of juvenile periodontitis (Savitt & Socransky 1984), advancing adult peri-

odontitis (Van Palenstein Helderman 1981), periodontal abscesses (Newman & Sims 1979) and in periodontitis exacerbated by diabetes mellitus (Mashimo et al. 1983). More recently, 2 new species *C. granulosa* and *C. haemolytica*, were added to this genus (Yamamoto et al. 1994). These latter species were isolated from supragingival plaque. This paper documents their isolation from subgingival plaque.

Materials and Methods

Subgingival plaque was obtained from 29 adult patients with chronic periodontitis (age range 36–61 years, mean=45.93 years±6.54) attending the Periodontology Department, Eastman Dental Hospital and Institute, London. Patients were recruited after a diagnosis of chronic adult periodontitis. True periodontal pocketing (≥ 5 mm) with alveolar bone loss was confirmed by standardised long-cone periapical radiographs. The medical history was non-contributory, and the absence of periodontal treatment and antibiotic therapy during the previous three months was ascertained. After obtaining voluntary informed patient consent, the deepest periodontal sites were selected for sampling subgingival plaque. These sites were isolated with cotton wool rolls and clinically-evident supragingival plaque removed with a periodontal probe. Subgingival plaque was obtained by inserting a sterile Gracey curette (Hu Friedy, USA), to the full depth of the periodontal pocket and subsequently moving it vertically along the side of the root to the gingival margin. The plaque sample was placed in a vial containing trypticase soy broth and immediately transferred to the laboratory for processing. Samples were serially diluted, inoculated onto fastidious anaerobic agar (FAA, Lab M, UK) and incubated anaerobically (MACS Anaerobic Workstation, Don Whitely Scientific, Yorkshire, UK) for 5 days. *Capnocytophaga* species, selected on the basis of their distinctive colonial morphology and negative Gram staining, were subcultured to purity on FAA incubated in 5% (v/v) CO_2 at 37°C for 3 days.

In addition, *Capnocytophaga* Type strains, namely *C. gingivalis* ATCC 33624, *C. ochracea* ATCC 27872, *C. sputigena* ATCC 33612, *C. granulosa* ATCC 51502 and *C. haemolytica* ATCC 51501, were grown on FAA under the same incubation conditions. Bacterial
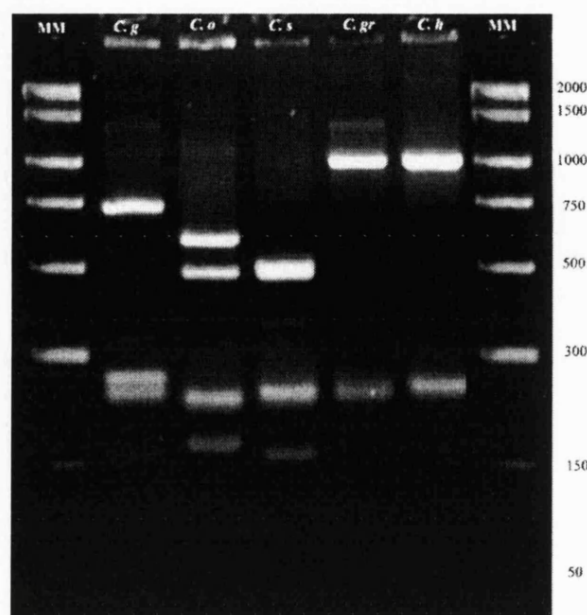

Fig. 1. 16S rRNA PCR RFLP analysis of ATCC *Capnocytophaga* Type strains using *Cfo* I as restriction enzyme. MM=molecular weight marker; *C.g.*=*C. gingivalis, C.o*=*C. ochracea, C.s*=*C. sputigena, C.gr*=*C. granulosa, C.h*=*C. haemolytica.*

DNA was extracted (after boiling the bacterial suspension for 2 min) and the 16S ribosomal RNA (rRNA) gene amplified using global primers (27f and 1492r, Genosys, Sigma, UK) via the polymerase chain reaction (PCR) (Lane 1991, Wilson et al. 1995). The ca. 1500 basepair PCR product was subsequently digested using the restriction enzyme *Cfo* I (Promega, WI, USA). The restriction digests were subjected to electrophoresis and separated on a 2% superfine resolution agarose gel (Amresco, Ohio, USA) stained with ethidium bromide and visualised using UV light.

Definitive identification of *Capnocytophaga* isolates was performed by using these molecular techniques. The restriction fragment length polymorphisms (RFLP) patterns were then compared to those obtained for the corresponding ATCC Type strains. Identification of representative isolates was confirmed by subjecting the PCR product to 16S rRNA gene sequencing (ABI Prism, BigDye™ Terminator Cycle Sequencing ready reaction kit, Applied Biosystems, Warrington, UK). Sequencing took place in an automated DNA sequencer (ABI Prism, 310 Genetic Analyzer, Applied Biosystems). Bacterial identification was determined by comparing the subsequent nucleotide sequence with the 16s rRNA gene

database at the Ribosomal Database Project at Michigan State University, MI, USA (Maidek et al. 2000).

Results

All 5 human species of *Capnocytophaga* were isolated from subgingival plaque. Clinical strains of *C. granulosa* and/or *C. haemolytica* were isolated from 16 of the 29 (55%) patients. 15 (51%) patients tested positive for *C. granulosa* and 3 (10%) patients tested positive for *C. haemolytica*.

The 16S rRNA PCR-RFLP digests with *Cfo* I for the 5 ATCC Type strains yielded 5 different patterns (Fig. 1). Gram-staining for the ATCC Type strains and clinical isolates confirmed their cellular morphology as gram-negative, slender rods with fusiform ends. Characteristic gliding motility, evidenced by spreading, was observed for some of the isolates (Leadbetter et al. 1979). Identification of the clinical isolates was performed by comparing the 16S rRNA PCR-RFLP patterns with those of the Type strains (Fig. 2). The clinical isolates which exhibited the most spreading on FAA also displayed a green/lilac fluorescence, a feature not reported previously. Microscopic examination of the gram stained preparations of these isolates revealed in-
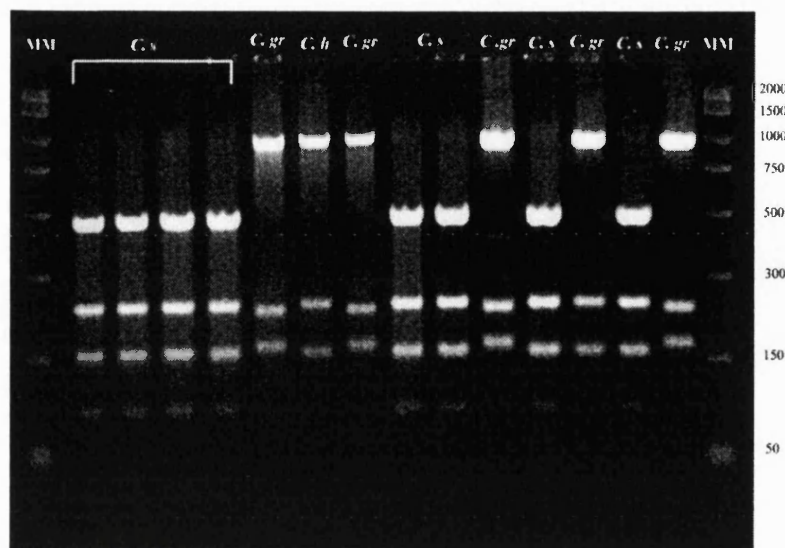
Fig. 2. 16S rRNA PCR RFLP analysis of *Capnocytophaga* clinical isolates using *Cfo* I as restriction enzyme. MM=molecular weight marker; *C.s*=*C. sputigena*, *C.gr*=*C. granulosa*, *C.h*=*C. haemolytica*.

clusions characteristic of *C. granulosa* (Yamamoto et al. 1994). These granules were more discernible after crystal violet staining (Fig. 3). Confirmation of their identification as *C. granulosa* was secured via 16S rRNA gene sequencing.

C. haemolytica colonies displayed a golden yellow hue distinct from the lilac colour of *C. gingivalis* or the grey colour of *C. sputigena*. On average, the diameter of the *C. haemolytica* colonies

was larger than that of *C. ochracea*. Some of the *C. haemolytica* isolates exhibited haemolysis on FAA; however, this feature was lost on subsequent subculture. Gram stain preparations showed long slender fusiform rods which were indistinguishable from *C. ochracea*, *C. sputigena* and *C. gingivalis*, and which lacked the granular inclusions typical of *C. granulosa*. Furthermore, *C. haemolytica* isolates

showed minimal spreading when compared to *C. granulosa*.

Discussion

5 *Capnocytophaga* species (*C. gingivalis, C. ochracea, C. sputigena, C. granulosa* and *C. haemolytica*) have been recovered from the human oral cavity (Leadbetter et al. 1979, Yamamoto et al. 1994). *C. gingivalis, C. ochracea* and *C. sputigena* have been isolated from both supragingival and subgingival dental plaque, the latter implicating them as putative periodontal pathogens (Van Palenstein Helderman 1981). *C. granulosa* and *C. haemolytica* have been isolated from supragingival plaque only (Yamamoto et al. 1994). This is apparently the 1st report to date of their isolation from subgingival plaque from adult periodontitis patients.

During plaque sampling, care was taken to avoid contamination of subgingival plaque with supragingival plaque. This was done by prior removal of the supragingival plaque to the level of the gingival margin with a periodontal probe or with a cotton wool roll when the adjacent tooth was missing. The samples were collected by scraping the side of the root from the base of the pocket to the gingival margin. Care was taken not to collect plaque present on the crown of the tooth. Curettes were used in preference to paper points, as the former are capable of removing a larger proportion of the subgingival biofilm (Kiel & Lang 1983, Moore et al. 1985).

FAA was used in preference to other media as the results of a comparative study by this group revealed that FAA was the only culture medium which supported the growth of all five *Capnocytophaga* species (Ciantar et al. 2001). Furthermore, since it can support the growth of most periodontopathogens (Griffiths & Wade 1987), it should be possible to determine the relative proportions of all 5 *Capnocytophaga* species in subgingival plaque (data not yet available).

Identification of the clinical isolates was performed using 16S rRNA PCR-RFLP analysis using *Cfo* I as the restriction enzyme (Wilson et al. 1995). This method of identification was chosen as the Type strains demonstrated 5 different RFLP patterns after digestion with the restriction enzyme *Cfo* I. The identification of random isolates was confirmed using 16S rRNA
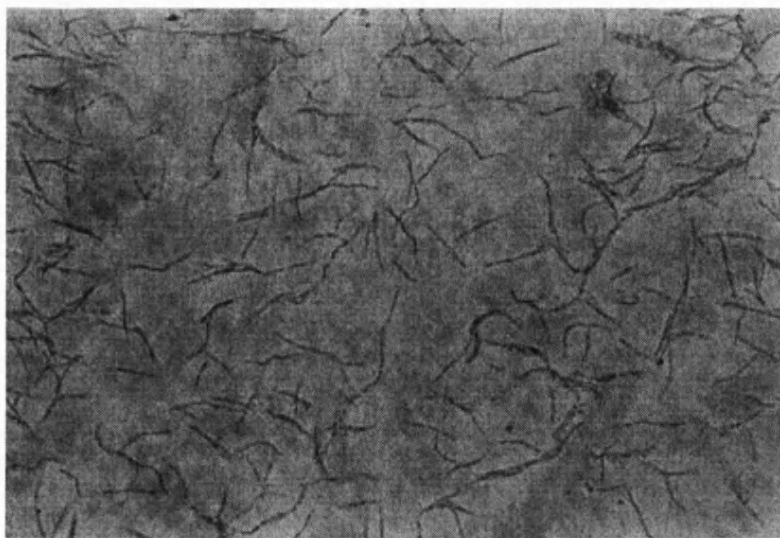

Fig. 3. Gram stain of *C. granulosa* clinical isolate showing the characteristic intracellular granular inclusions.

gene sequencing. Since RFLP analyses are quicker and more cost-effective than gene sequencing, and accurate enough to enable bacterial identification, it was the preferred method in this investigation. Differentiation between isolates (*C. ochracea* and *C. sputigena*) is not possible using biochemical methods alone (Laughon et al. 1982).

The clinical significance of *C. granulosa* and *C. haemolytica* in subgingival plaque has not as yet been ascertained. Current work by this group is targeted at the frequency distribution of these species in subgingival plaque. It is also relevant that *C. gingivalis*, *C. ochracea* and *C. sputigena* have been implicated as focal infection agents in systemic disease (Parenti and Snydman 1985, Warren et al. 1986). A recent paper reports the isolation of *C. granulosa* from an abscess in the lumbosacral area (Ebinger et al. 2000). The authors suggest that this isolate could have reached the sacral region only via anachoresis from the mouth. No systemic infection due to *C. haemolytica* has been reported.

Conclusion

This study would appear to be the first to report the isolation of *C. granulosa* and *C. haemolytica* from human subgingival plaque from sites with chronic periodontitis. Further work is indicated to evaluate their pathogenic nature orally and systemically.

Acknowledgement

Zusammenfassung

Capnocytophaga granulosa und Capnocytophaga haemolytica; neue Arten in subgingivaler Plaque.
Hintergrund: In der Mundhöhle findet sich eine der komplexesten Mikrofloren des gesamten Körpers. Kenntnisse dieser Mikroflora, insbesondere der parodontalen Mikroflora, sind eine entscheidende Voraussetzung für das Verständnis der Wechselbeziehungen zwischen Mikroorganismen und Wirt, die zur Entstehung infektiöser entzündlicher Parodontalerkrankungen führen. Die *Capnocytophaga*-Arten werden zu den vermutlich parodontalpathogenen Keimen gezählt. Bisher sind nur 3 Mitglieder dieses Genus (*C. gingivalis*, *C. ochracea*, *C. sputigena*) aus subgingivaler Plaque isoliert worden.
Zielsetzung: Bericht über die Isolation der 2 erst kürzlich identifizierten Stämme *C. granulosa* und *C. haemolytica* aus subgingivaler Plaque bei Patienten mit Erwachsenenparodontitis.
Material und Methoden: Bei 29 Patienten mit chronischer Erwachsenenparodontitis wurde subgingivale Plaque gewonnen. Die Plaqueproben wurden auf einem sehr anspruchsvollen anaeroben Agar inokkuliert und 5 Tage lang anaerob bebrütet. Die Identifizierung der klinischen Isolate erfolgte mittels 16S rRNS PCR-RFLP-Analyse mit dem Restriktionsenzym *Cfo* I und wurde durch 16S rRNS Gensequenzierung unterstützt.
Ergebnisse: Bei 16 von 29 Patienten (55%) konnten entweder für *C. granulosa* oder *C. haemolytica* gefunden werden. Insgesamt wurden 70 Proben (63 *C. granulosa*; 7 *C. haemolytica*) aus subgingivaler Plaque isoliert. 15 (51%) Patienten waren positiv für *C. granulosa* und 3 (10%) für *C. haemolytica*.
Schlussfolgerungen: Dies ist der erste Bericht über das Vorkommen von *C. granulosa* oder *C. haemolytica* in subgingivaler Plaque. Weitere Untersuchungen sind erforderlich, um den relativen Anteil dieser Arten in subgingivaler Plaque gesunder und erkrankter Stellen zu bestimmen.

Résumé

Le capnocytophaga granulosa et le capnocytophaga haemolytica: espèces nouvelles dans la plaque dentaire sous-gingivale
Origine: La cavité buccale abrite une des microflores les plus variées du corps humain. La connaissance des ces microflores, et de la microflore parodontale en particulier, s'avère indispensable pour comprendre les interactions hôte-bactéries qui guident le développement de la maladie parodontale inflammatoire infectieuse. Les espèces *capnocytophaga* ont été impliquées comme pathogénes parodontaux putatifs. Jusqua'à présent seul 3 membres de ce genre (*C. gingivalis*, *C. ochracea et C. sputigena*) ont été isolés de la plaque dentaire sous-gingivale.
But: Ce manuscript décrit l'isolation des deux espèces récentes à savoir le *C. granulosa* et le *C. haemolytica* de la plaque dentaire sous-gingivale de patients avec parodontite de l'adulte.
Méthodes: La plaque dentaire sous-gingivale a été prélevée chez 29 patients avec parodontite chronique adulte. Ces échantillons de plaque ont été inoculés dans de l'agar anaérobie et incubés anaérobiquement durant 5 jours. Une identification de routine des isolats cliniques a été effectuée par l'analyse 16S ARNr PCR-RFLS en utilisant CfO I comme enzyme de restriction et corroborée par la séquence de gènes 16S ARNr.
Résultats: 16 des 29 patients (55%) étaient positifs pour soit le *C. granulosa*, soit le *C. haemolytica*. Un total de 70 isolats (63 *C.* granulosa et 7 *C. haemolytica*) ont été cultivés à patir de la plaque dentaire sous-gingivale. 15 (51%) patients étaient positifs pour *C. granulosa* et 3 (10%) pour *C. haemolytica*.
Conclusion: Cesi est le premier rapport qui démontre la présence de *C. granulosa* et *C. haemolytica* dans la plaque dentaire sous-gingivale. Davantage de recherche s'avère nécressaire pour établir les proportions relatives de ces espèces sous-gingivales en présence de parodonte sain et malade.

References

Birkedal-Hansen, H., Caufield, P. W., Wanne-Muehler, Y. M. & Pierce, R. (1982) A sensitive screening assay for epitheliotoxins produced by oral microorganisms. *Journal of Dental Research* **61** (abs no. 125).

Ciantar, M., Spratt, D. A., Newman, H. N. & Wilson, M. (2001) Assessment of 5 culture media for the growth and isolation of *Capnocytophaga* species. *Clinical Microbiology and Infection* in-press.

Ebinger, M., Nichterlein, T., Schumacher, U. K., Mannake, B., Schmidt, D. & Bohn, I. (2000) Isolation of *Capnocytophaga granulosa* from an abscess in an immunocompetent patient. *Clinical Infectious Diseases* **30**, 606–607.

Griffiths, M. & Wade W., 1987 (Comparison of 6 media for culture of subgingival bacteria.) Journal of Dental Research **66** (abs no. 334).

Irving, J. T., Socransky, S. S. & Tanner, A. C. (1978) Histological changes in experimental periodontal disease in rats monoinfected with gram-negative organisms. *Journal of Periodontal Research* **13**, 326–332.

Kiel, R. A. & Lang, N. P. (1983) Effect of subgingival sampling techniques on periodontal microbiological culturing. *Journal of Dental Research* **62** (abs no. 247).

Killian, M. (1981) Degradation of immunoglobulins A1, A2 and G by suspected principal pathogens. *Infection & Immunity* **34**, 757–765.

Lane, D. J. (1991) 16S/23S rRNA sequencing. In: *Nucleic Acid techniques in bacterial systematics*, eds. E. Stackebrandt and M. Goodfellow. John Wiley Sons Ltd., ch. 6, 115–175.

Laughon, B. E., Syed, S. A. & Loesche, W. J. (1982) API ZYM system for identification of *Bacteroides* spp., *Capnocytophaga* spp., and spirochetes of oral origin. *Journal of Clinical Microbiology* **15**, 97–102.

Leadbetter, E. R., Holt, S. C. & Socransky, S. S. (1979) *Capnocytophaga*: new genus of gram-negative bacteria (I). General characteristics, taxonomic consideration and significance. *Archives of Microbiology* **122**, 9–16.

Maidek, B. L., Cole, C. R., Lilburn, T. G., Parker, C. T., Saxman, P. R., Stredwick, J. M., Garrity, G. M., Li, B., Olsen, G. J., Pramanik, S., Schmidt, T. M. & Tiedje, J. M. (2000) The RDP (Ribosomal Database Project) continues. *Nucleic Acids Research* **28**, 173–174.

rodontalpathogenen Keimen gezählt. Bisher sind nur 3 Mitglieder dieses Genus (*C. gingivalis*, *C. ochracea*, *C. sputigena*) aus subgingivaler Plaque isoliert worden.

Mashimo, P. A., Yamamoto, Y., Slots, J., Park, B. H. & Genco, R. J. (1983) The periodontal microflora of juvenile diabetics: culture, immunofluorescence and serum antibody studies. *Journal of Periodontology* **54**, 420–430.

Mashimo, P. A., Yamamoto, Y., Nakamura, M. & Slots, J. (1983) Selective recovery of oral *Capnocytophaga* species with sheep blood agar containing bacitracin and polymixin B. *Journal of Clinical Microbiology* **17**, 187–191.

Moore, W. E. C., Holdeman, L. V., Cato, E. P., Siebert, R. M., Burmeister, J. A., Palcanis, K. G. & Ranney, R. R. (1985) Comparative bacteriology of juvenile periodontitis. *Infection & Immunity* **48**, 507–519.

Newman, M. G. & Sims, T. N. (1979) The predominant cultivable microbiota of the periodontal abscess. *Journal of Periodontology* **50**, 350–354.

Parenti, D. M. & Snydman, D. R. (1985) *Capnocytophaga* species: infections in non-immunocompromised and immunocompromised hosts. *Journal of Infectious Diseases* **151**, 140–147.

Savitt, E. D. & Socransky, S. S. (1984) Distribution of certain subgingival microbial species in selected periodontal conditions. *Journal of Periodontal Research* **19**, 111–123.

Socransky, S. S. (1963) The microbiota of the gingival crevice area of man – I. Total microscopic and viable counts and counts of specific organisms. *Archives of Oral Biology* **8**, 275–280.

Socransky, S. S. & Haffajee, A. (1994) Evidence of bacterial aetiology: a historical perspective. *Periodontology 2000* **5**, 7–25.

Stevens, R. H., Sela, M. N., Shapira, J., Hammond, B. F. (1980) Detection of a fibroblast proliferation inhibitory factor from *Capnocytophaga* sputigena. *Infection & Immunity* **27**, 271–275.

Van Palenstein Helderman, W. H. (1981) Microbial aetiology of periodontal disease. *Journal of Clinical Periodontology* **8**, 261–280.

Warren, J. S., Stephen, D & Allen, M. D. (1986) Clinical pathogenic and laboratory features of *Capnocytophaga* infections. *American Journal of Clinical Pathology* **86**, 513–518.

Wilson, M. J., Wade, W. G. & Weightman, A. J. (1995) Restriction fragment length polymorphism analysis of PCR-amplified 16S ribosomal DNA of human *Capnocytophaga*. *Journal of Applied Bacteriology* **78**, 384–401.

Yamamoto, T., Kajiura, S., Hirai, Y., Watanabe, T. (1994) *Capnocytophaga haemolytica* sp. nov. and *Capnocytophaga granulosa* sp. nov., from human dental plaque. *International Journal of Systematic Bacteriology* **44**, 324–329.

Address:

D. A. Spratt
Department of Microbiology
Eastman Dental Institute for
Oral Health-Care Sciences
University College London
256 Gray's Inn Rd.
London WC1X 8LD
UK

Fax: +44(0)207 915 1127
e-mail: d.spratt@eastman.ucl.ac.uk

Development of an *in vitro* microassay for glucose quantification in submicrolitre volumes of biological fluid

M. Ciantar[1], D. A. Spratt[1], H. N. Newman[2], M. Wilson[1]

[1]Department of Microbiology, Eastman Dental Institute for Oral Health Care Sciences, University College London, and
[2]Periodontology & Preventive Dentistry, University of London, London, UK

Ciantar M, Spratt DA, Newman HN, Wilson M: Development of an in vitro *microassay for glucose quantification in submicrolitre volumes of biological fluid. J Periodont Res 2002; 37: 79–85. © Blackwell Munksgaard 2002.*

Glucose quantification in serum or plasma is traditionally based on a colourimetric enzymatic assay using commercially available assay kits. Sample volumes of blood or serum are usually in the range of a few microlitres to a few millilitres. However, for biological fluids such as gingival crevicular fluid (GCF), which can only be sampled in submicrolitre volumes, such assays have proven unsuitable. The aim of this study was to develop a reliable and reproducible assay for quantifying glucose in submicrolitre samples of GCF. The assay involved modification of a commercially available kit for glucose quantification. Test solutions of (i) serum and (ii) serum with added glucose at known concentrations (range 50–400 mg/dl) were prepared to simulate GCF and GCF enriched with glucose, respectively. Submicrolitre volumes (range 0.2 μl to 0.8 μl) of the test solutions were added to the reagent solution (200 μl) using a Hamilton syringe. The reaction was performed under standard conditions of time and temperature. The colour change was assayed spectrophotometrically at 492 nm. The results showed that this microassay is sufficiently sensitive to detect 50 mg/dl glucose in 0.2 μl of sample and indicate that the accuracy and sensitivity of this assay make it suitable for glucose quantification in submicrolitre volumes of GCF, particularly relevant to investigations of the relationship between diabetes mellitus and chronic inflammatory periodontal disease. *In vivo* evaluation of this novel microassay was performed using GCF samples taken from periodontally healthy and chronic periodontitis patients. Using non-parametric analysis, the results showed that the assay detected statistically significant differences in glucose concentrations between the two patient groups ($p < 0.05$). Higher glucose levels were detected at the periodontally diseased sites. For each patient, the GCF-glucose:blood-glucose ratio was calculated. The results show that this ratio was higher in the periodontitis group (1:2) when compared to the healthy group (1:9). In conclusion, the results of this investigation have shown that this microassay can quantify glucose in GCF and that GCF-glucose levels are higher at periodontitis sites.

The determination of blood glucose is one of the most important and fundamental biochemical tests undertaken for the detection and management of disorders associated with abnormal carbohydrate metabolism. The determination of glucose levels by the simultaneous use of two enzymes, glucose oxidase and peroxidase, was developed by Keston in 1956 (1). The reaction is based on a two-stage

colourimetric change. In the presence of glucose oxidase, glucose is converted to gluconolactone, which reacts with water to form gluconic acid and hydrogen peroxide. Subsequently, in the presence of peroxidase, hydrogen peroxide acts as an oxidising agent and reacts with a colourless oxygen acceptor, converting it into a coloured oxygen acceptor.

Trinder (2) originally used 4-amino phenazone as the chromogenic oxygen acceptor, but this has been replaced by 4-aminoantipyrine and p-hydroxybenzene sulphonic acid in a commercially available assay kit. The resulting colour is due to the formation of a quinoneimine dye which can be quantified spectrophotometrically and is directly proportional to the original quantity of glucose.

A limitation of this assay is that the minimum sample volume should be 5 μl. Thus, to date, glucose quantification in samples in the submicrolitre range, e.g. in gingival crevicular fluid (GCF), is impossible. GCF originates from serum due to microvascular (postcapillary venule) leakage in the gingival vessels. It exudes through the junctional epithelium and into the gingival crevice in health and into the periodontal pocket in disease (3, 4). The constituents of GCF are derived from a number of sources namely serum, connective tissue and epithelium, which it passes through on its way to the crevice. It also contains inflammatory cells and bacteria present within the crevice or pocket (5). Thus its biochemical composition reflects the physiological/pathological state prevailing within the crevice/pocket, respectively, at that point in time. Quantification of glucose in GCF would give further insight into the environmental conditions which prevail in the periodontal pockets of patients with, for example, diabetes mellitus (DM). Glucose acts as a carbon source for a wide variety of microorganisms. Furthermore, it acts as a potent reducing agent and may therefore reduce the redox potential within the periodontal pocket, thereby encouraging the growth of putative anaerobic periodontal pathogens. Increased glucose levels in GCF may support the growth of a more saccharolytic flora. The level of GCF-glucose may also affect the physiological functioning of periodontal ligament cells, thereby contributing to the development of chronic inflammatory periodontal diseases (6). Collectively, these environmental changes may be selected for a periodontitis-related microflora, which may partly explain the increased periodontal destruction observed in DM patients. The aim of this investigation was to develop a novel, standardised and sensitive microassay which could be used to quantify the glucose content in submicrolitre volumes of GCF. Furthermore, GCF glucose levels were compared to blood glucose levels.

Material and methods

Reagents

A commercially available assay kit indicated for serum estimation (Glucose Trinder, procedure number 315; Sigma Diagnostics, Poole, Dorset, UK) was used in this microassay. A series of glucose (Analar; Merck, Poole, Dorset, UK) solutions were prepared over a concentration range of 50–400 mg/dl. A glucose/urea nitrogen standard containing a glucose concentration of 100 mg/dl (Sigma Diagnostics) was used as a control. Human serum (male, type AB; Sigma Diagnostics) was used as a substitute for GCF.

Procedure

Trinder's reagent was reconstituted according to the manufacturer's instructions. The absorbance of the freshly reconstituted solution was checked prior to each assay. The solution was discarded if the absorbance, when measured spectrophotometrically (model Pharmacia, Ultrospec 2000, Pharmacia Biotech Ltd, Cambridge, UK) in a 1 cm light path at 492 nm vs. water as reference, exceeded 0.2. Trinder's reagent was pipetted into the wells of a 96-well round-bottomed microtitre plate. A 1 μl Hamilton syringe (700 series; Bonaduz, Switzerland) with attached Cheney adaptor was used to dispense the samples (i.e. either glucose solutions or human serum spiked with glucose) into the respective wells. The volume of glucose sample was equal to 0.5 μl. The volume of human serum spiked with glucose ranged between 0.2 μl and 0.8 μl. All reactions took place under standard conditions of temperature (20°C) and time (18 min). The colour change of the microassay was assessed spectrophotometrically at 492 nm using a microtitre plate reader (MRX Microplate Reader; Dynex Technologies UK Ltd, Billingshurst, West Sussex, UK).

The manufacturer's recommendations for this particular assay are to use: (i) a minimum sample volume of 5 μl, and (ii) a sample to reagent ratio of 5 μl to 1 ml. Since this assay was aimed at determining the volume of glucose in submicrolitre volumes of fluid (GCF), this ratio was proportionally reduced to 0.5 μl sample to 100 μl reagent. The effect of alteration in sample and reagent volumes on the biochemical characteristics, and hence linearity, of the assay, was tested using a range of glucose solutions with a sample:reagent ratio of 0.5 μl:100 μl. The concentration of the glucose solutions used ranged from 50 mg/dl to 400 mg/dl (Fig. 1).

The microassay was subsequently repeated using human serum spiked with glucose (concentration
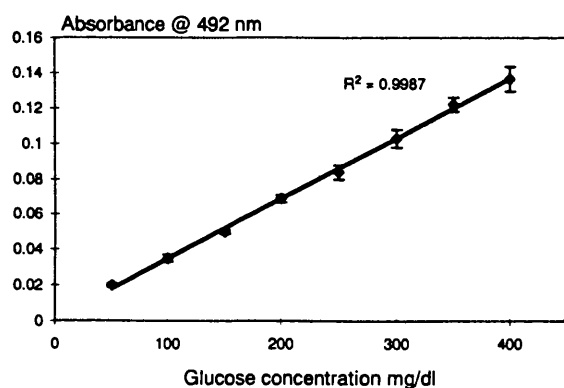
Fig. 1. Absorbance of reaction mixture (glucose solution and Trinder's reagent) measured at 492 nm vs. increasing glucose concentration. Glucose sample = 0.5 μl; Trinder's reagent = 100 μl; reaction time = 18 min. Each datum point represents mean values (n = 4); error bars represent standard deviations.
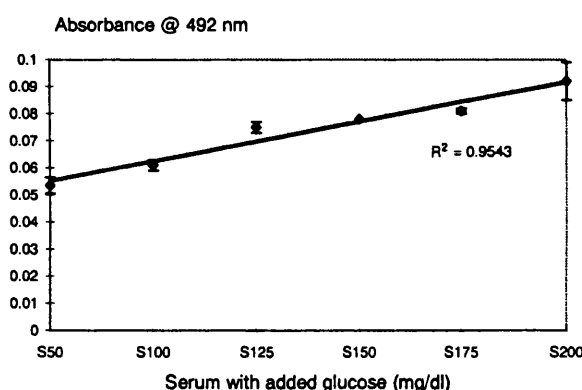

Fig. 2. Absorbance of reaction mixture (serum with added glucose and Trinder's reagent) measured at 492 nm vs. increasing glucose concentration. Sample volume = 0.5 μl; Trinder's reagent = 100 μl; reaction time = 18 min. Each datum point represents mean values (n = 4); error bars represent standard deviations. Intercept = inherent serum glucose.

range 50–200 mg/dl). The sample (serum/glucose mixture) to reagent ratio was maintained at 0.5 μl : 100 μl (Fig. 2).

The assay was also performed using human serum spiked with glucose as the sample, the volume of which ranged from 0.2 μl to 0.8 μl while the reagent volume was increased to 200 μl (Fig. 3). In order to simulate clinical sampling of GCF, the latter part of the study was performed by applying known volumes of the serum/glucose sample to Periopaper strips. These commercially available filter paper strips are specifically intended for GCF collection (Ora Flow Inc., Amityville, New York, USA).

GCF samples

Ethical approval for this project was obtained from the Joint Ethics and Research Committee, Eastman Dental Institute & Hospital, London. Nineteen systemically healthy subjects (mean age 35 yr ± 11.8; range 23–61 yr; 9 men and 10 women) were recruited from the Periodontology Department, Eastman Dental Hospital, London. The patients' medical histories were non-contributory and none had received any periodontal treatment or antibiotics in the previous three months. After obtaining the patients' voluntary written informed consent, clinical findings were recorded by a single examiner.

Periodontal pocket probing depth (PPD) was recorded to the nearest mm using an EN15 probe (Dentsply UK, Weybridge, Surrey, UK). The patients were divided into 2 groups: periodontally healthy subjects (n = 11), with PPD ≤ 3 mm, and periodontitis patients (n = 8) with PPD ≥ 5 mm. In the latter case, the presence of bone loss was assessed from bite-wing radiographs. Plaque Index (7) and Bleeding Index (8) were also recorded.

GCF collection and processing

Sites selected for GCF collection were isolated with cotton wool rolls. A saliva ejector was used to avoid saliva contamination of the samples. Any clinically evident supragingival plaque was removed with a periodontal probe, care being taken not to elicit gingival bleeding, and the area was briefly and gently dried with air. GCF was collected from three sites in periodontally healthy subjects and from six sites in periodontitis patients. In the latter case, the samples were collected as follows: 3 samples from healthy sites (PPD ≤ 3 mm) and 3 from diseased sites (PPD ≥ 5 mm). Periopaper strips used for GCF collection were inserted into the crevice or pocket until mild resistance was felt and left *in situ* for 2 min. Thereafter, the volume of fluid collected was determined using a chair-side located Periotron 8000 (Ora Flow Inc.).

The filter paper strips were then immediately placed in separate, dry, sterile, prelabelled Eppendorf tubes. The tubes were in turn placed in liquid nitrogen and transported to the laboratory for glucose analysis. Quantification of glucose in GCF was performed using the modified microassay. Any blood-stained GCF strips were excluded. Briefly, each strip was placed in a separate well of the microtitre plate with each well containing 100 μl Trinder's reagent. The reaction was allowed to proceed at room temperature for 18 min. A blank assay, a standard reagent and the patient's plasma sample were run in parallel with the GCF samples. The optical density (OD) of the samples was noted. The background OD was then subtracted from the actual OD value and the quantity of glucose per 2-minute sample was calculated from a previously established calibration graph.
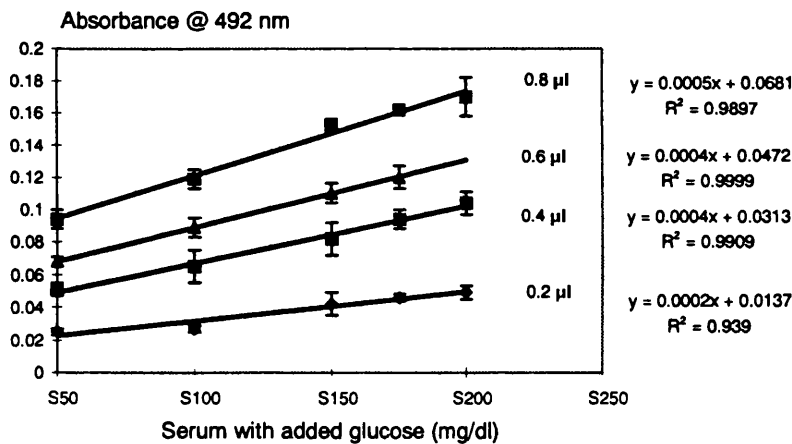
Absorbance @ 492 nm

$y = 0.0005x + 0.0681$
$R^2 = 0.9897$

$y = 0.0004x + 0.0472$
$R^2 = 0.9999$

$y = 0.0004x + 0.0313$
$R^2 = 0.9909$

$y = 0.0002x + 0.0137$
$R^2 = 0.939$

Serum with added glucose (mg/dl)

Fig. 3. Absorbance of reaction mixture (serum plus added glucose and Trinder's reagent) measured at 492 nm vs. increasing glucose concentration. The sample volume varied between 0.2 μl and 0.8 μl; volume of Trinder's reagent used for all assays was 200 μl; reaction time = 18 min. Each datum point represents mean values ($n = 4$); error bars represent standard deviations; intercept = inherent serum glucose.

Blood samples

A blood sample was withdrawn by venepuncture from the antecubital fossa of each patient using the Vacutainer system (Beckton Dickinson, Oxford, UK). The blood sample was centrifuged and the plasma separated. Using a Hamilton syringe, 0.5 μl of plasma was added to the 100 μl of Trinder's reagent in the respective wells in the microtitre plate. For each patient, the assays for the blood samples were run in parallel with the GCF samples.

Statistical analysis

Differences in absolute glucose amounts per 2-minute sampling period for the three different groups were analysed using the non-parametric Kolmorogov-Smirnov test. The Kolmorogov-Smirnov test is based on differences in terms of both the median and the spread of data distributions (9). Statistical significance was set at the 5% level.

Results

Microassay

The results (Fig. 1) show that the linearity of the assay was not affected when the sample (glucose solution) to reagent volume was lowered from 5 μl per ml to 0.5 μl per 100 μl, respectively. Furthermore, the linearity of the assay was also maintained when human serum spiked with glucose (over a concentration range of 50–200 mg/dl) was used as the sample inoculum (Fig. 2). This part of

the investigation was repeated twice. The linear equations which best described the results for the different sample volumes consistently showed that the intercept was equivalent to the inherent serum glucose (i.e. human serum without added glucose).

The results of the microassay when performed with human serum spiked with glucose (concentration range 50–200 mg/dl) over a volume range of 0.2 μl to 0.8 μl are shown in Figure 3. The reagent volume was equal to 200 μl. The results of these assays showed that the linearity of each assay was maintained even though the sample to reagent volume was altered. Furthermore, the intercept given by each of the linear equations (Fig. 3) was consistently equal to inherent serum glucose values for the specified assay as shown in Table 1.

Clinical samples

A total of 81 GCF samples were collected (Table 2). Thirty-three samples were collected from the periodontally healthy patients (mean probing depth 2.06 mm ± 0.7 and a mean GCF volume of 0.46 μl ± 0.34). Two of these samples were blood-stained and were thus excluded from the analysis. A total of 48 samples were collected from the periodontitis subjects as follows: 24 samples were collected from the healthy sites in periodontitis patients (mean probing depth 2.3 mm ± 0.47, mean GCF volume 0.5 μl ± 0.39); and 24 GCF samples were collected from diseased sites (mean probing depth 6 mm ± 0.93, mean GCF volume 1.09 μl ± 0.47). The absolute amount of glucose per strip (i.e. per 2-minute sampling) was calculated from a previously established calibration graph. Data for

Table 1. Comparison of absorbance at 492 nm of inherent serum glucose (as assayed spectrophotometrically) with the calculated value obtained from the linear equation in Fig. 3

Sample volume (μl)	Absorbance at 492 nm of inherent serum glucose (serum without added glucose)	Intercept as calculated from equation in Fig. 3
0.2	0.021	0.014
0.4	0.038	0.031
0.6	0.048	0.047
0.8	0.071	0.068

Table 2. Summary of clinical data

	Periodontally healthy ($n=11$)	Periodontitis ($n=8$)	
		Healthy pockets	Diseased pockets
Sites (n)	31	24	24
PPD (mm)*	2.1$\pm$0.7	2.3$\pm$0.47	6$\pm$0.93
GCF (μl)*	0.46$\pm$0.34	0.5$\pm$0.39	1.09$\pm$0.47
PI*	0.3$\pm$0.4	0.8$\pm$0.8	1.0$\pm$0.75
BII*	0.12$\pm$0.4	0.28$\pm$0.56	0.90$\pm$0.99
GCF-g†	0.017$\pm$0.05	0.07$\pm$0.19‡	0.19$\pm$0.29‡
Blood-g*	78.03$\pm$17.12	107.70$\pm$27.65	
GCF g:b	1:9	1:4	1:2

PPD = periodontal pocket probing depth; GCF = gingival crevicular fluid; PI = Plaque Index; BI = Bleeding Index; GCF-g = absolute GCF glucose (μg/2 min sample); Blood-g = Blood glucose (mg/dl); GCF g:b = GCF-glucose:blood-glucose.
*Mean ± SD; †Median ± interquartile range; ‡Statistically significantly different from periodontally healthy sites ($p<0.05$).

plotting this graph were those obtained when performing the microassay *in vivo*.

Discussion

Microassay

Serum glucose determination is routinely undertaken when disorders in carbohydrate metabolism, e.g. diabetes mellitus, are suspected. Biological fluids derived from serum, e.g. GCF, are thought to reflect the glucose concentration in serum (10). However, unlike a blood sample which would consist of a few microlitres or millilitres of blood, a sample of GCF from any one site can only be collected in submicrolitre quantities.

A few studies around the 1970s investigated the levels of glucose in GCF with disparate results (10–12). Direct comparison of these studies is impractical as the methods of GCF sampling and volume determination varied between the studies. Two studies (10, 12) used microlitre pipettes to collect GCF. No detail in these studies was given as

to the amount of GCF collected or the unit time of the sampling process. Calibrated microcapillary tubes have been used by other investigators to collect GCF (13). However, the drawback with this method is that long sampling times (10–15 min) are required in order to collect a quantifiable amount of fluid. This would in turn increase the possibility of sampling error. Furthermore, stroking the gingival margin as suggested by Ficara *et al.* (10) would stimulate GCF flow, leading to elevated volumes per unit time. Hara & Löe (11) used filter paper strips which were weighed pre- and post-sampling. In addition, the biochemical assays for glucose estimation have varied between studies, e.g. Ficara *et al.* (10) used the hexokinase rather than the glucose oxidase method.

The aim of this *in vitro* investigation was to develop a standardised and sensitive microassay for glucose determination in submicrolitre samples. A commercially available assay kit which, according to the manufacturer, required a minimum sample volume of 5 μl was modified to enable quantification of glucose in submicrolitre volumes of GCF. The results of this investigation show that this assay, as modified, was sensitive enough to quantify glucose in submicrolitre volumes of samples. The linear characteristics of the assay were not altered, even when the sample to reagent ratio was altered. Although variation in the sample to reagent ratio deviated from the manufacturer's instructions, this was intentionally undertaken since the volume of GCF will vary *in vivo*, thus resulting in a variation in the sample to reagent ratio.

It would have been ideal to use GCF for the *in vitro* evaluation of the microassay, however this biological fluid cannot be collected in sufficiently large quantities to undertake such *in vitro* studies. The alternative was to use human serum, since GCF originates from serum. Glucose was added to serum in an attempt to simulate GCF collected from patients diagnosed with diabetes mellitus. Preliminary studies (data not shown), which involved performing the assay with and without the glucose solution applied to the filter paper strips, showed that the glucose did not irreversibly bind to the filter paper strips and was released unreservedly into the assay solution.

GCF volume quantification in the studies referred to above (10–12) was performed rather inaccurately, i.e. by weighing strips or by volumetric quantification in capillary tubes. A recent and more accurate method for GCF quantification employs the Periotron 8000. This device is designed to quantify submicrolitre volumes of fluid applied to a filter paper strip (14).

The results obtained from this *in vitro* investigation show that this microassay is sensitive enough to

quantify glucose over a concentration range of 50–200 mg/dl in submicrolitre volumes of biological fluids.

Quantification of GCF-glucose

Quantification of GCF components can be expressed in different ways (15). The most commonly used methods are either the total (absolute) amount per standardised collection time or concentration (amount/unit volume). In this study, the absolute amount of glucose per unit sampling time was used to calculate the respective quantity of glucose per strip. This method was chosen in preference to other methods as it has the advantage of excluding GCF volumes during subsequent calculations. Minimal variation during GCF collection or its subsequent quantification via the Periotron (considered accurate in the assessment of GCF volume) would lead to disproportionately large errors in quantification of GCF components (16).

The recommended time for GCF collection varies between 5 and 30 s. In this study, a 2-minute GCF collection time was used to avoid the possibility of insufficient GCF volumes, in particular from the healthy patients.

Statistical analysis showed that a parametric test was inappropriate because the data were not normally distributed. Furthermore, the Mann–Whitney test could not be employed because the spread of the data varied considerably between the three groups.

This preliminary study has shown that the absolute amount of GCF-glucose can be quantified using this microassay. This absolute amount differed between periodontally healthy and periodontitis subjects, and also between healthy and diseased sites in the periodontitis group. The GCF-glucose per unit time was statistically significantly greater ($p < 0.05$) in the chronic periodontitis group, thus suggesting that more GCF-glucose is available at the diseased sites. Sufficient care was implemented to avoid contamination with saliva during the sampling procedure. In addition, the possibility of contamination of GCF with glucose from saliva was ruled out as the patients had not had anything by mouth for at least one hour prior to GCF collection.

This preliminary study has shown that there is a difference between GCF-glucose at different sites, with higher glucose levels at diseased sites. Furthermore, the GCF-glucose: blood-glucose ratio increased at the more periodontally diseased sites. The higher glucose levels at such sites may act as a selective pressure on the subgingival microflora. It may not only lead to a quantitative change by

encouraging the growth of subgingival species, but also to a qualitative change by encouraging the growth of a more saccharolytic subgingival microflora. The environmental pressure exerted by glucose may potentially induce alterations in bacterial genetic expression, leading to up- or down-regulation of gene expression which may subsequently produce enzymes relevant to periodontal destruction. Host cells may also be affected by the altered glucose levels. This could undermine the host defence mechanisms.

Further work incorporating larger patient groups and diabetes mellitus patients is required before major conclusions can be drawn.

Acknowledgements

References

1. Keston AS. Specific colorimetric enzymatic analytical reagents for glucose. Abstracts of papers, 129th Meeting of the American Chemical Society, Dallas, TX, USA. April 1956:31.
2. Trinder P. Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. *Ann Clin Biochem* 1969;**6**:24–27.
3. Egelberg J. The topography and permeability of vessels at the dentogingival junction in dogs. *J Periodont Res* 1967; **20**(suppl 1):1–39.
4. Cimasoni G. Crevicular fluid updated. *Monographs in Oral Science.* Basel: Karger, 1983.
5. Lamster IB. Evaluation of components of gingival crevicular fluid as diagnostic tests. *Ann Periodontol* 1997;**2**:123–137.
6. Sung-Jim K, Jong-Hyuk C, Joon-Bong P, Young-Hyuk K. High glucose stimulates production of PGE_2 in human periodontal ligament cells and human gingival fibroblasts. *J Dent Res, IADR Abstracts* 1998;**77**(Issue B):Abstract No: 1873.
7. Löe H. The gingival index, plaque index and retention index systems. *J Periodontol* 1967;**38**:610–616.
8. Saxton CS, van der Oudeeraa F. The effect of a dentifrice containing zinc citrate and Triclosan on developing gingivitis. *J Periodont Res* 1989;**24**:75–80.
9. Gibbons JD. *Nonparametric methods for quantitative analysis*, 3rd edn. Ohio: American Sciences Press, 1997.
10. Ficara AJ, Levin MP, Grower MF, Kramer GD. A comparison of the glucose and protein content of gingival fluid from diabetics and nondiabetics. *J Periodont Res* 1975;**10**:171–175.
11. Hara K, Löe H. Carbohydrate components of the gingival exudate. *J Periodont Res* 1969;**4**:202–207.
12. Kjellman O. The presence of glucose in gingival exudate and resting saliva of subjects with insulin-treated diabetes mellitus. *Swed Dent J* 1970;**63**:11–19.

13. Kaslick RS, Chasens AJ, Weinstein D, Wakdman R, Pluhar T, Lazarra R. Quantitative analysis of sodium, potassium and calcium in gingival fluid from gingiva with varying degrees of inflammation. *J Periodontol* 1970;**41**:93–97.

14. Ciantar M, Caruana DJ. Periotron 8000: calibration characteristics and reliability. *J Periodont Res* 1998;**33**:259–264.

15. Lamster IB, Oshrain RL, Fiorello LA, Celenti RS, Gordon JM. A comparison of 4 methods of data presentation for lysosomal enzyme activity in gingival crevicular fluid. *J Clin Periodontol* 1988;**15**:347–352.

16. Lamster IB, Hartley LJ, Vogel RI. Development of a biochemical profile for gingival crevicular fluid. *J Periodontol* 1985;**56**:13–21.