CAPNOCYTOPHAGA SPP. AND
DIABETES MELLITUS-PERIODONTITIS

Thesis submitted by
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for the degree of
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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 HbA1c = 9.3%) & 25 non-DM-Periodontitis (median HbA1c = 5.3%) subjects. Subgingival plaque samples were collected from 3 healthy (PPD ≤ 3 mm) and 3 diseased sites (PPD ≥ 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.
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.
Dedication

To my Mama & Daddy, Sister, Brothers
& their respective families
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<tr>
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<td>16S ribosomal Ribonucleic Acid gene</td>
</tr>
<tr>
<td>AAP</td>
<td>American Academy of Periodontology</td>
</tr>
<tr>
<td>ADA</td>
<td>American Diabetes Association</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced Glycation End products</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>BI</td>
<td>Bleeding Index</td>
</tr>
<tr>
<td>bp</td>
<td>basepair</td>
</tr>
<tr>
<td>C. cn</td>
<td>C. canimorsus</td>
</tr>
<tr>
<td>C. cy</td>
<td>C. cynodegmi</td>
</tr>
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<td>C. gingivalis</td>
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<td>C. granulosa</td>
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<td>C. haemolytica</td>
</tr>
<tr>
<td>C. o</td>
<td>C. ochracea</td>
</tr>
<tr>
<td>C. s</td>
<td>C. sputigena</td>
</tr>
<tr>
<td>Cap Medium</td>
<td><em>Capnocytophaga</em> culture medium (Rummens <em>et al.</em> 1985)</td>
</tr>
<tr>
<td>Cl</td>
<td>Confidence Intervals</td>
</tr>
<tr>
<td>CIPD</td>
<td>Chronic Inflammatory Periodontal Diseases</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>DM</td>
<td>Diabetes Mellitus</td>
</tr>
<tr>
<td>DM-Periodontitis</td>
<td>Periodontitis in patients with Diabetes Mellitus</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>FAA</td>
<td>Fastidious Anaerobe Agar</td>
</tr>
<tr>
<td>GCF</td>
<td>Gingival crevicular fluid</td>
</tr>
<tr>
<td>GT</td>
<td>Glucose transport proteins</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HbA₁c</td>
<td>Glycated haemoglobin</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulins</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>INF-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>kb</td>
<td>kilobasepair</td>
</tr>
<tr>
<td>LB Medium</td>
<td>Leadbetter culture medium (Leadbetter <em>et al.</em> 1979)</td>
</tr>
<tr>
<td>LCAL</td>
<td>Lifetime cumulative attachment loss</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<th>Description</th>
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<tr>
<td>Mac</td>
<td>Macrophage</td>
</tr>
<tr>
<td>Mash</td>
<td>Mashimo medium (Mashimo et al. 1983b)</td>
</tr>
<tr>
<td>mg dL⁻¹</td>
<td>Milligrams per decilitre</td>
</tr>
<tr>
<td>MIC₉₀</td>
<td>Minimum inhibitory concentration for 90% of strains (µg ml⁻¹)</td>
</tr>
<tr>
<td>MLM</td>
<td>Multilevel modelling</td>
</tr>
<tr>
<td>mmol⁻¹</td>
<td>Millimoles per litre (mM)</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix Metalloproteinases</td>
</tr>
<tr>
<td>Mo</td>
<td>Monocyte</td>
</tr>
<tr>
<td>MV MLM</td>
<td>Multivariate Multilevel modelling</td>
</tr>
<tr>
<td>NCTC</td>
<td>National Culture Type Collection</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor-κB</td>
</tr>
<tr>
<td>Non-DM-Periodontitis</td>
<td>Periodontitis in non-Diabetes Mellitus patients</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>OGTT</td>
<td>Oral Glucose Tolerance test</td>
</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PDL</td>
<td>Periodontal Ligament</td>
</tr>
<tr>
<td>PI</td>
<td>Plaque Index</td>
</tr>
<tr>
<td>PMNL(s)</td>
<td>Polymorphonuclear Leucocyte(s)</td>
</tr>
<tr>
<td>PPD</td>
<td>Pocket probing depth</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for Advanced Glycation End products</td>
</tr>
<tr>
<td>RE</td>
<td>Restriction enzyme</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
</tr>
<tr>
<td>RI</td>
<td>Random Intercepts</td>
</tr>
<tr>
<td>RIGLS</td>
<td>Restricted Iterative Generalised Least Squares</td>
</tr>
<tr>
<td>RTF</td>
<td>Reduced Transport Fluid</td>
</tr>
<tr>
<td>rcf</td>
<td>Rotational centrifugal force</td>
</tr>
<tr>
<td>Slots Medium</td>
<td>Slots medium for A. a (Slots et al. 1982)</td>
</tr>
<tr>
<td>TAC</td>
<td>Total Anaerobic Count (obligate &amp; facultative, cfu ml⁻¹)</td>
</tr>
<tr>
<td>TCapno</td>
<td>Total <em>Capnocytophaga</em> counts (cfu ml⁻¹)</td>
</tr>
<tr>
<td>TIMPs</td>
<td>Tissue Inhibitors of Matrix Metalloproteinases</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor-α</td>
</tr>
<tr>
<td>VC</td>
<td>Variance Components</td>
</tr>
<tr>
<td>µg</td>
<td>Micrograms</td>
</tr>
</tbody>
</table>
List of grants awarded in support of this project


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**.


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 Ciantar** (£637).

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

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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.

List of publications arising from this thesis

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

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

   

4. GCF-glucose: potential differences between DM- and non-DM-Periodontitis subjects?
   
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 ≥ 11.1 mmol\(^{-1}\). 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 ≥ 7 mmol\(^{-1}\). Fasting is defined as no caloric intake for at least 8 hours.

3. 2-hr post-prandial glucose ≥ 11.1 mmol\(^{-1}\) 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 mmol\(^{-1}\) = normal fasting glucose.
2. FPG ≥ 6.1 mmol\(^{-1}\) and ≤ 7 mmol\(^{-1}\) = impaired fasting glucose.
3. FPG ≥ 7 mmol\(^{-1}\) = 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 mmol\(^{-1}\) = normal glucose tolerance.
2. 2 hr PG ≥ 7.8 mmol\(^{-1}\) and < 11.1 mmol\(^{-1}\) = impaired glucose tolerance.
3. 2 hr PG > 11.1 mmol\(^{-1}\) = 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)

<table>
<thead>
<tr>
<th>HbA1c (%)</th>
<th>diabetic control</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 to 6%</td>
<td>Normal</td>
</tr>
<tr>
<td>&lt; 7%</td>
<td>Good diabetes control</td>
</tr>
<tr>
<td>7 to 8%</td>
<td>Moderate Diabetes control</td>
</tr>
<tr>
<td>&gt; 8%</td>
<td>Poor control</td>
</tr>
</tbody>
</table>

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 (Koltermann 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.

![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

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


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).
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.
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

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

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.
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).
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, Tierney 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

<table>
<thead>
<tr>
<th>Hyperglycaemia</th>
<th>Hypoglycaemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slow onset</td>
<td>Sudden onset</td>
</tr>
<tr>
<td>Disorientation</td>
<td>Mental confusion</td>
</tr>
<tr>
<td>Rapid and deep breathing</td>
<td>Sudden mood change</td>
</tr>
<tr>
<td>Hot, dry skin</td>
<td>Tachycardia</td>
</tr>
<tr>
<td>Loss of consciousness</td>
<td>Nausea</td>
</tr>
<tr>
<td></td>
<td>Hunger</td>
</tr>
<tr>
<td></td>
<td>Increased gastric motility</td>
</tr>
<tr>
<td></td>
<td>Cold clammy skin</td>
</tr>
<tr>
<td></td>
<td>Hypothermia</td>
</tr>
<tr>
<td></td>
<td>Loss of consciousness</td>
</tr>
</tbody>
</table>

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, Loe 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 (Loe 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).
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 (Løe 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
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

<table>
<thead>
<tr>
<th>I Adult Periodontitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>II Early-Onset Periodontitis</td>
</tr>
<tr>
<td>A. Prepubertal Periodontitis</td>
</tr>
<tr>
<td>2. Localised</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>III Periodontitis Associated with Systemic Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Primary neutrophil disorder</td>
</tr>
<tr>
<td>Agranulocytosis</td>
</tr>
<tr>
<td>Cyclic neutropenia</td>
</tr>
<tr>
<td>Chediak Higashi Syndrome</td>
</tr>
<tr>
<td>Neutrophil Adhesion abnormalities</td>
</tr>
<tr>
<td>Job's Syndrome</td>
</tr>
<tr>
<td>Lazy leukocyte Syndrome</td>
</tr>
<tr>
<td>Neutrophil functional abnormalities</td>
</tr>
<tr>
<td></td>
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</tbody>
</table>

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

<table>
<thead>
<tr>
<th>IV Necrotising Ulcerative Periodontitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>V Refractory Periodontitis</td>
</tr>
<tr>
<td>VI Miscellaneous conditions</td>
</tr>
</tbody>
</table>
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 Løe 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 & Løe 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

<table>
<thead>
<tr>
<th>Human studies</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical suppression of bacteria</td>
<td>Soh et al. 1982, Haskel et al. 1986</td>
</tr>
<tr>
<td>Immune response to periodontal bacteria</td>
<td>Ebersole et al. 1982, 1987</td>
</tr>
<tr>
<td>Correlation of certain bacteria with disease</td>
<td>Dzink et al. 1985, 1988</td>
</tr>
</tbody>
</table>
Table 1.8 Evidence for a bacterial aetiology in periodontitis (cont’d)

<table>
<thead>
<tr>
<th>Animal studies</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Gnotobiotic animals</td>
<td>Irving et al. 1975, Heijl et al. 1980</td>
</tr>
<tr>
<td>Ligature induced periodontitis</td>
<td>Sallay et al. 1982</td>
</tr>
<tr>
<td>Indigenous plaque</td>
<td>Isogai et al. 1985</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Microbial Pathogenicity</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial virulence factors</td>
<td>Slots &amp; Genco 1984, Fives-Taylor et al. 1999</td>
</tr>
</tbody>
</table>

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.

![Diagram of the dento-gingival interface](image)

**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)
Chapter 1 Introduction: Periodontitis

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).

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 (Dahlén 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; Loe 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

<table>
<thead>
<tr>
<th>Adult Periodontitis</th>
<th>Juvenile Periodontitis</th>
<th>Rapidly Progressive Periodontitis</th>
<th>Refractory Periodontitis</th>
<th>Prepubertal periodontitis</th>
<th>Necrotising Ulcerative Periodontitis</th>
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<tr>
<td><em>Por. gingivalis</em></td>
<td><em>A. a</em></td>
<td><em>A. a</em></td>
<td><em>A. a</em></td>
<td><em>A. a</em></td>
<td><em>Treponema</em> spp.</td>
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<td><em>A. a</em></td>
<td><em>Prev. intermedia</em></td>
<td><em>Por. gingivalis</em></td>
<td><em>Pre. intermedia</em></td>
<td><em>Prev. intermedia</em></td>
<td><em>Selenomonas</em> spp.</td>
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<tr>
<td><em>Prev. intermedia</em></td>
<td><em>E. corrodens</em></td>
<td><em>T. forsythensis</em></td>
<td><em>T. forsythensis</em></td>
<td><em>Prev. intermedia</em></td>
<td><em>Fusobacterium</em> spp.</td>
</tr>
<tr>
<td><em>E. corrodens</em></td>
<td><em>Capno</em> spp.</td>
<td><em>C. rectus</em></td>
<td><em>C. rectus</em></td>
<td><em>P. micros</em></td>
<td><em>Candida</em> spp.</td>
</tr>
<tr>
<td><em>F. nucleatum</em></td>
<td><em>C. rectus</em></td>
<td><em>Capno. spp</em></td>
<td><em>Capno. spp.</em></td>
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<td><em>C. rectus</em></td>
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<td><em>T. forsythensis</em></td>
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<td><em>P. micros</em></td>
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<td><em>Treponema</em> spp.</td>
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<td><em>Selenomonas</em> spp.</td>
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<td><em>Eubacterium</em> spp.</td>
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<tr>
<td><em>Capno. spp (?)</em></td>
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</tbody>
</table>

*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.

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).

![Cytokine Diagram](image)

**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 immunoinflammatory response which may account for most of the destruction seen in
Chapter 1 Introduction: Periodontitis

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 immune-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).

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, PGE₂ 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.
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 (H₂O₂, O₂⁻) as well as MMPs and PGE₂ and Leukotriene B₄. PGE₂ 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).

1.3.2 Epidemiology
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.
Chapter 1 Introduction: DM-Periodontitis

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
  
  **In cell membrane**
  
  - Perturbs cell membrane lipid composition
  
  **Altered cell biology**
  
  - AGE formation
  
  **Inside cell**
  
  - AGE-RAGE interaction
  
  - Altered cellular phenotype
  
  - Inflammatory phenotype
  
  - Oxidant stress
  
  **DNA damage? mutation**
  
  **Reactive oxygen intermediates**
  
  **Interferes with lipid and glucose metabolism**
  
  **Exacerbates periodontal tissue destruction**

**DM-Periodontal pathology**

- basement membrane thickening
- collagen breakdown
- endothelial proliferation
- haematological abnormalities

- tissue perfusion & $\downarrow$ $O_2$
- $\downarrow$ Mo & Mac migration
- $\uparrow$ risk of infection with Gram-negative anaerobes (LPS)

**Periodontitis**

- Host - bacteria interaction
- Immuno-inflammatory response
- PMNL, Mo, Mac accumulation
- Cytokine release

- $\downarrow$ TNF$\alpha$, IL-1, IL-6

**Mac** = Macrophages, **Mo** = Monocytes, **PMNL** = Polymorphonuclear Leucocytes, **LPS** = Lipopolysaccharide, **NF-κB** = Nuclear Factor-κB.

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 HbA1c levels after
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, PGE2, & 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 & Løe 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.

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 CO₂-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
Chapter 1 Introduction: *Capnocytophaga* spp.

"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).
Chapter 1 Introduction: *Capnocytophaga* spp.

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).
**Chapter 1 Introduction: Capnocytophaga spp.**

*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>
<thead>
<tr>
<th>Enzyme</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminopeptidases</td>
<td>Laughon <em>et al.</em> 1982, Nakamura &amp; Slots 1982,</td>
</tr>
<tr>
<td></td>
<td>Spratt <em>et al.</em> 1994,1995,</td>
</tr>
<tr>
<td>Arylaminopeptidase</td>
<td>Suido <em>et al.</em> 1986</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>Nakamura &amp; Slots 1982, Poirier &amp; Holt 1983,</td>
</tr>
<tr>
<td></td>
<td>Spratt <em>et al.</em> 1994</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>Nakamura &amp; Slots 1982, Poirier &amp; Holt 1983,</td>
</tr>
<tr>
<td></td>
<td>Spratt <em>et al.</em> 1994</td>
</tr>
<tr>
<td>Collagenase</td>
<td>Söderling <em>et al.</em> 1991</td>
</tr>
<tr>
<td>α-glucosidase</td>
<td>Slots 1981, Laughon <em>et al.</em> 1982</td>
</tr>
<tr>
<td>β-glucosidase</td>
<td>Slots 1981, Laughon <em>et al.</em> 1982</td>
</tr>
<tr>
<td>Phospholipase A$_2$</td>
<td>Sandholm <em>et al.</em> 1988</td>
</tr>
<tr>
<td>Dextranase</td>
<td>Igarashi <em>et al.</em> 1998</td>
</tr>
<tr>
<td>Elastase</td>
<td>Utito <em>et al.</em> 1988, Grenier &amp; Turgeon 1994,</td>
</tr>
<tr>
<td></td>
<td>Gazi <em>et al.</em> 1997</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>Gregory <em>et al.</em> 1978</td>
</tr>
<tr>
<td>Sialidase</td>
<td>Moncla <em>et al.</em> 1990</td>
</tr>
<tr>
<td>Immunoglobulin A1 protease</td>
<td>Kilian <em>et al.</em> 1981, Frandsen <em>et al.</em> 1991,</td>
</tr>
</tbody>
</table>

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 A2 (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 (Moncia et al. 1990). Bacterial sialidases have been considered as virulence factors in many pathogenic species that colonise mucosal surfaces (Moncia 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 (lino & 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, Frielings 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 \textit{Capnocytophaga} spp. as putative periodontal pathogens

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

Studies investigating the association of \textit{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 \textit{Capnocytophaga} spp. and chronic inflammatory periodontal conditions

<table>
<thead>
<tr>
<th>Periodontal Diagnosis</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pubertal gingivitis</td>
<td>Gusberti et al. 1983, Mombelli et al. 1990</td>
</tr>
<tr>
<td>Rapidly progressive periodontitis</td>
<td>Kamma et al. 1995</td>
</tr>
<tr>
<td>Juvenile periodontitis</td>
<td>Page et al. 1985, Kim et al. 1992</td>
</tr>
<tr>
<td>Refractory periodontitis</td>
<td>Listgarten et al. 1993</td>
</tr>
<tr>
<td>Peri-implantitis</td>
<td>Augthun et al. 1997, Fardal et al. 1999</td>
</tr>
<tr>
<td>Pericoronitis</td>
<td>Leung et al. 1993</td>
</tr>
</tbody>
</table>

In some of these conditions there prevails an underlying immune defect e.g. decreased PMNL chemotaxis, which may potentially lead to \textit{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

<table>
<thead>
<tr>
<th>Systemic condition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papillon-Lefèvre Syndrome</td>
<td>Tinanoff et al. 1986, Robertson et al. 2001</td>
</tr>
<tr>
<td>Down’s syndrome</td>
<td>Barr-Agholme et al. 1992, Amano et al. 2000</td>
</tr>
<tr>
<td>Hypophosphatasia</td>
<td>Baab et al. 1986,</td>
</tr>
<tr>
<td>Neutropenia</td>
<td>Kamma et al. 1998</td>
</tr>
<tr>
<td>HIV-seropositive</td>
<td>Okuda et al. 1994</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>Mashimo et al. 1983a</td>
</tr>
</tbody>
</table>

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>
<thead>
<tr>
<th>Infection</th>
<th>Oral disease</th>
<th>Name of species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteraemia</td>
<td>Yes</td>
<td>Species not identified$^5$</td>
<td>Montejo Baranda et al. 1984</td>
</tr>
<tr>
<td>Bacteraemia</td>
<td>Yes</td>
<td><em>C. ochracea, C. sputigena</em></td>
<td>Martino et al. 2001</td>
</tr>
<tr>
<td>Bacteraemia - NIC*</td>
<td>Yes</td>
<td>Species not identified</td>
<td>Parenti &amp; Snydman 1985</td>
</tr>
<tr>
<td>Bacteraemia - IC**</td>
<td>Yes</td>
<td>Species not identified</td>
<td>Parenti &amp; Snydman 1985</td>
</tr>
<tr>
<td>Peritonitis</td>
<td>NA†</td>
<td><em>C. sputigena</em></td>
<td>Testilliano Tarrero et al. 1988, Esteban et al. 1995</td>
</tr>
<tr>
<td>Peritonitis</td>
<td>NA</td>
<td><em>C. ochracea</em></td>
<td>Mortensen et al. 1985</td>
</tr>
<tr>
<td>Endophthalmitis</td>
<td>NA</td>
<td><em>C. ochracea</em></td>
<td>Rubsam en et al. 1993</td>
</tr>
<tr>
<td>Keratitis</td>
<td>NA</td>
<td>Species not identified</td>
<td>Alexandrakis et al. 2000</td>
</tr>
<tr>
<td>Liver abscess</td>
<td>NA</td>
<td>Species not identified</td>
<td>Mortensen et al. 1985</td>
</tr>
<tr>
<td>Septic arthritis</td>
<td>No‡</td>
<td><em>C. ochracea</em></td>
<td>Winn et al. 1984</td>
</tr>
<tr>
<td>Empyema</td>
<td>Yes</td>
<td>Species not identified</td>
<td>Parenti &amp; Snydman 1985</td>
</tr>
<tr>
<td>Lung abscess</td>
<td>No</td>
<td>Species not identified</td>
<td>Parenti &amp; Snydman 1985</td>
</tr>
<tr>
<td>Sinusitis</td>
<td>No</td>
<td>Species not identified</td>
<td>Parenti &amp; Snydman 1985</td>
</tr>
<tr>
<td>Conjunctivitis</td>
<td>No</td>
<td>Species not identified</td>
<td>Parenti &amp; Snydman 1985</td>
</tr>
<tr>
<td>Subphrenic abscess</td>
<td>No</td>
<td>Species not identified</td>
<td>Parenti &amp; Snydman 1985</td>
</tr>
<tr>
<td>Wound abscess</td>
<td>No</td>
<td>Species not identified</td>
<td>Parenti &amp; Snydman 1985</td>
</tr>
<tr>
<td>Osteomyelitis</td>
<td>No</td>
<td>Species not identified</td>
<td>Parenti &amp; Snydman 1985</td>
</tr>
<tr>
<td>Amniotic fluid infection</td>
<td>NA</td>
<td>Species not identified</td>
<td>McDonald &amp; Gordon 1988</td>
</tr>
<tr>
<td>Chorioamnionitis</td>
<td>NA</td>
<td>Species not identified</td>
<td>Mercer 1985</td>
</tr>
<tr>
<td>Endocarditis</td>
<td>NA</td>
<td>Species not identified</td>
<td>Montejo Baranda et al. 1984</td>
</tr>
<tr>
<td>Endocarditis</td>
<td>Dental treatment</td>
<td><em>C. ochracea</em></td>
<td>Buu-Hoi et al. 1988,</td>
</tr>
<tr>
<td>Endocarditis</td>
<td>Dog bite</td>
<td><em>C. canimorsus</em></td>
<td>Ngage et al. 1999</td>
</tr>
<tr>
<td>Lumbosacral abscess</td>
<td>Possibly oral source</td>
<td><em>C. granulosa</em></td>
<td>Ebinger et al. 2000</td>
</tr>
<tr>
<td>Portal-systemic shunt</td>
<td>Dental treatment</td>
<td>Species not identified</td>
<td>Mello et al. 1990</td>
</tr>
<tr>
<td>Septicaemia</td>
<td>Yes</td>
<td>Species not identified</td>
<td>Warren &amp; Allen 1986</td>
</tr>
</tbody>
</table>
Table 1.14 Systemic infections caused by *Capnocytophaga* spp. (cont’d)

<table>
<thead>
<tr>
<th>Infection</th>
<th>Oral disease</th>
<th>Name of species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Septicaemia</td>
<td>Cat scratch</td>
<td><em>C. canimorsus</em></td>
<td>Mahrer &amp; Raik 1992</td>
</tr>
<tr>
<td>Septicaemia</td>
<td>Periodontal abscess</td>
<td>Species not identified</td>
<td>Alberio &amp; Lammle 1998</td>
</tr>
<tr>
<td>Acute renal failure</td>
<td>NA</td>
<td><em>C. canimorsus</em></td>
<td>Bateman <em>et al.</em> 1992</td>
</tr>
</tbody>
</table>

*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)

<table>
<thead>
<tr>
<th>Reference</th>
<th>Subjects</th>
<th>DM Control (HbA1c)</th>
<th>Mean Age (yrs)</th>
<th>No of sites</th>
<th>AL (mean, mm)</th>
<th>PPD (mean, mm)</th>
<th>Data analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mashimo et al. 1983</td>
<td>9 IDDM</td>
<td>NA</td>
<td>17.4</td>
<td>3-8 D sites / patient (n = 38)</td>
<td>NA</td>
<td>7.05</td>
<td>% Microbial counts</td>
</tr>
<tr>
<td>Zambon et al. 1988</td>
<td>8 NIDDM</td>
<td>OGTT</td>
<td>NA</td>
<td>2 D sites / patient</td>
<td>NA</td>
<td>≥ 5</td>
<td>Coverage analysis of Good</td>
</tr>
<tr>
<td>Sastrowijoto et al. 1989</td>
<td>22 IDDM</td>
<td>Good (7.7%, n = 12)</td>
<td>37.2</td>
<td>1H &amp; 1D site / patient</td>
<td>NA</td>
<td>2.8</td>
<td>Mann-Whitney U test</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poor (9.9%, n = 10)</td>
<td>31.8</td>
<td>1H &amp; 1D site / patient</td>
<td>NA</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Sastrowijoto et al. 1990</td>
<td>6 IDDM</td>
<td>Poor (12.9%)</td>
<td>32.3</td>
<td>1H &amp; ID site / patient</td>
<td>NA</td>
<td>H = 1.8</td>
<td>Friedman 2-way ANOVA</td>
</tr>
<tr>
<td>Mandell et al. 1992</td>
<td>15 IDDM</td>
<td>Poor (10.4%)</td>
<td>34.6</td>
<td>1H &amp; 1D site / patient</td>
<td>H = 0.6</td>
<td>H = 2.8</td>
<td>Mean data, Wilcoxon signed rank test</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>D = 2.8</td>
<td>D = 5.8</td>
<td></td>
</tr>
<tr>
<td>Sbordone et al. 1995</td>
<td>16 IDDM, 16 nonDM sibs</td>
<td>Moderate (8.76%)</td>
<td>Non-DM: 13.2</td>
<td>1H &amp; 1D site / patient</td>
<td>H = 2.19*</td>
<td>H = 2.2*</td>
<td>Mean data, Wilcoxon signed rank test</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IDDM: 11.3</td>
<td></td>
<td>DM = 1.8*</td>
<td>DM = 2.29*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thorstensson et al. 1995</td>
<td>28 IDDM, 34 NonDM</td>
<td>NA</td>
<td>IDDM: 40-59</td>
<td>4 pooled samples /patient</td>
<td>NA</td>
<td>≥4 - ≥6</td>
<td>Student t-test; χ² test; Mann-Whitney U test</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Non-DM: 40-59</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sbordone et al. 1998</td>
<td>16 IDDM, 16 nonDM sibs</td>
<td>Moderate (value NA)</td>
<td>NA</td>
<td>1H &amp; 1D site / patient</td>
<td>H = 2.2*</td>
<td>H = 2.2*</td>
<td>Mean data for DM/nonDM groups; % of total anaerobe counts</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>DM = 2.3*</td>
<td>DM = 2.3*</td>
<td></td>
</tr>
</tbody>
</table>

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).

HbA1c: 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).
Chapter 1 Introduction: *Capnocytophaga* spp. & DM-Periodontitis

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 HbA1c (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 HbA1c 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>
<thead>
<tr>
<th>Reference</th>
<th>Plaque Samples</th>
<th>Culture Media</th>
<th>ID tests</th>
<th>Microflora</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mashimo et al. 1983</td>
<td>Subgingival plaque collected with paper</td>
<td>TSBA</td>
<td>Colony morphology &amp;</td>
<td><em>Capnocytophaga</em> spp. = 24% of microflora</td>
</tr>
<tr>
<td></td>
<td>points; Ringer's; vortexed x 20 sec</td>
<td></td>
<td>biochemical tests</td>
<td></td>
</tr>
<tr>
<td>Zambon et al. 1988</td>
<td>Subgingival plaque collected with paper</td>
<td>ETSA</td>
<td>Colony morphology, Gram reaction &amp;</td>
<td>43.8% sites positive for <em>Capnocytophaga</em> spp.</td>
</tr>
<tr>
<td></td>
<td>points; Ringer's; vortexed x 60 sec</td>
<td></td>
<td>biochemical tests</td>
<td></td>
</tr>
<tr>
<td>Sastrowijoto et al. 1989</td>
<td>Subgingival plaque collected with cotton</td>
<td>Mashimo &amp; Cap;</td>
<td>Colony morphology &amp;</td>
<td>Low levels of <em>Capnocytophaga</em> spp. in H &amp; D</td>
</tr>
<tr>
<td></td>
<td>wool on broach; BM broth; vortexed x 10 sec</td>
<td>BA for total</td>
<td>biochemical tests</td>
<td>sites</td>
</tr>
<tr>
<td>Sastrowijoto et al. 1990</td>
<td>Subgingival plaque collected with cotton</td>
<td>TSBV, Mashimo,</td>
<td>Colony morphology &amp;</td>
<td>Low levels of <em>Capnocytophaga</em> spp. in H &amp; D</td>
</tr>
<tr>
<td></td>
<td>wool on broach; BM broth; vortexed x 10 sec</td>
<td>Cap, BA for</td>
<td>biochemical tests</td>
<td>sites</td>
</tr>
<tr>
<td></td>
<td></td>
<td>total counts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mandell et al. 1992</td>
<td>Subgingival plaque collected with scaler;</td>
<td>Mashimo for</td>
<td>Based on growth on</td>
<td><em>Capnocytophaga</em> spp not significantly different</td>
</tr>
<tr>
<td></td>
<td>Ringer's solution; sonicated x 5 sec</td>
<td><em>Capno.</em></td>
<td>selective media.</td>
<td>between H &amp; D</td>
</tr>
<tr>
<td>Sbordone et al. 1995</td>
<td>Subgingival plaque collected with curette;</td>
<td>TSA for total</td>
<td>Colony morphology, Gram reaction &amp;</td>
<td><em>Capnocytophaga</em> spp. similar in both groups</td>
</tr>
<tr>
<td></td>
<td>Ringer's solution; vortexed x 10 sec;</td>
<td>counts; TSBA</td>
<td>biochemical tests</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sonicated x 20 sec</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thorstenssson et al. 1995</td>
<td>Subgingival plaque from all teeth</td>
<td>BBA</td>
<td>Colony morphology &amp;</td>
<td><em>Capnocytophaga</em> spp. not different between</td>
</tr>
<tr>
<td></td>
<td>pooled; few kept separate; paper points;</td>
<td></td>
<td>Gram reaction</td>
<td>groups</td>
</tr>
<tr>
<td></td>
<td>VGMA III and mailed to lab</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sbordone et al. 1998</td>
<td>Subgingival plaque collected with curette;</td>
<td>TSBA</td>
<td>Colony morphology, Gram reaction &amp;</td>
<td><em>Capnocytophaga</em> spp. not a risk factor for</td>
</tr>
<tr>
<td></td>
<td>Ringer's; vortexed x 10 sec; sonicated x 20</td>
<td></td>
<td>biochemical tests</td>
<td>periodontal breakdown</td>
</tr>
<tr>
<td></td>
<td>sec.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ETSA = Enriched Trypticase Soy Agar; TSBA = Trypticase Soy Agar + 5% blood; BA = Blood Agar; BBA = Brucella Blood Agar; BM = pre-reduced 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.* 1978a, 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, Hajajee & 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](image)

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 analyzed 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.
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 sites clustered within teeth within subjects. 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 \( \pm 2\text{SE} \)) 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
Chapter 1 Introduction: Statistical issues in Periodontal Research

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 n\textsuperscript{th} 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

\( \beta_0 = \) intercept

\( \beta_1 = \) estimate for slope of the explanatory variable \( x \) (differential, \( dy/\text{dx} \)).

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, \( \beta_0 \) is the overall mean of all site values across all sites and subjects; \( u_{0j} \) is the residual difference between the overall mean (\( \beta_0 \)) 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 (\( \beta_0 \)) 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 \( \beta_0 \). 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, \( x_i \)):

\[ y_{ij} = \beta_{0ij} + \beta_1 x_i \]

where \( \beta_{0ij} = \beta_0 + e_{0ij} + u_{0j} \), with again \( \beta_0 = \) the overall mean intercept; \( e_{0ij} = \) the random variation across sites (within subjects); \( u_{0j} = \) the random variation across subjects, and \( \beta_1 = \) the estimated change in outcome \( (y_{ij}) \) per unit change in the covariate \( (x_i) \).
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 multivariate) (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 subgingival plaque & blood from DM- & non-DM-Periodontitis subjects  
   (Chapter 5)

   Antimicrobial sensitivity testing of clinical isolates  
   (Chapter 6)

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

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

<table>
<thead>
<tr>
<th>Patient’s age</th>
<th>≥35, ≤65 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical evidence of CAP:</td>
<td></td>
</tr>
<tr>
<td>Probing pocket depth (true pockets, generalised)</td>
<td>≥ 5 mm</td>
</tr>
<tr>
<td>Radiographic evidence of bone loss*</td>
<td>yes</td>
</tr>
<tr>
<td>Minimum number of teeth (excluding third molars)</td>
<td>20</td>
</tr>
<tr>
<td>Diabetes Mellitus-Periodontitis</td>
<td>test group</td>
</tr>
<tr>
<td>Non-Diabetes Mellitus-Periodontitis</td>
<td>control group</td>
</tr>
</tbody>
</table>

* Standardised full-mouth long cone periapical radiographs
Table 2.2 Patient exclusion criteria

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

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 Ouderea 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) CO₂ 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, HbA₁₀, 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.
Chapter 3: Isolation media

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) 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% CO₂ 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*.

![Growth of *Capnocytophaga* ATCC strains on various media.](image)

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" (Rummmens 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

<table>
<thead>
<tr>
<th>Media</th>
<th>ATCC strains (n = 7) 7 species</th>
<th>Clinical isolates (n = 7) 3 species</th>
<th>Plaque isolates (n = 5) 5 species</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cg</td>
<td>Co</td>
<td>Cs</td>
</tr>
<tr>
<td>FAA</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Cap</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>LB</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Mashimo</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Slots</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

✓ Indicates growth (not absolute number)
Table 3.2 Comparison of media constituents

<table>
<thead>
<tr>
<th>FAA (gL⁻¹)</th>
<th>Mashimo (gL⁻¹)</th>
<th>Cap (gL⁻¹)</th>
<th>LB (gL⁻¹)</th>
<th>Slots (gL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone mix 23.0</td>
<td>Tryptone Soy Agar (Oxoid) 40.0</td>
<td>Columbia Blood Agar: Trypticase Soy Broth (Difco) 44.0</td>
<td>Trypticase Soy Agar (Difco) 30.0</td>
<td>Trypticase Soy Agar (Difco) 40.0</td>
</tr>
<tr>
<td>Sodium chloride 5.0</td>
<td>Tryptone 15.0</td>
<td>Bacto Pantone 10.0</td>
<td>Bacto 15.0</td>
<td>Bacto</td>
</tr>
<tr>
<td>Soluble starch 1.0</td>
<td>Soya peptone 5.0</td>
<td>Bacto Bitone 10.0</td>
<td>Bacto 5.0</td>
<td>Tryptone 17.0</td>
</tr>
<tr>
<td>Agar No.2 12.0</td>
<td>Na Cl 5.0</td>
<td>Tryptic digest of beef heart</td>
<td>Na Cl 5.0</td>
<td>Bacto 3.0</td>
</tr>
<tr>
<td>Sodium bicarbonate 0.4</td>
<td>Agar 15.0</td>
<td>Corn starch 1.0</td>
<td>agar 15.0</td>
<td>Bacto dextrose 2.5</td>
</tr>
<tr>
<td>Glucose 1.0</td>
<td></td>
<td></td>
<td></td>
<td>NaCl 5.0</td>
</tr>
<tr>
<td>Sodium pyruvate 1.0</td>
<td>Yeast extract 1.0</td>
<td>Bacto Agar 15</td>
<td>Bacto Agar 30.0</td>
<td>K₂PO₄ 2.5</td>
</tr>
<tr>
<td>Cysteine HCl, H₂O 0.5</td>
<td>Glucose 2.0</td>
<td>Haemoglobin (ml) 100.0</td>
<td>Haemoglobin (ml) 100.0</td>
<td>Yeast extract 1.0</td>
</tr>
<tr>
<td>haemin 0.01</td>
<td>bacitracin 0.05</td>
<td>Polymyxin (ml) 10.0</td>
<td>Polymyxin (ml) 10.0</td>
<td>Bacitracin 0.075</td>
</tr>
<tr>
<td>Vitamin K 0.001</td>
<td>polymyxin B 0.1</td>
<td>Polymyxin B 0.15</td>
<td>Polymyxin B 0.005</td>
<td>Vancomycin 0.005</td>
</tr>
<tr>
<td>L-arginine 1.0</td>
<td>Sheep Blood (ml) 50.0</td>
<td>Vancomycin 0.005</td>
<td>Vancomycin 0.005</td>
<td>Horse serum (ml) 100.0</td>
</tr>
<tr>
<td>Soluble pyrophosphate 0.25</td>
<td></td>
<td></td>
<td>Trimethoprim 0.0025</td>
<td></td>
</tr>
<tr>
<td>Sodium succinate 0.5</td>
<td></td>
<td></td>
<td>Amphotericin B 0.0025</td>
<td></td>
</tr>
<tr>
<td>Horse blood (ml) 50.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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:
Chapter 4: Identification of *Capnocytophaga* spp.

Chapter 4

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

Laughon *et al.* 1982), protein profiles (Khwaja *et al.* 1990, Vandamme *et al.* 1996), multilocus enzyme electrophoresis and serotyping of immunoglobulin 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 µ⁻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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>27f</td>
<td>5′ AGAGTTTGATCMTGGCTCAG</td>
</tr>
<tr>
<td>1492r</td>
<td>5′ TACGGYTACCTTTAGACTTT</td>
</tr>
<tr>
<td>357f</td>
<td>5′ CTCCTACGGGAGGCAGCAG</td>
</tr>
</tbody>
</table>

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 Multilmage Light Cabinet (Alphalnnotech Corp., Staffordshire, UK) and the resulting image captured by a computer software programme (AlphaEase™, Alphalnnotech).

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
agrose gel (Anachem) containing ethidium bromide (0.5 µg mL⁻¹). The same molecular weight marker (10 µl) 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 MgCl₂) 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 electrophoretograms 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 (GGG*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.
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](image)

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).

![Figure 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](image)

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*. 

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

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

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 (*Cg* 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](image)

*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
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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.
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).
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### 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.*
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*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
Chapter 4: Identification of Capnocytophaga spp.

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:

Chapter 5

Capnocytophaga spp. isolated from subgingival plaque & blood from DM- & non-DM-Periodontitis subjects
Chapter 5: *Capnocytophaga* spp. isolated from 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).
Chapter 5: Capnocytophaga spp. isolated from DM-Periodontitis subjects

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 & Snyder 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 (TCapno) 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 K₃ 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% N₂, 10% H₂, 10% CO₂; 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).
Chapter 5: \textit{Capnocytophaga} spp. isolated from DM-Periodontitis subjects

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 \textit{Capnocytophaga} spp. (Mashimo \textit{et al.} 1983a, Zambon \textit{et al.} 1988, Sastrowijoto \textit{et al.} 1989, Sbordone \textit{et al.} 1995) or number of colony forming units per ml (Müller \textit{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 TCapno 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. HbA₁c, age, PPD, LCAL and GCF volume. Two-sample independent t-tests were used for comparing patients' age and HbA₁c, 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 HbA₁c 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):
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 TCapno 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,
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:

\[ y_{ij} = \beta_0 + \epsilon_{oij} + u_{oj} \]

where \( \beta_0 \) = mean value of the outcome variable \( y_{ij} \). The total variance is partitioned
across each level:

\( \epsilon_{oij} \sim N(0, \sigma^2_{oe}) \) where zero is the mean value and \( \sigma^2_{oe} \) is the variance across sites;

\( u_{oj} \sim N(0, \sigma^2_{ou}) \) where zero is the mean value and \( \sigma^2_{ou} \) 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 \( \chi^2 \) 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:
Chapter 5: Capnocytophaga spp. isolated from DM-Periodontitis subjects

\[ y^{(k)}_i = \beta_0^{(k)} \]

where \( y^{(k)}_i \) = logit \( (p^{(k)}_i) = \ln \left( \frac{p^{(k)}_i}{1- p^{(k)}_i} \right) \) where \( p^{(k)}_i \) 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).

\( k = 1, \ldots, 7 \) for all seven outcomes; \( \beta_0^{(k)} = \text{intercept} = \beta_0^{(k)} + u_0^{(k)} + e_0^{(k)} \), where \( e_0^{(k)} \) = site-level residuals for each outcome, which are normally distributed and have a mean of zero; \( u_0 \) = 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:

\[
\text{Corr} (0_k,0_l) = \frac{\text{Cov} (0_k,0_l)}{\sqrt{\text{var} (0_k) \text{var} (0_l)}} \\
\text{.................Equation (1)}
\]

where \( 0_k \) = outcome variable \( k \) (1, ..., 7) and \( 0_l \) = 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.

<table>
<thead>
<tr>
<th>Subject status</th>
<th>DM-Periodontitis (n = 21)</th>
<th>Non-DM-Periodontitis (n = 25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of sites</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>Male</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>52 (46.5, 57.0)</td>
<td>45.0 (43.0, 55.0)</td>
</tr>
<tr>
<td>HbA1c (%)†^</td>
<td>9.3 (8.0, 10.0)</td>
<td>5.3 (4.97, 5.72)</td>
</tr>
<tr>
<td>Duration (yrs)</td>
<td>8.0 (5.0, 13.5)</td>
<td>0</td>
</tr>
<tr>
<td>Ethnic group: Caucasian</td>
<td>6</td>
<td>19</td>
</tr>
<tr>
<td>Asian</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Other</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>

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 HbA1c and age.

The level of HbA1c was significantly greater in DM-Periodontitis subjects (P < 0.001). No significant difference was observed for age.

<table>
<thead>
<tr>
<th>Subject status</th>
<th>DM-Periodontitis</th>
<th>Non-DM-Periodontitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site-status</td>
<td>Healthy‡ #</td>
<td>Diseased‡</td>
</tr>
<tr>
<td>No. of sites</td>
<td>63</td>
<td>63</td>
</tr>
<tr>
<td>PPD‡ (mm)</td>
<td>3.0 (2.0, 3.0)</td>
<td>7.0 (6.0, 8.0)</td>
</tr>
<tr>
<td>LCAL‡ (mm)</td>
<td>0.0 (0.0, 3.0)</td>
<td>7.0 (6.0, 10.0)</td>
</tr>
<tr>
<td>PI*</td>
<td>1.0 (1.0, 1.0)</td>
<td>1.0 (1.0, 2.0)</td>
</tr>
<tr>
<td>BI‡</td>
<td>0.0 (0.0, 0.0)</td>
<td>2.0 (1.0, 2.0)</td>
</tr>
</tbody>
</table>

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)
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.

<table>
<thead>
<tr>
<th>Species</th>
<th>Actual Number (n = 848)</th>
<th>Percentage of total isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. gingivalis</td>
<td>44</td>
<td>5.2</td>
</tr>
<tr>
<td>C. gingivalis variant</td>
<td>34</td>
<td>4.0</td>
</tr>
<tr>
<td>C. ochracea</td>
<td>366</td>
<td>43.8</td>
</tr>
<tr>
<td>C. ochracea variant</td>
<td>136</td>
<td>15.4</td>
</tr>
<tr>
<td>C. sputigena</td>
<td>52</td>
<td>6.1</td>
</tr>
<tr>
<td>C. granulosa</td>
<td>210</td>
<td>24.8</td>
</tr>
<tr>
<td>C. haemolytica</td>
<td>6</td>
<td>0.7</td>
</tr>
</tbody>
</table>

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 TCapno 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 TCapno counts and TAC

The results from the VC model (i.e. explanatory variables not included; Table 5.4) for the outcome variable TCapno 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.

<table>
<thead>
<tr>
<th>Estimate (95%CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept (mean)</td>
<td>8.47 (7.83, 9.10)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Random structure</th>
<th>Variance (SE)</th>
<th>Proportion of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level-2 (subject)</td>
<td>3.45 (1.00)</td>
<td>&lt;0.001 31%</td>
</tr>
<tr>
<td>Level-1 (site)</td>
<td>7.65 (0.72)</td>
<td>&lt;0.001  69%</td>
</tr>
</tbody>
</table>

Table 5.4 VC model for TCapno 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).
Chapter 5: *Capnocytophaga* spp. isolated from DM-Periodontitis subjects

<table>
<thead>
<tr>
<th></th>
<th>Estimate (95%CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixed structure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept (mean)</td>
<td>13.31 (12.83, 13.79)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Random structure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Level-2 (subject)</td>
<td>1.83 (0.58)</td>
<td>0.002</td>
</tr>
<tr>
<td>Level-1 (site)</td>
<td>5.23 (0.50)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total</td>
<td>7.06</td>
<td>100%</td>
</tr>
</tbody>
</table>

Table 5.5 Variance components model for TAG (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 TCapno and TAG 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 TCapno.](image-url)
Fig. 5.5 Residuals from Variance Components Models for TAC

5.4.2.2 RI models TCapno counts and TAC

The RI models for TCapno (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.

<table>
<thead>
<tr>
<th>Fixed effects (covariates)</th>
<th>Estimate (95%CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>7.83 (7.01, 8.65)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>DM</td>
<td>1.39 (0.17, 2.61)</td>
<td>0.025</td>
</tr>
</tbody>
</table>

Random Effect

<table>
<thead>
<tr>
<th>Variance (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subject (level-2 $\sigma^2_{ow}$)</td>
</tr>
<tr>
<td>Site (level-1 $\sigma^2_{oe}$)</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

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

The RI model for the effect of DM on the TCapno 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
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 TCapno counts in the DM-Periodontitis subjects were significantly higher than the TCapno counts in the non-DM-Periodontitis subjects ($P < 0.025$; Fig. 5.6).

![Graph showing mean counts for TCapno (cfu ml^-1) in non-DM-Periodontitis & DM-Periodontitis subjects. Bars represent 95% CI.]

The Rl model for the effect of site-status on the TCapno counts is shown in Table 5.7

<table>
<thead>
<tr>
<th>Fixed effects (covariates)</th>
<th>Estimate (95%CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>7.80 (7.09, 8.52)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Site-status</td>
<td>1.31 (0.67, 1.96)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Random Effect</th>
<th>Variance (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subject (level-2 $\sigma_{\mu}^2$)</td>
<td>3.59 (1.01)</td>
</tr>
<tr>
<td>Site (level-1 $\sigma_{\epsilon_0}^2$)</td>
<td>7.16 (0.68)</td>
</tr>
<tr>
<td>Total</td>
<td>10.75</td>
</tr>
</tbody>
</table>

Table 5.7 Rl model for the effect of site-status in the outcome variable TCapno counts (log transformed)

The Rl 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 $T_{\text{Capno}}$ counts in diseased sites were significantly higher than in the healthy sites ($P < 0.001$; Fig.5.7).

![Graph showing mean counts for $T_{\text{Capno}}$ (cfu ml$^{-1}$) in healthy and diseased sites. Bars represent 95% CI.](image)

**Fig.5.7** Mean counts for $T_{\text{Capno}}$ (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)
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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 TCapno 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.

<table>
<thead>
<tr>
<th>Fixed effects (covariates)</th>
<th>Estimate (95%CI)</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>6.98 (6.04, 7.92)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Subject-level (level-2) DM</td>
<td>1.78 (0.39, 3.16)</td>
<td>0.012</td>
</tr>
<tr>
<td>Site-level (level-1) Site-status</td>
<td>1.64 (0.76, 2.52)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Interaction DM &amp; site-status</td>
<td>-0.68 (-1.98, 0.62)</td>
<td>0.302</td>
</tr>
</tbody>
</table>

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

The results of the RI model for TCapno 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.

<table>
<thead>
<tr>
<th>Fixed effects</th>
<th>Estimate (95%CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>11.62 (10.92, 12.32)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Subject-level (level-2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>1.76 (0.72, 2.81)</td>
<td>0.001</td>
</tr>
<tr>
<td>Site-level (level-1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Site-status</td>
<td>2.15 (1.47, 2.83)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Interaction: DM &amp; site-status</td>
<td>-0.81 (-1.83, 0.21)</td>
<td>0.118</td>
</tr>
</tbody>
</table>

| Random Effects                     |                  |         |
| Subject-level (level-2 \(\sigma^2_{\omega}\)) | 1.64 (0.50) | 0.001   |
| Site-level (level-1 \(\sigma^2_{\omega}\))    | 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.
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Model assumptions for the combined and interaction models for TCapno 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 TCapno.](image)

![Fig.5.11 Residuals from Combined & Interaction model for TAC.](image)
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The TCapno 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)
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**Total *Capnocytophaga* counts:** Pair-wise contrasts for TCapno counts between groups for significance values (generated through Main Effects & Interactions model in *MlwiN*) are presented in Table 5.10.

<table>
<thead>
<tr>
<th></th>
<th>Non-DM-P H</th>
<th>Non-DM-P D</th>
<th>DM-P H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-DM-P D</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM-P H</td>
<td>0.013</td>
<td>0.888</td>
<td></td>
</tr>
<tr>
<td>DM-P D</td>
<td>&lt;0.001</td>
<td>0.135</td>
<td>0.050</td>
</tr>
</tbody>
</table>

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 *MlwiN*) and the data presented in Table 5.11.

<table>
<thead>
<tr>
<th>Pair-wise contrasts</th>
<th>Non-DM-P H</th>
<th>Non-DM-P D</th>
<th>DM-P H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-DM-P D</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM-P H</td>
<td>0.001</td>
<td>0.421</td>
<td></td>
</tr>
<tr>
<td>DM-P D</td>
<td>&lt; 0.001</td>
<td>0.084</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>C. g</th>
<th>C. o</th>
<th>C. s</th>
<th>C. gr</th>
<th>C. h *</th>
<th>C. gv</th>
<th>C. ov</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. g</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. o</td>
<td>0.440</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. s</td>
<td>0.323</td>
<td>0.447</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. gr</td>
<td>0.080</td>
<td>0.692</td>
<td>0.407</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. h *</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. gv</td>
<td>-0.092</td>
<td>0.133</td>
<td>-0.354</td>
<td>0.092</td>
<td>-</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>C. ov</td>
<td>-0.349</td>
<td>0.537</td>
<td>0.608</td>
<td>0.531</td>
<td>-</td>
<td>0.346</td>
<td>1.000</td>
</tr>
</tbody>
</table>

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.
Chapter 5: *Capnocytophaga* spp. isolated from DM-Periodontitis subjects

<table>
<thead>
<tr>
<th>Sites</th>
<th>C. g</th>
<th>C. o</th>
<th>C. s</th>
<th>C. gr</th>
<th>C. h *</th>
<th>C. gv</th>
<th>C. ov</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. g</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. o</td>
<td>-0.106</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. s</td>
<td>0.091</td>
<td>-0.134</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. gr</td>
<td>0.141</td>
<td>0.041</td>
<td>0.221</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. h *</td>
<td>0.074</td>
<td>0.077</td>
<td>0.083</td>
<td>0.213</td>
<td>1.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. gv</td>
<td>-0.137</td>
<td>-0.116</td>
<td>0.115</td>
<td>0.016</td>
<td>0.082</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>C. ov</td>
<td>0.031</td>
<td>-0.082</td>
<td>0.263</td>
<td>0.287</td>
<td>0.268</td>
<td>0.074</td>
<td>1.000</td>
</tr>
</tbody>
</table>

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

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.
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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.](image)

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.
Capnocytophaga spp. isolated from DM-Periodontitis subjects

*Capnocytophaga ochracea* variant were increased at HDM and DDM sites and decreased at DnonDM sites.

![Graph showing prevalence of Capnocytophaga spp.](image)

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...
Chapter 5: *Capnocytophaga* spp. isolated from DM-Periodontitis subjects

(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 Staphytec 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 HbA1c 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 not 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 its 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 $T_{\text{Capno}}$ count was also observed in the diseased sites when compared to 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 $T_{\text{Capno}}$ 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 \textit{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 \textit{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) \textit{C. gingivalis} and its variant showed an overall decrease in OR

ii) \textit{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) \textit{C. sputigena} and \textit{C. granulosa} showed an increased trend in all sites, whilst

iv) \textit{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) \textit{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 microflora. The decreased prevalence of \textit{C. gingivalis} in DM subjects was unexpected. Investigations by Spratt \textit{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 \textit{C. gingivalis} decreased. Direct comparison between these two studies is precluded as the experimental conditions of the \textit{in vitro} (Spratt \textit{et al.} 1996) and \textit{in vivo} (this) studies were completely different. The subgingival microbial biofilm present in the \textit{in vivo} situation is known to exhibit different physiological properties to bacteria grown in batch culture (Marsh & Martin 1999). The findings for \textit{C. granulosa} and \textit{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 \textit{et al.} 2001). Furthermore, comparison of the prevalence of \textit{C. gingivalis} and \textit{C. ochracea} \textit{C. sputigena
Chapter 5: 

Capnocytophaga spp. isolated from DM-Periodontitis subjects

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, 175)
Chapter 5: *Capnocytophaga* spp. isolated from DM-Periodontitis subjects

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 HbA1c. 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.
6.2 Materials & methods

6.2.1. Bacterial strains

The 7 \textit{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 \textit{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% CO₂.

Plate inoculation was performed using a multi-point inoculator (Mast Group, Merseyside, UK). Each run consisted of 30 \textit{Capnocytophaga} clinical isolates, 4 control strains (β-haemolytic \textit{Streptococcus} spp., Oxford \textit{Staphylococcus aureus} NCTC 6571, \textit{E. coli} NCTC 10418, Vancomycin B resistant \textit{Enterococcus 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 (log₂) 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:

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>Solvent</th>
<th>Diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin V</td>
<td>97% ethanol</td>
<td>Deionised water</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Deionised water</td>
<td>Deionised water</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>Phosphate buffers*</td>
<td>Phosphate buffers*</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>Deionised water</td>
<td>Deionised water</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Deionised water</td>
<td>Deionised water</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>Deionised water</td>
<td>Deionised water</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>97% ethanol + water (1:1)</td>
<td>Deionised water</td>
</tr>
</tbody>
</table>

#### ii) Agents incorporated in selective media†

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>Solvent</th>
<th>Diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>Dimethylsulphoxide</td>
<td>Deionised water</td>
</tr>
<tr>
<td>Polymyxin B sulphate</td>
<td>Deionised water</td>
<td>Deionised water</td>
</tr>
<tr>
<td>Bacitracin</td>
<td>Deionised water</td>
<td>Deionised water</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>Propylene glycol</td>
<td>Deionised water</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>Deionised water</td>
<td>Deionised water</td>
</tr>
</tbody>
</table>

† 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)

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

*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:

$$42.5 \text{ ml agar} + 2.5 \text{ ml lysed blood} + 5 \text{ ml antimicrobial agent}$$

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 $$10^8 \text{ cfu ml}^{-1}$$. This suspension was diluted 10-fold (1:10) with MHB giving a final adjusted concentration of $$10^7 \text{ cfu ml}^{-1}$$. 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 $$10^4 \text{ cfu ml}^{-1}$$ 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.
6.2.5 Incubation
The plates were incubated anaerobically (80% N\textsubscript{2}, 10% H\textsubscript{2}, 10% O\textsubscript{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\textsubscript{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.
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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\textsubscript{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\textsubscript{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.
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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\textsubscript{90} (\(\mu g\) ml\(^{-1}\)) values for ATCC strains of \textit{Capnocytophaga} spp.

<table>
<thead>
<tr>
<th></th>
<th>Antimicrobials used in clinical practice</th>
<th>Agents incorporated into selective media</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clind</td>
<td>Amox</td>
</tr>
<tr>
<td>\textit{C. gingivalis}</td>
<td>(\leq 0.125)</td>
<td>(\leq 0.125)</td>
</tr>
<tr>
<td>\textit{C. ochracea}</td>
<td>(\leq 0.125)</td>
<td>(\leq 0.125)</td>
</tr>
<tr>
<td>\textit{C. sputigena}</td>
<td>(\leq 0.125)</td>
<td>(\leq 0.125)</td>
</tr>
<tr>
<td>\textit{C. granulosa}</td>
<td>(\leq 0.125)</td>
<td>(\leq 0.125)</td>
</tr>
<tr>
<td>\textit{C. haemolytica}</td>
<td>(\leq 0.125)</td>
<td>0.5</td>
</tr>
<tr>
<td>\textit{C. canimorsus}</td>
<td>(\leq 0.125)</td>
<td>NG</td>
</tr>
<tr>
<td>\textit{C. cynodegmi}</td>
<td>(\leq 0.125)</td>
<td>(\leq 0.125)</td>
</tr>
</tbody>
</table>

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\textsubscript{90} for ATCC \textit{Capnocytophaga} strains were greater than the corresponding MIC\textsubscript{90} values for \textit{Capnocytophaga} clinical isolates (see Table 6.4)
Table 6.4 MIC$_{90}$ (μg ml$^{-1}$) values for clinical isolates of *Capnocytophaga* spp.

<table>
<thead>
<tr>
<th></th>
<th>Clind</th>
<th>Amox</th>
<th>Amp</th>
<th>Te</th>
<th>PenV</th>
<th>Eryth</th>
<th>Mz</th>
<th>Vanc</th>
<th>PolyB</th>
<th>Bacit</th>
<th>AmphB</th>
<th>Trimeth</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. gingivalis</em></td>
<td>≤0.125</td>
<td>≤0.125</td>
<td>-</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>64</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>C. gingivalis v</em></td>
<td>≤0.125</td>
<td>≤0.125</td>
<td>-</td>
<td>0.5</td>
<td>0.25</td>
<td>0.25</td>
<td>64</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>C. ochracea</em></td>
<td>≤0.125</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>512</td>
<td>64</td>
<td>16</td>
<td>&gt;512</td>
<td>&gt;512</td>
<td>128</td>
<td>≤0.125</td>
</tr>
<tr>
<td><em>C. ochracea v</em></td>
<td>≤0.125</td>
<td>≤0.125</td>
<td>-</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>256</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td><em>C. sputigena</em></td>
<td>≤0.125</td>
<td>≤0.125</td>
<td>≤0.125</td>
<td>2</td>
<td>1</td>
<td>128</td>
<td>128</td>
<td>4</td>
<td>256</td>
<td>64</td>
<td>256</td>
<td>≤0.125</td>
</tr>
<tr>
<td><em>C. granulosa</em></td>
<td>≤0.125</td>
<td>≤0.125</td>
<td>-</td>
<td>0.5</td>
<td>2</td>
<td>1</td>
<td>128</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>C. haemolytica</em></td>
<td>≤0.125</td>
<td>≤0.125</td>
<td>-</td>
<td>0.25</td>
<td>0.5</td>
<td>0.5</td>
<td>16</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Species</th>
<th>Number</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. gingivalis</em></td>
<td>19</td>
<td>4.8</td>
</tr>
<tr>
<td><em>C. gingivalis</em> variant</td>
<td>11</td>
<td>2.8</td>
</tr>
<tr>
<td><em>C. ochracea</em></td>
<td>180</td>
<td>45.2</td>
</tr>
<tr>
<td><em>C. ochracea</em> variant</td>
<td>72</td>
<td>18.0</td>
</tr>
<tr>
<td><em>C. sputigena</em></td>
<td>42</td>
<td>10.6</td>
</tr>
<tr>
<td><em>C. granulosa</em></td>
<td>70</td>
<td>17.6</td>
</tr>
<tr>
<td><em>C. haemolytica</em></td>
<td>4</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>398</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>
Table 6.6 Numbers of clinical isolates of *Capnocytophaga* spp. tested for antimicrobial / antifungal sensitivity.

<table>
<thead>
<tr>
<th></th>
<th>Clind</th>
<th>Amox</th>
<th>Amp</th>
<th>Te</th>
<th>PenV</th>
<th>Eryth</th>
<th>Mz</th>
<th>Vanc</th>
<th>PolyB</th>
<th>Bacit</th>
<th>AmphB</th>
<th>Trimeth</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. gingivalis</em></td>
<td>17</td>
<td>14</td>
<td>-</td>
<td>17</td>
<td>17</td>
<td>17</td>
<td>17</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>C. gingivalis v</em></td>
<td>11</td>
<td>11</td>
<td>-</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>C. ochracea</em></td>
<td>144</td>
<td>142</td>
<td>5</td>
<td>143</td>
<td>142</td>
<td>144</td>
<td>139</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td><em>C. ochracea v</em></td>
<td>67</td>
<td>68</td>
<td>-</td>
<td>68</td>
<td>68</td>
<td>68</td>
<td>68</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>C. sputigena</em></td>
<td>43</td>
<td>43</td>
<td>17</td>
<td>43</td>
<td>43</td>
<td>43</td>
<td>43</td>
<td>17</td>
<td>17</td>
<td>17</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td><em>C. granulosa</em></td>
<td>59</td>
<td>59</td>
<td>-</td>
<td>59</td>
<td>59</td>
<td>59</td>
<td>55</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>C. haemolytica</em></td>
<td>3</td>
<td>3</td>
<td>-</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>344</td>
<td>340</td>
<td>22</td>
<td>341</td>
<td>343</td>
<td>345</td>
<td>336</td>
<td>22</td>
<td>22</td>
<td>22</td>
<td>22</td>
<td>22</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Number of Capnocytophaga clinical isolates for which the MIC (µg ml⁻¹) was:</th>
<th>≥512</th>
<th>256</th>
<th>128</th>
<th>64</th>
<th>32</th>
<th>16</th>
<th>8</th>
<th>4</th>
<th>2</th>
<th>1</th>
<th>0.5</th>
<th>0.25</th>
<th>≤0.125</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clind</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>1</td>
<td>2</td>
<td>-</td>
<td>1</td>
<td>338</td>
<td>344</td>
</tr>
<tr>
<td>Amox</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>34</td>
<td>14</td>
<td>12</td>
<td>277</td>
<td>340</td>
</tr>
<tr>
<td>Te</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6</td>
<td>2</td>
<td>89</td>
<td>154</td>
<td>90</td>
<td>341</td>
<td></td>
</tr>
<tr>
<td>PenV</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>1</td>
<td>6</td>
<td>7</td>
<td>35</td>
<td>116</td>
<td>59</td>
<td>114</td>
</tr>
<tr>
<td>Mz</td>
<td>24</td>
<td>27</td>
<td>14</td>
<td>30</td>
<td>15</td>
<td>17</td>
<td>64</td>
<td>78</td>
<td>23</td>
<td>17</td>
<td>9</td>
<td>3</td>
<td>15</td>
<td>336</td>
</tr>
<tr>
<td>Eryth</td>
<td>-</td>
<td>-</td>
<td>23</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>3</td>
<td>7</td>
<td>33</td>
<td>84</td>
<td>44</td>
<td>150</td>
<td>345</td>
</tr>
</tbody>
</table>

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...
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\textsubscript{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\textsubscript{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\textsubscript{90} for the \textit{Capnocytophaga} ATCC strains and clinical isolates is shown in Table 6.8.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Antimicrobial agent used in selective medium</th>
<th>Conc. of antimicrobial agent (µg ml(^{-1}))</th>
<th>Range of MIC\textsubscript{90} (µg ml(^{-1})) for ATCC strains(^\dagger)</th>
<th>Range of MIC\textsubscript{90} (µg ml(^{-1})) for clinical isolates*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mashimo</td>
<td>Bacitracin</td>
<td>50.0</td>
<td>1 - &gt;512</td>
<td>64 - &gt;512</td>
</tr>
<tr>
<td>Cap</td>
<td>Polymyxin B</td>
<td>1.96</td>
<td>1 - &gt;512</td>
<td>256 - &gt;512</td>
</tr>
<tr>
<td></td>
<td>Vancomycin</td>
<td>5.0</td>
<td>&lt;0.125 - 32</td>
<td>4 - 16</td>
</tr>
<tr>
<td></td>
<td>Trimethoprim</td>
<td>2.5</td>
<td>&lt;0.125 - 0.25</td>
<td>&lt;0.125</td>
</tr>
<tr>
<td></td>
<td>Amphotericin B</td>
<td>2.5</td>
<td>8 - &gt;512</td>
<td>128 - 256</td>
</tr>
<tr>
<td>Slots</td>
<td>Bacitracin</td>
<td>75.0</td>
<td>1 - &gt;512</td>
<td>64 - &gt;512</td>
</tr>
<tr>
<td></td>
<td>Vancomycin</td>
<td>5.0</td>
<td>&lt;0.125 - 32</td>
<td>4 - 16</td>
</tr>
</tbody>
</table>

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

\(^\dagger\) from Table 6.3

\(*\) from Table 6.4 (data refer to \textit{C. ochracea} and \textit{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 \textit{Capnocytophaga} strains. In the case of the \textit{Capnocytophaga} clinical isolates, the concentrations of bacitracin, vancomycin and trimethoprim would affect recovery of \textit{C. ochracea} and \textit{C. sputigena}. The results for bacitracin and vancomycin are in agreement with those of Sutter \textit{et al.} (1981).

The high susceptibility of \textit{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 \textit{Capnocytophaga} spp. (Montejo Baranda \textit{et al.} 1984, Buu-Hoi \textit{et al.} 1988, Ngaage \textit{et al.} 1999). Pencillin V and erythromycin (also used for endocarditis prophylaxis) showed slightly higher MIC\textsubscript{90} values. The exception in the case of erythromycin was \textit{C. sputigena} which yielded an MIC\textsubscript{90} of 128 µg ml\(^{-1}\) (Table 6.4).
Chapter 6: Antimicrobial Sensitivity Testing

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).

<table>
<thead>
<tr>
<th>Dose* (mg)</th>
<th>GCF conc. (µg ml⁻¹)</th>
<th>MIC₉₀ (µg ml⁻¹) for \textit{Capnocytophaga} clinical isolates*#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin 500</td>
<td>ND</td>
<td>&lt;0.25 - 2</td>
</tr>
<tr>
<td>Amoxicillin 500</td>
<td>3 - 4</td>
<td>&lt;0.125 - 1</td>
</tr>
<tr>
<td>Tetracycline 500</td>
<td>5 - 12</td>
<td>&lt;0.25 - 2</td>
</tr>
<tr>
<td>Clindamycin 150</td>
<td>1 - 2</td>
<td>&lt;0.125</td>
</tr>
<tr>
<td>Metronidazole 500</td>
<td>8 - 10</td>
<td>64 - 256</td>
</tr>
<tr>
<td>Erythromycin 250</td>
<td>ND</td>
<td>0.25 - 128</td>
</tr>
</tbody>
</table>

Table 6.9 Comparison of GCF concentration of antimicrobial agent versus MIC₉₀ 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 \textit{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.
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 microorganisms 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.
Chapter 7: GCF-glucose

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 (Ultraspec 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).
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 ≤ 3 mm and periodontitis (n = 8) with PPD ≥ 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 ≤ 3 mm) and 3 from diseased sites (PPD ≥ 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 haemoglobin levels (HbA1c). For each patient, 3 healthy sites (PPD ≤ 3 mm) and 3 diseased sites (PPD ≥ 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
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.

![Absorbance @ 492nm](image)

**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 \mu l; Trinder's reagent = 100 \mu l; 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 \mu l to 0.8 \mu l, are shown in Fig. 7.4. The reagent volume was equal to 200 \mu l. 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.

### 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.

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

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
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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.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Healthy</th>
<th>Periodontitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>site status</td>
<td>healthy</td>
<td>healthy⁺</td>
</tr>
<tr>
<td>No. of sites</td>
<td>31</td>
<td>24</td>
</tr>
<tr>
<td>PPD⁺ (mm)</td>
<td>2.0 (1.8, 3.0)</td>
<td>2.0 (2.0, 3.0)</td>
</tr>
<tr>
<td>LCAL⁺ (mm)</td>
<td>0.0 (0.0, 0.0)</td>
<td>0.0 (0.0, 4.0)</td>
</tr>
<tr>
<td>GCF⁺ (μl)</td>
<td>0.4 (0.3, 0.6)</td>
<td>0.4 (0.3, 0.9)</td>
</tr>
<tr>
<td>Plaque⁺ Index</td>
<td>0.0 (0.0, 1.0)</td>
<td>0.5 (0.0, 1.3)</td>
</tr>
<tr>
<td>Bleeding Index</td>
<td>0.0 (0.0, 0.0)</td>
<td>0.0 (0.0, 1.0)</td>
</tr>
<tr>
<td>Blood glucose (mg dl⁻¹)</td>
<td>72.7 (69.2, 81.8)</td>
<td>100.0 (88.0, 125.0)</td>
</tr>
</tbody>
</table>

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 & Bl.

⁺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).
Table 7.3 VC model for GCF-glucose concentration (µg µl⁻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 (µg µl⁻¹) in periodontally healthy and periodontitis groups.](image-url)
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).

<table>
<thead>
<tr>
<th>Fixed effects (covariates)</th>
<th>Estimate (95%CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept (Healthy non-Periodontitis patients/sites)</td>
<td>0.08 (0.03,0.19)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Subject/site-level</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy sites in Periodontitis group</td>
<td>0.14 (0.03,0.58)</td>
<td>0.389</td>
</tr>
<tr>
<td>Diseased sites in Periodontitis group</td>
<td>0.18 (0.04,0.75)</td>
<td>0.227</td>
</tr>
<tr>
<td>Random Effects</td>
<td>Variance (SE)</td>
<td></td>
</tr>
<tr>
<td>Subject (level-2 $\sigma^2_{\omega_0}$)</td>
<td>1.64 (0.68)</td>
<td>0.018</td>
</tr>
<tr>
<td>Site (level-1 $\sigma^2_{\alpha_0}$)</td>
<td>1.60 (0.30)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total</td>
<td>1.16</td>
<td></td>
</tr>
</tbody>
</table>

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).
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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.7.6 Residuals for RI Model for GCF-glucose concentration (µg µl⁻¹) in periodontally healthy and periodontitis subjects (validation study).
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.

<table>
<thead>
<tr>
<th>Subject status</th>
<th>Healthy</th>
<th>Periodontitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy</td>
<td>Healthy</td>
<td>Diseased</td>
</tr>
<tr>
<td>Blood-glucose (mg dL⁻¹)</td>
<td>72.7</td>
<td>100.0</td>
</tr>
<tr>
<td>GCF-glucose (mg dL⁻¹)</td>
<td>8</td>
<td>14</td>
</tr>
<tr>
<td>GCF-glucose:Blood-glucose</td>
<td>1:9</td>
<td>1:7.1</td>
</tr>
</tbody>
</table>

Table 7.5 GCF-glucose: Blood-glucose ratios in Healthy & non-DM-Periodontitis subjects. N = 77.
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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.

<table>
<thead>
<tr>
<th>Subject status</th>
<th>DM-Periodontitis</th>
<th>Non-DM-Periodontitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site status</td>
<td>Healthy(^\d)</td>
<td>Diseased(^\d)</td>
</tr>
<tr>
<td>No. of sites</td>
<td>41</td>
<td>44</td>
</tr>
<tr>
<td>PPD(^\d) (mm)</td>
<td>3.0 (2.0, 3.0)</td>
<td>6.0 (0.5, 8.0)</td>
</tr>
<tr>
<td>LCAL(^\d) (mm)</td>
<td>0.0 (0.0, 3.0)</td>
<td>7.0 (5.3, 8.0)</td>
</tr>
<tr>
<td>GCF(^\d) vol(^\d) (μl)</td>
<td>0.5 (0.3, 1.2)</td>
<td>1.3 (0.7, 1.6)</td>
</tr>
<tr>
<td>PI(^\d)(^\d)</td>
<td>1.0 (1.0, 1.0)</td>
<td>1.0 (1.0, 2.0)</td>
</tr>
<tr>
<td>BI(^\d)(^\d)</td>
<td>0.0 (0.0, 0.0)</td>
<td>2.0 (0.0, 2.0)</td>
</tr>
<tr>
<td>Blood-glucose (mg d(^-1))</td>
<td>205.4 (145.6, 257.8)</td>
<td>100.8 (86.0, 120.7)</td>
</tr>
</tbody>
</table>

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.

\(^\d\)Significant difference between healthy & diseased sites within groups \((P < 0.050)\)

\(^\d\)Significant difference between healthy & diseased sites, non-DM-Periodontitis group only \((P = 0.001)\)

\(^\d\)No significant difference between diseased sites, between groups \((P = 0.200)\)

\(^\d\)No significant difference between healthy sites, between groups

\(^\d\)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.

<table>
<thead>
<tr>
<th>Level</th>
<th>Estimate (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept (mean)</td>
<td>0.18 (0.12, 0.25)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Random structure</td>
<td>Variance (SE)</td>
<td>Proportion of total</td>
</tr>
<tr>
<td>Level-2 (subject)</td>
<td>0.83 (0.23)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Level-1 (site)</td>
<td>1.03 (0.11)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total</td>
<td>1.86</td>
<td>100%</td>
</tr>
</tbody>
</table>

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).
<table>
<thead>
<tr>
<th>Fixed effects (covariates)</th>
<th>Estimate (95%CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept (Healthy non-DM, non-Periodontitis patients / sites)</td>
<td>0.09 (0.04, 0.19)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Subject/site-level</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy non-DM-Periodontitis</td>
<td>0.16 (0.06, 0.37)</td>
<td>0.249</td>
</tr>
<tr>
<td>Healthy DM-Periodontitis</td>
<td>0.41 (0.17, 0.96)</td>
<td>0.001</td>
</tr>
<tr>
<td>Diseased non-DM-Periodontitis</td>
<td>0.14 (0.05, 0.34)</td>
<td>0.343</td>
</tr>
<tr>
<td>Diseased DM-Periodontitis</td>
<td>0.23 (0.09, 0.53)</td>
<td>0.057</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Random Effects</th>
<th>Variance (SE)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Subject (level-2 (\sigma^2_{oo}))</td>
<td>0.71 (0.20)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Site (level-1 (\sigma^2_{oe}))</td>
<td>1.01 (0.11)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Total</td>
<td>1.72</td>
<td></td>
</tr>
</tbody>
</table>

Table 7.8 RI model for GCF-glucose concentration (µg µl⁻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.
Chapter 7: GCF-glucose

<table>
<thead>
<tr>
<th>Pair-wise contrasts</th>
<th>Controls</th>
<th>HP-NDM</th>
<th>DP-NDM</th>
<th>HP-DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP-NDM</td>
<td>0.249</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DP-NDM</td>
<td>0.343</td>
<td>0.681</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HP-DM</td>
<td>0.001</td>
<td>0.017</td>
<td>0.008</td>
<td></td>
</tr>
<tr>
<td>DP-DM</td>
<td>0.057</td>
<td>0.378</td>
<td>0.262</td>
<td>0.012</td>
</tr>
</tbody>
</table>

Table 7.9 P-values resulting from pair wise contrasts between groups for GCF-glucose concentration (µg µL⁻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 healthy sites.

<table>
<thead>
<tr>
<th>Subject status</th>
<th>DM-Periodontitis</th>
<th>Non-DM-Periodontitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site status</td>
<td>Healthy</td>
<td>Diseased</td>
</tr>
<tr>
<td>Blood glucose (mg dL⁻¹)</td>
<td>205.4</td>
<td>100.8</td>
</tr>
<tr>
<td>GCF-glucose (mg dL⁻¹)</td>
<td>41</td>
<td>23</td>
</tr>
<tr>
<td>GCF-glucose:Blood-glucose (mg dL⁻¹)</td>
<td>1:5.0</td>
<td>1:8.9</td>
</tr>
</tbody>
</table>

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).

\textit{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 \textit{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 & Lôe 1969, Kjellman 1970, Ficara \textit{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 \textit{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 \textit{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 \textit{et al.} (1975) would stimulate GCF flow leading to elevated volumes per unit time. Hara & Lôe (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 \textit{et al.} (1975) used the hexokinase while Kjellman \textit{et al.} 1970 used the glucose oxidase method.

The aim of the \textit{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 & Loe 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.
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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 & Loe 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
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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 & Løe (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 Loe 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. forsythenis, 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.
Chapter 8: General discussion & conclusions

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 subgingival 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 (Table 1.14), 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$_{90}$ ($\mu$g 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 subgingival 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


Chapter 9: References


Chapter 9: References


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SPSS Sample Power: http://www.SPSS.com/software/Spower/


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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: Patient information sheet

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.................................................................Name .............................................................
Date...........................................
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.:  
Address:  
BPA Nos.:  
Tel No:  

<table>
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<th>Patient Information sheet given</th>
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<td>Mouthwash</td>
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<td>Perio Rx</td>
<td>Yes</td>
<td>No</td>
<td>Date:</td>
</tr>
<tr>
<td>Blood sampled</td>
<td>Yes</td>
<td>No</td>
<td>LF</td>
</tr>
</tbody>
</table>

Sites sampled for plaque  
PD (mm)  

Sites sampled for GCF  
PD (mm)  

NOTES:
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

1. Prepare stock buffers (A & B):
   A. Stock buffer A: KH$_2$PO$_4$ (0.2 M)
      1. Add 13.6 g of KH$_2$PO$_4$ (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$_2$HPO$_4$ (0.2 M)
      1. Add 22.82 g of K$_2$HPO$_4$.3H$_2$O (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.
Appendix 9: MLM glossary

**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).
Assessment of five culture media for the growth and isolation of Capnocytophaga spp.

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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. spuigena, 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. spuigena 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
yields 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

<table>
<thead>
<tr>
<th>ATCC type strains</th>
<th>FAA</th>
<th>Cap</th>
<th>LB</th>
<th>Mashimo</th>
<th>Slots</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cg</td>
<td>5.2 x 10^6 (3.6 x 10^5)</td>
<td>0</td>
<td>4.9 x 10^6 (1.8 x 10^5)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Co</td>
<td>2.4 x 10^6 (6.6 x 10^5)</td>
<td>0</td>
<td>0</td>
<td>6.5 x 10^7 (11 x 10^6)</td>
<td>0</td>
</tr>
<tr>
<td>Cs</td>
<td>1 x 10^7 (2 x 10^6)</td>
<td>3.1 x 10^6 (3.4 x 10^5)</td>
<td>4.9 x 10^6 (2 x 10^5)</td>
<td>1.6 x 10^7 (2.5 x 10^6)</td>
<td>0</td>
</tr>
<tr>
<td>Cgr</td>
<td>8.1 x 10^6 (7.2 x 10^5)</td>
<td>0</td>
<td>4.2 x 10^7 (6.9 x 10^6)</td>
<td>1 x 10^8 (1 x 10^7)</td>
<td>0</td>
</tr>
<tr>
<td>Ch</td>
<td>1.4 x 10^7 (3.3 x 10^6)</td>
<td>1 x 10^6 (1.6 x 10^5)</td>
<td>6.4 x 10^7 (2.6 x 10^6)</td>
<td>1.3 x 10^8 (3.3 x 10^7)</td>
<td>6.8 x 10^7 (1.2 x 10^8)</td>
</tr>
<tr>
<td>Cn</td>
<td>1.9 x 10^8 (4.4 x 10^7)</td>
<td>1.2 x 10^8 (1.6 x 10^7)</td>
<td>2.5 x 10^9 (3.8 x 10^8)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cco</td>
<td>3.4 x 10^7 (5.7 x 10^6)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Clinical isolates

| 74 (Cp) | 7.3 x 10^7 (5 x 10^7) | 0   | 1.1 x 10^8 (1.7 x 10^8) | 5.8 x 10^8 (1.7 x 10^8) | 0 |
| 92 (Co) | 3 x 10^8 (8.2 x 10^7) | 1.6 x 10^7 (4.6 x 10^6) | 6 x 10^8 (1.6 x 10^7) | 4.6 x 10^8 (2.8 x 10^7) | 5 x 10^9 (0.0) |
| 87 (Co) | 3.2 x 10^8 (5.1 x 10^7) | 1.9 x 10^7 (2.7 x 10^6) | 4.1 x 10^8 (1.3 x 10^7) | 4.4 x 10^8 (8.4 x 10^6) | 4.1 x 10^9 (2.2 x 10^8) |
| 76 (Co) | 2.1 x 10^9 (4.1 x 10^8) | 1.8 x 10^8 (1.7 x 10^8) | 3 x 10^9 (8.5 x 10^7) | 1.3 x 10^9 (2 x 10^8) | 1.3 x 10^9 (6.1 x 10^8) |
| 61 (Co) | 7.5 x 10^7 (2.7 x 10^6) | 5.3 x 10^7 (4.4 x 10^6) | 7.1 x 10^8 (5.5 x 10^7) | 2.8 x 10^9 (1.8 x 10^8) | 0 |
| 62 (Co) | 9.3 x 10^7 (1.2 x 10^6) | 1.9 x 10^8 (1.3 x 10^7) | 2 x 10^9 (4.8 x 10^7) | 1 x 10^10 (2.6 x 10^8) | 1.2 x 10^10 (4.4 x 10^8) |
| 97 (Cg) | 2.7 x 10^8 (5.5 x 10^7) | 3.8 x 10^9 (5.1 x 10^8) | 1.3 x 10^10 (1.2 x 10^9) | 0 |

Plaque samples

| 1 | 3.8 x 10^8 (4.8 x 10^7) | 6.5 x 10^8 (3.7 x 10^7) | 7.5 x 10^8 (3.5 x 10^7) | 5 x 10^9 (1.3 x 10^8) | 0 |
| 2 | 0 | 0 | 0 | 0 |
| 3 | 7.5 x 10^8 (5 x 10^7) | 8 x 10^9 (31 x 10^8) | 7.5 x 10^9 (3.5 x 10^8) | 1.8 x 10^10 (1.3 x 10^9) | 0 |
| 4 | 2 x 10^9 (71 x 10^8) | 1.2 x 10^9 (6 x 10^8) | 1.5 x 10^9 (11 x 10^8) | 6 x 10^10 (1.9 x 10^9) | 0 |
| 5 | 3.8 x 10^9 (2.5 x 10^8) | 3.1 x 10^9 (6.4 x 10^8) | 2.9 x 10^10 (4.8 x 10^9) | 7.9 x 10^10 (11 x 10^9) | 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; Con, C. canimorsus; Cco, C. cynodegmi. *CFU/mL significantly different from FAA values at P < 0.05. © 2001 Copyright by the European Society of Clinical Microbiology and Infectious Diseases, CMI, 7, 158–166
investigations which set out to test the antimicrobial susceptibility of \textit{Capnocytophaga} spp. was that the bacterial strains used were not identified. This precludes accurate information being obtained regarding the antibiotic sensitivity of the different \textit{Capnocytophaga} strains and which could explain the results obtained in this investigation. Although Mashimo medium was formulated to be specific for \textit{Capnocytophaga} spp., Mashimo et al [11] found that only 80% of the recovered strains were \textit{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 \textit{Capnocytophaga} species from contaminated clinical specimens' [12].

The literature regarding the sensitivity of \textit{Capnocytophaga} spp. to the antibiotics used in Cap medium is confusing, with some authors suggesting growth inhibition [18,19] and others implying intrinsic resistance [20]. Since these studies did not identify the isolates, such conclusions cannot be made. Therefore, antibiotic susceptibility testing of the different \textit{Capnocytophaga} spp. warrants further investigation.

LB medium was included in the study on the basis of recommendations by Leadbetter et al [1], who used it during the original \textit{Capnocytophaga} isolation and characterization studies. In our studies, this medium performed nearly as well as FAA.

Slots' medium [13], a selective medium for \textit{Actinobacillus actinomycetemcomitans}, has been used in several investigations in which \textit{Capnocytophaga} spp. were concurrently isolated with \textit{A. actinomycetemcomitans}. Its selectivity is imparted by the antibiotics bacitracin and vancomycin.

The third part of this investigation was undertaken to rule out the possibility that maintaining subcultures of \textit{Capnocytophaga} spp. on FAA could potentially bias the results in favor of FAA. It was claimed to be an 'excellent medium for the recovery of \textit{Capnocytophaga} species from contaminated clinical specimens' [12].

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

REFERENCES

Capnocytophaga granulosa and Capnocytophaga haemolytica: novel species in subgingival plaque


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 CfoI 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.

Key words: periodontitis; subgingival microflora; RFLP; 16S rRNA

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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 periosteal elevator. 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 sample plaque 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 Whitey Scientific, Yorkshire, UK) for 3 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) CO₂ 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 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% super fine 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 (Leedbetter 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-
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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.

elusions 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.
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 Szymanki 1985, Warren et al. 1986). A recent paper reports the isolation of C. granulosa from an abscess in the lumbar sacral 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

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Zusammenfassung

Capnocytophaga granulosa und Capnocytophaga haemolytica: neue Arten in subgingivaler Plaque.


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.


Résumé

Le capnocytophaga granulosa et le capnocytophaga haemolytica : espèces nouvelles dans la plaque dentaire sous-gingivale

Origine: La cavité buccale abrite une des microflets les plus variées du corps humain. La connaissance des ces microflets, et de la microflore parodontale en particulier, s'avère indispensable pour comprendre les interactions hôte-bactériennes qui guident le développement de la maladie parodontale inflammatoire infectieuse. Les espèces capnocytophaga ont été impliquées comme pathogènes parodontaux putatifs. Jusqu'à présent seuls 3 membres de ce genre (C. gingivalis, C. ochracea et C. sputigena) ont été isolés de la plaque dentaire sous-gingivale.

But: Ce manuscrit 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 plaques ont été inoculés dans de l’agar anaérobie et incubés anaérobie durant 5 jours. Une identification de routine des isolats cliniques a été effectuée par l’analyse 16S ARBNr PCR-RFLS en utilisant CfoI comme enzyme de restriction voile ponorée par la séquence de gênes 16S ARBNr.

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 partir de la plaque dentaire sous-gingivale. 15 (51%) patients étaient positifs pour C. granulosa et 3 (10%) pour C. haemolytica.

Conclusion: C’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 nécessaire pour établir les proportions relatives de ces espèces sous-gingivales en présence de parodontite aigu et malade.

References


Novel species in subgingival plaque


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Development of an in vitro microassay for glucose quantification in submicrolitre volumes of biological fluid


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 \(\mu l\) to 0.8 \(\mu l\)) of the test solutions were added to the reagent solution (200 \(\mu l\)) 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 \(\mu l\) 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
Microassay for glucose quantification

Absorbance @ 492 nm

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.

Glucose concentration (mg/dL)

Absorbance @ 492 nm

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.

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.
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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 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
collect GCF. No detail in these studies was given as
Two studies (10, 12) used microlitre pipettes to
volume determination varied between the studies.
collected in submicrolitre quantities.
consist of a few microlitres or millilitres of blood,
impractical as the methods of GCF sampling and
(10-12). Direct comparison of these studies is
e.g. diabetes mellitus, are suspected. Biological
levels of glucose in GCF with disparate results
plotting this graph were those obtained when
performing the microassay \textit{in vivo}.

\textbf{Discussion}

\textbf{Microassay}

Serum glucose determination is routinely under­
taken 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 & Loe (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 \textit{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 \( \mu l \) was modified to enable quantifica­
tion 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 \textit{in vivo}, thus resulting in a variation
in the sample to reagent ratio.

It would have been ideal to use GCF for the
\textit{in vitro} evaluation of the microassay, however
this biological fluid cannot be collected in suffi­
ciently large quantities to undertake such \textit{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 \textit{in vitro}
investigation show that this microassay is sensitive enough to

\begin{table}
\centering
\caption{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}
\begin{tabular}{|c|c|c|}
\hline
Sample volume (\( \mu l \)) & Absorbance at 492 nm of inherent serum glucose (serum without added glucose) & Intercept as calculated from equation in Fig. 3 \\
\hline
0.2 & 0.021 & 0.014 \\
0.4 & 0.038 & 0.031 \\
0.6 & 0.048 & 0.047 \\
0.8 & 0.071 & 0.068 \\
\hline
\end{tabular}
\end{table}

\begin{table}
\centering
\caption{Summary of clinical data}
\begin{tabular}{|c|c|c|}
\hline
 & Periodontally healthy (\( n=11 \)) & Periodontitis (\( n=8 \)) \\
\hline
Sites (\( n \)) & 31 & 24 & 24 \\
PPD (mm) & 2.1 ± 0.7 & 2.3 ± 0.47 & 6 ± 0.93 \\
GCF (\( \mu l \)) & 0.46 ± 0.34 & 0.5 ± 0.39 & 1.09 ± 0.47 \\
PI & 0.3 ± 0.4 & 0.8 ± 0.8 & 1 ± 0.75 \\
BI & 0.12 ± 0.4 & 0.28 ± 0.56 & 0.9 ± 0.99 \\
GCF-glucose* & 0.017 ± 0.005 & 0.07 ± 0.191 & 0.19 ± 0.29 \\
Bleeding index & 78.03 ± 17.12 & 107.70 ± 27.65 & \\
GCF-glucose : blood-glucose & 1 ± 0.9 & 1 ± 0.2 & 1 ± 0.2 \\
\hline
PPD = periodontal pocket probing depth; GCF = gingival crevicular fluid; PI = Plaque Index; BI = Bleeding Index; GCF-glucose = absolute GCF glucose (\( \mu g/2 \) min sample); Blood-glucose = Blood glucose (mg/dl); GCF-glucose : blood-glucose.
\begin{tabular}{*{3}{c}}
*Mean ± SD; †Median ± interquartile range; ‡Statistically significantly different from periodontally healthy sites (\( p<0.05 \)).
\end{tabular}
\end{table}
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.

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