
**PRESENCE OF PUTATIVE
PERIODONTOPATHIC ORGANISMS
AT TOOTH AND IMPLANT SITES**

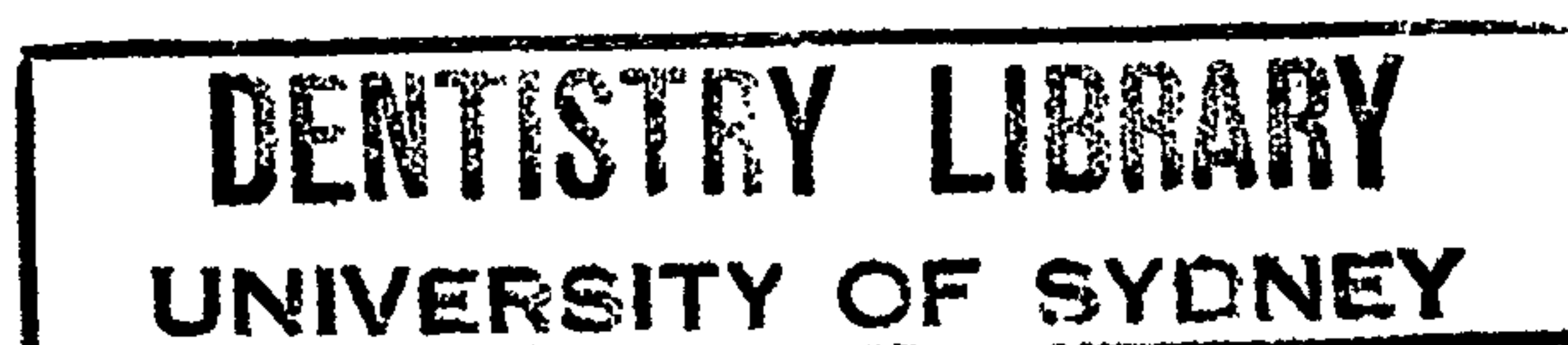
JOY GOUVOUSSIS (BDS)

**A thesis submitted in partial fulfilment
of the requirements for the degree of**

MASTER OF DENTAL SCIENCE (PERIODONTICS)

**Department of Preventive Dentistry
University of Sydney**

October, 1993



ABSTRACT

Dental implant systems have been used successfully to aid the retention of full and partial dentures, fixed bridges and single tooth restorations. The success of osseointegrated titanium implant systems has attracted attention from the dental profession and provided new options for the replacement of missing teeth. Despite successful osseointegration initially, implant failures may occur during subsequent clinical function. One of the reasons proposed for these failures has been local microbial infections, resulting in the loss of osseointegration of the implant fixture. This problem has serious implications for 'transitional' denture patients who retain their teeth despite a hopeless periodontal prognosis until osseointegration has occurred and the superstructure has been inserted. A better understanding of implant failures in these circumstances would provide a rational clinical approach to the management of these patients.

The present study investigated the possibility of cross-infection from periodontally involved teeth to dental implants. Twenty patients with one or more osseointegrated implants were assessed for the presence of seven putative periodontopathic organisms. DNA probes specific for *A. actinomycetemcomitans* (Aa), *P. gingivalis* (Pg), *P. intermedia* (Pi), *E. corrodens* (Ec), *F. nucleatum* (Fn), *T. denticola* (Td) and *C. recta* (Cr) were used to identify the presence of each organism at sampled sites. Tooth sites were divided into healthy (TH), gingivitis (TG) and periodontitis (TP) categories using clinical criteria. Similarly, implant sites were grouped into healthy (IH), periimplantitis (IPi) and failing (IA) categories. Analysis of data from tooth sites identified a significant increase in the presence of the seven periodontopathic organisms from TH to TG to TP sites ($p \leq 0.0001$). At implant sites a similar

increase in the presence of periodontopathic organisms from IH to IPi and IH to IA sites was noted ($p \leq 0.0001$), but the difference between IPi and IA sites was not significant. When individual organisms within parallel categories: TH/IH; TG/IPi; TP/IA, were examined *Pg*, *Pi* and *Cr* occurred more frequently at TP than IA sites ($p \leq 0.05$). The possibility of cross-infection of each test organism was examined within subjects independently and this data pooled for each organism across all subjects. When tooth and implant sites in the same mouth were examined, the absence of *Pg*, *Td*, and *Cr* from implant sites despite their presence at tooth sites was significant ($p \leq 0.0003$). In subjects where these three organisms were absent from tooth sites, they were also absent from implant sites ($p \leq 0.0003$). It is concluded that although cross-infection from tooth sites to implant sites is possible, ailing implants are not associated with an increased presence of periodontopathic organisms.

STATEMENT OF AUTHORSHIP

The contents of this thesis consist of original work carried out by the author (unless otherwise stated and duly acknowledged). No part of this thesis has been submitted in whole or in part for any other degree.

ACKNOWLEDGEMENTS

I would like to express my thanks to my supervisor, Dr Stephen Yeung for his support, patience and encouragement as well as the many selfless hours of guidance and help throughout this study. I am indebted to Dr Braham Pearlman, Head of Periodontics at Westmead Hospital who supported me throughout my course and allowed me the time to carry out this research. Thanks also to Dr John Highfield (Head of Periodontics, University of Sydney) for his direction and to Associate Professor Barnard (Preventive Dentistry, University of Sydney) in whose department the work was carried out.

I am grateful to Susanna Gordon for her help and persistence in anaerobic culturing and to Dr Neil Hunter, Dr Tom Higgins, Dr Phil Bird and the Department of Clinical Microbiology - Institute of Clinical Pathology and Medical Research, Westmead Hospital, for reference strains of organisms. My thanks also to Poppy Sindhusake for her assistance with the statistical analysis.

Gratitude is also extended to Drs Norton Duckmanton, Alistair Stevenson, William Jackson, David Grossberg, Gregory Charlesworth, Richard Chan, and Stephen Yeung for providing the subjects for this study and to the Society of Implant Dentistry for their generous financial support and their interest in this study.

Special thanks to my good friends Dr Roderick Marshall, for proof-reading the manuscript, and Dr Peter Serb for his helpful and calming nature throughout the course.

I would like to thank my parents for their patience with me and whose support make this work possible.

TABLE OF CONTENTS

ABSTRACT: ii

STATEMENT OF AUTHORSHIP: iv

ACKNOWLEDGEMENTS: v

CHAPTER ONE: REVIEW OF THE LITERATURE 1.1

CHAPTER TWO: METHODS AND MATERIALS 2.1

CHAPTER THREE: RESULTS 3.1

CHAPTER FOUR: DISCUSSION 4.1

REFERENCES R.1

APPENDIX 1 A.1

APPENDIX 2 A.2

APPENDIX 3 A.3

APPENDIX 4 A.4

CHAPTER ONE

REVIEW OF THE LITERATURE

1.1 Introduction 1.1

1.2 Anatomy and histology 1.4

1.2.1 Epithelial attachment to teeth and implants 1.5

 (i) Tooth - Epithelium: Health and disease 1.5

 (ii) Implant - Epithelium: Health and disease 1.8

 (a) *in vitro* studies 1.8

 (b) Animal *in vivo* studies 1.10

 (c) Human *in vivo* studies 1.13

1.2.2 Connective tissue attachment to teeth and implants 1.16

 (i) Tooth - Connective tissue: Health and disease 1.16

 (ii) Implant - Connective tissue: Health and disease 1.19

 (a) *in vitro* studies 1.19

 (b) Animal *in vivo* studies 1.20

 (c) Human *in vivo* studies 1.24

1.2.3 Summary 1.26

1.3 Support and Function 1.27

1.3.1 Tooth in periodontium 1.27

1.3.2 Osseointegration 1.29

1.4 Wound healing and cell adhesion 1.31

 (i) Animal studies 1.33

 (ii) Human studies 1.34

1.5 Physical characteristics of implants 1.35

1.5.1 Surface chemistry 1.35

1.5.2 Surface texture 1.36

 (i) Effect on tissue attachment 1.36

 (ii) Effect on plaque accumulation 1.38

1.6	Clinical parameters used to evaluate health and disease	1.40
1.6.1	Teeth	1.40
1.6.2	Implants	1.42
1.6.3	Validity of clinical criteria	1.45
	(i) Probing depth	1.45
	(ii) Marginal erythema	1.47
	(iii) Bleeding on probing	1.48
	(iv) Mobility	1.49
	(v) Radiography	1.50
	(vi) Summary	1.51
1.6.4	Disease definitions	1.52
	(i) Ailing implant	1.52
	(ii) Failing implant	1.52
1.7	Microbiology	1.52
1.7.1	Plaque fomation	1.52
1.7.2	Plaque associated with disease	1.55
1.7.3	Partially edentulous studies	1.58
1.8	Summary	1.61
1.9	Hypotheses	1.62

CHAPTER TWO

MATERIALS AND METHODS

2.1 Research plan 2.1

2.2 Study population 2.2

2.2.1 Selection criteria 2.3

2.2.2 Oral examination 2.3

 (i) Plaque index 2.3

 (ii) Periodontal charting 2.4

2.2.3 Clinical grouping 2.4

2.2.4 Plaque sampling 2.7

2.3 DNA probes 2.8

2.3.1 Introduction 2.8

2.3.2 Validation of specificity of DNA probes 2.11

 (i) *Actinobacillus actinomycetemcomitans* 2.11

 (ii) *Porphyromonas gingivalis* 2.15

 (iii) *Prevotella intermedia* 2.18

 (iv) *Eikenella corrodens* 2.21

 (v) *Fusobacterium nucleatum* 2.24

 (vi) *Campylobacter recta* 2.27

2.3.3 DNA probe methodology 2.30

2.4 Statistical analyses 2.33

2.4.1 Statistical method for testing hypothesis #1 2.33

2.4.2 Statistical method for testing hypothesis #2 2.35

CHAPTER THREE

RESULTS

3.1 DNA probe validation results 3.1

3.2 Hypothesis #1 - Population characteristics 3.3

3.3 Results - Hypothesis #1 3.5

3.3.1 Descriptive statistics 3.5

3.3.2 Analysis of variance 3.8

3.3.3 t-tests on mean variety of organisms 3.11

3.3.4 Percentage presence of test organisms 3.13

3.3.5 Summary (hypothesis #1) 3.16

3.4 Results hypothesis #2 3.17

3.4.1 Population characteristics - hypothesis #2 3.17

3.4.2 Model used to test hypothesis #2 3.19

3.4.3 Analysis of cross-infection within each subject 3.22

3.4.4 Summary (hypothesis #2) 3.26

CHAPTER FOUR

DISCUSSION		4.1
CONCLUSION		4.11

REFERENCES		R.1
-------------------	-------	-----

APPENDIX I

FORMULATION OF GROWTH MEDIA		A.1
------------------------------------	-------	-----

APPENDIX 2

FORMULATION OF STOCK SOLUTIONS		A.2
---------------------------------------	-------	-----

APPENDIX 3

TESTS FOR IDENTIFICATION OF BACTERIAL SPECIES		A.3
--	-------	-----

APPENDIX 4

LABORATORY IDENTIFICATION OF 6 ORGANISMS		A.4
---	-------	-----

LIST OF FIGURES

Figure 2.1	DNA probe methodology	2.32
Figure 3.1	Mean variety of organisms in each clinical category	3.7
	Figure 3.1a (tooth sites)	3.7
	Figure 3.1b (implant sites)	3.7
Figure 3.2	Combinations of implant cross-infection from tooth sites . . .	3.21
Figure 3.3	Routes of cross-infection considered	3.21
Figure 3.4	Relative support or rejection of hypothesis #2 for each of the seven test organisms	3.25

LIST OF PLATES

Plate 2.1a	<i>Actinobacillus actinomycetemcomitans</i> on agar	2.14
Plate 2.1b	<i>Actinobacillus actinomycetemcomitans</i> gram stain	2.14
Plate 2.2a	<i>Porphyromonas gingivalis</i> on agar	2.17
Plate 2.2b	<i>Porphyromonas gingivalis</i> gram stain	2.17
Plate 2.3a	<i>Prevotella intermedia</i> on agar	2.20
Plate 2.3b	<i>Prevotella intermedia</i> gram stain	2.20
Plate 2.4a	<i>Eikenella corrodens</i> on agar	2.23
Plate 2.4b	<i>Eikenella corrodens</i> gram stain	2.23
Plate 2.5a	<i>Fusobacterium nucleatum</i> on agar	2.26
Plate 2.5b	<i>Fusobacterium nucleatum</i> gram stain	2.26
Plate 2.6a	<i>Campylobacter recta</i> on agar	2.29
Plate 2.6b	<i>Campylobacter recta</i> gram stain	2.29

LIST OF TABLES

Table 2.1	Clinical criteria used to categorise tooth and implant sites . . .	2.6
Table 2.2	Sensitivity and specificity of OmniGene's DNA probe tests for periodontal pathogens	2.10
Table 2.3	Chi square grid for testing hypothesis #2	2.36
Table 3.1	Correlation between cultural and DNA probe results for six organisms	3.2
Table 3.2	Population demographics (hypothesis #1)	3.3
Table 3.3	Percentage of sites in each clinical category (hypothesis #1) .	3.4
Table 3.4	Descriptive statistics for mean variety of organisms in each clinical category	3.5
Table 3.5a	One way ANOVA results at tooth sites	3.9
Table 3.5b	One way ANOVA results at implant sites	3.9
Table 3.5c	Indication of significant groups using Scheffés technique of multiple analysis comparisons	3.10
Table 3.6	Independent sample t-test results	3.11
Table 3.7a	Percentage of sites within each clinical category at which individual organisms were detected	3.13
Table 3.7b	Percentage of sites at which individual organisms were detected	3.15
Table 3.8	Population demographics (hypothesis #2)	3.17
Table 3.9	Percentage of sites in each clinical category (hypothesis #2) .	3.18
Table 3.10	Chi square grid for testing raw data of possible cross-infection (hypothesis #2)	3.22
Table 3.11	Chi square for organisms at tooth and implant sites in the same mouth	3.23

ABBREVIATIONS

+ +	organism present at both tooth and implant site
- -	organism absent from both tooth and implant site
+ -	organism present at tooth site but absent from implant site
- +	organism absent from tooth site but present at implant site
#1	number 1
#2	number 2
μL	microlitres
μm	micrometres
Å	Angström
Aa	<i>Actinobacillus actinomycetemcomitans</i>
ANOVA	analysis of variance
Bf	<i>Bacteroides forsythus</i>
Cr	<i>Campylobacter recta</i>
°C	degrees Celsius
DNA	deoxyribonucleic acid
Ec	<i>Eikenella corrodens</i>
ELISA	enzyme linked immunosorbent assay
Fn	<i>Fusobacterium nucleatum</i>
gms	grams
HA	hydroxyapatite
IA	ailing implant site
IH	healthy implant site
IPi	periimplantitis site
L	litres
LJE	long junctional epithelium
M	molar (molarity)
mg	milligrams
mL	millilitres
mm	millimetres
Pg	<i>Porphyromonas gingivalis</i>

Pi	Prevotella intermedia
PI	plaque index
SEM	scanning electron microscopy
Td	Treponema denticola
TEM	transmission electron microscopy
TG	gingivitis tooth site
TH	healthy tooth site
TP	periodontitis tooth site

CHAPTER ONE

REVIEW OF THE LITERATURE

1.1 INTRODUCTION

The concept of osseointegration as proposed by Brånemark advocates the "unloaded" healing of titanium implants to the alveolar bone prior to clinical loading for function (Adell et al. 1981). The success rate of osseointegration in the fully edentulous patient is high (Adell et al. 1986, Albrektsson et al. 1986). The subsequent clinical failure of a small proportion of these implants has been attributed to "over loading", microbial infection or both (Mombelli et al. 1987, Rosenberg et al. 1991).

The observation that failing implants share a similar microbial profile in the periimplant tissues as periodontally involved teeth (Mombelli et al. 1987, Becker et al. 1990), raises a concern in the partially dentate mouth. Active periodontal pockets associated with natural teeth are a potential source of infection for dental periimplant tissues and may contribute to the loss of osseointegration (Lindhe et al. 1992).

Osseointegration has been defined as "a direct structural and functional connection between ordered, living bone and the surface of a load-carrying implant..."(Brånemark 1985) The use of osseointegrated implants as abutments for various prostheses has become a predictable adjunct in the treatment of partially and fully edentulous patients (Albrektsson et al. 1986).

A well functioning muco-periosteal-osseous barrier zone has been considered important for the long term prognosis of fixtures (Adell et al. 1986). However the contribution of the periimplant tissue response to microbial plaque and its effect on the clinical longevity of dental implants is not well understood.

Osseointegrated implants are being used more frequently in partially dentate patients. The practice of placing implants into mouths potentially harbouring putative periodontopathic microorganisms in the sulci of the remaining teeth has raised a concern about the potential of cross-infection to the implant site. If this were so, recognition and early intervention may prevent peri-implant tissue breakdown. An understanding of the relationship between the clinical appearance of peri-implant tissues and the microflora harboured beneath the soft tissue cuff may serve to permit early interception when pathology is at a reversible stage. This is particularly desirable because many aspects of conventional periodontal therapy are not applicable to the implant undergoing progressive attachment loss.

This chapter will review the comparative anatomy of the soft tissue to tooth or implant junction (in health and disease) and discuss the features of the implant abutment which affect the nature of this junction. The physiology and functional characteristics of the two structures will be compared. The relevant methods of clinical assessment and the microbiology of implant associated plaque will also be discussed. Finally, recent reports linking the presence of teeth with increased periodontopathic organisms at implant sites will be reviewed and hypotheses will be stated.

1.2 ANATOMY AND HISTOLOGY

Teeth and dental implants are both transmucosal structures. Periimplant "gingiva" will be referred to as periimplant mucosa, as gingiva implies periodontal mucosa with its inherent fibre systems attaching it to the tooth and alveolus. Such fibre systems have not been routinely described in periimplant mucosa (Adell et al. 1986), hence the term gingiva is not appropriate.

The periimplant tissue interface has presented technological and physical problems in its study due to difficulty in sectioning the implants for microscopic examination without disruption of adjacent biologic tissues. Several techniques have been developed to overcome this problem, each with their own inherent problems (Fletcher et al. 1979, Gould et al. 1981 and 1984).

The average dimensions of the connective tissue, junctional and crevicular epithelium, have been well established for natural teeth (Garguilo et al. 1961). This attachment apparatus develops from a biological system of tooth eruption and is guided by physiologic influences of remodelling. In contrast, the dimensions of the implant-soft tissue attachment are determined by the placement site, surgical technique and possibly the age of the patient. The average dimensions of soft-tissue implant attachment have not been reported to date.

The dentogingival junction is the region of soft tissue attachment to the tooth coronal to the alveolar crest. It is composed of two regions which can be distinguished under low power microscopy. The coronal region consists of a tapering band of non-keratinised epithelial cells, continuous with those lining the gingival sulcus, which adapt to the adjacent tooth surface and seal off the underlying tissues from the oral/sulcular environment. Apical to the epithelial attachment is the zone of connective tissue attachment. Collagen fibres inserting into the root cementum fan out into the gingiva and other nearby structures (Schröder and Listgarten 1977). The dimension of the dentogingival junction has been termed the "biologic width" with an average height of 2.04mm (Garguilo et al. 1961, Baima 1986).

1.2.1 EPITHELIAL ATTACHMENT TO TEETH AND IMPLANTS

(i) TOOTH-EPITHELIUM: Health and Disease

In the natural dentition, the primary epithelial attachment is derived from the fusion of the reduced enamel epithelium with the oral epithelium. This junctional epithelium is composed of large cells held together by relatively few desmosomes and separated by wide intercellular spaces. At its coronal extent, the junctional epithelium is approximately 15 - 20 cells wide and forms the base of the gingival sulcus. It is this free surface at the base of the gingival sulcus from which epithelial cells are desquamated as the junctional epithelium is renewed through cell division in the basal layer. More apically, it tapers

along a relatively smooth basement membrane to terminate at the insertion of the first intact gingival connective tissue fibres. The epithelium is supported by collagen fibre bundles in the deeper gingival connective tissues (Ten Cate 1975).

The ultrastructural features of the dentogingival junction have been described in electron microscopic studies by Listgarten (1966). Interposed between the cell membrane of the junctional epithelial cells and the enamel surface is a bilaminar region similar in appearance to the basement membrane (seen in light microscopy). This internal basement lamina is composed of an electron lucent zone (lamina lucida) and an electron dense zone (lamina densa). The amorphous layer between enamel and the lamina densa has been described as the sublamina lucida (Kobayashi et al. 1976). The electron dense region is believed to be type IV collagen (Genco 1990). The internal basement lamina is continuous with the (external) basement membrane which lies between the junctional epithelium and the underlying connective tissue. The junctional epithelium is unique because it has two basement membranes. Within the cells adjacent to the internal basement lamina, hemidesmosomes may be seen. These tufted structures are similar to the desmosomes seen between adjacent cells, and are believed to have a role in mediating the epithelial-enamel adhesion (Schröder and Listgarten 1977). New junctional epithelium and hemidesmosomal attachments have been shown to form after injury to the

gingival attachment epithelium (Taylor and Campbell 1972), surgery (Frank et al. 1972), or its complete removal (Listgarten 1967).

Hemidesmosomes are not unique to the enamel or connective tissue interfaces in gingival tissue. Epithelial attachment has been observed to form over calculus covered by cuticle but not plaque (Listgarten and Ellegard 1973).

Morphologic changes of epithelium during inflammation include intercellular oedema, evident through widened intercellular spaces, decreased numbers of desmosomes, disruption of the basal lamina and cellular disruptions. (Takarada et al. 1974). The junctional epithelium has been shown to contain an increased but variable number of transmigrating neutrophils, and infiltrating monocytes. In addition the epithelium proliferates and the previously straight basement membrane becomes convoluted, extending deep into the underlying connective tissue. Following loss of attachment and resolution, the epithelium has been observed to re-unite with the root surface in the form of a long junctional epithelium (LJE) (Listgarten 1967).

Lavelle (1981) proposed the concept of a mucosal seal around dental implants. Support for this has come from clinical data showing that the periimplant tissues can be maintained in health for 18 months or longer (Lekholm et al. 1986). Considerable literature has been published in the last decade investigating the ultrastructure of the union between oral epithelium and

materials used for transmucosal dental implants. For simplicity a review of these studies has been subdivided into *in vitro* as well as animal and human *in vivo* studies.

(ii) IMPLANTS-EPITHELIUM: Health and Disease

(a) *In vitro* studies

The strength of adhesion between human epithelial cells and titanium was tested by Fletcher et al. (1979). Test materials were placed onto a tissue culture monolayer of epithelial cells and incubated for four hours. The strength of adhesion was tested using compressed air. The titanium-epithelial cell adhesion was superior to glass, polystyrene, gold, Vitallium and copper. The first study to describe the ultrastructure of the attachment between epithelial cells and a titanium metal substructure was by Gould et al. (1981). Porcine periodontal epithelial cell rests were grown on epoxy discs coated with a film of titanium approximately 300Å thick for fourteen days. Transmission electron microscopy (TEM) showed a confluent layer of cells covering the metal surface. Hemidesmosomal attachments and an extracellular matrix resembling the basement membrane seen under epithelial cells *in vivo* was visible between the culture cells and the metal surface. It was concluded that epithelial cells attach to titanium surfaces in a similar manner to that seen in natural teeth.

Not all investigators have found the attachment apparatus between cultured cells and titanium as described by Gould et al. (1981). Jansen et al. (1985), in a similar study to Gould's, investigated the ultrastructure of guinea pig epithelial cell attachment to titanium, gold, carbon, apatite, and polystyrene substrates. The findings disputed those of Gould et al. (1981), noting hemidesmosomal-like attachments only on apatite and polystyrene but not on metal or carbon surfaces. In contrast, they found epithelial cells adapted very closely to the metal surface and were interposed by a basement membrane like material. The authors attributed the difference in results to differences in cell populations, culture conditions, or treatment of titanium substrates. It is known that, *in vivo*, substrates are covered with an adsorbed layer of serum proteins, so direct cell-substrate contact is unlikely to occur. The authors hypothesised that the adsorbed serum component and/or substrate influences the type of contacts, hemidesmosomal or focal, that will form. The failure to demonstrate hemidesmosomes is not proof of their absence (Gipson et al. 1983). These findings have recently been supported in a study by Kasten et al. (1990). Human epithelial cells were seeded onto titanium and hydroxyapatite (HA) surfaces. The authors found an increased frequency of adhesion to the HA surface.

In vitro studies into the adhesion of oral epithelial cells to titanium seem to indicate that a physical union is possible. When human epithelial cells are used, the nature of this union appears to be similar to that seen between

epithelial cells and tooth structure. However, caution should be exercised when extrapolating these results to the clinical situation. Unlike the controlled conditions *in vitro*, implant materials in the oral cavity are subject to many influences. Such things as plaque accumulation, adsorption of salivary glycoproteins or gingival crevicular fluid, and potentially the build-up of corrosion products may alter the surface characteristics of the material and prevent the formation of the attachment apparatus observed *in vitro*. To ascertain the effect of these influences, *in vivo* studies have been carried out on both animal and human subjects.

(b) Animal *in vivo* studies

The attachment of epithelial cells *in vivo* to Vitallium subperiosteal implants with transmucosal abutments via hemidesmosomes was first demonstrated by James and Schultz (1974). Although a non-titanium material was used and the subjects were monkeys rather than humans, this paper demonstrated that the potential for soft tissue adhesion to inert materials existed in a biological system. This potential was confirmed by Listgarten and Lai (1975). In their study both a basal lamina and hemidesmosomal adhesions could be demonstrated on epoxy resin tooth replicas retrieved from previous extraction sockets in monkeys .

Dog studies have also shown epithelial attachments to implant surfaces *in vivo*. Crystal-sapphire implants in place for up to two years were examined by light

microscopy as well as TEM and scanning electron microscopy (SEM) by McKinney et al. (1985). They demonstrated that regenerated crevicular gingiva develops epithelial cells with organelles, tonofilaments, vesicles and hemidesmosomes with a similar morphological structure to that which interfaces the natural tooth. Two distinct electron dense layers were noted within the lamina densa as well as a region analogous to the dental cuticle and the sublamina lucida as described by Kobayashi et al. (1976). An unexpected finding was the numerous secretory vesicles throughout the cytoplasm of epithelial cells and fused with the outer plasma membrane. They suggest this may partially explain how the basal lamina and other extracellular proteoglycans on the external surface of the junctional epithelial cells are produced (McKinney et al. 1985).

These studies have demonstrated epithelial adhesion *in vivo* to materials other than titanium. However, similar studies have only recently been published demonstrating adhesion to a titanium abutment in an animal model. Berglundh et al. (1991) reported the soft tissue attachment to 27 titanium implants in 5 beagle dogs. Teeth were maintained on the contralateral side to serve as comparisons. The clinical appearance of the gingival cuff around the implants was very similar to that of the natural tooth. Bleeding on light probing could not be elicited. Histological examinations of the inflammation free gingiva revealed similar epithelial characteristics to the junctional epithelium around healthy mature teeth i.e. epithelium lining the implant surface showed 2-5 cell

layers, few rete pegs, a smooth surface without keratinisation and no elongation toward the apex. This epithelium extended apically along the abutment surface to a point approximately 1-1.5mm coronal to the crest of the investing bone. Hence, the spatial relationship of the epithelial attachment to a titanium implant abutment appeared very similar to that of teeth. Berglundh et al. (1991) speculated that epithelium functions as a biological seal around implants as in the case of natural teeth (Kurashina et al. 1984).

Pathologic changes *in vivo* have been described in association with the presence of bacterial plaque. Berglundh et al. (1992) described similar epithelial changes adjacent to teeth and implants in dogs following plaque accumulation over three weeks. Epithelial proliferation accompanied by increased rete ridge formation were principle changes. In addition, increased transmigration of mononuclear phagocytic cells was described. This finding has been supported by other investigators, although some have noted differences in the epithelial cell responses to advanced marginal inflammation between implants and teeth. Lindhe et al. (1992), using ligature induced marginal tissue breakdown in a dog model, showed that implant associated epithelium invariably demonstrated ulcerated regions unlike its tooth counterpart. This finding was confirmed in a monkey model by Schou et al. (1993), who also noted a faster formation of pocket epithelium and faster apical down-growth around implants than around teeth. It appears that the epithelial response to plaque bacteria and the associated subjacent inflammation is

initially very similar for implants and teeth in several animal models. However, when the degree of inflammation is increased, a difference in response may become evident. More research into this difference is necessary.

In conclusion, animal and *in vitro* studies indicate that a biologic seal exists between the titanium implant surface and adjacent epithelial cells in health and that a similar plaque associated inflammation is observed in the connective tissues adjacent to teeth and titanium implants. However, the response of animals cannot be equated with that of humans. Therefore, evaluation of the results from human trials is necessary to ensure similar adhesion and response occurs.

(c) Human *in vivo* studies

Human *in vivo* studies on soft tissue to implant attachment in health are complicated by ethical considerations. Hence, few papers have been published examining the ultrastructure of this union in man.

Pure titanium implants were removed en-bloc with trephines from humans after reported successful clinical function up to 7 years by Hansson et al. (1983). These samples were examined using light microscopy and SEM. The authors observed a tight collar of epithelial cells around the titanium implants. The

cells were attached to the titanium surface by hemidesmosomes. The authors concluded that titanium was the metal of choice for use in implant surgery.

The findings of Hansson et al. (1983) were supported by Gould et al. (1984). The latter authors placed titanium coated epoxy replicas into the palatal gingiva of a human volunteer for a period of 4 weeks. The tissue encapsulated implants were retrieved during periodontal surgery in the region and analysed using TEM. The pattern of hemidesmosomal attachments and basement membrane formation found was analogous to that found at teeth. (Gould et al. 1984).

Adell et al. (1986) studied the periimplant mucosa around titanium implants in 16 edentulous patients. Biopsies of the mucosa adjacent to one implant in each mouth were taken after 30 months of service and examined histologically. The epithelial cells previously abutting the implant strongly resembled those of normal junctional epithelium. This finding is in support of a previous report from the same group of investigators using TEM. In that paper, gingival cells of normal size and shape were densely packed against the titanium surface and attached by hemidesmosomes and a thin layer of ground substance. (Albrektsson et al. 1981).

From the above studies it can be concluded that the epithelial attachment to titanium implants in humans is similar histomerically to that seen around teeth

in the absence of plaque associated inflammation. However, as in the animal models described previously, these similarities may not hold true in the presence of inflammation.

Several studies have examined the histologic changes associated with inflammation within the periimplant soft tissues in man. Sanz et al. (1991) examined biopsy specimens taken from the marginal tissue around titanium implants with clinical signs of periimplantitis in 6 subjects as well as six subjects with no apparent inflammation. They found that in the inflamed sites exhibited a proliferation of the sulcular epithelium as well as acanthosis and "papillomatosis". Within the epithelium, individual cells appeared enlarged and sometimes vacuolated. In addition, there was increased transmigration of mononuclear phagocytic cells through the junctional epithelium which also showed increased intercellular spaces. These findings are similar to those found in the junctional epithelium of teeth during inflammation (Page and Schröder 1976). The only difference appears to be in the presence of vacuolisation within the epithelial cells. This is explained by the authors as resulting from mechanical trauma to the epithelial cells, presumably due to the mobility of the implants at the time of the biopsy. Comparable studies to those investigating histologic changes in inflammation around teeth and implants in the same mouths of animals do not appear to have been conducted in humans. Thus, it remains to be seen whether acute inflammation causes similar

differences in epithelial response as those reported by Lindhe et al. (1992) and Schou et al. (1993).

From the above discussion it is apparent that there is a biologic potential of animal and human oral epithelial cells to form an attachment to titanium surfaces *in vitro*. In addition when oral hygiene is maintained, a similar attachment can be achieved *in vivo*. This union resembles that seen around teeth both clinically and histologically. The findings of these reports have also indicated that inflammation can be minimised if microbial plaque is not allowed to accumulate within the crevice. When plaque associated inflammation does occur, the epithelial tissues around the implant respond in a manner similar to that seen in the junctional epithelium of teeth.

1.2.2 CONNECTIVE TISSUE ATTACHMENT TO TEETH AND IMPLANTS

(i) TOOTH-CONNECTIVE TISSUE: Health and Disease

The connective tissues of the dentogingival junction provide structural support for the gingival and junctional epithelia. Collagen fibres within the tissues are densely packed and contribute to the toughness and rigidity of the free gingival margin. The dentogingival fibres are arranged into discrete bundles and extend into the gingival connective tissue, periosteum or the roots of adjacent teeth.

The insertion of the dentogingival fibres into the root surface has been examined under high power magnification. Type I collagen fibre bundles insert perpendicularly into the acellular radicular cementum as Sharpey's fibres, and intermingle with the intrinsic collagen fibres of the cementum (Schröder 1988).

The dentogingival collagen fibre bundles have been grouped in the following manner:

CIRCULAR - in the free gingiva and encircle the tooth like a cuff. They do not insert into the tooth itself.

DENTOGINGIVAL - originate in supracrestal cementum and fan out to end in the connective tissue of the free gingiva.

DENTOPERIOSTEAL - originate in supracrestal cementum fan out apically to end in periosteum.

TRANSEPTAL - extend between supra-alveolar cementum of approximating teeth.

In health, the gingival connective tissue is comprised not only of the dentogingival fibres, but also of blood and lymph vessels, neural tissue, oxytalan, elastin and reticulin fibres as well as numerous cells such as mast cells and pluripotential undifferentiated mesenchymal cells. Interspersed between these structures is the ground substance, comprising various proteins, glycosaminoglycans and proteoglycans. Inflammatory cells are almost

completely absent in health, although a slow, continuous, efflux of polymorphonuclear leukocytes is seen through the junctional epithelium.

The changes in the subepithelial connective tissue with the onset of gingival inflammation have been reviewed by Page and Schröder (1976). Dilation of blood vessels below the junctional epithelium and the disruption of the perivascular connective tissue fibres are early signs of gingival inflammation. With progression of disease more collagen breakdown is seen near the site of bacterial build up while deposition and fibrosis is seen at some distance to the site (Melcher et al. 1962). Fibroblasts undergo various pathologic changes indicative of a disturbance in collagen synthesis (Gavin 1970). The rate of loss of collagen with the onset of inflammation indicates that dissolution of collagen as well as a reduction in its production is involved. Pocket formation results from the loss of sub-epithelial collagen through lysis of collagen fibres inserting into cementum with breakdown and subsequent reconstitution of the basal lamina. In addition, an increased density of inflammatory cell infiltrate can be observed. Hence the supportive architecture of the gingiva is replaced by inflammatory cells. This situation is reversible unless it has resulted in breakdown of alveolar bone and the apical migration of the epithelial attachment.

(ii) IMPLANT-CONNECTIVE TISSUE: Health and Disease

Unlike the root surface, which gains its connective tissue attachment during tooth formation, the implant surface does not permit insertion of fibres into its structure. Despite this limitation, the clinical appearance and tone of the periimplant tissues is remarkably similar to that surrounding teeth (Stallard 1985, Adell et al. 1986). The lack of a periodontal ligament not only alters the functional characteristics of the implant but prevents the establishment of a normal vascular supply to the supra-alveolar connective tissue. Egelberg (1966) demonstrated the presence of a dentogingival plexus of venules beneath the junctional epithelium around teeth. These vessels normally drain into larger vessels within the periodontal ligament. No studies to date have investigated the alterations in vascular supply in soft tissues induced by the presence of a transmucosal implant. Furthermore, it has been observed by many authors that the epithelium attached to the titanium abutment surface does not extend apically to reach the alveolar bone (Berglundh et al. 1991, Listgarten et al. 1991). These findings imply that the connective tissues may be attached to the abutment surface deep within the tissues. Several lines of investigation have attempted to ascertain the nature of the connective tissue attachment to the implant.

(a) *in vitro* studies

To achieve an attachment with the connective tissue fibres in the periimplant mucosa, the surface of the titanium abutment must be biocompatible. Hence, it

must permit a close association with the collagen producing cells. Several studies have investigated the acceptance of pure titanium and titanium alloys by human gingival fibroblasts *in vitro*. Lowenberg et al. (1987) demonstrated that titanium alloy discs were at least as compatible as demineralised and non-demineralised root slices which were used as controls. Similar affinity for titanium was shown by Inoue et al. (1987). Recently, Guy et al. (1993) demonstrated greater fibroblast attachment to pure titanium than to either porous or non-porous hydroxy apatite. The affinity for these substrates was enhanced by the application of fibronectin to the material.

These studies therefore imply that collagen producing fibroblasts can survive and function normally when closely adapted to the abutment surface. However, this does not mean that the collagen thus produced will be in any way adherent to the material, but illustrates the potential for fibroblast products to come into close contact with the substrate.

(b) Animal *in vivo* studies

Many animal studies have examined the connective tissue apposition to titanium and other transmucosal dental implants. In general, the observation is that mucosal connective tissue fibres are closely adapted though not demonstrably adherent to the implant surface. The fibres are usually arranged into discrete bundles which are either distributed circumferentially (Buser et al. 1992), parallel (Berglundh et al. 1991, Listgarten et al. 1991), haphazardly

(Fartash et al. 1990, Arvidson et al. 1990) or a combination of these (Ruggeri et al. 1992). In addition, many authors have published reports indicating that connective tissue fibre bundles can lie perpendicular to the abutment surface and in some instances be attached to them in a "functional orientation" (Schröder et al. 1981, Kurashina et al. 1984). Buser and co-workers (1990a) postulated that the orientation of the periimplant gingival fibres was determined by the surface properties of the implant material and the nature of the epithelium through which the abutment passes. Berglundh et al. (1991), compared healthy supra-alveolar connective tissues in dogs at tooth and titanium implant sites. At the implant sites, the junctional epithelium ended at a varying distance from the gingival margin consistently leaving a connective tissue portion coronal to the bone crest in direct contact with the titanium surface. The connective tissue was devoid of inflammatory cell infiltrates and harboured a dense network of collagen fibres which, in major bundles, extended from the alveolar bone crest to the gingival margin. In the periimplant tissue, the vast majority of the coarse fibre bundles were attached to the marginal bone, not to the marginal titanium surface, and mainly arranged in a direction parallel to the surface of the titanium abutment. The marginal portion of the periimplant mucosa contained significantly more collagen and fewer fibroblasts than corresponding gingival tissue. Analysis of soft tissues revealed that while the composition of the free gingiva differed from that of the adjacent supra-alveolar connective tissue with respect to collagen, fibroblasts, leucocytes and vessels, the periimplant mucosa had a more uniform composition. This may

support the hypothesis that the supra-alveolar tissue at teeth is comprised of two different units but the periimplant mucosa originates from one tissue, the mucosa of the edentulous ridge, and that the introduction of a titanium abutment failed to alter the characteristics of this masticatory mucosa.

From these studies it is apparent that in the absence of a root cementum, the connective tissue fibres surrounding the implant abutment adopts an orientation similar to that of the non-inserting dentogingival fibre groups around teeth. The consequence of the different fibre structure has been investigated in experimental periimplant inflammation studies. Kurashina et al. (1984) assessed the soft tissues around 27 two year old hydroxyapatite implants in 5 beagle dogs. Despite weekly cleaning, plaque and calculus accumulation took place on the surfaces of implants and neighbouring teeth. Clinical and histologic investigations showed a large area of collagen fibre destruction and inflammatory cell infiltrate at tooth and implant sites. Some areas revealed a perpendicular orientation of connective tissue fibres and a "saw tooth" pattern at the implant-connective tissue junction. This arrangement may be related more to the implant material than to the pathologic process itself. No comparison was made regarding the extent of the inflammatory infiltrate between the implant and tooth sites.

A similar comparison between inflamed implant and tooth sites in dogs was reported by Ericsson et al. (1992). Three months after discontinuation of oral

hygiene around teeth and titanium implants, the animals were sacrificed and the areas examined histologically. The authors found that the inflammatory infiltrate around implants extended deeper into the tissues than around teeth (2.1mm and 0.9mm respectively). From these reports it may be postulated that the lack of attached gingival fibres around implants in some way promoted deeper extension of microbial irritants or a wider inflammatory infiltrate. Further support for this hypothesis was provided by Lindhe et al. (1992). In this paper ligature induced periodontitis and periimplantitis were compared in a dog model. The authors noted that the inflammatory infiltrate extended to the bone only in the periimplant tissues. Furthermore, after removal of the ligatures, healing was considerably delayed around the implant only. In contrast to implants, the vascular supply to the tissues immediately below the junctional epithelium in the teeth of monkeys is derived in part from vessels originating from the periodontal ligament space (Egelberg 1966). This relationship does not exist at osseointegrated implant sites and hence the different tissue response may be a reflection of this. Schou et al. (1993) proposed that the presence of periodontal ligament cells was in some way protective of the tissues. In their study experimental periodontitis was induced around ankylosed and normal teeth in monkeys. The tissue response and presence of osteoclasts was compared to that seen in similar conditions around titanium implant abutments. The authors noted an increased presence of osteoclasts in the bone adjacent to implants only.

Therefore, studies in animals have shown that connective tissue fibres can closely adapt to the implant abutment surface. However, in the absence of a root cementum, these fibres cannot attach to the implant surface. While some materials promote perpendicular alignment of these fibres, titanium generally promotes circular or parallel arrangements. In the presence of inflammation, this connective tissue arrangement does not appear to provide the same protection to the deeper tissues as seen adjacent to teeth with intact dentogingival fibres. These findings are in contrast with those of Klinge et al (1991) who reported on the findings of an unpublished study. It was claimed that substantially more bone loss occurred around teeth with ligature induced periodontitis than at implants in a dog model. Thus, it appears that the inflammatory response to bacterial plaque in the periimplant cuff of animals is different to that found around natural teeth. These findings have important implications for the placement and maintenance of implants in clinical implantology. Several studies have investigated the implant-connective tissue association in health and disease in humans.

(c) Human *in vivo* studies

The union between the collagenous fibrous tissues surrounding titanium implant abutments in health has been investigated by Thomsen and Ericsson (1985). These investigators found a close adaptation of collagen fibres to the titanium in the region most near the periosteum. More coronally, epithelial cell remnants and a fibrillar material separated the metal from the collagen. Thus a

junctional epithelial region over a connective tissue zone was described. This relationship is similar to those described in experimental animals (Lindhe et al. 1992, Berglundh et al. 1991). A similar relationship was noted by Hansson et al. (1983) when examining the tissues adjacent to clinically healthy titanium implants in humans. Fibroblasts were reported to be the dominant cell type close to the implant abutment near the alveolar bone crest, although macrophages and lymphocytes were also observed. Bundles of collagen filaments and fibrils were seen to approach the implant although a proteoglycan layer 20nm thick always separated both the cells and fibrils from the metal oxide surface. No indication of a toxic reaction could be seen in the cells bordering the implant or the adjacent connective tissues. The authors concluded that the connective tissue accepts the titanium surface rather than isolating it as a foreign body.

Early inflammatory changes in the periimplant connective tissues in humans were described by Lekholm et al. (1986). These changes resembled those seen in gingival tissues used for comparison. Seymour et al. (1989) investigated the inflammatory infiltrate within inflamed periimplant and gingival tissues in 13 patients. Using immunohistochemical analyses adapted from the study of periodontitis lesions, these authors could not identify differences in the lymphocytic composition of infiltrates adjacent to teeth or implants. The CD4 to CD8 ratio and the proportion of T cells to B cells indicated that the

periimplant lesion was a stable and well controlled response to microbial plaque.

In contrast, Sanz et al. (1991) reported the presence of an infiltrate dominated by mononuclear and plasma cells in biopsy specimens taken from around titanium implants exhibiting soft tissue inflammation. Transmission electron microscopy revealed a loss of periimplant fibres and fibroblasts, being replaced by the inflammatory infiltrate. Such histologic changes are akin to those described around natural teeth by Page and Schröder (1976) and implants in animals (Kurashina et al. 1984). The relative extent of inflammatory infiltration within periimplant tissues and gingiva has not been reported to date.

1.2.3 SUMMARY

Despite the obvious differences in the anatomy of teeth and titanium transmucosal implants, the soft tissue reaction to these surfaces appears quite similar. Oral epithelium attaches to the titanium abutment surface in health to form a junctional epithelium which extends only some of the way towards the alveolar bone. A shallow crevice is formed as the epithelium approaches the abutment surface. Connective tissue abuts to the implant surface beneath the epithelial surface. Direct attachment to the implant has only been described for titanium sprayed implants to date. In the presence of plaque associated

inflammation, epithelial cells respond by proliferation and thickening in a manner similar to that observed adjacent to teeth. The connective tissues are disrupted and replaced by an infiltrate containing lymphocytes. Animal studies indicate that the degree of tissue involvement in the presence of inflammation was greater at the implant than at the natural tooth. This may be a result of the lack of connective tissue attachment to the implant surface directly below the epithelium. In addition, the different vascular supply to the periimplant tissues may promote a different response to inflammatory mediators. More research into human tissue response to microbial plaque around implants is required to ascertain the potential for marginal bone destruction at these sites.

1.3 SUPPORT AND FUNCTION

1.3.1 TOOTH IN PERIODONTIUM

The gingiva *per se* does not contribute significantly to tooth support. Instead, it provides a biological seal for the underlying tissues from the oral environment through the junctional epithelium and the gingival connective tissue fibres. Support for the tooth is derived from its position within the alveolar bone socket. The attachment is mediated by collagen fibres of the periodontal ligament inserting into the cementum at an oblique angle.

Mühlemann (1967) described the periodontal ligament as a shock absorber for sudden forces applied to teeth. Experimental results suggest that shock absorption is mediated at various levels. Light forces are absorbed by compression of vascular channels forcing fluid out of the periodontal ligament and into vascular lumens within the fenestrated alveolar bone plate of the tooth socket. Three phases of recovery following a load on a tooth were described by Picton et al. (1974). The first phase is relatively fast and is the result of recovery following deformation of the bone. A slower second phase is associated with refilling of blood vessels. Finally, extrusion of the tooth results from the rehydration and polymerisation of proteoglycan molecules within the ligament. Hence a characteristic Voigt pattern is formed when displacement versus force is charted for a natural tooth. In contrast, ankylosed teeth produce a linear relation between force and displacement because they are merely supported by an elastic alveolus with a high spring constant and very little dampening effect.

Other functions of the periodontal ligament include proprioception and the provision of cells for the synthesis and maintenance of the mineralised tissues. It also acts as a 'double periosteum' which effectively interferes with ankylosis of bone to cementum, and permits physiologic and orthodontically induced movement of teeth through the bone (Listgarten et al. 1991).

In the absence of appropriate progenitor cells, it is not reasonable to expect a functional periodontal ligament to develop. Under conditions where progenitor cells were present, an attachment apparatus similar to that around natural teeth formed on titanium dental implants in monkeys (Buser et al.1990b). The authors concluded that a true periodontal ligament can be achieved by placing implants in close proximity to submerged roots. This finding, however, was based on a single case in a monkey and cannot be used to justify similar therapeutic measures in humans.

1.3.2 OSSEOINTEGRATION

The attachment of an implant to bone does not resemble that of the teeth. Bone tissue forms directly around osseointegrated implants to fit their contour precisely. At the molecular level the cells are always separated from the metal surface oxide of the implant by a thin layer of proteoglycans. Bower (1987) cautioned that periimplant is not periodontium and should not be expected to behave as such. This is illustrated clinically in that teeth have a slight physiological mobility and can be relocated using orthodontic forces. In contrast, osseointegrated implants are firmly united to the alveolus and cannot be relocated using orthodontics (Roberts et al. 1984). Super-eruption in teeth to re-establish occlusal contacts is also not seen with implants.

Mechanically, a threaded fixture prevents initial instability during the healing phase and provides a form of interlocking of the bone on a macroscopic scale that allows full development of the strength of the bone to shear and compressive forces. This close apposition of bone to the titanium implant is the essential feature that allows transmission of stress (a force per unit area) from the implant to the bone without any appreciable relative motion (Skalak 1983).

Mechanical properties of commonly used implant biomaterials are generally adequate to withstand physiologic loads without failure of the implant. Bone, however, is known to remodel in response to applied stress and the manner in which endosteal implants distribute stress to their supporting tissues is of importance for their stability and long term prognosis. Long term success of implants may be influenced by such parameters as the type and duration of loading, the macro-design of the implant and any superstructure devices. Biomechanical considerations were reviewed by Kasemo (1983), Skalak (1983) and Kasemo and Lausmaa (1985). However, a paucity of information exists in the published literature on these and other biomechanical variables.

1.4 WOUND HEALING AND CELL ADHESION

One of the factors contributing to a successful implant integration appears to be the nature of the soft tissue healing. An understanding of the soft tissue healing process may enable future clinicians to improve the prognosis of their implants. Brånemark osseointegrated titanium dental implants require two separate healing stages. The first stage of healing occurs with the exclusion of epithelium and involves the biologic union of alveolar bone to the implant structure (osseointegration). Many studies have examined the nature and ultrastructure of this healing event (Kasemo et al. 1985, Roberts et al. 1987). In contrast, little is known about the biochemical and molecular mechanisms which control or influence the second, soft tissue, healing phase at the implant abutment after its insertion.

During wound repair, the basal cells of the epithelium divide and migrate over a granulation tissue bed filling the defect. In order for junctional epithelial cells to retain this ability it is felt that they should remain relatively undifferentiated (Ten Cate 1985). Several studies have attempted to manipulate the soft tissue healing process against titanium implants in an effort to understand the mechanisms involved. Control of cellular functions is often mediated by local extracellular glycoproteins. Laminin and fibronectin are produced by epithelial cells and fibroblasts respectively. These extracellular matrix glycoproteins determine the activity of cells in the immediate vicinity. Lowenberg et al.

(1985) demonstrated improved fibroblastic attachment to titanium alloys coated with bovine collagen when compared to uncoated alloy. These findings support the work of Kleinman et al. (1981) who demonstrated that collagen-bound fibronectin and other glycoproteins provided a useful substratum for various cell attachments *in vitro*. Based on this work Von Recum et al. (1985) applied fibronectin and collagen to titanium abutment surfaces *in vivo* in an effort to promote fibroblastic proliferation and attachment. They found that the fibronectin actually retarded healing to the metal in both human and animal models. Such unexpected findings are occasionally found with in studies on use of this extracellular glycoprotein. In an extensive review of fibronectin and laminin by Hakomori et al. (1984) fibronectin was shown to have a wide range of effects on many cell types. Unfortunately, at this time the combined effects of these molecules on healing have yet to be completely elucidated and hence their clinical applications restricted.

Our understanding of the healing process within the soft tissues around transmucosal titanium implants is restricted to that which can be observed using light and electron microscopy. Some theories regarding the biochemical processes guiding the observed changes have been proposed though little proof exists at this time. The features of soft tissue healing to implants is reviewed below.

(i) Animal studies

McKinney et al. (1984) described the formation of a junctional epithelium from previously oral epithelium adjacent to newly placed implants in dogs. The epithelium extended pseudopodia towards and then along the material surface until a junctional epithelium was formed. The same group examined the epithelial attachment to dental implants in mongrel dogs with TEM the following year (McKinney et al. 1985). From these observations they theorised on the nature of the attachment formation. It was proposed that the glycosaminoglycans hyaluronic acid, heparin sulfate and heparin, which are produced by fibroblasts during the healing phase following implant insertion, coat the implant surface. Fibronectin, also produced by fibroblasts, is present in the extracellular matrix and could act to hold the cells to the nearby macromolecules. As the junctional epithelium regenerates, producing its own basal lamina and the glycoprotein laminin, fibronectin could provide the bond between the proteoglycans on the implant surface and the lamina densa of the basal lamina, hence the union of the tissues to the implant surface.

The effect of plaque on the healing of soft tissues following titanium implant placement in dogs was reported by Gotfredsen et al. (1991). The authors found an increased length of junctional epithelium in plaque infected compared to non-plaque infected implants. These findings are in keeping with those seen around teeth of humans following periodontal surgery (Nyman et al. 1977).

(ii) human studies

Schmid et al. (1991) and (1992) obtained biopsies of healthy periimplant mucosa adjacent to titanium implants and gingiva from human subjects. The tissues were assayed for plasminogen activity, which acts as a marker for junctional epithelial cells. The authors found that keratinised oral epithelium, when grown into a wound adjacent to a titanium dental implant, may form an epithelium that resembles junctional epithelium not only morphologically, but also with respect to plasminogen activator activity. They hypothesised that components of crevicular fluid act as trigger mechanisms for some epithelial cell behaviour including plasminogen activator production, irrespective of the cell's development origin or connective tissue base. This could explain how the epithelial attachment apparatus at implants may be expected to behave in a similar fashion to that of the dentogingival junction.

Therefore, healing studies have shown that a junctional epithelium can form *de novo* following the insertion of titanium implant abutments. The histologic processes involved and the biochemical controls of this process are presumed to be similar to those associated with healing of gingiva following its disruption. This data and the comparative histologic data regarding the anatomy of the gingival sulcus and the periimplant cuff presented earlier, support the notion that a similar environment may exist within the two sites in health and that these sites respond in a similar way to irritants derived from plaque bacteria within the crevice.

1.5 PHYSICAL CHARACTERISTICS OF IMPLANT SURFACES

1.5.1 SURFACE CHEMISTRY

Ceramic and metallic implant materials are, from a practical viewpoint, very closely related, because the chemical properties of metallic implants are governed by their surface oxides, which form a thin ceramic like coat on the metal surface.

A surface oxide layer, approximately 10Å thick, forms within seconds of exposure of the pure metal to air at room temperature. This film is formed due to the implant not being in thermal equilibrium with its surroundings, and because it is able to dissociate oxygen molecules in the air (Kasemo and Lausmaa. 1985). The oxide layer thickness is determined by kinetic factors such as the speed of oxygen and metal ion transport through the developing film. Titanium offers the attractive combination of a mechanically strong core (the metal) and a biologically compatible, very thin (oxide) surface coating. The proven biocompatibility of titanium is related to the high corrosion resistance of the surface oxide, its high dielectric constant, and the preparation procedure that produces a relatively clean surface (Kasemo 1983).

The possibility of titanium ion release from the implant *in vivo* was raised by Berry et al. (1992). Little is known about the interaction of different metals

from implants on specific oral micro-organisms. The precise antimicrobial action of many inhibitory compounds including the metals remains unclear. If the implant surface was able to selectively inhibit the adhesion of some microbial species while promote others, it could presumably alter the course of plaque formation and microbial succession. Hence, a whole new plaque ecology may develop on the implant surface which could either prove beneficial or destructive to the adjacent tissues. This hypothesis is in part supported by Berry et al. (1992) who found that various metals suppressed the growth and adhesion of disease associated bacteria on their surface *in vitro*. Insufficient data exists at this time to speculate further on the rôle surface chemistry may have in microbial colony selection at implants. Plaque formation studies have, however, failed to identify a clear difference in the primary colonisation and succession of bacterial plaques on newly placed implant abutments fabricated from various materials (Siegrist et al. 1991)

1.5.2 SURFACE TEXTURE

(i) Effect on Soft Tissue Attachment

The effect of implant surface texture depends on the scale at which it occurs. Irregularities of $\geq 1 \mu\text{m}$ enhance the macroscopic interlocking between bone and the implant surface. On the other hand, irregularities approaching the molecular dimension may interfere with atomic interaction due to steric interferences.

Soft tissue attachment to the implant abutments may also be affected by the surface roughness. Different surface roughness was believed to be the cause of greater epithelial cell attachment to hydroxyapatite than titanium in the *in vitro* study by Kasten et al. (1990). Conceivably, contact guidance of epithelial cells migrating across the clean abutment surface could be affected by an altered surface texture. Brunette et al. (1983) found that microscopic grooves made on a titanium coated silicone block were able to guide the migration of epithelial cells *in vitro*. The significance of this may not be borne out clinically because epithelial cell migration during healing of a gingival separation is along the tissue rather than the tooth (or implant) surface.

Schröder et al. (1981) described a perpendicular collagen fibre orientation to their plasma sprayed titanium implants while others described connective tissues running parallel to the implant surface (Gould et al. 1984). Listgarten et al. (1991) attributed this phenomenon to the different surface textures between these implants. Fibroblasts grown *in vitro* around titanium discs tend to become oriented perpendicular to porous discs, but assume a more parallel orientation to the surface of smooth discs (Inoue et al. 1987). Burchard et al. (1991), found that plasma sprayed titanium specimens consistently exhibited a higher number of attached fibroblast cells than either smooth or hydroxyapatite specimens. Fibroblasts attached to smooth surfaced specimens typically exhibited an orientation parallel to grooves in the titanium cylinder resulting from machine polishing. Hypothetically, surface grooves, machined for specific size and

orientation, could be used to encourage preferential attachment and migration of cells during the healing phase of newly placed implants (Brunette 1988).

The surface roughness of an abutment may have clinical implications in terms of the type of tissue attachment and seal achieved. No data has been published to date regarding the relative weaknesses of epithelial attachments to materials differing in their roughness only.

(ii) Effect on Plaque Accumulation

Factors such as surface roughness, contour and the general design of an implant system are of considerable clinical importance as they may contribute to plaque colonisation and retention both supra and submucosally, and hence affect the adequacy of patient performed plaque control.

Siegrist et al. (1991) conducted an *in vivo* study of early plaque formation against enamel, dentine, amalgam, gold, silver-palladium, chrome and ceramic pieces inserted into the mid-buccal area of a bridge pontic. Following microbial culture and SEM assessment, they concluded that the degree of surface roughness affected early quantitative plaque accumulation only. No effect was seen on the final quality or quantity of the plaque. These findings are in agreement with those of Nakazato et al. (1989) who found equivocal microbial colonisation on discs made of various implant materials and worn in the mouth for 48 hours. A small and transient difference in the 2 hour colonization was

noted. This difference was attributed to surface texture rather than surface free energies. Recently, Quirynen et al. (1993) performed an *in vivo* study on patients with fixed prostheses supported by endosseous titanium implants. Two titanium abutments (the transmucosal component) in each subject were replaced with either an unused standard smooth abutment or a roughened (by sandblasting) titanium abutment. Abutments were left *in situ* and retrieved after three months of habitual oral hygiene for examination of plaque by phase contrast microscopy, DNA probe analysis and culturing. Results indicated that supragingival rough surfaces harboured significantly fewer coccoid organisms (64% vs 81%) which is indicative of mature plaque. The presence and density of subgingival pathogens was more related to the patient's dental status rather than the surface characteristics of the abutments. Gatewood et al. (1993) compared supragingival plaque maturation on smooth and rough tooth and titanium surfaces. Small pieces of enamel and root were bonded to teeth of volunteers such that the root part was located within a natural periodontal pocket. A similar protocol was followed using a titanium piece which had been plasma sprayed at one end to simulate the rough root surface. Evaluation was done with a scanning electron microscope. The authors were unable to find a difference in morphotypic colonisation or succession on both supra and subgingival plaque regardless of surface roughness.

The combined findings of these studies indicate early microbial colonisation of titanium implant abutment surfaces follows approximately the same sequence

and succession as teeth. The texture of the material does not appear to have a significant effect on the quality or quantity of bacterial plaque formation. Although Siegrist et al. (1991) concluded that a smooth abutment design was more desirable, this may have to be weighed against the apparent connective tissue attachment advantages seen by Schröder et al. (1981). If a smooth surface provides no less resistance to plaque formation yet induces unfavourable connective tissue topography adjacent to the implant, our current preferences for machined abutments may require revision. Surface free energy variations between materials do not appear to influence plaque accumulation *in vivo*. Further research is needed to determine the effect of surface roughening by professional and self performed home care on plaque and soft tissue adhesion (Fox et al. 1990).

1.6 CLINICAL PARAMETERS USED TO EVALUATE HEALTH AND DISEASE

1.6.1 TEETH

Clinical parameters such as probing depth, plaque index, bleeding on probing and mobility have failed to reliably predict active periodontal disease (Haffajee et al. 1983). When these clinical tests were used on a selected subgroup of patients presumed to have active periodontitis, the predictive value of the

clinical tests improved only slightly (Halazonetis et al. 1989). Lang et al. (1990) was able to show that the absence of bleeding on probing was a reliable indicator of health.

Analysis of disease progression data against individual clinical signs does not encompass the true clinical situation where several tests and the operator's intuition are combined to make an assessment. In a recent study by Vanooteghem et al. (1990), three experienced periodontitis were asked to examine 11 patients and use all available clinical parameters as well as their clinical judgement to predict which sites will improve following treatment and which will show further attachment loss. Their findings after two to three years showed a positive predictive value of only 10%. This study was a test not only of a single indicator, but of an integrated collection of objective parameters and subjective interpretation of these by clinicians familiar with their limitations.

Thus, clinical assessment using conventional instruments does not afford the clinician an insight into current and future disease activity at a particular site. However, they do serve as a provisional indicator for establishing the previous disease experience. In addition, changes over time in these signs can indicate progression of disease, retrospectively.

1.6.2 IMPLANTS

Clinical criteria used to classify the periodontal condition have been widely adapted to the periimplant tissues. Although implant failures have been associated with the same clinical signs seen in periodontally involved gingiva (such as: gingivitis, suppuration, soft tissue oedema, bone loss on radiographs, and increased probing depths) a relationship between some of these observations and disease could not be established by Apse et al. (1991). Hence, these parameters can only be used as descriptive data, useful for comparative studies, but are no more likely to predict implant longevity than the progression of periodontal disease (Adell et al. 1981).

The evaluation of an implant differs to that of a tooth in several fundamental ways. Firstly, anatomical differences exist between the way in which these two structures interface with the surrounding soft tissues. These comparative differences were described in sections 1.2 and 1.3. Therefore, the interpretation of clinical signs used to define health and disease in teeth must be done with caution when applied to implants.

Secondly, the criteria for health and success in the implant do not match those of teeth. Albrektsson et al. (1986) proposed the following criteria for defining successful osseointegrated implants:

1. That an individual, unattached implant is immobile when tested clinically.
2. That a radiograph does not demonstrate any evidence of periimplant radiolucency.
3. That vertical bone loss is less than 0.2mm annually, following the implant's first year of service.
4. That individual implant performance be characterised by an absence of persistent and/or irreversible signs and symptoms such as pain, infections, neuropathies, paraesthesia or violation of the mandibular canal.
5. That in the context of the above, a success rate of 85% at the end of a five-year observation period and 80% at the end of a ten-year period be the minimum criterion for success.

Clearly, the lack of physiologic mobility in a tooth would alert the clinician to the possibility of ankylosis and replacement root resorption. In the converse, a mobile tooth may still be considered periodontally healthy if the degree of mobility can be accounted for by the functional load and periodontal support. Similarly, radiographic evidence of a space between the implant and the investing bone would not be interpreted as healthy periodontal ligament in the same manner as it would in a tooth.

Finally, the location of the soft tissue margin in the alveolar mucosa and the tendency for a larger area of inflammatory infiltrate preclude the comparison of marginal redness with teeth positioned within the attached gingiva. No evidence exists at this time to suggest that this larger area of infiltrate represents a more aggressive disease. Therefore, signs of marginal redness around an implant may give the clinician a false impression of the degree of tissue destruction occurring (Becker et al. 1990). A similar consideration became apparent in studies of the minimal width of attached gingiva in teeth (Myasato et al. 1977).

Albrektsson and Sennerby (1991) pointed out that implant survival status is often misinterpreted as success. A distinction was made between *survival*, which implied implants remaining in the jaws irrespective of the quality of survival and function and *success*, which relates to a predefined success criteria. Without such criteria, the patient's failure to return could be interpreted as continued satisfaction and hence success.

1.6.3 VALIDITY OF CLINICAL CRITERIA

The significance of common clinical tests for implants are reviewed below. The validity of these parameters for implants has only recently begun to be investigated.

(i) Probing Depth

Newman and Flemming (1988) and Haanaes (1990) both agree that periimplant pocketing does occur in disease. Cross sectional periodontal probing studies found that the average sulcus depth adjacent to titanium implants in man was approximately 2.8mm in the absence of inflammation and 3.8mm in the presence of minor inflammation and bleeding on probing (Lekholm et al. 1986, Bower et al. 1989, Buser et al. 1990a). Variation between studies is presumably due to the soft tissue management at stage II surgery. This is similar to the average probing depths measured by van der Velden and de Vries (1978) in human gingiva (2.1mm-3.7mm). This finding is in accord with the data presented by Listgarten et al. (1976), since in health the probe tip does not penetrate the epithelium. It could be presumed that in the presence of inflammation, probing depths should be deeper around implant sites as they do not have the inserting connective tissues which restrict probing depths in the natural dentition (Fowler et al. 1982).

Some implants and abutment designs do not facilitate accurate probing depth measurements, and the precise location of the probe tip in its apical placement is not known. Klinge (1991) studied the relationship of the periodontal probe to the radiographic bone level at fixture sites. Generally, he found that the tip of the probe was located several millimetres coronal to the radiographic bone level. Accuracy of periodontal probing around implants is generally higher than at teeth because of clearly defined landmarks (Newman and Flemming 1988). However, the connective tissue that forms around the threads has a tendency to direct the probe tip into the upper surface of each thread, making apical passage of the probe tip extremely difficult (Bauman et al. 1992). The mucoperiosteal flap thickness at surgery will influence the future pocket depth. Hence, absolute pocket depth is not necessarily indicative of implant failure (van Steenberghe 1988).

The reliability of probing depth changes at implants was reviewed by Newman and Flemming (1988). They felt that probing depth measurements appeared to have similar specificity, but lower sensitivity for disease activity around implants compared to teeth. Apse et al. (1991) reported a progressive reduction in probing depths around Brånemark titanium implants over a three years period. This finding is supported by the similar conclusions of Lekholm et al. (1986) and Bower et al. (1989). Quirynen et al. (1991) compared probing depth values around Brånemark implants with radiographic bone loss over 4-5 years using duplicate measuring with a pressure sensitive probe. They found a high

intra-examiner correlation (standard deviation = 0.4mm) as well as a Pearson correlation with bone heights of 0.76. In addition, they found that the average distance of the probe tip to bone was 1.4mm. This value presumably corresponds to the height of connective tissue apposition to the implant.

Periodontal probing around osseointegrated titanium implants has been shown to be highly reproducible and correlated with radiographic changes. Periimplant probing depths will increase with disease progression. Periodontal probing retains its rôle as a diagnostic aid but is not indicated at this time for assessing disease severity or progression.

(ii) Marginal Erythema

Controversy over the need for keratinised mucosa around abutments has arisen from observations of clinically healthy movable mucosa appearing traumatised by marginal fixture threading following some bone resorption (Adell et al. 1981). There may, however, be no justification in such concern where adequate hygiene can be achieved and maintained. The clinical health of gingival tissue was independent of the presence or width of keratinised gingiva or the height of periodontal support (Wennström and Lindhe 1983). Similar observations were described for osseointegrated implants in humans and dogs (Adell et al. 1986, Strub et al. 1988).

A longitudinal prospective and a cross sectional retrospective study was undertaken to investigate marginal tissue reactions at osseointegrated titanium fixtures and their abutments supporting fixed bridges. Conventional clinical periodontal examination methods failed to provide the examiner with a full description of the condition of the soft tissues adjacent to fixture abutments (Adell et al. 1985).

Becker et al. (1990) were unable to correlate the amount of plaque accumulation around implants with soft tissue inflammation. A wide variation appeared to exist for both parameters between subjects. Similarly, Apse et al. (1991) found an inverse relationship between the presence of plaque at the cuff margin and the presence of inflammation. The nature of the tissues surrounding abutments and the often extensive suprastructures fixed to these implants makes this diagnostic criteria somewhat subjective in nature. Other information gathered along with the visual examination would improve the diagnostic sensitivity for periimplant infection.

(iii) Bleeding on Probing.

The use of bleeding on probing as an indicator of disease at implant sites has not been standardised. Hence, every paper reports the use of different criteria and scoring for this observation. The Gingival index of Löe and Silness (1963) is used by Rams et al. (1984) and Becker et al. (1990) while others simply note the presence or absence of bleeding on probing (Lekholm et al. 1986, Rams et

al. 1991 and Lindhe et al. 1992). Mombelli et al. (1987) and Ong et al. (1992) used a modification of the Gingival Sulcus Bleeding Index of Mühlemann and Son (1971) while Rams and Link (1983) did not record the gingival condition at all. Therefore, the choice of bleeding on probing as an assessment of gingival condition is widely varied among the current literature. Although comparisons cannot be made across studies using different indices, the consensus of these papers is that frank bleeding on probing and suppuration can be correlated with breakdown around implants. Lekholm et al. (1986) reported a correlation between the presence of bleeding on probing and radiographic changes around Brånemark implants.

(iv) Mobility

Albrektsson et al. (1986) described the absence of clinically detected mobility as a criteria for success. Most studies examining implants in health or disease do not describe the mobility of the implants being examined at all (Rams and Link 1983, Adell et al. 1986, Apse et al. 1989 and 1991, Rams et al. 1991, Steflik et al. 1991, Lindhe et al. 1992). In addition, some authors could not report the mobility of implants due to their connection to other teeth and implants (Rams et al. 1991). Koth et al. (1985) report the use of a Wasserman et al. (1973) modification of the Miller index to assess mobility but admit they did not remove the prosthesis prior to this assessment.

Mobility of osseointegrated implants was assessed by Sekine et al. (1986). Minor movement, presumably a function of the flexion of the investing bone could be detected. This movement did not appear to vary with fixture length. Thus, implants may sustain extensive bone loss without increased clinical changes in mobility. Hence, the sensitivity of this test is considered low although its specificity is very high. The implication is that longitudinal studies using change in mobility alone may underestimate the number of failing implants. The lack of physiologic mobility of implants has meant the development of a variety of indices. Becker et al. (1990) simply recorded the presence or absence of mobility while Mombelli et al. (1987) described a three level criteria. A Score of 0 represented a "rock solid" implant. A score of 1 was assigned to an implant showing minimal displacement with the application of 1N force. A score of 2 was assigned to an implant exhibiting apparent mobility.

(v) Radiography

Radiographic interpretation of bone loss at the implant periphery is highly sensitive and specific for progressing attachment loss at implant sites. Stability of bone levels has been used as a criteria for success (Adell et al. 1981, Adell et al. 1985, Albrektsson et al. 1986). The definite topographical structures on the implant allow a high degree of comparison with previous radiographs. Adell et al. (1981) and Lekholm et al. (1986) approximated that 1mm of marginal bone is lost in the first year following clinical loading and subsequently 0.1mm is

lost each year after that. However, greater bone loss is often observed around Bränemark implants in the maxillary than the mandibular arch (Quirynen et al. 1992).

Recently, Jeffcoat and co-workers (1992) described the adaptation of quantitative digital subtraction radiography for the assessment of *in vitro* periimplant bone change. They were able to detect bone loss of less than 25mg with a sensitivity and specificity of 0.94 and 0.97 respectively. Such an application will have significant benefit in the early detection of failing implants when readily available.

(vi) Summary

Nine year results from edentulous patients have lead Apse et al. (1991) to conclude that periodontal indices (plaque index, keratinised mucosa index, gingival index, and probing depth), when applied to osseointegrated periimplant mucosa, are of limited value and may be of little clinical significance when considered as measures of success or failure of osseointegrated dental implants. Mobility tests of each fixture and radiographic examinations remain the most reliable longitudinal examination methods. Success should reflect maintenance of a stable level of osseointegration around fixtures in clinical function. Excessive occlusal loading, microbial infection or a combination of these factors, have been associated with loss of implant integration during clinical service.

1.6.4 DISEASE DEFINITIONS

(i) Ailing Implant

Meffert (1992) defined the ailing implant as one which exhibits bone loss with pocketing although currently appearing static at the 3 - 4 month maintenance checks. Other clinical signs of inflammation may also be present. There is no clinically detectable mobility in the ailing implant. The term "ailing" implies that there is still some way left to manage or improve the prognosis of the fixture.

(ii) Failing Implant

The failing implant may show bone loss, pocketing, bleeding on probing, purulence, and indications that the bone loss patterns are progressing irrespective of therapy. There is clinically detectable mobility associated with the failing or failed implant which is analogous to a hopeless prognosis.

1.7 MICROBIOLOGY

1.7.1 PLAQUE FORMATION

The clean tooth surface is colonised by oral micro-organisms within an hour of the formation of the acquired pellicle. Within 4 hours there are approximately 10^3 - 10^4 bacteria per mm^3 on the tooth surface (Nyvad and Kilian 1987).

Socransky et al. (1977) described the bacterial succession during plaque formation on human teeth. Early colonisation was by Gram positive cocci (*S. sanguis*) and short rods (*Actinomyces* species and *Veillonella*). Gram positive rods increase to comprise about 25% of 1-3 day old plaque. Anaerobic Gram negative species and spirochetes were virtually absent in early plaque, comprising about 4-7% in 1-3 day old plaques (Löe et al. 1965). The acquisition of a bacterial plaque on the tooth surface is unavoidable. However, the presence of this plaque can be associated with health. Slots (1977a) demonstrated that a plaque containing predominantly facultative anaerobic cocci and rods such as *Actinobacillus viscosus*, and *Streptococci* species was associated with healthy gingiva.

After a week of undisturbed plaque accumulation the plaque composition begins to change. Anaerobic species and filamentous organisms begin to appear and their relative proportions increase just prior to the onset of gingivitis (Löe et al. 1965, Theilade et al. 1966). Gingivitis is believed by van Palenstein-Helderman (1981) to be a result of an increased total plaque mass at the gingival margin rather than a specific plaque composition.

The colonisation of titanium implant materials *in vivo* has been studied in humans by Gatewood et al. (1993) (see section 1.5.2(ii)). They reported a similar sequence of microbial adhesion and succession on tooth and titanium pieces for up to 10 days. Early plaque on the titanium surface was composed

of coccoid and short rod forms. Filamentous forms appeared within 5 days and spirochetes within 7 days. No difference was seen in the rate of colonisation between the tooth and the implant surfaces. Hence, plaque micro-organisms appear to be able to persist in the periimplant crevice in a similar manner to those found in the gingival sulcus. These findings are in agreement with *in vitro* studies showing the plaque formation and succession on titanium surfaces (Nakazato et al. 1989, Siegrist et al. 1991). Rams et al. (1991) evaluated the microbial plaque around titanium and HA-coated implants after 7-10 months of function. They found a predominance of *Streptococcal* species in healthy/stable implant sites. This is in agreement with Mombelli and Mericske-Stern (1990) who reported over 70% of cultivable species in healthy periimplant sites comprised of Gram positive facultative anaerobic cocci and rods 2 years after implantation. Periodontopathic species accounted for only 7.3% of the total flora. This is consistent with Ong et al. (1992) who found *Porphyromonas gingivalis* (Pg), *Actinobacillus actinomycetemcomitans* (Aa), and *Prevotella intermedia* (Pi) were very infrequently associated with healthy implant sites. Similar findings were reported after a three year study by Bower et al (1989). Thus, the periimplant crevice appears to harbour similar facultative micro-organisms in health as seen in the gingival sulcus.

1.7.2 PLAQUE ASSOCIATED WITH DISEASE

Cross sectional studies have shown that the microflora associated with periodontal disease is quite different to those associated with health or gingivitis. The predominant cultivable micro-organisms in the periodontal pocket were identified as Gram negative anaerobic rods such as *Fusobacterium* species and *Bacteroides* species (Slots 1977b). Morphologic studies have shown a predominance of motile rods and spirochetes (Listgarten and Helldén 1978). Normally associated with juvenile periodontitis (Zambon et al. 1983), recent reports have isolated *Aa* from adult periodontitis sites (Savitt and Socransky 1984). A more detailed analysis of the anaerobic species in the periodontal pocket has been provided by Moore (1987) and Dzink et al. (1988).

The microbiota at failing implants was compared to healthy implant sites by Mombelli et al (1987). In this study, coccoid forms accounted for 95% of total microbial count in healthy sites and spirochetes were never found. In contrast, failing implant sites demonstrated a significantly increased proportion of spirochetes, fusiform and motile rods. The predominant species were determined to be *Pi* and *Fusobacterium* species. These findings are in agreement with previous studies on the predominant microflora around failing implants (Rams and Link 1983). Rams et al. (1984) found the proportion of spirochetes in health and disease was 2.3% and 32% of total counts respectively. Recent cross sectional studies have also shown an increased

proportion of periodontopathic organisms associated with failing implant sites. Alcoforado et al. (1991) reported on the microbial findings at 26 failing implants from 17 patients using culturing techniques. They found *Bacteoides* species, *Fusobacterium* species, *Wolinella* species as well as *Aa* in the crevices of some implants while others harboured enteric Gram negative rods. Becker et al. (1990) used DNA probes to identify the presence of *Aa*, *Pi* and *Pg* in the crevices around 15 failing implants. They found these micro-organisms to be present in 27.8%, 35.4% and 37.5% of the sites respectively. However, only 5 Brånemark implants from three patients were examined in this study. These did not harbour *Pi* or *Pg* at all. This may represent a difference in the Brånemark system or simply be the result of low numbers of samples. Larger study populations are required to clarify this difference.

A longitudinal study of one edentulous patient who had plaque samples taken regularly from initially healthy implant sites for 120 days was reported by Mombelli et al. (1988). This study found *Fusobacterium* species appeared within 42 days and small spirochetes appeared within 4 months. These changes corresponded with changed clinical signs such as pocketing, erythema and suppuration. Hence, some periodontopathic organisms can colonise the periimplant crevice within 2-3 months. This microbial succession is in agreement with observed microbial changes in the periimplant crevice of animals during experimental periimplantitis. Leonhardt et al. (1992) compared the microbial changes from health to gingivitis and health to experimental

periodontitis in dogs. These microbial changes were compared to those seen at Brånemark implant sites under-going similar experimental conditions. They found the proportions of *Streptococci* species at healthy tooth and implant sites decreased from 60.6% and 40.2% respectively to less than 1% in disease. On the other hand, *Pi* and *Pg* counts increased at both tooth and implant sites in the presence of disease. The authors concluded that, "microbial colonisation and establishment on titanium implants with healthy gingiva, experimental gingivitis and periodontitis follow the same patterns as on teeth".

The microbial etiology of periimplantitis has recently been further supported by Mombelli and Lang (1992). In this report, periimplantitis was suppressed by the topical application of 0.5% chlorhexidine in addition to a 10 day course of 1000mg Ornidazole (a long acting Metronidazole analogue). Clinical signs of disease improved over the first six months and were accompanied by a change in the microflora. In the second six months clinical signs returned and the microflora once again resembled that prior to antibiotic therapy.

Therefore, studies examining the microbiota of healthy and disease associated implants show a marked similarity to teeth at a comparable clinical stage. However, these studies do not address the issue of acquisition of these micro-organisms at the implant sites. Many studies do not mention the clinical status of other teeth in the mouths of patients with failing implants or do not mention the presence of teeth at all (Rams and Link 1983, Rams et al. 1984, Becker et

al. 1990). Recent attention has turned to the issue of cross-infection between periodontally infected teeth and implant abutment crevices in partially edentulous patients.

1.7.3 PARTIALLY EDENTULOUS STUDIES

Early studies on the microflora associated with the periimplant cuff were conducted on patients who were edentulous. Therefore, they had for some time, an altered oral environment and microflora devoid of such micro-organisms as spirochetes (Adell et al. 1986). With the development of single tooth and short span implant techniques, the opportunity of completely eliminating periodontal pathogenic micro-organisms from the oral cavity has been lost. The potential for colonisation of the periimplant cuff by micro-organisms whose normal ecological niche is the periodontal pocket may be greater. Several studies have investigated this potential.

Leonhardt et al. (1992) evaluated the crevicular and periimplant microbiota in health, gingivitis, and periodontitis using microbial culture techniques. No significant difference in the proportions of micro-organisms between teeth and implants at each stage of the experiment was found in this dog model. This data suggest a similar dynamics in the bacterial colonisation of these two types of lesion. The question of a causal relationship of the microbial flora and the pathology of the lesions remained unanswered. The extrapolation of this

animal data to the human situation has not been confirmed. However, a considerable body of evidence from cross sectional studies demonstrate a similarity in the type of micro-organisms occurring in lesions around natural teeth compared with those around implants (Mombelli et al. 1987). These findings appear to indicate that the environment in the periimplant cuff is the same as that in the gingival crevice and one may be a source of micro-organisms for the other.

A longitudinal assessment of plaque composition at newly placed Brånemark implant abutment sites was recently reported by Koka et al. (1993). The microflora at 10 osseointegrated implants from 4 patients were sampled at baseline, 14 and 28 days and the presence of six plaque organisms compared to that at nearby teeth. The authors found subgingival colonisation occurred between 14 and 28 days for *Pg*, *Pi*, *Fusobacterium nucleatum* (*Fn*), *Actinomyces viscosus*, and *Treponema socranskii* but not *Treponema denticola* (*Td*). The results of this study cannot be easily compared with other studies describing the acquisition of periodontopathic microflora in edentulous patients due to the many differences in study design (Mombelli et al. 1988). Such a comparison would be of great value in the assessment of the role of infected tooth sites in the acquisition of periodontopathic organisms at implant sites.

Recent studies have attempted to distinguish the microbial plaque composition at implant sites between edentulous and partially dentate patients. Apse et al. (1989) compared the subgingival flora of 15 partially dentate patients with implants to 6 edentulous patients with implants using culture and dark field microscopy. They found more motile rods in the edentulous patients, but more *Bacteroides* species and "wet spreaders" in the partially dentate cases. Notably, no statistically significant difference was found in the crevicular microflora between samples derived from teeth and those derived from implants in partially dentate patients whereas there was between implants in dentate versus edentulous cases. It was concluded by the authors that the gingival crevice of teeth acts as a reservoir of periodontal pathogens for the periimplant cuff.

In a similar study, Quirynen et al. (1990) found bacterial morphotypes at teeth and implants from partially dentate mouths were in approximate proportion whereas those taken from implants in edentulous mouths contained proportionately fewer motile rods and spirochetes and more coccoid groups. All patients selected for this study received Brånemark implants following periodontal hygiene and maintenance therapy. However, the pooling of data across patients did not allow analysis of the relative microflora at teeth and implants within the same mouths.

From the cross-sectional studies demonstrating a greater presence of periodontopathic organisms at implant sites of partially dentate versus

edentulous patients, it may be predicted that plaque associated implant failure would be more prevalent in the presence of teeth. This hypothesis was confirmed by Rosenberg et al. (1991) who found 6 of 22 (27.3%) failing implants in fully edentulous patients were lost due to infection compared with 6 of 10 (60%) failing implants in partially dentate patients. Such preliminary findings, although suggestive of a relationship between failing implants and the presence of teeth, require support from further studies in which the patient rather than the site category is the unit of investigation. In this way it can be determined whether the failing implants are occurring in the same mouth as periodontally involved teeth.

1.8 SUMMARY

The predictable long-term success of osseointegrated dental implants has been attributed to a number of factors (Adell et al. 1986), including the use of biocompatible materials such as titanium (Albrektsson et al. 1982), gentle surgical techniques, a long healing time and proper stress distribution when in function (Adell et al. 1981). Despite successful osseointegration in the first instance, implant failures have occurred for no immediately obvious reason during subsequent clinical function. Several reports (Mombelli et al. 1987, Becker et al. 1990, Rosenberg et al. 1991) have indicated that following successful osseointegration dental implants can suffer from progressive bone loss and eventual exfoliation in a manner reminiscent of that seen in chronic

inflammatory periodontal disease. Clinical criteria used for monitoring periodontal diseases have some value in assessing the health of the periimplant tissues although the interpretation of these signs must be done cautiously due to the differences in anatomy between teeth and implants. Recently, several reports have identified an association between the presence of periodontopathic micro-organisms in the periimplant crevice and the presence of teeth.

Taken together, the data support the concept that periodontal pockets around natural teeth can harbour a variety of periodontopathic organisms which may act as a source of cross-infection for other tooth or implant sites. In order to answer these important questions, the study described in this thesis proposes to examine the dynamics of colonisation of periodontopathic organisms at implant sites and the possibility of cross infection from tooth to implant sites in the same mouth.

1.9 HYPOTHESES

This thesis examines the following hypotheses:-

- (1) That there is an increasing presence of putative periodontopathic organisms with increasing clinical inflammation of soft tissues at both tooth and implant sites.
- (2) That the periodontopathic organisms from tooth sites can cross-infect implant sites in the same mouth.

CHAPTER TWO

MATERIALS AND METHODS

2.1 RESEARCH PLAN

The hypotheses as stated in Section 1.9 are:

- (1) That there is an increasing presence of putative periodontopathic organisms with increasing clinical inflammation of soft tissues at both tooth and implant sites.
- (2) That the periodontopathic organisms from tooth sites can cross-infect implant sites in the same mouth.

Hypothesis 1 was tested by comparing plaque samples from tooth and implant sites in three parallel (total of six) clinical categories for the presence of seven putative periodontopathic organisms: *Actinobacillus actinomycetemcomitans* (Aa), *Porphyromonas gingivalis* (Pg), *Prevotella intermedia* (Pi), *Eikenella corrodens* (Ec), *Fusobacterium nucleatum* (Fn), *Treponema denticola* (Td) and *Campylobacter recta* (Cr).

Hypothesis 2 was tested by comparing the presence of the seven putative periodontopathic organisms between tooth and implant sites in the same mouth. A model was used for testing "support" or "rejection" of the second hypothesis of cross-infection from tooth to implant sites in the same mouth.

2.2 STUDY POPULATION

Subjects with at least one Brånemark osseointegrated titanium dental implant were recruited from the Dental Implant Centre (United Dental Hospital Sydney), the private practices of members of the Australian Society of Osseointegration (NSW) Inc. and Australian Society of Implant Dentistry (NSW Branch).

A total of 20 subjects (13 male, 7 female) aged 45 to 73 years (mean $\pm$ standard deviation, 56.7 ± 8.8 ; median 54 years) participated in the study. These 20 subjects were used to test the first hypothesis (31 teeth and 43 implant). A subpopulation of 16 subjects (31 teeth and 28 implants) who had both natural teeth and implant sites in the same mouth was selected to test the second hypothesis. Informed consent was obtained from all subjects and their respective clinicians after explanation of the research plan.

2.2.1 SELECTION CRITERIA

Subjects were required to have at least one Brånemark titanium implant present a minimum of two weeks post stage II surgery. Access to periimplant tissues for plaque sampling using endodontic paper points was imperative. Test sites were selected from the maxilla and mandible to reflect the range of soft tissue health and/or disease at tooth and implant sites in each mouth.

Subjects with a history of rheumatic fever, congenital heart conditions, diabetes, thyroid disorder, blood dyscrasias, chronic liver or kidney disease, chemotherapy or radiotherapy (immunosuppressive therapy) were not accepted into the study. All subjects were in good medical health and had not received antibiotics in the previous three months.

2.2.2 ORAL EXAMINATION

Oral examination was carried out for each test site and the following information was recorded:

(i) Plaque Index

The Plaque Index (PI) (Silness and Løe 1964) was used to score all test sites where:

0 = no plaque

1 = no visible plaque, detection by sharp probe in gingival third

2 = visible plaque in gingival third

3 = abundant plaque on tooth or implant

(ii) Periodontal Charting

A periodontal examination was performed for all teeth and implants present.

Periodontal probing was carried out with a Williams^a periodontal probe and furcations assessed with an unmarked 2N Nabers^a probe. Eflux of exudate from the base of the sulcus within 10 seconds of probing was recorded as either bleeding or suppuration.

(iii) Soft Tissue Redness

Soft tissue redness of each test site was recorded as present or absent at the time of examination. Colour photographic slides of all test sites were taken at the time of examination and later used to check and standardise recordings for the various clinical parameters.

2.2.3 CLINICAL GROUPING

Clinical criteria were used to categorise tooth sites into healthy (TH), gingivitis (TG), and periodontitis (TP). Similarly, implant sites were grouped as healthy (IH), periimplantitis (IPi) and ailing (IA).

TOOTH SITES

Healthy (TH)

Gingivitis (TG)

Periodontitis (TP)

IMPLANT SITES

Healthy (IH)

Periimplantitis (IPi)

Ailing (IA)

^aHu-Friedy - Chicago USA

Each of the test sites were assigned a clinical category. Healthy teeth (TH), showed no clinical signs of soft tissue inflammation. Similarly, healthy implants (IH) showed no clinical signs of soft tissue inflammation.

Teeth with gingivitis (TG), had gingival erythema, oedema and bleeding on probing from the base of the sulcus within 10 seconds after probing, but no periodontal pocketing $\geq 5\text{mm}$. Implants placed in the periimplantitis (IPi) category clinically exhibited periimplant soft tissue inflammation, and bleeding within 10 seconds after probing to the base of the sulcus. The depth of the periimplant sulcus taken at one point in time was not used to categorise implant sites as a single measurement of periimplant sulcus may merely represent soft tissue thickness.

Teeth in the periodontitis category (TP) exhibited gingival erythema, bleeding/suppuration from the base of the sulcus and one or more of: a periodontal probing depth of $\geq 5\text{mm}$; radiographic evidence of bone loss; or mobility; or pain. Soft tissue erythema and bleeding on probing plus one or more of the other criteria were sufficient to categorise teeth into TP category. Implants were categorised as ailing (IA) if there was evidence of mobility, pain or radiographic evidence of bone loss around implants of $>0.2\text{mm}$ after the first year in addition to periimplant soft tissue inflammation. The clinical classification is summarised in Table 2.1.

Table 2.1: Clinical criteria used to categorise tooth and implant sites

	TH	TG	TP [†]	IH	IPi	IA [†]
REDNESS ^{††}		✓	✓		✓	✓
B.O.P. [§]		✓	✓		✓	✓
SUPPURATION			†			†
PROBING DEPTH ≥ 5MM			✓	N/A	N/A	N/A
RADIOGRAPHIC [‡]			✓			✓
MOBILITY			†			†
PAIN						†

✓= denotes the presence of clinical sign as identified in the left column.
Blank box= denotes the absence of clinical sign as identified in the left column.

TH = healthy teeth

IH = healthy implants

TG = teeth with gingivitis

IPi = implants with periimplantitis

TP = periodontitis teeth

IA = ailing implants

†† Soft tissue redness at each test site was noted at the time of periodontal examination and compared with the other test sites using the colour slides.

§ Bleeding on probing from the base of the sulcus up to 10 seconds after probing.

‡ Radiographic evidence of bone loss around teeth; a periimplant radiolucency or >0.2mm annual vertical bone loss around implants after the first year.

† Soft tissue redness and bleeding on probing, plus one or more of the other criteria were sufficient to categorise teeth into TP and implants into IA categories.

2.2.4 PLAQUE SAMPLING

Supragingival hard and soft deposits were removed at tooth sites using a 4L/4R Columbia Universal curette^a. Plaque was removed from implant abutment surfaces using cotton pellets held by College tweezers^a.

For the sampling of subgingival plaque, the test site was isolated with cotton rolls and gently air dried. Two #50 (medium) sterile endodontic absorbent paper points were inserted simultaneously into the bottom of the sulcus until resistance was felt. The paper points were left *in situ* for 10 seconds prior to transferring to a specimen collection vial (1.5mL Eppendorf tube). The specimen collection vial was identified by bar code labelling.

If specimens were to be stored longer than one month prior to being dispatched, 150µL of a transport medium was placed into each Eppendorf tube prior to specimen collection. Following specimen collection, paper points were placed into the Eppendorf tubes and frozen at -70°C. Specimens were sent in batches to the laboratory^b packed in dry ice.

^aHu-Friedy - Chicago USA

^bOmniGene®, Inc. Cambridge, MA. USA.

2.3 DNA PROBES

2.3.1 INTRODUCTION

DNA probes specific for *A. actinomycetemcomitans* (Aa), *P. gingivalis* (Pg), *P. intermedia* (Pi), *E. corrodens* (Ec), *F. nucleatum* (Fn), *T. denticola* (Td) and *C. recta* (Cr) were used to identify the presence of each organism at sampled sites. Each DNA probe consists of a single stranded radioactively labelled sequence of nucleic acids that is complementary to a unique region of the target organism's genome. Hence, this labelled probe has the ability to bind to complementary sequences of DNA to form a double helix. The hybridised DNA segment (double helix) is then allowed to incubate and expose a photographic film. The size and intensity of the resultant captured zone/band can be compared with a known standard, thus allowing for an estimation of the concentration of the organism in the sample.

Nucleic acid probes can be whole-genomic or cloned. Whole genomic probes are constructed from the entire genome of the target microorganism and are more sensitive because the entire genome is used as the possible hybridisation sites. Cloned probes are isolated sequences of DNA that do not show cross-reactivity and are produced in quantity by cloning in a plasmid vector within a temporary host microorganism such as *Escherichia coli* (Savitt et al. 1990).

Cloned probes can approach the sensitivity of whole genomic probes (French et al. 1986), and can be manufactured cost effectively.

The selected DNA probes for putative periodontal pathogens are highly specific in activity showing minimal (<1%) cross-reactivity with similar organisms (Savitt et al. 1988). They are a more rapid and more specific method than conventional culturing techniques or monoclonal antibodies using ELISA for detection these organisms (Kornman et al. 1992). The detection limit of ^{32}P labelled DNA probes is approximately 10^3 - 10^4 organisms. Sensitivity and specificity of the selected DNA probes for periodontal pathogens are shown in Table 2.2

Quantitation of target organisms reported by the laboratory was derived from the degree of hybridisation between DNA probes and their complementary strands in each sample. It is estimated that paper point samples become saturated with approximately 10^6 bacterial cells (Slots and Rosling 1983), hence the relative proportions of each organism can be estimated.

Table 2.2: Sensitivity and Specificity[†] of OmniGene's DNA Probe Tests for Periodontal Pathogens.

BACTERIAL SPECIES	PROBE TYPE	SENSITIVITY *	SPECIFICITY **
Aa	cloned	100 % (15/15)	92 % (114/124)
Pg	whole	96 % (14/15)	92 % (96/104)
Pi	whole	100 % (5/5)	97 % (110/111)
Ec	cloned	100 % (5/5)	82 % (66/81)
Fn	cloned	100 % (7/7)	99 % (80/81)
Td	whole	ND	ND
Cr	cloned	100 % (4/4)	98 % (79/81)

[†] Sensitivity and specificity are determined using *in vitro* grown cultures.

* Sensitivity calculated as TRUE POSITIVE/TRUE POSITIVE + FALSE NEGATIVE.

**Specificity calculated as TRUE NEGATIVE/TRUE NEGATIVE + FALSE POSITIVE.

ND = Not done

2.3.2 VALIDATION OF SPECIFICITY OF DNA PROBES

A series of six pure and three mixed samples derived from pure *in vitro* cultures of six of the seven test organisms were forwarded to the laboratory^a as mock patient samples for validation of DNA probe specificity. Pure cultures of *Aa*, *Pg*, *Pi*, *Ec*, *Fn* and *Cr* were isolated, grown and identified more definitively in the following manner:

(i) *Actinobacillus actinomycetemcomitans*

Culture medium: BHV^b and Blood Agar^c plates.

Growth conditions: Both CO₂ (5% CO₂, temperature 37°C and humid conditions) enriched environment and anaerobic chamber (5% CO₂, 10% Hydrogen, 85% Nitrogen and temperature 37°C).

Colony morphology: Small grey convex colonies that appeared to grow better under CO₂ conditions. At 24 hours colony size was 0.1 - 0.5mm diameter by 72 hours colony size under CO₂ conditions had increased to ≥1mm diameter. Plate

2.1a

^aOmniGene®, Inc. Cambridge, MA. USA.

^bSee appendix 1 for formulation

Gram stain: Small Gram negative coccobacilli (short rods) Plate 2.1b

Motility: Non motile (using hanging drop technique)

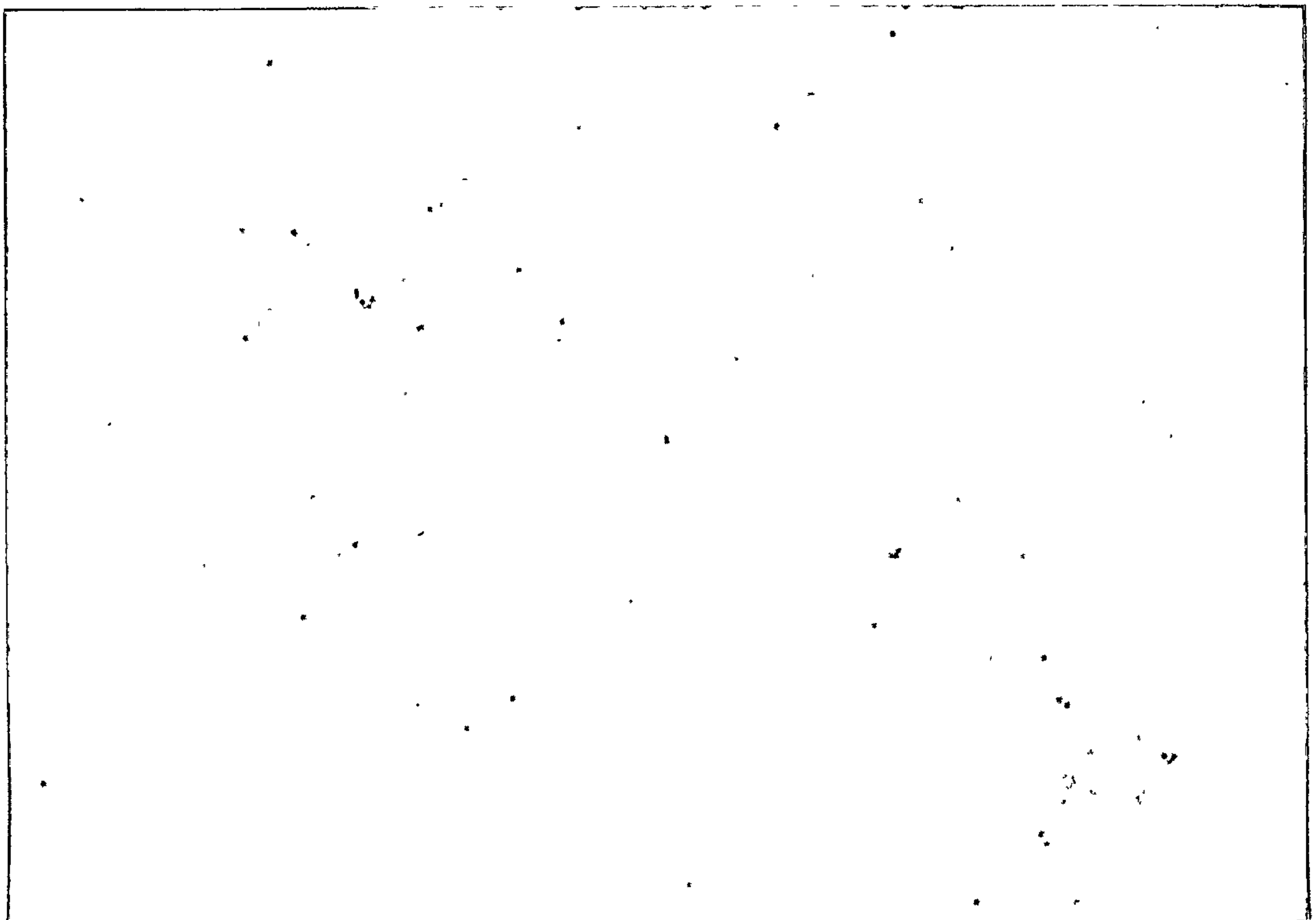
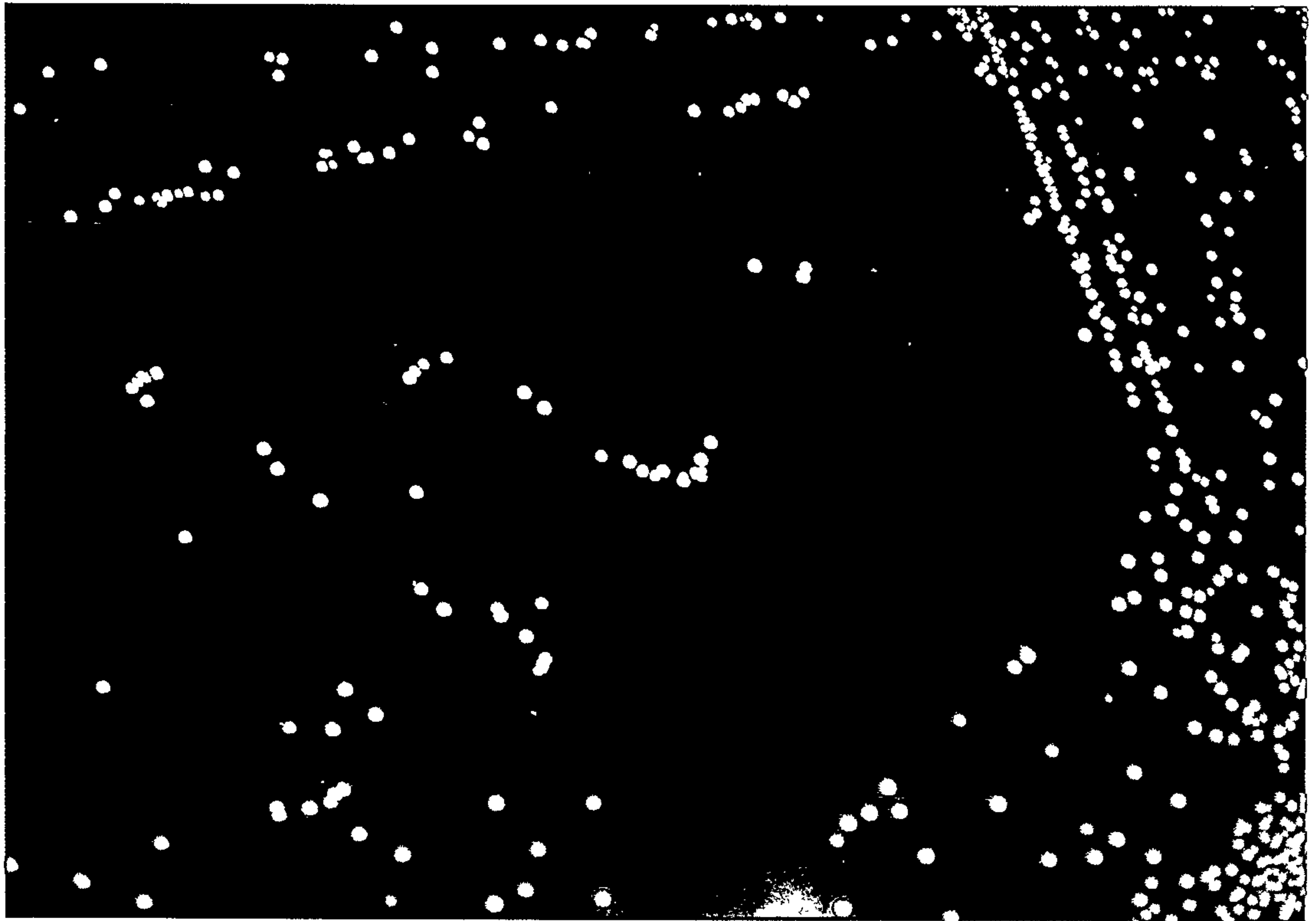
Biochemical tests for identification: Glucose +, Maltose + Catalase -, Indole +,

Nitrate reducing, Oxidase + colonies were definitively identified as *A.*

actinomycetemcomitans (see also Table 3 appendix 4).

Plate 2.1a: *Actinobacillus actinomycetemcomitans* colonies grown on BHV agar plates for 48 hours in a CO₂ environment.

Plate 2.1b: Gram stain of *Actinobacillus actinomycetemcomitans*. A Gram negative coccobacillus, it is characteristic for the individual *Aa* organisms to "clump" the Gram stain.



(ii) *Porphyromonas gingivalis* (W50)

Culture medium: BHV^a plates.

Growth conditions: In an anaerobic chamber (5% CO₂, 10% Hydrogen, 85% Nitrogen and temperature 37°C).

Colony morphology: Fine 0.1mm diameter buff coloured colonies appeared at 24 - 48 hours. At 72 hours colony size was approximately 1mm diameter, colony colour was brown to black, and haemolysis of the agar was evident. Plate 2.2a

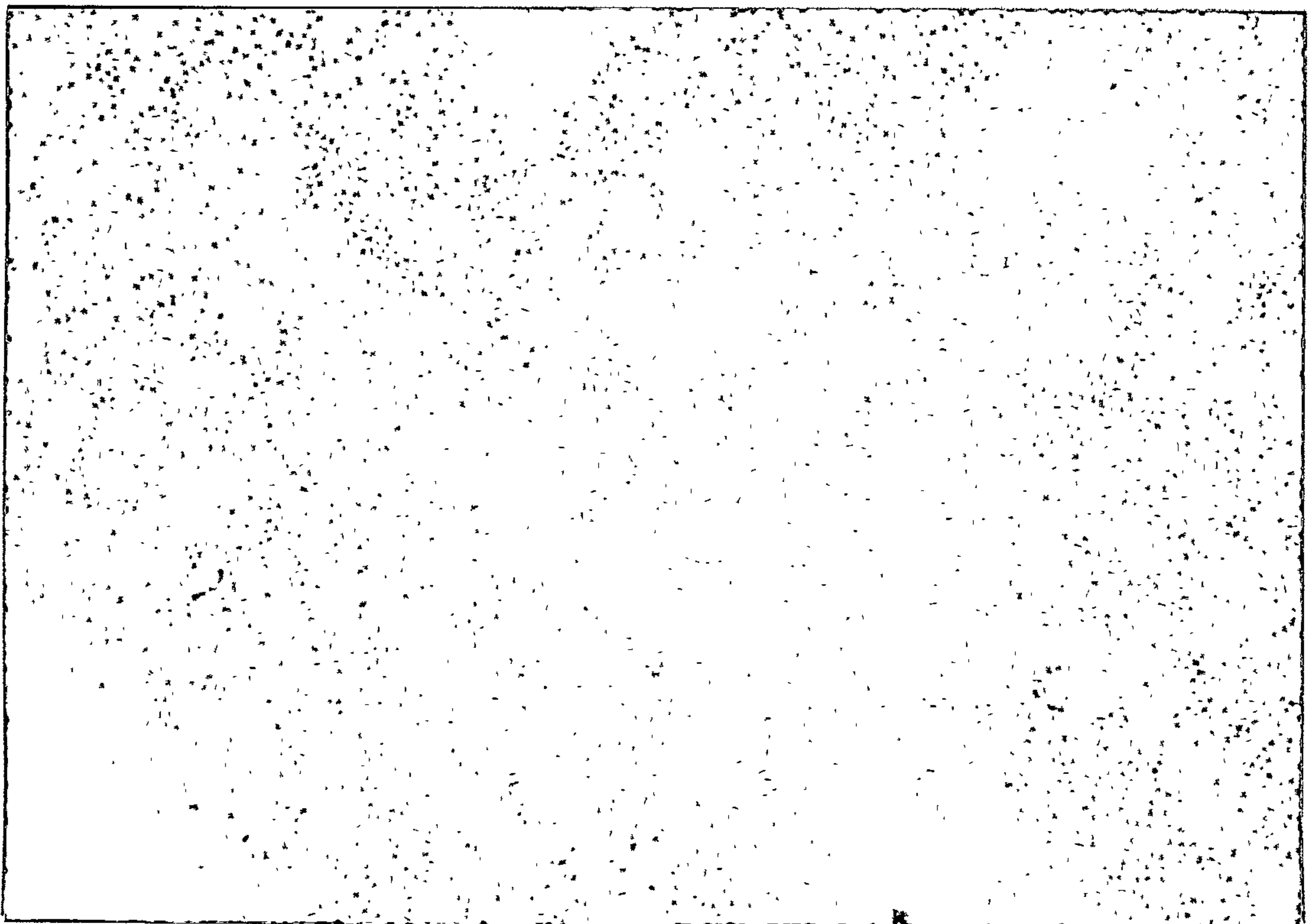
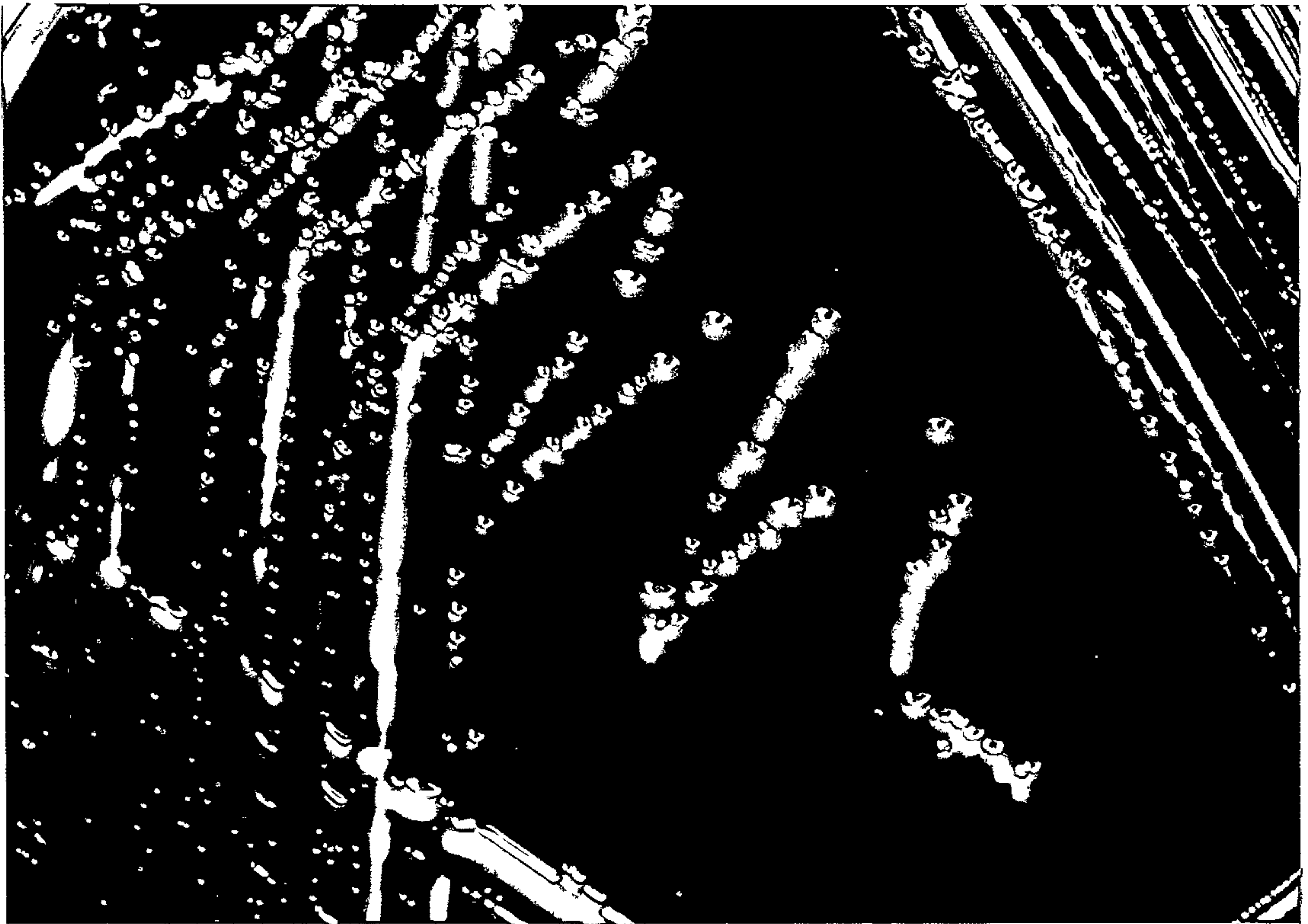
Gram stain: Gram negative rods Plate 2.2b

Biochemical tests for identification: Rapid ID 32 A kit (see Appendix B) was used and results from API identification program were as follows: urease -, arginine dihydrolase -, β -galactosidase +, β -galactosidase-6-phosphate -, α -glucosidase -, β -glucosidase -, α -arabinosidase -, β -glucuronidase -, β -N-acetyl-glucosaminidase +, Mannose -, Raffinose -, Nitrate -, Indole +, Alkaline Phosphatase +, Arginine Arylamidase +, Proline Arylamidase -, Leucyl Glycine Arylamidase +, Phenylalanine Arylamidase -, Leucine Arylamidase -, Alanine Arylamidase +. Identification was 99.9% as *P. gingivalis*.

^aSee appendix 1 for formulation.

Plate 2.2a: Appearance of *Porphyromonas gingivalis* colonies grown on BHV agar plates after 5 days of growth in anaerobic conditions. Note early black pigmentation of colonies.

Plate 2.2b: Gram stain of *Porphyromonas gingivalis* . A Gram negative rod.



(iii) *Prevotella intermedia*

Culture medium: BHV^a plates.

Growth conditions: In an anaerobic chamber (5% CO₂, 10% Hydrogen, 85% Nitrogen and temperature 37°C).

Colony morphology: 0.5 - 1mm diameter grey colonies appeared at 24 - 48 hours. At 72 hours colony size was approximately 1.5 - 2mm diameter, colony colour was brown to black. Plate 2.3a

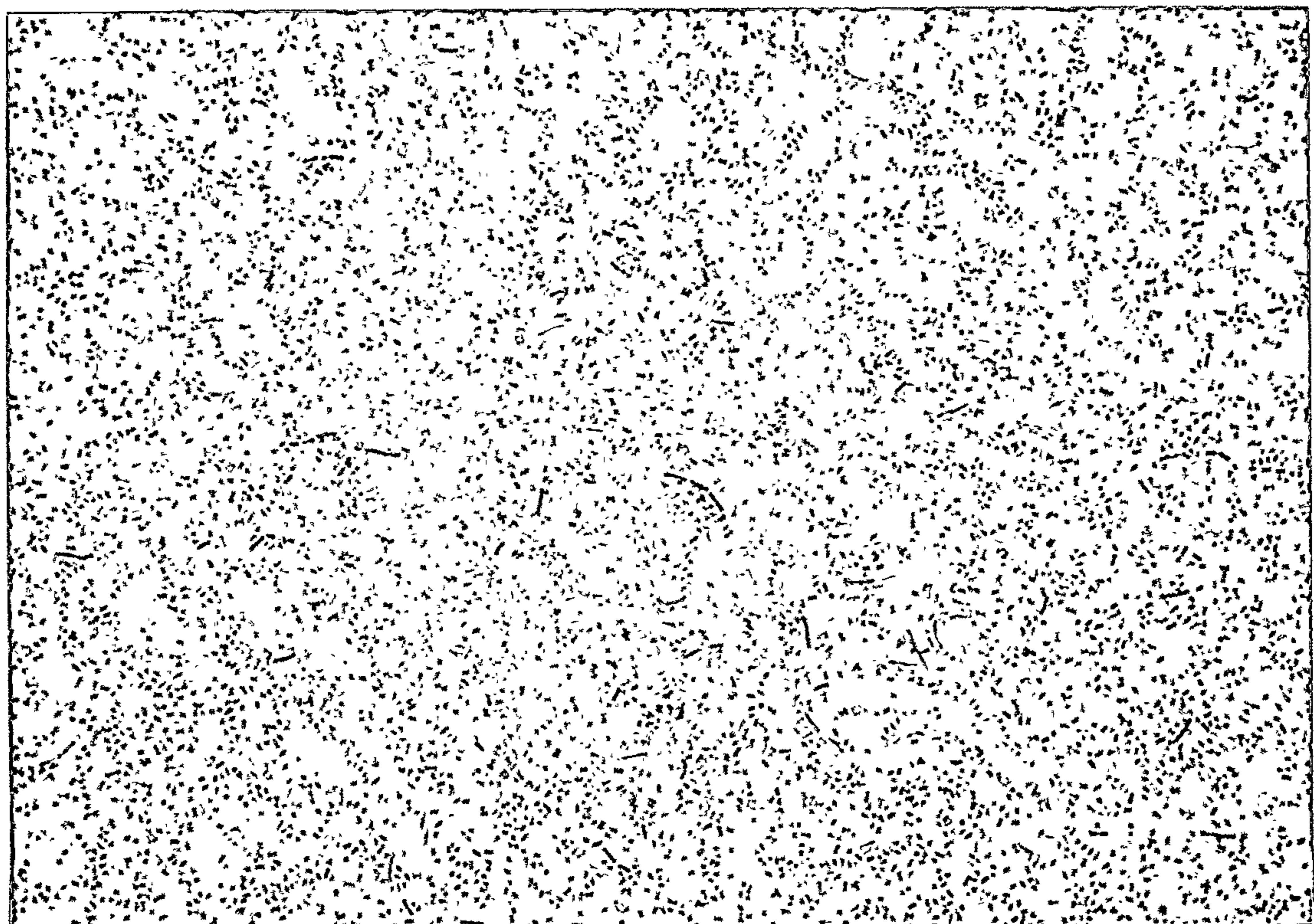
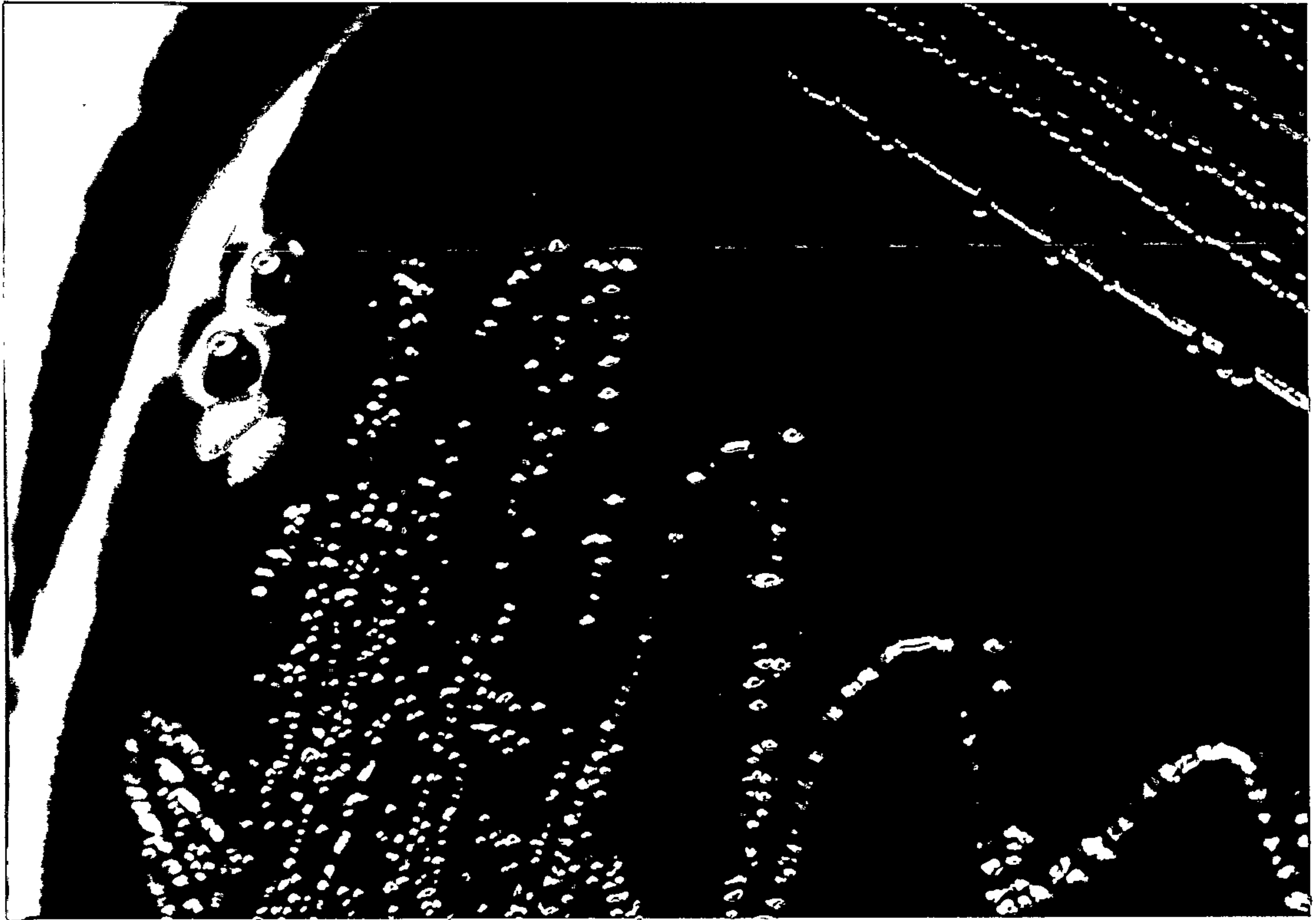
Gram stain: Gram negative rods. Plate 2.3b

Biochemical tests for identification: Glucose +, Lactose -, Maltose +, Catalase -, Indole +, non Nitrate reducing, Oxidase -, lipase + colonies were definitively identified as *P. intermedia* (see also Table 3 appendix 4).

^aSee appendix 1 for formulation.

Plate 2.3a: Appearance of *Prevotella intermedia* colonies grown on BHV agar plates after 5 days of growth in anaerobic conditions. Note black pigmentation of colonies.

Plate 2.3b: Gram stain of *Prevotella intermedia*. A Gram negative rod.



(iv) *Eikenella corrodens*

Culture medium: BHV^a and Blood Agar^a plates.

Growth conditions: CO₂ (5% CO₂, temperature 37°C and humid conditions) enriched environment.

Colony morphology: Small centrally moist clear colonies surrounded by a flat spreading growth that pits the agar. At 24 hours colony size was 0.5 - 1mm diameter by 72 hours colony size had increased to 2mm diameter and the spreading growth was apparent. Plate 2.4a

Gram stain: Slender bacillus with straight unbranched Gram negative rods (1.5 - 4µm in length) Plate 2.4b

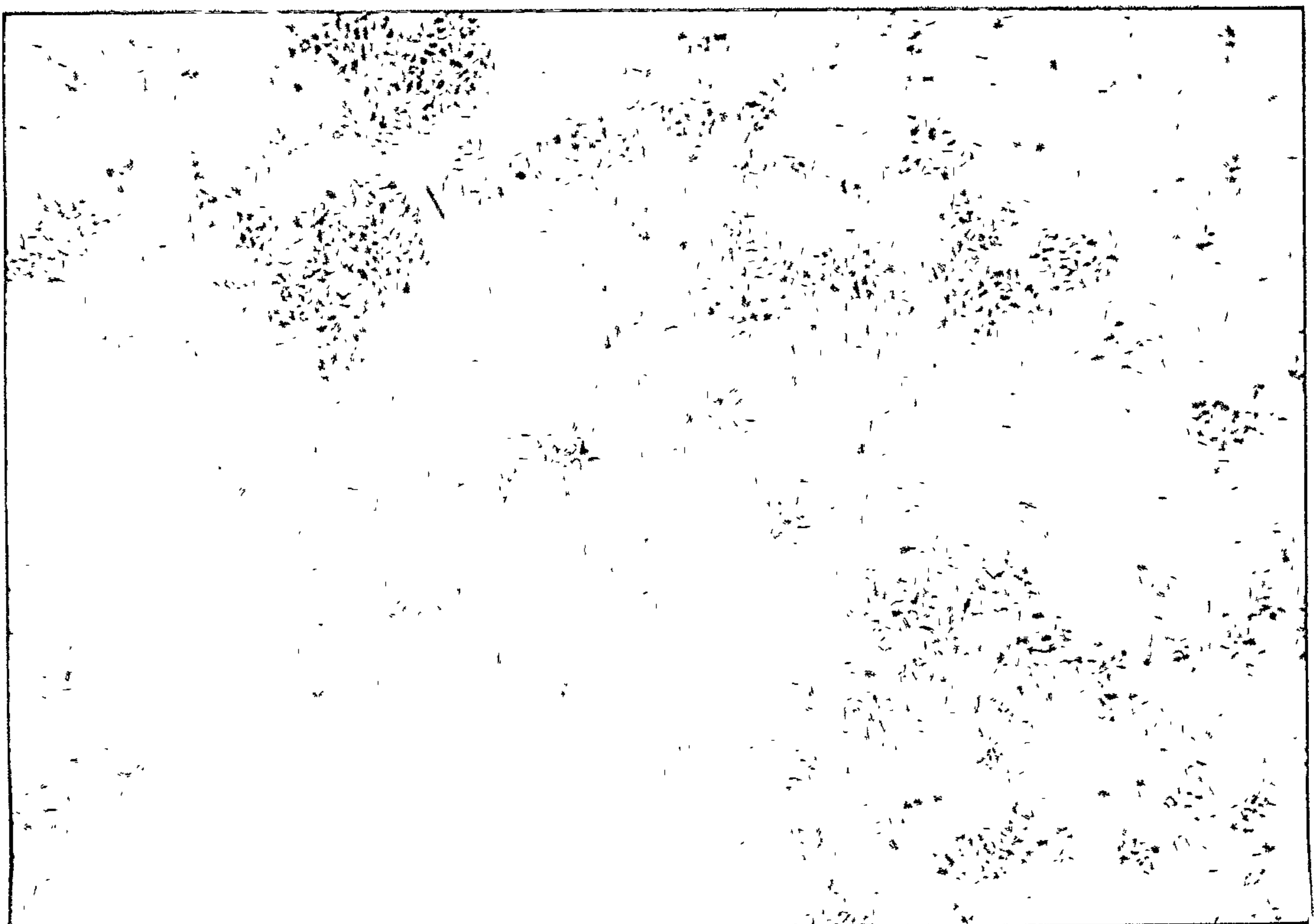
Motility: Non motile (using hanging drop technique)

Biochemical tests for identification: Nonsaccharolytic, Glucose -, Catalase -, Indole -, Nitrate reducing, Oxidase + colonies were definitively identified as *E. corrodens* (see also Table 3 appendix 4).

^aSee appendix 1 for formulation.

Plate 2.4a: Appearance of *Eikenella corrodens* colonies grown on Blood agar plates for 48 hours in a CO₂ environment. Note clear central colony surrounded by a spreading "halo" that pits the agar.

Plate 2.4b: Gram stain of *Eikenella corrodens*. A straight slender Gram negative rod.



(v) *Fusobacterium nucleatum* (ATCC^a 10953)

Culture medium: BHV^b plates.

Growth conditions: In an anaerobic chamber (5% CO₂, 10% Hydrogen, 85% Nitrogen and temperature 37°C).

Colony morphology: White round convex, 'breadcrumb' surface texture, slightly α haemolytic colonies with a distinctive odour. At 24 hours colony size was 0.1 - 0.5mm diameter by 72 hours colony size increased to 1mm diameter. Plate 2.5a

Gram stain: Thin Gram negative rods with tapering ends (often referred to as needle-like). Plate 2.5b

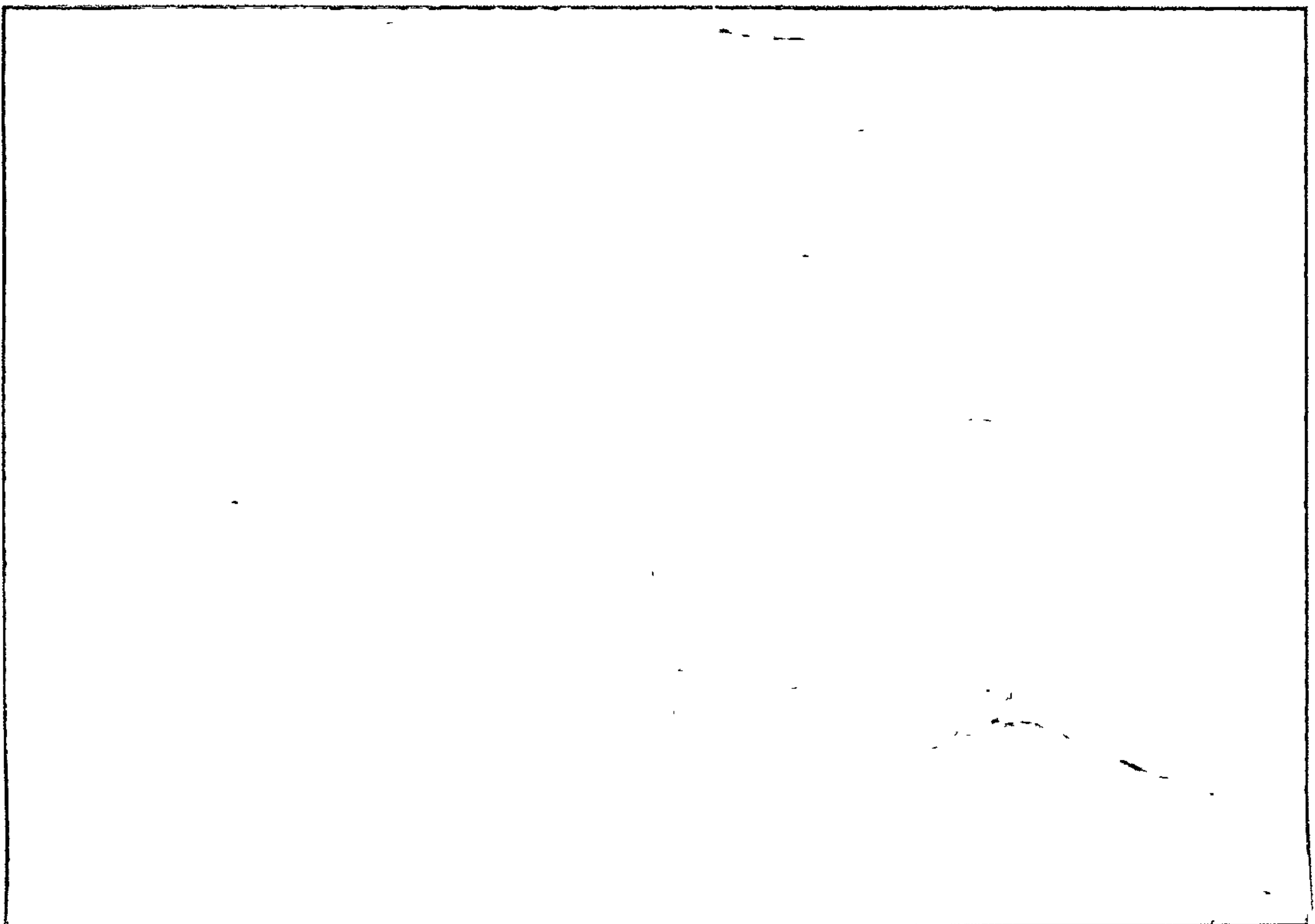
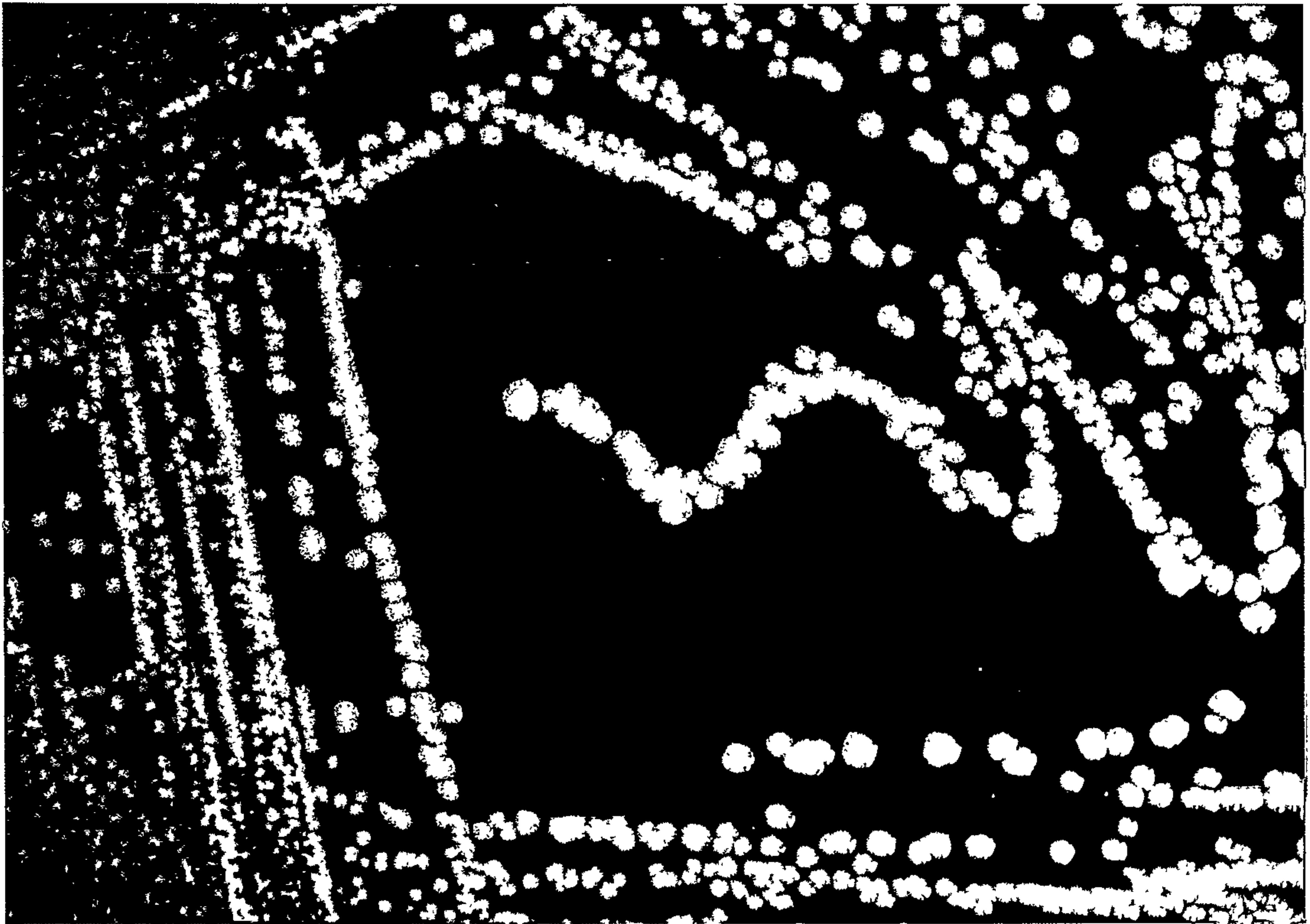
Biochemical tests for identification: Glucose -, Fructose -, Mannose -, Indole +, Oxidase -, lipase - colonies were definitively identified as *F. nucleatum* (see also Table 3 appendix 4).

^aATCC - American Type Culture Collection

^bSee appendix 1 for formulation.

Plate 2.5a: Appearance of *Fusobacterium nucleatum* colonies grown on BHV agar plates for 48 hours in an anaerobic environment. Note 'breadcrumb' appearance of colonies.

Plate 2.5b: Gram stain of *Fusobacterium nucleatum*. A Gram negative rod with slender tapering ends.



(vi) *Campylobacter recta* (FDC^a 371)

Culture medium: BHV plates with added formate and fumarate^b.

Growth conditions: In an anaerobic chamber (5% CO₂, 10% Hydrogen, 85% Nitrogen and temperature 37°C).

Colony morphology: Clear round non haemolytic colonies. At 24 hours colony size was 0.1 - 0.5mm diameter by 72 hours colony size increased to 1mm diameter. Plate 2.6a

Gram stain: Thin Gram negative rods. Plate 2.6b

Motility: Motile (using hanging drop technique)

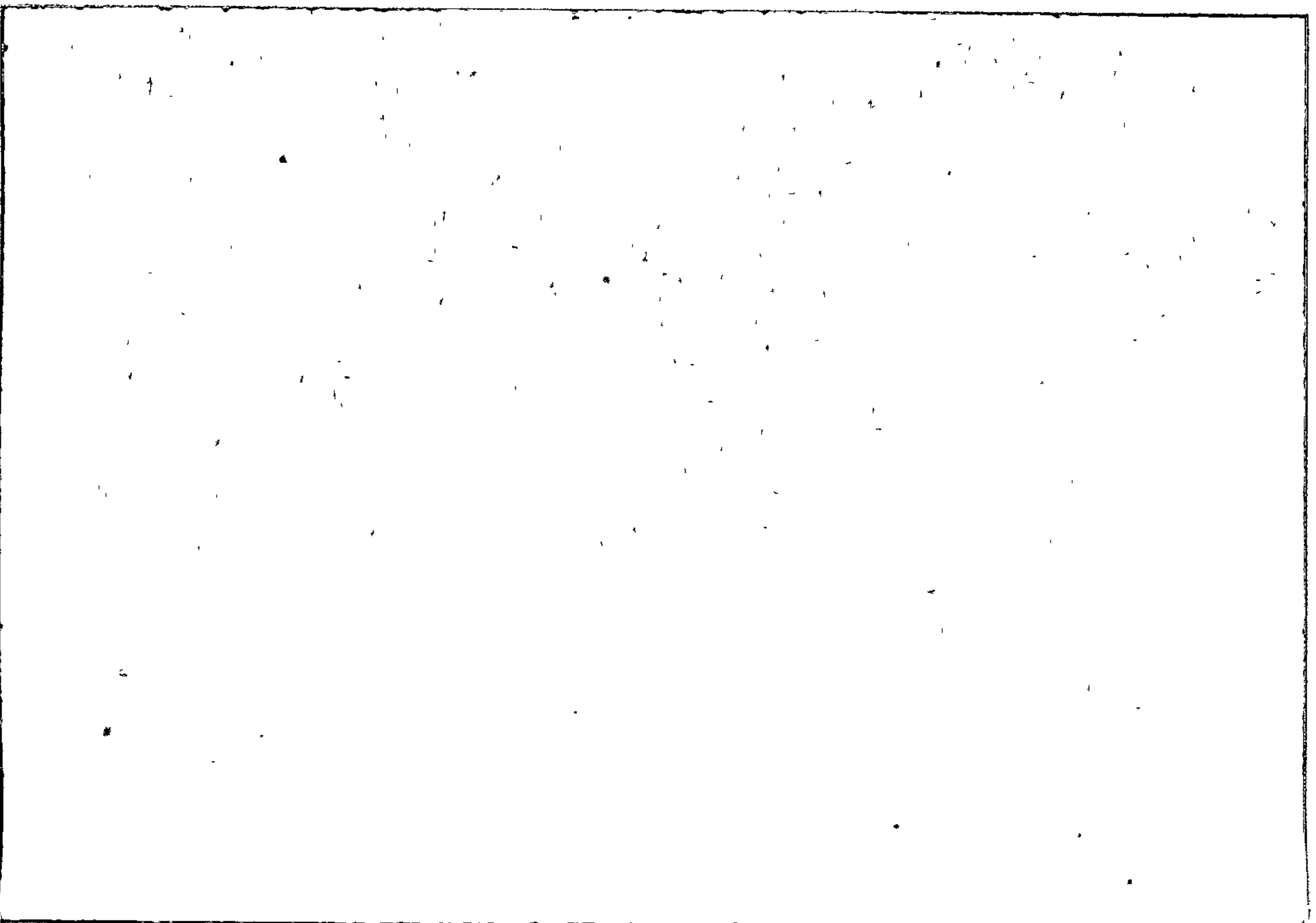
Biochemical tests for identification: Glucose -, Maltose -, Lactose -, Sucrose -, Fructose -, Mannose -, Catalase -, Nitrate reducing, Oxidase - colonies were definitively identified as *C. recta* (see also Table 3 now appendix 4).

^aFDC - Forsyth Dental Centre

^bSee appendix 1 for formulation.

Plate 2.6a: Appearance of *Campylobacter recta* colonies grown on BHV agar plates for 48 hours in an anaerobic environment. Note clear round small colonies.

Plate 2.6b: Gram stain of *Campylobacter recta*. A Gram negative rod.



2.3.3 DNA PROBE METHODOLOGY

DNA Probes specific for *Aa* (cloned), *Pg* (whole), *Pi* (whole), *Ec* (cloned), *Fn* (cloned), *Td* (whole) and *Cr* (cloned) were developed (French et al. 1986, Lippke et al. 1991), and selected for use in this study to detect the presence of the test organisms in subgingival plaque samples collected. Samples were eluted from the paper points and the DNA was denatured as described by French et al. (1986), and Murray and French (1989).

Briefly, the processing of plaque samples required the disruption of the bacterial cell wall and dissolution of non DNA components in each sampled specimen. This was achieved by treatment of the sample with an alkaline solution of enzymes and detergents. The resulting mixture was boiled in a high pH solution which denatured the DNA to a single stranded form to expose the binding site for the single stranded probes. (Figure 2.1).

Dry plaque samples were solubilized in an elution buffer, boiled for 10 minutes and aliquoted. Aliquots of samples (plaque and standard controls) were equilibrated with an equal volume of loading buffer (1:1 3M NaCl, 0.3M NaOH-2M NH₄OAc) and applied to a nitrocellulose filter. A vacuum was

applied to attach the samples onto the filters^a. The nitrocellulose filters were then rinsed in 1mL 0.5M NaCl (loading buffer), air dried and baked at 80°C for 1 hour. The ³²P radiolabelled single stranded nucleic-acid probe of a known specificity was then added and adequate time allowed for hybridisation^b in the same buffer with the addition of 10% dextran sulphate. The nitrocellulose filters were then washed thoroughly^c to remove unhybridised probes and air dried. Kodak X-OMAT AR film with an intensifying screen was laid over the washed, dried filters at -70°C for 2-18 hours. Specimens containing hybridised radioactive probes appear as dark spots on the film due to the radioactivity of the probes degrading and being absorbed onto the film.

The intensity of the dark spot on the film reflects the amount of organism present in each specimen. The density and width of the test signal is compared to the signal generated by the control and hence an approximate quantitation derived.

^a The spotting of the specimen onto the filter allows the single stranded DNA molecules to present sequences for binding by standing up from the solid surface (Murray and French 1989). Filters containing sample blots are pre-hybridised with salmon-sperm DNA and Dehardts solution for 1 hour at 65°C.

^b Probes will hybridise with complementary strands of the test organisms DNA if present in the plaque sample.

^c Filters were washed in two stages: 10 minutes in 1×Standard saline citrate, 0.5% Sodium dodecyl sulfate at room temperature, followed by 30 minutes in 0.1×Standard saline citrate, 0.5% Sodium dodecyl sulfate at 60°C.

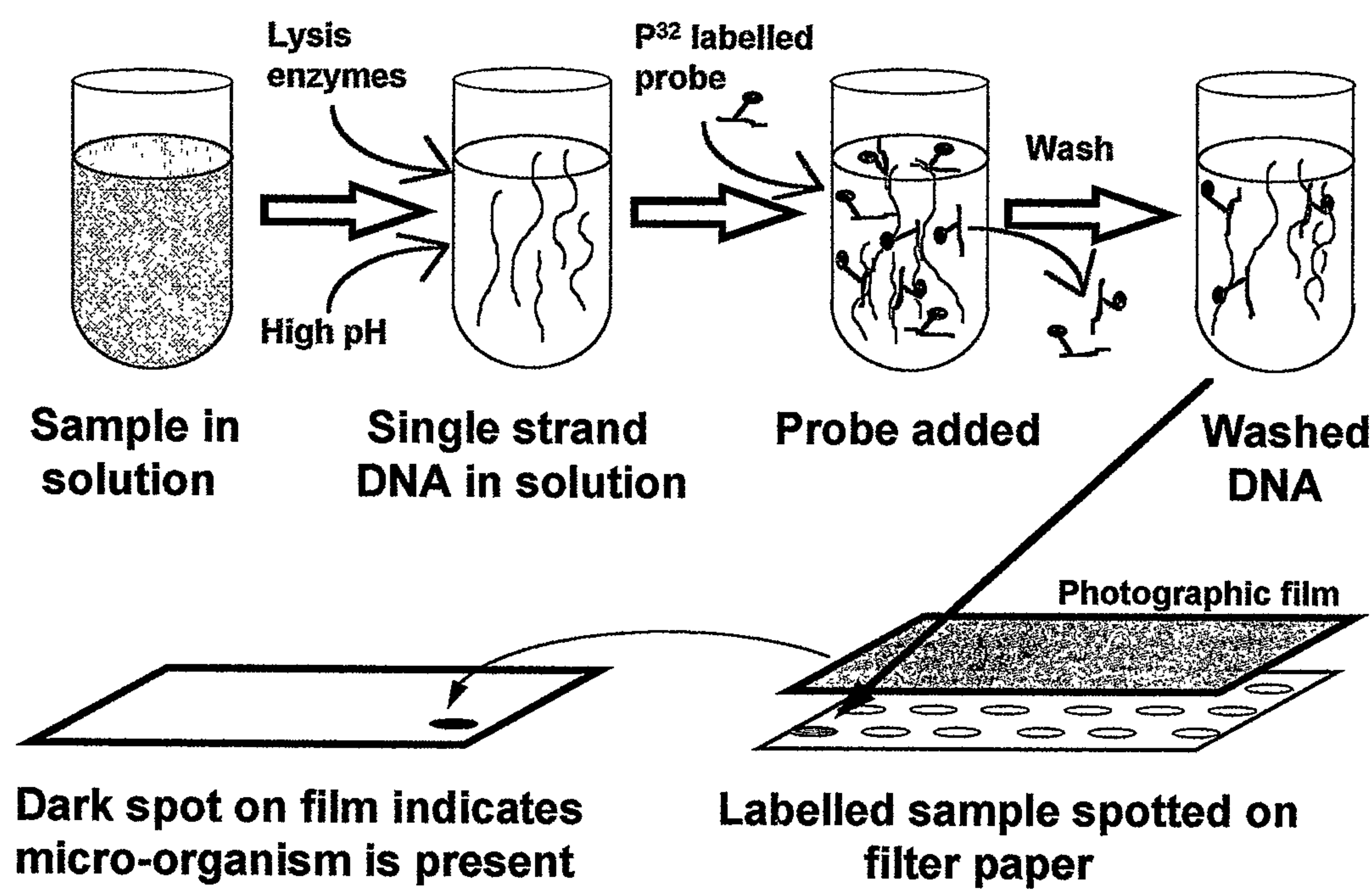


Figure 2.1: DNA Probe methodology

A schematic representation of the processing of plaque samples for the detection of target organisms using DNA Probe technology.

2.4 STATISTICAL ANALYSES

2.4.1 STATISTICAL METHOD FOR TESTING HYPOTHESIS #1

To test the first hypothesis that there is increasing presence and variety of test organisms with increasing clinical inflammation at tooth and implant sites, 74 sites from 20 subjects were used. Sites sampled were categorised as teeth or implants, and further into a clinical category within this group.

TOOTH SITES

Healthy (TH)

Gingivitis (TG)

Periodontitis (TP)

IMPLANT SITES

Healthy (IH)

Periimplantitis (IPi)

Ailing (IA)

Each site sampled received a score 0-7 depending on how many of the seven putative periodontopathic organisms were present at the site. The average score (number of types of organisms) was calculated for each of the six clinical categories, and expressed as a mean with a standard error.

One way analysis of variance (ANOVA) was used to compare means of TH/TG/TP categories. One way ANOVA was also used to compare means from IH/IPi/IA categories. Scheffé's technique of multiple analysis (chosen over the Duncan technique of multiple comparisons for its greater statistical power) was

then used to identify possible differences between the clinical categories at tooth sites, and any differences between clinical categories at implant sites. Analysis of the data in this manner would identify any significant trend in the mean variety of test organisms found between categories of increasing clinical inflammation at tooth sites and similarly at implant sites.

Clinical categories were then paired into parallel subgroups of increasing clinical inflammation across tooth and implant sites: TH/IH; TG/IPi; TP/IA. Comparisons of mean variety of the 7 test organisms across parallel clinical categories were performed using independent sample t-tests. This analysis would result in identification of any trends in variety of test organisms at tooth and implant sites across categories of parallel clinical inflammation.

The proportion of tooth sites at which each organism occurred for a clinical category was compared with the proportion of implant sites at which the same organism occurred for the parallel clinical category. This comparison was undertaken to answer the question whether for a given clinical status, the 7 test organisms that appeared at tooth and implant sites were similar. Chi square tests were used to test the association of each organism at each pair of parallel clinical categories.

2.4.2 STATISTICAL METHOD FOR TESTING HYPOTHESIS #2

To test the hypothesis that test organisms from tooth sites can cross-infect implant sites in the same mouth, 59 sites from 16 subjects were used. These subjects were selected on the basis that they had both tooth and implant sites.

Tooth and implant sites sampled from the same mouth were assessed for the presence or absence of each of the seven test organisms. The presence of each organism at a site was scored as (+) and its absence from a site as (-).

Four combinations of cross-infection from tooth to implant sites exist for each of the test organisms:

1. Presence at both tooth and implant sites was scored (+ +)
2. Absence from both tooth and implant sites was scored (- -)
3. Presence at tooth but absence from implant site was scored (+ -)
4. Absence from tooth but presence at implant site was scored (- +)

Possibilities 1 and 2 (above) support the hypothesis of cross-infection from tooth to implant sites in the same mouth, while possibilities 3 and 4 disagree with this hypothesis (support the null hypothesis).

All the combinations of cross-infection in the direction from tooth to implant sites within each mouth were investigated for each organism separately. Each of the combinations of cross-infection was scored according to the manner in which the second hypothesis was supported or rejected and data was collected in the format of Table 2.3. Chi square tests with the Fischer exact correction were employed to compare the proportions in each of the cells.

Table 2.3: Chi Square grid for testing hypothesis #2

FOR EACH OF THE 7 ORGANISMS	AGREES WITH HYPOTHESIS	DISAGREES WITH HYPOTHESIS
PRESENT AT TOOTH	+ +	+ -
ABSENT FROM TOOTH	- -	- +

- + + Presence of each organism at both tooth and implant sites
- - Absence of each organism from both tooth and implant
- + - Presence of each organism at tooth but absence from implant site
- + Absence of each organism from tooth but presence at implant site

CHAPTER THREE

RESULTS

3.1 DNA PROBE VALIDATION RESULTS

The specificity of the DNA probes used in this study was confirmed by testing against known pure cultures of six of the test organisms *Aa*, *Pg*, *Pi*, *Ec*, *Fn* and *Cr* (*Td* was not used in the validation of DNA probes due to the culturing difficulties associated with spirochetes). Cultures of reference strains were grown according to the method outlined in section 2.3.2. These cultures were sampled with paper points and DNA probe analysis carried out according to the method described in section 2.3.3. Test samples taken from pure culture plates consisted of one or four of the test organisms. A close correlation was found in all cases, confirming the ability of DNA probes to identify the presence of the particular organism in pure and mixed samples (Table 3.1).

Table 3.1: Correlation of cultural and DNA probe results for 6 organisms.

ORGANISMS IN TEST SAMPLE	DETECTION BY DNA PROBE
Aa	Aa +
Pg	Pg +
Pi	Pi +
Ec	Ec +
Fn	Fn +
Cr	Cr +
Aa, Ec, Cr, Fn	Aa + Ec + Cr + Fn +
Aa, Fn, Cr, Pg	Aa + Fn + Cr + Pg +
Cr, Pg, Pi, Fn	Cr + Pg + Pi + Fn +

+ Denotes positive correlation of known culture sample with detection by DNA Probe.

Aa - *Actinobacillus actinomycetemcomitans*

Pg - *Porphyromonas gingivalis*

Pi - *Prevotella intermedia*

Ec - *Eikenella corrodens*

Fn - *Fusobacterium nucleatum*

Cr - *Campylobacter recta*

3.2 HYPOTHESIS #1 - POPULATION CHARACTERISTICS

A total of 20 subjects consented to participate in the study, and were used to test the first hypothesis. A subpopulation of 16 patients, who had subgingival plaque samples taken from both tooth and implant sites in the same mouth, were selected from the total population to test the second hypothesis of cross-infection of periodontopathic organisms (hypothesis #2).

All subjects had a minimum of one Brånemark osseointegrated oral implant present at least two weeks after stage II surgery. The population demographics for the total population are summarised in Table 3.2.

Table 3.2: Population demographics (hypothesis #1).

AGE:	Mean:	56.7 years
	Standard Deviation:	8.8 years
SEX:	Male:	65%
	Female:	35%
SITES SAMPLED:	Total:	74
	Tooth:	41.8%
	Implant:	58.2%
MONTHS SINCE STAGE II:	Mean:	14 months
	Standard Deviation:	13 months
	Mode:	4 months

Each subject had between 2 and 5 sites sampled of which one was always an implant. Clinical criteria (see section 2.2.3) were used to categorise test sites into parallel subgroups across tooth and implant sites. The percentage of sites in each clinical category are shown in Table 3.3.

Table 3.3: Percentage of sites in each clinical category (hypothesis #1)

TOOTH SITES		IMPLANT SITES	
Healthy (TH)	29%	Healthy (IH)	42%
Gingivitis (TG)	35.5%	Periimplantitis (IPi)	39.5%
Periodontitis (TP)	35.5%	Ailing (IA)	18.5%

3.3 RESULTS - HYPOTHESIS #1

3.3.1 DESCRIPTIVE STATISTICS

The presence of putative periodontopathic organisms at tooth or implant sites was scored (0-7) according to the number of species of test organisms detected at each site. Descriptive statistics of mean and standard error were performed for each clinical category and are shown in Table 3.4. In Figure 3.1a & 3.1b, the clinical category means are expressed as vertical bars with standard errors.

Table 3.4: Descriptive statistics for mean variety of organisms in each clinical category.

DESCRIPTIVE STATISTICS	TH	TG	TP	IH	Ipi	IA
TOTAL COUNT	9	11	11	18	17	8
MEAN	0.22	2.64	5.27	0.44	3.41	3.38
STANDARD ERROR	0.15	0.75	0.36	0.17	0.54	0.75

TH Healthy tooth sites

TG Gingivitis tooth sites

TP Periodontitis tooth sites

IH Healthy implant sites

Ipi Periimplantitis sites

IA Ailing implant sites

Figure 3.1: Mean variety of test organisms in each clinical category

Figure 3.1a: Variety of test organisms at tooth sites

- TH Healthy tooth sites
- TG Gingivitis tooth sites
- TP Periodontitis tooth sites

Figure 3.1b: Variety of test organisms at implant sites.

- IH Healthy implant sites
- Ipi Periimplantitis sites
- IA Ailing implant sites

Figure 3.1a: Variety of test organisms at tooth sites.

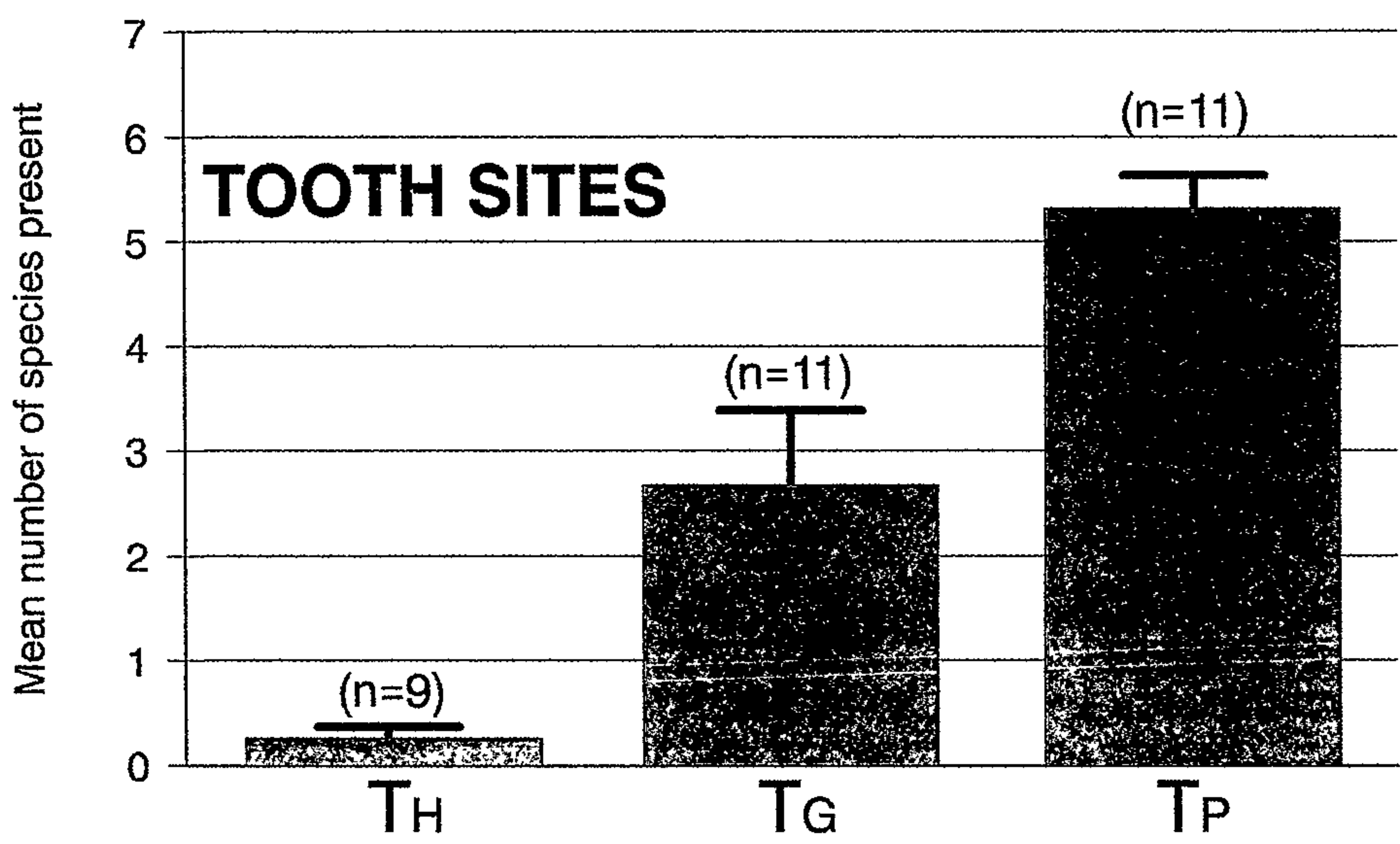
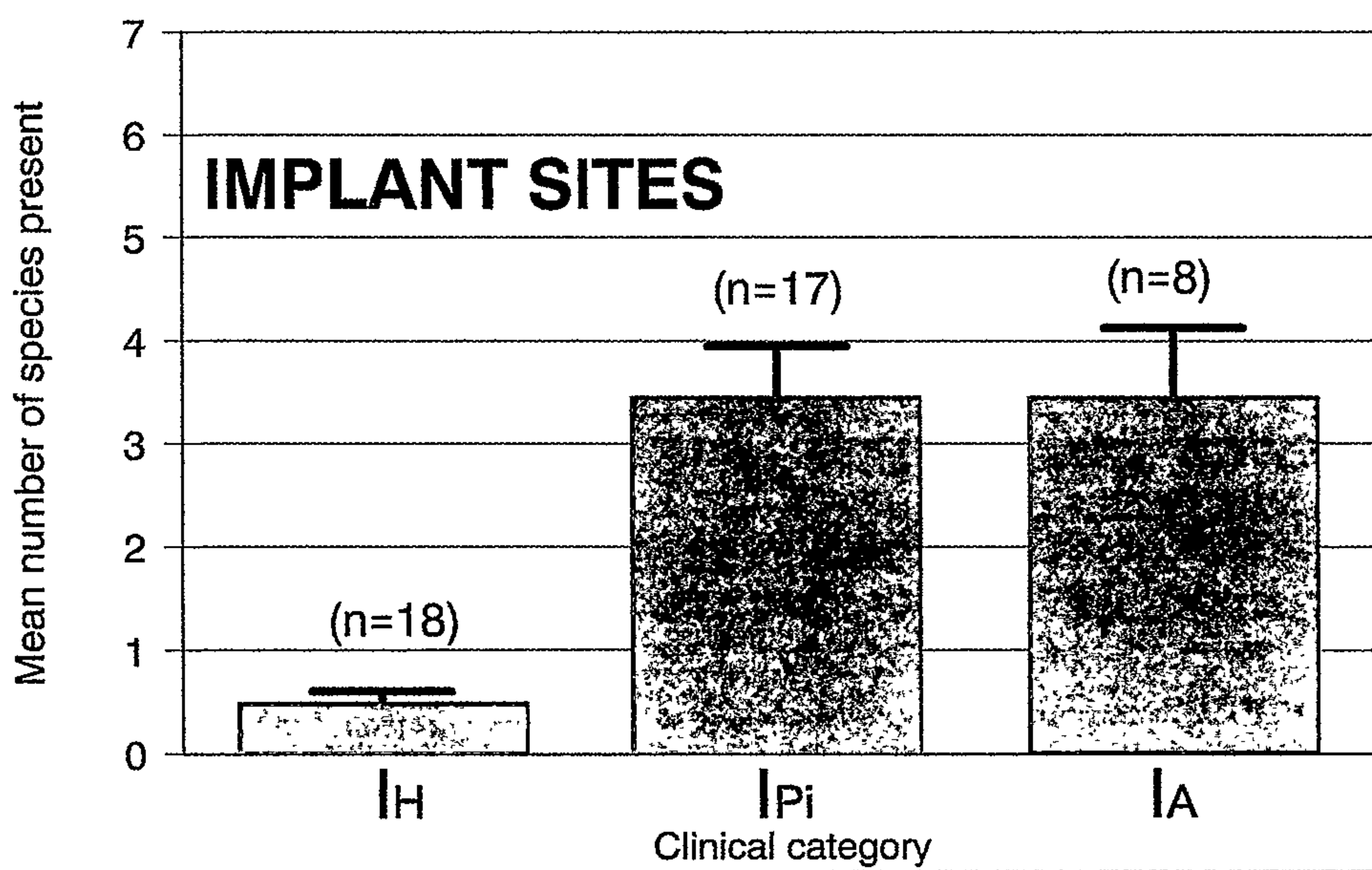


Figure 3.1b: Variety of test organisms at implant sites.



3.3.2 ANALYSIS OF VARIANCE

One way analysis of variance (ANOVA) was used to compare the mean variety of test organisms in categories of increasing clinical inflammation at tooth sites (Figure 3.1a). Similarly, the mean variety of test organisms in categories of increasing clinical inflammation at implant sites were compared using one way ANOVA (Figure 3.1b). The null hypothesis of the ANOVA assumed the means between the clinical groups within each of the categories, tooth or implant, were the same. If the between group variance was large, and within group variance was small then the null hypothesis was rejected as the underlying group means were different (Tables 3.5a and 3.5b). The null hypothesis was rejected for clinical groups in the tooth category ($p=0.00000136$), and for clinical groups in the implant category ($p=0.0000124$).

Scheffé's technique of multiple comparisons was used to identify the populations which contributed to the rejection of the null hypothesis (Table 3.5c). A significant difference between the mean number of species present in TH/TG, TH/TP and TG/TP groups was detected ($p\leq 0.05$). Similarly, a significant difference was detected between mean number of species present in IH/IPi and IH/IA groups but not between IPi/IA groups ($p\leq 0.05$). This data suggested that there was significant difference between all three tooth categories, and between healthy implants sites and periimplantitis sites. However, no difference was detected between the ailing implant sites and periimplantitis sites.

Table 3.5a: One way ANOVA results at tooth sites.

TEETH			
SOURCE OF VARIATION	MEAN SQUARES	f VALUE	p VALUE
BETWEEN GROUPS	63.60	22.75	1.36×10^{-6}
WITHIN GROUPS	2.80		SIG.

Table 3.5b: One way ANOVA results at implant sites.

IMPLANTS			
SOURCE OF VARIATION	MEAN SQUARES	f VALUE	p VALUE
BETWEEN GROUPS	45.71	15.18	1.24×10^{-5}
WITHIN GROUPS	3.01		SIG.

SIG. denotes a statistically significant p-value ($p \leq 0.01$)

Table 3.5c: Indication of significant groups using Scheffé's technique of multiple comparisons analysis.

	TH	TG	TP		IH	Ipi	IA
TH				IH			
TG	*			Ipi	*		
TP	*	*		IA	*		

* Denotes pairs of groups significantly different at the p<0.05 level

TH Healthy tooth sites
TG Gingivitis tooth sites
TP Periodontitis tooth sites

IH Healthy implant sites
Ipi Periimplantitis sites
IA Ailing implant sites

3.3.3 t-TESTS ON MEAN VARIETY OF ORGANISMS

Analysis of the mean variety of the 7 test organisms at tooth and implant sites across parallel clinical categories TH/IH; TG/IPi; TP/IA was performed using independent sample t-tests (Table 3.6).

Table 3.6: Independent sample t-test results

STATISTIC	TH (n=9)	IH (n=18)	TG (n=11)	IPi (n=17)	TP (n=11)	IA (n=8)
Mean	0.22	0.44	2.64	3.41	5.27	3.38
St.Deviation	0.44	0.71	2.50	2.24	1.19	2.13
St. Error	0.15	0.17	0.75	0.54	0.36	0.75
t - value	-0.86		-0.86		2.48	
p - value	0.398		0.400		0.024*	

* Denotes statistically significant result at $p \leq 0.05$.

St. Deviation - Standard Deviation

St. Error - Standard Error

TH Healthy tooth sites

IH Healthy implant sites

TG Gingivitis tooth sites

IPi Periimplantitis sites

TP Periodontitis tooth sites

IA Ailing implant sites

A significant difference was detected in the mean variety of organisms only between TP and IA sites. This result implied that the mean variety of test organisms at TG sites does not vary significantly from that at Ipi sites.

Similarly, that healthy tooth sites TH, harbour a similar variety of the test organisms as do healthy implant sites (IH), but that periodontitis sites (TP) and ailing implant sites (IA) do harbour a different mean variety of test organisms.

The direction of this association can be determined from the mean values of test organisms for each of these categories. TP sites on average would harbour 5.2 of the 7 test organisms, and IA only 3.3 of the test organisms. Hence, a greater variety of putative periodontopathic organisms can be expected to be associated with TP than IA sites.

3.3.4 PERCENTAGE PRESENCE OF TEST ORGANISMS

The results of mean variety of organisms mask information on the individual test organisms. The proportion of tooth and implant sites at which each of the test organisms was detected was compared across parallel clinical categories. This was done to investigate the association of the individual test organisms in each of the clinical categories. The percentage of sites within each clinical category at which individual organisms were detected is shown in Table 3.7a.

Table 3.7a: Percentage of sites within each clinical category at which individual organisms were detected.

ORGANISM	TH (n=9)	IH (n=18)	TG (n=11)	Ipi (n=17)	TP (n=11)	IA (n=8)
Aa	0.0	0.0	18.2	23.5	9.1	25.0
Pg	0.0	0.0	36.4	41.2	81.9	37.5*
Pi	0.0	11.1	36.4	23.5	90.9	50.0*
Ec	22.2	11.1	63.6	82.4	100.0	87.5
Fn	0.0	16.7	36.4	70.6	81.9	62.5
Td	0.0	0.0	27.3	47.1	72.8	50.0
Cr	0.0	5.6	45.5	52.9	90.9	25.0*

*denotes statistical significance at p≤0.05.

TH Healthy tooth sites
TG Gingivitis tooth sites
TP Periodontitis tooth sites

IH Healthy implant sites
Ipi Periplantitis sites
IA Ailing implant sites

Analysis identified *Pg*, *Pi* and *Cr* to be present at a significantly higher number of TP than IA sites ($p \leq 0.05$). *Pg* was detected at 81.9% of periodontally involved tooth sites compared with only 37.5% of ailing implant sites. *Pi* was detected at 90.0% of periodontally involved tooth sites and 50% of ailing implant sites. Similarly, *Cr* was detected at 90.0% of periodontally involved tooth sites and only 25.5% of ailing implant sites.

IA sites showed a comparable mean variety of test organisms with TG and IPI sites (see Table 3.4). Chi square tests were used to compare the percentage presence of each test organism at ailing implant sites to those at periimplantitis sites and also gingivitis sites. This analysis investigated whether any one of the test organisms were predominantly associated with each of these three clinical categories. (Table 3.7b)

There was no significant association between the percentage of sites at which each of the test organisms was detected at gingivitis, periimplantitis and ailing implant sites.

Table 3.7b: Percentage of sites at which individual organisms were detected.

ORGANISM	Ipi (n=17)	IA (n=8)	p-value	TG (n=11)	IA (n=8)	p-value
Aa	23.5	25.0	p≥0.05	18.2	25.0	p≥0.05
Pg	41.2	37.5	p≥0.05	36.4	37.5	p≥0.05
Pi	23.5	50.0	p≥0.05	36.4	50.0	p≥0.05
Ec	82.4	87.5	p≥0.05	63.6	87.5	p≥0.05
Fn	70.6	62.5	p≥0.05	36.4	62.5	p≥0.05
Td	47.1	50.0	p≥0.05	27.3	50.0	p≥0.05
Cr	52.9	25.0	p≥0.05	45.5	25.0	p≥0.05

TH Healthy tooth sites

TG Gingivitis tooth sites

TP Periodontitis tooth sites

IH Healthy implant sites

Ipi Periimplantitis sites

IA Ailing implant sites

3.3.5 RESULTS SUMMARY (HYPOTHESIS #1)

The mean variety of seven putative periodontopathic organisms tested were found to significantly increase from health to gingivitis and periodontitis at tooth sites. A similar increase in the mean variety of the seven test organisms was detected from healthy to periimplantitis sites, but no further increase was detected to ailing implant sites. Analysis of the percentage of sites in each clinical category which harboured the seven test organisms identified *Pg*, *Pi* and *Cr* to be present at significantly more periodontitis tooth sites than ailing implant sites.

3.4 RESULTS HYPOTHESIS #2

3.4.1 POPULATION CHARACTERISTICS - HYPOTHESIS #2

The hypothesis that a microorganism from a tooth site within a subject's mouth may cross-infect an implant site in the same mouth was investigated using 16 subjects. A total of 31 tooth and 28 implant sites in these subjects were sampled. The population demographics for the subpopulation used to test the hypothesis of cross-infection are summarised in Table 3.8.

Table 3.8: Population demographics (hypothesis #2).

AGE:	Mean:	58.1 years
	Standard Deviation:	9.1 years
SEX:	Male:	68.8%
	Female:	31.2%
SITES SAMPLED:	Total:	59
	Tooth:	52.5%
	Implant:	47.5%
MONTHS SINCE STAGE II:	Mean:	10.8 months
	Standard Deviation:	8.1 months
	Mode:	4 months

Each subject had between 2 and 5 sites sampled of which one was always an implant. Clinical criteria (see section 2.2.3) were used to categorise test sites into parallel subgroups across tooth and implant sites. The percentage of sites in each clinical category are shown in Table 3.9.

Table 3.9: Percentage of sites in each clinical category (hypothesis #2)

TOOTH SITES		IMPLANT SITES	
Healthy (TH)	29%	Healthy (IH)	46.4%
Gingivitis (TG)	35.5%	Periimplantitis (IPi)	28.6%
Periodontitis (TP)	35.5%	Ailing (IA)	25%

3.4.2 MODEL USED TO TEST HYPOTHESIS #2

A model was developed to analyse the data in the subpopulation of subjects who were used to test the second hypothesis. Within each of the subjects (who had both tooth and implant sites sampled), all sites were scored for the presence (+) or absence (-) of each test organism. As the second hypothesis proposes cross-infection of putative periodontopathic organisms from tooth to implant sites in the same mouth, each tooth site was considered a possible source of organisms for cross-infection. (Figure 3.2) Four combinations of implant cross-infection from each tooth within a particular mouth were possible:

1. + + Organism present at both tooth and implant sites
2. - - Organism absent from both tooth and implant sites
3. + - Organism present at tooth but absent from implant site
4. - + Organism absent from tooth but present at implant site

The presence of an organism at both the tooth and implant site or its absence from both the tooth and implant site was considered to support the hypothesis of cross-infection. The other two possibilities were noted as rejection of the hypothesis. For all sampled sites the possibility of cross-infection from tooth to implant sites within each mouth were investigated for each organism (Figure 3.3).

Figure 3.2: Combinations of implant cross-infection from tooth sites.

- Possible route of cross-infection from tooth to implant site
- + → + Organism present at both tooth and implant sites
- → - Organism absent from both tooth and implant sites
- + → - Organism present at tooth but absent from implant site
- → + Organism absent from tooth but present at implant site

Figure 3.3: Routes of cross-infection considered.

TOOTH_{1....n} Possible tooth sites

IMPLANT_{1....n} Possible implant sites

Figure 3.2: Combinations of implant cross-infection from tooth sites.

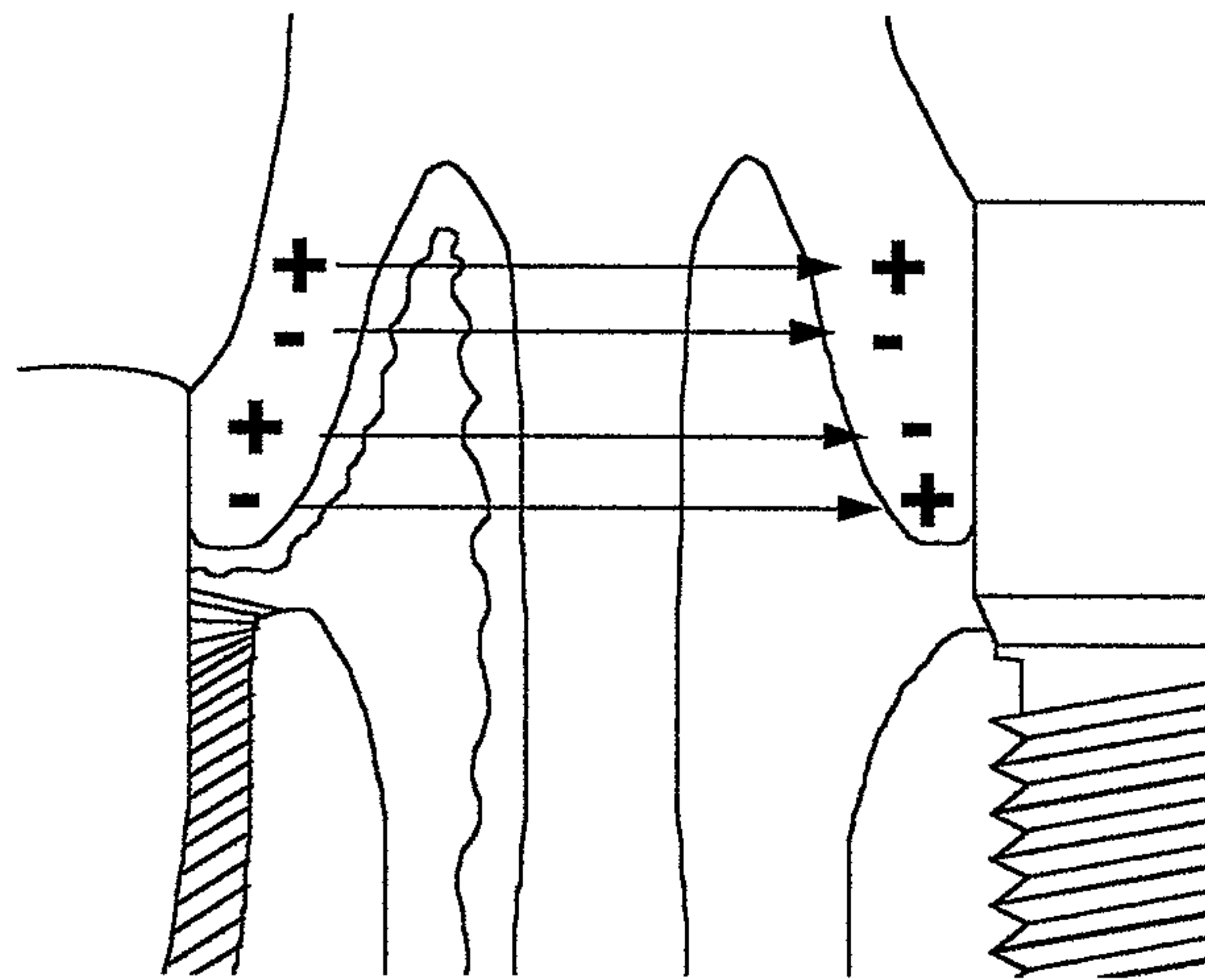
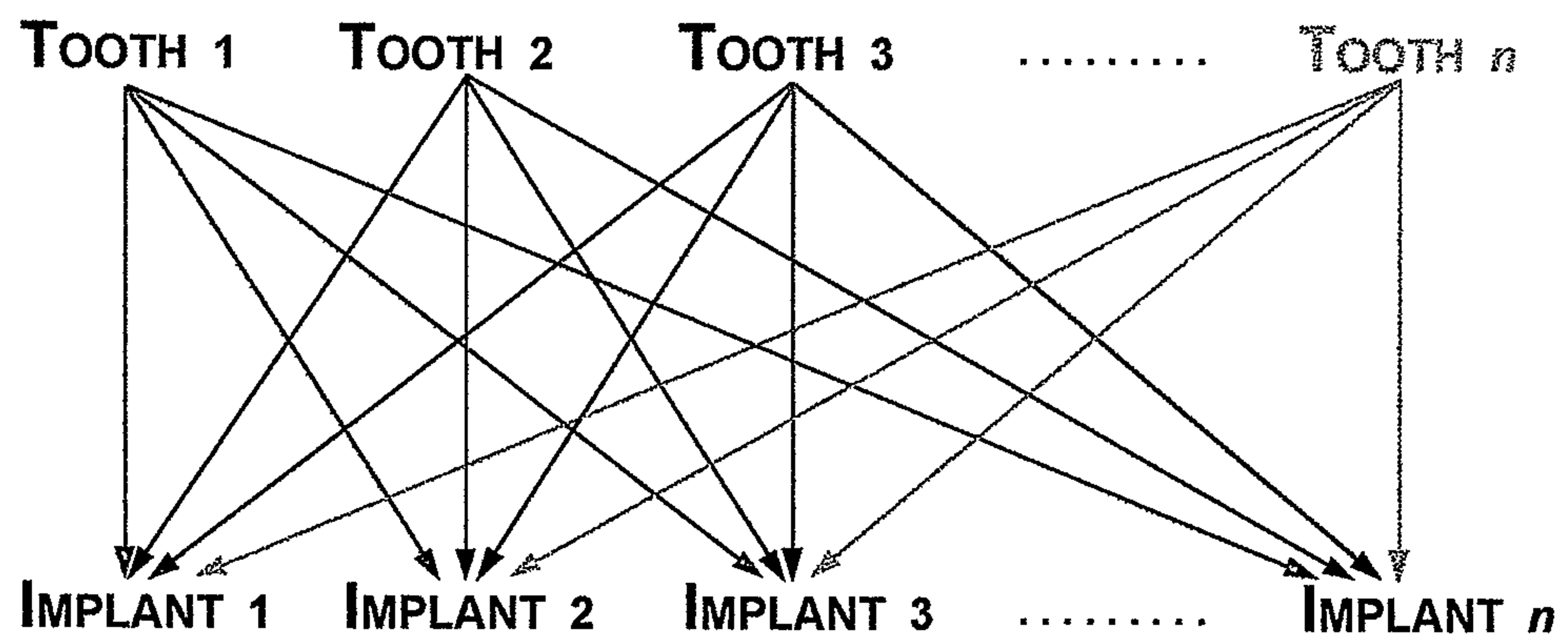


Figure 3.3: Routes of cross-infection considered.



3.4.3 ANALYSIS OF CROSS-INFECTION WITHIN EACH SUBJECT.

To maintain independence of the data, the probability of cross-infection was calculated for each subject independently and this data then pooled for all patients and each organism (Table 3.10).

Results of proportional Chi square tests for each of the 7 putative periodontopathic organisms used to test the hypothesis that microorganisms at tooth sites infect implant sites in the same mouth are shown in Table 3.11. The total number of times each organism is present (detected) plus the number of times it is absent should add to 100%. The Chi square tests examined for a significant association between hypothesis #2 and the presence of the microorganism at the tooth site. The values in Table 3.11 represent the proportion (percentage) of sites at which the hypothesis of cross-infection was supported, and what percentage of sites at which it was rejected.

Table 3.10: Chi Square grid for testing possible cross-infection.

FOR EACH OF THE 7 ORGANISMS	HYPOTHESIS SUPPORTED	HYPOTHESIS REJECTED
PRESENT AT TOOTH	+ +	+ -
ABSENT FROM TOOTH	- -	- +

- + + Organism present at both tooth and implant sites
- - Organism absent from both tooth and implant sites
- + - Organism present at tooth but absent from implant site
- + Organism absent from tooth but present at implant site

Table 3.11: Proportional Chi square for organisms at tooth and implant sites in the same mouth.

Aa	HYPOTHESIS SUPPORTED	HYPOTHESIS REJECTED	P VALUE
PRESENT AT TOOTH	10.5	16.7	0.538
ABSENT FROM TOOTH	89.5	83.3	

Pg	HYPOTHESIS SUPPORTED	HYPOTHESIS REJECTED	P VALUE
PRESENT AT TOOTH	27.3	90.9	0.0003 ***
ABSENT FROM TOOTH	72.7	9.1	

Pi	HYPOTHESIS SUPPORTED	HYPOTHESIS REJECTED	P VALUE
PRESENT AT TOOTH	33.3	63.6	0.093
ABSENT FROM TOOTH	66.7	36.4	

Ec	HYPOTHESIS SUPPORTED	HYPOTHESIS REJECTED	P VALUE
PRESENT AT TOOTH	60.0	71.4	0.521
ABSENT FROM TOOTH	40.0	29.6	

Fn	HYPOTHESIS SUPPORTED	HYPOTHESIS REJECTED	P VALUE
PRESENT AT TOOTH	41.9	46.2	1.528
ABSENT FROM TOOTH	58.1	53.8	

Td	HYPOTHESIS SUPPORTED	HYPOTHESIS REJECTED	P VALUE
PRESENT AT TOOTH	18.2	81.8	0.0003 ***
ABSENT FROM TOOTH	81.8	18.2	

Cr	HYPOTHESIS SUPPORTED	HYPOTHESIS REJECTED	P VALUE
PRESENT AT TOOTH	20.8	80.0	0.0003 ***
ABSENT FROM TOOTH	79.2	20.0	

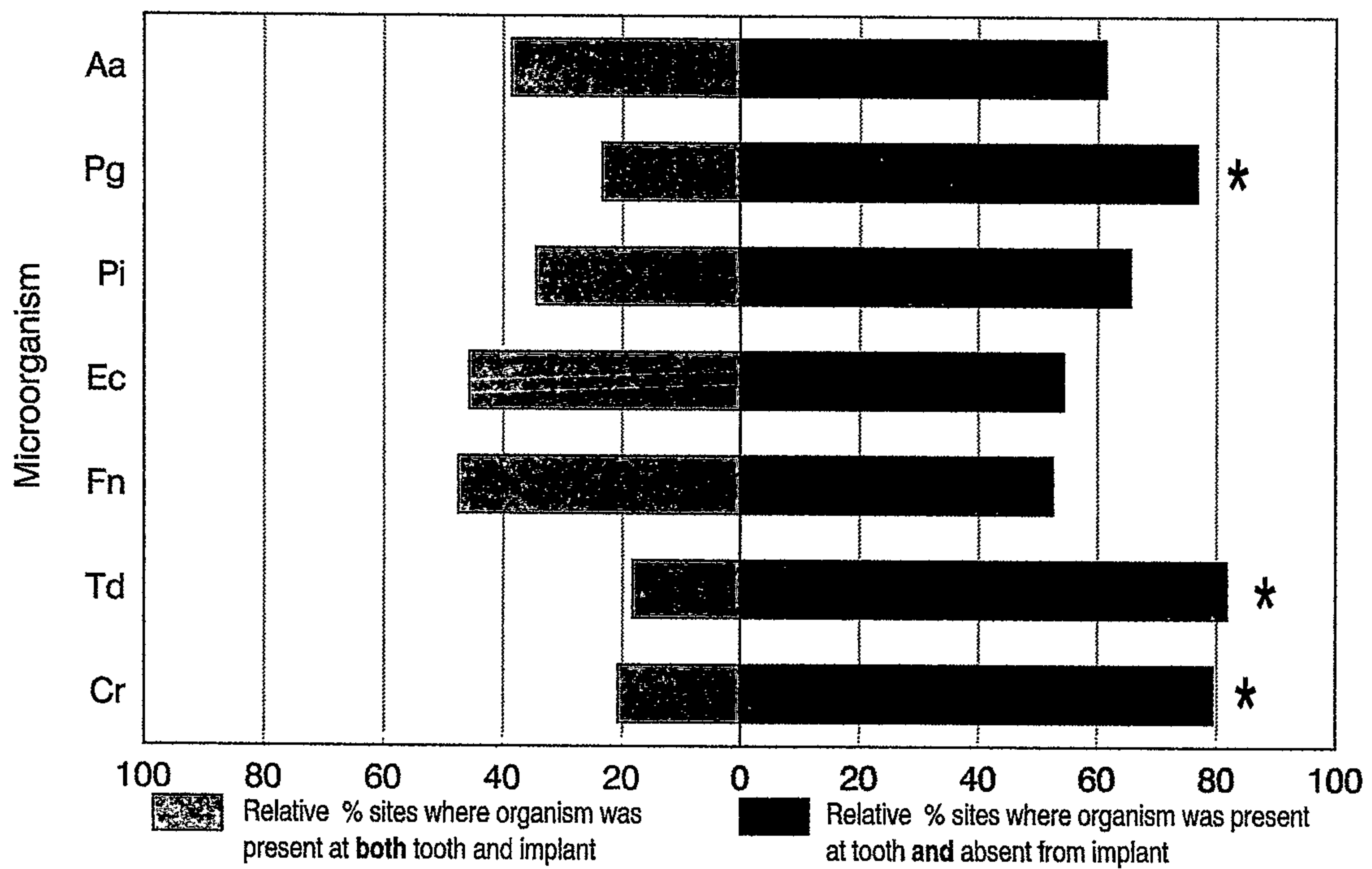
*** Denotes highly significant result (p<0.001)

Shaded cells in Table 3.11 represent the percentage of sites at which each microorganism was absent from the tooth sites and thus was unable to cross-infect implants in the same mouth. There were occasions where a microorganism was not detected at a tooth site, but it was detected at implant sites in the same mouth (organism absent from tooth, and hypothesis rejected).

For each hypothetical possibility at a tooth site, (support or rejection of hypothesis #2), the presence and absence of each microorganism should add to 100%. Results that directly supported (organism detected at both tooth and implant site), or directly rejected (organism detected at tooth site but not at implant site) the hypothesis of cross-infection, were then standardised across all species and graphed (Figure 4).

When *Pg*, *Td* and *Cr* were present at tooth sites these organisms were mostly absent from implant sites in the same mouth ($p=0.0003$). Rejecting the hypothesis of cross-infection. Similarly, when *Pg*, *Td* and *Cr* were absent from tooth sites these organisms were also absent from implant sites in the same mouth ($p=0.0003$). This would appear to support the hypothesis of cross-infection, but the absence of the organism at the tooth site automatically negates any possibility of cross-infection of the organism from that tooth to implant sites. The possibility of cross-infection from another source would arise in the scenario where although absent from tooth sites, organisms were detected at implant sites within a mouth.

Figure 4: Relative support or rejection of hypothesis #2 for each of the seven test organisms.



* Denotes highly significant result (p < 0.001)

Aa - *Actinobacillus actinomycetemcomitans*

Pg - *Porphyromonas gingivalis*

Pi - *Prevotella intermedia*

Ec - *Eikenella corrodens*

Fn - *Fusobacterium nucleatum*

Td - *Treponema denticola*

Cr - *Campylobacter recta*

3.4.4 RESULTS SUMMARY (HYPOTHESIS #2)

The second hypothesis states that cross-infection occurs from tooth to implant sites within each subject. Development of a model was required to maintain statistical independence of the data. The model allowed the unit of analysis to become the individual subjects rather than each of the sites sampled.

Results from the present study reject the hypothesis of cross-infection from tooth to implant sites within a mouth for the population studied. All of the seven test organisms were present at tooth sites and absent from implant sites more often than they were present at both tooth and implant sites in the same mouth. This association was only significant for *Pg*, *Td* and *Cr*. This finding suggests that cross-infection by putative periodontopathic organisms from tooth to implant sites is unlikely.

CHAPTER FOUR

DISCUSSION

Numerous microbial species have been identified in association with human periodontal diseases (Moore 1987) and failing implants (Mombelli et al. 1987). The potential for cross infection from tooth sites to osseointegrated implant sites is raised by Quirynen and Listgarten (1990), Rosenberg and co-workers (1991). The present study investigated the incidence of putative periodontopathic organisms at tooth sites and compared their presence at implant sites in the study population and within individual subjects.

The sensitivity and specificity of DNA probes used in the present study was established by French et al. (1986), Maiden et al. (1991). The technique compares favourably to conventional culturing due to its cost effectiveness, and the assay speed. The DNA probes chosen for the present study are believed to be specific for organisms associated with disease in the teeth and implants of this study group (Dzink et al. 1988, Maiden et al. 1991).

The first hypothesis sought to investigate the increase in pathogenic species diversity across three clinical categories at both teeth and implants. The cross sectional data derived from sampling the entire study population showed a progressive increase in the mean variety of the seven putative periodontopathic (test) organisms at tooth sites for each clinical category. The observed changes from health to gingivitis were in accord with the findings of L  e and Theilade (1965) who showed increased species diversity in the plaque adjacent to the gingival margins of teeth from health to gingivitis. In this study, the increase in the variety of test organisms was statistically significant between healthy (TH), gingivitis (TG) and periodontitis (TP) sites. Cultural and morphologic studies had shown that the microflora at periodontitis sites of teeth comprises a greater number and variety of Gram negative anaerobic rods and spirochetes than healthy sites or sites with gingivitis (Slots 1977(a),(b), Listgarten and Helld  n 1978). On the other hand, increased number, but not diversity, of organisms at tooth sites with varying degrees of inflammation was recently reported by Socransky et al. (1991).

The present study detected few putative periodontal pathogens at healthy implant sites. This is in agreement with Mombelli and Mericske-Stern (1990) who reported a low occurrence of *Prevotella intermedia* and *Fusobacterium* species and no *Porphyromonas gingivalis* and spirochetes at healthy implant sites in edentulous mouths. Similar findings were reported by Quirynen and Listgarten (1990) who analysed plaque samples from tooth and implant sites in partially dentate subjects using darkfield microscopy.

In the present study a significant increase in the variety of test organisms from clinically healthy (IH) to periimplantitis (IPi) sites was noted. The significant increase in mean variety of test organisms from healthy (IH) to ailing implants (IA) was consistent with data from other cross sectional studies on edentulous (Lekholm et al. 1986, Mombelli et al. 1987) and partially dentate (Apse et al. 1989) subjects using cultural method and dark field microscopy. The observed changes in this study represent increases in the variety of pathogenic test organism species rather than absolute numbers of organisms as in other cross sectional studies. Hence, a direct comparison with studies reporting an increase in percentage of sites present or number of colony forming units (CFU) cannot be made. The method of analysis used in the present study was chosen to avoid possible errors of assumption regarding the viability of detected organisms and to accentuate the possible detection of changes in species occurrence rather than the degree of their predominance. The aim of the present study was primarily to observe increased species diversity.

The variety of test organisms at ailing implant sites (IA) was comparable to, but not significantly greater than, periimplantitis (IPi) sites. This finding suggested there was no increased species diversity between these two clinical categories as there were between gingivitis and periodontitis. In contrast, longitudinal animal studies from "healthy" to "experimental periimplantitis" reported progressive microbial diversity throughout (Hickey et al. 1991, Leonhardt et al. 1992). However, ligature induced periodontitis (and presumably periimplantitis) in animals may not be analogous to human periodontal disease. The lack of change in the species diversity in the implant situation is an

unexpected finding and suggests that either a different mechanism to periodontitis is in operation at implant sites or that the clinical criteria used to group the implants sites do not reflect changes in the microbial flora.

To determine whether the observed similarity in mean variety of test organisms from (IPi) to (IA) was significantly different to the changes from (TG) to (TP) the two data sets were compared across parallel clinical categories. Parallel group comparison showed no significant difference between the mean variety of test organisms at healthy tooth or implant sites, nor between gingivitis and periimplantitis categories. However, there was a significant but negative association between periodontitis and ailing implant sites ($p < 0.05$) (Table 3.6). This negative association implied that there was a true difference between the variety of test organisms at ailing implants and teeth with periodontal disease. The micro-organisms responsible for the difference were *P. gingivalis*, *P. intermedia*, and *Campylobacter recta*. In periodontitis, the percentage presence of these three organisms increased considerably from (TG) to (TP) sites. On the other hand, no significant difference was noted in the percentage presence of the same organisms between (IA) sites and (IPi) and (TG) sites ($p > 0.05$) (Table 3.7b).

This comparison further supports the notion that the microbial composition of plaque around the ailing implants of this study is more akin to that of a gingivitis than a periodontitis. Clinically, the sampled ailing implants do not resemble the failing and failed implants described by Rams and Link (1983) or Mombelli et al. (1987). The

findings of the present study do not dispute previous data regarding the similarity of plaque in periodontitis and failing implants. Since evidence of bone loss was one of the criteria for classifying an implant into this category, it may be presumed that a number of these failing implants were undergoing attachment breakdown for reasons other than plaque associated marginal inflammation (Roberts et al. 1984). Ahqvist et al. (1990) reported a correlation in the bone loss around fixtures with long cantilevers and found a significant difference between the marginal bone loss at the most distal fixture and other fixtures in the same mouth. Quirynen et al. (1992) examined the marginal bone loss around Brånemark fixtures under various loading conditions. They concluded that increased marginal bone loss can definitely be associated with occlusal overloading. Bone quality and the type of fixture used had important influences on the amount of bone loss seen.

A microbial difference between 48 failing osseointegrated implants was reported by Rosenberg et al. (1991). They identified two distinct groups of implant failure, infectious and traumatic. Characterisation of the microbial flora associated with each type of failure clearly demonstrated distinct bacteriologic profiles. Implants which failed due to infection had an increased presence of spirochetes and motile rods. Microbial culturing also found a predominance of *Fusobacterium* species. In contrast, the microbiota associated with implants failing due to trauma (either surgical or functional) resembled that of "healthy" implant sites. Steflik et al. (1991) reported on the cause of failure of seven endosseous implants. Periimplant infection was associated

with one case. Encapsulations and fixture fractures were the predominant causes of failure of these implants.

The observations made in the present study support the argument that a distinction may be made between ailing implants experiencing failure due to trauma and those due to chronic marginal inflammation by assessing the percentage presence of each of seven periodontopathic micro-organisms. The validity of such a test requires further evaluation with larger numbers of ailing implants in both categories. Furthermore, a revision of the classification of ailing implants may be required to distinguish between the two pathologic processes. This would be beneficial for early intervention since ailing implants losing attachment due to trauma may not improve following antibiotic therapy in the same manner as those with microbial periimplantitis (Mombelli and Lang 1992).

The second part of the study investigated the hypothesis that cross infection of periodontal pathogens may occur in subjects with both teeth and implants. The hypothesis of teeth acting as a reservoir of putative periodontal pathogens remains untested. Previous data indicate a possible link between the presence of periodontal pathogens at implants and teeth using pooled samples from both edentulous and partially dentate mouths (Quirynen and Listgarten 1990, Rosenberg et al. 1991).

The main problem in experimental design is to provide data which can be analysed, yet remain statistically independent on a per subject basis. To conclusively test this hypothesis in humans requires large numbers of multiple samples taken from all teeth and implants in the same mouth. The present study, however, assumed that cross infection did exist and aimed only to demonstrate evidence of it at a single time point. Statistical independence problems were overcome by developing a model whereby the issue of cross-infection was addressed from tooth to implant sites in each mouth. The analysis from each mouth was then pooled and treated as independent data. The model allowed for four possibilities of cross infection: ++, --, + -, - +. The hypothesis of cross-infection was supported in the case of ++ and rejected in the case of + -. Occasionally there were incidences where an organism was absent from the tooth site(s) sampled yet detected at implant site(s) in the same mouth. Several explanations for this are possible including unsampled teeth in the same mouth harbouring the organism or exogenous sources of infection (van Steenberg et al. 1993). Multiple samples were not taken in the present study to avoid the risk of type 1 errors (false positives).

The present study found that, for a given micro-organism, a significant association between its presence or absence at teeth and implants in the same mouth occurs only with *P. gingivalis*, *T. denticola* and *C. recta*. However, the nature of this association is such that when they were present at tooth sites, these organisms are mostly absent from implant sites, rejecting the hypothesis of cross-infection. This finding must be viewed in light of the limitations of the study design. Ideally all tooth and implant sites should be tested and subjected to the same analytical model. The choice of a representative

tooth in each clinical category aims to avoid sample bias without resorting to this extreme. In addition, the failure to identify a particular species does not mean it is completely absent from the site, but rather that its presence is below the detection limits of the DNA probes (3×10^6 organisms per paper point). Alternatively, sampling error could also account for such discrepancies (Baker et al. 1991).

An alternative explanation for the apparent lack of evidence of cross infection from teeth to implants in the present study may involve the anatomical differences between the implant and tooth sulcus. It is feasible that the local environment of the implant associated crevice may result in a preferential selection of some organisms at the expense of others. Hillman and Socransky (1982) have described an example where the local overgrowth of *A. actinomycetemcomitans* is suppressed by hydrogen peroxide produced by *Streptococcus sanguis* (*S. Sanguis*), an early coloniser of teeth (Socransky et al. 1977). A delay in the establishment of a protective microflora may give rise to pathologic changes without the normal succession of organisms seen at teeth. The lack of a cementum into which endotoxin may penetrate, or the lack of a true connective tissue attachment are two such anatomical differences (Listgarten et al. 1991). Hence, there is a need to develop an animal model to test the hypothesis in a controlled manner and to provide direct proof of such cross infection. The validity of any detected cross infection using this model can also be enhanced by genotyping the organism found at the tooth and implant sites. This offers more information regarding the source of the micro-organism at the implant site (ie. endogenous or exogenous). A recent report by van Steenberg et al. (1993) described the use of DNA restriction

endonuclease analysis of *P. gingivalis* isolates to demonstrate cross infection of this micro-organism within 7 of 8 couples. Hence, a similar comparison could be made between an isolate found in an implant crevice and one intentionally implanted into the gingival sulcus of a laboratory animal. The clinical implications of a lack of cross infection would include a revision of the justification for the prophylactic extraction of periodontally involved teeth prior to abutment connection (Stage II) surgery.

Validity testing of the DNA probe methodology indicated a 100% agreement with the cultured organisms. Hence, the sensitivity and specificity is equal to 1. This result is not unexpected given the method used, and confirms that no cross reactivity occurs between species which could alter analysis of the results. A clinical comparison of DNA and culture methods was conducted by Maiden et al. (1991) for the same six periodontal pathogens used in the validation of this study. *T. denticola* can not be cultured readily so could not be tested against the DNA probes. They found an overall sensitivity for the six organisms ranged from 0.26 for *A. actinomycetemcomitans* to 0.90 for *F. nucleatum*. The poor sensitivity for some species was attributed to the lower detection limit of culture methods. The specificity of DNA probes ranged from 0.21 for *P. gingivalis* to 0.93 for *A. actinomycetemcomitans*. Low specificity was due to large numbers of sites testing positive by the probe but negative by culture. This highlights the difficulty in culturing these fastidious anaerobes. The value of DNA probes in the detection of periodontal pathogens over other methods was recently reviewed by Loesche et al. (1992). DNA probes, ELISA and immunofluorescence were significantly superior to culturing for the detection of *P. gingivalis*, *A.*

actinomycescomitans, *Bacteroides forsythus* and were comparable to the microscopic approach in the detection of *T. denticola*. Strezempko et al. (1987) did not find cross reactivity of DNA probes for *P. gingivalis* and *A. actinomycescomitans* with over 40 bacterial species that may be expected to be found in plaque. For these reasons, DNA probes are the preferred analytical tool in the present study.

Paper point sampling is used in the present study for convenience despite known shortcomings (Baker et al 1991). Paper points attract microorganisms by capillary action of the paper, thereby selecting bacteria from crevicular fluid and not those strongly adherent to the surface. Renvert et al. (1992) showed that sampling obtained with a paper point resulted in higher numbers of CFU irrespective of the order in which sampling was performed. The paper point method was found to recover significantly more CFU and numbers of spirochetes, while the sampling order had no significant influence on any of the bacterial variables. Kiel and Lang (1983) reported 10 to 1,000 fold more CFU obtained with curette sampling compared to paper points. They reported that the microorganisms obtained with paper points increased with the number of paper points used. Moore et al. (1985) detected similar numbers of CFU for samples collected by a Morse scaler 00 and one paper point. The number of paper points inserted in the pocket and the technique used for scaler sampling could explain the differences reported in various studies. Similar criticism of the technique has been made by Baker et al. (1991). Using an *in vitro* method they found that 90% of microorganisms obtained by paper point sampling were from the top layer, possibly because the paper points became saturated by the top layer and were incapable of

absorbing organisms from lower layers. It is difficult to extrapolate such an *in vitro* findings to *in vivo* situation. In the present study it was possible that more bacteria at the gingival margin were harvested than at the depth of the pocket. Fortunately, the absolute number of organisms present at the sampled site had no influence on the outcome of data analysis.

CONCLUSION

Using DNA probe analysis, the present study demonstrated a progressive increase in periodontopathic organisms from healthy to gingivitis to periodontitis tooth sites. This finding is consistent with our current knowledge of the pathogenesis of plaque related gingivitis and periodontal disease. However, no difference could be detected between the periimplantitis and ailing implant sites suggesting bacterial plaque may have a very limited role in the development and progression of ailing implants. The routine use of DNA probe analysis may assist in the differential diagnosis of ailing implants from biomechanical causes or that from bacterial infection.

The data also did not support the hypothesis of cross-infection of implant sites by periodontopathic organisms from periodontally involved teeth in the same mouth.

REFERENCES

ADELL R, LEKHOLM U, BRÅNEMARK P-I, LINDHE J, ROCKLER B, ERIKSSON B, LINDVALL A, YONEYAMA T, SBORDONE L. (1985)

Marginal tissue reactions at osseointegrated titanium fixtures. Proceedings of the Gothenberg Conference September 1983.

Swed Dent J 28(suppl.):175-181.

ADELL R, LEKHOLM U, ROCKLER B, BRÅNEMARK P-I. (1981)

A 15-year study of osseointegrated implants in the treatment of the edentulous jaw.

Int J Oral Surg 10:387-416.

ADELL R, LEKHOLM U, ROCKLER B, BRÅNEMARK P-I, LINDHE J, ERIKSSON B, SBORDONE L. (1986)

Marginal tissue reactions at osseointegrated titanium fixtures: (I). A 3-year longitudinal study.

Int J Oral Maxillofac Surg 15:39-52.

AHQVIST J, BORG K, GUNNE J, NILSON H, OLSSON M, ÅSTRAND P. (1990)

A long term follow up study of osseointegrated implants in edentulous jaws. A 2 year longitudinal study.

Int J Oral Maxillofac Implant 5:155-163.

ALBREKTSSON T, BRÅNEMARK P-I, HANSSON H, IVARSSON B, JÖNSSON U. (1982)

Ultrastructural analysis of the interface zone of titanium and gold implants. In: Lee A, Albrektsson T, Brånemark P-I, editors: Clinical applications of Biomaterials. John Wiley & Sons Ltd, New York, 1982:pp 167-177.

ALBREKTSSON T, BRÅNEMARK P-I, HANSSON H, LINDSTRÖM J. (1981)

Osseointegrated titanium implants. Requirements for ensuring a long lasting, direct bone-to-implant anchorage in man.

Acta Orthop Scand 52:55-70.

ALBREKTSSON T and SENNERBY L. (1991)

State of the art in oral implants.

J Clin Periodontol 18:474-481.

- ALBREKTSSON T, ZARB G, WORTHINGTON P, ERIKSSON A. (1986)**
The long-term efficacy of currently used dental implants: A review and proposed criteria of success.
Int J Oral Maxillofacial Implant 1:11-25.
- ALCOFORADO G, RAMS T, FEIK D, SLOTS J. (1991)**
Microbial aspects of failing osseointegrated dental implants in humans.
J Paradontol 10:11-18.
- APSE P, ELLEN R, OVERALL C, ZARB G. (1989)**
Microbiota and crevicular fluid collagenase activity in the osseointegrated dental implant sulcus: a comparison of sites in edentulous and partially edentulous patients.
J Periodont Res 24:96-105.
- APSE P, ZARB G, SCHMITT A, LEWIS D. (1991)**
The longitudinal effectiveness of osseointegrated dental implants. The Toronto study: Peri-implant mucosal response.
Int J Perio Resto Dent 11:95-111.
- ARVIDSON K, BAYSTEDT H, ERICSSON I. (1990)**
Histomeric and ultrastructural studies of tissues surrounding Astra dental implants in dogs.
Int J Oral Maxillofac Implant 5:127-134.
- BAIMA R. (1986)**
Extension of clinical crown length.
J Prosthet Dent 55:547-551.
- BAKER P, BUTLER R, WIKESJÖ U. (1991)**
Bacterial sampling by absorbent paper points. An in vitro study.
J Periodontol 62:142-146.
- BAUMAN G, MILLS M, RAPLEY J, HALLMON W. (1992)**
Clinical parameters of evaluation during implant maintenance.
Int J Oral Maxillofac Implant 7:220-227.
- BECKER W, BECKER B, NEWMAN M, NYMAN S. (1990)**
Clinical and microbiologic findings that may contribute to dental implant failure.
Int J Oral Maxillofac Implant 5:31-38.

BERGLUNDH T, LINDHE J, ERICSSON I, MARINELLO C, LILJENBERG B, THOMSEN P. (1991)

The soft tissue barrier at implants and teeth.
Clin Oral Implant Res 2:81-90.

BERGLUNDH T, LINDHE J, MARINELLO C, ERICSSON I, LILJENBERG B. (1992)

The soft tissue reaction to de novo plaque formation on implants and teeth. An experimental study in the dog.
Clin Oral Impl Res 3:1-8.

BERRY C, MOORE T, SAFAR J, HENRY C, WAGNER M. (1992)

Antibacterial activity of dental implant metals.
Implant Dent 1:59-65.

BOWER R. (1987)

Periodontal considerations of endosseous implants: A philosophy for understanding the tissue-implant interface.
Aust Pros J 25-28.

BOWER R, RADNY N, WALL C, HENRY P. (1989)

Clinical and microscopic findings in edentulous patients three years after incorporation of osseointegrated implant supported bridgework.
J Clin Periodontol 16:580-587.

BRÅNEMARK P-I. (1985)

Introduction to osseointegration. In: Brånemark P-I, Zarb G, Albrektsson T, editors: Tissue-Integrated Prostheses: Osseointegration in Clinical Dentistry. Quintessence Publishing Co, Chicago, 1985; Ch 1:11-76.

BRUNETTE M, KENNER G, GOULD T. (1983)

Grooved titanium surfaces orient growth and migration of cells from human gingival explants.
J Dent Res 62:1045-1048.

BURCHARD W, COBB C, DRISKO C, KILLOY W. (1991)

Effects of chlorhexidine and stannous fluoride on fibroblast attachment to different implant surfaces.
Int J Oral Maxillofac Implant 6:418-426.

BUSER D, WARRER K, KARRING T, STICH H. (1990b)

Titanium implants with a true periodontal ligament: An alternative to osseointegrated implants?
Int J Oral Maxillofac Implant 5:113-116.

BUSER D, WEBER H, DONATH K, FIORELLINI J, PAQUETTE D, WILLIAMS R. (1992)

Soft tissue reactions to non-submerged unloaded titanium implants in beagle dogs.

J Periodont 63:225-235.

BUSER D, WEBER H, LANG N. (1990a)

Tissue integration of non submerged implants. 1 year results of a prospective study with 100 ITI hollow-cylinder and hollow-screw implants.

Clin Oral Implant Res 1:33-40.

DZINK D, SOCRANSKY S, HAFFAJEE A. (1988)

The predominant cultivable microbiota of active and inactive lesions of destructive periodontal disease.

J Clin Periodontol 15:316-323.

EGELBERG J. (1966)

The blood vessels of the dentogingival junction.

J Periodont Res 1:163-179.

ERICSSON I, BERGLUNDH T, MARINELLO C, LILJENBERG B, LINDHE J. (1992)

Long standing plaque and gingivitis at implants and teeth in the dog.

Clin Oral Implant Res 3:99-103.

FARTASH B, ARVIDSON K, ERICSSON I. (1990)

Histology of tissues surrounding single crystal sapphire endosseous dental implants. An experimental study in the beagle dog.

Clin Oral Implant Res 1:13-21.

FLETCHER R, SCHNEIDER G, LABANT M, ALBERTSON J. (1979)

An in vitro technique for measuring cell adhesion to rigid materials.

J Dent Res 58:1750-1751.

FOWLER C, GARRETT S, CRIGGER M, EGELBERG J. (1982) Histologic probe position in treated and untreated human periodontal tissues.

J Clin Periodontol 9:373-385.

FOX S, MORIARTY D, KUSY R. (1990)

The effect of scaling a titanium implant surface with metal and plastic instruments: An in vitro study.

J Periodontol 61:485-490.

- FRANK R, FIORE-DONNO G, CIMISONI G, OGILVIE A. (1972)**
Gingival reattachment after surgery in man. An electron microscopic study.
J Periodontol 43:597-607.
- FRENCH K, SAVITT E, SIMON S, EKLUND S, CHEN M, KLOTZ L, VACCARO K. (1986)**
DNA probe detection of periodontal pathogens.
Oral Microbiol Immunol 1:58-62.
- GARGUILO A, WENTZ F, ORGAN B. (1961)**
Dimensions and relations of the dento-gingival junction in humans.
J Periodontol 32:261-267.
- GATEWOOD R, COBB C, KILLOY W. (1993)**
Microbial colonisation on natural tooth structure compared with smooth and plasma-sprayed dental implant surfaces.
Clin Oral Implant Res 4:53-64.
- GAVIN J. (1970)**
Ultrastructural features of chronic marginal gingivitis.
J Periodont Res 5:19-25.
- GIPSON I, GRILL S, SPURR S, BRENNAN S. (1983)**
Hemidesmosome formation in vitro.
J Cell Biol 97:849-857.
- GOTFREDSEN K, ROSTRUP E, HOJORTING-HANSEN E, STOLTZE K, BUDTZ-JORGENSEN E. (1991)**
Histologic and histomorphometrical evaluation of tissue reactions adjacent to endosteal implants in monkeys.
Clin Oral Implant Res 2:30-37.
- GOULD T, BRUNETTE D, WESTBURY L. (1981)**
The attachment of epithelial cells to titanium in vitro.
J Periodont Res 16:611-616.
- GOULD T, WESTBURY L, BRUNETTE D. (1984)**
Ultrastructural study of the attachment of human gingiva to titanium in vivo.
J Prosthet Dent 52:418-420.
- GUY S, McQUADE M, SCHEIDT M, McPHERSON J. (1993)**
In vitro attachment of human gingival fibroblasts to endosseous implant materials.
J Periodontol 64:542-546.

HAANAES H. (1990)

Implant and infections with special reference to oral bacteria.
J Clin Periodontol 17:516-524.

HAFFAJEE A, SOCRANSKY S, GOODSON J. (1983)

Clinical parameters as predictors of destructive periodontal disease activity.
J Clin Periodontol 10:257-265.

HAKOMORI S, FAKUDA M, SEKIGUCHI K, CARTER W. (1984)

Fibronectin, laminin and other extracellular glycoproteins. In: Piez K, Reddi A, editors: Extracellular matrix biochemistry. Elsevier, New York 1984:pp 229-275.

HALAZONETIS T, HAFFAJEE A, SOCRANSKY S. (1989)

Relation of clinical parameters to attachment level loss in subsets of subjects with destructive periodontal disease.
J Clin Periodontol 16:563-568.

HANSSON H, ALBREKTSSON T, BRÅNEMARK P-I. (1983)

Structural aspects of the interface between tissue and titanium implants.
J Prosthet Dent 50:108-113.

HICKEY J, O'NEAL R, SCHEIDT M, STRONG S, TURGEON D, VAN DYKE T. (1991)

Microbiologic characterisation of ligature-induced peri-implantitis in the microswine model.
J Periodontol 62:548-553.

HILLMAN J and SOCRANSKY S. (1982)

Bacterial interference in the oral ecology of *Actinobacillus actinomycetemcomitans* and its relationship to human periodontosis.
Arch Oral Biol 27:75-77.

INOUE T, COX J, PILLIAR R, MELCHER A. (1987)

Effect of the surface geometry of smooth and porous-coated titanium alloy on the orientation of fibroblasts in vitro.
J Biomed Material Res 21:107-126.

JAMES R and SCHULTZ R. (1974)

Hemidesmosomes and the adhesion of junctional epithelial cells to metal implants. A preliminary report.
Oral Implantol 4:294-302.

JANSEN J, DE WIJN J, WOLTERS-LUTGERHORST J, VAN MULLEM P. (1985)

Ultrastructural study of epithelial cell attachment to implant materials.
J Dent Res 64:891-896.

JEFFCOAT M, REDDY M, VAN DEN BERG H, BERTENS E. (1992)

Qualitative digital subtraction radiography for the assessment of periimplant bone change.
Clin Oral Implant Res 1:22-27.

KASEMO B. (1983)

Biocompatibility of titanium implants: Surface science aspects.
J Prosthet Dent 49:832-837.

KASEMO B and LAUSMAA J. (1985)

Metal selection and surface characteristics. In: Brånemark P-I, Zarb G, Albrektsson T, editors: Tissue-Integrated Prostheses: Osseointegration in Clinical Dentistry. Quintessence Publishing Co, Chicago, 1985:pp 99-116.

KASTEN F, SOILEAU K, MEFFERT R. (1990)

Quantitative evaluation of human gingival epithelial cell attachment to implant surfaces in vitro.
Int J Perio Rest Dent 10:69-79.

KIEL R and LANG N. (1983)

Effect of subgingival sampling techniques on periodontal microbiological culturing.
J Dent Res 62(Special Issue):247(Abstr.702).

KLEINMAN H, KLEBE R, MARTIN G. (1981)

Role of collagenous matrices in the adhesion and growth of cells.
J Cell Biol 88:473-485.

KLINGE B. (1991)

Implants in relation to natural teeth.
J Clin Periodontol 18:482-487.

KOBAYASHI K, ROSE G, MAHAN C. (1976)

Ultrastructure of the dentoepithelial junction.
J Periodont Res 11:313-330.

KOKA S, RAZOOGME, BLOEM T, SYED S. (1993)

Microbial colonisation of dental implants in partially edentulous subjects.
J Prosthet Dent 70:141-144.

KORNMAN K, JONES A, NOLAN P, ALVARADO R, MANWELL M, WOOD R. (1992)

Culture, DNA probes and monoclonal antibodies for the detection of bacteria in periodontitis.

J Dent Res 71(Special Issue A):121(Abstr. 126).

KOTH D, McKINNEY R, STEFLIK D. (1985)

A clinical evaluation of the implant-gingival tissue interface. In:McKinney R, Lemons J, editors: The Dental Implant. Clinical and biological response of oral tissues. PSG Publishing Co. Inc. Littleton, 1985:pp 114-130.

KURASHINA K, DE LANGE G, DE PUTTER C, DE GROOT K. (1984)

Reaction of surrounding gingiva to permucosal implants of dense hydroxyapatite in dogs.

Biomaterials 5:215-220.

LANG N, ADLER R, JOSS A, NYMAN S. (1990)

Absence of bleeding on probing: An indicator of periodontal stability.

J Clin Periodontol 17:714-721.

LEKHOLM U, ERICSSON I, ADELL R, SLOTS J. (1986)

The condition of soft tissues at tooth and fixture abutments supporting fixed bridges. A microbial and histological study.

J Clin Periodontol 13:558-562.

LAVELLE C. (1981)

Mucosal seal around endosseous dental implants.

J Oral Implantol 9:357-371.

LEONHARDT A, BERGLUNDH T, ERICSSON I, DAHLEN G. (1992)

Putative periodontal pathogens on titanium implants and teeth in experimental gingivitis and periodontitis in beagle dogs.

Clin Oral Implant Res 3:112-119.

LINDHE J, BERGLUNDH T, ERICSSON I, LILJENBERG B, MARINELLO C. (1992)

Experimental breakdown of peri-implant and periodontal tissues. A study in the beagle dog.

Clin Oral Implant Res 3:9-16.

LIPPKE J, PEROS W, KEVILLE M, SAVITT E, FRENCH C. (1991)

DNA probe detection of *Eikenella corrodens*, *Wolinella recta* and *Fusobacterium nucleatum* in subgingival plaque.

Oral Microbiol Immunol 6:81-87.

LISTGARTEN M. (1966)

Electron microscopic study of the dentogingival junction in man.
Am J Anat 119:147-154.

LISTGARTEN M. (1967)

Electron microscopic features of the newly formed epithelial attachment after gingival surgery.
J Periodontol 2:46-51.

LISTGARTEN M. (1976)

Structure of the microbial flora associated with periodontal disease and health and disease in man. A light and electron microscopic study.
J Periodontol 47:1-18.

LISTGARTEN M and ELLEGAARD B. (1973)

Electron microscopic evidence of cellular attachment between junctional epithelium and dental claculus.
J Periodont Res 8:143-155

LISTGARTEN M and HELLDÉN L. (1978)

Relative distribution of bacteria at clinically healthy and periodontally diseased sites in humans.
J Clin Periodontol 5:115-132.

LISTGARTEN M, LAI C-H. (1975)

Ultrastructure of the intact interface between an endosseous epoxy resin dental implant and the host tissue.
J Biol Buccale 3:13-28.

LISTGARTEN M, LANG N, SCHRÖEDER H, SCHRÖEDER A. (1991)

Periodontal tissues and their counterparts around endosseous implants.
Clin Oral Implant Res 2:1-19.

LISTGARTEN M, MAO R, ROBINSON P. (1976)

Periodontal probing and the relationship of the probe tip to periodontal tissues.
J Periodontol 47:511-513.

LÖE H, LISTGARTEN M, TERRANOVA V. (1990)

The gingiva. Structure and function. In: Genco R, Goldnman H, Cohen D, editors: Contemporary Periodontics. C.V.Mosby, St Louis, 1990:p 25.

LÖE H and SILNESS J. (1963)

Periodontal disease in pregnancy. I. Prevalence and severity.
Acta Odont Scand 21:533-551.

LÖE H, THEILADE E, JENSEN S. (1965)

Experimental gingivitis in man.
J Periodontol 36:177-187.

LOESCHE W, LOPATIN D, STOLL J, VAN POPERIN N, HUJOEL P. (1992)

Comparison of various detection methods for periodontopathic bacteria: Can culture be considered the primary reference standard?
J Clin Microbiol 30:418-426.

LOWENBERG B, AUBIN J, DEPORTER D, SODEK J, MELCHER A. (1985)

Attachment, migration and orientation of human gingival fibroblasts to collagen-coated, surface-demineralized and non-demineralized dentin in vitro.
J Dent Res 64:1106-1110.

LOWENBERG B, PILLIAR R, AUBIN J, FERNIE G, MELCHER A. (1987)

Migration, attachment and orientation of human gingival fibroblasts to root slices, naked and porous-surfaced titanium alloy discs, and Zircalloy 2 discs in vitro.
J Dent Res 66:1000-1005.

MAIDEN M, TANNER A, McARDLE S, NAJAPAUER K, GOODSON J. (1991)

Tetracycline fibre therapy monitored by DNA probe and cultural methods.
J Periodont Res 26:452-459.

McKINNEY R, STEFLIK D, KOTH D. (1984)

The biologic response to the single crystal sapphire endosseous dental implant: Scanning electron microscopic observations.
J Prosthet Dent 51:372-379.

McKINNEY R, STEFLIK D, KOTH D. (1985)

Evidence for a junctional epithelial attachment to ceramic dental implants. A transmission electron microscopic study.
J Periodontol 56:579-591.

MEFFERT R. (1992)

How to treat ailing and failing implants.
Implant Dent 1:25-33.

MELCHER A. (1962)

The pathogenesis of chronic gingivitis.
Dent Pract 12:2-1

MOMBELLI A and LANG N. (1992)

Antimicrobial treatment of peri-implant infections
Clin Oral Implant Res 3:162-168.

MOMBELLI A and MERICSKE-STERN R. (1990)

Microbiological features of stable osseointegrated implants used as abutments for overdentures.
Clin Oral Implant Res 1:1-7.

MOMBELLI A, VAN OOSTEN M, SCHÜRCH E, LANG N. (1987)

The microbiota associated with successful or failing osseointegrated titanium implants.
Oral Microbiol Immunol 2:145-151.

MOMBELLI A, BUSER D, LANG N. (1988)

Colonisation of osseointegrated titanium implants in edentulous patients. Early results.
Oral Microbiol Immunol 3:113-120.

MOORE W. (1987)

Microbiology of periodontal disease.
J Periodont Res 22:335-341.

MOORE W, HOLDERMAN L, CATO E. (1985)

Comparative bacteriology of juvenile periodontitis.
Infect Immun 48:507-519.

MÜHLEMANN H, SON S. (1971)

Gingival sulcus bleeding-a leading symptom in initial gingivitis.
Helv Odontol Acta 15:107-113.

MÜHLEMANN H. (1967)

Tooth Mobility: A review of clinical aspects and research findings.
J Periodontol 38:686-708.

MURRAY P and FRENCH C. (1989)

DNA probe detection of periodontal pathogens. In: Myers H, editor: New Biotechnology in Oral Research. Karger, Basel, 1989:pp 33-53.

MYASATO M, CRIGGER M, EGELBERG J. (1977)

Gingival condition in areas of minimal and acceptable width of keratinised gingiva.
J Clin Periodontol 4:200-209.

NAKAZATO G, HIRONORI T, SATO M, YAMAUCHI M. (1989)

In vivo plaque formation on implant materials.

Int J Oral Maxillofac Implant 4:321-326.

NEWMAN M and FLEMMING T. (1988)

Periodontal considerations of implants and implant associated microbiota.

J Dent Educ 52:737-744.

NYMAN S, LINDHE J, ROSLING B. (1977)

Periodontal surgery in plaque infected dentitions.

J Clin Periodontol 4:240-249.

PAGE R and SCHRÖEDER H. (1976)

Pathogenesis of inflammatory periodontal disease. A summary of current work.

Lab Invest 33:235-247.

NYVAD B and KILIAN M. (1987)

Microbiology of the early colonisers of human enamel and root surfaces in vivo. Scand J Dent Res 95:369-380.

ONG E, NEWMAN H, WILSON M, BULMAN J. (1992)

The occurrence of periodontitis related micro-organisms in relation to titanium implants.

J Periodontol 63:200-205.

PICTON D, JOHNS R, WILLS D, DAVIES W. (1974)

The relationship between the mechanisms of tooth and implant support.

Oral Sci Rev 5:3-22.

QUIRYNEN M and LISTGARTEN M. (1990)

The distribution of bacterial morphotypes around natural teeth and titanium implants ad modum Brånemark.

Clin Oral Implant Res 1:8-12.

QUIRYNEN M, NAERT I, VAN STEENBERGHE D. (1992)

Fixture design and overload influence marginal bone loss and fixture success in the Brånemark system.

Clin Oral Implant Res 3:104-111.

QUIRYNEN M, VAN DER MEI H, BOLLEN C, SCHOTTE A, MARECHAL M, DOORNBUSCH G, NAERT I, BUSCHER H, VAN STEENBERGHEN D. (1993)

An in vivo study of the influence of the surface roughness of implants on the microbiology of supra- and subgingival plaque.

J Dent Res 72:1304-1309.

QUIRYNEN M, VAN STEENBERGHE D, JACOBS R, SCHOTTE A, DARIUS P. (1991)

The reliability of pocket probing around screw type implants.
Clin Oral Implant Res 2:186-193.

RAMS T and LINK C. (1983)

Microbiology of failing dental implants in humans. Electron microscopic observations.

J Oral Implantol 11:93-100.

RAMS T, ROBERTS T, FEIK D, MOLZAN A, SLOTS J. (1991)

Clinical and microbiological findings on newly inserted hydroxyapatite-coated and pure titanium human dental implants.

Clin Oral Implant Res 2:121-127.

RAMS T, ROBERTS T, TATUM H, KEYES P. (1984)

The subgingival microflora associated with human dental implants.

J Prosthet Dent 51:529-534.

RENVERT S, WILKSTRÖM M, HELMERSSON M, DAHLÉN G, CLAFFEY N. (1992)

Comparative study of subgingival sampling techniques.

J Periodontol 63:797-801.

ROBERTS W, SMITH R, ZILBERMAN Y, MOZSARY P, SMITH R. (1984)

Osseous adaptation to continuous loading of rigid endosseous implants.

Am J Orthodont 86:95-110

ROBERTS W, TURLEY P, BREZNIAK N, FIELDER P. (1987)

Bone physiology and metabolism.

J Calif Dent Assoc 15:54-63.

ROSENBERG E, TOROSIAN J, SLOTS J. (1991)

Microbial differences in two clinically distinct types of failures of osseointegrated implants.

Clin Oral Implant Res 2:135-144.

RUGGERI A, FRANCHI M, MARINI N, TRIST P, PAITELLI A. (1992)

Supracrestal circular collagen fibre network around osseointegrated non-submerged titanium implants.

Clin Oral Implant Res 3:169-175.

SANZ M, ALANDEZ J, LAZARO P, CALVO JL, QUIRYNEN M, VAN STEENBERGHE D. (1991)

Histopathologic characteristics of peri-implant soft tissues in Brånemark implants with 2 distinct clinical and radiographic patterns. A histomeric and ultrastructural study.

Clin Oral Implant Res 2:128-134.

SAVITT E, KEVILLE M, PEROS W. (1990)

DNA probes in the diagnosis of periodontal microorganisms.

Arch Oral Biol 35(Suppl. 1):1535-1595.

SAVITT E and SOCRANSKY S. (1984)

Distribution of certain subgingival microbial species in selected periodontal conditions.

J Periodont Res 19:111-123.

SAVITT E, STRZEMPKO M, VACCARO K, PEROS W, FRENCH C. (1988)

Comparison of cultural methods and DNA probe analyses for the detection of *Actinobacillus actinomycetemcomitans*, *Bacteriodes gingivalis*, and *Bacteriodes intermedius* in subgingival plaque samples.

J Periodontol 59:431-438.

SCHMID J, COHEN R, CHAMBERS D. (1991)

Plasminogen activator in human periodontal health and disease.

Arch Oral Biol 36:245-250.

SCHMID B, SPICHER I, SCHMID J, LANG N (1992)

Plasminogen activator in human gingival tissue adjacent to dental implants.

Clin Oral Implant Res 3:85-89.

SCHOU S, HOLMSTRUP P, REIBEL J, JUHL M, HJORTING-HANSEN E, KORNMAN KS. (1993)

Ligature induced marginal inflammation around teeth: Stereologic and histologic observations in Cynomolgus monkeys (*Macaca Fascicularis*).

J Periodontol 64:529-537.

SCHRÖEDER H. (1988)

Origin, structure and distribution of cementum and its possible role in local periodontal treatment. In: Periodontology Today. Basel, Zurich, 1988:pp 32-40.

SCHRÖEDER H and LISTGARTEN M. (1977)

Fine structure of the developing epithelial attachment of human teeth. Monographs of Developmental Biology. Krager, Basel, 1977:pp 44-112

SCHRÖEDER A, VAN DER ZYPEN E, STICH H, SUTTER F. (1981)

The reaction of bone, connective tissue and epithelium to endosteal implants with sprayed titanium surfaces.

J Oral Maxillofac Surg 9:15-25.

SEKINE H, KOMIYAMA Y, HOTTA, YOSHIDA K. (1986)

Mobility characteristics and tactile sensitivity of osseointegrated fixture-supporting systems. In: van Steenberghe D, Albrektsson T, Brånemark P-I, editors: Tissue-integration in oral and maxillofacial reconstruction.

Amsterdam, Excerpta Medica, 1986:pp 60-71.

SEYMOUR G, GEMELL E, LENZ L, HENRY P, BOWER R, YAMAZAKI K. (1989)

Immunohistologic analysis of the inflammatory infiltrates associated with osseointegrated implants.

Int J Oral Maxillofac Implant 4:191-198.

SIEGRIST B, BRECX M, GUSBERTI F, JOSS A, LANG N. (1991)

In vivo early human plaque formation on different supporting substances. A scanning electron microscopic and bacteriological study.

Clin Oral Implant Res 2:38-46.

SILNESS J and LÖE H. (1964)

Periodontal disease in pregnancy. II. Correlation between oral hygiene and periodontal condition.

Acta Odontol Scand 24:747-759.

SKALAK R. (1983)

Biomechanical considerations in osseointegrated prostheses.

J Prosthet Dent 49:843-848.

SLOTS J. (1977a)

Microflora in the healthy gingival sulcus in man.

Scand J Dent Res 85:247-254.

SLOTS J. (1977b)

The predominant cultivable microflora of advanced periodontitis.

Scand J Dent Res 85:114-121.

SLOTS J and ROSLING B. (1983)

Suppression of periodontopathic microflora in localised juvenile periodontitis by systemic tetracycline.

J Clin Periodontol 10:465-486.

SOCRANSKY S, HAFFAJEE A, SMITH C, DIBART S. (1991)

Relation of counts of microbial species to clinical status at the sampled site.
J Clin Periodontol 18:766-775.

SOCRANSKY S, MAGANIELLO A, PROPAS D, ORAM V, VAN HOUTE J. (1977)

Bacteriological studies of developing supragingival dental plaque.
J Periodont Res 12:90-106.

STALLARD R. (1985)

The periodontal implant junction. In: McKinney R, Lemons J, editors: The dental implant clinical and biological response of oral tissues.
PSG Publishing Co. Inc. Littleton, 1985:pp 131-142.

STEFLIK D, MCKINNEY R, SISK A, PARR G, MARSHALL B. (1991)

Dental implants retrieved from humans: A diagnostic light microscopic review of the findings in seven cases of failure.
Int J Oral Maxillofac Implants 6:147-153.

STRUB J, GARBERTHUEL T, SCHARER P. (1988)

Role of attached gingiva for peri-implant health in dogs
J Dent Res 67(special issue):287(Abstr 1396).

STRZEMPKO M, SIMON S, FRENCH C, LIPPKE J, RAIA F, SAVITT E, VACCARO K. (1987)

A cross-reactivity study of whole genomic DNA probes for *Haemophilus actinomycetemcomitans*, *Bacteroides intermedius* and *Bacteroides gingivalis*.
J Dent Res 66:1543-1546.

TAKARADA H, CATTONI M, SUGIMOTO A, ROSE G. (1974)

Ultrastructural studies of human gingiva II: The lower part of the pocket epithelium in chronic periodontitis.
J Periodont Res 45:30-43.

TAYLOR A and CAMPBELL M. (1972)

Reattachment of gingival epithelium to the tooth.
J Periodontol 43:281-293.

TEN CATE A. (1975).

The dentogingival junction. A review of the literature.
J Periodontol 46:475-477.

TEN CATE A. (1985)

The Gingival Junction In: Brånemark P-I, Zarb G, Albrektsson T, editors: Tissue-Integrated Prostheses: Osseointegration in Clinical Dentistry Eds. Quintessence, Chicago: 1985:pp 145-153.

THEILADE E, WRIGHT W, JENSEN B, LÖE H. (1966)
Experimental gingivitis in man. II Longitudinal clinical and bacteriological investigation.
J Periodontol Res 1:1-13.

THOMSEN P and ERICSSON I. (1985)
Light and transmission electron microscopy used to study the tissue morphology close to implants.
Biomaterials 6:42-424.

VANOOTEGHEM R, HUTCHENS L, BOWERS G, KRAMER G, SCALLHORN R, KIGER R, CRIGGER M, EGELBERG J. (1990)
Subjective criteria and probing attachment loss to evaluate the effects of plaque control and root debridement.
J Clin Periodontol 17:580-587.

VAN PALENSTEIN-HELDERMAN W. (1981)
Microbial etiology of periodontal disease.
J Clin Periodontol 8:261-280.

VAN STEENBERGEN T, PETIT M, SCHOLTE L, VAN DER VELDEN U, DE GRAFF J. (1993)
Transmission of *Porphyromonas gingivalis* between spouses.
J Clin Periodontol 20:340-345.

VAN STEENBERGHE D. (1988)
Periodontal aspects of osseointegrated oral implants ad modum Brånemark.
Dent Clin North Am 32:355-370.

VAN DER VELDEN U and DE VRIES J. (1978)
Introduction of a new periodontal probe: the pressure probe.
J Clin Periodontol 5:188-197.

VON RECUM A, SCHREUDERS P, POWERS D. (1985)
Basic healing phenomena around permanent percutaneous implants. In: Proceedings of the International Congress of Tissue Integration in Oral and Maxillofacial Reconstruction. Brussels. May 1985:pp 159-169.

WASSERMAN B, GEIGER A, TURGEON L. (1973)

Relationship of occlusion and periodontal disease.
J Periodontol 44:572-578.

WENNSTRÖM J and LINDHE J. (1983)
Role of attached gingiva for maintenance of periodontal health.
J Clin Periodontol 10:206-221.

ZAMBON J, CHRISTERSSON L, SLOTS J. (1983)
Actinobacillus actinomycetemcomitans in human periodontal disease:
Prevalence in patient groups and distribution of biotypes and serotypes within
families.
J Periodontol 54:707-711.

APPENDIX 1

FORMULATION OF GROWTH MEDIA

BHV Plates

To make up 3L

117gms Oxoid Columbia Agar Base^a

15gms Oxoid Yeast Extract

Suspend in 3L of sterile distilled water

Add: 3mL Vitamin K solution^a

30mL Haemin/Cystine solution^a

pH = 7.5 after addition of the above.

Autoclave at 121°C for 15 minutes with a pouring temperature of 40°C.

At pouring temperature add 150mL fresh horse blood.

Dispense the medium into 90mm petri dishes with approximately 20mL per plate.

Blood Agar Plates

To make up 1L

40gms Oxoid Columbia Agar Base

Suspend in 1L of sterile distilled water

Autoclave at 121°C for 15 minutes with a pouring temperature of 40°C.

At pouring temperature add 50mL defibrinated horse blood.

Dispense the medium into 90mm petri dishes with approximately 20mL per plate.

Formate and Fuamrate enriched Plates

Sodium Formate 2gms/L (final concentration)

Sodium Fumarate 3gms/L (final concentration)

Add to dry ingredients of BHV plates and proceed as for BHV plates.

^aSee Appendix 2 for formulation

APPENDIX 2

FORMULATION OF STOCK SOLUTIONS

Vitamin K (Menadione) Solution

Final concentration in media is 10mg/L

1mL Vitamin K (stock solution) dissolved in 99mL absolute ethanol.

Filter this stock solution.

Aliquot into 3mL amounts and store at 20°C.

NOTE: As vitamin K is light sensitive precautions to minimise light exposure must be taken.

Haemin / Cystine Solution

50mg Haemin

4gms L-cystine

Dissolve in 40mL of 1M NaOH

Make up to 100mL with sterile distilled water.

NOTE: Haemin final concentration in the media should be 5mg/L

L-cystine final concentration in the media should be 400mg/L

This stock solution is made up fresh because it is light sensitive. A better quality of media is produced when prepared fresh.

Oxoid Columbia Agar Base (CM331)^a

Special Peptone 23gms/L

Starch 1gm/L

Sodium Chloride 5gms/L

Agar No. 1 10gms/L

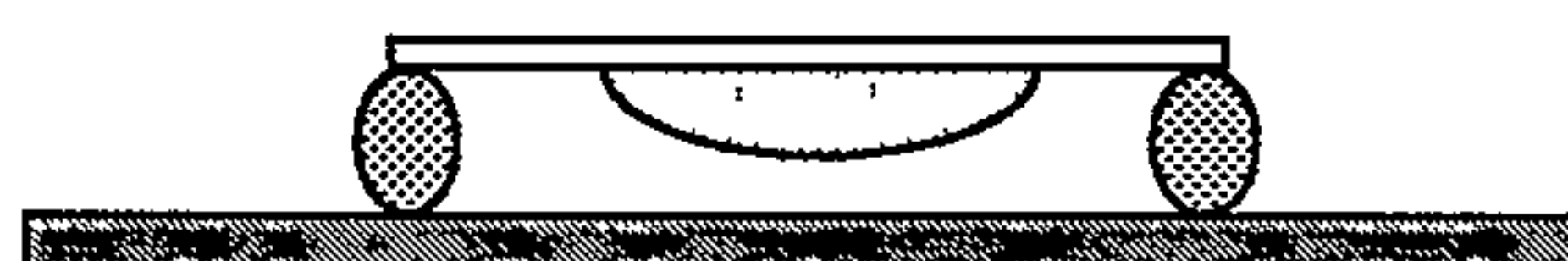
pH 7.3 ± 0.2

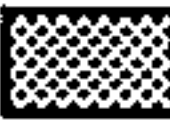
^aUnipath Ltd., Hampshire England

APPENDIX 3

TESTS FOR IDENTIFICATION OF BACTERIAL SPECIES

MOTILITY - (hanging drop method)



-  Cover slip
-  Hanging drop
-  Paraffin
-  Microscope slide

Place paraffin on each of the four corners of a cover slip and a drop of meat broth inoculated with the test organism in the centre. Place a microscope slide over the top, invert and view under a light microscope. Look for evidence of motility of the organisms.

INDOLE

The purpose of this test is to determine the ability of an organism to split indole from the tryptophan molecule.

Tryptophan deamination is reductive whereby ammonium (NH_2) is removed and released as ammonia (NH_3) and energy which is utilised by the bacterium. The degradation of tryptophan releases indole, pyruvic acid, ammonia and energy. The pyruvic acid can be further metabolised by the glycolytic cycle or by entering Krebs's cycle to release CO_2 , water and a large yield of energy. The NH_3 can be used to synthesise new amino acids by using the energy present for the anabolic reaction.

The indole, split from the tryptophan molecule can be detected by a reagent which involves a chemical combination producing a distinct colour.

NITRATE

To detect the ability of an organism to reduce nitrate to nitrite or nitrogen gas.

OXIDASE

To determine the presence of production of an intracellular oxidase enzyme. Oxidases catalyze removal of hydrogen from a substrate but use only oxygen as a hydrogen acceptor. Obligate anaerobic organisms lack oxidase activity since they are unable to live in the presence of atmospheric oxygen and do not possess a cytochrome oxidase system.

CATALASE

The purpose of this test is to identify the presence of the enzyme catalase. The enzyme catalase is present in most cytochrome - containing aerobic and facultative anaerobic bacteria. Usually, organisms which lack the cytochrome system also lack the catalase enzyme and therefore are unable to break down hydrogen peroxide.

Most anaerobic bacteria possess a peroxidase enzyme in lieu of catalase. A limitation of the catalase test is that it is not specific and may interfere with the peroxidase enzymes. Also, red blood cells contain catalase and their presence gives a false positive result, so this test cannot be done off blood agar.

MacFaddin Jean F. Biochemical Tests for identification of Medical Bacteria. Second Edition (Williams and Wilkins Baltimore USA)1980.

APPENDIX 4

LABORATORY IDENTIFICATION OF 6 ORGANISMS

Laboratory identification of 6 putative periodontopathic organisms.

	Aa	Pg	Pi	Ec	Fn	Cr
Gram stain	—	—	—	—	—	—
Facultative anaerobe (F)/Anaerobe (A)	F	A	A	F	A	A
Brown/Black colony	—	+	+	—	—	—
Formate/Fumarate needed for growth	—	—	—	—	—	+
Motility using hanging drop technique	—	N/A	N/A	—	N/A	+
Sugars - Glucose	+	—	+	—	—	—
- Maltose	+	—	+	—	—	—
- Lactose	—	—	—	—	—	—
- Mannose	+	—	V	—	—	—
- Fructose	N/A	—	+	N/A	—	—
- Sucrose	—	—	+	—	—	—
Nitrate reducing	+	—	—	+	N/A	+
Oxidase	+	—	—	+	—	+ ^w
Catalase	+	—	—	—	N/A	—
Lipase	N/A	—	+	N/A	—	N/A
Indole	—	+	+	—	+	N/A
β N-acetyl-glucosaminidase	N/A	+	N/A	N/A	N/A	N/A

— negative

+ positive

+^w weak positive

N/A not applicable

V variable

F facultative anaerobe

A obligate anaerobe

DENTISTRY LIBRARY
UNIVERSITY OF SYDNEY