OCCURRENCE OF RED-COMPLEX AND AGGREGATIBACTER ACTINOMYCETEMCOMITANS IN SUBGINGIVAL PLAQUE AMONG PATIENTS WITH PERIODONTAL DISEASE AT THE UNIVERSITY OF NAIROBI DENTAL HOSPITAL

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V60/70343/2011

A THESIS SUBMITTED IN PARTIAL FULFILLMENT FOR THE AWARD OF MASTER OF DENTAL SURGERY IN PERIODONTOLOGY DEGREE, SCHOOL OF DENTAL SCIENCES, UNIVERSITY OF NAIROBI

2014

DECLARATION

I declare that this thesis is my original work and it has not been submitted to any other institution for the award of a degree.

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DEDICATION

This thesis is dedicated to my wife Elizabeth Wambui and my daughter Isabelle Wanjiru.

ACKNOWLEDGEMENTS

I thank the almighty God for giving me the strength and perseverance to carry out this research to completion. I also want to thank the University of Nairobi for awarding me the scholarship that enabled me to pursue this master's degree course. I am also thankful to the Ministry of Defence- Kenya Navy for granting me study leave. I am especially grateful to Dr Nelson Matu for his guidance, support and assistance in handling and analysing the data. I also sincerely thank my other supervisors Dr Tonnie Mulli and Prof Loice Gathece for their guidance, positive criticism and encouragement all through the various stages of the research. I am greatly indebted to my wife Elizabeth Wambui and my daughter Isabelle Wanjiru for always being there for me and other members of my family for their ever-present encouragement and support in various ways.

I thank Teddy Amuge of the International Institute of Tropical Agriculture (IITA) Kenya and Cisily Kariuki of the University of Nairobi Institute of Tropical and Infectious Diseases (UNITID) for their valued technical advice on PCR. I also thank Prof. Jaoko, Chairman, Department of Medical Microbiology, University of Nairobi for his assistance and Dr Oyugi, Laboratory Director, UNITID for his advice, guidance and positive criticism.

I am also thankful to laboratory technicians Mr. Ernest Lutomia of UNITID for his assistance, Mr. John Kariuki of Department of Biochemistry, University of Nairobi, Mr. Simon Ogolla of the Kenya AIDS Vaccine Initiative and Mr. Peter Muthoga of UNITID. I also thank William Nyakundi and Jose Geda, both of Inqaba Forensics East Africa for helping with DNA quantification.

I also want to thank Dr Gitonga and Dr Mwamlole for their assistance in data collection as well as Dr Kagereki for his assistance in data analysis. Finally I want to

thank all patients for their willingness to participate in this study outside their regular and scheduled appointments.

TABLE OF CONTENTS

DECLARATION i
SUPERVISORS' APPROVAL ii
DEDICATION iii
ACKNOWLEDGMENT iv
TABLE OF CONTENTS vi
LIST OF TABLES ix
LEGEND OF FIGURES x
ABBREVIATIONS xi
ABSTRACT xiii

CHAPTER ONE 1
1.1 Introduction 1
1.2 Literature Review 4
  1.2.1 Definition and types of periodontal diseases 4
  1.2.2 Epidemiology of periodontal diseases 4
  1.2.3 Aetiology of periodontal disease 5
  1.2.4 Aggregatibacter actinomycetemcomitans 6
  1.2.5 Red complex 7
  1.2.6 Risk factors for periodontal disease 9
  1.2.7 Methods of detection of oral bacteria 10
1.3 Statement of the research problem and justification 14
  1.3.1 Problem statement 14
  1.3.2 Justification 14
1.4 Objectives 15
  1.4.1 Main objective 15
  1.4.2 Specific objectives 15
1.5 Null Hypothesis 16
1.6 Alternate Hypothesis 16
1.7 Study Variables 16

CHAPTER TWO 17
2.1 Materials and Methods **17**
2.1.1 Study Design 17
2.1.2 Study Area 17
2.1.3 Study Population 17
2.1.3.1 Inclusion criteria 17
2.1.3.2 Exclusion criteria 17
2.1.4 Sample Size Determination 18
2.1.5 Sampling Procedure 19
2.1.6 Data Collection and Management 19
2.1.6.1 Clinical examination 19
2.1.6.2 Subgingival plaque sample collection 20
2.1.6.3 Cross infection control 20
2.1.6.4 Laboratory procedure 20
2.1.6.4.1 DNA extraction 21
2.1.6.4.2 DNA quantification 21
2.1.6.4.3 Multiplex PCR 22
2.1.6.4.3.1 Optimisation of PCR conditions 22
2.1.6.4.4 PCR product detection 25
2.1.7 Data Analysis and Presentation 25
2.1.8 Data Reliability and Validity 26
2.1.9 Ethical Considerations 26
2.1.10 Benefits 27

CHAPTER THREE 28
3.1 Results 28
3.1.1 Sociodemographic characteristics 28
3.1.1.2 Oral hygiene status 31
3.1.1.3 Gingivitis 32
3.1.1.4 Periodontal status among participants 33
3.1.1.5 Occurrence of periodontal pathogens 35
3.1.1.5.1 Porphyromonas gingivalis 37
3.1.1.5.2 Aggregatibacter actinomycetemcomitans 39

3.1.1.6 DNA quantification 41
CHAPTER FOUR 42
4.1 Discussion 42
4.1.1 Sociodemographic characteristics 42
4.1.2 Oral health practices 43
4.1.3 Oral hygiene status 43
4.1.4 Gingivitis 44
4.1.5 Periodontal status 44
4.1.6 Risk factors and periodontal disease 45
4.1.7 Occurrence of periodontal pathogens 46
4.1.8 Associations between periodontal pathogens and other variables 50
4.1.8.1 Aggregatibacter actinomycetemcomitans 50
4.1.8.2 Porphyromonas gingivalis 51
4.1.9 Quantitative/real-time PCR 51
4.1.10 Bacterial DNA quantification 52
4.1.11 Limitations 52
4.1.12 Conclusion 52
4.1.13 Recommendations 53

REFERENCES 54
APPENDICES 64
APPENDIX I 64
APPENDIX II: 65
APPENDIX III: 66
APPENDIX IV:: 67
APPENDIX V: 69
APPENDIX VI: 70
APPENDIX VII : 72
APPENDIX VIII: 74

LIST OF TABLES

Table 1.1: Study variables.. 16

Table 2.1: Periodontal Diseases Case Definition (CDC/AAP case definition (2007) 20

Table 2.2: Volumes of various PCR reagents per reaction tube per sample....................... 24

Table 3.1: Sociodemographic characteristics 27

Table 3.2: Frequency of brushing by sociodemographic and other variables 29

Table 3.3: Oral hygiene status by sociodemographic and other variables.......................... 31

Table 3.4: Gingivitis by sociodemographic and other variables.. 32

Table 3.5: Periodontitis by sociodemographic and other variables 34

Table 3.6: Association between P. gingivalis and other variables..................................... 38

Table 3.7: A.a by sociodemographic and other variables.. 40

Table 3.8: Association between DNA concentration and other variables 41

LEGEND OF FIGURES

Fig 2.1: Gradient PCR to optimise annealing temperature. 22

Fig 2.2: PCR to optimise Taq concentration... 23

Fig 2.3: PCR to optimise primer concentration 24

Fig 3.1: Occurrence of periodontal pathogens using PCR.. 35

Fig 3.2: Detection of periodontal pathogens using PCR 35

Fig 3.3: Detection of periodontal pathogens using PCR 36

Fig 3.4: Detection of periodontal pathogens using PCR... 36

ABBREVIATIONS

A.a	*Aggregatibacter actinomycetemcomitans*
AAP	American Academy of Periodontology
AHEA	Associate of the Higher Education Academy
BDS	Bachelor of Dental Surgery
Bp	Base pair
CDC	Centres for Disease Control and Prevention
CDE	Certificate in Dental Education
CAL	Clinical Attachment Loss
DNA	Deoxyribonucleic acid
dNTP'-s	Deoxynucleotides
GCAP	Graduate Certificate in Academic Practice
Jap	Japan
Kb	Kilo base
KNH	Kenyatta National Hospital
MClinDent	Master in Clinical Dentistry
MPH	Masters in Public Health
MSc.Dent	Master of Science in Dentistry
P. gingivalis	*Porphyromonas gingivalis*

P. intermedia	*Prevotella intermedia*
PCR	Polymerase Chain Reaction
PhD	Doctor of Philosophy
RNA	Ribonucleic acid
S. mutans	*Streptococcus mutans*
SPSS	Statistical Package for Social Sciences
Taq	*Thermus aquaticus*
T. denticola	*Treponema denticola*
T. forsythia	*Tannerella forsythia*
UK	United Kingdom
UoN	University of Nairobi
UWC	University of the Western Cape

ABSTRACT

Background: Periodontal diseases are common worldwide with chronic periodontitis affecting 80% of Kenyans. These diseases carry high morbidity if left untreated. Many microorganisms are involved in periodontal disease causation and progression including a combination of bacteria, which include gram negative bacteria such as the 'red complex' (*Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia*) and *Aggregatibacter actinomycetemcomitans*.

Studies that have described microorganisms in patients with periodontitis in Kenya are based on conventional culturing techniques. PCR was used in this study to better characterise microbial profiles of periodontal disease in patients with periodontal disease at the University of Nairobi Dental Hospital.

Aim: To determine the occurrence of red complex and *Aggregatibacter actinomycetemcomitans* and determine the relationship between the detection frequency of these organisms in subgingival plaque with the severity of periodontal disease among patients with periodontitis attending the University of Nairobi Dental Hospital.

Materials and methods: A descriptive cross-sectional study was carried out between the months of July 2013 and March 2014 at the University of Nairobi Dental Hospital. Using convenience sampling, a total of 92 persons were recruited into the study. After collection of participants' biodata, periodontal examination including plaque score and gingival index measurement as well as full mouth periodontal probing was done on patients with periodontal diseases and conditions and subgingival plaque collected. DNA extraction from collected plaque was done in the laboratory and presence of target bacteria including *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* was assessed in the laboratory using PCR by utilising

species-specific primers. Data collected was coded, entered and analysed using SPSS version 20, Microsoft Excel, and R software. Descriptive statistics were done using means and standard deviations. Chi square, Analysis of Variance, t- tests and correlation statistics were also done.

Results: Using the AAP/CDC classification, majority of participants were found to have moderate or severe periodontitis. *Aggregatibacter actinomycetemcomitans* was found in 15.20% of participants whereas *Porphyromonas gingivalis* was present in 17.40% of study participants. Associations were found between presence of periodontal pathogens and age, DNA concentration in samples, the gingival index, and increasing periodontal disease severity.

Discussion: There was positive association found between detection frequency of *P. gingivalis* and detection frequency of *A. a* in this study. This compared with findings of other similar microbiologic cross sectional studies that utilised molecular techniques. Positive association was also found between gender and the level of education and between frequency of tooth brushing and time of last dental visit. There was also positive association found between severity of periodontal disease and both age and time of last dental visit.

Conclusion: Majority of participants in this study were found to have either moderate or severe periodontitis as defined by the AAP and CDC. Statistically significant differences were found between the severity of periodontitis in those participants who were found to have *Aggregatibacter actinomycetemcomitan*s and *Porphyromonas gingivalis* and those in whom the bacteria were absent. Association was therefore drawn between occurrence of the bacteria and periodontitis.

Recommendation: Polymerase chain reaction should be considered as an alternative to other methods of periodontal microbiology investigation. There is further need to map out microorganisms associated with periodontal disease in the Kenyan setting.

CHAPTER ONE

1.1 Introduction

Periodontal diseases are common illnesses occurring worldwide with periodontitis reported to affect 80% of Kenyans[(1)] and gingivitis reported at 79% in Tanzania[(2)] whereas Aggressive periodontitis has been reported at 28.8% in Uganda[(3)]. The causes of these diseases are multifactorial in that several bacterial species are involved, interacting with host tissues and cells leading to release of many inflammatory mediators. This in turn leads to destruction of tooth-supporting structures. The diseases are classified into several groups with gingival diseases affecting gingival tissues with no loss of attachment whereas periodontitis is accompanied by inflammation within the supporting tissues of the teeth, attachment loss, and bone loss[(4)] and is clinically characterised by symptoms such as bleeding gums, bad breath, gingival recession, tooth mobility and migration.

Studies have reported that a combination of bacteria which include gram negative anaerobic bacteria such as *Porphyromonas gingivalis (P. gingivalis), Treponema denticola (T. denticola)* and *Tannerella forsythia (T. forsythia)* are associated with the pathogenesis of chronic periodontitis while *Aggregatibacter actinomycetemcomitans (A.a)* is associated with both aggressive periodontitis[(5, 6)] and chronic periodontitis. *A.a* has also been isolated in healthy human individuals[(5)]. The 'red complex' comprises of bacteria that are considered to be periodontal pathogens. These include *P. gingivalis, T. denticola* and *T. Forsythia* (previously called *Bacteroides forsythus* or *Tannerella forsythensis*). The 'complex' has been found to be a portion of the climax community in the biofilms at sites expressing progressing periodontitis[(7)]. *A.a* (previously called *Actinomyces actinomycetemcomitans*) is a non-motile, rod-shaped gram-negative bacterium, which is a facultative anaerobe. It does not belong to the

red complex and it has been designated as an aetiological agent for chronic periodontitis together with *P. gingivalis* and *T. forsythia*. It has further been reported to have a lipopolysaccharide that is a toll-like receptor 4 agonist and which regulates bone sialoprotein by suppressing its transcription, and thus is an early marker of osteoblast differentiation and hence an indicator for impending aggressive periodontitis[8].

To the best of the investigator's knowledge, no Kenyan studies describing the microbial profile of periodontal diseases utilising molecular techniques such as Polymerase Chain Reaction (PCR) are known. The two known microbiological studies in Kenya used culturing technique to investigate putative periodontal pathogens in subgingival plaque in a rural population. One study had a detection frequency of 79% of *P.gingivalis* whereas *A.a* was detected in 29% of participants[9]. Another study in Kenya depicted that the strains of *A.a, P. gingivalis* and *P. intermedia* from subgingival plaque among Kenyan adults were similar to those obtained from Swedish adults[10]. However *A.a, P. gingivalis* and *P. intermedia* were described to be more frequently found in Kenyans than European and North American populations. In an Australian study using enzyme-linked immunosorbent assay (ELISA) technique, *A.a* was detected in 23%, *P. gingivalis* in 15% and *P. intermedia* in 10% of volunteer subjects[11]. Of those subjects found to have chronic periodontitis, 46% were found to have detectable *A.a.* In contrast, an American study utilising PCR technique found the prevalence of *A.a* in healthy subjects to be 4.5% while it was 23.5% in subjects with aggressive periodontitis[12]. PCR is a highly sensitive technique and has the ability to detect pathogens more rapidly. It is also less labour intensive than other techniques[13] such as culture that has been used in the Kenyan

study. Owing to its advantages, PCR was used in this study to identify the bacterial components in subgingival plaque.

From the aforementioned, the current study thus aimed at establishing the occurrence of *Aggregatibacter actinomycetemcomitans* and red complex microorganisms in subgingival plaque among patients with periodontal disease attending the University of Nairobi Dental Hospital. The detection frequency of *A.a* and red complex and their relationship with severity of periodontal disease was studied.

1.2 Literature Review

1.2.1 Definition and types of periodontal diseases

Periodontal diseases have undergone various historical ways of definition and classification with most forms of periodontitis being characterised by the presence of gingival inflammation, pocket formation and loss of alveolar bone and periodontal ligament as a bottom line(14). The diseases are broadly classified into several groups including gingival diseases, chronic periodontitis, aggressive periodontitis, periodontitis as a manifestation of systemic disorders, necrotizing periodontal diseases, abscesses of the periodontium, and periodontitis associated with endodontic lesions (15). These diseases have various clinical presentations and different rates of progression with the overall eventual result being compromised periodontium with possibility of impaired masticatory function and tooth loss.

1.2.2 Epidemiology of periodontal diseases

Periodontal disease in its various forms is common among different populations and regions of the world at varying prevalence, extent and severity. Periodontal disease progresses from an initial inflammation of the gingiva and subsequently may progress to involve the periodontal ligament as well as the alveolar bone. Destruction of periodontal tissue support is said to occur through cyclic periods of linear and 'burst' episodic periods of heightened disease activity and subsequent repair but with a net total gradual loss of supporting tissue(16). The classification currently generally used in classifying periodontal diseases and conditions is based on the recommendations of the 1999 International Workshop for a Classification of Periodontal diseases and conditions(15). However, lack of uniformity in the definition of periodontitis used in epidemiologic studies exists, and findings from different research groups has been

reported with the suggestion that longitudinal prospective studies that address hypotheses emerging from the cross-sectional data be carried out so as to better understand the epidemiology of periodontitis[16]. This lack of uniformity led to a working group with representatives from both the Centres for Disease Control and Prevention (CDC) and the American Academy of Periodontology (AAP) being formed in 2003 to develop and standardise clinical case definitions for population-based studies of periodontitis. Owing to the fact that it takes into consideration both the probing depth and clinical attachment loss in its classification of disease, this CDC/AAP case definition (2007)[17] was used in classifying periodontitis in this study. It has stringent criteria for defining severe periodontitis to ensure that patients identified by the criteria actually do have the disease. Other categories in this classification are "moderate" periodontitis and "no or mild" periodontitis.

A study done in Germany reported a prevalence rate of 90% of periodontitis[18] while in Australia, a 61% prevalence of moderate and severe periodontitis has been reported[19]. Periodontal disease in Brazil has been reported to be significantly more prevalent among males, lighter and darker-skinned black individuals, those with lower family per capita incomes and those with lower schooling level[20]. In Thailand, periodontitis as characterized by clinical attachment loss of at least 1 mm has been reported at 100% in an adult population with attachment loss of ≥4 mm being 92% among 30–39-year-olds and 100% among 50–59-year-olds[21].

1.2.3 Aetiology of periodontal disease

Periodontal diseases are caused by many types of microorganisms especially bacteria in complex biofilms mostly found in the gingival crevice[22]. These biofilms adhere to the tooth and sulcular epithelium and cause a gradual progression from periodontal health to gingivitis and eventual periodontitis whereby there is destruction of the tooth

supporting structures. Dental plaque and gingivitis affect more than 90% of people but prevalence of moderate periodontitis is relatively low, affecting about 20% of the population[23].

Unlike many other types of infections that are caused by a single exogenous microorganism exerting its destructive properties through its virulence factors, periodontal infections are normally of a mixed type. Bacteria are primarily the cause of the initial inflammatory lesion that leads to gingivitis although it is the host response that determines whether disease progresses or not[24]. Apart from bacteria, viruses especially herpesviridae are also thought to have a role in aetiopathogenesis of periodontal disease[25].

Aetiopathogenesis of periodontal disease has been described as being initiated and perpetuated by microbes. The mostly predominant are gram-negative anaerobic or microaerophilic bacteria, which cause antigenic and other virulence factors on the host's immune-inflammatory response. This leads to connective tissue and bone metabolism that causes clinical signs of disease initiation and progression[24]. Many microorganisms destructive to the periodontium are normally found as part of normal oral flora in health and exert their destructive effects when combined with other organisms especially when conditions in the mouth are favourable or when the host's immune response is impaired.

1.2.4 Aggregatibacter actinomycetemcomitans

Aggregatibacter actinomycetemcomitans is a gram negative facultative, rod-shaped non-motile, non-sporing bacteria closely related to the *Pasteurellaceae* family[26]. It is a commensal but is also commonly isolated in localised aggressive periodontitis lesions[27]. This organism has been described to be more prevalent in patients with moderate or severe periodontitis compared to healthy control subjects[28]. As a

periodontal pathogen it has several virulence factors that include leukotoxins[29], proteins that inhibit eukaryotic cell cycle progression, proteins and peptides that induce proinflammatory cytokine networks and yet other proteins that interact with the host in various ways[30]. A high prevalence of 57% of systemic antibodies against *A.a* that might contribute to limit systemic infection with the bacteria has been reported in a Swedish population[31]. The bacteria comprise six serotypes based on structural characteristics of the O-antigen. The JP2 clone of serotype b has a 530 base pair (bp) deletion in the promoter region of the leukotoxin operon, is highly virulent, causing increased cytokine production in human dendritic cells[32] and it is linked to development of aggressive periodontitis in adolescents[12, 26, 33-36] and healthy subjects as well[37]. It adheres tightly to a variety of host tissues including the oral mucosa in the absence of teeth prior to tooth eruption. It has a cytolethal-distending toxin that kills fibroblasts and also produces leukotoxins that destroy host polymorphonuclear cells such as neutrophils[30, 38]. The JP2 clone has been associated with toothbrush sharing as well as eating from the same plate and drinking from the same glass[39].

1.2.5 Red complex

The 'red complex' of bacteria is one of the closely related groups of bacteria found in subgingival plaque and are especially strongly associated with increased pocket depths in chronic periodontitis[40, 41]. It comprises of *Porphyromonas gingivalis, Treponema denticola* and *Tannerella forsythia* (previously called *Bacteroides forsythus* or *Tannerella forsythensis*). Together with the 'Orange complex', they are the late colonisers of subgingival plaque. The 'red complex' has been found to be a portion of the community of microorganisms in the biofilms at sites expressing progressing periodontitis[7, 42] and are also described as markers for destructive periodontal disease in adults as their levels have been found to be higher in patients

with periodontal disease than in healthy control subjects[28]. In Asia, the red complex of bacteria has been reported to be four times as likely to colonise patients with ≥ 10 sites with attachment loss of ≥ 5 mm, and 4.3 times as likely in those with with ≥ 30 such sites compared to sites with < 5 mm attachment loss in a rural population with limited access to dental care from Southern Thailand[43].

Studies have reported that *P . gingivalis,* perhaps the most investigated periodontal pathogen, is more prevalent in diseased sites with more than 5mm loss of attachment and more than 4mm pocket depth in comparison with non-diseased sites with less than 1mm attachment loss and pocket depth of less than 3mm[10, 44]. Cumulative risk scores for advanced periodontitis have been described to be strongly associated with a summation of salivary *P. gingivalis* concentration, salivary interleukin-1 β as well as salivary matrix metalloproteinase-8 levels[45]. Higher *P. gingivalis* levels have also separately been reported in plaque from teenagers with periodontitis compared to healthy control subjects[46]. No difference has however been reported in the occurrence of the various periodontopathogens including total viable count between elderly Chinese patients with ≥ 2mm attachment loss and those with < 2mm attachment loss[47]. The K1 and K2 serotypes of *P. gingivalis* have been associated with an increased production of the osteoclastogenesis-related factor RANKL[48]. The bacterium contains virulence factors of the cysteine protease group called gingipains that are associated with host tissue destruction [49, 50]. Pathogenicity of the organism has been reported to be associated with its ability to multiply in iron-limiting conditions in animal models[51]. It has also been reported to exhibit a competitive advantage over *A. a* in biofilm formation and proteolytic capabilities when the two organisms were co-cultured in the same biofilm[52]. Recent research points to *P. gingivalis* possibly having a role in a mediating development of orodigestive cancers

especially oral squamous cell carcinoma[53]. Presence of *P.gingivalis* and *T. denticola* in placental tissue has been associated with occurrence of hypertensive disorders of pregnancy and therefore these two members of the red complex could have a role in adverse pregnancy outcomes in pregnant individuals with periodontal disease[50]. *T. forsythia* has been described in sites progressing from periodontal health to disease[54]. The levels of the three microorganisms in the red complex have been reported not to differ significantly between subgingival plaque samples analysed immediately on the same day of collection, those stored at $+4^0$C for 6 weeks before processing and those stored at -20^0C for 6 months before processing for analysis using checkerboard DNA-DNA hybridization technique[55]. However, the red complex has additionally been reported to be detectable at higher amounts in subgingival plaque than supragingival plaque derived from adults with periodontitis[56].

1.2.6 Risk factors for periodontal disease

Various risk factors for periodontal disease have been described. These include non-modifiable factors such as age advancement. In this, it is claimed that rather than periodontal breakdown being an inevitable consequence of ageing, prolonged exposure to true risk factors such as periodontopathic bacteria and systemic diseases may be the more plausible explanation. Prevalence, extent and severity of periodontal attachment loss, the hallmark of periodontal disease, has separately been associated with increasing age[57, 58].

Genetic polymorphisms, gender differences whereby females have been considered to have better oral hygiene habits hence less periodontal disease experience than their male counterparts; and racial differences may also predispose one to periodontal disease to some extent[59]. Environmental risk factors for periodontal disease may include mouth colonization by certain specific microorganisms, cigarette smoking,

obesity, osteoporosis, Human Immunodeficiency Virus (HIV) infection, psychosocial factors including stress as well as diabetes mellitus[60]. It has, however, been reported that microbiota in subgingival plaque may not differ significantly between type II diabetics and non-diabetic individuals[61].

In a study done in Tanzania, low levels of education, age advancement and male gender were identified as risk factors for gingivitis[2]. This adds to findings of an Asian study which reported poor oral hygiene, overgrowth of certain periodontopathogenic bacteria, tobacco smoking and uncontrolled diabetes mellitus as the risk factors associated with periodontal disease[16]. Male gender, wearing partial dentures and reporting less frequent tooth brushing were reported to be associated with greater attachment loss in a study done in Southern China[62]. Moreover, colonization by specific bacteria at high levels, smoking, and poorly controlled diabetes have been separately established as risk factors for periodontitis[16].

1.2.7 Methods of detection of oral bacteria

Several methods of detection of microorganisms responsible for various forms of periodontal disease from dental plaque, tissue biopsy, gingival crevicular fluids and blood samples exist. These methods differ regarding detection time, minimum number of cells that can be detected and the ability to quantify the number of cells present in a sample. The methods include bacterial culture-based cultivation, microscopy, DNA probe hybridisation, specific antibody immunofluorescence, fluorescent oligonucleotide probe hybridisation, gene amplification via Polymerase Chain Reaction, loop-mediated isothermal amplification and cloning and sequencing of 16S rRNA libraries[26, 63].

These traditional methods such as culture-based cultivation and microscopy could have limitations in their diagnostic ability and reliability due to factors such as poor

sensitivity, their complex way of interpretation, cross-reactions between organisms and poorly viable or slow-growing nature of some organisms[64]. For example, it has been reported that only 50-60% of the subgingival microbiota can be grown in the laboratory even when using state-of-the-art culturing techniques[65].

Molecular methods that are more superior in diagnosis have, as a matter of necessity, been developed. These include checkerboard DNA hybridisation, DNA amplification by loop-mediated isothermal amplification (LAMP), and phylogenetic 16S rRNA analysis among others [26, 38, 66].

Polymerase chain reaction (PCR) is a quick and reliable molecular technique. It utilises the function of natural enzymes called polymerases whose job is to copy, proofread, and correct the copies of genetic material in form of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). It works on complex combinations of genetic material characterising, seeking out, identifying, and duplicating a particular bit of genetic material from blood, hair, or other tissue specimens, from microbes, animals, or plants. It has variously been referred to as molecular photocopying.

Further, PCR is normally useful in diagnosis by searching out disease-causing organisms that are difficult to culture such as bacteria, fungi and viruses; has a high sensitivity and specificity as well as the ability to generate analysable amounts of organisms' genetic material for identification of copy numbers of a particular microorganism's genetic material[65].

PCR as a diagnostic method can therefore essentially identify most disease–causing organisms including those implicated in periodontal disease[67]. PCR results are not normally affected by transportation conditions unlike anaerobic culture techniques. Several samples can be tested simultaneously with the technique also having higher specificity than other diagnostic techniques[63]. For instance, it has previously been

reported to be more sensitive in detection of *P. gingivalis* with a sensitivity of 86% when compared to conventional culturing that showed a sensitivity of 76%[68]. PCR has also been reported to have a higher detection frequency of 27% of *A.a* compared to a prevalence of 22% reported utilising culture technique[69]. In another study to compare copy numbers of *Streptococcus mutans* from oral samples, copy numbers for *S. Mutans* were higher than those from colony forming units on standard culture plate counts. PCR also detected *S. mutans* 84% of the time that standard plate count did not, with standard plate count detecting *S. Mutans* 33% of the time that PCR did not[70]. PCR has additionally reportedly detected *Aggregatibacter actinomycetemcomitans* in 44% of periodontitis subjects and 16% of healthy subjects compared to detection frequency of 18% in periodontitis cases and 2% in healthy subjects reported in healthy subjects using culture[37]. However, PCR cannot differentiate between viable and non-viable organisms, it cannot be used to monitor response to therapy, it requires sequences of the proteins to be detected to be known and it can also be quite expensive[63].

Multiplex PCR utilises multiple species-specific primers that target various genes of different organisms in the same reaction. It therefore saves on time and number of reactions and can be used to target different bacteria in the same reaction without having to carry out many reactions[13, 71].

It is therefore evident that PCR is a more reliable diagnostic tool compared to other diagnostic methods in detecting pathogenic microorganisms. The detection frequency can in turn allow for association to be made between the detection frequency of pathogens and severity of the disease caused by the said pathogens.

In conclusion, therefore, PCR may be a more superior technique in detection of periodontal pathogens and periodontal pathogen characterisation compared to traditional methods such as microbial culture.

1.3 STATEMENT OF THE RESEARCH PROBLEM AND JUSTIFICATION

1.3.1 Problem statement

Periodontal disease has a high prevalence in Kenya; with periodontitis affecting 80% of the adult population[1]. Periodontitis is also highly prevalent worldwide with various microorganisms being implicated in its causation and progression. If left untreated, periodontal disease usually leads to weakening and loss of tooth attachment apparatus and eventual loss of teeth. The true picture of the putative microorganisms responsible for periodontal disease in the Kenyan population still remains elusive since current literature is based on studies that were carried out using techniques that are prone to errors. Newer and more reliable detecting methods exist.

1.3.2 Justification

Existing local data on the microbiology of periodontal disease in the Kenyan and African population relied on culturing techniques[1, 10] which may be quite unreliable. This is because these techniques have poor sensitivity, small detection windows, are often tedious, labour intensive and time consuming not to mention their complex interpretation. Besides, these techniques are not quantitative and thus may not be relied upon to predict the association of these microorganisms with the severity of periodontal disease. The use of PCR can be used to detect subspecies of the putative periodontopathogenic microorganisms in plaque further clarifying the pathogenesis of periodontal disease. PCR also enables not only detection but the quantification of the amounts of microorganisms in plaque samples, has more specificity and uses less time. It is therefore possible to further relate the levels of microorganisms with clinical findings such as severity of clinical attachment loss and therefore also the severity of periodontal disease. The aim of this study therefore was to determine the

presence of *A.a* and red complex in subgingival plaque samples taken from patients with periodontal disease and to relate the detection frequency of these bacteria with the severity of periodontal disease using PCR. Previous microbiologic studies on periodontal pathogens in Kenya had been carried out using less reliable methods such as culturing. Detection frequency of periodontopathogenic microorganisms using PCR can be related to clinical findings such as severity of Clinical Attachment Loss and hence also the severity of periodontal disease in patients. The results of this study may help clinicians to better understand and manage periodontal disease.

1.4 Objectives

1.4.1 Main objective

To determine the occurrence of *Aggregatibacter actinomycetemcomitans* and red complex in subgingival plaque among patients with periodontal disease attending the University of Nairobi Dental Hospital.

1.4.2 Specific objectives

1. To determine the periodontal status among patients at the University of Nairobi Dental Hospital Periodontology Clinic.
2. To determine the occurrence of *Aggregatibacter actinomycetemcomitans* among patients with periodontal disease attending the University of Nairobi Dental Hospital.
3. To determine the occurrence of red complex in subgingival plaque among patients with periodontal disease attending the University of Nairobi Dental Hospital.
4. To determine whether there is an association between detection frequency of *Aggregatibacter actinomycetemcomitans* and severity of periodontal disease.

5. To determine whether there is an association between detection frequency of red complex and severity of periodontal disease in the study population.

1.5 Null Hypothesis

There is no association between the detection frequency of *Aggregatibacter actinomycetemcomitans* and red complex and severity of periodontal disease.

1.6 Alternate Hypothesis

There is association between the detection frequency of *Aggregatibacter actinomycetemcomitans* and red complex and severity of periodontal disease.

1.7 Study Variables

Table 1.1: Study variables

Variable	Measurement
Sociodemographic	
Age	Number of years
Gender	Whether male or female
Occupation	Type of occupation
Marital status	Whether married, single, divorced, widowed or separated
Level of education (highest)	Highest level of education attained
Residence	Place of residence(Nairobi or outside Nairobi)
Independent variables	
Aggregatibacter actinomycetemcomitans	Present or absent
Red complex-*Tannerella forsythia*	Present or absent
-*Porphyromonas gingivalis*	Present or absent
-*Treponema denticola*	Present or absent
Dependent variables	
Severity of gingivitis	Gingival Index
Severity of periodontitis	Clinical Attachment Loss (CAL)
Oral health status	Plaque score
Bacterial DNA concentration	Amount of DNA

CHAPTER TWO

2.1 Materials and Methods

2.1.1 Study Design

This was a descriptive cross-sectional study.

2.1.2 Study Area

The study was carried out between July 2013 and March 2014 at the University of Nairobi Dental Hospital in Nairobi, which offers undergraduate and postgraduate training in dentistry in Kenya. The hospital manages patients with various orofacial conditions and diseases including periodontal diseases and conditions in patients including those referred from other public hospitals and private dental clinics in Nairobi and other parts of the country.

2.1.3 Study Population

The study population consisted of patients presenting at the University of Nairobi Dental Hospital during the period of the study.

2.1.3.1 Inclusion criteria

Patients treated at the Oral Diagnosis and Periodontology clinics and who gave consent.

2.1.3.2 Exclusion criteria

- Patients with a history of use of antibiotics in the three months preceding the study.
- Pregnant patients.
- Lactating patients
- Periodontal therapy in the six months preceding the study.

2.1.4 Sample Size Determination

The prevalence of *Aggregatibacter actinomycetemcomitans* infection has been reported to be 46%[10]. Using Fishers formula for prevalence cross sectional studies;

$$n = \frac{z^2pq}{d^2}$$

Where n = desired size (for population greater than 10,000).

z = the standard normal deviation at the required confidence level (1.96 for 95% confidence level).

p = proportion of the population estimated to have the characteristic being measured.

q = 1-p

d = level of statistical significance set.

$$n = \frac{1.96 \times 1.96 \times 0.46 \times 0.54}{0.05 \times 0.05}$$

$$= \mathbf{381}$$

The calculated sample size for a population > 10, 000 was 381. However the average number of new patients with periodontal disease seen at the Periodontology Clinic is 4 per day giving 120 patients in one month. Using the formulae for a population of less than 10 000

$$nf = \frac{n}{1+n/N}$$

Where nf = desired sample size (for population <10,000)

n = desired sample size (for population >10,000)

N = estimate of population size

$$nf = \frac{381}{1 + (381/120)}$$

$$= \mathbf{\underline{91.25}}$$

The minimum sample size was determined to be **92** participants.

2.1.5 Sampling Procedure

Convenience sampling method was used to select the study participants whereby all patients presenting at the oral diagnosis and periodontology clinics of the UoN Dental Hospital and who met the inclusion criteria and consented to inclusion were recruited into the study.

2.1.6 Data Collection and Management

2.1.6.1 Clinical examination

Data concerning sociodemographic variables was collected from participants and filled in a form. Clinical examination was then done on a dental chair by the investigator using disposable gloves, masks, gauze, dental mirrors and sterile Michigan 'O' periodontal probes. Supragingival plaque scores were taken using Silness-Loe index (1964) and gingival index using the Loe and Silness index (1963) on Ramfjord's index teeth (**Appendix I**). Full mouth periodontal examination was done and probing pocket depth, bleeding on probing and recession in millimetres was measured at six sites per tooth (mesiobuccal, mid-buccal, distobuccal, mesiolingual, midlingual and distolingual) at all teeth excluding third molars if present. Pocket depth and clinical attachment level measurements were done using a Michigan 'O' sterile periodontal probe and recorded in the periodontal probing depth chart (**Appendix IV**) to the nearest millimetre. Periodontal diagnosis was done using the CDC/AAP case definition criteria as shown in table 2.1 below.

Table 2.1: Periodontal Diseases Case Definition (CDC/AAP case definition (2007)[17])

Disease category	*CAL*	*PPD*
Severe periodontitis	$\geq$2 interproximal sites with CAL of $\geq$6mm (not on the same tooth)	**AND** $\geq$1 interproximal site with PPD of $\geq$5mm
Moderate periodontitis	$\geq$2 interproximal sites with CAL of $\geq$4mm (not on the same tooth)	**OR** $\geq$2 interproximal sites with PPD of $\geq$5mm (not on the same tooth)
No or Mild periodontitis	Neither ''moderate'' nor ''severe'' periodontitis	

**Third molars excluded*. *PPD*: periodontal probing depth, *CAL*: clinical attachment loss.*

2.1.6.2 Subgingival plaque sample collection

Removal of supragingival plaque with sterile cotton pellets and isolation of teeth with cotton rolls was done. Subgingival plaque was then collected with sterile paper points (Meta Biomed® Co. Ltd, Cheongju, South Korea) inserted into the gingival sulcus of Ramfjord's index teeth for 15 seconds[5, 13, 26]. All six paper points from each patient were pooled in a microcentrifuge tube and stored at -20^0C until laboratory testing.

2.1.6.3 Cross infection control

All instruments and the gauze used in examination and sample collection were sterilised using an autoclave to prevent cross infection. Clean examination gloves were also used for examination of every patient.

2.1.6.4 Laboratory procedure

For PCR to be carried out, the requirements include the DNA to be copied (template), sequences at the borders of the DNA molecule to be copied (forward and reverse primers) and deoxynucleotides (dNTPs). The primers which are duplicates of DNA

sequences on either side of the DNA segment of interest (gene) must be known and can be manufactured in the lab or bought commercially from suppliers.

The three basic steps in PCR are denaturation or unwinding of DNA by heating to 90-96°C, hybridization or annealing in which the primers bind to their complementary bases on the now single-stranded DNA and extension or DNA synthesis by a polymerase.

2.1.6.4.1 DNA extraction

The plaque samples were let to thaw at room temperature for 30 minutes before further processing. DNA extraction was then done using a commercial DNA extraction kit (Mericon® DNA bacterial extraction kit (Qiagen GmbH, Hilden, Germany)) as per protocol from Mericon® DNA bacteria handbook: 200 µL of fast lysis buffer was added to the microcentrifuge tube containing the pooled paper points from each participant. Brief, vigorous vortexing of the tube contents was then done. The microcentrifuge tubes were then placed into a heating block and heated at 100^{0}C for 10 minutes followed by removal and cooling to room temperature (15-25^{0}C) for 2 minutes. The tubes were then centrifuged at 13,000 X g for 5 minutes. 100 µL of the supernatant was finally transferred to a fresh 1.5 mL microcentrifuge tube and aliquots of the collected supernatant used directly in PCR reaction. Some of the supernatant was stored for 3 weeks at -20^{0}C as PCR and pathogen detection went on.

2.1.6.4.2 DNA quantification

The amount of extracted DNA in each sample was quantified using the Nanodrop 2000 spectrophotometer (Thermo scientific, Germany).

2.1.6.4.3 Multiplex PCR

2.1.6.4.3.1 Optimisation of PCR conditions

Gradient PCR to optimize the annealing temperature was done using DNA from sample 02 as shown in figure 2.1 below. In this, well 1 had the 1 kb DNA ladder with temperatures being as follows from well 3-14 (left to right): 56.1, 56.4, 57.1, 58.0, 58.7, 59.6, 60.4, 61.3, 62.1, 63.0, 63.5, and 63.8^{0}C respectively. Gradient PCR showed no preferential amplification of isolates and several annealing temperature ranges could therefore be utilised between 56.1 and 63.8 ^{0}C for the PCR.

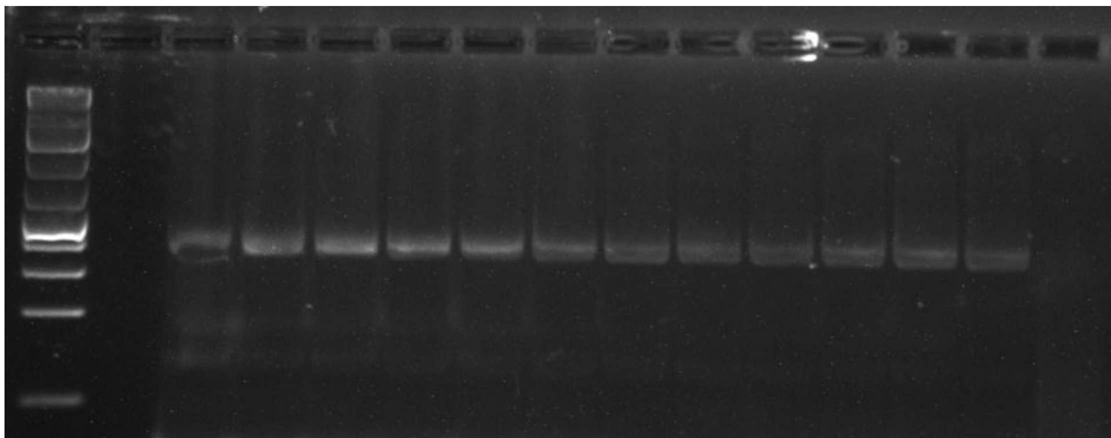

Fig 2.1: Gradient PCR to optimise annealing temperature**:** Sample 02 was used.

Gel 2 was then done to optimise the volume of Taq polymerase required for every reaction. A volume of 0.1 µL of Taq polymerase was utilised in the first set of wells with a 0.2 µL volume being utilised in the second set of wells. DNA was drawn from sample 03. A 0.5 µL volume of primer was used in 20µL reaction mix with primer drawn directly from the stock solution of constituted primer. The results are shown in figure 2.2 below.

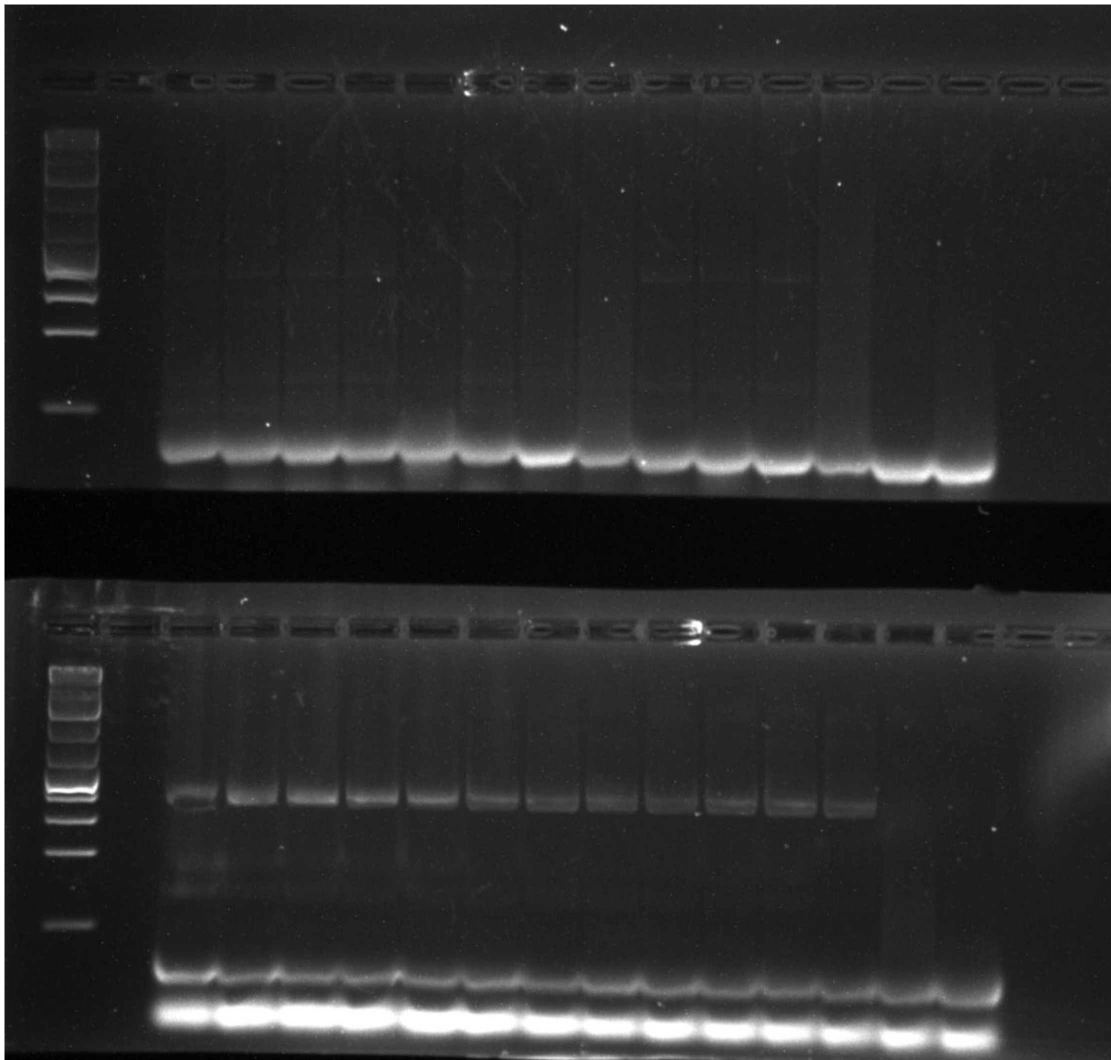

Fig 2.2: PCR to optimise Taq concentration: *Sample 03 was used with 0.5 µL of primer being used in a 20 µL volume of reaction mix. The primer was drawn directly from stock solution. The upper set of wells was done using 0.1µL of Taq with the lower set of wells utilising 0.2 µL of Taq.*

The volume of Taq polymerase utilised was therefore set at 0.2 µL due to the relative prominence of the bands as shown in figure 2.2.

Optimisation of primer concentration was also done in gel 3 as figure 2.3 below shows.

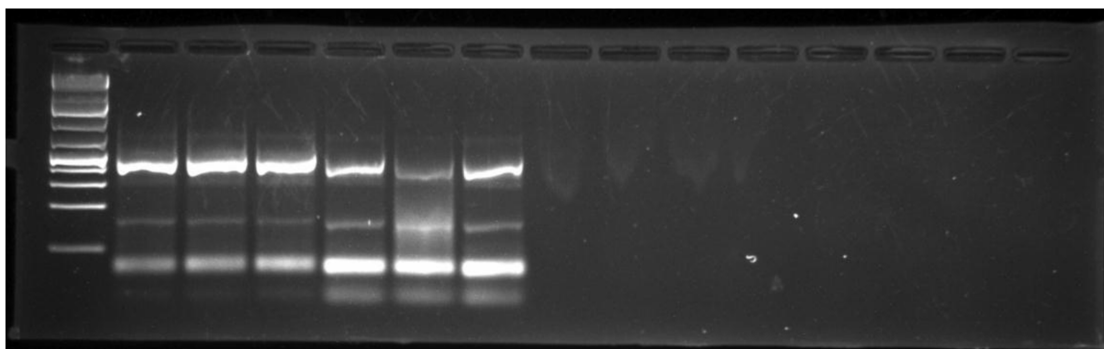

Fig 2.3: PCR to optimise primer concentration: *(Wells 2, 3 and 4 have 0.05ul of stock primer; well 5, 6 and 7 have 0.1µL of stock primer). Reactions were set in 20µL, using sample 03.*

DNA amplification through multiplex Polymerase Chain Reaction was then done. The volumes and concentrations of the various reagents were utilised according to the findings of the optimisation reactions in a total volume of 20µL as shown in table 2.2 below:

Table 2.2: Volumes of various PCR reagents per reaction tube per sample.

Reagent	**Volume (µL)**
Template DNA	2.0
PCR buffer	7.2
Forward primer	0.05
Reverse primer	0.05
Taq polymerase	0.2
Sterile distilled nuclease-free water	10.5
Total volume	20

Another reaction to check for reproducibility and validity of the results was done using a commercially acquired pre-mixed Thermo Scientific DreamTaq Green PCR

mastermix (2X)®, (Inqaba Biotec East Africa, Kenya): a mixture of all PCR primer sets (0.05μL each, the specific primer pairs are in **Appendix V**), 25 μL of DreamTaq Green PCR mastermix (2X)®, 2 μL of template DNA topped up with nuclease-free water to a total volume of 50 μL. PCR assay was performed using a GeneAmp® PCR system 9700 thermal cycler (Applied Biosystems, Thermo Fisher Scientific Inc, Germany) using recommended and optimised conditions: one cycle of initial denaturation at 95^0C for 3 minutes, 35 cycles of touch-down PCR (denaturation at 94^0C for 45 seconds, Annealing at 59^0C for 45 seconds, extension at 72^0C for 1 minute, and final extension at 72^0C for 20 minutes).

2.1.6.4.4 PCR product detection

The PCR products then underwent electrophoresis on a 1.8% Ultrapure™ Agarose gel (Invitrogen™, Thermo Fisher Scientific Corporation) whereby 7μL of PCR product per lane was used. Visualisation of the migrated PCR products was done under UV light following staining with ethidium bromide for detection of *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis, Treponema denticola* and *Tannerella forsythia*. Utmost care in handling ethidium bromide including use of protective eyewear, face masks and gloves was taken due to the known toxic effects of the chemical. The used gels were also discarded appropriately. A Syngente Ingenius® documentation system was utilised to capture and save photographs of the PCR results.

2.1.7 Data Analysis and Presentation

The detection frequency of bacteria was calculated from PCR data and given as a percentage of bacteria-positive subjects. Data collected was coded and entered into a computer using Microsoft Excel. Data cleaning was done by checking frequencies and missing data entered. Analysis was done using Microsoft Excel, R software, and

Statistical Packages for Social Sciences (SPSS) version 20.0 for Windows. Confidence level was set at 95%.

Data was presented in form of tables, bar charts and photographs.

2.1.8 Data Reliability and Validity

To determine intra-examiner consistency, the principal investigator re examined every tenth patient on the same day of examination to reduce intra-examiner variability. The principal investigator also carried out all the clinical examinations and measurements. A Kappa value of 0.7 was obtained in this study with a score of $\leq$ 0.4 being considered poor to fair agreement, 0.41-0.60 moderate agreement, 0.61-0.80 substantial agreement and 0.8-1.00 almost perfect agreement. There was therefore substantial agreement in this study. Data processing included data cleaning and validation. Sample collection and handling including transportation to the lab was also done in consultation with a microbiologist to ensure safety and viability. All the equipment and machines used in this study were calibrated and had passed quality assurance and quality control checks. Also, all procedures entailing DNA handling were done on ice to prevent repeated thawing and freezing of both the DNA as well as the reagents.

2.1.9 Ethical Considerations

Approval to conduct the study was given by the Kenyatta National Hospital (KNH) and University of Nairobi (UoN) Ethics, Research and standards Committee. Permission to conduct the study was sought from School of Dental Sciences of the University of Nairobi. Written informed consent was obtained from subjects before commencement of the study by signing a consent form (**Appendix VI**). All the subjects who met the inclusion criteria had an equal chance of being included in the study. Participation in the study was however voluntary and no coercion or forceful

inclusion of subjects into the study was done. The participants were at liberty to terminate their participation at any time without victimization. Data collected was treated with confidentiality and was only used for purposes of this study and not for any other reason. No subject's personal details including names were revealed to unauthorised persons. There are no financial benefits either to the investigator or the participants from this study. All instruments were autoclaved before being used on participants. There were no risks posed to participants during examination and plaque sample collection as these were carried out within the setting of a dental clinic with all cross infection measures being strictly followed. Plaque samples were also transported to the molecular biology lab in proper transportation armamentaria and handled with high cross infection control standards hence no risk was posed.

2.1.10 Benefits

The participants were managed for their various periodontal diseases and conditions and appropriate referrals done for other orofacial conditions requiring other expert management.

The results on periodontal pathogens from this study will be published in the hope that they will provide new information on the existence of and levels of various periodontopathic bacteria in the Kenyan population and their association with severity of periodontal disease.

CHAPTER THREE

3.1 Results

3.1.1 Sociodemographic characteristics

Ninety-two participants were included in the study. Of these, 46 (50%) were male with 46 (50%) being female. The age ranged between 18-76 with a mean of 39.6 (±14.58 S.D) years. The females were slightly older (mean 40.35± 15.29 S.D years) than males (mean 38.93± 14.00 S.D years). However, the difference was not statistically significant (t= 0.46, p=0.65). As table 3.1 below shows, males had statistically significant higher level of education compared to their female counterparts (X^2= 9.65, p=0.008). Chi square test showed no statistically significant association between gender and marital status, and no association between marital status and level of education.

Table 3.1: Sociodemographic characteristics

Variable		Gender			X^2	P-Value
		Male n (%)	Female n (%)			
Level of education	Primary	2(14.3)	12(85.7)		9.65	0.008
	Secondary	14(48.3)	15(51.7)			
	Tertiary	30(61.2)	19(38.8)			
Marital status	Married	27(58.7)	22(47.8)		2.54	0.281
	Single	17(37.0)	18(39.1)			
	Others	2(4.3)	6(13.0)			
		Marital status				
		Single n(%)	**Married n(%)**	**Others n(%)**		
Level of education	Primary	3(21.4)	10(71.4)	1 (7.1)	9.08	0.059
	Secondary	7(24.1)	20(69.0)	2 (6.9)		
	Tertiary	25(51.0)	19(38.8)	5 (10.2)		

Residence

A total of 61(66.3%) participants were from Nairobi county with the remaining 31(33.7%) residing outside Nairobi.

Occupation

Of the 92 participants, 33(35.9%) were in formal employment, 40(43.5%) were in informal employment whereas 19(20.7%) were unemployed. Males were more likely to be in formal employment compared to females with no statistically significant difference between occupation and gender (X^2= 2.90, p= 0.234).

Risk factors for periodontal disease

On assessment of known risk factors for periodontal diseases, 17.4 % of the respondents reported a positive history of smoking with 4.3% having diabetes whereas 9.8% had hypertension.

Last dental visit

Twenty-five participants (27.2%) reported to have last visited a dentist less than 1 year prior to the time of carrying out this study, 27(29.3%) reported having visited a dentist 1-5 years prior, whereas 40(43.5%) reported either to have last visited a dentist more than five years prior or never having visited a dentist at all.

3.1.1.1 Oral health practices

More than half of the participants (n=59, 64.1%) reported brushing their teeth once daily with the remaining 33 (35.9%) brushing their teeth twice or thrice daily. Table 3.2 below shows the frequency of tooth brushing by sociodemographic variables. Using chi square test, there was a statistically significant difference between the

frequency of tooth brushing and the time of last visit to a dentist with many of those participants who brushed their teeth once daily being more likely to have been to a dentist more than 5 years ago or never having been to a dentist at all (X^2=16.27, df=8, p= 0.041).

Table 3.2: Frequency of brushing by sociodemographic and other variables

Variable		**Brushing frequency**		**X^2**	**DF**	**P-Value**
		Once daily n (%)	**Twice or thrice daily n (%)**			
Gender	Male	31(67.4)	15(32.6)	0.46	2	0.793
	Female	28(60.9)	18(39.1)			
Level of education	Primary	11(78.6)	3(21.4)	4.82	4	0.307
	Secondary	17(58.6)	12(41.4)			
	Tertiary	31(42.5)	42(57.5)			
Marital status	Married	35(71.4)	14(28.6)	4.65	8	0.326
	Single	20(57.1)	15(42.9)			
	Others	4(50)	4(50)			
Last dental visit	< 1 year	15(60.0)	10(40.0)	16.27	8	0.041
	1-5 years	12(48.0)	13(52.0)			
	> 5 years/ Never	32 (84.2)	6(15.8)			
Smoking	Yes	9(56.3)	7(43.7)	0.57	2	0.753
	No	50(65.8)	26(34.2)			

3.1.1.2 Oral hygiene status

The prevalence of plaque was 100% with the mean plaque score being 1.73 (±0.35 S.D). Table 3.3 below shows oral hygiene status by sociodemographic variables. Married participants had a statistically significant higher mean plaque score than other participants (F=2.60, df= 4, p= 0.041). There was no statistically significant association between oral hygiene status and age. Age contributed only 0.04% of the oral hygiene status which was not statistically significant (r^2= 0.0004, p =0.86).

Table 3.3: Oral hygiene status by sociodemographic and other variables

Variable		Plaque score	Test	df	P-value
Gender	Male	1.79 (± 0.33 S.D)	t=1.62	90	0.110
	Female	1.68 (± 0.37 S.D)			
Frequency of brushing	Once a day	1.77(± 0.36 S.D)	F= 2.69	2	0.073
	≥Twice ≤thrice daily	1.54(± 0.28 S.D)			
Marital status	Married	1.83(± 0.27 S.D)	F= 2.60	4	0.041
	Single	1.64(± 0.41 S.D)			
	Others	1.69(± 0.21 S.D)			
Level of education	Primary	1.68(± 0.54 S.D)	F= 0.43	2	0.652
	Secondary	1.78(± 0.31 S.D)			
	Tertiary	1.72(± 0.31 S.D)			
Last dental visit	< 1 year ago	1.65(± 0.45 S.D)	F= 1.03	4	0.397
	1-5 years ago	1.72(± 0.37 S.D)			
	>5 years ago/never been to a dentist	1.78(± 0.29 S.D)			

3.1.1.3 Gingivitis

All participants were found to have gingival inflammation. The mean gingival index was 1.34(±0.37 S.D) with a range of 0.25- 2.17. A total of 18 respondents (19.6%) were found to have mild gingivitis, 73 (79.3%) had moderate gingivitis whereas 1 (1.1%) had severe gingivitis. Table 3.4 below shows the distribution of gingivitis by sociodemographic and other variables. Married participants had a statistically significant higher mean gingival index than other participants (F= 1.04, p= 0.022). Among the participants, 54% of cases of gingivitis were attributable to plaque score and this was statistically significant (r^2= 0.54, p< 0.001).

Table 3.4: Gingivitis by sociodemographic and other variables

Variable/ Characteristic		Gingival index	Test	df	P- value
Gender	Male	1.36 (± 0.35 S.D)	t= 0.61	2	0.602
	Female	1.32 (± 0.39 S.D)			
Frequency of brushing	Once a day	1.39(± 0.38 S.D)	F= 1.55	2	0.217
	≥Twice ≤thrice daily	1.26(± 0.40 S.D)			
Marital status	Married	1.42(± 0.34 S.D)	F= 3.01	4	0.022
	Single	1.28(± 0.37 S.D)			
	Others	1.37(± 0.18 S.D)			
Level of education	Primary	1.29(± 0.56 S.D)	F= 1.04	2	0.358
	Secondary	1.42(± 0.33 S.D)			
	Tertiary	1.31(± 0.33 S.D)			
Last dental visit	< 1 year ago	1.28(± 0.48 S.D)	F= 1.00	4	0.420
	1-5 years ago	1.30(± 0.33 S.D)			
	>5 years ago/never been to a dentist	1.42(± 0.35 S.D)			
Smoking					
	Yes	1.29(± 0.35 S.D)	F=0.37		0.545
	No	1.35(± 0.38 S.D)			

3.1.1.4 Periodontal status among participants

Definition of periodontitis was based on the current recommended CDC/AAP case definition (2007)[17]. Using this standard, 19 of the participants (20.7%) were found to have severe periodontitis, 38 (41.3%) had moderate periodontitis, whereas 35 (38.0%) had mild or no periodontitis. As table 3.5 below shows, statistically significant positive association was found between the severity of periodontitis based on the participants' last visit to a dentist. In this, periodontitis was more likely to be to a greater extent among those who visited the dentist less frequently (X^2=23.01, p= 0.003). Statistically significant association was also found between age and periodontitis with disease more likely to be severe with increasing age (F= 11.70, $p < 0.001$). Association was also found between periodontitis and mean gingival index (F= 3.46, p= 0.036) but not with mean plaque score (F= 1.04, p= 0.435).

Table 3.5: Periodontitis by sociodemographic and other variables

Variable		DISEASE			Test	DF	P-Value
		No/Mild Disease n (%)	Moderate n (%)	Severe n (%)			
Gender	Male	16 (34.8)	22(47.8)	8 (17.4)	X^2=1.68	2	0.432
	Female	19 (41.3)	16(34.8)	11 (23.9)			
Level of education	Primary	2 (14.3)	7 (50)	5 (35.7)	X^2=7.25	4	0.123
	Secondary	9 (31)	13(44.8)	7 (24.1)			
	Tertiary	24 (49)	18(36.7)	7 (14.3)			
Last dental visit	< 1 year	12(46.2)	11(42.3)	3(11.5)	X^2=23.01	8	0.003
	1-5 years	12(42.9)	12(42.9)	4(14.3)			
	> 5 years/ Never	11(54.7)	15(68.5)	12(76.8)			
Frequency of brushing	Once a day	22 (37.3)	25(42.4)	12 (20.3)	X^2=3.89	4	0.422
	≥Twice ≤thrice daily	13 (39.4)	13(39.4)	7 (21.2)			
Marital status	Married	14(28.6)	23(46.9)	12(24.5)	X^2=14.04	8	0.081
	Single	19(54.3)	10(28.6)	6(17.1)			
	Others	2(25)	5(62.5)	1(12.5)			

3.1.1.5 Occurrence of periodontal pathogens

A.a was present in 14 (15.2%) of participants whereas *P.gingivalis* was present in 16 (17.4%) of study participants.

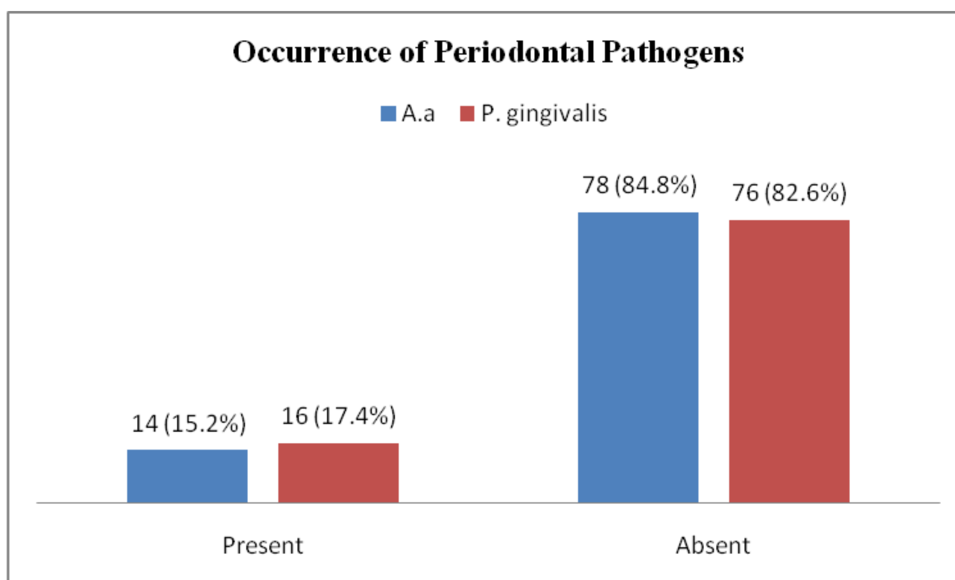

Fig 3.1: *Occurrence of the periodontal pathogens Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis*

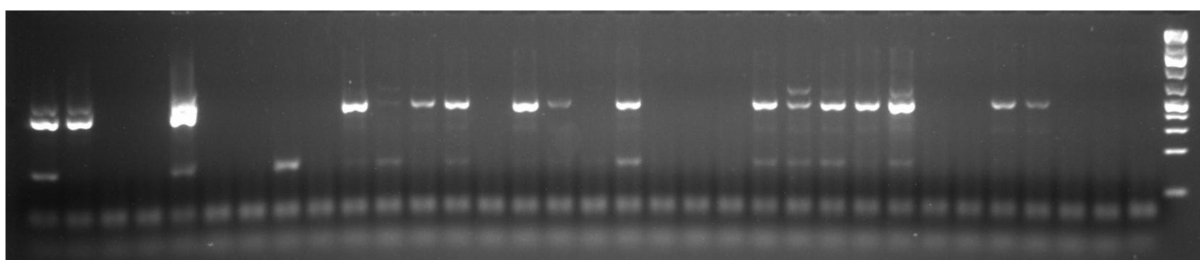

Fig 3.2: Detection of periodontal pathogens A.a and red complex using multiplex PCR: *Photograph of gel showing PCR product detection for samples 33-64. The second last well on the right contained nuclease-free sterile distilled water (negative control) whereas the last well on the right contained the 1,000 bp ladder*

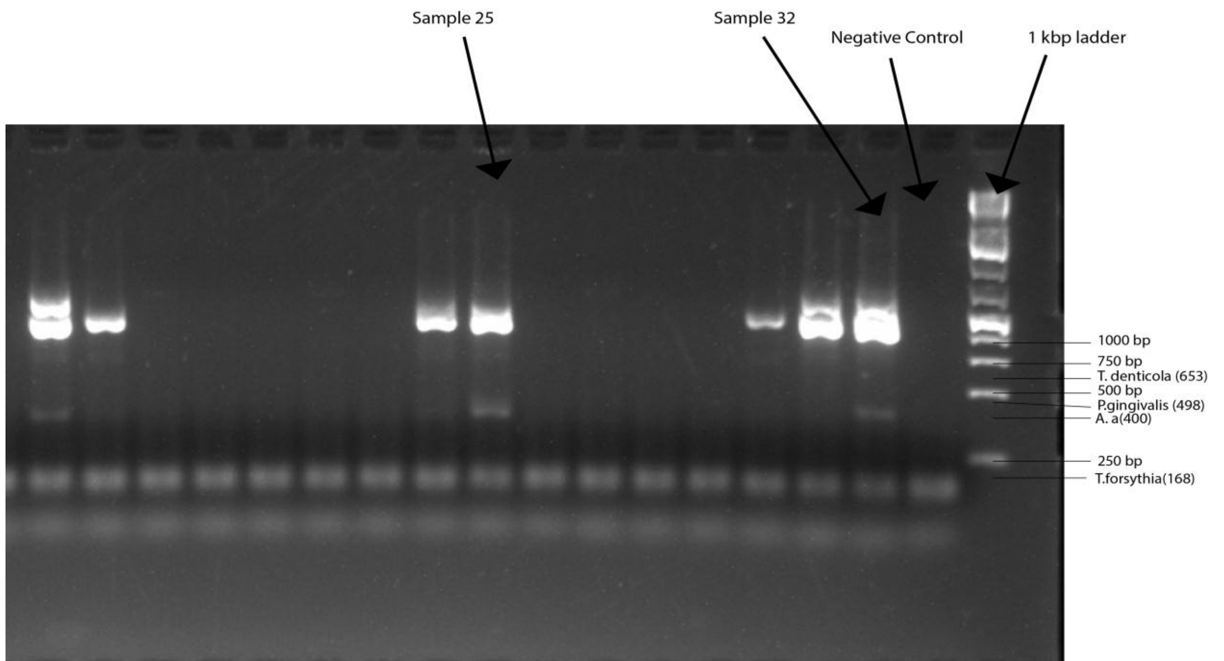

***Fig 3.3**: **Detection of periodontal pathogens A.a and red complex using multiplex PCR:** Sample 25 was positive for both A. a and P. gingivalis whereas sample 32 was positive only for A. a. The rightmost well contained the 1000 bp ladder with the second lane from right being a negative control (sterile distilled nuclease-free water).*

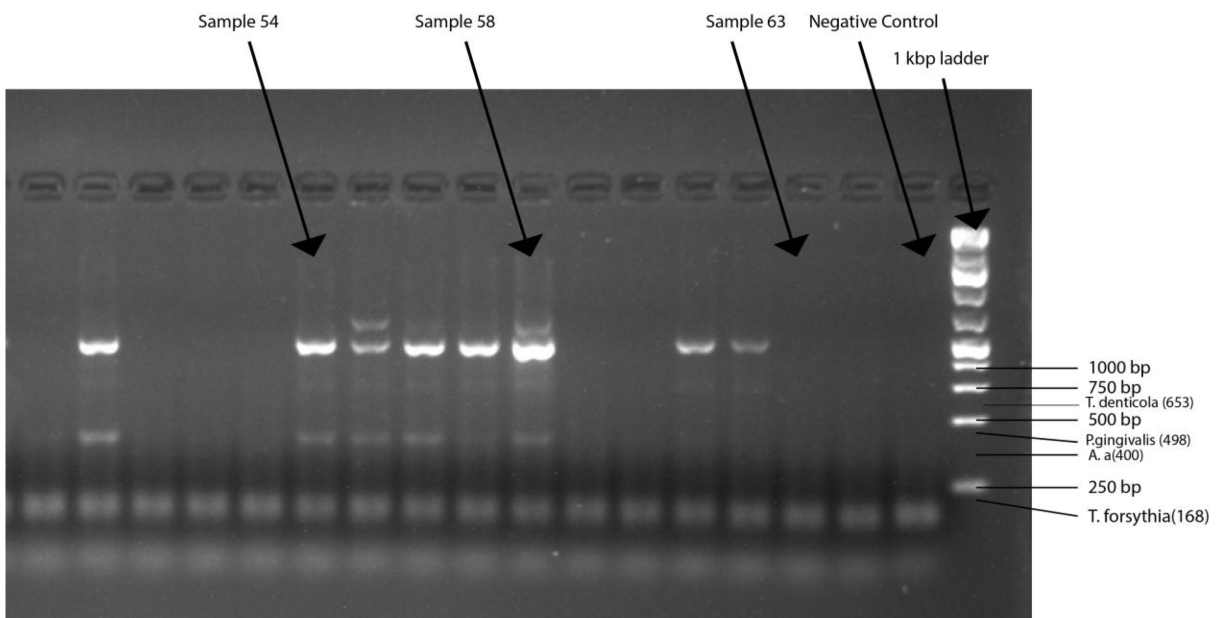

***Fig 3.4: Detection of periodontal pathogens using multiplex PCR**: Samples 54 and 58 were positive for A.a only. Sample 63 did not have any periodontal pathogen.*

In this study, occurrence of *Tannerella forsythia* was not assessed due to presence of primer dimers at the approximate location of the bands for the organism (168 bp) that obscured any bands that may have been present. Also, no discernible bands for *Treponema denticola* (band size 653 bp) were identified for all the samples despite utilisation of primers specific to this organism. A repeat PCR to determine if *T. Denticola* and *T. Forsythia* could be detected in a second reaction was not done due to limitation of finances to procure new primers as well as time limitation. The two microorganisms investigated in detail therefore were *P. gingivalis* and *A. a*.

3.1.1.5.1 Porphyromonas gingivalis

The organism was present in 16 (17.4%) of the participants with no statistically significant difference between males and females. As table 3.6 below shows, statistically significant associations were found between detection frequency of *P. gingivalis* and plaque score (t= 2.47, p= 0.015), gingival index (t= 3.24, p= 0.022), and periodontal disease severity (X^2=11.23, p= 0.042) but not with clinical attachment loss.

Table 3.6: Association between P. gingivalis and other variables

Variable		*P. gingivalis* Present n(%)	*P. gingivalis* Absent n(%)	Test	DF	P-Value
Age	Years	16(17.4)	76(82.6)	t= 1.87	90	0.065
Gender	Male	7 (43.8)	39 (51.3)	X^2= 0.30	1	0.582
	Female	9 (56.2)	37 (48.7)			
Plaque score		16(17.4)	76(82.6)	t= 2.47	90	0.015
Gingival index		16(17.4)	76(82.6)	t= 3.24	90	0.022
CAL		16(17.4)	76(82.6)	t= 1.90	90	0.061
Disease severity	No/mild	4(11.43)	31(88.57)	X^2= 6.34	2	0.042
	Moderate	5(13.16)	33(86.84)			
	Severe	7(36.84)	12(63.16)			
Frequency of brushing	Once a day	11(68.8)	48(63.2)	X^2= 0.40	2	0.451
	Twice or thrice daily	5(31.2)	28(36.9)			
Marital status	Married	11 (68.8)	38 (50.0)	X^2= 3.36	2	0.174
	Single	3 (18.8)	32 (42.1)			
	Others	2 (12.5)	6 (7.9)			
Level of education	Primary	3 (18.8)	11 (14.5)	X^2= 0.94	2	0.693
	Secondary	6 (37.5)	23 (30.3)			
	Tertiary	7 (43.8)	42 (55.3)			
Last dental visit	< 1 year ago	4 (25.0)	21 (27.6)	X^2= 0.14	2	0.971
	1-5 years ago	5 (31.2)	22 (28.9)			
	>5years ago/ never been to a dentist	7 (43.8)	33 (43.4)			
Smoking	Yes	3 (18.8)	13 (17.1)	X^2=0.025	1	0.876
	No	13 (81.2)	63 (82.9)			

3.1.1.5.2 Aggregatibacter actinomycetemcomitans

A. a was present in 14 (15.2%) of participants. Table 3.7 below shows association between detection of *A.a* and other variables. Statistically significant association was found between *A.a* detection and age (t=2.19, p= 0.031), clinical attachment loss (t= 4.61, p<0.001) and periodontal disease severity (X^2=11.23, p= 0.004).

Table 3.7: A.a by sociodemographic and other variables

Variable		*A.a*		Test	DF	P-Value
		Present n(%)	**Absent** n(%)			
Age	Years	14(15.2)	78(84.8)	t= 2.19	90	0.031
Gender	Male	4 (28.6)	42 (53.8)	X^2= 3.03	1	0.082
	Female	10 (71.4)	36 (46.2)			
Plaque score		14(15.2)	78(84.8)	t= 1.00	90	0.320
Gingival index		14(15.2)	78(84.8)	t= 1.46	90	0.148
CAL		14(15.2)	78(84.8)	t= 4.61	90	<0.001
Disease severity	No/mild/moderate	8(10.96)	65(89.04)	X^2=11.23	2	0.004
	Severe	6(31.58)	13(68.42)			
Frequency of brushing	Once a day	9(64.3)	50(64.1)	X^2= 0.82	2	0.859
	Twice or thrice daily	5(35.7)	28(35.9)			
Marital status	Married	8 (57.1)	41 (52.6)	X^2= 1.31	2	0.613
	Single	4 (28.6)	31 (39.7)			
	Others	2 (14.3)	6 (7.7)			
Level of education	Primary	3 (21.4)	11 (14.1)	X^2= 2.33	2	0.358
	Secondary	6 (42.9)	23 (29.5)			
	Tertiary	5 (35.7)	44 (56.4)			
Last dental visit	< 1 year ago	1 (7.1)	24 (30.8)	X^2= 4.05	2	0.136
	1-5 years ago	4 (28.6)	23 (29.5)			
	>5years ago/ never been to a dentist	9 (64.3)	31 (39.7)			
Smoking	Yes	2 (14.3)	14 (17.9)	X^2=0.116	1	0.734
	No	12 (85.7)	64 (82.1)			

3.1.1.6 DNA quantification

The mean DNA concentration was found to be 111.45 ng/µL with a standard deviation of 67.08 and a range between 10.90 to 369.60 ng/µL. As seen in table 3.8 below, statistically significant differences were found between DNA concentration among samples positive for *A.a* and *P. gingivalis* and those in which the bacteria were not present. Positive correlations were found between DNA concentration and plaque score with 15% DNA concentration attributed to plaque score (r^2= 0.15, p< 0.001); gingival index with 11% DNA concentration attributed to gingival index (r^2= 0.11, p< 0.001); probing depth with 10% of DNA concentration attributed to probing depth (r^2= 0.10, p= 0.02); and CAL with 49% of DNA concentration being attributed to CAL (r^2= 0.49, p< 0.001).

Table 3.8: Association between DNA concentration and other variables

Variable/ Characteristic			**n (%)**	**DNA concentration(ng/µL)**	**Test**	**df**	**P-value**
Periodontal pathogens	*P.gingivalis*	Yes	16 (17.4)	173.76 (S.D± 64.79)	F= 20.243	90	<0.001
		No	76 (82.6)	98.34 (S.D± 60.14)			
	A.a	Yes	14 (15.2)	173.29 (S.D± 50.08)	F=16.412	90	<0.001
		No	78 (84.8)	100.35 (S.D± 63.83)			
			Mean				
Plaque score			1.73 (±0.35 S.D)		r^2= 0.15		<0.001
Gingival index			1.34 (±0.37 S.D)		r^2= 0.11		<0.001
Probing depth			1.93 (± 0.44 S.D) mm		r^2= 0.10		0.002
CAL			2.29 (± 0.64 S.D) mm		r^2= 0.49		<0.001

CHAPTER FOUR

4.1 Discussion

4.1.1 Sociodemographic characteristics

The age of participants ranged from 18-76 years with a mean of 39.64 years and a wide standard deviation of 14.58 years, implying that ages of adult persons seeking dental treatment for various illnesses and conditions at the study area and in the general population may be diverse and varied.

Concerning the level of education, majority of the participants (53%) had tertiary education (college, undergraduate or postgraduate education); with 32% having secondary education whereas only 15% had schooling up to primary level. This high percentage of participants with tertiary education could be due to the fact that this study was done in an urban setting whereby the urban population is probably more educated with respondents being aware of their oral health status. Low income levels and rural residence have been reported as risk indicators for clinical attachment loss(57). The urban population is probably also more enlightened on the need for better oral health seeking behaviour as opposed to rural populations. The population in this study is different from that in a study done to determine the prevalence of gingival recession, level of oral hygiene and oral hygiene practices and associated factors among women at a maternity ward in Tanzania(72). It was found out that 85.7% of the study population of 14-44 year old women had informal to primary education with only 14.3% having secondary to college education. In another study done in Tanzania, gingival recession in subjects from a rural population was reported at 20% with recession in subjects from suburban areas being 15% whereas recession in subjects from urban areas was 11%(2). This may imply that access to healthcare may be better in urban setups than in the rural areas. Socio-economic status including level of

education and income levels have been associated with periodontal diseases with more prevalence reported in persons with low education level and low income[73].

Majority of study participants were found to reside in Nairobi. This could be explained by the fact that the study was carried out in Nairobi hence most participants were likely to be drawn from the city and its environs. Majority of the participants were also found to be in formal or informal employment compared to those who were unemployed.

4.1.2 Oral health practices

Participants' frequency of tooth brushing was found to be a predictor of their oral health seeking behaviour with statistically significant association being found between brushing frequency and time of last dental visit. Those brushing their teeth less frequently were more likely to have visited a dentist a long time ago or to have never visited a dentist at all.

4.1.3 Oral hygiene status

In this study, the plaque score correlated with the gingival index as well as probing depth but not with the clinical attachment loss. The mean plaque score of 1.73 in this urban population was reported despite majority of participants (64.1%) reporting brushing their teeth once daily. In a study done in a rural population in Kenya, Mwacharo (2004) found out that 54% of the study population utilised multiple cleansing practices, 28% brushed their teeth once daily, 16% brushed twice daily while 11.1% brushed their teeth thrice daily[74]. Frequency of tooth brushing has separately been reported at 38.8% (once daily), 51.1% (twice daily), 61.2% (twice or more daily) and 9.2% (thrice daily) in a population of women attending a maternity ward in Tanzania[72].

The associations found between clinical attachment loss and probing depth as well as detection frequency of *A.a* are in agreement with findings by Morikawa et al whereby detection frequency of periodontal pathogens was elevated in parallel with higher pocket probing depth and clinical attachment loss[13]. Increased probing depth may make subgingival plaque control by brushing or even scaling and prophylaxis difficult. The subgingival plaque may then subsequently get colonised by the periodontally destructive microorganisms, leading to further periodontal tissue destruction.

4.1.4 Gingivitis

The mean gingival index measured with the Loe-Silness index of 1963 was not statistically significant between the two genders as opposed to the 85% prevalence rate reported in males compared to 74% among females in a Tanzanian population with male gender being described as a risk factor for periodontal disease [2]. Correlations were found between the gingival index and plaque score as well as the probing depth in the current study but surprisingly not with clinical attachment loss. A high percentage of sites with attachment loss>7mm was predicted by a high percentage of sites with abundant plaque in a Thai population[21].

4.1.5 Periodontal status

Using the CDC/AAP classification[17], 38% of participants were found to have mild or no periodontitis whereas 41.3% had moderate periodontitis with 20.7% having severe periodontitis. The high number of moderate and severe cases could be explained by the fact that the participants were drawn from a population of patients seeking treatment at a dental hospital. The convenience sampling method utilized therefore indicated that dental disease experience in the participants was more likely to be higher than a randomly selected study population because participants in this study

were drawn from a hospital setup. A study in Southern China reported attachment loss of > 4mm in 56% of respondents from an urban population compared to attachment loss of > 4 mm in 61% of 35-44 year old respondents from a rural population[(62)].

Mean clinical attachment loss in this study was found to be 2.29mm with 72% participants having CAL of ≥3mm. This figure is comparatively lower than the prevalence of attachment loss of >4mm in 92% of 30-39-year olds and in 100% of 50-59-year-olds in a rural Thai population[(21)]. Attachment loss of 4mm or more on at least one tooth has separately been reported in 56% of urban 35-44-year-old Chinese[(62)].

4.1.6 Risk factors and periodontal disease

The finding that none of the diabetic participants harboured any of the two microorganisms under investigation may be because the number of participants with diabetes was too low to allow confirmation of any statistical association between diabetes and periodontal pathogen detection. By utilising quantitative real-time PCR, Field and co-workers (2012) found no statistically significant difference in bacterial counts of *P. gingivalis* between diabetic and non-diabetic subjects. Similarly, the counts of *A.a* did not differ significantly between type 2 diabetics and non diabetic subjects in their study[(61)].

Concerning age as a risk factor for periodontal disease, a statistically significant difference was found between the mean age of participants who had *Aggregatibacter actinomycetemcomitans* and the mean age of participants who did not have the organism. The high mean age of pathogenic bacteria- positive subjects compared to the low mean age of *A.a*- free subjects seems to tally with results of studies assessing risk factors for periodontal disease in which age has been described as a risk factor for

periodontal disease in Tanzania[2], North America[58], Southern China[62], Southern Thailand[21], Germany[18] and globally[57].

4.1.7 Occurrence of periodontal pathogens

Aggregatibacter actinomycetemcomitans was found in 15.2% of participants whereas *Porphyromonas gingivalis* was present in 17.4% of participants in this study population that included subjects with various forms of periodontal diseases and conditions.

In a Kenyan study evaluating putative periodontal pathogens in subgingival plaque utilizing culture technique by Dahlen and co-workers (1992), *P. gingivalis* was detected in 79% of "diseased" persons compared to a detection rate of 18% in persons without periodontal disease. *A. a* was detected in 29% of "diseased" persons compared to a detection rate of 18% in participants without periodontal disease[9]. "Diseased" persons in this case were defined as those with loss of attachment of ≥4mm with an additional 1 interproximal site in a lower central incisor having at least 5 mm loss of attachment and accompanied by a pocket of 4 mm or deeper. "Non-diseased persons" were defined as those who had loss of attachment not exceeding 2mm and no pockets of >4 mm. This high detection rate of *P. gingivalis* led the investigators to conclude that occurrence of the bacteria could predict or discriminate between sites with periodontal destruction hence predicting periodontal disease. The current study therefore had a comparatively low detection frequency of *P. gingivalis* and *A.a*.

Another microbiologic study by Dahlen and co-workers (1989) in Kenya utilizing culture method found *P. gingivalis* in 70% and *A. a* in 40% of subgingival plaque from participants from a rural population[10]. These detection rates are still higher than

those in the current study that was carried out in an urban population and which utilised PCR technique.

A possible explanation to the relatively low detection rate of *P. gingivalis* and *A.a* in the current study compared to the findings by Dahlen et al in their two studies could be due to some of the factors enumerated by Sanz et al (2004). In a review paper on various methods of detection of *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* and *Tannerella forsythia* in periodontal microbiology[(68)], they noted some limitations in the use of PCR. Among them is the risk of contamination. However, the current study was carried out in conditions meant to minimize as much as possible the possibility of this occurring by carrying out sample collection and handling with sterile equipment, utilization of protective gear such as face masks and gloves and observation of strict laboratory practices. Sanz and co workers also mentioned the possibility of difficulties when working with small DNA quantities. This is because some reagents necessary for PCR such as Taq polymerase may be exhausted before an adequate detectable amplicon is attained from the sample DNA during the reaction. The DNA quantities in the current study were however quite adequate with most samples having >20 ng/µL with only one sample having 10 ng/µL. Also, the PCR process entails many complex and intricate interrelated factors each of which may affect the outcome of the overall procedure if a problem is encountered with it. These factors include buffer salt concentration, primer size and annealing temperature. To mitigate for problems with these factors, primer sequences for target bacteria in the current study were derived from the reputed Genbank database. Optimization of PCR conditions was also done and the annealing temperature used for the reaction was the one that showed the most prominent bands during optimization with optimization for primer concentration also being done.

The detection rates for *P.gingivalis* and *A.a* in this study are also lower compared to those described in a rural population in southern Thailand in which *P.gingivalis* was present in 99.7% of study subjects with *A.a* being present in 92.7% of participants in a study that utilized ‘checkerboard’ DNA-DNA hybridization technique[43]. A possible explanation for this might be that rural populations may not perform regular or thorough plaque control measures such as tooth brushing or flossing as compared to their urban counterparts. This may in turn lead to more plaque accumulation with minimal plaque disruption or removal. There is therefore a likelihood of having the ecological status change to a pathogenic one containing pathogenic organisms such as *A.a* and *P. gingivalis*. The high rate of pathogen recovery might also point to ‘checkerboard’ DNA-DNA hybridization being a more superior technique in periodontal pathogen recovery as compared to regular PCR but it must be borne in mind that ‘checkerboard’ DNA-DNA hybridization is not applicable for individual plaque sample analysis hence is not normally used for diagnostic but rather for epidemiological research and ecologic studies[63]. The two microorganisms may also possibly constitute the normal oral flora of the population in southern Thailand bearing in mind that yet a different study in southern Thailand utilising culture technique to investigate periodontal pathogens in a randomly selected stratified probability sample found a high detection rate of 88% of *A. a* in study participants[75].

In Cameroon, a study investigating prevalence of several species of oral bacteria found significantly more ($p<0.01$ or $p<0.001$) periodontitis patients than healthy subjects having either *P. gingivalis* or *A.a.* The study reported a prevalence of 85.7% of *P.gingivalis* in subjects with periodontitis compared to a 19.0% prevalence in healthy controls. For *A.a*, the prevalence in subjects with periodontitis was 52.3% compared to 9.5% in healthy subjects[76]. *A.a* has separately been detected in 70.6%

of Sudanese patients with aggressive periodontitis[5] and also at higher detection rates in diseased sites of Chinese children with localized aggressive periodontitis compared to healthy sites[27]. In the current study, *A.a* was detected in one participant who had generalized aggressive periodontitis but *P. gingivalis* was not detected in the same participant contrary to expectation that *P. gingivalis* would be found in generalized aggressive disease with *A. a* expected to be detected more in localized aggressive periodontitis lesions.

In another study assessing the detection frequency of various periodontal pathogens in persons with periodontal destruction compared to healthy control subjects in a Dutch population, van Wilkenhoff et al (2002) found that *A. a* was present in 31% of persons with periodontal destruction compared to 12.8% of control subjects who were found to harbour the bacteria[28]. Although they utilized culturing technique in their study, this figure is still higher than that of 15.2% found in the current study that utilized the presumably superior PCR technique, possibly due to the fact that the two studies involved different types of populations that may have different genetic compositions that may predispose to different reactions to periodontopathic bacterial insult. In the same study, *P. gingivalis* was detected in 59.5% of diseased subjects compared to a detection frequency of 10.6% of control subjects, still a higher detection rate compared to that in the current study.

The use of PCR to assess various periodontal pathogens such as *A.a*, *P. gingivalis* and *T. denticola* can be traced back to Watanabe and Frommel's research in 1996 where they set out to investigate various primers and their ability to amplify gene segments specific to the periodontal pathogens[67]. Morikawa et al in 2008 investigated periodontal pathogens in a Japanese population utilizing multiplex PCR just like in the current study in which all primer pairs for the target bacteria were

incorporated in each reaction tube. They found *P.gingivalis* in 31.3% of participants at the supportive periodontal therapy stage of treatment whereas *A.a* was found in 1.2% of participants in the same study[13]. The detection frequency of *P. gingivalis* in the current study incorporating participants at first presentation at a periodontal clinic was therefore comparatively lower than that of persons at the maintenance phase of management, a rather surprising finding as it may be expected that persons undergoing periodontal maintenance would have stable periodontal status and therefore would have lower levels of periodontopathogens. These detection rates in the study by Morikawa almost equal those of a volunteer non-diseased Australian population in which distribution of putative periodontal pathogens were investigated. *A.a* was detected in 22.8% of subjects while *P. gingivalis* was detected in 14.7% of respondents[11].

4.1.8 Associations between periodontal pathogens and other variables

4.1.8.1 Aggregatibacter actinomycetemcomitans

Detection frequency of *A.a* was positively associated with increasing participant age (p= 0.031). This may indicate that with age increase, associated plaque accumulation over time coupled with less than optimal tooth brushing may transform to one harbouring pathogenic microorganisms such as *A.a*. Association was also found between detection of *A.a* with the detection of *P.gingivalis* (p= 0.042).

As concerns association with severity of periodontitis, none of the respondents in whom *A. a* was present fell within the mild or no disease category. Eight (57.14%) had moderate periodontitis whereas six (42.86%) had severe periodontitis. All the participants who had the bacteria therefore either had moderate or severe periodontal disease. Severe disease can therefore be associated with a higher likelihood of

detection of *A. a* according to the findings of this study. Conversely, of the seventy eight participants who were found not to have the bacteria, thirty five (44.87%) had mild or no disease, thirty (38.46%) had moderate disease whereas only thirteen (16.67%) had severe disease. Majority of the participants who did not have the bacteria therefore had mild or no periodontal disease with only a small number having severe disease. A statistically significant difference (p= 0.004) was found between detection frequency of *A. a* among the various classes of periodontal disease severity with detection frequency being higher with increasing periodontal disease severity.

4.1.8.2 Porphyromonas gingivalis

Detection frequency of *P.gingivalis* was associated with severity of periodontitis with 75% of participants in whom the organism was present having either moderate or severe periodontitis. Detection frequency of *P. gingivalis* has been reported to be elevated in persons with increased probing depths and increased clinical attachment loss[(13)]. A statistically significant difference (p= 0.042) was found between detection frequency of *P. gingivalis* and that of *A.a*, indicating the role these two organisms play together in periodontal destruction. A statistically significant difference was also found between mean DNA concentrations of bacteria-positive participants ($p \leq 0.001$) and those in whom the bacteria was absent.

4.1.9 Quantitative/real-time PCR

In a study comparing detection and quantification of periodontal pathogens between real-time PCR quantitative and anaerobic culture techniques, a higher detection frequency of 27.4 % compared to 21.6% for *A.a* was reported with real-time PCR compared to anaerobic culture[(69)], giving credence to the idea that real-time PCR is probably a more superior technique than other techniques including regular PCR that

was utilized in the current study. Real-time PCR can therefore be considered the gold standard in periodontal molecular diagnosis[63, 64, 70, 77].

4.1.10 Bacterial DNA quantification

Statistically significant differences were found in DNA concentration in samples from participants in whom *P. gingivalis* ($p < 0.001$) and *A.a* ($p < 0.001$) were present. Strong correlations were also found between DNA concentration and plaque score ($p < 0.001$), gingival index ($p < 0.001$), probing depth ($p=0.002$) and clinical attachment loss ($p < 0.001$). Whereas this DNA may have been from any of the several hundred species of bacteria found in the gingival sulcus, the statistically significant associations and correlations may possibly still implicate the two bacteria under investigation in destructive periodontal disease. Densitometry for the bands in the gel or real-time/quantitative PCR could have helped with quantification of the two bacteria but this was not done due to financial constraints.

4.1.11 Limitations

Due to the small number of new patients seen at the Periodontology clinic of the University of Nairobi Dental Hospital, convenience sampling instead of random sampling was used to select the study population.

The study required reagents and materials that are costly with the cost being borne by the investigator.

4.1.12 Conclusion

Majority of participants in this study were found to have either moderate or severe periodontitis as defined by the AAP and CDC. The detection frequency of known periodontal pathogens *A. a* and *P. gingivalis* in this study done using regular Polymerase Chain Reaction was found to be relatively lower than that reported using

similar techniques in previous studies. Statistically significant associations were found between detection frequency of *A. a* and *P. gingivalis* and the severity of periodontitis. Association could then be drawn between the bacteria and periodontitis. The null hypothesis was therefore rejected within the limitations of this study.

4.1.13 Recommendations

In order to conclusively associate the occurrence of *P. gingivalis* and *A.a* with periodontal disease in the current population, there is need for a randomised study including subjects who are free from disease and those with different severities of periodontitis. Owing to the limitations of regular PCR compared to real-time/quantitiative PCR, the latter should be preferred in future studies as it would be helpful in not only detection but quantification of the pathogens. It may also be useful to carry out microbiologic tests for patients with severe periodontitis with the aim of determining the need for adjunctive chemotherapeutic therapy for periodontal disease

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APPENDICES

APPENDIX I

Plaque score: Silness and Loe Plaque index 1964

Scores	Criteria
0	No plaque
1	A film of plaque adhering to the free gingival margin and adjacent area of the tooth. The plaque may be seen in situ only after application of disclosing solution or by using the probe on the tooth surface.
2	Moderate accumulation of soft deposit s within the gingival pocket, or the tooth and gingival margin which can be seen with the naked eye.
3	Abundance of soft matter within the gingival pocket and/or on the tooth and gingival margin

Gingival index: Loe and Silness Index 1963

0- Normal, absence of oedema and no bleeding on probing
1- Presence of oedema with absence of bleeding
2- Oedema present, glazing and bleeding on probing
3- Oedema, ulcerations with spontaneous bleeding

APPENDIX II

SCREENING FORM

Date..Serial no..

Age..File no..

	Yes	No
Antibiotic use in past 3 months		
Pregnant		
Lactating patients		
Periodontal treatment in the past three months		

APPENDIX III

OCCURRENCE OF RED-COMPLEX AND AGGREGATIBACTER ACTINOMYCETEMCOMITANS IN SUBGINGIVAL PLAQUE AMONG PATIENTS WITH PERIODONTAL DISEASE ATTENDING A PERIODONTOLOGY CLINIC IN NAIROBI

1.2 BIODATA:

Date...Serial number...

Age (Years)..File number..

Sex ☐ Male Female ☐

Marital status Single ☐ Married ☐ Divorced ☐ Widowed ☐ ☐ Separated

Residence...

Occupation..

Highest level of educatio ☐ Primary

☐ Secondary

☐ College

☐ University (Undergraduate)

☐ Postgraduate

Tooth brushing habit ☐ Never

☐ Occasionally

☐ Once daily

☐ Twice daily

☐ Thrice daily

☐ More than thrice daily

☐ Other

Last dental visit: ☐ Less than 3 months ago ☐ 3-6 months ago

☐ 6 months -1 year ago ☐ >1 year ago ☐ > 5 years ago

☐ >10 years ago ☐ Never been to a dentist

Risk factors : Smoking ☐ Diabetes ☐ Hypertension ☐

APPENDIX IV

CLINICAL EXAMINATION FORMS:

PLAQUE SCORE : Silness and Loe Index 1964

Tooth	16		11		24		36		31		44	
Surface	F	P	F	P	F	P	F	L	F	L	F	L
score												

Total..............Mean..............

GINGIVAL INDEX : Loe and Silness index (1963)

Tooth	16		11		24		36		31		44	
Surface	F	P	F	P	F	P	F	L	F	L	F	L
score												

Total..............Mean..............

Periodontal probing depth chart

Maxillary arch:

Tooth	**17**			**16**			**15**			**14**			**13**			**12**			**11**			**21**			**22**			**23**			**24**			**25**			**26**			**27**		
Palatal																																										
Recession(mm)																																										
CAL(mm)																																										
Facial																																										
Recession(mm)																																										
CAL(mm)																																										
Mobility																																										

Average CAL........

Mandibular arch:

Tooth	**47**			**46**			**45**			**44**			**43**			**42**			**41**			**31**			**32**			**33**			**34**			**35**			**36**			**37**		
Lingual																																										
Recession(mm)																																										
CAL(mm)																																										
Facial																																										
Recession(mm)																																										
CAL(mm)																																										
Mobility																																										

Average CAL...........

APPENDIX V

Primer sequences:

Organism	Genbank access number	Sequence	Size(bp)
Aggregatibacter actinomycetemcomitans	X16829	Forward 5’-GAAGGCGACGACCACTTAGC-3’ Reverse 5’-GTGCACGATCCTTTTCAGGT-3’	400
Treponema denticola	AJ272339	Forward 5’-CAAATAATGCCGATTACGGGCTTT-3’ Reverse 5’-GCCTTCGTTACCCATCGCAA-3’	653
Tannerella forsythia	AY546489	Forward 5’-CTGGAGCAGTCTTGGAATCTG-3’ Reverse 5’-GCAGCCTGAGTCAGGCTTTTT-3’	168
Porphyromonas gingivalis	D26470	Forward 5’-CGAAGTCTTCATCGGTCGTT-3’ Reverse 5’-GTACCTGTCGGCTTACCATCTT-3’	498

APPENDIX VI

CONSENT FORM

This research entitled "Occurrence of red-complex and Aggregatibacter actinomycetemcomitans in subgingival plaque among patients with periodontal disease at the University of Nairobi Dental Hospital" is being carried out by Dr.Wambugu Jethro J. Chege, a postgraduate student in the department of Periodontology, Community and Preventive Dentistry, School of Dental Sciences, University of Nairobi towards his attainment of a Master of Dental Surgery in Periodontology degree of the University of Nairobi.

Part of the research will entail examination of participants using instruments on all their teeth and this will include measurement of the amount of plaque debris and the extent of gum disease in the participants' mouths. There are no health risks posed to participants during the examination.

All information gathered during this study will be treated with utmost confidentiality and will not be revealed to any other person and will only be used for purposes of this research. Participants will participate in the study voluntarily without being forcefully made to participate in the research and they may opt out the study at their own free will without any threats or adverse ramifications. No payment of any form will be given to participants. The findings of this research may be useful to doctors in understanding of and planning for treatment of gum diseases and supporting structures of the teeth.

Having read and understood the above information, and with any questions I may have had having been answered satisfactorily by the investigator (Dr Wambugu), I do hereby voluntarily consent to participate in this research.

.... Date............................

Signed by participant

<u>Declaration by the principal investigator</u>

I have clearly explained to the participant the purpose and expected benefits of this study and have answered his/her questions regarding this research on the date on this consent form.

.... Date.......................................

Signed by investigator

For clarifications and enquiries on the consent please contact

The KNH/UoN Ethics and Research Standards Committee Secretariat

Email: uonknh_erc@uonbi.ac.ke

Telephone Number +254-20 2726300 Ext 44355

APPENDIX VII

FOMU YA KUTAFUTA IDHINI KUTOKA KWA WAHUSIKA KATIKA UTAFITI:

Utafiti huu unaitwa "Kuweko kwa viini vya bacteria aina ya *Aggregatibacter actinomycetemcomitans* na red-complex baina ya watu walio na ugonjwa wa ufizi katika Hospitali ya Matibabu ya Meno katika Chuo Kikuu cha Nairobi." Unafanywa na daktari Jethro J. Chege Wambugu ambaye ni mwanafunzi katika chuo kikuu cha Nairobi.

Utafiti huu unahusisha wahusika kuangaliwa katika midomo yao na vifaa vilivyo safi. Pia idadi ya uchafu ulio katika meno na ufizi wa wahusika utachunguzwa. Hakuna madhara ya aina yo yote kwa wahusika katika utafiti huu.

Habari zote zitakazotolewa na wahusika kuwahusu hazitajulishwa mtu yeyote na zitatumiwa kwa utafiti huu peke yake. Pia zitawekwa kwa njia ya kibinafsi na kisiri katika rekodi ambazo mchunguzi mkuu ataziweka vyema. Habari za kibinafsi kama vile majina na umri wa wahusika hazitaandikwa po pote katika rekodi zo zote za umma wala kujadiliwa katika mikutano ama kongamano za kisayansi. Wahusika watashiriki kwa utafiti huu kwa hiari yao bila kushurutishwa au kulazimishwa na ye yote. Wahusika pia wanaweza kujiondoa kutoka kwa utafiti huu wakati wo wote bila vitisho au madhara yo yote. Hakuna malipo ya kifedha au aina nyingine ambayo washirika watapewa kwa kushiriki katika utafiti huu. Matokeo ya utafiti huu yanaweza kusaidia madaktari na wanasayansi kuelewa magonjwa ya ufizi na jinsi ya kuyatibu vyema zaidi.

Baada ya kusoma na kuelewa maelezo haya, na baada ya maswali yoye niliyokuwa nayo kuhusu utafiti huu kujibiwa na Daktari Wambugu, ninakubali kuhusishwa katika utafiti huu kwa kutia sahihi hapa chini.

.... Tarehe..

Sahihi ya mshiriki

Maelezo ya mchunguzi mkuu:

Nimemweleza mshiriki kuhusu maudhui na manufaa ya uchunguzi huu na nimejibu maswali aliyokuwa nayo siku ambayo imetiwa sahihi hapa chini.

....Tarehe...

Sahihi ya mchunguzi mkuu

Kwa maelezo zaidi wasiliana na

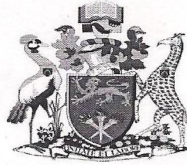
The KNH/UoN Ethics and Research Standards Committee Secretariat

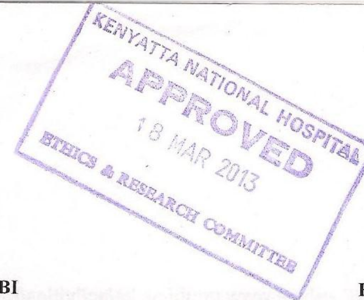
Email: uonknh_erc@uonbi.ac.ke

Nambari ya simu +254-20 2726300 Ext 44355

APPENDIX VIII

ETHICAL APPROVAL





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Ref: KNH-ERC/A/55

18th March 2013

Dr. Jethro J. Chege Wambugu
Dept.of Periodontology/Community and Preventive Dentistry
School of Dental Sciences
University of Nairobi

Dear Dr. Wambugu

Research proposal: Occurrence of Red-complex and Aggregatibacter Actinomycetemcomitans in Subgingival plaque among patients with Periodontal Disease at the University of Nairobi dental Hospital (P617/11/2012)

This is to inform you that the KNH/UoN-Ethics & Research Committee (KNH/UoN-ERC) has reviewed and **approved** your above revised proposal. The approval periods are 18th March 2013 to 17th March 2014.

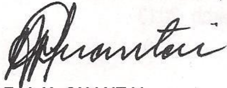
This approval is subject to compliance with the following requirements:

a) Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
b) All changes (amendments, deviations, violations etc) are submitted for review and approval by KNH/UoN ERC before implementation.
c) Death and life threatening problems and severe adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH/UoN ERC within 72 hours of notification.
d) Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH/UoN ERC within 72 hours.
e) Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (*Attach a comprehensive progress report to support the renewal*).
f) Clearance for export of biological specimens must be obtained from KNH/UoN-Ethics & Research Committee for each batch of shipment.
g) Submission of an *executive summary* report within 90 days upon completion of the study
This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/or plagiarism.

"Protect to Discover"

For more details consult the KNH/UoN ERC website **www.uonbi.ac.ke/activities/KNHUoN**

Yours sincerely



PROF. A.N. GUANTAI
SECRETARY, KNH/UON-ERC

c.c. The Deputy Director CS, KNH
The Principal, College of Health Sciences, UoN
The Dean, School of Dental Sciences, UoN
The Chairman, Dept.of Periodontology/Community and Preventive Dentistry, UoN
The HOD, Records, KNH
Supervisors: Dr. Nelson Matu, Dr. Tonnie K. Mulli, Prof. L. W. Gathece

"Protect to Discover"