Objectives: To determine the occurrence of Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans in patients with periodontal disease using polymerase Chain Reaction.

Design: A descriptive cross-sectional study.

Setting: The University of Nairobi Dental Hospital.

Subjects: Patients presenting at the Oral Diagnosis and Periodontology clinics during the period of the study. The patients had periodontal examination done followed by subgingival plaque collection from selected teeth. DNA extraction from the plaque samples was then done followed by PCR based on Taqman probes using commercially available kits.

Results: A total of 92 participants were recruited in the study. Aggregatibacter actinomycetemcomitans (A.a) was found in 14 (15.20%) while Porphyromonas gingivalis (P.g) was present in 16 (17.40%) of the study participants. Based on independent sample t-test, statistically significant positive associations were found between detection frequency of Porphyromonas gingivalis and plaque score (t= 2.47, p= 0.015), gingival index (t= 3.24, p= 0.022), but not with clinical attachment loss (t= 1.90, p= 0.061). Chi square test revealed a positive association between detection rate of P.g and periodontal disease severity (X²= 6.34, p= 0.042). Similar association was also found between detection rate of Aggregatibacter actinomycetemcomitans and increasing age (t=2.19, p= 0.031), clinical attachment loss (t= 4.61, p<0.001) and periodontal disease severity (X²=11.23, p= 0.004).

Conclusion: The multiplex polymerase chain reaction technique utilised in this study enabled detection of target bacteria in the same reaction mixture as opposed to other periodontal microbiological techniques that require each bacteria to be investigated individually therefore PCR should be considered as an alternative to other methods of periodontal microbiology investigation. The association between periopathogenic microorganisms, A.a and P.g with occurrence and severity of periodontal disease were also confirmed in this study.

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 many microorganisms including 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 ‘red complex’ of bacteria that include Porphyromonas gingivalis (P. gingivalis), Treponema denticola and Tannerella forsythia (previously called Bacteroides forsythus or Tannerella forsythensis) has been found to be a portion of the climax community in the biofilms at sites expressing progressing periodontitis
Aggregatibacter actinomycetemcomitans (A.a; previously called Actinomyces actinomycetemcomitans) is a non-motile, rod-shaped gram-negative bacterium, which is a facultative anaerobe. It is a commensal but is also commonly isolated in localised aggressive periodontitis lesions (5). Detection of these periodontal pathogens is important in the diagnosis and management of periodontal disease. However, traditional methods such as culture-based cultivation and microscopy are tedious, time consuming and costly and especially for fastidious microorganisms such as A.a. Molecular methods such as polymerase chain reaction (PCR) have been developed in an effort to overcome these limitations in other fields of medicine. 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 (6,7). The main objective of this study was therefore to determine the occurrence of the periodontal pathogens Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans in patients with periodontal disease at the University of Nairobi Dental Hospital using PCR.

MATERIALS AND METHODS

Ninety-two (92) patients presenting at the Oral Diagnosis and Periodontology clinics of the School of Dental Sciences of the University of Nairobi between July 2013 and March 2014 were selected by convenience sampling in a descriptive cross-sectional study. There was no age restriction in participant selection. However it was assumed that most participants would be adults since plaque-induced periodontal destruction normally occurs in permanent dentition and therefore all patients who fitted the inclusion criteria were all least eighteen years old. Patients had not used antibiotics in the three months preceding the study. They also had not received periodontal therapy in the six months preceding the study. Written informed consent was obtained from each participant prior to plaque sample collection. Ethical approval to carry out the study was sought from Kenyatta National Hospital and University of Nairobi Ethics, Research and Standards Committee (Approval ref no KNH-ERC/A/55) as well as from the School of Dental Sciences of the University of Nairobi.

Clinical examination: Clinical measurements made included probing pocket depth, clinical attachment loss, plaque score using Silness-Loe index (1964) and gingival index using Löe and Silness index (1963). Measurement data were taken in millimeters at six sites per tooth (mesiobuccal, mid-buccal, distobuccal, mesiolingual, midlingual and distolingual) at all teeth excluding third molars.

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(7-9). All six paper points from each patient were pooled in a micro centrifuge tube and stored at -20°C until laboratory testing.

Lab procedure
DNA extraction: Plaque samples were let to thaw at room temperature for 30 minutes and DNA extraction 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.

Multiplex PCR: Following optimisation of PCR conditions, a multiplex PCR was done in a total volume of 20µL using 0.05 µL of all primer sets, 2.0 µL of template DNA, 0.2 µL of taq polymerase, 7.2 µL of PCR buffer and topped up with 10.4 µL nuclease-free sterile distilled water in each reaction tube. 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), 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°C for 3 minutes, 35 cycles of touch-down PCR (denaturation at 94°C for 45 seconds, annealing at 59°C for 45 seconds, extension at 72°C for 1 minute, and final extension at 72°C for 20 minutes). The sequences of forward and reverse primers for the bacteria and predicted sizes of PCR products as accessed from the reputable Genbank database are shown in table 1 below.
Table 1
**Primer sequences and predicted sizes of PCR products BLASTED from the genome bank**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Genbank access number</th>
<th>Sequence Forward</th>
<th>Size(bp)</th>
<th>Sequence Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggregatibacter actinomycetemcomitans</td>
<td>X16829</td>
<td>5’- GAAGGCGACGACCACTTAGC-3’</td>
<td>400</td>
<td>5’- GTGCACGATCCCTTTTCAGGT-3’</td>
</tr>
<tr>
<td>Treponema denticola</td>
<td>AJ272339</td>
<td>5’- CAAATAATGCCGATTACGGGCTTT-3’</td>
<td>653</td>
<td>5’- GCCTTCGTTACCCATCGCAA-3’</td>
</tr>
<tr>
<td>Tannerella forsythia</td>
<td>AY546489</td>
<td>5’- CTGGAGCAGTCTTGGAATCTG-3’</td>
<td>168</td>
<td>5’- GCAGCCTGAGTCAGGCTTTT-3’</td>
</tr>
<tr>
<td>Porphyromonas gingivalis</td>
<td>D26470</td>
<td>5’- CGAAGTCTTCATCGGCGTT-3’</td>
<td>498</td>
<td>5’- GTACCTGTCGGCATTACCATCTT-3’</td>
</tr>
</tbody>
</table>

**Statistical analyses:** 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% therefore a p-value of less than 0.05 was considered significant.

**RESULTS**

A total of 92 participants (46 male and 46 female) were included in the study. The age ranged between 18-76 years 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, independent sample t-test did not reveal a statistically significant difference in mean ages between the two genders (t= 0.46, p=0.65). *A.a was*
present in 14 (15.2%) of participants whereas *P. gingivalis* was present in 16 (17.4%) of study participants. Figure 1 below shows the occurrence of the two periodontal pathogens under investigation.

**Figure 1**  
Occurrence of the periodontal pathogens *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* among 92 participants

<table>
<thead>
<tr>
<th></th>
<th>Present</th>
<th>Absent</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.a</td>
<td>78 (84.8%)</td>
<td>14 (15.2%)</td>
</tr>
<tr>
<td>P. gingivalis</td>
<td>76 (82.6%)</td>
<td>16 (17.4%)</td>
</tr>
</tbody>
</table>

Occurrence of two other members of the red complex, *Tannerella forsythia* and *Treponema denticola* was not assessed due to presence of primer dimers at the approximate location of the bands for *Tannerella forsythia* (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. Figures 2, 3 and 4 below are images showing sections of the gel demonstrating PCR product detection for some of the samples.

**Figure 2**  
Detection of periodontal pathogens A.a and P. gingivalis using multiplex PCR

Photograph of gel showing PCR product detection from sample 33 on the left to sample 64 in the third-right well. 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.
Sample 25 was positive for both A. a and P. gingivalis whereas sample 32 was positive only for A. a. The well on the right contained the 1000 bp ladder with the second lane from right being a negative control (sterile distilled nuclease-free water).

Samples 54 and 58 were positive for A.a only. Sample 63 did not have any of the periodontal pathogens under investigation.
Porphyromonas gingivalis: The organism was present in 16 (17.4%) of the participants with no statistically significant difference between males and females. As shown in table 2 below, statistically significant associations were found between detection frequency of P. gingivalis and plaque score (t= 2.47, p= 0.015) as well as gingival index (t= 3.24, p= 0.022) but not with clinical attachment loss (CAL). Chi square test revealed a statistically significant association between detection frequency of P. gingivalis and periodontal disease severity ($X^2=11.23$, p= 0.042).

Table 2
Association between P. gingivalis and periodontal disease parameters

<table>
<thead>
<tr>
<th>Variable</th>
<th>Present n(%)</th>
<th>Absent n(%)</th>
<th>Test</th>
<th>DF</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque score</td>
<td>16(17.4)</td>
<td>76(82.6)</td>
<td>t= 2.47*</td>
<td>90</td>
<td>0.015</td>
</tr>
<tr>
<td>Gingival index</td>
<td>16(17.4)</td>
<td>76(82.6)</td>
<td>t= 3.24*</td>
<td>90</td>
<td>0.022</td>
</tr>
<tr>
<td>CAL</td>
<td>16(17.4)</td>
<td>76(82.6)</td>
<td>t= 1.90*</td>
<td>90</td>
<td>0.061</td>
</tr>
<tr>
<td>Disease severity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No/ Mild</td>
<td>4(11.43)</td>
<td>31(88.57)</td>
<td>$X^2=6.34#$</td>
<td>2</td>
<td>0.042</td>
</tr>
<tr>
<td>Moderate</td>
<td>5(13.16)</td>
<td>33(86.84)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>7(36.84)</td>
<td>12(63.16)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* – Independent t test
# – Chi square test

Aggregatibacter actinomycetemcomitans: A.a was present in 14 (15.2%) of participants. Table 3 below shows association between detection of A.a and other variables. Independent sample t-test revealed a statistically significant positive association between A.a detection and age (t=2.19, p= 0.031) as well as clinical attachment loss (t= 4.61, p<0.001). Chi square statistics demonstrated a statistically significant positive association between A.a detection frequency and periodontal disease severity ($X^2=11.23$, p= 0.004) as demonstrated in table 3 below.

Table 3
Association between A.a and periodontal disease parameters

<table>
<thead>
<tr>
<th>Variable</th>
<th>Present n(%)</th>
<th>Absent n(%)</th>
<th>Test</th>
<th>DF</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>14(15.2)</td>
<td>78(84.8)</td>
<td>T=2.19$</td>
<td>90</td>
<td>0.031</td>
</tr>
<tr>
<td>Plaque score</td>
<td>14(15.2)</td>
<td>78(84.8)</td>
<td>t= 1.00*</td>
<td>90</td>
<td>0.320</td>
</tr>
<tr>
<td>Gingival index</td>
<td>14(15.2)</td>
<td>78(84.8)</td>
<td>t= 1.46*</td>
<td>90</td>
<td>0.148</td>
</tr>
<tr>
<td>CAL</td>
<td>14(15.2)</td>
<td>78(84.8)</td>
<td>t= 4.61*</td>
<td>90</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Disease severity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No/ Mild/Moderate</td>
<td>8(10.96)</td>
<td>65(89.04)</td>
<td>$X^2=6.34#$</td>
<td>2</td>
<td>0.004</td>
</tr>
<tr>
<td>Severe</td>
<td>6(31.58)</td>
<td>13(68.42)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*– Independent t test
# – Chi square test
$–$ ANOVA
DIAGNOSIS

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(10). Morikawa et al in 2008 investigated periodontal pathogens in a Japanese population utilising multiplex PCR just like in the current study in which all primer pairs for the target bacteria were incorporated in each reaction tube(7).

The current study in which Aggregatibacter actinomycetemcomitans was found in 15.2% of participants and Porphyromonas gingivalis was present in 17.4% of participants had a comparatively low detection frequency of P. gingivalis and A.a in comparison to findings by Dahlén and co-workers (1992). In their study utilizing culture technique, they found a detection rate of P. gingivalis of 79% in “diseased” Kenyans 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(11). “Diseased” persons in their 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 Dahlén 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(12). There was therefore a relatively low detection rate of P. gingivalis and A.a in the current study compared to the findings by Dahlén et al in their two microbiologic studies in Kenya. This could partly be explained by limitations in the use of PCR as a diagnostic tool as enumerated by Sanz et al (2004). Factors such as plaque sample contamination, the small volumes of reagents and DNA quantities being used in PCR, the many complex and intricate interrelated factors such as buffer salt concentration, primer size and annealing temperature can also possibly to result in exhaustion in some reagents necessary for PCR such as Taq polymerase before an adequate detectable amplicon is attained from the sample DNA during the reaction (13). To mitigate for problems with these factors, primer sequences for target bacteria in the current study were derived from the reputed Genbank database. Optimisation of PCR conditions was also done and the annealing temperature used for the reaction was the one that showed the most prominent bands during optimisation with optimisation for primer concentration also being done.

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. Concerning 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 eighty 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.

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(7). A Cameroonian 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(14) although it utilised ‘checkerboard’ DNA-DNA hybridization technique(15) which is, however, not applicable for individual plaque sample analysis hence is not normally used for diagnostic but rather for epidemiological research and ecologic studies.(16).

Quantitative/real-time PCR may be considered for use in periodontal molecular microbiological as has been suggested by Malathi (2014) and Mackay (2004)
compared to the regular PCR that was utilized in this study(16,17).

ACKNOWLEDGEMENTS

To thank the patients who consented to participate in this research outside their regular and scheduled appointments. We also acknowledge Ms Teddy Amuge of the International Institute of Tropical Agriculture (IITA) Kenya for her valued technical advice on and carrying out the PCR analysis. Finally, we wish to acknowledge the University of Nairobi Dental Hospital for allowing us to use the facility for data collection.

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