J. J. C. Wambugu, BDS, MDS, N. K. Matu, BDS, MSc. Dent, T. K. Mulli, CDE, AHEA, GCAP, BDS, MClinDent, PhD and L. W. Gathece, BDS, MPH, PhD, Department of Periodontology / Community and Preventive Dentistry, School of Dental Sciences, P. O. Box 19676, Nairobi, Kenya

Request for reprints to: Dr. J. J. C. Wambugu, Department of Periodontology / Community and Preventive Dentistry, School of Dental Sciences, P. O. Box 19676, Nairobi, Kenya

OCCURENCE OF PORPHYROMONAS GINGIVALIS AND AGGREGATIBACTER ACTINOMYCETEMCOMITANS IN SUBGINGIVAL PLAQUE AMONG PATIENTS WITH PERIODONTAL DISEASE AT THE UNIVERSITY OF NAIROBI DENTAL HOSPITAL

J. J. C. WAMBUGU, N. K. MATU, T. K. MULLI and L. W. GATHECE

ABSTRACT

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 (X^2= 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 (X^2=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

(4). *Aggregatibacter actinomycetemcomitans* (*A.a*; previously called *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 Loe 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 -20^0C 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 $0.05\,\mu$L of all primer sets, $2.0\,\mu$L of template DNA, $0.2\,\mu$L of taq polymerase, $7.2\,\mu$L of PCR buffer and topped up with $10.4\,\mu$L 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), $25\,\mu$L of DreamTaq Green PCR mastermix (2X)®, $2\,\mu$L of template DNA topped up with nuclease-free water to a total volume of $50\,\mu$L.

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). 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

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

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 ($\pm$14.58 S.D) years. The females were slightly older (mean 40.35$\pm$15.29 S.D years) than males (mean 38.93$\pm$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
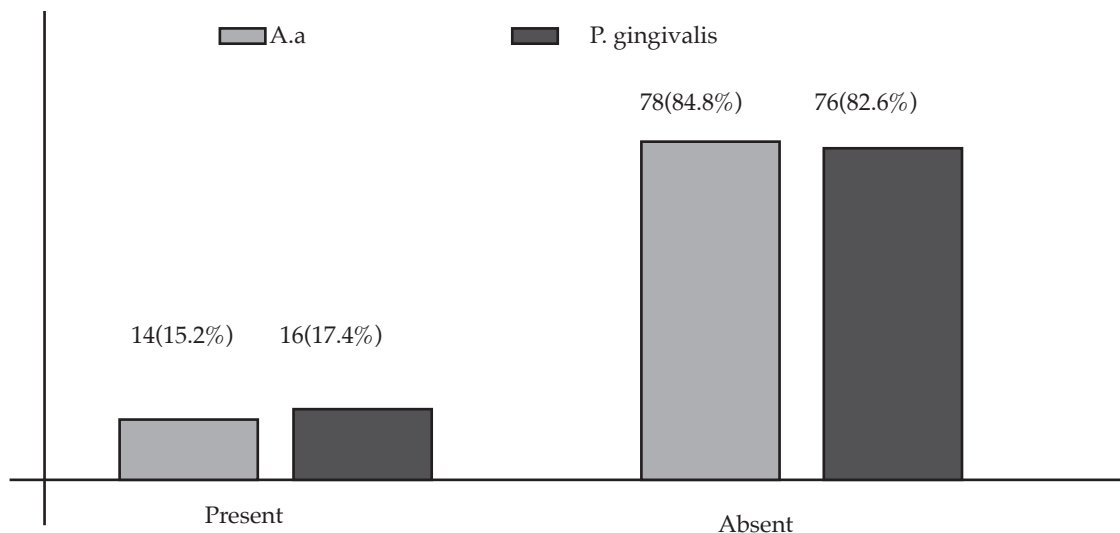
Occurrence of two other members of the red complex, *Tannerella forsythia* and *Treponema denticola* was not assessed due to presence of primer dimers at the approximate location of the bands for *Tannerella forsythia* (168 bp) that obscured any bands that may have been present. Also, no discernible 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
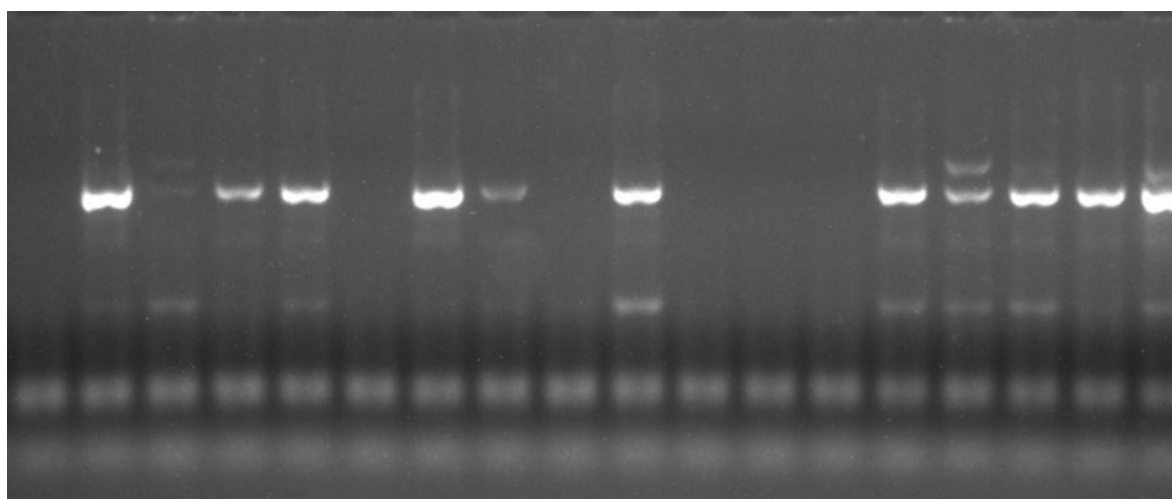

Photograph of gel showing PCR product detection from sample 33 on the left to sample 64 in the third-right well. The second last well on the right contained nuclease-free sterile distilled water (negative control) whereas the last well on the right contained the 1,000 bp ladder.

Figure 3
Detection of periodontal pathogens A.a and red complex using multiplex PCR
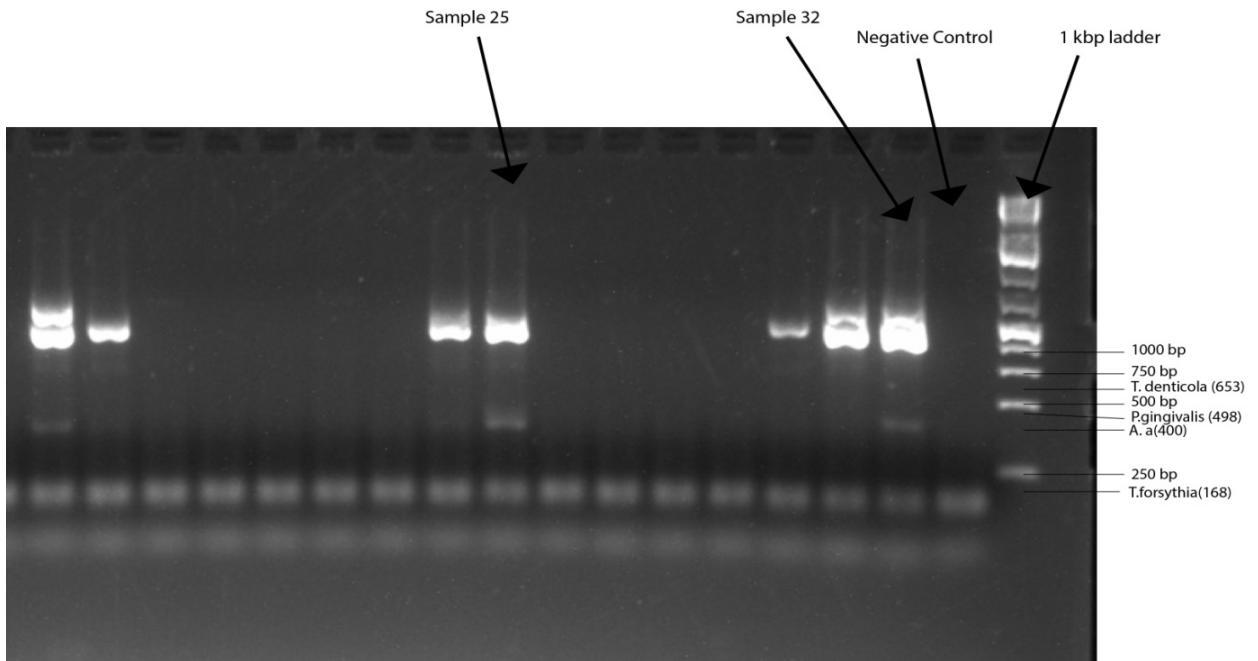
Sample 25 was positive for both *A. a* and *P. gingivalis* whereas sample 32 was positive only for *A. a*. The well on the right contained the 1000 bp ladder with the second lane from right being a negative control (sterile distilled nuclease-free water).

Figure 4
Detection of periodontal pathogens using multiplex PCR
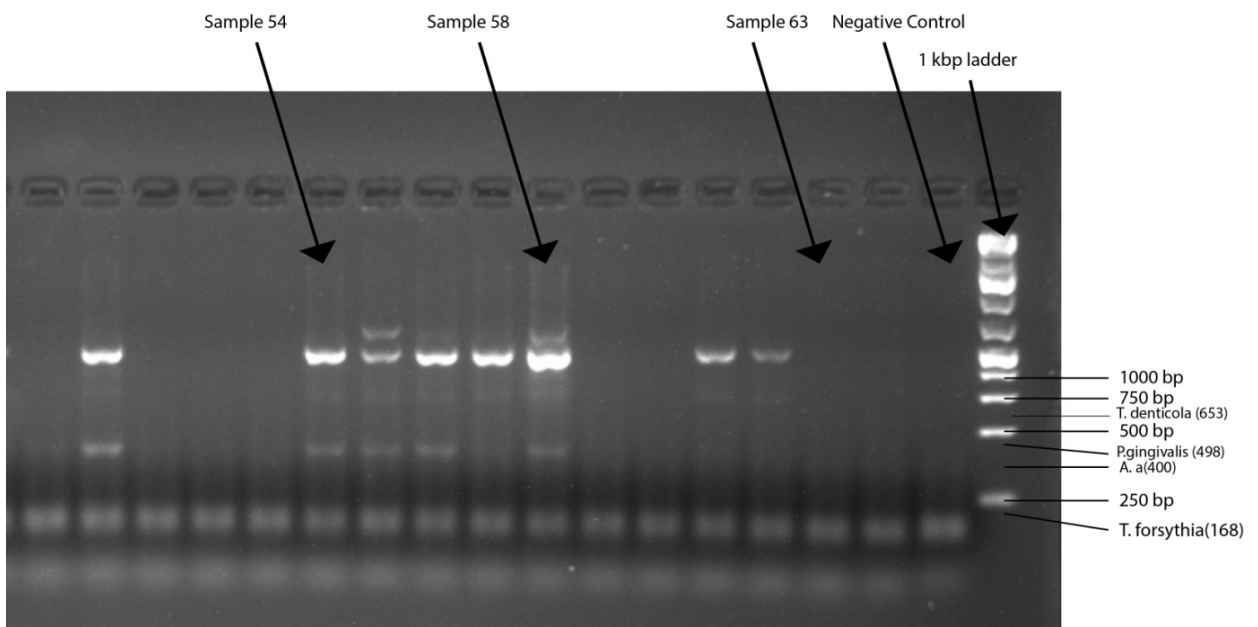
Samples 54 and 58 were positive for *A.a* only. Sample 63 did not have any of the periodontal pathogens under investigation.

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

Variable		P. gingivalis Present n(%)	Absent n(%)	Test	DF	P-value
Plaque score	(Mean=1.73±0.35 S.D)	16(17.4)	76(82.6)	t= 2.47*	90	0.015
Gingival index	(Mean=1.34±0.37 S.D)	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)			

* – 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

Variable		A.a Present n(%)	Absent n(%)	Test	DF	P-value
Age	Years	14(15.2)	78(84.8)	T=2.19$	90	0.031
Plaque score	(Mean=1.73±0.35 S.D)	14(15.2)	78(84.8)	t= 1.00*	90	0.320
Gingival index	(Mean=1.34±0.37 S.D)	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= 6.34#	2	0.004
	Severe	6(31.58)	13(68.42)			

*– Independent t test

\# – Chi square test

$ – ANOVA

DISCUSSION

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 utilised in this study(16,17).

REFERENCES

1. Baelum, V., Fejerskov, O. and Manji. F. Periodontal diseases in adult Kenyans. *J Clin Periodontol.* 1988; **15**: 445-452.
2. Mumghamba, E. G. S., Markkanen, H. A. and Honkala, E. Risk factors for periodontal diseases in Ilala, Tanzania. *J Clin Periodontol.* 1995; **22**: 347-354.
3. Albandar, J. M., Muranga, M. B. and Rams, T. E. Prevalence of aggressive periodontitis in school attendees in Uganda. *J Clin Periodontol.* 2002; **29**: 823-831.
4. Holt, S.C. and Ebersole, J.L. Porphyromonas gingivalis, Treponema denticola, and Tannerella forsythia: the red complex, a prototype polybacterial pathogenic consortium in periodontitis. *Periodontology.* 2005; **38**: 72-122.
5. Shaddox, L. M., Huang, H., Lin, T., Hou, W., Harrison, P. L., Aukhil, I., *et al.* Microbiological Characterization in Children with Aggressive Periodontitis. *J Dent Res.* 2012; **91**: 927-933.
6. Tran, S. D. and Rudney, J. D. Multiplex PCR Using Conserved and Species-Specific 16S rRNA Gene Primers for Simultaneous Detection of Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis. *Journal of Clinical Microbiology.* 1996; **34**: 2674–2678.
7. Morikawa, M., Chiba, T., Tomii, N., Sato, S., Takahashi, Y., Konishi, K., *et al.* Comparative analysis of putative periodontopathic bacteria by multiplex polymerase chain reaction. *J Periodont Res.* 2008; **43**: 268-274.
8. Henderson, B., Ward, J. M. and Ready, D. Aggregatibacter (Actinobacillus) actinomycetemcomitans: a triple A* periodontopathogen? *Periodontology, 2000.* 2010; **54**: 78-105.
9. Elamin, A., Albandar, J. M., Poulsen, K., Ali, R. W. and Bakken, V. Prevalence of Aggregatibacter actinomycetemcomitans in Sudanese patients with aggressive periodontitis: a case–control study. *J Periodont Res.* 2011; **46**: 285-291.
10. Watanabe, K. and Frommel, T. O. Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans and Treponema denticola detection in oral plaque samples using the polymerase chain reaction. *J Clin Periodontol.* 1996; **23**: 212-219.
11. Dahlen, G., Manji, F., Baelum, V. and Fejerskov, O. Putative periodontopathogens in "diseased" and "non-diseased" persons exhibiting poor oral hygiene. *J Clin Periodontol.* 1992; **19**: 35-42.
12. Dahlen, G., Manji, F., Baelum, V. and Fejerskov, O. Black-pigmented Bacteroides species and Actinobacillus actinomycetemcomitans in subgingival plaque of adult Kenyans. *J Clin Periodontol.* 1989; **16**: 305-310.
13. Sanz, M., Lau, L., Herrera, D., Morillo, J. M. and Silva, A. Methods of detection of Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, and Tannerella forsythensis in periodontal microbiology, with special emphasis on advanced molecular techniques: a review. *J Clin Periodontol.* 2004; **31**: 1034-1047.
14. Ali, R. W., Johannessen, A. C., Dahlen, G., Socransky, S. S. and Skaug, N. Comparison of the subgingival microbiota of periodontally healthy and diseased adults in Northern Cameroon. *J Clin Periodontol.* 1997; **24**: 830-835.
15. Papapanou, P. N., Teanpaisan, R., Obiechina, N. S., Pithpornchaiyakul, W., Pongpaisal, S., Pisuithanakan, S., *et al.* Periodontal microbiota and clinical periodontal status in a rural sample in southern Thailand. *Eur J Oral Sci.* 2002; **110**: 345–352.
16. Malathi, K., Sharmila, K., Sable, D. and Ahamed, S. Microbial Diagnosis in Periodontics: Merits and Demerits: A Review. *Journal of Dental and Medical Sciences.* 2014; **13**: 104-107.
17. Mackay, I. M. Real-time PCR in the microbiology laboratory. *Clinical Microbiology and Infection.* 2004; **10**: 190-212.