Periodontopathogenic bacterial species among patients with periodontal diseases at Mulago Hospital Dental Clinic in Kampala, Uganda: A cross-section study

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Received 8 July, 2014; Accepted 22 August, 2014

Periodontal diseases (PD) are disorders of the supporting structures of the teeth caused by components of the plaque that develop on the hard root surface adjacent to the soft tissues. Periodontal disease is caused by bacteria living in plaque biofilms. These bacteria also affect dental implants, causing peri-implantitis, and eventually implant failure. Early diagnosis and treatment to eliminate or significantly reduce these bacterial loads enables clinical success with dental implants. However, culture-based methods for detecting these bacteria are difficult to implement therefore making DNA–based techniques more efficient. WHO adds that at present, the distribution and severity of periodontal infections vary in different parts of the world and within the same region or country. In Uganda, data on prevalence of common bacteria species causing periodontal diseases is limited. The aim of this study was to determine the prevalence of periodontopathogenic bacteria in patients attending Mulago Hospital Dental School Clinic. Paper point periodontal specimens were collected from 100 periodontal diseases patients. These specimens were directly tested for 11 selected periodontopathogenic bacteria using two molecular tests: Micro-IDent® and Micro-IDent® plus (HAIN Lifescience GmbH, Nehren, Germany). It was found that 86% of the studied participants were with periodontopathogenic bacteria species. Fusobacterium nucleatum, Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans and Prevotella intermedia prevalence was 86, 7, 1 and 1%, respectively. Some patients had more than one periodontopathogenic bacteria detected. Average age and pocket depths of the study participants were 32 years and 3.5 mm, respectively. Age, pocket depth and gender were not statistically associated with any of the isolated periodontopathogenic bacteria species (p>0.05).

Key words: Periodontal disease, periodontopathogenic, multiplex-polymerase chain reaction (PCR), pocket depth, DNA, reverse hybridization.

INTRODUCTION

Periodontal diseases are disorders of supporting structures of the teeth (e.g., gingivae, periodontal ligament, and supporting alveolar bone). The disease is due to an inflammatory process that results in destruction of supporting
tissue of the teeth which is a result of mixed microbial infection (Haffajee et al., 2004; Kim and Amar, 2006; Darveau, 2010; Hasan and Palmer, 2014). These diseases are initiated by components of plaque that develop on the hard root surface adjacent to soft tissues of the supporting periodontium and this leads to inflammation of the gingiva, periodontal tissue destruction and in severe cases alveolar bone loss with tooth exfoliation (Pihlstrom et al., 2005). Periodontal diseases have been reported to affect up to 90% of the world’s population (Petersen et al., 2005; Moeen et al., 2008; Borjevic, 2012). In addition, more recent evidence has suggested a potential role of periodontal infections in more serious systemic diseases including cardiovascular disease, respiratory infections, diabetes and low-birth weight complications (Li et al., 2000; Igari et al., 2014). A consensus report concerning periodontal diseases and microbial etiology designated Porphyromonas gingivalis, Tannerella forsythia and Aggregatibacter actinomycetemcomitans as the most important causative organisms for the various forms of periodontal diseases (Genco et al., 1996). In addition, a dozen other oral bacterial species including Prevotella intermedia, Eikenella corrodens, Campylobacter rectus and spirochetes are closely associated with periodontitis (Genco et al., 1996).

Studies employing molecular genetics techniques for bacterial identification have demonstrated that both prevalence and level of colonization of these pathogens vary significantly between populations of different racial or geographical origin (Haffajee et al., 2004; Nibali et al., 2009). However, majority of studies determining the characteristics of these pathogens in different populations have been carried out in developed countries and little is known about the occurrence and profile of periodontal pathogens in populations of developing countries. Periodontal diseases are rampant in Uganda (Albandar et al., 2002; Muwazi et al., 2005). World Health Organization (WHO) adds that at present, the distribution and severity of periodontal infections varies in different parts of the world and within the same region or country (Petersen et al., 2005). Therefore, it is imperative to identify periodontopathogenic bacterial species in a specific population.

Culture techniques require viable bacteria, capnophilic requirements, labour intensive, time consuming and expensive. Recently, PCR-based methods for the detection of periodontopathogenic species in subgingival plaque samples have become commercially available (Genco et al., 1996; Urban et al., 2010). One such product is MicroIDent® and Micro-IDent®plus assay (HAIN Lifescience GmbH, Nehren, Germany), which can be used in microbiological laboratories. This assay is a multiplex PCR assay designed to detect and identify 11 selected periodontopathogenic bacterial species directly from paper points of periodontal specimens. The aim of this study was to determine the prevalence of periodontopathogenic bacteria among patients with periodontal disease that visited Mulago Hospital Dental Clinic.

**METHODOLOGY**

**Ethical issues**

Ethical clearance was obtained from the IRB (SBSREC-068), School of Biomedical Sciences, Makerere University, College of Health Sciences as well as the Uganda National Council for Science and Technology (UNCST). Parents/Guardians were informed about the study and written informed consent was obtained from the parents or legal guardians were applicable.

**Inclusion criteria**

The study recruited both female and male patients who provided written informed consent, above 10 years of age and presented with periodontal disease of different categories. Furthermore, study participants had not received periodontal surgery and had at least 20 natural teeth.

**Study participants and clinical specimens**

A total of 100 participants presented to the Mulago Dental School Clinic with periodontal diseases had dry paper point subgingival specimens collected between March and May, 2013. Patients with periodontal disease were diagnosed according to the criteria of the American Academy of Periodontology International Classification of 1999. The specimens were transported to MBN Clinical laboratories from where specimen processing was performed (Figure 1). Specimens were collected from pocket depths ranging from 1 to 6 mm of clinical attachment loss (CAL). For each patient with periodontitis, two pockets with the highest depths were chosen for bacterial sampling. Where possible, pockets were chosen from different quadrants. Samples were pooled to single vial per patient. For collection, the paper points were inserted into the periodontal pockets for at least 10 seconds and subsequently transferred into a sterile vial.

**DNA extraction and manipulation from dry paper points**

Genolyse DNA extraction kit (HAIN Lifescience) was used to extract bacterial DNA from dry paper points according to the manufacturer’s instructions. Briefly, 100 µl of lysis solution was added to the vial containing each periodontal sample and vigorously vortexed for 10 s to elute the bacteria cells from the paper points. This was incubated at 95°C on PeQLab heating block (Biotechnologie, GmbH, Germany) for 5 min with vigorous vortexing at 3 min interval. The mixture was then cooled to room temperature. A volume of 100 µl neutralizing buffer was then added and then spun in Mikro 200 microfuge (Centrifugen, Hettich, Germany) for 10 min at 14,000 rpm. A volume of 5.0 µl of the supernatant was used as template for the subsequent amplification step. This was stored at -20°C while waiting hybridization.

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Dental Clinic, Patient with Periodontal disease

Consent patient

Yes

Enroll and collect subgingival specimens

DNA Extraction by Genolyse DNA Isolation Kit

Micro-Ident® and Micro-IDent® plus-Multiplex PCR of 16S rDNA

Reverse Hybridization of the multiplex PCR products

Pasting and Scanning of the Strips

• *A. actinomycetemcomitans*
• *P. gingivalis*
• *T. forsythia*
• *T. denticola*
• *P. intermedia*
• *P. micra*
• *F. nucleatum/periodonticum*
• *C. rectus*
• *E. nodatum*
• *E. corrodens*
• *Capnocytophaga* species

CC: Conjugate Control
AC: Amplification Control

Figure 1. Study methodological summary.

**Micro-Ident® and Micro-IDent®** plus-Multiplex PCR of 16S rDNA

PCR amplification was carried out in a reaction volume of 50 µl consisting of 5.0 µl of template DNA and 45 µl of reaction mixture containing 35 µl of primer-nucleotide mix-PNM (microDent), 5.0 µl of 10x PCR buffer, 5.0 µl of 2.5 mM MgCl₂ and 0.2 U Hot start Taq (Qiagen, GmbH, Germany). PCR cycling was carried out in GTQ- Cycler 96 thermal cycler (HAIN Lifescience, Germany). The cycling conditions comprised an initial denaturation step at 95°C for 5 min, 10 cycles at 95°C for 30 s and at 60°C for 2 min, 20 cycles at 95°C
for 10 s, at 55°C for 30 s and at 72°C for 30 s, and a final extension step at 72°C for 10 min. Negative control was included in batch of samples tested. The negative control was 5.0 µl of sterile PCR water, each added to 45 µl of reaction mixture. The subsequent reverse hybridization was performed according to the Micro-IDent® and Micro-IDent® plus assay (HAIN Lifescience GmbH, Nehren, Germany).

Reverse hybridization

The subsequent reverse hybridization was performed according to the MicroIdent® and Micro-IDent® plus. Briefly, the biotinylated amplicons were denatured and incubated in Twincubator® (HAIN Lifescience) at 45°C with hybridization buffer and strips coated with two control lines (Conjugate and Amplification control) and five (Micro-IDent®) or six (Micro-IDent® plus) species-specific probes. After PCR products had bound to their respective complementary probe, a highly specific washing step removed any unspecifically bound DNA. Streptavidin conjugated alkaline phosphatase was added, the samples were washed and hybridization products were visualized by adding a substrate for alkaline phosphatase. Bands developed and the strips were pasted onto the paper for interpretation (Table 1). A total of 11 selected periodontopathogenic bacteria species can be identified using the assay.

Analysis of results

Data was collected using study participant forms. It was extracted and entered into an Excel (Microsoft Office, 2010) database, proof-read for entry errors and cleaned, coded and imported into SPSS software, version 16.0 for Windows (SPSS Inc., Chicago, IL, USA) formatted and analysed. Cross tabulation was done to establish the relationship between each putative factor and periodontopathic bacterial species. The association between age, gender and selected periodontopathic bacterial species was tested using a chi-square test ($p < 0.05$), bivariate and multivariate analyses were also performed in SPSS software. A descriptive analysis was performed for each variable (means, standard deviation). A level of significance of 95% ($p<0.05$) was set.

RESULTS

In a total of 100 participants, the study found that 86 (86%) participants had at least one periodontopathic bacteria. The young adults (19 to 45 years) made 66% of the study participants while 62% of the study participants were female making more than half of the study participants (Table 2). Eighty-six (86.0%) patients with Fusobacterium nucleatum were detected, 7 (7%) had P. gingivalis and also 7 patients out of 86 had both F. nucleatum and P. gingivalis. One (1.0%) patient had been co-infected by F. nucleatum and A. actinomycetemcomitans; however, only one patient had been infected with three periodontopathic bacteria, that is, F. nucleatum, P. gingivalis and P. intermedia. F. nucleatum was identified in at least 86 study participants (Table 3). The study participants ranged from 10 to 62 years (average age 32) and average pocket depth of 3.6 mm. The study found F. nucleatum to be more abundant at pocket depth of 3 mm (Table 4).

This study found no statistically significant association between age, gender, pocket depths and the different periodontopathogenic bacterial species that were identified from the study participants ($p > 0.05$) by both bivariate and multivariate analysis.

DISCUSSION

The presence of specific periodontal pathogenic bacteria in subgingival plaque has been used as an indicator for active periodontal disease (Eick and Pfister, 2002). This study has documented a high prevalence of F. nucleatum (86%) in the studied population. This is in agreement with the study done by Eick and Pfister (2002) who analysed 33 early onset periodontitis patients and 15 periodontally healthy subjects by cultivation and Micro-IDent® kit. They concluded that F. nucleatum was found to be more frequent in cases of periodontitis. Deep pockets contained more P. intermedia, F. nucleatum and spirochetes than did shallow pockets (Eick and Pfister, 2002). Another study by Avila and Velasquez (2002) involving 50 participants with periodontal disease documented prevalence of F. nucleatum, P. gingivalis, A. actinomycetemcomitans and P. intermedia at 96, 78, 90, and 84%, respectively in periodontal disease patients in Sao Paulo, Brazil; however, the sample size was the limitation of their study (Farias et al., 2012).

Our study found low prevalence of P. gingivalis (7%) and very low prevalence of P. intermedia and A. actinomycetemcomitans at 1% each. Eick and Pfister (2002) reported isolating these organisms from deeper pockets of >4 mm, P. intermedia and A. actinomycetemcomitans were isolated from pocket depth of ≥4.5 mm (Eick and Pfister, 2002). In our study, 53% of the pockets sampled were <4 mm in depth, and this might explain the differences in the findings. A study in Australia by Hamlet et al. (2001) found high frequency of A. actinomycetemcomitans, a facultative anaerobic bacterium at shallow pockets than deeper ones. Our study reported a prevalence of 1% amongst participants enrolled. We probed averagely shallow pocket depths. This may further be explained by the fact these studies are carried out in different racial groups. Epidemiological studies involving adults have demonstrated significant variation in the prevalence of periodontal pathogens depending on race, ethnicity, and geographic location (Torrungruang et al., 2009). In patients where we isolated more than one periodontopathic bacterium, probably these organisms exhibited symbiotic relationship to cause disease.

Pocket depths, age and gender did not appear to be statistically associated with different periodontopathic bacteria species detected in this study. This is in agreement with Farias et al. (2012). However, our study findings were not in agreement with Eick and Pfister (2002) who documented positive correlations between...
Table 1. Bacteria species identified by Micro-IDent® and Micro-IDent® plus assay.

<table>
<thead>
<tr>
<th>Micro-IDent® assay (Five species)</th>
<th>Micro-IDent® plus assay (Six species)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Actinobacillus actinomycetemcomitans</em></td>
<td><em>Peptostreptococcus micros</em></td>
</tr>
<tr>
<td><em>Porphyromonas gingivalis</em></td>
<td><em>Fusobacterium nucleatum</em></td>
</tr>
<tr>
<td><em>Bacteroides forsythus</em></td>
<td><em>Campylobacter rectus</em></td>
</tr>
<tr>
<td><em>Prevotella intermedia</em></td>
<td><em>Eubacterium nodatum</em></td>
</tr>
<tr>
<td><em>Treponema denticola</em></td>
<td><em>Eikenella corrodens</em></td>
</tr>
<tr>
<td>-</td>
<td><em>Capnocytophaga</em> sp.</td>
</tr>
</tbody>
</table>

Table 2. Characteristics of studied patients.

<table>
<thead>
<tr>
<th>Age group</th>
<th>Number (n)</th>
<th>Female</th>
<th>Male</th>
<th>Number patients with bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-12</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>13-18</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>19-45</td>
<td>66</td>
<td>41</td>
<td>25</td>
<td>60</td>
</tr>
<tr>
<td>46-64</td>
<td>19</td>
<td>12</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>65 and above</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>62</td>
<td>38</td>
<td>86</td>
</tr>
</tbody>
</table>

86/100 of the study participants had periodontopathogenic bacteria.

Table 3. Prevalence of periodontopathogenic bacteria in the studied population

<table>
<thead>
<tr>
<th>Parameter</th>
<th><em>F. nucleatum</em></th>
<th><em>P. gingivalis</em></th>
<th><em>P. intermedia</em></th>
<th><em>A. actinomycetemcomitans</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>55</td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>M</td>
<td>31</td>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>86</td>
<td>7</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

*F. nucleatum* was more frequently isolated from study participants.

Table 4. Distribution of the different periodontopathogenic bacteria species at different pocket depths.

<table>
<thead>
<tr>
<th>Variable</th>
<th><em>F. nucleatum</em></th>
<th><em>P. gingivalis</em></th>
<th><em>P. intermedia</em></th>
<th><em>A. actinomycetemcomitans</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Pocket depth (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>23</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3.5</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>21</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4.5</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>14</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>-</td>
<td>86</td>
<td>7</td>
<td>1</td>
</tr>
</tbody>
</table>

pocket depths, frequency and quantity of periodontopathogenic bacteria. However, there sample size was statistically small (50 patients studied) and further this may also be explained by the fact that the population they studied was different from ours in terms of host genetics, socioeconomic status, lifestyle, age groups, diets, cigarette smoking habits, oral hygiene habits, geographical settings, underlying health conditions and methodology
used. Socransky et al. (1998) also compared the microflora of pockets with different depths and found a higher prevalence of *P. gingivalis*, in deep pockets than in shallow pockets. Our study found 82.6% of the periodontopathogenic bacteria isolated from pocket depth ≥3 mm. This fact can be explained by the deeper pockets that offer a better medium for anaerobes and consequently, greater colonization of these pathogens (Hamlet et al., 2001; Socransky et al., 1998). Age group 19 to 45 years comprised 66 of the enrolled participants; however, 91% (n=60) of these were positive for periodontopathogenic bacteria species. This age group is made up of young adults and factors responsible for this high prevalence of periodontopathogenic bacterial species can further be investigated. World Health Organization reports severe periodontal (gum) disease, which may result in tooth loss (found in 15 to 20% of middle-aged (35 to 44 years) adults) (WHO, 2012).

**Conclusion**

Using Multiplex PCR-based technique, *F. nucleatum* was found to be the most common bacteria detected among patients present with periodontal disease at Mulago Hospital Dental School Clinic.

**Competing interests**

All authors declare that they have no competing interests.

**ACKNOWLEDGEMENTS**

This study was carried out with the financial support obtained from MBN Clinical laboratories, Kampala, Uganda. The authors acknowledge the contributions of the dentist and study participants from Mulago Hospital Dental School Clinic.

**REFERENCES**


