PERIODONTIUM or periodontal tissues, are tissues that surround, support and maintain the teeth in the maxillary and mandibular bones. Like other tissues, the periodontal tissues are subject to a number of diseases. The most periodontal pathogens associated with periodontal disease are Tannerella forsythia, Porphyromonas gingivalis, Treponema denticola, Prevotella Intermedia, Fusobacterium nucleatum and Aggregatibacter actinomyctecomumans. Female hormones have been suggested to play an important role in periodontal disease infection. The objective of this study was to identify the above periodontal pathogens associated with periodontal disease in a population of Rwandan women. This study requested the participation of randomly selected women admitted in the department of obstetric-gynecology of the teaching hospital of Butare in Rwanda. Gingival crevice fluid was collected from four teeth (16, 26, 36, 46) with filter paper strips by inserting the strips into the base of the pocket for one minute per tooth. PCR was used for the detection of the presence of the 6 target bacteria in GCF. F. nucleatum was the most prevalent with 86.2%, P. intermedia (73.5%), T. forsythia (47.6%), A. actinomyctecomumans (45%), P. gingivalis (28.4%) and T. denticola with (24.3%). One hundred and eighty six (93.0%) of the patients harboured at least one of the six periodontopathogens. This study showed that there is an urgent need to improve oral health care and research in Rwanda, on the African continent in general and especially in women who are more exposed to periodontal diseases than men.

Key Words: Prevalence – Periodontopathogens - Women - Gingival Crevicular Fluid - Rwanda.

RESUME

Les tissus paradontals sont des tissus qui entourent, supportent et maintiennent les dents sur les os maxillaires et mandibulaires. Comme tous les autres tissus, les tissus paradontals sont exposés à de nombreuses maladies. Les bactéries qui sont associées le plus souvent avec les maladies paradontales sont: Tannerella forsythia, Porphyromonas gingivalis, Treponema denticola, Prevotella Intermedia, Fusobacterium nucleatum et Aggregatibacter actinomyctecomumans. Les hormone femelles ont été suggérées avoir joué un grand rôle dans les infections des maladies paradontales. L'objectif de cette étude était d'identifier la prévalence des bactéries ci-haut citées qui sont associées le plus souvent avec les maladies paradontales dans une population des femmes au Rwanda. Les participants de cette étude étaient des femmes admises à l'hôpital universitaire de Butare dans le département de gynécologie-obstétrique. Le liquide créviscule gingival provenant des quatre dents (16, 26, 36, 46) était collecté à l'aide du papier filtre en insérant ce dernier dans la base des poches pendant une minute par dent. Le PCR était utilisé pour détecter la présence de ces 6 bactéries dans le liquide créviscule gingival. F. nucleatum était le plus prévalent avec 86.2 %, P. intermedia (73.5 %), T. forsythia (47.6 %), A. actinomyctecomumans (45 %), P. gingivalis (28.4 %) et T. denticola avec (24.3 %). Cent quatre-vingt six (93,0 %) des patients avaient au moins un des six bactéries paradontales. Cette étude montre qu’il y a un besoin urgent pour améliorer l’hygiène dentaire et la recherche dans ce domaine au Rwanda et en Afrique en général et ceux-ci surtout sur les femmes qui sont plus exposées à ces maladies que les hommes.

Mots-clés : Prévalence - bactéries paradontales - femmes - Rwanda
**Prevalence of Six Periodontal**

Intermedia, Fusobacterium nucleatum and Aggregatibacter actinomycetemcomutans [6-11].

Although women take more care of their teeth than men, three-quarter of periodontal office visits are made by Women [12]. Female hormones during puberty, menses, pregnancy, contraceptive use and menopause have been suggested to play an important role in periodontal disease infection [13-17]. The increase of estrogen and progesterone concentration in plasma stimulate bacterial growth and are associated with periodontal disease progression [18-20]. To our knowledge, the prevalence of periodontal pathogens in Rwanda and in other Sub Saharan Africa is almost unknown. This is due to a lack of adequate laboratories for their identification. The objective of this study was to identify the prevalence of the six most periodontal pathogens (Tannerella forsythia, Porphyromonas gingivalis, Treponema denticola, Prevotella Intermedia, Fusobacterium nucleatum and Aggregatibacter actinomycetemcomutans) associated with periodontal disease in a population of Rwandan women.

**MATERIAL AND METHODS**

This study requested the participation of randomly selected pregnant women admitted in the department of obstetric-gynecology of the teaching hospital of Butare in Rwanda. Informed consent was obtained from the participants in verbal and written. They were informed of the purpose of the study and were required to sign the form if they agree to participate in the study and were assured of confidentiality of any disclosures. Gingival crevice fluid (GCF) was collected from four teeth [16, 26, 36, 46] with filter paper strips (PropFlow, Inc., Amityville, NY) by inserting the strips into the base of the pocket for one minute per tooth [21]. The filter paper strips were handled carefully to prevent saliva or blood contamination in the mouth when collecting GCF. Each paper strip was placed into 50 µl phosphate buffered saline sampling buffer in an Eppendorf tube that was supplemented with 0.05 % tween-20 (PBS-T) and stored at -80°C [22]. Samples were transported on dry ice to South Africa and stored once again at -80°C before analysis.

Polymerase Chain Reaction (PCR) was used for the detection of the presence of the 6 target bacteria of this study. Samples from the freezer were thawed by incubation at 37°C for 10 min. After thawing the frozen GCF samples, each sample contained in an Eppendorf tube was centrifuged (10.000 X g) for 15 minutes at 4°C. The supernatants of the 4 tubes of 1 patient were pooled [23]. Samples were vortexed for 30 seconds and centrifuged at 2500X g for 2 minutes. The supernatant was removed and the pellet resuspended in 100 µl of distilled water. Another step of vortexing and centrifugation was done and the pellet was resuspended in 500 µl of distilled water. The suspension was heated at 94°C for 10 min and the vials immediately chilled on ice for 5 min. Reference DNA from the following trains Tannerella forsythia strain ATCC 43037, Porphyromonas gingivalis strain ATCC 33277, Treponema denticola strain 521, Prevotella Intermedia strain ATCC 25611, Fusobacterium nucleatum strain NTCC 10562, and Aggregatibacter actinomycetemcomutans strain ATCC 33396 were used as positive control. Chilled samples were centrifuged for 10 seconds at 9000X and 5 µl aliquots of the supernatants were used in the PCR assay. Twenty five of the DreamtaqTM Green PCR Master Mix(2X) (FE K1081, Inqaba biotec), 0.1-1.0 µM of each primer and 18 µl of water nuclease were added to the 5 µl of template DNA. Species-specific primers (Inqaba biotec) were used to detect the presence of the 6 target periodontal organisms in this study. The expected product lengths were 641 bp for T. forsythia, 404 bp for P. gingivalis, 316 bp for T. denticola, 307 bp for P. Intermedia, 500 bp for A. actinomycetemcomitans, and 705 bp for F. nucleatum. A pair of ubiquitous primers product length (602 bp) which matches most bacterial 16S rRNA genes at the same position was used as a positive control for the PCR reaction. Nucleotide sequences of selected and modified 16S rDNA primer pairs are listed in Table 1.

The negative control contained 5 µl of distilled water in place of the sample and the positive control consisted of 49 µl from the master mix and 1 µl (100ng) of the reference genomic DNA. A brief vortexing of samples was done. PCR amplifications was performed as follows: P. gingivalis [24], an initial denaturation step at 94°C for 2 minutes, followed by 36 cycles of a denaturation step at 94°C for 30 seconds, a primer annealing step at 60°C for 1 minute, an extension step at 72°C for 1 minute, and a final step at 72°C for 10 minutes; T. forsythia, T. denticola and ubiquitous primers [24]. An initial denaturation step at 95°C for 2 minutes, followed by 36 cycles of a denaturation step at 95°C for 30 seconds, a primer annealing step at 60°C for 1 minute, an extension step at 72°C for 1 minute, and a final step at 72°C for 2 minutes; Prevotella intermedia [25]: an initial denaturation step at 95°C for 2 minutes, followed by 36 cycles as one cycle at 94°C for 30 s (denaturation) followed by 55°C for 1 min (annealing) with an elongation of 72°C for 1 minute, and a final step at 72°C for 10 minutes; A. actinomycetemcomitans and F. nucleatum: Same conditions as described previously by Rocas et al [24]. The PCR products were analyzed by electrophoresis in 1 % gel using Tris-Borate EDTA buffer at 90 V. A 100 bp size ladder (O’GeneRuler 100 bp DNA ladder, Fermentas) was used as the molecular weight marker. The DNA was stained with ethidium bromide and visualized under UV light. Data were analyzed using SPSS 14.0. All questionnaires, oral examination and laboratory data were entered into Excel 2003 and then were transferred in SPSS for analysis. Frequencies were calculated using descriptive statistics.
Prevalence of Six Periodontal Diseases

RESULTS AND DISCUSSION

The target of this study was to identify the prevalence of six periodontopathogenic bacteria (Tannerella forsythia, Porphyromonas gingivalis, Treponema denticola, Prevotella Intermedia, Fusobacterium nucleatum, and Aggregatibacter actinomycetemcomitans) most associated with periodontal disease in a population of Rwandan women.

Examples of PCR detection of the six periodontopathogens are demonstrated in Figures 1-6. Table 2 reports the prevalence of the six periodontopathogens. F. nucleatum was the most prevalent with 86.2 %, the second was P. intermedia (73.5 %), the third T. forsythia (47.6 %), followed by A. actinomycetemcomitans (45 %), then P. gingivalis (28.4%) and the last was T. denticola with (24.3 %). Only 3 (1.5 %) patients were negative to all 6 periodontopathogens and 186 (93.0 %) of the patients harboured at least one of the six periodontopathogens. Choi et al [26] found that F. nucleatum was present in all diseased sites and in 58 % of healthy sites while Treponema sp, P. gingivalis and T. forsythia were detected in more than 96 % of diseased sites and were present in 22 %, 18 % and 18 % of healthy sites respectively. A. actinomycetemcomitans and P. intermedia were present in 74 % and 71 % of diseased sites and in 1 % and 2 % of healthy sites respectively. Our study examined random sites whether or not they were diseased.

Research has shown that periodontal disease which is a chronic inflammatory disease can act as the site of origin for dissemination of periodontopathogens and their toxins as well as induce inflammatory mechanisms to distant body sites, thus linking periodontal diseases to other serious health risk such as: osteoporosis, hearth disease and stroke, pregnancy problems, diabetes and respiratory diseases [27-30]. Therefore treating periodontal disease may help also the management of many other chronic inflammatory conditions.

The result of this study showed that the prevalence of the six peridontopathogens most associated with periodontal disease in Rwandan women is high. Therefore there is an urgent need to improve oral health care and research in Rwanda and on the African continent in general and especially in women who are more exposed to periodontal diseases than men.

Table I. PCR primer sequences used for detection of our target bacteria

<table>
<thead>
<tr>
<th>Target</th>
<th>PCR primer pairs (5'-3')</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porphyromonas gingivalis:</td>
<td>- Forward AGG CAG CTT GCC ATA CTG CG</td>
<td>Rocas et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>- Reverse ACT GTT AGC AAC TAC CGA TGT</td>
<td></td>
</tr>
<tr>
<td>Tannerella forsythia:</td>
<td>- Forward GCG TAT GTA ACC TGC CCG CA</td>
<td>Rocas et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>- Reverse TGC TTC AGT GTC AGT TAT ACC T</td>
<td></td>
</tr>
<tr>
<td>Treponema denticola:</td>
<td>- Forward TAA TAC CGA ATG TGC TCA TTT ACA T</td>
<td>Rocas et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>- Reverse TCA AAG AAG CAT TCC TTC TTA</td>
<td></td>
</tr>
<tr>
<td>Prevotella Intermedia:</td>
<td>- Forward CAA AGA TTC ATC GGT GGA</td>
<td>Kook et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>- Reverse GCC GGTT CTT TAT TCG AAG</td>
<td></td>
</tr>
<tr>
<td>Fusobacterium nucleatum:</td>
<td>- Forward ATT GTG GCT AAA AAT TAT AGT T</td>
<td>Mayanagi et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>- Reverse ACC CTC ACT TTG AGG ATT ATA G</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Reverse ATT TCA CAC CTC ACT TAA AGG T</td>
<td></td>
</tr>
<tr>
<td>Ubiquitous primers:</td>
<td>- Forward GAT TAG ATA CCC TGG TAG TCC AC</td>
<td>Rocas et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>- Reverse CCC GGG AAC GTA TTC ACC G</td>
<td></td>
</tr>
</tbody>
</table>
Prevalence of Six Periodontal

Table II. : Prevalence of the six periodontopathogens in GCF

<table>
<thead>
<tr>
<th>PCR Results</th>
<th>Positives N (%)</th>
<th>Negatives N (%)</th>
<th>Missings N (%)</th>
<th>Valid Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>F. nucleatum</td>
<td>163 (81.5)</td>
<td>26 (13.0)</td>
<td>11 (5.5)</td>
<td>86.2</td>
</tr>
<tr>
<td>P. intermedia</td>
<td>139 (69.5)</td>
<td>50 (25.0)</td>
<td>11 (5.5)</td>
<td>73.5</td>
</tr>
<tr>
<td>T. forsythia</td>
<td>91 (45.5)</td>
<td>100 (50.0)</td>
<td>9 (4.5)</td>
<td>47.6</td>
</tr>
<tr>
<td>A. actinomycetemcomutans</td>
<td>85 (42.5)</td>
<td>104 (52.0)</td>
<td>11 (5.5)</td>
<td>45</td>
</tr>
<tr>
<td>P. gingivalis</td>
<td>54 (27.0)</td>
<td>136 (68.0)</td>
<td>10 (5.0)</td>
<td>28.4</td>
</tr>
<tr>
<td>T. denticola</td>
<td>46 (23.0)</td>
<td>143 (71.5)</td>
<td>11 (5.5)</td>
<td>24.3</td>
</tr>
<tr>
<td>Positives cases</td>
<td>186 (93.0)</td>
<td>3 (1.5)</td>
<td>11 (5.5)</td>
<td>98.4</td>
</tr>
</tbody>
</table>

Figure 1: PCR amplification of P. gingivalis using Species-specific primers
Expected product size: 404 bp, lane 1: DNA marker 100 bp, lane 2 (Gel 1): positive control, lane 3 (Gel 1): negative control.

Figure 2: PCR amplification of T. forsythia using Species-specific primers
Expected product size: 641 bp, lane 1: DNA marker 100 bp, lane 2 (Gel 1): positive control, lane 3 (gel 1): negative control.

Figure 3: PCR amplification of T. denticolas using Species-specific primers
Expected product size: 316 bp, lane 1: DNA marker 100 bp, lane 2 (gel 1): positive control, lane 3 (gel 1): negative control.

Figure 4: PCR amplification of P. intermedia using Species-specific primers
Expected product size: 307 bp, lane 1: DNA marker 100 bp, lane 2 (gel 1): positive control, lane 3 (gel 1): negative control.
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