Article

PREVALENCE OF SIX PERIODONTAL PATHOGENS IN RWANDAN WOMEN'S GINGIVAL CREVICULAR FLUID

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ABSTRACT

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 Agregatibacter actinomycetemcomutans. Female hormones have been suggested to play an important role in periodontal disease infection. The objective of this study was to identify the prevalence of 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. actinomycetemcomutans (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 mandibullaires. Comme tous les autre 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 and Agregatibacter actinomycetemcomutans.
Les hormone femelles ont été suggérées avoir joué un grand rôle dans les infections des maladies paradontales. L'objectif de cette étude était d'identidier 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'hopital universitaire de Butare dans le department de gynécologie-obstétrique. Le liquide créviculaire 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 créviculaire gingival. F. nucleactum était le plus prévalent avec 86,2 %, P. intermedia (73,5 %), T. forsythia (47,6 %), A. actinomycetemcomutans (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 parandontales. 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 parandotales - femmes - Rwanda

INTRODUCTION

Periodontium or periodontal tissues, are tissues that surround, support and maintain the teeth in the maxillary and mandibular bones. The periodontium is formed by the gingivae, the alveolar bone, the periodontal ligament and the cementum [1]. Like other tissues, the periodontal tissues are subject to a number of diseases. The disease process may be limited to the gingivae or involve the deeper periodontal structures [2].

The first stage of periodontal disease is called gingivitis. At this stage, the gingivae are red, swollen and can bleed easily resulting in false pocket formation. Gingivitis can be treated by improving oral hygiene practice. If it is not treated, poison from bacteria can penetrate deep tissues of the periodontium and destroy the periodontal membrane and the alveolar bone. At this stage, periodontal disease is called periodontitis. A true periodontal pocket is formed, caused by the migration of the junctional epithelial tissue at the base of the gingivae down the root of the tooth [3]. At the late stage of periodontitis, gingivae and alveolar bone can be seriously damaged resulting in tooth loss [4, 5].

Most of periodontal diseases are associated with the presence or overgrowth of anaerobic bacteria either alone or in association. The most periodontal pathogens associated with periodontal disease are Tannerella forsythia, Porphyromonas gingivalis, Treponema denticola, Prevotella

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Intermedia, Fusobacterium nucleatum and Agregatibacter 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 Saharans 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 Agregatibacter 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 -800 C [22]. Samples were transported on dry ice to South Africa and stored once again at -800 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 37o 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 4o 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 supernatent 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 940 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 Agregatibacter 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 peridontal 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 perfomed 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 600C for 1 minute, an extention step at 720C for 2 minutes, 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 and extention step at 720C 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. actinomycetemcomutans 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.

RESULTS AND DISCUSSION

The target of this study was to identify the prevalence of six periodontopathic bacteria (Tannerella forsythia, Porphyromonas gingivalis, Treponema denticola, Prevotella Intermedia, Fusobacterium nucleatum, and Agregatibacter actinomycetemcomutans) 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. actinomycetemcomutans (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. actinomycetemcomutans 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

Target		PCR primer pairs (5'-3')	Source
Porphyromonas gingivalis:	- Forward	AGG CAG CTT GCC ATA CTG CG	Rocas *et al.* (2001)
	-Reverse	ACT GTT AGC AAC TAC CGA TGT	
Tannerella forsythia :	- Forward	GCG TAT GTA ACC TGC CCG CA	Rocas *et al.* (2001)
	- Reverse	TGC TTC AGT GTC AGT TAT ACC T	
Treponema denticola:	- Forward	TAA TAC CGA ATG TGC TCA TTT ACA T	Rocas *et al.* (2001)
	- Reverse	TCA AAG AAG CAT TCC CTC TTC TTC TTA	
Prevotella Intermedia:	- Forward	CAA AGA TTC ATC GGT GGA	Kook *et al.* (2005)
	- Reverse	GCC GGT CCT TAT TCG AAG	
Fusobacterium nucleatum:	- Forward	ATT GTG GCT AAA AAT TAT AGT T	Mayanagi *et al.* (2004)
	- Reverse	ACC CTC ACT TTG AGG ATT ATA G	
Actinobacillus actinomycetemcomutans:			Avila-campos and Julio (2003)
	- Forward	GCT AAT ACC GCG TAG AGT CGG	
	-Reverse	ATT TCA CAC CTC ACT TAA AGG T	
Ubiquitous primers:	- Forward	GAT TAG ATA CCC TGG TAG TCC AC	Rocas *et al.* (2001)
	- Reverse	CCC GGG AAC GTA TTC ACC G	

Table II. : Prevalence of the six peridontopathogens in GCF

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

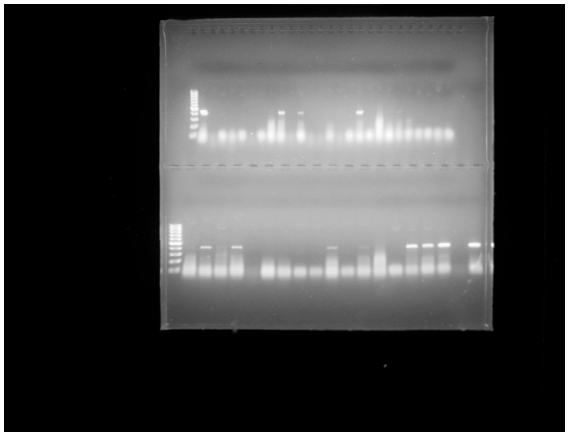

Figure 1: PCR amplification of P. gingivalis using Species-specific primers

Expected product size: 404 bp, lane 1: DNA marker 100 bp, lane 2 (Gel 1): positive control, lane 3 (Gel 1): negative control.

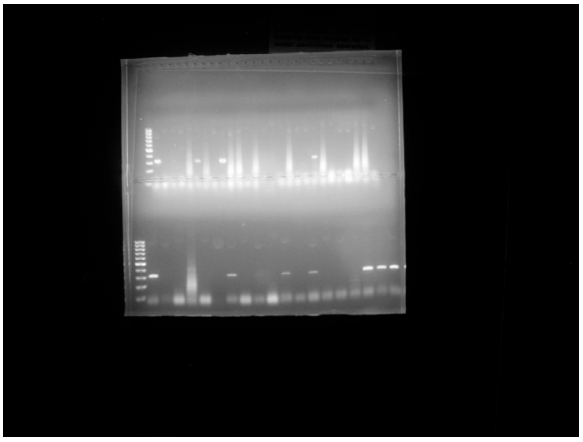

Figure 2: PCR amplification of T. forsythia using Species-specific primers

Expected product size: 641 bp, lane 1: DNA marker 100 bp, lane 2 (Gel 1): positive control, lane 3 (gel 1): negative control.

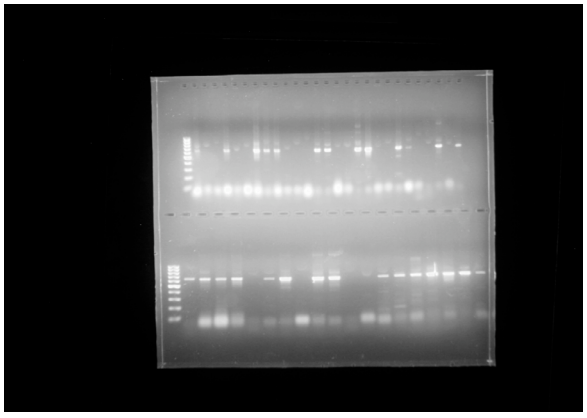

Figure 3: PCR amplification of T. denticolas using Species-specific primers

Expected product size: 316 bp, lane 1: DNA marker 100 bp, lane 2 (gel 1): positive control, lane 3 (gel 1): negative control.

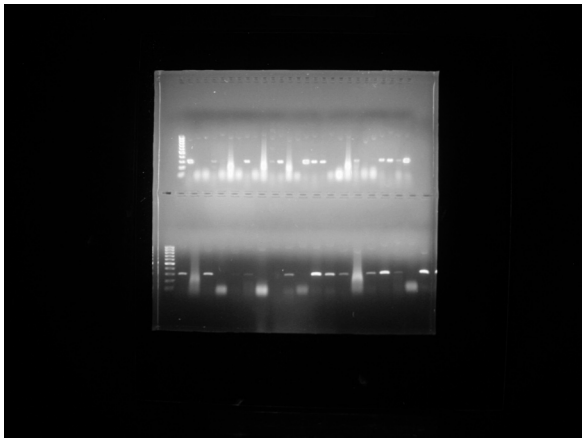

Figure 4: PCR amplification of P. intermedia using Species-specific primers

Expected product size: 307 bp, lane 1: DNA marker 100 bp, lane 2 (gel 1): positive control, lane 3 (gel 1): negative control.

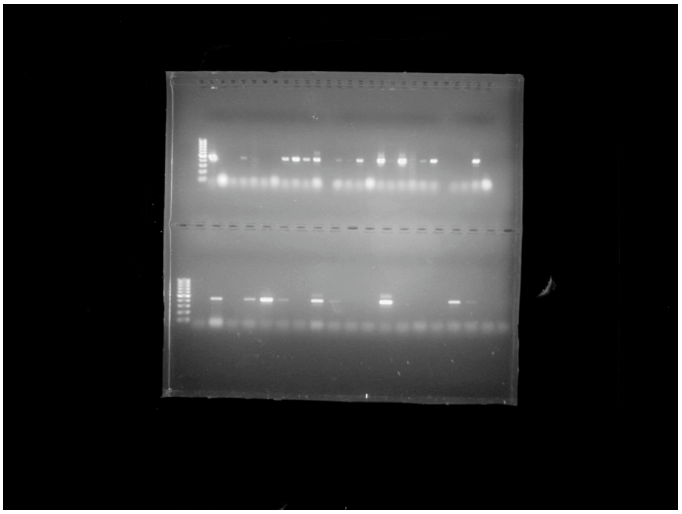

Figure 5: PCR amplification of A. actinomycetemcomutans using Species-specific primers

Expected product size: 500 bp, lane 1: DNA marker 100 bp, lane 2 (gel 1): positive control, lane 3 (gel 1): negative control.

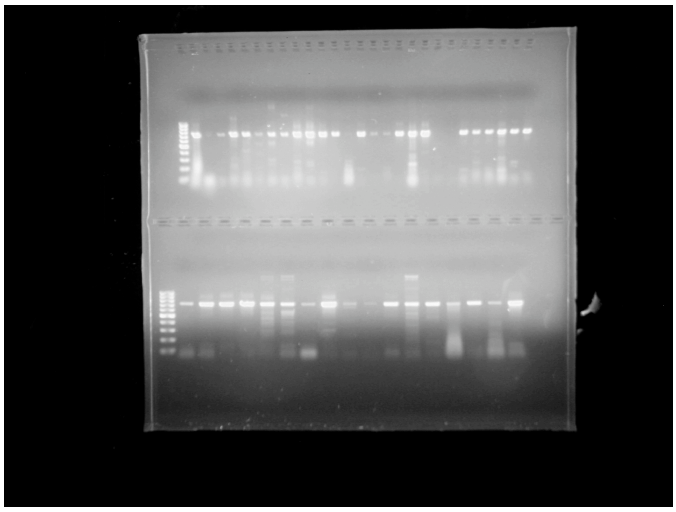

Figure 6: PCR amplification of F. nucleatum using Species-specific primers

Expected product size: 705 bp, lane 1: DNA marker 100 bp, lane 2 (gel 1): positive control, lane 3 (gel)

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