Characterization of Periodontal Biofilm in Down Syndrome Patients: A Comparative Study

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*The **aim** of this study was to characterize the main periodontal bacterial species in Down syndrome (DS) patients with and without periodontitis. **Method:** This cross-sectional study involved 75 DS patients, 45 with and 30 without periodontitis. Informed consent, health and dental questionnaires and periodontitis diagnosis were performed. PCR and LAMP assays were performed on subgingival dental plaque sample. **Results:** Tannerella forsythia was the most frequent bacteria detected in the group with and without periodontitis (95.5 and 63.3%) followed by Treponema denticola (88.8 and 50%) and Porphyromonas gingivalis (53.3 and 25% respectively). There were statistical differences between groups ($p<0.05$). Pg fimA type I was the most frequent Porphyromonas gingivalis genotype. Two different sets o primers (Aa-F/Aa-R and ltx3/ltx4) were used to detect Aggregatibacter actinomycetemcomitans and different frequencies were obtained, (68% and 14.6% respectively), they had a weak correlation (Cohen Kappa=0.16). After sequencing of PCR products, ltx3/ltx4 showed more specificity. JP2 clone of A. actinomycetemcomitans was not detected in any sample. **Conclusions:** The composition of oral biofilm is fundamental for the development of periodontal disease independently of immunological alterations associated with DS. The frequency of detection of A. actinomycetemcomitans reported in the literature has a wide range, because the primers and probes applied.*

Keywords: *Down syndrome; trisomy 21, periodontitis; periodontal bacteria; dental plaque.*

INTRODUCTION

Down syndrome (DS) is a genetic disorder, characterized by retarded generalized growth and mental deficiencies and different systemic manifestations, such as cardiovascular, auditive, visual and immunological disorders.[1,2] They show distinctive oral features such as: protrusive tongue, hipodontia, microdontia, hypoplastic defects, and high incidence of severe, early-onset periodontal disease.[3-5] DS patients show a severe periodontal breakdown similar to aggressive periodontitis; however, the etiology of this condition is not fully understood, and the immunological component is believed to be as the most important factor.[1] The quantitative and qualitative composition of oral biofilm are considered the main etiological factor in periodontal disease, in DS patients, the quality of periodontal biofilm has not been explored deeply.[6]

Aggregatibacter actinomycetemcomitans is considered a main pathological agent for aggressive periodontitis, this gram-negative microorganism produces a leukotoxin that leads to aggressive periodontal tissue destruction.[7] A particular variant of *A. actinomycetemcomitans*, termed the JP2 clone is strongly associated with severe aggressive periodontitis. This clone has a deletion in the 530 bp promoter region of the *ltx* operon encoding the leucotoxin, and it translates a more efficient toxin, the main virulence factor of *A. actinomycetemcomitans*.[8]

On the other hand, *Porphyromonas gingivalis* has been considered as the main species associated with chronic periodontitis. It has been reported that extentions from the cell wall named fimbriae promote initial biofilm development and allow adhesion to periodontal tissues.[9] An association between specific alleles of the *fimA* gene encoding the structural subunit of fimbriae and severe periodontitis has been reported.[10,11] Other species, such as *Treponema denticola, Tannerella forsythia*, together with *P. gingivalis*, constitute the red complex; this consortium of bacteria has been associated with destructive periodontitis, and it leads to development of more severe clinical signs of periodontitis.[12] The aim of this study was to characterize the main periodontal bacterial species in Down syndrome patients with and without periodontitis.

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MATERIALS AND METHOD

This cross-sectional study involved 75 subjects with DS, 45 of them with periodontitis and 30 without periodontitis, all of them living in San Luis Potosi (northern-central region), Mexico. Subjects were recruited from the Oral Medicine Clinic of San Luis Potosi University. Subjects completed a health questionnaire that included information about systemic health and periodontal status. Informed and voluntary written consent from the tutor of the patients was obtained prior to clinical examination according to the ethical principles of declaration of Helsinki (Version 2008). Subjects involved in this study were cooperative males and females with permanent dentition. Patients with antibiotic therapy in three months prior to the study were excluded.

Diagnosis of periodontitis

Periodontitis was determined by a calibrated examiner measuring pocket depth and clinical attachment level indexes.[13] These indexes were obtained by using a Michigan periodontal probe (Hu-Friedy Instrument Co, Chicago, IL, USA). The probe was inserted parallel to teeth long axis and crossed each tooth's surface circumferentially and clinical attachment loss index was measured from the epithelial attachment to the cement-enamel junction. The diagnosis of periodontitis was determined when the pocket depth was $\geq$ 3 mm and the attachment loss was $\geq$ 2 mm in at least 10 sites measured.[14]

Subgingival plaque sample

After cleaning of tooth crown with a sterile sponge, subgingival plaque samples were collected from all the present teeth with a Gracey curette from the vestibular, mesial, palatine and distal sulcus and placed into an eppendorf tube with 1 ml of Phosphate Buffer Saline. The samples were divided into anterior and posterior teeth. Each sample was taken 2 hours after the last meal and tooth brushing.[14] Samples were stored at -40°C, until the assays were carried out.

DNA extraction and PCR

All samples were processed aseptically to prevent contamination from environment and during DNA extraction method and PCR assays. DNA extraction was performed in accordance to previous report.[15] PCR assay was carried out in 25 µl of a reaction mixture containing 1 U Taq DNA Polymerase (Roche, Indianapolis, IN, USA), 0.3 µM of oligonucleotides, 0.2 mM of dNTP´s, 1.5 mM of $MgCl_2$ and 10 ng of DNA template. Specific primers for each periodontal species and genotypes of *P. gingivalis* used in the study were reported previously.[11,16,19] For *A. actinomycetemcomitans* detection, two different set of primers were used: Aa-F/Aa-R[20] and ltx3/ltx4.[21] PCR reactions were performed in a thermal cycler (iCycler, BIO-RAD laboratories, Hercules, CA, USA) with the cycling parameters previously reported according to each set of primers. A positive control was included in each PCR set by using DNA of the following bacterial strains: *P. gingivalis* (ATCC 33277 and 53997) *T. forsythia* (ATCC 43037), *A. actinomycetemcomitans* (ATCC 29523 and JP2), *T. denticola* (ATCC 35405). A negative control was also included in each PCR assay. The PCR products were analyzed by electrophoresis in a 2% agarose gel using Tris-acetate-EDTA buffer, using a 100-bp DNA ladder marker (New England Biolab, Beverly, MA, USA) to estimate the molecular size. Each gel was stained with ethidium bromide (0.5µg/ml) and photographed under UV light (Chemi Doc, BIO-RAD laboratories, Hercules, CA, USA).

Detection of the JP2 clone of A. actinomycetemcomitans with LAMP Method

When samples were positive for *A. actinomycetemcomitans* by PCR, the presence of JP2 clone of *A. actinomycetemcomitans* was determined by loop mediated amplification method (LAMP). The LAMP reaction was performed in a master mix of 25 µl containing 1.6 µM each of the primers FIP and BIP, 0.2 µM of primers F3 and B3, 0.4 µM of primers LF and LB, 8 U of the *Bst* DNA polymerase large fragment (New England Biolabs, Ipswich, Massachusetts), 1.4 mM each of the four deoxynucleoside triphosphates, 0.8 M betaine (Sigma, St. Louis, MO, USA), 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)2SO_4$, 8 mM $MgSO_4$, 0.1% Tween 20, and template DNA in a volume of up to 5 µl.[22] The mixture was incubated at 63°C for 60 min and then heated at 80°C for 2 min to terminate the reaction. Samples were considered positive when a white precipitate was detected by visual inspection and/or an amplification product was observed by electrophoresis in a 2% agarose gel stained with ethidium bromide.

Sequencing of PCR products for A. actinomycetemcomitans with two different sets of primers

PCR products obtained with ltx3/ltx4 and Aa-F/Aa-R primers were purified with a commercial kit, (NucleoSpin Extract II, Macherey Nagel Düren, Germany), and sequenced using the Big Dye Terminator method (Applied Biosystems, Applera, France). The thermocycling protocol was: 96°C for 5 min followed by 25 cycles of 96°C for 30 sec, 59°C for 15 sec, and 60°C for 4 min. DNA was precipitated with isopropanol and analyzed on an ABI Prism 3700 DNA Analyser automated sequencer (Applied Biosystems, Applera, France). The DNA sequences were compared with known sequences included in the NCBI database and aligned by BLAST program (http://www.ncbi.nlm.nih.gov/BLAST).

Statistical Analysis

The examiner was calibrated in all variables with an expert in periodontology through intraclass correlation coefficient. All data are expressed as mean, standard deviation and range. Qualitative data are expressed as frequency and proportion. A Levene and Shapiro Wilk tests were used to know the variables distribution. To detect statistical differences in detection of periodontal bacterial DNA between groups in quantitative variables the Mann-Whitney U test and for qualitative samples Chi-square test were used. For the correlation in the use of different primers a Cohen Kappa test was used. JMP program version 9.0 (SAS Institute, Cary, NC, USA) and Stata version 11.0 (Stata Corp LP, College Station, TX, USA) were used for statistical analysis. Statistical significance was set at $\alpha = 0.05$.

RESULTS

The inter-observer and intra-observer reproducibility regarding the diagnosis of periodontitis (probing depth and attachment loss indexes) were analyzed by intraclass correlation coefficient obtaining 0.90.

The mean age of the DS patients with periodontitis was: 24.7 ± 7.7 years (30 males and 15 females). In the group without periodontitis the mean age was 21 ± 4.3 years (17 males and 13 females). In age and gender, there were no statistical differences between the two groups ($p > 0.05$).

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Table 1. Periodontal status of Down syndrome patients

Group	Probing depth* (mm)			Attachment level** (mm)			Tooth loss*** (teeth number)		
	$\bar{x}$	SD	Range	$\bar{x}$	SD	Range	$\bar{x}$	SD	Range
Without Periodontitis n=30	2.0	0.3	1.3-3.1	0.8	1.0	1.3-2.1	1.1	1.8	0-6
With Periodontitis n=45	3.6	1.0	2.1-6.3	4.1	1.1	2.5-7.4	3.0	2.4	0-10
Upper anterior teeth	3.4	1.0	1.8-6.2	4.0	1.2	2.4-7.2	0.3	0.7	0-3
Lower anterior teeth	3.3	1.3	1.5-6.8	3.9	1.4	2.1-7.9	0.1	0.7	0-5
Upper posterior teeth	3.8	0.9	2.2-6.0	4.4	1.0	2.4-6.8	1.3	1.2	0-5
Lower posterior teeth	3.5	1.0	1.4-6.4	4.1	1.1	2.1-7.5	1.3	1.0	0-4

*p<0.0001, **p<0.0001, ***p<0.0003 Mann-Whitney U test. SD: Standard deviation

Periodontal status (table 1)

The mean probing depth in the group with periodontitis was 3.6 ±1.0 mm, and the upper posterior teeth were the most affected (3.8 ± 0.9 mm). In the group without periodontitis the mean probing depth was 2.0 ± 0.3 mm. Regarding the attachment level, in the group with periodontitis the mean was 4.1 ± 1.1 mm, the upper posterior teeth were the most affected with a mean of 4.4 ± 1.0 mm, on the other hand, the group without periodontitis, showed a mean attachment level of 0.8 ±1.0 mm. In both variables there was statistical differences between groups ($p<0.05$). The frequency of tooth loss in the group without periodontitis was 1.1 ± 1.8 lost teeth and in the periodontitis group was 3.0 ± 2.4 being the posterior teeth the most frequently absent (1.3 lost teeth). There were statistical differences between groups ($p < 0.05$).

Detection of periodontal bacterial DNA by PCR assay (table 2)

PCR performed on subgingival dental plaque samples was used to assay for the presence of *P. gingivalis*, *P. gingivalis fimA genotypes*, *T. forsythia*, *T. denticola* and *A. actinomycetemcomitans*.The species most frequently detected in both groups were *T. forsythia* (95.5% and 63.3% respectively), followed by *T. denticola* (88.8%, 50%) and *P. gingivalis* (53.3%, 26.6%). A significant statistical difference between groups for these species was found ($p < 0.05$). The frequency of positive samples for *A. actinomycetemcomitans* using Aa-F and Aa-R primers, which amplify part of the 16S rRNA gene, was 75.5% in the group with periodontitis and 56.6% in the group without periodontitis, there was significant statistical differences (p=0.0455). On the other hand, the frequency obtained with ltx3/ltx4 primers which amplify part of the *ltx* promoter region, was 17.7% in the group with periodontitis and 10% in the group without periodontitis. No statistically significant difference was found between groups (p=0.3509). No one of the samples positive for *A. actinomycetemcomitans* showed presence of the JP2 clone by PCR or LAMP methods.

Detection of bacterial combinations (table 2)

In the group with periodontitis, the most frequent bacterial combination identified was the presence of the red complex (*P. gingivalis, T. forsythia*, *T. denticola*) representing 46.7% of the patients, the opposite scenario was found in the group without periodontitis where only 16.7% showed the presence of the combination of the three bacterial species of the red complex. In the group without periodontitis the most frequent combination was when the three species were absent (20%) or only *T. forsythia* was present (20%). The deepest probing depth (>4 mm) in the group with periodontitis was obtained when *A. actinomycetemcomitans* was present in the combinations. In the group without periodontitis the deepest measure was obtained when the red complex was present (2.2 mm).

Table 2. Detection of periodontal bacteria in Down syndrome patients

Species	With periodontitis	Probing Depth	Without periodontitis	Probing Depth	P*
	Frequency (%)	mean ±SD	Frequency (%)	mean ±SD	
Tannerella forsythia	43 (95.5)	3.6 ±1.0	19 (63.3)	2.2 ±0.3	0.0008
Treponema denticola	40 (88.8)	3.7 ±1.0	15 (50)	2.1 ±0.4	<.001
Porphyromonas gingivalis	24 (53.3)	3.6 ±0.9	8 (26.6)	1.9 ±0.3	0.0097
Aa (Aa-F/Aa-R primers**)	34 (75.5)	3.8 ±1.1	17 (56.6)	1.9 ±0.2	0.0455
Aa (ltx3/ltx4 primers***)	8 (17.7)	4.3 ± 1.1	3 (10)	2.0 ±1.1	0.3509
Aa JP2 clone (PCR and LAMP)	0	-	0	-	
Red complex (*Pg, Td, Tf*)	21(46.7)	3.5 ±1.0	5 (16.7)	2.2 ±0.3	
Td – Tf	10 (22.2)	3.5 ±1.0	5 (16.7)	1.9 ±0.4	
Td – Tf – Aa	6 (13.3)	4.3 ±1.2	0	-	
Tf	4 (8.9)	2.9 ±0.6	6 (20)	1.8 ±0.7	
Pg – Tf – Td – Aa	2 (4.4)	4.2 ±0.4	0	-	
Negative to all species	0	0	6 (20)	2.0 ±0.3	
Td	0	0	3 (10)	2.2 ±0.8	
Other combinations	2 (4.4)	-	5 (16.7)	-	

*Chi-square, SD: Standard deviation, *Pg: Porphyromonas gingivalis, Td: Treponema denticola, Tf: Tannerella forsythia, Aa: Aggregatibacter actinomycetemcomitans.* **Conrads et al, 1996, ***Poulsen et al, 2003

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Table 3. Genotyping of *Porphyromonas gingivalis*

Genotype	With periodontitis n=24		Without periodontitis n=8	
	Frequency (%)	PD (mm)	Frequency (%)	PD (mm)
I	18 (75%)	3.7	3 (37.5%)	2.3
Ib	3 (12.5%)	4.2	2 (25%)	2.5
II	13 (54.1%)	3.4	6 (75%)	2.3
III	2 (8.3%)	3.1	0	-
IV	4 (16.6%)	3.2	3 (37.5%)	2.0
V	0	-	0	-
Pg genotypes profiles				
I	8 (33.3%)	3.7	1 (12.5%)	2.0
Ib	1 (4.2%)	3.4	0	0
II	1 (4.2%)	2.8	2 (25%)	2.1
II-IV	2 (8.3%)	3.3	2 (25%)	2.0
II-III	1 (4.2%)	3.3	0	-
II-III- IV	1 (4.2%)	3.3	0	-
I-1b-II	1 (4.2%)	5.3	2 (25%)	2.5
I - Ib	1 (4.2%)	3.4	0	-
I -II	7 (29.2%)	2.9	0	2.3
I-IV	1 (4.2%)	3.2	0	-
IV	0	-	1 (12.5%)	1.8

*p<0.05 Chi-square test. PD: Mean of Probing depth in millimeters.

Genotyping of Porphyromonas gingivalis (table 3)

PCR was used to type the *fimA* gene in the samples positive for *P. gingivalis*. Some samples were positive for multiple *fimA* alleles indicating that various genotypes of *P. gingivalis* were present. Among the 24 (53.3%) positive samples to *P. gingivalis* in the group with periodontitis, Pg *fimA* type I represented 75%, as the most frequent genotype detected, followed by Pg *fimA* type II detected in 54.1%. On the other hand, in the group without periodontitis, from the 8 (26.6%) positive subjects to Pg, 75% were Pg *fimA* type II positive followed by Pg *fimA* type II and IV (37.5%). However the deepest probing depth in both groups was found in the presence of Pg *fimA* type Ib (4.2 mm and 2.5 mm, in groups with and without periodontitis, respectively).

The profile most frequently detected in the group with periodontitis was the presence of Pg *fimA* type I alone (33.3%). In the group without periodontitis the most frequent profiles were the presence of Pg *fimA* type II alone (25%) and the combination of Pg *fimA* type I-Ib-II and the combination II-IV (25%). The highest probing depth in both groups was detected in the combination Pg *fimA* I-Ib-II (5.3 mm in the periodontitis group, 2.5 mm in the group without periodontitis).

Sequencing of PCR products identified by different primers (table 4)

When Aa-F/Aa-R primers were used to detect *A. actinomycetemcomitans*, it was obtained a frequency of 51 (68%) positive samples in the 75 Down syndrome patients. On the other hand, the frequency of positive samples obtained with ltx3/ltx4 primers was 11 (14.6%). There was significant statistical difference (p=0.0001). There was inconsistency in 40 of 75 samples, all of them were positive with Aa-F/Aa-R primers and negative with ltx3/ltx4 primers. The Aa-F/Aa-R PCR amplicons from 10 of these samples along with 10 samples where the two PCR methods agreed were sequenced. The obtained 16S rRNA sequences were used to search in GenBank and were aligned using the BLAST program. The results demonstrated that the Aa-F/Aa-R primers were not specific for detection of *A. actinomycetemcomitans*. PCR products from samples positive with Aa-F/Aa-R primers and negative with ltx3/ltx4 showed low sequence homology with *A. actinomycetemcomitans*. In contrast, among these samples four showed 99% sequence similarity to Haemophilus genomospecies P3 oral cloneMB3_38, three showed 99% sequence similarity to uncultured bacterium clone E53-98, one showed 99% similarity to Peptostreptococcus sp. Oral clone BP1-73, and one was 98% similar to uncultured bacterium clone Ax3_313. On the other hand, the sequences of the amplicons from samples positive in the ltx3/ltx4 PCR were identical to those of *A. actinomycetemcomitans*. Thus, the 40 samples that showed inconsistency between the two primer sets used for detection of *A. actinomycetemcomitans* were considered false positives. Statistical analysis for detection of the correlation between both primers showed a weak correlation (Cohen Kappa= 0.16).

DISCUSSION

Down syndrome is a genetic alteration with clinical features such as oral, facial, cardiovascular and immunological disturbances. Periodontal disease has a high prevalence in early stages in DS patients. It affects about 92% of young population and 96% before 30 years.[23,24] This fact have been associated with some factors such as immunological deficiency, poor oral hygiene, fragile periodontal tissue, early senescence, poor masticatory function and mouth

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Table 4. Correlation between different primers for detection of Aa.

Primers	Frequency (%) n=75		P*
Aa-F/Aa-R	51 (68%)		0.0001
ltx3/ltx4	11 (14.6%)		
Correlation **	0.16		
Sequencing with Big Dye method, Alignment with BLAST program			
	Aa-F/Aa-R PCR products		
Sample	**Species*** **	**Access number**	**Homology**
1	Peptostreptococcus sp. Oral clone BP1-73 gene for 16S ribosomal RNA	>dbj|AB121857.1	99%
2,4,5,9	Haemophilus genomosp. P3 oral clone MB3_C38 16S ribosomal RNA gene	>gb|DQ003635.1|	99%
3,7,8	Uncultured bacterium clone E53-98 16S ribosomal RNA gene	>gb|DQ639550.1|	99%
6	Uncultured bacterium clone Ax3_313 16S small subunit ribosomal RNA gene.	>gb|EU762966.1|	98%
	ltx3 and ltx4 PCR products		
11-20	Uncultured actinobacillus sp. Partial 16SrRNA gen, clone 603007 (oral)	>emb|AM420240.1|	100%
	Actinobacillus actinomycetemcomitans strain ATCC 43718 16S ribosomal RNA gene	>gb|AY362884.1|	100%
	Actinobacillus actinomycetemcomitans 16S small subunit ribosomal RNA gen	>gb|M75035,1|ACN RRNAN	100%

*Chi-square test **Cohen Kappa ***Species with the best homology

breathing but the microbiological aspect have been not explored extensively. *A. actinomycetemcomitans* has been considered as the main species responsible of aggressive periodontitis and the pattern of periodontitis observed in DS patients is very similar to this type of disease but in the classification of periodontal disease from AAP (1999).[25] The condition affecting DS subjects is classified as periodontitis associated with systemic diseases. The 75 DS patients with (45) and without (30) periodontitis included in the present study had a mean age of 24.7 and 21 years old respectively and the most of subjects studied were males, (66.6% and 56.6% respectively), this finding is in accordance with the suggestion that DS affects more males than females with a gender ratio of about 1.42.[26]

Regarding periodontal status, in the group without periodontitis the mean probing depth was 2.0 mm, and the attachment level was 0.8 mm, this finding is in accordance with healthy periodontal tissues parameters. On the other side, in the group with periodontitis the mean probing depth was 3.6 mm for all tooth surfaces but when the deepest pocket of each tooth was measured, the mean was 4.35 mm. It has been reported a pocket depth of 6 mm but the mean age of the patients included was 38 years old, the difference in age of samples could explain the difference.[24] The most affected teeth were the upper molars, they showed the highest pocket depth (3.8 mm), highest attachment loss (4.4 mm) and they were the most frequently absent teeth (1.35 lost teeth).

In respect of the composition of periodontal biofilm in the group with and without periodontitis, the bacterial species most frequently detected was T. forsythia (95.5%, 63.3%), followed by *T. denticola* (88.8%, 50%) and *P. gingivalis* (53.3%, 26.6% respectively); these bacteria are associated with chronic periodontitis. Since these three bacterial species of the red complex showed statistical difference between groups, these findings indicate that the presence of these species are determinant for periodontal disease development in accordance with a previous report in DS subjects at the same group of age.[6] They obtained a percentage of detection of 89.7%, for *T. forsythia*, 53.8% for *T. denticola* and 76.9% for *P. gingivalis*. The sets of primers used in this study were the same except for *T. denticola*.

For detection of *A. actinomycetemcomitans*, the main periodontal pathogen associated with aggressive periodontitis, two different sets of primers were used, Aa-F/Aa-R and ltx3/ltx4.[20,21,26] There are studies in healthy and DS patients reporting different frequencies of detection of *A. actinomycetemcomitans* with PCR from 17 to 82%.[6,11,28] In the present study, the frequency obtained with Aa-F and Aa-R was 75.5% in the group with periodontitis, and 56.6% in the group without periodontitis, there was statistical difference between groups. This finding is similar to *A. actinomycetemcomitans* frequency (82.1%) in a previous report in a group of DS patients with periodontitis with similar age (25.4 years old) and using the same set of primers.[6] However when ltx3 and ltx4 primers were used, the positive samples to *A. actinomycetemcomitans* in the group with periodontitis were 17.7% and 10% in the group without periodontitis; there was not statistical difference.

There was an inconsistency in 40 of the 75 samples showing a weak correlation between both set of primers. Therefore, it was decided to sequence the PCR products from 10 samples. There was not homology with *A. actinomycetemcomitans* genome when Aa-F and Aa-R primers were used, 4 samples had a 99% homology with Haemophilus species, 4 samples corresponded to uncultured bacteria, and 1 showed homology with Peptostreptococcus species. On the other side, the PCR products obtained with ltx3 and ltx4 for the 10 samples had a 100% homology with *Actinobacillus actinomycetemcomitans* genome (now named *Aggregatibacter actinomycetemcomitans*). Therefore we could suggest that ltx3/ltx4 are more specific to detect *A. actinomycetemcomitans* than Aa-R/Aa-R. This finding explains the difference of isolation frequency in the different clinical studies reported.[6,11]

The presence of JP2 clone of *A. actinomycetemcomitans* was also investigated, it is a member of a particular clonal lineage that

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shows a 530 bp deletion in the promoter region of the ltx operon resulting in enhanced production of leucotoxin, the main virulence factor of *A. actinomycetemcomitans*. Therefore, JP2 clone is very aggressive, it has been isolated only from patients with aggressive periodontitis of West and Northwest African descent, including both Africans and Arabs.[8] Since the periodontitis affects to DS patients and had similar clinical parameters to aggressive periodontitis, the identification of JP2 clone was included in this study. LAMP method is very sensitive, simple and low-technology method for detection of JP2 clone of *A. actinomycetemcomitans*; however we did not obtained any positive sample by both PCR and LAMP methods.

The most frequent combination in the group with periodontitis was the presence of the three species of red complex (*P. gingivalis*, *T. forsythia*, *T. denticola)*, the opposite scenario was found in the group without periodontitis where the three species (20%) of the red complex were absent or only the presence of *T. forsythia* (20%) was detected. In the group without periodontitis the deepest measure was obtained only when the red complex was present (2.23 mm), confirming that the red complex is the main bacterial group involved in periodontal disease development in DS patients. When the frequency of *P. gingivalis* and *A. actinomycetemcomitans* were compared, there were statistical differences ($p<0.05$). This finding could suggest that *P. gingivalis* have a more important role in the initial development of periodontitis in DS patients than *A. actinomycetemcomitans*. It is important to notice that the deepest probing depth (>4 mm) in the group with periodontitis was obtained when *A. actinomycetemcomitans* was present in the combination, therefore *A. actinomycetemcomitans* could be associated with periodontitis severity.

There are six different genotypes of *P. gingivalis* based in *fimA* gen (I-V and Ib), from the 24 (53.3%) positive Down syndrome subjects to *P. gingivalis* in the group with periodontitis: *Pg fimA* type I (75%), was the most frequent genotype detected, followed by Pg *fimA* type II. On the other hand in the group without periodontitis, 8 (26.6%) subjects were positive to Pg, most of the samples (75%) were positive to Pg *fimA* type II. These results were different to data obtained in a previous study where Pg *fimA* II was detected in 46% of DS patients with periodontitis and in 14.3% of without periodontitis DS patients.[6] The sample size of positive patients to Pg mainly in the group without periodontitis was small. Some reports had affirmed that Pg *fimA* type II is closely associated with periodontitis development and Pg *fimA* type I with healthy periodontal tissues; However, in this study Pg *fimA* type I was the most prevalent genotype in periodontitis samples, which suggest that the impairment of the immune system play a role in the severity of DS periodontitis. Similar findings were found in patients affected by Diabetes Mellitus.[14]

In combination, the profile most frequently detected in the group with periodontitis was the presence of Pg *fimA* type I alone (33.3%) and the combination of types I-II (29.2%) on the other side, in the group without periodontitis were combinations includind Pg *fimA* type II. The deepest probing depth in both groups was detected in the combination Pg *fimA* I-Ib-II (5.3 mm in the periodontitis group, 2.5 mm in the group without periodontitis).

CONCLUSIONS

Results suggest that the composition of oral biofilm is fundamental for the development of periodontal disease independently of immunological alterations associated with Down syndrome, because there were statistical differences in the three species that conform the periodontal bacterial red complex. In spite of the clinical pattern of periodontitis in DS patients is similar to the aggressive type, *Porphyromonas gingivalis* more closely related with periodontitis than *A. actinomycetemcomitans*, but the presence of *A.actinomycetemcomitans* could be associated with higher severity of the disease. JP2 clone of *A. actinomycetemcomitans* was not present in this group of Mexican DS patients. The frequency of detection of *A. actinomycetemcomitans* reported in the literature has a wide range, because the method used, specially the primers and probes applied when *A. actinomycetemcomitans* is identified by PCR and other molecular assays

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