Anaerobe 52 (2018) 50–58

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Clinical microbiology

Qualitative, quantitative and genotypic evaluation of *Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum* isolated from individuals with different periodontal clinical conditions

Viviane Aparecida Arenas Rodrigues [a, 1], Erica Dorigatti de Avila [b, c, 1], Viviane Nakano [a], Mario Julio Avila-Campos [a, *]

[a] *Anaerobe Laboratory, Department of Microbiology, Institute of Biomedical Sciences, University of Sao Paulo - USP, Av. Prof. Lineu Prestes, 1374, Sao Paulo, SP, Brazil*
[b] *Postdoctoral Research Fellow, Department of Dental Materials and Prosthodontics, School of Dentistry of Araraquara, Sao Paulo State University - UNESP, Rua Humaita, 1680, Araraquara, SP, Brazil*
[c] *Department of Biomaterials, Radboud University Medical Center, Philips van Leydenlaan 25, Nijmegen, the Netherlands*

A R T I C L E I N F O

Article history:
Received 24 December 2017
Received in revised form
26 April 2018
Accepted 28 May 2018
Available online 29 May 2018

Handling Editor: Elisabeth Nagy

Keywords:
Aggregatibacter actinomycetemcomitans
Fusobacterium nucleatum
Oral microbiology
Periodontal disease
Bacterial typing

A B S T R A C T

Aggregatibacter actinomycetemcomitans and *Fusobacterium nucleatum* are strongly associated with periodontitis, and their evaluations are relevant to understand their role in the etiology and progression of periodontal diseases. In this study, the qualitative and quantitative detection of *A. actinomycetemcomitans* and *F. nucleatum,* as well as their genetic diversity, were evaluated in individuals with gingivitis, chronic periodontitis and periodontally healthy. In addition, the biotyping, serotyping, and prevalence of the *ltx* and *cdt* genes in *A. actinomycetemcomitans* were also determined. Subgingival biofilms obtained from gingivitis (70), periodontitis (75) and healthy (95) individuals were analyzed by cultures and PCR. Bacterial typing and presence of *ltx* and *cdt* genes in *A. actinomycetemcomitans* were also verified. DNA from *A. actinomycetemcomitans* and *F. nucleatum* was detected respectively, in 65.7% and 57.1% of gingivitis, 80% and 68% of periodontitis, and 57.8% and 37.8% of healthy. *A. actinomycetemcomitans* from gingivitis were biotypes I, II, IV, V, and X, and serotypes a, c, and e. In periodontitis, biotypes II, VI, and X, and serotypes a, b, and c were found. In healthy subjects, biotypes II and X, and serotypes b and c were found. The LTX and *ltxA* were observed in strains from gingivitis and periodontitis pockets. Subsequently, our data also showed no direct relationship between *ltxA* gene expression and leukotoxin gene 530-bp presence. On the other hand, *cdt* gene predominated during the inflammatory disease process. Our results strongly support a role of *A. actinomycetemcomitans* and *F. nucleatum* in advanced stage of periodontal disease.

1. Introduction

Periodontal disease expression involves intrinsic interactions of the biofilm with the host's immune inflammatory response and subsequent alterations in bone and connective tissue homeostasis [1,2]. Although this complex disease is dependent on contexts, such as age [3], oral hygiene habits [4], and acquired behavioral factors, periodontal bacteria are considered to be the most important etiologic factors that are able to initiate the inflammatory response [5].

Among the oral pathogenic bacteria, *Aggregatibacter actinomycetemcomitans*, a Gram-negative bacterium, is particularly known to be involved with aggressive and chronic periodontal disease [5–9]. The drastic inflammatory response is a

* Corresponding author. Anaerobe Laboratory, Department of Microbiology, University of Sao Paulo - USP, Av. Prof. Lineu Prestes 1374, 05508-900, São Paulo, SP, Brazil.
E-mail address: mariojac@usp.br (M.J. Avila-Campos).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.anaerobe.2018.05.015
1075-9964/

consequence of interactions between virulence factors from *A. actinomycetemcomitans* and the host's cells, mainly, fibroblasts and epithelium cells. To this date, seven serotypes of *A. actinomycetemcomitans* were identified [10], of which, serotypes a, b and c are detected most frequently in periodontal sites [11,12]. Interestingly, in a recent report, it was found that there is a high prevalence of strains entirely lacking serotype b of *A. actinomycetemcomitans* from deep pockets with progressive periodontitis [13], contradicting previous outcomes. These findings suggest a potential difference in subgingival colonization or the antigenic composition of these pathogens depending on the geographical regions from different individuals populations [14]. Among the proteins expressed by *A. actinomycetemcomitans,* leukotoxin is considered to be the major toxic virulent factor [15]. Its participation in periodontal disease progression is due to the capacity to activate and intensify bone resorption by caspase-1 stimulation in human monocytes/macrophages. Although all *A. actinomycetemcomitans* strains display the *ltx* gene, which is responsible to codify leukotoxin, variances in the *ltx* region might affect the protein expression among individual strains [16]. Another virulence factor involved in the pathogenesis of periodontal diseases is cytolethal-distending toxins (CDTs) [17]. This protein targets a wide spectrum of eukaryotic cells, including fibroblasts and T lymphocytes [18], and induces RANK L expression to further promote osteoclastogenesis. Similar to leukotoxin, the CDT expression can also vary among strains of *A. actinomycetemcomitans.* Furthermore, the presence of *cdt* genes may explain why some individuals are carriers and why there is an increased prevalence of *A. actinomycetemcomitans* associated with periodontitis depending on the geographical localization.

Another important Gram-negative bacterium involved with periodontal disease pathogenesis is *Fusobacterium nucleatum* [19,20]. This bacterial species is known to coaggregate with early and late colonizers by its proteins and receptors available in the out membrane, therefore contributing to periodontal disease progression [21]. In this context, the adhesins RadD and Fap2 were identified as two important virulence factors [22] that have binding properties for Gram-positive species and *Porphyromonas gingivalis*, respectively. Studies have shown that *F. nucleatum,* an anaerobic bacterium, is able to survive in high proportions of oxygen. This particular property may explain why fusobacteria are found in both healthy and diseased sites. Thus, *F. nucleatum* can have an important indirect role in the etiology of periodontal diseases by supporting the growth of some pathogenic bacteria, such as *P. gingivalis*, and possibly other oral anaerobes in oxygenated and CO_2-depleted environments [23]. The multiple roles and complexity of *F. nucleatum* delineate it as an important microorganism involved in periodontal disease pathogenesis.

Because *A. actinomycetemcomitans* and *F. nucleatum* produce endogenous infections and are strongly associated with periodontitis, their evaluations are relevant to understand their role in the etiology, development and progression of periodontal diseases. Therefore, the purpose of this study was to detect *A. actinomycetemcomitans* and *F. nucleatum,* as well as their genetic diversity, in Brazilian individuals with gingivitis, chronic periodontitis and periodontally healthy subjects. In addition, the biotyping, serotyping, and prevalence of the *ltx* and *cdt* genes in *A. actinomycetemcomitans* were also determined. The null hypothesis established here is that periodontal conditions are related with the prevalence of *A. actinomycetemcomitans* and *F. nucleatum*. In an attempt to help understand carriage and disease processes, such microbiological data may also contribute to better microbial characterization and add the knowledge of the global distribution of both pathogenic bacteria.

2. Materials and methods

2.1. Study design

A cross-sectional study was performed in the Clinic of Periodontology, Dental School, at the University of Sao Paulo. Two hundred forty individuals between 20- and 60-years-old with at least 14 teeth were included. On the other hand, as exclusion criteria, this study did not select individuals who received antibiotic prophylaxis within the last 6 months before analysis for dental examination, had uncontrolled systemic diseases, were immunologically compromised, had prior periodontal treatment, were diagnosed with gingivitis or chronic periodontitis three months before the beginning of the study, were smokers, had edentulous or had prosthetics.

All individuals were informed about the study design and procedures and signed informed consent forms. The ethical principles were conducted consistent with the Declaration of Helsinki and the design was approved by the Ethics in Human Research Committee of the Institute of Biomedical Sciences (Process No. 910/CEP).

2.2. Selected population and periodontal examination

The individuals were assigned to three groups. Group I (control): 95 subjects with a healthy periodontium, attached gingivae dotted with a pink coral color, no bleeding on probing, and a probing depth ≤ 3 mm; Group II: 70 individuals displaying clinical signs of gingivitis with gingival inflammation, light red erythema, loss of dotted surface (change in the texture of the gum surface), gingival recession without alveolar bone loss and a probing depth ≤ 3 mm; and Group III: 75 individuals displaying chronic periodontitis with clinical and radiographic signs of bone loss, mild or moderately swollen gums, flat or shaped craters papillae, gingival spontaneous bleeding by probing, change of the gingival staining, tooth mobility and a probing depth ≥ 5 mm.

A single trained examiner performed periodontal examination. The probing depth and gingival recession were measured and used to determine the clinical attachment level. The measures were made from six sites per tooth (mesio-vestibular, vestibular, disto-vestibular, meso-lingual, lingual and disto-lingual), excluding the third molars. The presence of supragingival biofilm and bleeding on probing were also recorded using the visible plaque and gingival plaque index, respectively.

2.3. Sample collection, bacterial isolation and DNA extraction

Prior to subgingival sampling, the supragingival biofilm was removed from the surfaces of the affected teeth with curettes. Mesio-buccal root of maxillary first permanent molars or central incisors were selected from healthy and subjects with gingivitis. Subgingival samples were collected from the deepest pockets of individuals with chronic periodontal disease. Then, two sterile paper points (No. 30-Dentsply, Ind Co Ltd, RJ, Brazil) were placed on the apical portion of the periodontal pocket or gingival crevice for 60 s and were then transported immediately to the laboratory in VMGA III medium [24]. Each clinical sample was washed twice with sterile Milli-Q water at 12,000 rpm for 10 min and the pellet re-suspended in Milli-Q water [25].

Subgingival samples for assessment of genotype evaluation were dispersed by vortex, and aliquots of 0.1 mL from pre-established dilutions (10^{-1} to 10^{-5}) were plated in duplicate onto selective trypticase soy-serum-bacitracin-vancomycin agar prior to further processing. After 72 h of incubation in anaerobic conditions (90% N_2 + 10% CO_2) at 37 °C, colonies of *A. actinomycetemcomitans* and *F. nucleatum* were identified using different approaches:

stereoscopic microscope, Gram staining, catalase production and fermentation of carbohydrates according to standardized methods [26,27].

Total bacterial DNA from subgingival samples was isolated using a phenol-chloroform method [30] and stored at −80 °C until use. The concentration of DNA was determined with a UV spectrophotometer ($A_{260} = 1.8$, and $A_{280\ nm} = 2.0$). Then, the DNA integrity was confirmed by loading 10 μL of each DNA sample in a 1% agarose gel before use.

2.4. Biotyping and serotyping of A. actinomycetemcomitans strains

The differentiable physiologic characteristics of *A. actinomycetemcomitans* were evaluated for biotyping based on the fermentation of dextrin, maltose, mannitol, and xylose [27]. The distinct variation of serotypes within bacterial species was determined by multiplex PCR with specific primers (Supplemental data - Table 1) [28]. Briefly, cycling conditions were programmed for 1 cycle at 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 52 °C for 1 min and 72 °C for 1 min. After the last amplification samples were incubated at 72 °C for 5 min to fill in the protruding ends of newly synthesized PCR products. DNA from *A. actinomycetemcomitans* ATCC 29523 (serotype a), *A. actinomycetemcomitans* (serotype b), *A. actinomycetemcomitans* ATCC 33384 (serotype c), and *A. actinomycetemcomitans* CU 1000 (serotype f) were used as positive controls. The specific segments of DNA were analyzed using electrophoresis in 1% agarose gels, and images were digitally recorded using the Molecular Imager Gel Documentation system (Kodak Digital System DC-120).

2.5. Detection of the ltx and cdt genes in A. actinomycetemcomitans

PCR assays were performed using specific primers (Supplemental data - Table 1) [6,14,17, 29]. Amplifications were performed in volumes of 25 μL containing 10 X PCR buffer, 50 mM $MgCl_2$, 0.2 mM dNTP, 0.4 μM each primer, 0.5 U Platinum *Taq* DNA polymerase, and 1 ng DNA. The thermocycler was programmed for 94 °C (5 min) followed by 30 cycles of 94 °C (1 min), 50 °C–65 °C (1 min), 72 °C (1 min), and then 72 °C (5 min) to allow DNA extension. Ultrapure water instead of DNA was used as a negative control. PCR products were analyzed as mentioned above.

2.6. Bacterial detection by PCR

The PCR methodology used here was carried out as described in a previously published protocol [30]. The specific primers (Supplemental data - Tables 1 and 2) [6,14,17, 28–31] were used for PCR amplification of each bacterial species (*A. actinomycetemcomitans* and *F. nucleatum*). The final concentrations for a 25-μl PCR reaction are described in item 2.5. The thermal cycling conditions were programmed to 1 cycle of 94 °C (5 min) and 30 cycles of 94 °C (30 s), 56 °C (30 s) and 72 °C (30 s) for *A. actinomycetemcomitans*, and for *F. nucleatum*, 1 cycle of 94 °C (5 min) and 30 cycles of 94 °C (30 s), 40 °C (30 s) and 72 °C (30 s); and 1 cycle of 72 °C (5 min) for final extension of the DNA were used. DNA from reference strains of *A. actinomycetemcomitans* ATCC 29523 and *F. nucleatum* ATCC 25586 served as positive controls while an ultrapure water template was used as background controls. The specific segments of DNA were analyzed by electrophoresis in 1% agarose gels, as mentioned above.

2.7. Quantitative bacterial detection by qPCR assays

Initially, standard curves were generated by diluting the template control into a ten-fold dilution series. The amplification efficiency of the qPCR was based on the standard curve of distinct reference strains: *A. actinomycetemcomitans* ATCC 29523, *F. nucleatum* ATCC 25586, *Actinomyces israelii* ATCC 12102, *Dialister pneumosintes* ATCC 33048, *Enterococcus faecalis* ATCC 29212, *Porphyromonas gingivalis* ATCC 33277, *Porphyromonas endodontalis* ATCC 35406, *Prevotella intermedia* ATCC 25611, *Prevotella nigrescens* ATCC 33563, *Tannerella forsythia* ATCC 43037, and *Treponema denticola* ATCC 35405. The slope of the DNA standard curves was calculated by linear regression for each dilution using a system program Rotor Gene 6000 (Rotor Gene Series Software 1.7). The reaction efficiency was indicated by genomic copies of each bacterial species. Briefly, DNA quantification was determined using a SYBR Green system in a total of 20 μL, including 2X PCR Master Mix (Promega, Madison, USA) and 100 mM species-specific primers (Supplemental data - Table 3) [32–38]. The thermal cycler was programmed as follows for *A. actinomycetemcomitans*: 1 cycle of 95 °C (2 min), 40 cycles of 95 °C (45 s), 60 °C (2 min) and 56 °C (2 min) to *F. nucleatum*. For the other bacteria, we used annealing temperatures of 60 °C (2 min) for *P. gingivalis*, *P. endodontalis*, *T. forsythia* and *E. faecalis*; 55 °C (2 min) for *P. intermedia* and *D. pneumosintes*; 56 °C (2 min) for *P. nigrescens*; 59 °C (2 min) for *T. denticola*; and 50 °C (2 min) for *A. israelii*.

All amplification reactions were performed in triplicate in a thermal cycler Rotor Gene 6000 (Corbett Life Science, Mortlake, New South Wales, Australia). A dissociation curve (melting curve) was obtained to determine the primer specificity. A Master Mix without DNA served as a negative control.

Table 1
Demographic data of patients with gingivitis, chronic periodontitis and healthy. $p < 0.001$.

Characteristics	Patients (No.)		
	Gingivitis (70)	Chronic periodontitis (75)	Healthy (95)
Sex (M/F)	26/44	38/37	33/62
Age in years Mean ± SD (Median)	37.0 ± 13.6 (34.5)	46.5 ± 9.0 (47.0)	29.9 ± 9.7 (27.0)
Alcohol consumption (%)	2 (2.9)	1 (1.3)	1 (1.1)
Tobacco consumption (%)	3 (4.3)	2 (2.67)	0 (0.0)
Probing depth (PD) Mean ± SD (Median)	2.9 ± 0.3 (3.0)	5.9 ± 0.8 (6.0)	1.7 ± 0.6 (2.0)
Visible plaque sites (%)	41.75 ± 11.70	46.21 ± 10.20	20.40 ± 11.02
Bleeding on probing	41.30 ± 2.20	35.78 ± 0.53	11.40 ± 1.22
Bone loss	42.8 ± 2.33	40.05 ± 2.03	0.85 ± 0.23
Tooth mobility	20.02 ± 1.56	27.51 ± 1.11	1.01 ± 0.13
Gingival edema	39.34 ± 2.41	37.53 ± 1.15	8.22 ± 2.01

M/F: Male/Female. PD: Probing depth (in mm).
SD: Standard deviation.
Kruskal-Wallis test: $p < 0.05$ values were considered statistically significant.

Table 2
Prevalence of *Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum* in patients with gingivitis, chronic periodontitis and healthy. Values of $P < 0.05$ were considered statistically significant values by the Kruskal-Wallis test.

Bacteria	Clinical group (No.)	Culture		Conventional PCR		Real-time PCR		*p values*
		Positives samples		Positives samples		Positive samples		
		No.	%	No.	%	No.	%	
Aggregatibacter actinomycetemcomitans	Gingivitis (70)	2	2.8	46	65.7	50	71.4	
	Periodontitis (75)	4	5.3	60	80.0	69	92.0	<0.002
	Healthy (95)	5	5.26	55	57.8	62	65.2	
Fusobacterium nucleatum	Gingivitis (70)	13	18.6[b]	40	57.1	52	74.2	
	Periodontitis (75)	20	26.6[b]	51	68.0	68	90.6	<0.001
	Healthy (95)	19	20[b]	36	37.8	49	51.5	

Aggregatibacter actinomycetemcomitans = [a]Gingivitis ≠ Healthy and Periodontitis ≠ Healthy; *Fusobacterium nucleatum* = [b]Gingivitis ≠ Healthy and Periodontitis ≠ Healthy.

2.8. Typing A. actinomycetemcomitans and F. nucleatum by arbitrarily primed AP-PCR

Five arbitrary primers (Supplemental data - Table 1) were used for bacterial typing. DNA amplifications were performed in final volumes of 25 μL, as described in item 2.5. Thermal cycling parameters were initiated with a 5-min denaturation at 94 °C, followed by 30 cycles of 94 °C for 1 min, 34 °C for 1 min, 72 °C for 2 min and 1 cycle of 72 °C for 5 min to final extension of the DNA. PCR amplification products were evaluated eletrophoretically on 1% agarose gels. Profile images were digitally recorded using the Molecular Imager Gel Documentation system (Kodak Digital System DC-120). The gel analysis was based on the similarity of the amplitypes generated from *A. actinomycetemcomitans* and *F. nucleatum* from different origins. Templates without DNA served as negative controls and were included in each AP-PCR.

2.9. Statistical analyses

The statistical analyses were performed by using Chi-squared and Kruskal-Wallis tests. Dendrograms were constructed by using the NTSYS software (Applied Biostatistics, Inc. Version 1.7).

3. Results

3.1. Clinical findings

A total of 240 subjects participated in this investigation. The general parameters of the individuals who were evaluated according to the criteria established are shown in Table 1. A positive relationship between age and pocket depth ($p < 0.001$) was observed, but the genera did not influence periodontal disease stage ($p = 0.409$).

For culture assay, *Aggregatibacter actinomycetemcomitans* was isolated in 2 (2.8%) individuals with gingivitis, 4 (5.3%) with chronic periodontitis and 5 (5.26%) healthy individuals; and *F. nucleatum* in 13 (18.6%) of individuals with gingivitis, 20 (26.6%) with chronic periodontitis and 19 (20%) healthy. Both microorganisms were found in association in 5 (7.1%) individuals with gingivitis, 9 (12%) with chronic periodontitis and 3 (3.15%) healthy individuals.

3.2. Relationship between A. actinomycetemcomitans/F. nucleatum and periodontal disease stage

To investigate the relationship between *A. actinomycetemcomitans/F. nucleatum* and clinical parameters, subgingival biofilm samples were assessed from the distinct groups. To ensure the quality of the data, we used two different methods to detect the presence of both microorganisms. Both culture and PCR assessment were not consistent in showing the prevalence of bacteria depending on the disease stage, with the exception to *F. nucleatum* in healthy individuals. Using PCR, *A. actinomycetemcomitans* appeared more prevalently in gingivitis and periodontitis groups and *F. nucleatum* did not exhibit any relationship with the depth of the pockets. However, considering a co-dependent frame, both bacteria were prevalent in chronic periodontitis individuals (Tables 1 and 2).

In a second set of experiments, we obtained quantitative measurements of *A. actinomycetemcomitans* and *F. nucleatum* from all of groups. As expected, the proportion of both pathogenic bacteria increased significantly in samples from gingivitis and chronic periodontitis individuals compared with healthy individuals (Table 2).

3.3. Relation between biotyping and serotyping of A. actinomycetemcomitans

A summary of results for biotyping and serotyping of *A. actinomycetemcomitans* from samples is presented in Table 3. Considering that different serotypes of *A. actinomycetemcomitans* may influence its ability of co-aggregation with *F. nucleatum*, we identified the biotypes and serotypes expressed by *A. actinomycetemcomitans* strains isolated from all individuals. Although the biotypes II and X and serotype c were observed in all groups, only strains expressing biotype II and serotype c were detected from the deeper pockets (Table 3; Fig. 1).

Further detailed analysis determined the presence of the toxicity gene in *A. actinomycetemcomitans* isolated from individuals with different periodontal conditions (Fig. 2). LTX promoter and the *ltxA* gene were observed in 100% of the strains from periodontal individuals. Comparably, highly leukotoxic *A. actinomycetemcomitans* strains were detected in 82.8% of individuals with chronic periodontitis and minimally leukotoxic strains in 83% of healthy periodontal individuals. Significant percentages of *cdtA*, *cdtB* and *cdtABC* genes were found in individuals with gingivitis and chronic periodontitis.

3.4. Bacterial quantification

The possible bacterial associations among other potential periodontopathogens were determined. Table 4 shows the presence of most evaluated bacteria and the predominance of *P. intermedia* and *P. gingivalis* varying from 0 to 6.2×10^5 copies in the three individual groups. *T. forsythia*, *T. denticola*, *P. nigrescens*, *D. pneumosintes*, *P. endodontalis*, *E. faecalis* and *A. israelii* were detected in ranges from 0 to 28.2×10^4 copies.

Table 3
Distribution of genetic groups, leukotoxin genes, biotypes and serotypes of 70 *Aggregatibacter actinomycetemcomitans* isolated from patients with gingivitis (70), chronic periodontitis (75), and healthy individuals (95).

Clinical group (No)	Isolates groups (No)	Genetic groups (No)	Genes								Biotypes						Serotypes		
			530 bp	1022 bp	*ltxA*	*cdtA*	*cdtB*	*cdtC*	*cdtABC*	I	II[a]	IV	V	VI	X	a	b	c[a]	e
23	20	23	10	13	15	21	19	23	9	2 (9%)	3 (13%)	2 (9%)	3 (13%)	0 (0%)	13 (56%)	2 (8.7%)	0 (0%)	11 (47.8%)	10
35	20	35	29	6	35	25	23	27	26	0 (0%)	14 (40%)	0 (0%)	0 (0%)	1 (3%)	20 (57%)	2 (6%)	5 (14%)	28 (80%)	0 (0%)
12	11	11	1	10	11	1	3	3	3	0 (0%)	7 (58%)	0 (0%)	0 (0%)	0 (0%)	5 (42%)	0 (0%)	2 (17%)	10 (83%)	0 (0%)

Value of $p < 0.05$ was considered statistically significant by Chi-square test (χ^2).
[a]Gingivitis ≠ Chronic periodontitis and Gingivitis ≠ Healthy; $P < 0.05$ relative to biotype (II) and serotype (c). The biotypes III, VII, VIII an IX, and the serotype d and f, were not detected.

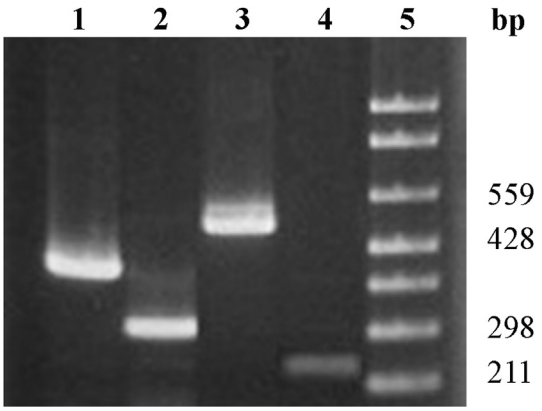

Fig. 1. Serotyping detection of *Aggregatibacter actinomycetemcomitans* by multiplex PCR. Lane 1. serotype A (individuals with gingivitis); Lane 2. serotype B (individuals with periodontitis); Lane 3. serotype C (individuals with periodontitis), and Lane 4. serotype E (individuals with gingivitis). Lane 5: 1 Kb Plus ladder (Invitrogen).

3.5. Differences crossed from A. actinomycetemcomitans and F. nucleatum independent strains

To obtain an overall estimation of bacterial genetic diversity within the clinical samples, AP-PCR was performed with oligonucleotide primers (OPA-3). The phylogenetic variations were clustered according to their similarity, as illustrated in the dendrograms. Consistent with previous data, most *A. actinomycetemcomitans* and *F. nucleatum* genetic variations were identified in individuals with gingivitis and chronic periodontitis. The AP-PCR method recorded 23 and 35 strains of *A. actinomycetemcomitans* in 7 and 13 clinical isolates from gingivitis and periodontitis sites, respectively (Supplemental data - Figs. 1 and 2). Of the total strains found, only 12 were detected in healthy individuals (Supplemental data - Fig. 3) and were grouped in 11 genetic groups with 75% of similarity.

For *F. nucleatum*, the OPA-5 primer identified 51 independent strains from 18 individuals with gingivitis, forming 9 genetic groups (78% of similarity; Supplemental Fig. 4). The genetic variation was even higher in the chronic periodontitis group; recognizing 62 strains grouped in 20 genetic groups (86% of similarity) from 29 individuals (Supplemental data - Fig. 5). Similar to *A. actinomycetemcomitans*, the frequencies of *F. nucleatum* strains detection was lower in healthy individuals than the other groups. Among the 40 strains identified, 16 genetic groups were formed with 85% of similarity (Supplemental data - Fig. 6).

4. Discussion

Periodontal disease constitutes a severe oral health problem worldwide [39]. Studies have reported that periodontal infections are not simply caused by biofilm accumulation on teeth surfaces but also by the interaction among several oral microbial community partners [40]. In this investigation, we extended our previous study [41–43] by providing scientific evidence about the genetic diversity of two pathogenic bacteria and their relationship to periodontitis aggressiveness. Similarly to previous outcomes [13, 44 , 45], quantitative analyses between *A. actinomycetemcomitans*/*F. nucleatum* and deeper probed cases showed significant differences in subjects with gingivitis and chronic periodontitis, compared to healthy subjects. Considering the ability of *A. actinomycetemcomitans* strains to secrete toxins involved in the

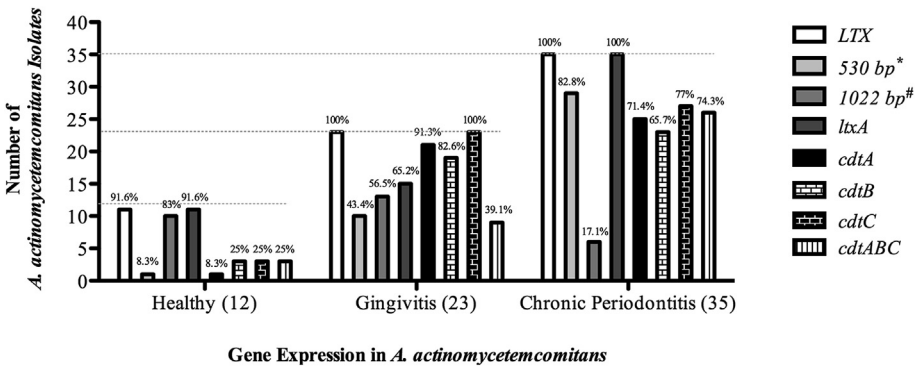

Fig. 2. Prevalence of toxicity genes in 70 *Aggregatibacter actinomycetemcomitans* isolated from individuals with different periodontal conditions. 530 bp*: presence of 530-bp in the LTX gene operon. 1022 bp#: presence of 1022-bp in the LTX gene operon.

Table 4
Bacterial quantitative determination (qPCR) in patients with gingivitis, chronic periodontitis and healthy individuals.

Bacteria	Clinical group (No.)	Real-time PCR	Number of copies (DNA/μL)		*p* values
		Positive samples (%)	Range of values	Median [interquartile deviation]	
Porphyromonas gingivalis	Gingivitis (70)	68 97.1	0–4.9×10^5	2.9×10^1 [1.3×10^2]	<0.001*
	Periodontitis (75)	74 98.6	0–5.5×10^5	6.7×10^3 [6.7×10^3]	
	Healthy (95)	35 36.8	0–4×10^5	1.1×10^1 [4.6×10^1]	
Aggregatibacter actinomycetemcomitans	Gingivitis (70)	50 71.4	0–1.14×10^8	2.2×10^2 [2.5×10^3][a]	<0.002*
	Periodontitis (75)	69 92.0	0–1.08×10^8	1.5×10^5 [9.9×10^5][a]	
	Healthy (95)	62 65.2	0–9.43×10^4	0.6×10^1 [1.1×10^2]	
Fusobacterium nucleatum	Gingivitis (70)	52 74.2	0–1.86×10^6	7.3×10^1 [2.2×10^3][b]	<0.001*
	Periodontitis (75)	68 90.6	0–3.98×10^6	4.0×10^2 [3.8×10^4][b]	
	Healthy (95)	49 51.5	0–2.63×10^5	0.0 [1.8×10^2]	
Porphyromonas endodontalis	Gingivitis (70)	13 18.5	0–12.2×10^4	0.0 [0.0]	<0.05
	Periodontitis (75)	21 28.0	0–12.2×10^4	0.0 [1.7×10^1][a]	
	Healthy (95)	6 6.3	0–2.8×10^5	0.0 [0.0]	
Prevotella intermedia	Gingivitis (70)	40 57.1	0–5.9×10^5	1.2×10^1 [8.5×10^1]	<0.001*
	Periodontitis (75)	56 74.6	0–6.2×10^5	8.6×10^2 [8.6×10^3][a]	
	Healthy (95)	43 45.2	0–5.2×10^5	1.1×10^1 [4.6×10^1]	
Prevotella nigrescens	Gingivitis (70)	23 33.8	0–14×10^4	0.0 [8.2×10^1]	<0.216
	Periodontitis (75)	31 41.3	0–12.3×10^4	0.0 [3.2×10^1]	
	Healthy (95)	22 23.1	0–28.2×10^4	0.0 [0.0]	
Tannerella forsythia	Gingivitis (70)	53 75.7	0–15.6×10^5	2.6×10^2 [3.8×10^3]	<0.001*
	Periodontitis (75)	62 82.6	0–6.2×10^5	6.2×10^2 [5.5×10^4][a]	
	Healthy (95)	65 68.4	0–5.2×10^5	9.1×10^1 [2.4×10^2]	
Dialister pneumosintes	Gingivitis (70)	66 94.2	0–25.9×10^3	1.0×10^6 [4×10^7]	<0.507
	Periodontitis (75)	72 96.0	0–21.4×10^4	9.0×10^5 [2.0×10^7]	
	Healthy (95)	87 91.5	0–24.0×10^4	5.7×10^5 [2.7×10^7]	
Treponema denticola	Gingivitis (70)	60 85.7	0–18.1×10^4	1.3×10^4 [1.1×10^6]	<0.05
	Periodontitis (75)	67 89.3	0–19.1×10^4	3.8×10^5 [8.0×10^6]	
	Healthy (95)	60 63.1	0–17.3×10^3	5.4×10^2 [7.6×10^3]	
Enterococcus faecalis	Gingivitis (70)	2 2.8	0–16.3×10^4	0.0 [0.0]	<0.605
	Periodontitis (75)	4 5.3	0–12.9×10^4	0.0 [0.0]	
	Healthy (95)	6 6.3	0–12.5×10^4	0.0 [0.0]	
Actinomyces israelii	Gingivitis (70)	30 42.8	0–11.4×10^4	0.0 [6.7×10^1]	<0.001*
	Periodontitis (75)	54 72.0	0–11.5×10^4	5.6×10^2 [5.9×10^2][a]	
	Healthy (95)	35 36.8	0–11.5×10^4	0.0 [3.0×10^1]	

*Values of $p < 0.05$ were considered statistically significant values by the Kruskal-Wallis test.
Actinomyces israelii = [a]Periodontitis ≠ Gingivitis; [a]Periodontitis ≠ Healthy.
Porphyromonas gingivalis = [a]Periodontitis ≠ Gingivitis; [a]Periodontitis ≠ Healthy.
Prevotella intermedia = [a]Periodontitis ≠ Gingivitis; [a]Periodontitis ≠ Healthy.
Tannerella forsythia = [a]Periodontitis ≠ Healthy.
Treponema denticola = [a]Periodontitis ≠ Gingivitis; [a]Periodontitis ≠ Healthy.
Porphyromonas endodontalis = [a]Periodontitis ≠ Gingivitis; [a]Periodontitis ≠ Healthy.
Aggregatibacter actinomycetemcomitans = [a]Gingivitis ≠ Healthy and Periodontitis ≠ Healthy.
Fusobacterium nucleatum = [b]Gingivitis ≠ Healthy and Periodontitis ≠ Healthy.

host immune response during the inflammatory process [16], we also demonstrated the distinctive surface structure gene expressions and related them to serotypes and probing depths.

A. actinomycetemcomitans and *F. nucleatum* are known as bacteria that are involved with periodontal diseases [21, 46]. The role of bacterial communication in the disease process outlines *F. nucleatum* as a key coadjuvant [22] that is able to co-aggregate with *A. actinomycetemcomitans* and intensifies damages around tooth-supporting tissues. This background knowledge corroborated our findings since a high prevalence *F. nucleatum* was observed in 90.6% of the total individuals with chronic periodontitis and 74.2% of the gingivitis group, while a lower percentage (51.5%) was detected in healthy subjects. However, regarding the clinical parameters, although no significant interaction between age and pocket depth was supported by the scientific literature, our work has shown a positive relationship between both variables.

To better understand the factors involved in the pathogenesis of periodontal diseases, we investigated the association between serotypes and disease stage. Additionally, we related the virulence factor genes expressions from different *A. actinomycetemcomitans* to an early development of periodontal disease. The allotment patterns of *A. actinomycetemcomitans* serotypes were assessed by numerous studies and by different experimental designs [13, 43,44,47,48]. Studies have suggested that highly pathogenic strains usually belong to serotype b; therefore, there may be an association between serotype b and aggressive periodontitis [15, 49 , 50]. Conversely, our study showed heterogeneity of bacterial biotypes and no relationship between serotypes and pocket depths was

observed. Indeed, a dominance of serotype c was observed in all groups, followed by serotypes a and b. Interesting, a recent study also did not detect serotype b even in relatively severe periodontal conditions among the subjects, and the authors interpreted the toxicity as being independent of serotypes [45]. Comparing our data with evidence from the literature and considering those studies were performed in different countries; we can suggest that gene expression is possibly driven by environmental conditions.

Taken into consideration the differences in *ltx* and *cdt* gene expressions among *A. actinomycetemcomitans* individual strains and their relationship with aggressive periodontitis [13, 51], further analyses were performed to correlate the presence of those genes with periodontal conditions. As expected, all strains of *A. actinomycetemcomitans* isolated from deep and shallow pockets had the *ltx* promoter and 91.6% of those bacterial species from healthy individuals were identified as carrying the *ltx* gene. Additionally, although only 8.3% of *A. actinomycetemcomitans* strains from healthy individuals exhibited a highly leukotoxic phenotype, almost 92% of the total *A. actinomycetemcomitans* found in those individuals produced protein toxins *ltxA*. In other words, our results suggested that *ltxA* gene expression might not serve as a risk marker for disease stage. Actually, it was quite surprising that deletion of 530 bp in the *ltx* promoter gene has been found in both periodontal conditions, and healthy and sick sites. With regard to additional important genes that were strongly associated with periodontal disease [17], our results revealed a high prevalence of the genes *cdtA*, *cdtB*, *cdtC*, and *cdtABC* in individuals with gingivitis and periodontal disease in contrast to the lower frequency observed in healthy conditions.

Wahasugui et al. [43] reported the presence of 64 *A. actinomycetemcomitans* strains as highly leukotoxic and only six strains as minimally leukotoxic. In addition, the presence of these leukotoxic strains had not association with the development of periodontitis, suggesting its transitory stage in oral cavity. It is possible that the evaluated strains here also showed this transitory characteristic in the analyzed population; since leukotoxic strains are associated with ecological and immunologic conditions of the examined individuals [15].

As mentioned above, the *cdt* gene is an immunosuppressive factor and its high expression during advanced stage of periodontal disease has been corroborated in recent reports. However, the distribution of *cdt* genes among the analyzed isolates did not show any serotype-dependent pattern, and it was not possible to demonstrate a specific correlation between them. These observations established the stage for a set of intriguing questions about which factors influence the expression of various toxins in bacterial cells of the subgingival biofilm *in vivo* in the presence of inflammation. Our results show a high frequency of the *cdtA*, *cdtB*, *cdtC* and *cdtABC* genes in gingivitis and periodontitis individuals, and a low frequency in healthy individuals. These results are in accordance with data previously reported by Wahasugui et al. [43] who suggested that the absence of amplicons to these genes might be due to the deletion of the genes or the lack of homology with the primers. These authors also reported the presence of only one strain from healthy individual harboring the *cdtC* gene suggesting the presence of an inactive gene.

To evaluate possible bacterial associations involved in the pathogenesis of periodontal disease, we also quantitatively determined the presence of other potential periodontal bacteria [34 , 52–58]. The predominance of *P. intermedia* and *P. gingivalis,* as well as the presence of *T. forsythia*, *T. denticola*, *P. nigrescens*, *D. pneumosintes*, *P. endodontalis*, *E. faecalis* and *A. israelii* in high values, allows us to suggest that there is some relationship among the presence of those bacteria including *A. actinomycetemcomitans* and *F. nucleatum* with the pathogenesis of periodontal disease. Since high numbers of these microorganisms were found in healthy individuals, it suggested that any host unbalance can modify the oral environment and develop infectious diseases.

Another important point to be discussed is the genetic variability observed in some pathogenic species as a reflex from pathogen-host-environment interactions. The high individual clonal diversity has been associated with advanced stage of periodontal disease and, as consequence of this mutation; the genetic types also may express different phenotypic characteristics and display an increase in virulence [59]. Consistent with the current information, our investigation identified, by a usefulness AP-PCR for fingerprinting pathogenic strains, most of genetic variations of *A. actinomycetemcomitans* and *F. nucleatum* in clinical isolates from individuals with gingivitis and chronic periodontitis. This is important information that will be addressed in future studies to uncover the bacterial genes involved in providing adaptation of the pathogens to the host environment, contributing to new target therapy development against resistant strains to antibiotics.

The results presented here strongly support a role of *A. actinomycetemcomitans* and *F. nucleatum* in advanced stage of periodontal disease. This study opens additional discussion about the role of the environment in the biotype and serotype prevalence depending on the periodontal conditions since the data revealed *A. actinomycetemcomitans* biotype II and serotype c in all clinical isolated samples. Subsequently, our data also showed no direct relationship between *ltxA* gene expression and leukotoxin gene 530-bp presence. This is in contrast to the *cdt* gene, which predominated during the inflammatory disease process. Possible bacterial synergisms can occur in diseased and healthy sites.

Acknowledgments

Strains were kindly provided by Dr. Jeffrey Kaplan (Department of Oral Pathology, Biology and Diagnostic Sciences, New Jersey Dental School, Newark, New Jersey). The authors thank Mrs. Marcia Harumi for her technical support. This study was supported by CAPES Foundation and FAPESP 2013/13652-6.

Conflict of interest: The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.anaerobe.2018.05.015.

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