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GENETIC DIVERSITY OF ORAL *Fusobacterium nucleatum* ISOLATED FROM PATIENTS WITH DIFFERENT CLINICAL CONDITIONS

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SUMMARY

The genetic diversity of 23 oral *Fusobacterium nucleatum* isolated from 15 periodontal patients, eight from seven healthy subjects, nine from nine AIDS patients and two from two *Cebus apella* monkeys were analyzed. *Eco*RI restricted the bacterial DNA and 28 ribotypes grouped from A to J groups were obtained. Isolates formed 24 ribotypes which were contained into A, B, C, D, E and F groups, and three reference strains and two clinical isolates of *A. actinomycetemcomitans,* and *E. coli* CDC formed four different ribotypes into the G, H, I and J groups. Moreover, from nine *F. nucleatum* from AIDS patients, six were ribotyped as group C and three as group D. By using ribotyping we distinguished *F. nucleatum* recovered from different sources. It is possible that isolates from AIDS patients may contain some phenotypic or genotypic factor did not observed in this study.

KEYWORDS: Anaerobic bacteria; Periodontopathogens; Periodontal disease; *Fusobacterium nucleatum*; Ribotyping.

INTRODUCTION

Fusobacterium nucleatum is one of the species that is statistically related to the periodontal disease, and it is the most common anaerobe present in human clinical infections such as sinusitis, pelvic infections, osteomyelitis, and brain and lung abscesses[5,7]. *F. nucleatum* constitutes a considerable part of the subgingival microbiota of gingivitis in children and adults and of periodontitis in juveniles and adults[13]. However, the role of this microorganism in periodontal disease remains undefined due to the heterogeneity within the species[3].

From an ecological point of view, there are few studies concerning the clonal diversity of gram-negative anaerobes indigenous to the human and the animal microbiota. Although many studies have examined the subgingival plaque microbiota by isolation, only few have characterized or subtyped *F. nucleatum* from different sources or clinical conditions. *F. nucleatum* has been recovered from 80% of periodontal patients and from 67.6% of healthy subjects[8] and a stable colonization in edentulous children was also observed[13].

The identification and the enumeration of the putative periodontal organisms from human dental biofilm samples present numerous technical difficulties due to the fastidious nature of the anaerobic bacteria[17]. An accurate identification of fusobacterial species is therefore very important not only for taxonomic reasons but also for the appropriate treatment of infections, since the susceptibility of different fusobacterial species to the wide variation of antibiotics[10].

Despite the large knowledge about the pathogenic potential and the medical aspects of fusobacteria, their metabolic and genetic aspects are little known[4,16]. In the last years, the use of tools as cloning, sequencing and PCR have given more information about the genetic aspects of *F. nucleatum*. Several methods of detecting phenotypic and genotypic characteristics have been used to distinguish those bacterial pathogens from commensal strains[6]. Different studies have underscored the heterogeneous nature of the genus *Fusobacterium* particularly among isolates of *F. nucleatum*. Moreover, species and strains of *Fusobacterium* have been studied and compared in several ways, such as by DNA-DNA hybridization, fatty acid analysis, constitution of the peptidoglycan layer, glutamate dehydrogenase electrophoretic patterns and AP-PCR[9,11,18]. In the present study, we have used ribotyping to examine the genetic diversity of oral *F. nucleatum* recovered from patients with different clinical conditions.

MATERIALS AND METHODS

Bacterial source: Fifteen patients with adult periodontitis (age range 18-40 years old), with clinical and radiographic evidence of the periodontal disease, including pockets of depth with equal or exceeding 5 mm; seven healthy subjects (age range 20-30 years old); and nine AIDS patients with periodontitis (age range 25-40 years old) were selected. The male: female ratio was 9:6 (periodontal patients), 5:2 (healthy subjects) and 9:0 (AIDS patients). All the individuals were patients of the Periodontology Clinic, University of São Paulo, São Paulo, SP, Brazil. Moreover, two healthy male *Cebus apella* monkeys

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from the State University of São Paulo, Araçatuba, SP, Brazil, were selected. All patients gave written informed consent to be recruited to the study and the approval was obtained from the Research Ethics Committee of the Biomedical Sciences Institute, University of São Paulo. Initially, the supragingival plaque was removed and subgingival biofilm samples were taken by using sterilized absorbent paper points (Dentsply Ind. & Co. Ltd., Rio de Janeiro, RJ, Brazil), which were introduced into the periodontal pocket or into gingival sulcus for 60 seconds and transported in a VMGA III medium[14]. Aliquots of 0.1 mL of undiluted and 10^{-2} diluted samples were inoculated within two hours after collection on Omata & Disraely agar, and incubated in anaerobic conditions (90% N_2/10% CO_2), at 37 ºC, for four days. *F. nucleatum* isolates were confirmed by the following characteristics: gram-negative, non-spore forming rods, indole positive, catalase negative and asaccharolytics, and identified by conventional biochemical tests[15,19]. Periodontal organisms such as *Actinobacillus actinomycetemcomitans* isolated from periodontal patients (PD1 and PD2), *A. actinomycetemcomitans* ATCC 29522, ATCC 29523 and ATCC 43718, *F. nucleatum* ATCC 10953 and ATCC 25583, and *Haemophilus aphrophilus* CDC and *Escherichia coli* CDC (Culture collection at the Anaerobe Laboratory, Hospital Infections Program, CDC, Atlanta, GA, USA) were also included.

Extraction and restriction of the bacterial DNA: Bacteria were grown in brain heart infusion broth (BHI - Difco Laboratories, Detroit, MI, USA) supplemented with 0.5% yeast extract. Cells were harvested and lysed with sodium dodecyl sulfate (SDS) and DNA was extracted as previously described by AVILA-CAMPOS *et al.*[1], with minor modifications. Each DNA sample was spectrophotometrically examined to determine the DNA concentration and purity. Restriction enzymes *Hind*III, *Hinc*II, *Eco*RI and *Eco*RV (New England Biolabs, Beverly, MA, USA) were used to digest DNA from *F. nucleatum* isolates and other bacteria to determine the most appropriate enzyme. Briefly, 1 µg of DNA was digested with each enzyme in a final volume of 20 µL, at 37 ºC, during seven hours, performed according to the manufacturer's specifications. Finally, the digested DNA was run in 0.9% agarose gels. TAE (40mM Tris-acetate, 1mM EDTA, pH 8.0) was used as the electrophoresis buffer in a BioRad unit (20 mA, overnight). DNA molecular-weight marker II and X digoxigenin (DIG)-labeled (Boehringer Mannheim, Germany) were used as size markers.

Southern blot: Southern transfer was performed as described by BOLSTAD *et al.*[3]. The DNA was blotted with a positively charged nylon membrane (Catalog No. 1209272; Boehringer Mannheim) recommended by the DIG-labeling method. The transfer was performed for 20 hours with 20 X SSC as the transfer buffer. All Southern blots were prehybridized at 65 ºC, for one hour, in hybridization buffer (5 X SSC [Gibco BRL], 0.5% wt/vol blocking reagent [Boehringer Mannheim], 0.1% wt/vol N-laurosylsarcosine, 0.02% wt/vol SDS). The same solution was used for hybridization, and the Southern blots were hybridized with a pKK3535 plasmid DNA nonradioactive probe at 65 ºC, overnight. The stringent washing and the chemiluminescent detection were mainly carried out in accordance with the manufacturer's protocol for the detection of DIG-labeled nucleic acids (Genius 1 Kit, Boehringer Mannheim). The membranes were washed twice, for five minutes each one, with washing buffer (2 X SSC-0.1% SDS), at room temperature and then twice, for 15 minutes each one, with 0.1 X SSC-0.1% SDS, at 65 ºC. Detection was performed by incubation for two hours in diluted anti-digoxigenin-AP Fab fragments (150 U, 1:3,000) and membranes were washed twice, for two minutes, in buffer solution (100 mM Tris-HCl, 100 mM $NaCl_2$, 50 mM $MgCl_2$, 0.02% sodium azide), and incubated for 30 minutes in NBT and X-phosphate in DMF (DIG DNA labeling and detection Kit, Boehringer Mannheim) in a dark place.

Statistical analysis: Ribotypes were determined by the observation of the banding patterns, and bands were analyzed by using a NTSYS program (Applied Biostatistic, Inc Version 1.7).

RESULTS

Restriction of the DNA: In this study, *Hind*II, *Hind*III and *Eco*RV enzymes failed to digest the DNA of all *F. nucleatum.* The *Eco*RI enzyme was the most efficient in cutting the bacterial DNA and in providing easily distinguishable 24 patterns among *F. nucleatum* isolates and the reference strains used, detected by using a pKK3535 probe. In addition, four more patterns were formed for the reference strains and clinical isolates of *A. actinomycetemcomitans* and *E. coli* CDC (Table 1).

Southern blot and ribotyping: Southern blots of *F. nucleatum* digested with *Eco*RI and hybridized with pKK3535 probe displayed similar band sizes in all the tested isolates from approximately 2.6 to 2.7 kb (data not shown). Moreover, *F. nucleatum* from AIDS patients produced additional bands ranging approximately from 7.0 to 9.0, which were not observed in isolates from periodontal patients, healthy subjects or monkeys (Fig. 1). Isolates displaying differences in hybridization

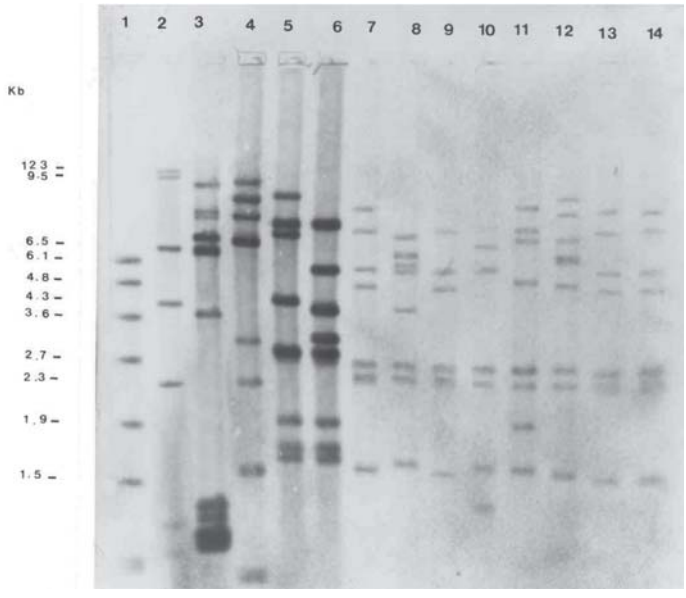

Fig. 1 - Ribotyping representative of *F. nucleatum*/EcoRI. Autoradiogram of Southern blot hybridized with pKK3535 probe and washed with 20 X SSC for two h, 15 min., at 65 ºC. DNA restricted with *Eco*RI. Lanes 1 and 2, DNA molecular weight marker (X and II, respectively); lane 3, *E. coli* CDC; lane 4, *H. aphrophilus*; lane 5, *A. actinomycetemcomitans* ATCC 29523; lane 6, *A. actinomycetemcomitans* isolated from periodontal patient; lane 7, *F. nucleatum* isolated from monkey; lane 8, *F. nucleatum* ATCC 10953; lane 9, *F. nucleatum* isolated from periodontal patient; lane 10, *F. nucleatum* isolated from healthy subject; lanes 11 and 12, *F. nucleatum* isolated from AIDS-patients; lanes 13 and 14, *F. nucleatum* isolated from periodontal patients.

Table 1
DNA hybridization, ribotype patterns and groups of the *F. nucleatum* and other bacteria restricted with *Eco*RI

Source/Isolate	Banding pattern	Ribotype (No.)	Ribotype group
Periodontal patients (23)			
P10, P14, P20, P22	8	Rb1	A
P6	9	Rb2	B
P1, P2, P3, P5, P15, P23	8	Rb8	D
P11, P12	10	Rb13	D
P4	3	Rb14	D
P9	6	Rb15	D
P8	6	Rb16	D
P7	4	Rb17	D
P13, P21	4	Rb18	D
P19	7	Rb9	D
P16	7	Rb21	E
P17, P18	8	Rb22	E
AIDS-patients (9)			
A7	9	Rb3	C
A4	9	Rb4	C
A1, A2	7	Rb5	C
A6	9	Rb6	C
A5	8	Rb7	C
A3, A8, A9	8	Rb10	D
Healthy individuals (8)			
H2, H3, H4	8	Rb8	D
H8, H9	8	Rb11	D
H6	8	Rb12	D
H10	5	Rb23	F
H11	8	Rb24	F
Monkey isolates (2)			
M1, M2	8	Rb1	A
Reference strains and other isolates (9)			
F. nucleatum ATCC 10953	8	Rb1	A
F. nucleatum ATCC 25586	7	Rb20	E
H. aphrophilus CDC	6	Rb19	D
A. actinomycetemcomitans ATCC 29522, ATCC 29523 and 43718	7	Rb26	H
A. actinomycetemcomitans isolate PD1	8	Rb25	G
A. actinomycetemcomitans isolate PD2	8	Rb27	I
E. coli CDC	10	Rb28	J

patterns by one or more bands were considered distinct strains. All the *F. nucleatum* isolates from different sources and reference strains were classified in ten ribotype groups (A to J) (Table 1). Table 1 shows the hybridization patterns and ribotypes in all tested bacteria restricted with the *Eco*RI enzyme. All the 23 *F. nucleatum* isolated from periodontal patients produced a range from three to 10 different hybridization patterns grouped in 12 ribotypes (Rb1, Rb2, Rb8, Rb9, Rb13, Rb14 to Rb18, Rb21 and Rb22). All nine *F. nucleatum* from AIDS-patients showed from seven to nine bands and they were clustered in six different ribotypes (Rb3 to Rb7 and Rb10). Of the eight *F. nucleatum* from healthy subjects, only two banding patterns (five and eight bands) were produced, then clustered in five different ribotypes (Rb8, Rb11, Rb12, Rb23, Rb24). Two *F. nucleatum* isolated from monkeys produced similar banding patterns (eight bands) than human isolates (Rb1). Interestingly, of nine HIV-patient isolates, six were clustered in group C and three isolates in the group D. It also was verified the similarities among *F. nucleatum* species isolated from AIDS-patients. In Fig. 2, it can be observed that *F. nucleatum* isolates from AIDS-patients displayed approximately 78% of similarity to A1, A2, A4, A5, A6, and A7 isolates, and 88% to the A3, A8 and A9 isolates. On the other hand, it was observed that two *A. actinomycetemcomitans* isolated from two periodontal patients (PD1, PD2) belonged to G (Rb25) and I (RB27) clusters, respectively, showing that they were different strains.

DISCUSSION

The significance of *F. nucleatum* in the development of the

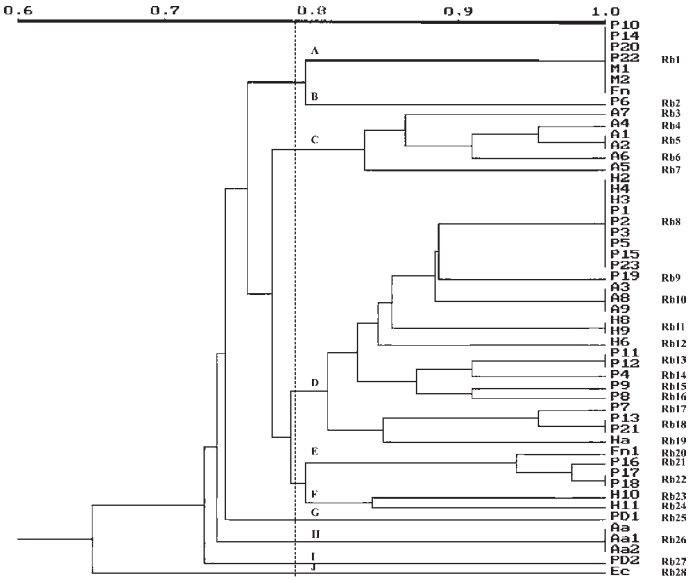

Fig. 2 - NTSYS dendrogram of genetic similarity for *Fusobacterium nucleatum* isolated from patients with different clinical conditions, analyzed by ribotyping. P: periodontal patients isolates; H: healthy individual isolates; A: AIDS patients isolates; M: monkey isolated; Ha: *H. aphrophilus*; Fn: *F. nucleatum* ATCC 10953; Fn1: *F. nucleatum* ATCC 25586; Aa, Aa1 and Aa2: *A. actinomycetemcomitans* reference strains; PD1 and PD2: *A. actinomycetemcomitans* clinical isolates; and Ec: *E. coli* CDC.

periodontal disease, as well as infections in other organs, has generated interest due to its pathogenic potential, its frequency in periodontal lesions, its synergism in mixed infections and its ability to form aggregates with other suspect pathogens in the periodontal disease[7,12]. Periodontal bacteria become pathogenic when 1% of the total organisms inhabit a dental biofilm sample, but their detection depends on the sensitivity and the specificity of the used method[2]. Then, an accurate identification is necessary, not only for taxonomic reasons, but also for the appropriate treatment of infections, since the susceptibility of different fusobacterial species to antibiotics, which varies widely[10].

The ribotyping method by using the probe pKK3535 and the *Eco*RI endonuclease showed a high degree of specificity when hybridized with a panel of potential cross-hybridizing species. On the other hand, ribotyping distinguished among closely related species such as *H. aphrophilus*, a common bacterium in healthy subgingival sulci and *A. actinomycetemcomitans,* a periodontal pathogen. Since both species share approximately 40% of the DNA-DNA homology using whole-cell probes, it can result in a high frequency of false-positive results with plaque samples or any other mixed population[18].

In this study, ribotyping appear as an efficient method to distinguish *F. nucleatum* isolated from patients with different clinical conditions, i.e., periodontal patients, healthy subjects, and AIDS patients and monkeys. However, six and three AIDS isolates, respectively, were localized in C and D groups, and it is possible that these isolates present maybe any phenotypic or genotypic factor not detected here. In addition, all the *F. nucleatum* were recovered from AIDS-patients with periodontal disease and they were not identified at the subspecies level.

There are many difficulties in the search of the etiologic agents of destructive periodontal diseases in HIV-positive patients with AIDS, including technical problems and difficulties in determining the activity of the periodontal disease. On the other hand, level of complexity exists because strains within species can differ in virulence, as it has been suggested in *F. nucleatum* subspecies[9]. By using the *Eco*RI enzyme it was possible to distinguish 28 ribotypes grouped in 10 groups including *F. nucleatum* from different sources and reference strains (Table 1). However, nine *F. nucleatum* recovered from AIDS patients were grouped in six different ribotypes (Rb3 to Rb7 and Rb10). In conclusion, the results obtained here show that ribotyping can be used for differentiating strains from different clinical conditions. Because only nine *F. nucleatum* strains recovered from AIDS patients were studied, it is suggested the need of examining a larger number of strains to determine the role of *F. nucleatum* play in the periodontal disease in that patients.

RESUMO

Diversidade genética de *Fusobacterium nucleatum* orais isolados de pacientes com diferentes condições clínicas

Neste estudo foi avaliada a diversidade genética de 23 amostras de *Fusobacterium nucleatum* isoladas da cavidade bucal de 15 pacientes com doença periodontal, de oito cepas isoladas de sete indivíduos sadios, de nove isoladas de nove pacientes com AIDS e de duas isoladas de dois macacos *Cebus apella*. Pela ação da enzima *Eco*RI sobre o DNA bacteriano foram reconhecidos 28 ribotipos agrupados de A a J. Os isolados testados formaram 24 ribotipos os quais foram contidos nos grupos A, B, C, D, E e F, e as três cepas de referência e dois isolados clínicos de *A. actinomycetemcomitans* e *E. coli* CDC formaram quatro diferentes ribotipos contidos nos grupos G, H, I e J. Em adição, as nove cepas de *F. nucleatum* isoladas de pacientes com AIDS, seis pertenciam ao grupo C e três ao grupo D. Usando-se a ribotipagem foi possível distinguir *F. nucleatum* isolados de diferentes origens.

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