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Distribution of Serotype-specific Genotypes of *Aggregatibacter actinomycetemcomitans* in Brazilian Patients with Down Syndrome with Different Periodontal Conditions

Distribución de los Serotipos Genotipos Específicos de *Aggregatibacter actinomycetemcomitans* en los Pacientes Brasileños con Síndrome de Down con Diferentes Condiciones Periodontales

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ABSTRACT: This study evaluated the occurrence of highly or minimally leukotoxic strains of *Aggregatibacter actinomycetemcomitans* (Aa) from patients with Down syndrome and the distribution of the different serotype-specific genotypes of this microorganism. Sixty-seven patients with Down syndrome were subjected to dental, periodontal and radiographic evaluations. Samples of subgingival biofilm were collected and plated onto TSBV agar and characteristic colonies of *A. actinomycetemcomitans* were identified by biochemical methods. The occurrence of this bacterium was also evaluated directly in the clinical specimens by PCR. The presence of 530 bp deletion in the promoter region was also determined by PCR in order to evaluate distribution of highly or minimally leukotoxic strains. *A. actinomycetemcomitans* was detected in 11.1% by culture and 22.2% by PCR from periodontally healthy subjects, 100% of the patients with Down syndrome with aggressive periodontitis, 50% and 75% of patients with chronic periodontitis by culture and PCR respectively. Only two patients with aggressive periodontitis were colonized by highly leukotoxic Aa. Serotype-specific genotypes a and c were the most prevalent. The results suggest the role of peculiar characteristics of Aa and patients with Down syndrome in the development of periodontitis and the influence of peculiar characteristics of the population in this process.

KEY WORDS: gingivitis, bacteria, periodontitis, down syndrome.

INTRODUCTION

Down syndrome (DS) is a genetic disorder caused by an extra chromosome 21, with an incidence of 1/700 live births, producing several effects on physical and psychological development. Patients with Down syndrome frequently present higher susceptibility to opportunistic infections, particularly gingivitis and periodontitis (Khocht *et al*., 2012). This susceptibility to aggressive periodontitis has been studied at the level of immune responsiveness, clinical and microbiological aspects (Sakellari *et al.*, 2005; Khocht *et al*.). In Latin America, due to the great diversity of influences related to human colonization, habits and geographic conditions, these peculiar characteristics are outstanding even within a single country (Herrera *et al.,* 2008).

Aggregatibacter actinomycetemcomitans (Aa) is frequently implicated with aggressive episodes of periodontitis and its distribution is influenced by ethnic and geographic characteristics (Haubek *et al.,* 2008). The virulence and distribution of different serotypes of Aa may vary significantly and some serotypes are more closely associated to the development of periodontal breakdown than others. This statement has been discussed particularly in patients with localized forms of aggressive periodontitis, where serotype b is frequently detected, while serotypes a and c have a stronger association with periodontal health or chronic forms of periodontitis (Kaplan *et al*.; Roman-Torres *et al.,* 2010). However, data on serotypes distribution of Aa in patients with Down syndrome are scarce,

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especially from patients subjected to dental preventive programs. In addition, this bacterium produces a heat-labile leukotoxin which belongs to the repeat-in-toxin (RTX) family. It has been observed that the leukotoxin expression is associated with the presence of a 530 bp deletion in the promoter region of the gene *ltxC*, although physiological factors may also be involved in this regulation (Gaetti-Jardim Jr. *et al.*, 2008).

Thus, this study evaluated the distribution of serotype-specific genotypes of Aa from children, adolescents and young adults with Down syndrome, living or not in public or private institutions, and the presence of a deletion in the ltx gene operon promoter, associated with highly leukotoxic strains.

MATERIAL AND METHOD

Casuistic and clinical evaluation. A total of 67 patients with Down syndrome, 27 children aged 6-11 years (mean age 8.03 ± 6.9 years), 23 adolescents aged 14-18 years (15.5 ± 6.9 years) and 17 young adults aged 19-31 years (23.5 ± 7.2 years) seen at the School of Dentistry of Araçatuba-UNESP, Araçatuba, SP, Brazil, from January 2000 to December 2009, were enrolled. Out of these patients, 37 were living at public and private institutions of Araçatuba and José Bonifácio municipalities and 30 were not institutionalized. Out of periodontally healthy patients with Down syndrome, 12 were children, 11 adolescents and 4 young adults, while 15 children, 10 adolescents and 4 young adults presented gingivitis, 2 adolescents and one young adult displayed clinical and radiographic evidences of aggressive periodontitis and 8 young adults had chronic periodontitis. A standardized questionnaire was applied to the caregivers and patients to collect information about socio-economic and dietary aspects.

The criteria for inclusion in the study are listed as follow: minimum of 10 teeth present, no periodontal or antibiotic treatment during the previous 6 months, good personal and parental levels of cooperation, and absence of systemic disease that could interfere with periodontal conditions or affect periodontal examinations and the collection of clinical samples. All patients and patient caregivers accepted to participate and gave written informed consent to be included in the study, which was approved by the institutional review boards of the UNESP (Proc. UNESP 63/2000 and 01369/2007).

Probing pocket depth and attachment level, bleeding on probing and hygiene index (presence or absence of plaque) were recorded at six points for each tooth. All measurements were performed using a manual probe and the examiner's clinical measurements were considered calibrated if the standard error of measurements was equal to 0.8 and the K value ranged between 0.82 and 0.94. Diagnosis of periodontitis followed the criteria of American Academy Periodontology.

Sample collection. Subgingival biofilm was collected after removal of supragingival biofilm. Samples from patients with periodontitis were obtained by using three sterile paper points (Dentsplay, Ind. Co. Ltd., RJ, Brazil) inserted to the apical portion of three deepest periodontal pockets presenting bleeding on probing, for 30 s. Two paper points were pooled and transported in VMGA III medium for culture, and one paper point was transferred to 300 μL ultrapure water, for molecular detection of the serotype-specific genotypes of A. actinomycetemcomitans. Samples from patients with gingivitis were obtained from three non contiguous periodontal sites with bleeding on probing and without clinical and radiographic evidences of bone loss, while samples from periodontally healthy subjects were collected from distolingual site of lower right first molar, distolingual site of lower left first molar and from distobuccal site of upper right first molar. Ten-fold dilutions of the clinical samples were plated in duplicate onto TSBV agar. After 72 h of incubation under anaerobiosis (90% N_2 + 10% CO_2) at 37ºC, characteristic colonies of A. actinomycetemcomitans were identified by biochemical methods (Gaetti-Jardim Jr. *et al.*).

Detection of Aa by PCR . Total bacterial DNA from the clinical samples and from isolates was extract by using a QIAamp DNA mini kit (QIAGEN, Hilden, Germany). Initially, PCR technique was used to identify samples positive for A. actinomycetemcomitans using a pair of primers for the 16S ribosomal DNA gene, as previously described (Ashimoto *et al*., 1996). Amplified products were analyzed by electrophoresis in agarose gel (1%) stained with 0.5 mg/ml ethidium bromide. For A. actinomycetemcomitans-positive samples, the detection of different serotype-specific genotypes was carried out using primers specific for each serotype (a–f), as previously described (Kaplan *et al*.). In addition, the DNA of 102 isolates was also genotyped.

The presence of the 530 bp deletion in the leukotoxin promoter of A. actinomycetemcomitans was

investigated in 65 isolates using a PRO primer pair (Gaetti-Jardim Jr. *et al.*), producing a 1,075 bp amplicon (minimally leukotoxic) or a 545 bp amplicon (highly leukotoxic). A. actinomycetemcomitans JP2 with a 530-bp deletion was used as a positive control, and A. actinomycetemcomitans ATCC 33383 as a negative control.

Statistical analysis. The results were expressed as median, standard deviation and percentages. Chi-square test, Mann-Whitney test and Fisher's exact test, and multiple comparison tests were performed to check differences in detection of Aa, as well as the relationships between microbial and clinical periodontal parameters. The statistical significance was established at 5% ($p < 0.05$). All tests were performed using statistical software (Biostat 5.0 and SPSS 15.0 for Windows Release 15.0, Chicago, IL-USA).

RESULTS

The occurrence of localized aggressive and localized chronic periodontitis was not associated with oral hygiene standards (Chi-square test, p= 0.127) or diet (Chi-square test, p= 0.301). It was verified associations between Aa and gingival bleeding (Mann-Whitney`s test, p=0.005), gingival edema (Chi-Square test, p<0.001), food retention (Chi-Square test, p=0.001), bone loss (Chi-square test, p= 0.018), and probing depth (Mann-Whitney test, p<0.001) was verified. Overall, the results obtained by culture and PCR were similar. The occurrence of highly leukotoxic strains (JP2-like strains) or minimally leukotoxic isolates is presented in Table I.

Table II presents the data on distribution of serotype-specific genotypes in the targeted population. Out of 67 subjects with Down syndrome, Aa was cultivated from 17 (25.4%) patients, and detected by PCR from 23 (34.3%) subgingival specimens, and this difference in microbial detection was not statistically significant. The occurrence of Aa was not associated with age, thus the results presented in Tables I and II do not discriminate the patients' age.

The JP2-like Aa strains were detected just from patients with aggressive periodontitis (N=3, 100%) and in one of these patients, a 17 years old adolescent, this microorganism shared the periodontal pocket with non-JP2 Aa strains. The distribution of serotype-specific genotypes obtained by culture showed infection by a single genotype (non-mixed colonization) in 16.4% of the patients, and serotype a was the most frequent whereas mixed infection was observed in 7.5% of the patients. The results obtained directly from the clinical samples by PCR showed non-mixed colonization of periodontal sites in 17.9% of the patients and mixed colonization in 14.9%; serotype c was the most common (Table II). No association was detected between serotype-specific genotypes and deletion in the promoter of ltx gene or clinical parameters. JP2 strains presenting this genetic deletion in leukotoxin promoter were consistently associated with aggressive forms of periodontitis in Down syndrome patients, since all the patients presenting this condition were colonized by this bacterium, which was not observed in patients with chronic periodontitis, gingivitis or healthy subjects.

Table I. Occurrence of highly leukotoxic (JP2) and minimally leukotoxic (non-JP2) A. actinomycetemcomitans in patients with Down's syndrome

Experimental group	Prevalence n (%)							
	Culture				PCR*			
	non-JP2 *Aa*	JP2 - *Aa*	non-JP2 *Aa* + JP2 *Aa*	Total	non-JP2 *Aa*	JP2 - *Aa*	non-JP2 *Aa* + JP2 *Aa*	Total
Periodontally healthy (N= 27)	3 (11.1)	0 (0.0)	0 (0.0)	3 (11.1)	6 (22.2)	0 (0.0)	0 (0.0)	6 (22.2)
Gingivitis (N=29)	7 (24.1)	0 (0.0)	0 (0.0)	7 (24.1)	8 (27.6)	0 (0.0)	0 (0.0)	8 (27.6)
Aggressive periodontitis (N= 3)	2 (66.7)	0 (0.0)	1 (33.3)	3 (100.0)	1 (33.3)	1 (33.3)	1 (33.3)	3 (100.0)
Chronic periodontitis (N=8)	4 (50.0)	0 (0.0)	0 (0.0)	4 (50.0)	6 (75.0)	0 (0.0)	0 (0.0)	6 (75.0)
Total (N= 67)	16 (23.9)	0 (0.0)	1 (1.5)	17 (25.4)	21 (31.3)	1 (1.5)	1 (1.5)	23 (34.3)

*Detection directly from clinical specimens.

Table II. Distribution of serotype-specific genotypes of A. actinomycetemcomitans in Aa-positive subjects with Down's syndrome

Serotype-specific genotypes	Prevalence n (%)									
			Culture					PCR		
	PHS[1]	PG[2]	PAP[3]	PCP[4]	Total	PHS	PG	PAP	PCP	Total
Nonmixed colonization	2 (7.4)	4 (13.8)	2 (66.7)	3 (37.5)	11 (16.4)	3 (11.1)	4 (13.8)	2 (66.7)	3 (37.5)	12 (17.9)
serotype *a*	1 (3.7)	2 (6.9)	0 (0.0)	2 (25.0)	5 (7.5)	1 (3.7)	1 (3.4)	0 (0.0)	1 (12.5)	3 (4.5)
serotype *b*	0 (0.0)	0 (0.0)	1 (33.3)	0 (0.0)	1 (1.5)	0 (0.0)	0 (0.0)	1 (33.3)	0 (0.0)	1 (1.5)
serotype *c*	1 (3.7)	1 (3.4)	1 (33.3)	1 (12.5)	4 (6.0)	2 (7.4)	2 (6.9)	1 (33.3)	1 (12.5)	6 (9.0)
serotype *d*	0 (0.0)	1 (3.4)	0 (0.0)	0 (0.0)	1 (1.5)	0 (0.0)	1 (3.4)	0 (0.0)	0 (0.0)	1 (1.5)
serotype *e*	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (12.5)	1 (1.5)
Mixed colonization	1 (3.7)	2 (6.9)	1 (33.3)	1 (12.5)	5 (7.5)	3 (11.1)	3 (10.3)	1 (33.3)	3 (37.5)	10 (14.9)
serotype *a + b*	1 (3.7)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.5)	0 (0.0)	1 (3.4)	0 (0.0)	1 (12.5)	2 (3.0)
serotype *a + c*	0 (0.0)	1 (3.4)	1 (33.3)	1 (12.5)	3 (4.5)	2 (7.4)	1 (3.4)	1 (33.3)	1 (12.5)	5 (7.5)
serotype *b +c*	0 (0.0)	1 (3.4)	0 (0.0)	0 (0.0)	1 (1.5)	1 (3.7)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.5)
serotype *a +b + c*	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.4)	0 (0.0)	1 (12.5)	2 (3.0)
Non-typeable samples	0 (0.0)	1 (3.4)	0 (0.0)	0 (0.0)	1 (1.5)	0 (0.0)	1 (0.0)	0 (0.0)	0 (0.0)	1 (1.5)

[1]Periodontally healthy subjects (n= 27); [2]Patients with gingivitis (n= 29); [3]Patients with aggressive periodontitis (n=3) [4]Patients with chronic periodontitis (n= 8).

DISCUSSION

Periodontitis is more frequent and severe among individuals with Down syndrome compared with non-syndromic age-matched individuals (Sakellari *et al.*). However, evidence suggests that severity of periodontitis in syndromic individuals is milder than previously reported, probably due to better dental care for these individuals in the last decades (Amano *et al*., 2001). Both immunological and microbiological approaches have focused the pathogenesis of the gingivitis and periodontitis on Down syndrome. Inconclusive results have also implicated a heavier colonization of gingival crevice or periodontal pockets by A. actinomycetemcomitans in the development of aggressive periodontitis in syndromic patients (Sakellari *et al*.). The results presented in Table I evidenced a similar frequency of colonization to that previously reported to age-matched non-syndromic patients with different periodontal status (Herrera *et al*.). Differences in the distribution of this microorganism between children, adolescents, and young adults were not found.

Aa is frequently related to aggressive episodes of periodontitis (Cunha *et al*., 2012), and its distribution is influenced by ethnic and geographic characteristics. For instance, this microorganism was detected in North American (35%), and Brazilian (52% to 90%) patients with periodontitis, besides its elevated prevalence in North African and Asian subjects (Haubek *et al*.). Other studies have demonstrated low frequency of this microorganism in Colombian, Spanish (Herrera *et al*.), and Dutch patients (Boutaga *et al*., 2006).

This bacterium was cultivated from 11.1% of samples from periodontally healthy patients with Down syndrome, 24.1% of patients with gingivitis, all patients with aggressive periodontitis and from half of the samples collected from patients with chronic periodontitis. By PCR, the detection was slightly improved, reaching 22.2% of the periodontally healthy subjects, 27.6% gingivitis patients, all patients with aggressive periodontitis, and 75% of the patients with chronic periodontitis. However, oral hygiene did not significantly interfere with occurrence of this microorganism. These values are similar to those reported by Khocht *et al.*, and relatively small when compared with those previously reported in children with Down syndrome, where the frequency of colonization varied from

nearly 25% in children aged 8-13 years to about 65% in young adults aged 19-28 years, irrespective of the periodontal status of the patients (Sakellari *et al*.). This heterogeneous distribution in different human populations is likely to be related to the virulence of the several serotypes of Aa.

In the present investigation, serotype-specific genotype f was not detected and serotypes d and e were just observed in 1.5% of the samples. Serotype e is more prevalent in the Far East than in the Americas or Europe, colonizing 18.6% Indonesians (van der Reijden *et al*., 2008), and 46.7% Japanese (Yoshida *et al*., 2003). The low prevalence of serotype d was also previously described in non-syndromic patients (van der Reijden *et al*.). Serotypes a and c are mostly detected in non-syndromic Brazilians with mild or moderate periodontitis whereas serotype c has been described in patients with severe periodontitis (Roman-Torres *et al*.). The results presented in Table I did not support any association among the serotypes in relation to periodontal status, though serotype a and c were the most commonly detected. Some studies have implicated serotype b with the development of localized aggressive periodontitis in non-syndromic patients in North America, Europe and some areas of the Far East (Yang *et al*., 2005), but this serotype distribution could not represent real association between a particular serotype and the severity of periodontal breakdown worldwide.

In the present study, the occurrence of multiple serotypes (serotype a + b + c) was observed in 3% of the samples, and this characteristic has been described in Indonesians (12.2% - 17%), Chinese (16.8%), Japanese (25%) and Finish (5.5%-7%), being more pronounced in the Far East in comparison to Europe and the Americas (Yoshida *et al*.; van der Reijden *et al.*). However, this was not detected in non-syndromic patients from Brazil (Roman-Torres *et al*.).

The frequency genetic deletion in the promoter is especially relevant in the microbial virulence since subjects harboring highly leukotoxic *A. actinomycetemcomitans* are more likely to convert from healthy status to localized aggressive periodontitis than those colonized by strains harboring full-length leukotoxin promoter region (Gaetti-Jardim Jr. *et al*.). The results presented here also suggest that this association is valid for Brazilian Down syndrome patients since strains with *ltxC* 530 bp deletion and positive samples were only observed from patients with aggressive periodontitis although the size of group was restrict.

CONCLUSIONS

The diversity and the distribution of serotype-specific genotypes of *A. actinomycetemcomitans* and strains with different genetic background to express leukotoxin in Down Syndrome patients with different periodontal status suggest the influence of peculiar microbial characteristics on host-parasite interactions in the development of periodontitis in syndromic patients.

GAETTI-JARDIM JR., E.; SUMIDA, D. H. & SCHWEITZER, C. M. Distribución de los serotipos genotipos específicos de *Aggregatibacter actinomycetemcomitans* en los pacientes brasileños con síndrome de Down con diferentes condiciones periodontales. ***Int. J. Odontostomat., 7(1)***:107-112, 2013.

RESUMEN: Este estudio evaluó la presencia de cepas altamente o mínimamente toxicas de *Aggregatibacter actinomycetemcomitans* (Aa) de los pacientes con síndrome de Down y la distribución de los serotipos genotipos específicos de este microrganismo por cultivo y PCR. Sesenta y siete pacientes con síndrome de Down fueron sometidos a un tratamiento dental y evaluaciones clínicas. Las muestras de biofilme subgingival fueron recogidas y cultivadas en agar TSBV y colonias características de Aa fueran identificadas mediante métodos bioquímicos. La presencia de esta bacteria se evaluó también directamente en las muestras clínicas por PCR. Los aislados y las muestras clínicas también se probaron con el fin de evaluar la distribución de serotipos de genotipos específicos por PCR, mientras que la presencia de delección de 530 bp en la región promotora del gen ltxC también fue determinado por PCR con el fin de evaluar de distribución de las cepas altamente o mínimamente toxicas. Aa fue aislado en 11,1% y 22,2% por PCR de pacientes periodontalmente sanos; en todos los pacientes con síndrome de Down con periodontitis agresiva, y en 50% y 75% de los pacientes con periodontitis crónica por cultivo y PCR, respectivamente. Sólo dos pacientes con periodontitis agresiva fueron colonizados por cepas altamente tóxicas. Los serotipos y genotipos específicos a y c fueron los más frecuentes. Los resultados sugieren una asociación de las peculiares características de Aa con las características de los pacientes con síndrome de Down en el desarrollo de la periodontitis.

PALABRAS CLAVE: gingivitis, bacterias, periodontitis, síndrome de Down.

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