DETECTION OF *MOGIBACTERIUM TIMIDUM* IN SUBGINGIVAL BIOFILM OF AGGRESSIVE AND NON-DIABETIC AND DIABETIC CHRONIC PERIODONTITIS PATIENTS

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ABSTRACT

The aim of the present study was to evaluate the frequency of detection of *Mogibacterium timidum* in subgingival samples of subjects with generalized aggressive periodontitis (GAgP) and uncontrolled diabetic and non-diabetic subjects with generalized chronic periodontitis (GChP). 48 patients with GAgP, 50 non-diabetic and 39 uncontrolled (glycated hemoglobin >7%) type 2 diabetic subjects with GChP were enrolled in this study. Subgingival biofilm were collected from deep pockets (probing depth > 7 mm). After DNA extraction, *M. timidum* was detected by Nested Polymerase Chain Reaction and chi-square test was used to data analysis (p>0.05). There were no differences in the frequency of detection of *M. timidum* between subjects with GAgP (35%) and non-diabetic subjects with GChP (40%) (p>0.05). The frequency of detection of *M. timidum* was significantly higher in deep pockets of diabetic subjects with GChP (56%) when compared to GAgP (p<0.05), but similar to non-diabetic subjects with GChP (p>0.05). The frequency of detection of *M. timidum* was higher in subjects GChP presenting uncontrolled type 2 diabetes mellitus, when compared to GAgP subjects.

Key words: *Mogibacterium timidum*, Aggressive periodontitis, Chronic periodontitis, Diabetes mellitus

INTRODUCTION

In 2000, the new genus *Mogibacterium* was proposed to include the novel species *Mogibacterium pumilum* and *Mogibacterium vescum*, as well as the former *Eubacterium timidum* by taxonomic reassignment (16). Members of the genus *Mogibacterium* are described as strictly anaerobic and asaccharolytic Gram-positive rod-shaped bacteria. Cells are non-motile, do not form spores and exhibit very poor growth in broth media. *Mogibacterium timidum* (formerly *Eubacterium timidum* (9)), nominated due to its slight or slow growth in clumps, is an example of this genera that are regular to slightly diphtheroid in shape. This bacterium has shown to be present in oral environments and to be involved in infectious oral

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diseases.

M. timidum was previously isolated from subgingival biofilm of periodontitis (9). In addition, detection of *M. timidum* increased as the severity of the clinical parameters of gingivitis increased (14), suggesting that this species could contribute to the increased susceptibility of adults to gingivitis and periodontitis. Subsequently, Moore *et al.* (13) demonstrated the presence of *M. timidum* periodontal pockets of "juvenile" and chronic periodontitis (CP). Moreover, *M. timidum* was also correlated to other forms of head and neck infections. Hill *et al.* (8) evaluated the microbial biodiversity of head and neck abscesses (Ludwig's angina) and acute lung and liver infections using culture and biochemical techniques, and identified *M. timidum* in many of the infected sites.

Although *M. timidum* has been shown to play an important role in periodontal disease, no study as of yet has compared the frequency of the detection of this bacterium between chronic and aggressive periodontitis (AgP). In addition, the levels of *M. timidum* in CP diabetic subjects, which present an increased risk for periodontitis and other infections (12), have not been previously evaluated. Diabetes mellitus (DM) and poor glycemic control are considered important risk factors for periodontitis and have exhibited a negative influence on subgingival microbiota (4,10). Therefore, the aim of this study was to evaluate the frequency of detection of *M. timidum* in subgingival samples of subjects with aggressive periodontitis (AgP) and uncontrolled type 2 diabetic and non-diabetic subjects with chronic periodontitis (ChP).

MATERIALS AND METHODS

Study population

Non-diabetic patients presenting generalized GAgP (n=48) and generalized GChP (n=50) were selected from population referred to Periodontal Clinic at Piracicaba Dental School, from December 2004 until February 2009. 39 subjects presenting GChP and type 2 DM for at least the past 5 years were selected from the population referred to the Periodontal Clinic at Guarulhos University, from July 2007 until January 2008. All eligible subjects were thoroughly informed of the nature, potential risks and benefits of their participation in the study and signed their informed consent.

GAgP and GChP were diagnosed based on the periodontal classification of the American Academy of Periodontology (1). Subjects had at least 15 teeth and needed to meet the following criteria in order to be included in this study:

GAgP:

- At least six permanent incisors and/or first molars with at least one site each with probing depth (PD) and clinical attachment level (CAL) > 4 mm and bleeding on probing (BoP);

- At least of three teeth other than first molars and incisors with at least one site each with PD and CAL > 4 mm and BoP

- At least 2 sites with PD and CAL $\geq$ 7 mm and BoP;

- Good general health;

- < 35 years old;

GChP (diabetic and non-diabetic)

- > 35 years old;

- At least 30% of the sites with PD and clinical attachment level (CAL) > 4 mm and BoP.

- At least 2 sites with PD and CAL $\geq$ 7 mm and BoP;

Exclusion criteria for all groups were pregnancy, lactation, current smoking and smoking within the past 5 years, periodontal or/and antibiotic therapies in the previous 6 months, use of mouthrinse containing antimicrobials in the preceding 3 months, any systemic condition (except DM) that could affect the progression of periodontal disease (e.g. immunological disorders) and long-term administration of anti-inflammatory and immunosuppressive medications. Subjects with periapical pathology, orthodontic appliances and multiple systemic complications of DM were also excluded from the study.

Glycemic status

A single laboratory (Clinical Analysis Laboratory, Guarulhos University) performed the glycated hemoglobin (HbA1c) monitoring of the diabetic subjects. HbA1c (%) was measured by high-performance liquid chromatography. Poorly-controlled diabetic subjects who had HbA1c values > 7% were included in the present study.

Clinical parameters

The following parameters were assessed at six sites of all teeth, excluding third molars (mesio-buccal, medio-buccal, disto-buccal, mesio-lingual, medio-lingual, disto-lingual), using a manual periodontal probe (UNC15, Hu-Friedy, Chicago, IL, USA): plaque index (PI), BoP, suppuration (SUP), PD (mm) and CAL (mm).

Experimental groups

Based on their glycemic and periodontal characteristics, the subjects were divided into one of the following groups: GAgP (n=48), non-diabetic with GChP (50) and poorly controlled type 2 diabetic subjects with GChP (n=39)

M. timidum detection

Subgingival biofilm were collected from 2 deep pockets (PD $\geq$ 7mm) per subject using a Gracey curette. Prior to sampling, supragingival plaque was removed with sterile cotton pellets and the sites were isolated with cotton rolls to avoid saliva contamination. DNA was extracted as previously described by Saito *et al.* (18). After DNA extraction, the presence of *M. timidum* was determined by a Nested Polymerase Chain Reaction (Nested PCR). The outer and inner primers were based on Mayanagi *et al.* (11). PCR products were then loaded on a 1% agarose gel stained with ethidium bromide and amplicons were detected under UV light.

Statistical Analysis

Demographic and clinical comparisons were performed by one way ANOVA. When there were significant differences by one way ANOVA, a pairwise comparison was performed by Tukey test. Gender distribution and *M. timidum* frequencies were compared using Chi-Square test. The level of significance was set at 5% for all statistical analysis.

RESULTS

Clinical and demographic results

Table 1 shows the demographic and clinical characteristics of study population according to periodontal and systemic diagnosis. No differences between groups were observed in all clinical parameters or gender distribution. GAgP subjects were younger (27.6 $\pm$ 0.9 years) than non-diabetic (43.6±8.3 years) and diabetic subjects with GChP (56.5 $\pm$ 9.5 years). Hb1Ac levels in diabetic group was 10.4 $\pm$ 2.6%

Table 1. Demographic characteristics of the study population and full-mouth clinical parameters (mean ± SD).

|	GAgP	Non-diabetic with GChP	Type 2 diabetic with GChP
Age	27.6±0.9 a	43.6 ± 8.3 b	56.4 ± 9.5 b
Gender (M/F)	13/35	16/34	21/22
PI (%)	55.4 ± 18,5	69.8 ± 33.7	76.5 ± 30.6
BoP (%)	39.5 ± 29.8	52.3 ± 34.6	54.1 ± 33.7
PD (mm)	3.3 ± 4.6	3.8 ± 4.5	3.6 ± 4.4
CAL (mm)	4.1 ± 1.0	4.4 ± 1.1	4.4 ± 0.9
Hb1Ac	-	-	10.4 ± 2.6

Different letters indicate statistical differences among groups (one way-ANOVA/Tukey; p<0.05). SD – Standard Deviation

M. timidum detection results

Figure 1 presents the frequency of detection of *M. timidum* among groups. There were no differences in the frequency of detection of M. *timidium* between non-diabetic subjects with GChP and GAgP and type 2 diabetic subjects with GChP (p>0.05). However, diabetic subjects with GChP presented higher frequency of detection of *M. timidium* when compared to GAgP (p<0.05). *M. timidum* was detected in 35% of subjects diagnosed with GAgP, while in 40% of the non-diabetic subjects with GChP harbored by this bacterium (p>0.05). GChP subjects presenting uncontrolled DM showed the highest detection frequency of *M. timidum* (56%)

No correlation to glycemic control was observed, with no relationship between presence of *M. timidum* and HbAc1 level (data no show).
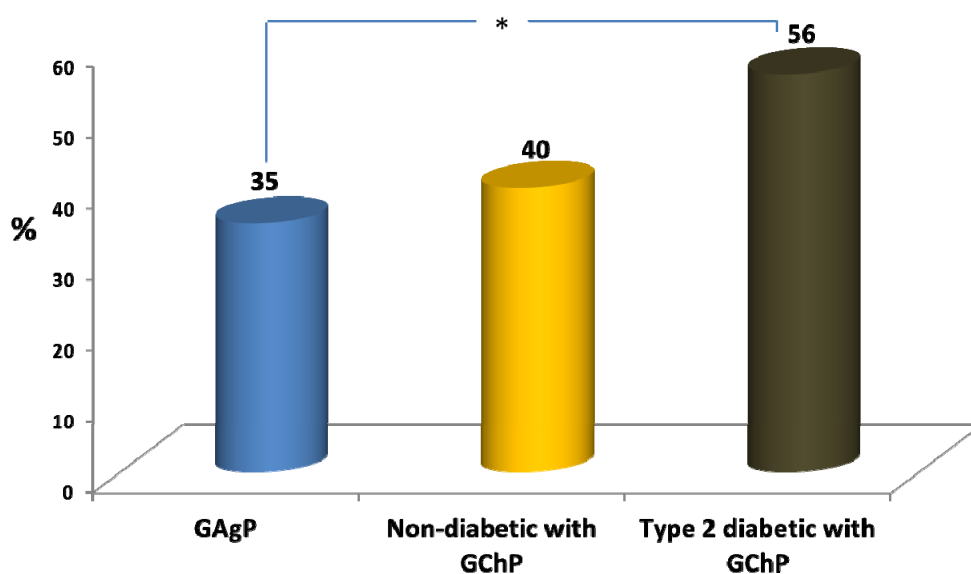
Figure 1. Frequency of *M. timidum* detection in all experimental groups (Chi-square test, p<0.05). GAgP: generalized aggressive periodontitis; GChP: generalized chronic periodontitis.

DISCUSSION

M. timidum has been described as an oral pathogen, usually detected in the subgingival environment and related to the severity of periodontal breakdown. Since there are no studies comparing the levels of *M. timidum* among different types of periodontitis and groups at risk for periodontitis, this study evaluated for the first time the frequency of detection of this species on subgingival samples from GAgP and ChP, associated and not associated with DM. Overall, our results confirm that deep periodontal pockets may shelter *M. timidium*, independent of the type of periodontitis. In addition, the present findings show that the frequency of *M. timidium* was similar between GAgP and GChP, but significantly increased when the GChP was associated with DM with poor glycemic control. Together, these data suggest that *M. timidum* may be associated with periodontitis and that GChP subjects under the challenge of hyperglycemia may be at increased risk for *M. timidium* colonization in deep pockets.

Several microbiological techniques have been used to identify *M. timidum* in different oral samples. By means of the PCR with species-specific oligonucleotides, *M. timidum* has

been detected in endodontic infections (7), periodontitis (11) and other head and neck infection foci (3). Other techniques have been also applied, such as checkerboard DNA-DNA hybridization and 16S rDNA clone library analysis with broad detection primers. In the present study, nested PCR was used for the detection of *M. timidum* in several samples from deep periodontal pockets, which is a modification of conventional PCR able to reduce the contamination of products due to the amplification of unexpected primer binding sites.

In 1980, Holdeman *et al.* (9) were the first to demonstrate the presence of *M. timidum* in periodontal pockets. After that, Moore et al. (13-15), in sequential studies, also determined the potential associations between *M. timidum* and periodontal disease. In their first study, the detection rates of such a species in periodontal pockets from ChP patients (formerly adult periodontitis) were 70% among the affected population (14). In addition, the abovementioned research group has also used the experimentally-induced gingivitis model, in which the prevalence of this microorganism was superior in adults than in youngsters (9 and 3%, respectively). Interestingly, *M. timidum* detection frequencies increased as the severity of the clinical parameters for gingivitis worsened (14). The authors suggested that *M. timidum* could contribute to adults' increased susceptibility to experimental gingivitis and to periodontitis. Later, Moore *et al.* (13) also detected *M. timidum* in juvenile periodontitis (now AgP). The frequencies of M. *timidum* detection were 58%, 70% and 11% for AgP, severe ChP and periodontally-healthy subjects, respectively. These results corroborate the findings of the present study, in which the levels of detection of *M. timidum* in AgP were also modestly less pronounced than in ChP (35 versus 40%, respectively). However, differences in the frequencies of *M. timidium* detection between the work of Moore et al. and the present study may be attributed to differences in the characteristics of the studies' populations, periodontal disease severity and microbiological techniques.

In the last decade, the role of *M. timidum* on periodontitis etiology has been investigated by a series of studies. In 2004, Booth et al. (2) detected *M. timidum* in shallow pockets of ChP patients by means of a non-cultivable technique (slot-blot hybridization). However, since the detection limit of this methodology was approximately 10^4 bacteria, the authors stated that their results should be carefully considered. Mayanagi *et al.* (11) evaluated the presence of *M. timidum* in sub- and supragingival biofilm from periodontitis patients and healthy individuals. Patients with periodontal disease presented high detection rates of *M. timidium* in comparison to healthy individuals. In supragingival biofilm, whilst only 15% of healthy individuals presented *M. timidum*, the species was detected in 65% of periodontitis patients. In addition, 10% and 70% of the subgingival biofilm from healthy and periodontitis patients, respectively, were infected with *M. timidum*. Recently, Colombo *et al.* (5), using a microarray methodology, revealed that periodontitis patients harboring higher amounts of *M. timidum* displayed a refractory type of periodontitis, resulting in lower gain of clinical attachment levels. In general, in agreement with the present results, all of these studies produced evidence that *M. timidum* may be an important pathogen in periodontitis.

Besides the confirmatory findings of the presence of *M. timidum* in periodontal pockets, the results of the present study contributed to the original information available regarding the increased colonization of *M. timidum* in periodontal pockets of diabetic subjects with GChP. We found that diabetic individuals with poor glycemic control presented the highest frequency of *M. timidum* detection. In fact, DM is a well-established risk factor for periodontitis, and an altered immune-inflammatory response seems to be the primary mechanism that could explain the increased severity and progression of periodontitis in diabetic subjects (12). Moreover, poorly controlled diabetic subjects, presenting elevated levels of HbA1c, show higher attachment and alveolar bone loss and exacerbated local inflammation when compared to well-controlled ones (6,17). In addition to the poorer inflammatory

immune response against pathogens, the subgingival microbial profile could also be a possible explanation for the increased risk of periodontitis in diabetic subjects. Intensified local inflammation in diabetic subjects with inadequate glycemic control could modify the subgingival environment and, consequently, the subgingival microbial profile. Recently, our research group has demonstrated that glycemic control positively correlates with the herpes virus colonization in periodontal pockets (4). In addition, Makiura *et al.* (10) showed that *Porphyromonas gingivalis* was detected more frequently in subjects with increased HbA1c values after periodontal treatment than in those patients with reduced HbA1c values.

Therefore, the study of specific microorganisms—such as *M. timidum* in the subgingival biofilm of groups susceptible to periodontitis, including diabetic subjects—seems to be important to improving knowledge about the relationship between DM and severe periodontitis development. Moreover, it is important attempt to the relatively low detection of *M. timidum* in periodontal sites. In all three groups included in this study, this bacterium was detected in approximately half of periodontal sites (although in diabetic subjects this frequency was higher than GAgP). However, it should be considered that more than 400 species are enrolled in periodontal disease and different biofilm composition has been associated to different conditions, for example, smokers, diabetics and systemic disorders. This way, different species could influence periodontal tissues destruction and, considering the results of the present study, *M. timidum* should be considered in future analysis. Therefore, considering that subgingival biofilm was collected from deep pockets of patients presenting generalized and severe periodontal destruction, and that *M. timidum* is almost absent in health sites, it could indicate the important role of *M. timidum* in sites exhibiting periodontal breakdown and could be enrolled in progression of disease in some cases. With this in mind, future studies should consider the significance of these results and identify the pathogenic pathway with which *M. timidum* could be associated. Thus, the

next step to consider in future analysis is the virulence factors and pathogen-associated molecular patterns of this species, determining its real role in periodontal breakdown and identifying a therapeutic protocol to control its infection. Altogether, considering its possible virulence factors and its high prevalence in periodontitis biofilm, *M. timidum* harbors high potential to initiate and/or to increase the inflammatory response in periodontal tissues, especially in patients presenting DM with poor glycemic control.

CONCLUSION

M. timidum was highly prevalent in both GAgP and GChP, but its prevalence was considerably increased when the GChP was associated with DM and poor glycemic control.

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