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Prevalence of periodontal pathogens in Brazilians with aggressive or chronic periodontitis

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Abstract

Objectives: Previous studies suggest differences between geographically and racially distinct populations in the prevalence of periodontopathic bacteria as well as greater periodontal destruction associated with infection by highly leucotoxic *Actinobacillus actinomycetemcomitans*. The present study examined these hypotheses in Brazilians with aggressive or chronic periodontitis.

Materials and Methods: Clinical, radiographical, and microbiological assessments were performed on 25 aggressive periodontitis and 178 chronic periodontitis patients including 71 males and 132 females, 15–69 years of age.

Results: The prevalence of *Porphyromonas gingivalis* was similar to that of other South American populations. The prevalence of *A. actinomycetemcomitans* and its highly leucotoxic subgroup was higher in Brazilians. Highly leucotoxic *A. actinomycetemcomitans* was more prevalent in aggressive periodontitis ($\chi^2 = 27.83$) and positively associated with deep pockets (>6 mm, $\chi^2 = 18.26$) and young age (<29 years, $\chi^2 = 18.68$). Greater mean attachment loss was found in subjects with highly leucotoxic *A. actinomycetemcomitans* than in subjects with minimally leucotoxic ($p = 0.0029$) or subjects not infected ($p = 0.0001$).

Conclusion: These data support the hypothesis of differences between populations in the prevalence of periodontopathic bacteria and of greater attachment loss in sites infected with highly leucotoxic *A. actinomycetemcomitans*. Detection of highly leucotoxic *A. actinomycetemcomitans* in children and adolescents may be a useful marker for aggressive periodontitis.

Key words: bacterial pathogens; leucotoxin; periodontal disease; periodontitis

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Numerous studies have examined the prevalence of periodontal pathogens in populations in Europe and the United States. However, fewer studies have examined the prevalence of periodontal pathogens in other parts of the world. Determining the global distribution of periodontal pathogens may be important since infectious agents frequently have limited geographic distributions. Notable examples of infectious agents with limited geographic distribution include *Rickettsia rickettsii*, the cause of Rocky Mountain Spotted Fever (Rizzo et al. 2004), *Borrelia burgdorferi*, the cause of Lyme Disease (Steere et al. 2004), and *Plasmodium falciparum*, the cause of malaria (Basco et al. 2002). Like these microorganisms, there may be differences in the global distribution of periodontopathic bacterial species putting some populations at greater risk for infection and periodontal disease than other populations. Such variations in the global distribution of periodontopathic bacteria could be important in the epidemiology and the treatment of periodontal diseases.

There is some evidence suggesting that there are, in fact, differences in the global distribution of periodontopathic bacteria and subspecies groups. For example, adults in rural Kenya and rural Thailand have higher rates of subgingival infection with species such as *Actinobacillus actinomycetemcomitans, Prevotella intermedia,* and *Porphyromonas gingivalis* than do populations in developed countries (Dahlen et al.

1989, 2002). The frequency of serotype b, *A. actinomycetemcomitans* is elevated in Americans (Zambon et al. 1983a, b, Yang et al. 2004), with aggressive periodontitis, but not in Koreans (Chung et al. 1989), Chinese (Wang et al. 1997), or Turks (Celenligil & Ebersole 1998). The highly leucotoxic clone of *A. actinomycetemcomitans* more likely infects people of African descent (Haubek et al. 1996, 1997, 2001) than in northern Europeans (Haubek et al. 1995) or Chinese (Mombelli et al. 1998, 1999, Wang et al. 2000).

In view of the limited data available on the global distribution of periodontopathic bacteria and subspecies groups, the present study examined the prevalence of *A. actinomycetemcomitans, Campylobacter rectus, P. gingivalis, P. intermedia,* and *Tannerella forsythensis* and the extent of periodontal destruction associated with infection by highly leucotoxic *A. actinomycetemcomitans* in Brazilians with aggressive or chronic periodontitis.

Materials and Methods

Subjects

Two hundred and three subjects, including 71 males and 132 females, 15–69 years of age, participated in this study. Subjects were categorized as having aggressive periodontitis or chronic periodontitis. Twenty-five subjects were diagnosed with aggressive periodontitis and had rapid attachment loss, bone loss, and familial aggregation (Lang et al. 1999). One hundred seventy-eight subjects were diagnosed with chronic periodontitis and had at least four sites with pocket depths >4 mm and at least four sites with attachment level measurements <4 mm, as well as radiographic evidence of alveolar bone loss.

Subjects were excluded if they (1) had fewer than 20 teeth; (2) had a chronic medical disease or condition; (3) required antibiotic pre-medication prior to dental treatment; (4) had taken antibiotics within 6 months prior to the clinical examination; or (5) had received periodontal therapy within the previous 6 months. The study protocol was reviewed and approved by the institutional review board at the University of Taubate, Sao Paulo, Brazil and was explained to potential subjects, who indicated their acceptance by signing a consent form.

Clinical procedures

Clinical measures of dental plaque, bleeding on probing, probing pocket depth, and clinical attachment level were made on the first molars (or, if missing, second molar) and maxillary right and mandibular left central incisors. Dental plaque and bleeding on probing were recorded as a ''1'' if present anywhere on these individual teeth or as a ''0'' if not present. Probing pocket depth was measured at six sites on each of these teeth using a Michigan ''O'' probe. Dental plaque, bleeding on probing, and probing pocket depth data were averaged for each subject.

Microbial sampling and laboratory procedures

Subgingival plaque samples were obtained from the mesial surface of the first molars (or, if missing, second molar) and maxillary right and mandibular left central incisors from each subject. Supragingival dental plaque was first removed using a sterile curette, and then one sterile paper point (fine, Johnson and Johnson, New Brunswick, NJ, USA) was inserted into the gingival sulcus/periodontal pocket and left in place for 10 s. The six paper points were placed into a single vial containing 1.0 ml of phosphate-buffered saline (pH 7.4). The bacterial cells were dispersed by vortexing at maximal setting for 1 min. and then maintained at -20°C until laboratory processing.

In the laboratory, the bacterial suspensions were thawed, centrifuged at $12{,}000 \times g$ for 1 min, and the DNA was extracted from the pellet (Instagen purification matrix, BioRad Laboratories, Hercules, CA, USA). Bacterial 16S rDNA was amplified from the extracted DNA by the polymerase chain reaction (PCR) using primers that amplify approximately 1500 bp of the 16S rDNA sequence. Between 30 and 100 ng of genomic DNA was added to a PCR mixture containing 1 µmol/l of the primers, 2.5 U of Taq polymerase in $1\times$ buffer, and 0.2 mmol/l of dCTP, dGTP, dATP, and dTTP in a total volume of 50 µl. The amplification was performed for 30 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C in thermocycler. A positive and a negative control were included with each set. The PCR product was transferred overnight onto charged nylon membranes using $10\times$ standard salt phosphate EDTA buffer (SSPE) and probed with digoxigenin-labelled species-specific oligonucleotides for *A. actinomycetemcomitans* (CACTTAAAGGTCC GCCTACGTG CC), *C. rectus* (CAAGCTACTTAATC TCCGTTCGAC), *P. gingivalis* (GC AGTTTCAACGGCAGGGCTGAACG), *P. intermedia* (GGTCCTTATTCGAA GGGTAAATGC), and *T. forsythensis* (CGTATCTCATTTTATTCCCCTGTA) (Dix et al. 1990, Dewhirst et al. 1992). Hybridization with digoxigenin-labelled oligonucleotide probes was performed at 45°C. The hybridized membranes were washed twice with high salt solution ($2\times$ SCC [0.15 M NaCl plus 0.015 trisodium citrate, pH 7.0] in 0.1% sodium dodecyl sulphate) at room temperature, and then twice with low salt solution ($0.1\times$ SCC in 0.1% sodium dodecyl sulphate) at 45°C. The membranes were reacted with anti-digoxigenin alkaline phosphatase conjugate. The colour reaction was produced with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salts.

To differentiate minimally leucotoxic from highly leucotoxic *A. actinomycetemcomitans*, PCR was performed on the extracted DNA using two oligonucleotide primers directed towards the *A. actinomycetemcomitans* leucotoxin promoter region (Brogan et al. 1994) – A - 5′-GCAGGATCCATATTAAATCT CCTTGT-3′ and B - 5′-GCGGTCGAC AACCTGATAACAGTATT-3′. These primers amplify either a 492 bp product characteristic of highly leucotoxic *A. actinomycetemcomitans* or a 1022 bp product characteristic of minimally leucotoxic *A. actinomycetemcomitans*. PCR was performed using standard conditions. The reaction mixture consisted of 100 ul of 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 100 µM each of dATP, dCTP, dGTP, dTTP (Amersham, Arlington Heights, IL, USA), 0.001% (w/v) gelatin, 100 ng of each primer (Invitrogen/Gibco, San Diego, CA, USA), 0.5 U Taq polymerase, and 100 ng of genomic DNA. The amplification was performed for 30 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C in a thermocycler. The amplification products were analysed by electrophoresis in 1.5% submarine agarose gels with ethidium bromide, and visualized by UV illumination staining. Positive (*A. actinomycetemcomitans* strain JP2) and negative (*A. actinomycetemcomitans* strain 652) controls were included.

Statistical analysis

Differences in the prevalence of the target bacteria were analysed through

the *Z*-test, $p<0.05$. Odds ratios were calculated to determine the relationship between bacterial species, and the χ^2-test was used to determine the statistical significance of the associations. The relationship between variables including age, gender, periodontal diagnosis periodontal pocket depth, bleeding on probing (0/1), plaque index (0/1), and the prevalence of target bacteria, including highly or minimally leucotoxic *A. actinomycetemcomitans,* was evaluated by multivariate logistic regression. The probability (p) of aggressive periodontitis (AP) was calculated as follows:

$$p(\mathrm{AP}) = \frac{1}{1 + \exp(11.22 - 0.42\,\mathrm{age} + 2.65\,A.actinomycetemcomitans)}$$

Mean clinical attachment levels were calculated from the microbiologically sampled teeth in each subject. Mean clinical attachment levels were compared in age- and sex-matched individuals infected with highly leucotoxic *A. actinomycetemcomitans*, infected with minimally leucotoxic *A. actinomycetemcomitans,* or not infected with *A. actinomycetemcomitans* using ANOVA.

Results

Two hundred three subjects were examined in this study. Twenty-five subjects, including six males and 19 females, were diagnosed with aggressive periodontitis and 178 subjects, including 65 males and 113 females, were diagnosed with chronic periodontitis. The mean age of subjects with aggressive periodontitis was 21.9 years, and the mean age of subjects with chronic periodontitis was 39.1 years. Mean values for dental plaque were significantly higher in subjects with chronic periodontitis than in subjects with aggressive periodontitis (0.65 *versus* 0.43, respectively, $p = 0.0004$). Mean probing pocket depths were significantly higher in subjects with aggressive periodontitis compared with subjects with chronic periodontitis (6.49 *versus* 5.59 mm, respectively, $p = 0.0001$).

Subjects with aggressive periodontitis were found almost exclusively in the 10–20 and 21–30 year age groups (Fig. 1). With the exception of the 61–70 age group, females outnumbered males in both the aggressive periodontitis and chronic periodontitis categories in each age group. *P. gingivalis* was most prevalent in both aggressive periodontitis and chronic periodontitis (Table 1). *A. actinomycetemcomitans* ($p = 0.001$) and *C. rectus* ($p = 0.047$) were found in higher prevalence in subjects with aggressive periodontitis compared with subjects with chronic periodontitis. The prevalence of each of the five target pathogens was higher in aggressive periodontitis subjects compared with chronic periodontitis subjects in each age group (data not shown).

Highly leucotoxic *A. actinomycetemcomitans* was most prevalent in subjects with aggressive periodontitis ($\chi^2 = 27.83$). Fifty-two percent of subjects with aggressive periodontitis harboured highly leucotoxic *A. actinomycetemcomitans,* while 11% harboured minimally leucotoxic *A. actinomycetemcomitans*. Eighty-three percent of the aggressive periodontitis subjects positive for *A. actinomycetemcomitans* harboured highly leucotoxic *A. actinomycetemcomitans,* while 17% of the positive subjects harboured minimally leucotoxic *A. actinomycetemcomitans*. By contrast, 8% of subjects with chronic periodontitis harboured highly leucotoxic *A. actinomycetemcomitans,* while 34% harboured minimally leucotoxic *A. actinomycetemcomitans*. Nineteen percent of the chronic periodontitis subjects positive for *A. actinomycetemcomitans* harboured highly leucotoxic *A. actinomycetemcomitans,* while 81% of the positive subjects harboured minimally leucotoxic *A. actinomycetemcomitans*. Odds ratio analysis showed a positive association between *A. actinomycetemcomitans* and *C. rectus* ($p<0.05$) in subjects with aggressive periodontitis. In subjects with chronic periodontitis, there were significant relationships among *A. actinomycetemcomitans*, *C. rectus*, *P. gingivalis*, and *T. forsythensis*; between *C. rectus*, *P. gingivalis*, and *P. intermedia*; and between both *P. gingivalis* and *P. intermedia* with *T. forsythensis*.

Multiple logistic regression analysis showed a statistically significant negative correlation between age and the presence of *A. actinomycetemcomitans* ($\chi^2 = -0.421$, $p = 0.0018$). That is, younger subjects were more likely to be infected with *A. actinomycetemcomitans* than were older subjects. There was no demonstrable effect of gender on either the prevalence of the target periodontal pathogens or on periodontal diagnosis. There were also significant positive correlations (Table 2) between the presence of highly leucotoxic *A. actinomycetemcomitans* and the diagnosis of aggressive periodontitis ($\chi^2 = 27.83$; $p = 0.013$), younger age ($\chi^2 = 18.68$; $p = 0.0015$), and probing pocket depths greater than 6 mm ($\chi^2 = 18.26$, $p = 0.019$). By contrast, minimally leucotoxic *A. actinomycetemcomitans* was more frequently detected in subjects with probing pocket depths of 4–6 mm. There was no association between the presence of either highly or

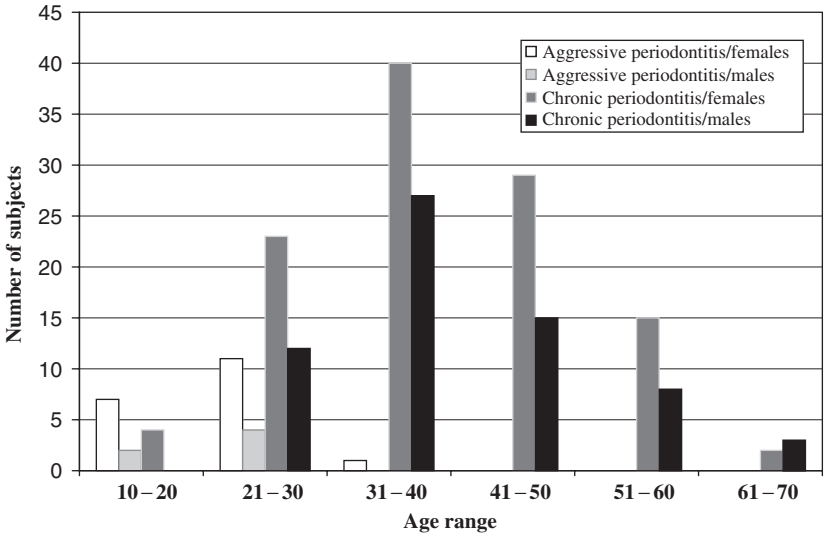

Fig. 1. Age and gender distribution of subjects.

Table 1. Prevalence of target pathogens

Target pathogen	Aggressive periodontitis (%)	Chronic periodontitis (%)	Z-value	*p*-value
*Actinobacillus actinomycetemcomitans**	72	41.6	3.134	0.001
*Campylobacter rectus**	48	30.3	1.671	0.047
Porphyromonas gingivalis	80	68.0	1.377	0.084
Prevotella intermedia	52	53.4	0.128	0.449
Tannerella forsythensis	56	45.5	0.989	0.161

*Statistically significant.

Table 2. Association between highly or minimally leucotoxic *Actinobacillus actinomycetemcomitans* and periodontal diagnosis, age, gender, and clinical variables

Variables	Highly leucotoxic	Minimally leucotoxic	χ^2	*p*-value
Diagnosis				
Chronic periodontitis	14	60	27.83	0.013
Aggressive periodontitis	15	3		
Gender				
Male	12	17	1.91	0.167
Female	17	46		
Age				
14–28 years of age	18	11	18.68	0.0015
29–39 years of age	7	22		
40–76 years of age	4	30		
Plaque				
+	17	36	0.02	0.893
–	12	27		
BOP				
+	18	28	2.47	0.116
–	11	35		
PD				
4–6 mm	8	47	18.26	0.019
⩾7 mm	21	16		

BOP, bleeding on probing; PD, pocket depth.

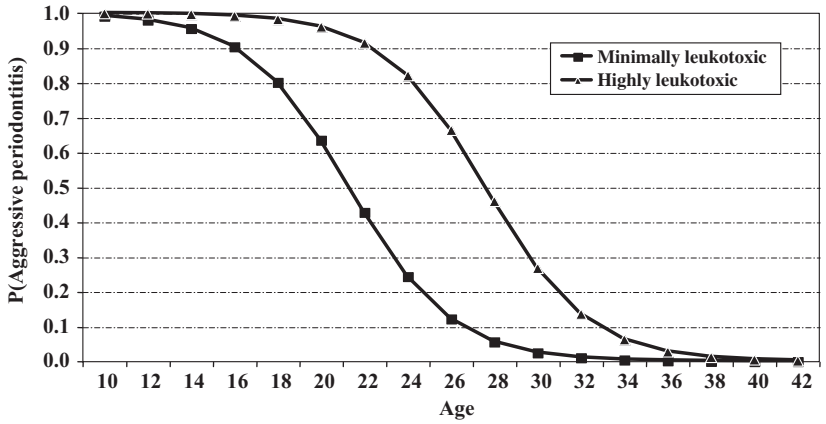

Fig. 2. Probability of aggressive periodontitis by the presence of highly leucotoxic or minimally leucotoxic *Actinobacillus actinomycetemcomitans*.

minimally leucotoxic *A. actinomycetemcomitans* and dichotomous measures of dental plaque, bleeding on probing, or gender. Comparing mean attachment levels for the microbiologically sampled teeth in age- and sex-matched individuals, subjects infected with highly leucotoxic *A. actinomycetemcomitans* demonstrated more clinical attachment loss than subjects infected with minimally leucotoxic *A. actinomycetemcomitans* ($p = 0.0029$) or subjects not infected with *A. actinomycetemcomitans* ($p = 0.0001$). Further, subjects infected with minimally leucotoxic *A. actinomycetemcomitans* demonstrated more clinical attachment loss than subjects ($p = 0.0037$) not infected with *A. actinomycetemcomitans.*

Young people harbouring *A. actinomycetemcomitans* had a significantly higher probability of having aggressive periodontitis compared with subjects of the same age without *A. actinomycetemcomitans*. Subjects 20 years of age or younger infected with highly leucotoxic *A. actinomycetemcomitans* had a nearly 100% probability of having aggressive periodontitis (Fig. 2) while subjects 30 years of age and older infected with highly leucotoxic *A. actinomycetemcomitans* had a greatly reduced probability of having aggressive periodontitis.

Discussion

We studied the prevalence of five periodontal pathogens in 203 Brazilians diagnosed with either aggressive or chronic periodontitis. As might be expected, subjects with aggressive periodontitis were found primarily in younger age groups, while subjects with chronic periodontitis were found in all age groups. Consistent with previous reports, the number of subjects between 10 and 30 years of age with aggressive periodontitis (Albandar et al. 2002) increased with age as did the number of older subjects with chronic periodontitis (Loe & Brown, 1991).

Previous studies of Brazilians report a similarly high prevalence of these pathogens, especially in cases of untreated aggressive and chronic periodontitis. For example, Tinoco et al. (1997) found that the prevalence of *A. actinomycetemcomitans* was 80% in 25 adolescents with localized juvenile periodontitis. However, the present study of Brazilians differs from studies of another South American population – Chile. Lopez et al. (1996) reported the prevalence of *A. actinomycetemcomitans* in Chileans with juvenile periodontitis patients at 29% or less than half that of our Brazilian population. Similarly, Gajardo et al. (2005) found the prevalence of *A. actinomycetemcomitans* in their Chilean aggressive periodontitis cohort to be 19% or about one-third that of our Brazilian population. Their chronic periodontitis cohort, in fact, had a higher prevalence of *A. actinomycetemcomitans* than did their aggressive periodontitis group – 35.3% *versus* 19.4%, respectively.

Similar to our Brazilian cohort, the prevalence of *P. gingivalis* in this Chi-

lean population was elevated in both the aggressive periodontitis and the advanced chronic periodontitis groups – 88.9% and 76%, respectively. The present study of Brazilians with chronic periodontitis is also consistent with a third study of Chileans with chronic periodontitis, indicating that they harbour higher proportions of ''red complex'' bacteria, particularly *P. gingivalis,* than do Americans with chronic periodontitis (Lopez et al. 2004). Thus, studies of Brazilians and Chileans with chronic periodontitis are consistent in their findings of a high prevalence of *P. gingivalis,* but differ in the findings for *A. actinomycetemcomitans* in aggressive periodontitis in which the prevalence in Brazilians is higher than that reported for Chileans.

Accordingly, differences between Brazil and Chile in the proportion of the population of African ancestry may account for differences in the prevalence of *A. actinomycetemcomitans*. Over 45% of the population of Brazil is of African ancestry, and Brazil has the largest population of African descendents in the Pan American region with nearly 75 million (Pan American, 2003). Even among the white population of Brazil, there is significant African ancestry. A study of mtDNAs in 247 white Brazilians demonstrated nearly equal amounts of Native American, African, and European matrilineal genetic contribution (Alves-Silva et al. 2000). Together with the United States' 36 million people of African descent, Brazil and the United States have the largest number of African descendents outside of Africa (Pan American, 2003). The population of Chile, by contrast, is less than 2% African descent. Since *A. actinomycetemcomitans* is reported to be found in higher prevalence in people of African descent and since the highly leucotoxic clone of *A. actinomycetemcomitans* is thought to be of African origin (Haubek et al. 1997), a population such as that of Brazil with a high proportion of people of African origin might be more likely to, as in the present study, demonstrate both a higher prevalence of *A. actinomycetemcomitans* and a higher prevalence of highly leucotoxic *A. actinomycetemcomitans* than would populations with lower proportions of people of African origin such as Chile. Significant differences in the prevalence of periodontal pathogens in racial groups living in the same geographic area have previously been reported. For example, Chinese living in the U.S. have a lower prevalence of *A. actinomycetemcomitans* (Cao et al. 1990), while African-Americans and Hispanic-Americans have a higher prevalence of *A. actinomycetemcomitans* and *P. gingivalis* (Schenkein et al. 1993, Beck et al. 1992, Umeda et al. 1998).

There appear to be clear differences in the prevalence of highly leucotoxic *A. actinomycetemcomitans* between patients with aggressive periodontitis and patients with chronic periodontitis. In the present study, 83% of the patients with aggressive periodontitis had highly leucotoxic *A. actinomycetemcomitans,* while 19% of the patients with chronic periodontitis had highly leucotoxic *A. actinomycetemcomitans*. Our previous studies of patients with aggressive periodontitis in the United States are consistent with the present study of patients with aggressive periodontitis in Brazil in finding a high prevalence of highly leucotoxic *A. actinomycetemcomitans*. We found then, as in the present study, that highly leucotoxic *A. actinomycetemcomitans* infected younger subjects (mean age 14 years), while minimally leucotoxic *A. actinomycetemcomitans* infected older subjects (mean age 35 years, Haraszthy et al. 2000). However, in contrast to our previous study in which we found highly leucotoxic *A. actinomycetemcomitans* only in subjects with localized juvenile or early-onset periodontitis (Haraszthy et al. 2000), in the present study, we also found highly leucotoxic *A. actinomycetemcomitans* in a small proportion of chronic periodontitis patients.

In this cross-sectional study, we found greater clinical attachment loss associated with infection by highly leucotoxic *A. actinomycetemcomitans,* as compared with infection by minimally leucotoxic *A. actinomycetemcomitans*. Our data are consistent with the results of a 2-year longitudinal study of 121 adolescents in Morocco (Haubek et al. 2004), reporting that subjects initially infected with highly leucotoxic *A. actinomycetemcomitans* had a significantly higher progression of clinical attachment loss than subjects harbouring minimally leucotoxic *A. actinomycetemcomitans*. These studies point to the importance of leucotoxin in the pathogenesis of *A. actinomycetemcomitans*-associated periodontitis, and suggest that the highly leucotoxic clone of *A. actinomycetemcomitans,* rather than the entire species, might be a particularly useful target for the diagnosis and/or treatment of aggressive periodontitis.

A systematic review of 11 published studies (Mombelli et al. 2002) found that the presence or absence of *A. actinomycetemcomitans* could not discriminate subjects with aggressive periodontitis from those with chronic periodontitis, but infection with highly leucotoxic *A. actinomycetemcomitans* was uniquely associated with aggressive periodontitis. Accordingly, it has been suggested that children at risk of harbouring highly leucotoxic *A. actinomycetemcomitans* by virtue of race or ethnicity might benefit from frequent periodontal examination (Haubek & Westergaard 2004) to detect incipient aggressive periodontitis. In the present study, subjects 20 years of age and younger infected with highly leucotoxic *A. actinomycetemcomitans* had a nearly 100% probability of having aggressive periodontitis. Together, the data point to the potential value of laboratory tests for subgingival infection with highly leucotoxic *A. actinomycetemcomitans* in children and adolescents of African descent for the diagnosis and, possibly, prevention of aggressive periodontitis.

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Clinical Relevance

Scientific rationale for the study: Recent data suggest that the pre-dilection for infection with periodontopathic bacteria may be related to age, race, or geographic area.

Principal findings: In 203 Brazilians with aggressive or chronic periodontitis, the prevalence of *P. gingivalis* was similar to other groups but the prevalence of *A. actinomycetemcomitans* was higher. Highly leucotoxic *A. actinomycetemcomitans* was associated with greater attachment loss, pockets >6 mm, and age <29 years compared with minimally leucotoxic.

Practical implications: Like risk factors such as diabetes and tobacco, risk for periodontitis and infection with periodontopathic bacteria may be related to age, race, and geographic location.