AGGREGATIBACTER ACTINOMYCETEMCOMITANS AND PREVOTELLA INTERMEDIA IN ADVANCED CHRONIC PERIODONTITIS PATIENTS


ABSTRACT

This cross-sectional study was carried out to identify A. actinomycetemcomitans and P. intermedia in the subgingival plaque of three ethnic groups (Malays, Chinese and Indians) in a selected group of adult Malaysians with advanced Chronic Periodontitis and to correlate these findings with their periodontal status. Thirty periodontally diseased adults were age, gender and ethnically matched with 30 healthy individuals. Clinical parameters were assessed for all. Subgingival plaque samples were collected for identification of A. actinomycetemcomitans and P. intermedia using polymerase chain reaction. Prevalence for P. intermedia (83.3%) was high and A. actinomycetemcomitans (6.7%) low in the total subject population. P. intermedia and A. actinomycetemcomitans were more prevalent in diseased (86.7%, 10% respectively) than in healthy (80%, 3.33% respectively) subjects. A. actinomycetemcomitans was detected in 15% Indians, 5% Malays but none of the Chinese subjects whereas P. intermedia was detected in 90% Malays, 85% Indians and 75% Chinese subjects. No significant association between presence of A. actinomycetemcomitans and P. intermedia with race and periodontal disease status was found. Only A. actinomycetemcomitans had a significant association with clinical attachment level (CAL) (p < 0.05). In conclusion, in this small subject group, none of the pathogens were associated with race and periodontal disease status and only A. actinomycetemcomitans had a significant association with CAL.

Key words: Aggregatibacter actinomycetemcomitans; chronic periodontitis; ethnicity; periodontopathogens; Prevotella intermedia

INTRODUCTION

Periodontitis is a multi-factorial disease with the dental biofilm as its essential component. Between 700 different types of bacteria have been detected in the mouth (1,2) out of which 400 or more species reside in the subgingival biofilm area (3). Most of these bacteria are thought to be an indigenous part of the normal oral flora and not associated with oral diseases. However, Aggregatibacter actinomycetemcomitans (A. Actinomycetemcomitans), Prevotella intermedia (P. intermedia), Porphyromonas gingivalis (P. Gingivalis), Tannarella forsythensis (T. forsythensis) (formerly known as Bacteroides forsythus), Campylobacter rectus, Eikenella corrodens, Fusobacterium nucleatum and some uncultivable spirochaetes have been implicated as major putative pathogens associated with the development and progression of periodontitis (4).

A susceptible host and/ or local environmental conditions that increase susceptibility in addition to the pathogenic bacteria may be required before disease progression occurs (5). Ethnicity, an important variable in the pathogenesis of periodontal disease, also has an influence on the presence and levels of certain bacteria in the subgingival microbiota (6). Differing prevalences of the various putative pathogens like A. actinomycetemcomitans, P. intermedia, P. gingivalis, T. forsythensis and other periodontopathogens have been reported from different parts of the world (7-10) suggesting that there may be specific distribution patterns in ethnically distinct populations. The Malaysian population, made up of 3 major ethnic groups which are the Malays, Chinese and Indians, presents with differences in the extent and severity of periodontal destruction possibly associated with different risk factors. Epidemiological investigations carried out in Malaysia showed that ethnicity may be an important risk factor as the Indian subjects have a higher prevalence of marked periodontal destruction as indicated by deep periodontal pocketing (11).

The presence of A. actinomycetemcomitans and P. intermedia have been established in advanced Chronic Periodontitis, but thus far, no known studies have been done in implicating its presence with ethnicity and clinical periodontal status in the Malaysian population. The objectives of this study are a) to identify the presence of A. actinomycetemcomitans and P. intermedia in the subgingival plaque of the three ethnic groups of selected adult Malaysians with Advanced Chronic Periodontitis and b) to correlate the
microbiological profile of the subjects in relation to A. actinomycetemcomitans and P. intermedia with their clinical periodontal status.

MATERIALS AND METHODS

Clinical examination of patient groups

Sample groups

This study is a cross-sectional study using a convenient sample of 60 adult subjects made up of 30 periodontally diseased patients (referred to the Periodontal Unit, Faculty of Dentistry, University of Malaya from its Primary Dental Care Unit) and 30 healthy subjects (patients from the Primary Dental Care Unit as well as the staff of the Faculty of Dentistry, University of Malaya). In each test and control group, there were 10 Malays, 10 Chinese and 10 Indians. The test and control groups were matched in age (within a range of ± 5 years), sex and ethnicity. Patients were within the age range of 35 – 65 years. For the test group, patients with advanced Chronic Periodontitis were chosen with probing pocket depths of 6 mm or more and clinical loss of attachment of 5mm or more at 4 or more teeth. For the control group, patients with a healthy periodontium were chosen with no clinical attachment loss greater than 3 mm. Patients with known systemic disease and patients who had been on antibiotic cover or who have had periodontal therapy within the past 6 months were excluded from the study. Ethical Clearance had been obtained for this study (Ethical Clearance No: DFPE 0301/0003[P]) from the Ethics Committee, University of Malaya. The nature of the study was explained and written consent was obtained from each patient.

Clinical parameters and measurements

Clinical parameters of the patients that were assessed were Plaque index (12), Gingival index (13), Bleeding index (14), probing pocket depth (PPD) and clinical attachment level (CAL). All measurements for PPD and CAL were recorded to the nearest millimeter with a Williams periodontal probe. The teeth selected were all canines, first or second incisors, first or second premolars and the first or second molars in each quadrant. For the incisors, premolars and molars, teeth with the higher probing pocket depths were chosen. The number of teeth present was also recorded. Clinical measurements were performed by two examiners (author included), and prior to this study a reproducibility study was done to validate intra and inter examiner reproducibility.

Sampling of plaque

Subgingival plaque samples were obtained by first isolating the sites to be sampled with cotton rolls and supragingival plaque was then removed with cotton pellets, before sampling of subgingival plaque was done with sterile curettes. In test subjects, sampling was done at 4 or more teeth with the deepest probing depths (>6mm) which showed bleeding on probing and these plaque samples were pooled. In control patients, sampling was done at interproximal sites that did not show any bleeding on probing. Subgingival scrapings were collected from the base of pockets up to the gingival margin. Scrapings were then resuspended in 1.5 ml of phosphate buffered saline and stored at -80°C until polymerase chain reaction (PCR) procedure was commenced.

PCR identification of bacterial strains

Bacterial strains and DNA template

Bacterial strains used as positive controls were ATCC (American Type Cell Culture) 43718 for A. actinomycetemcomitans and ATCC 25611 for P. intermedia which were obtained from Microbiology Department, King’s College, London. The strains were suspended in Brain Heart Infusion (BHI) media. The DNA extraction was done as follows: 100μl of bacterial sample was incubated for 10 minutes at 95°C. It was then cooled in ice for 5 minutes and centrifuged at 14,500 x g (maximum speed on the Eppendorf Mini spin plus microcentrifuge machine) for 2 minutes to obtain the DNA supernatant. The DNA was then stored at -80°C until used (15).

PCR amplification

The oligonucleotide primers targeting the leukotoxin gene ltx A of the leukotoxin operon (16) were used to detect A. actinomycetemcomitans. The primers used to detect P. intermedia were derived from the 16 S rRNA sequences of P. intermedia sensu strict (15) and were able to distinguish P. intermedia from Prevotella nigrescens.

The protocol used for A. actinomycetemcomitans (17) and P. intermedia were as shown in Table 1. For both A. actinomycetemcomitans and P. intermedia, PCR was carried out at 94°C for 3 minutes for 1 cycle, followed by 35 subsequent cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute (58°C for 40 seconds for P. intermedia) and extension at 72°C for 1 minute (72°C for 2 minute for P. intermedia). The final extension was carried out at 72°C for 10 minutes.

The PCR products were then visualized by illumination of the agarose gel with ultraviolet light and viewed under a transilluminator (Bio Rad, USA). The bands for the positive control for A. actinomycetemcomitans were at 285 bp and for P. intermedia were at 267 bp.

Statistical analysis

Fisher’s Exact test was used to obtain the significance level of association between presence of A. actinomycetemcomitans and P. intermedia and groups (diseased and healthy) as well as ethnicity. Cross tabulation was done to assess detection frequency of single and mixed bacterial infections in
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One way ANOVA was utilized to compare mean clinical scores with presence or absence of *A. actinomycetemcomitans* and *P. intermedia* in both the diseased and healthy groups. Since *A. actinomycetemcomitans* positive cases were low, ANOVA testing for mean CAL score was repeated using Mann-Whitney test. The statistical analysis for the study was done using the SPSS 11.5 for Windows.

RESULTS

A total of 60 patients, 30 males (50%) and 30 females (50%) were examined. Sociodemographic data of subjects is shown in Table 2. The mean age was 46.08±8.11 years. Out of the 60 subjects, 16.7% were smokers. In the diseased group, 23.3% were smokers while only 10% were smokers in the healthy group. In view of the difficulty in recruiting these matched subjects, smokers were not excluded, which may have been a potential cause of confounding bias. No significant difference was found between the clinical parameters of the diseased subjects in the three ethnic groups (Table 3).

As shown in Table 4, the prevalence level for *A. actinomycetemcomitans* in the total subject population was low (6.67%). In the diseased group the prevalence of *A. actinomycetemcomitans* was 10% whereas in the healthy group the prevalence of *A. actinomycetemcomitans* was 3.33%. The prevalence for *A. actinomycetemcomitans* in the total Indian subjects was the highest followed by total Malay subjects. *A. actinomycetemcomitans* was not detected in any of the Chinese subjects.

Table 4 also shows that the presence of *P. intermedia* in the total population as well as diseased and healthy groups were high. In the Indian and Chinese groups, *P. intermedia* was found to be slightly higher in the diseased groups as compared to the healthy population. However in the Malay group, *P. intermedia* was found equally in both diseased and healthy groups.

Race comparisons among the three ethnic groups showed there was no association between the presence of *A. actinomycetemcomitans* and *P. intermedia* and ethnicity. The odds ratio of detecting *A. actinomycetemcomitans* bacteria in diseased as compared to healthy was 3.22 (95% CI=0.32, 32.89). The odds ratio of detecting *P. intermedia* bacteria in diseased as compared to healthy was 1.63 (95% CI=0.41, 6.47).

Analysis of detection frequency of single and mixed bacterial infections showed that of the total 60 subjects, 54 (90%) subjects were found to harbour at least one of the two pathogens. Of these infected subjects harbouring the pathogens. One way ANOVA was utilised to compare mean clinical scores with presence or absence of *A. actinomycetemcomitans* and *P. intermedia* in both the diseased and healthy groups. Since *A. actinomycetemcomitans* positive cases were low, ANOVA testing for mean CAL score was repeated using Mann-Whitney test. The statistical analysis for the study was done using the SPSS 11.5 for Windows.

### Table 1. List of reagents used for PCR protocol

<table>
<thead>
<tr>
<th>Reagents</th>
<th><em>A. actinomycetemcomitans</em></th>
<th><em>P. intermedia</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial suspension</td>
<td>10µl</td>
<td>10µl</td>
</tr>
<tr>
<td>Forward primer (Qiagen Operon)</td>
<td>100pmol</td>
<td>100pmol</td>
</tr>
<tr>
<td>Reverse primer (Qiagen Operon)</td>
<td>100pmol</td>
<td>100pmol</td>
</tr>
<tr>
<td>dNTPs (Finnzymes Oy)</td>
<td>200µM</td>
<td>200µM</td>
</tr>
<tr>
<td>Thermophilic DNA polymerase 10x buffer (Promega, USA)</td>
<td>5µl</td>
<td>5µl</td>
</tr>
<tr>
<td>Magnesium Chloride</td>
<td>2 mM</td>
<td>2 mM</td>
</tr>
<tr>
<td>Taq DNA Polymerase (Promega, USA)</td>
<td>1.5 Units</td>
<td>2.5 Units</td>
</tr>
</tbody>
</table>

In the diseased group, 23.3% were smokers while only 10% were smokers in the healthy group. In view of the difficulty in recruiting these matched subjects, smokers were not excluded, which may have been a potential cause of confounding bias. No significant difference was found between the clinical parameters of the diseased subjects in the three ethnic groups (Table 3).

### Table 2. Socio-demographic characteristics of study subjects (Chi-square for income level and Fisher’s Exact Test for gender, smoking habits and regularity of dental visits)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Diseased</th>
<th>Healthy</th>
<th>Total</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong> Mean (sd)</td>
<td>46.53(8.68)</td>
<td>45.63(7.62)</td>
<td>46.08(8.11)</td>
<td>0.67</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male n(%)</td>
<td>15(50)</td>
<td>15(50)</td>
<td>30(100)</td>
<td>0.6</td>
</tr>
<tr>
<td>Female n(%)</td>
<td>15(50)</td>
<td>15(50)</td>
<td>30(100)</td>
<td></td>
</tr>
<tr>
<td><strong>Income level</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low n(%)</td>
<td>14(46.7)</td>
<td>6(20)</td>
<td>20(33.3)</td>
<td>0.03</td>
</tr>
<tr>
<td>Middle n(%)</td>
<td>14(46.7)</td>
<td>16(53.30)</td>
<td>30(50)</td>
<td></td>
</tr>
<tr>
<td>High n(%)</td>
<td>2(6.6)</td>
<td>8(26.7)</td>
<td>10(16.7)</td>
<td></td>
</tr>
<tr>
<td><strong>Smoking habits</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoker n(%)</td>
<td>7(23.3)</td>
<td>3(10)</td>
<td>10(16.7)</td>
<td></td>
</tr>
<tr>
<td>Non-smoker n(%)</td>
<td>23(76.7)</td>
<td>27(90)</td>
<td>50(83.3)</td>
<td></td>
</tr>
<tr>
<td><strong>Dental visits</strong></td>
<td></td>
<td></td>
<td></td>
<td>0.024</td>
</tr>
<tr>
<td>Regular n(%)</td>
<td>5(16.7%)</td>
<td>13(43.3%)</td>
<td>18(30%)</td>
<td></td>
</tr>
<tr>
<td>Irregular n(%)</td>
<td>25(83.3%)</td>
<td>17(56.7%)</td>
<td>42(70%)</td>
<td></td>
</tr>
</tbody>
</table>

sd= standard deviation
Table 3. Comparison of mean clinical parameters in diseased subjects by ethnicity (ANOVA)

<table>
<thead>
<tr>
<th>Clinical parameters</th>
<th>Malay (mean(sd))</th>
<th>Chinese (mean(sd))</th>
<th>Indian (mean(sd))</th>
<th>Total (mean(sd))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Missing teeth</td>
<td>4.4(3.24)</td>
<td>3.4(2.32)</td>
<td>2.7(2.0)</td>
<td>3.5(2.76)</td>
</tr>
<tr>
<td>Plaque Index</td>
<td>1.03(0.39)</td>
<td>1.09(0.40)</td>
<td>1.09(0.37)</td>
<td>1.07(0.36)</td>
</tr>
<tr>
<td>Gingival Index</td>
<td>1.16(0.31)</td>
<td>1.32(0.44)</td>
<td>1.23(0.22)</td>
<td>1.24(0.36)</td>
</tr>
<tr>
<td>Bleeding Index</td>
<td>68.2(21.5)</td>
<td>76(19.5)</td>
<td>68.7(18.5)</td>
<td>71.0(21.0)</td>
</tr>
<tr>
<td>PPD mean</td>
<td>4.08(0.77)</td>
<td>4.17(0.95)</td>
<td>4.09(2.12)</td>
<td>4.12(0.75)</td>
</tr>
<tr>
<td>CAL mean</td>
<td>5.03(0.99)</td>
<td>5.23(1.26)</td>
<td>5.99(2.19)</td>
<td>5.41(1.15)</td>
</tr>
</tbody>
</table>

All p values >0.05. sd = standard deviation

Table 4. Prevalence of *A. actinomycetemcomitans* and *P. intermedia* in the subgingival plaque of the three ethnic groups and the total population (Fisher’s Exact Test)

<table>
<thead>
<tr>
<th></th>
<th>Malays (n=20)</th>
<th>Chinese (n=20)</th>
<th>Indian (n=20)</th>
<th>All races (n=60)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D n(%)</td>
<td>H n(%)</td>
<td>D n(%)</td>
<td>H n(%)</td>
</tr>
<tr>
<td></td>
<td>Aa -ve</td>
<td>Aa -ve</td>
<td>Aa -ve</td>
<td>Aa -ve</td>
</tr>
<tr>
<td>Aa -ve</td>
<td>9(90)</td>
<td>10(100)</td>
<td>10(100)</td>
<td>27(90)</td>
</tr>
<tr>
<td>Aa -ve</td>
<td>1(10)</td>
<td>1(10)</td>
<td>2(20)</td>
<td>4(40)</td>
</tr>
<tr>
<td>P -ve</td>
<td>1(10)</td>
<td>2(20)</td>
<td>1(10)</td>
<td>6(2)</td>
</tr>
<tr>
<td>P -ve</td>
<td>9(90)</td>
<td>8(80)</td>
<td>9(90)</td>
<td>26(86.7)</td>
</tr>
</tbody>
</table>

Aa = *A. actinomycetemcomitans*  
P = *P. intermedia*  
-ve = present, ve = absent  
D = diseased, H = healthy

Discussion

The results of this study demonstrate that prevalence of *A. actinomycetemcomitans* was low and *P. intermedia* high in both the diseased and healthy subjects. There was also no significant association between the presence of *A. actinomycetemcomitans* and *P. intermedia* with ethnicity.

The low prevalence of *A. actinomycetemcomitans* detected in this study is similar to that reported previously by a number of workers who examined subjects based on disease status (10,18-20), although higher detection levels for *A. actinomycetemcomitans* have also been reported (8,9,17,21-23). In most studies it was found that the prevalence of *A. actinomycetemcomitans* in subgingival plaque decreases with increasing age and is predominantly associated with younger age groups (24-27). In the present study, subjects were from the 35 – 65 years age group. This may explain the low prevalence for *A. actinomycetemcomitans* in both the diseased and healthy subjects as it did not include younger age groups.
Studies comparing periodontitis patients in Spain and Netherlands (28) observed that *A. actinomycescomitans* was significantly more prevalent in the Dutch patients (25% versus 35%). van Winkelhoff et al (29) observed that this may be related to the significant difference in medical use of antibiotics in the two countries. Netherlands is the most restrictive country in Europe while Spain is among the countries that use the highest number of antibiotics per inhabitant (30). Differences in medical use of
antibiotics among Malaysian subjects as compared to countries where higher detection levels for *A. actinomycetemcomitans* have been reported may also exist.

In this study population, *A. actinomycetemcomitans* was not identified in Malaysian Chinese diseased and healthy groups. This finding is in agreement with Cao et al (31), where *A. actinomycetemcomitans* was not detected in visiting Chinese male students in USA who had periodontitis. The findings are however in contrast to the high detection levels of *A. actinomycetemcomitans* in the normal flora of young Chinese subjects in China (9) and Singaporean Chinese subjects (17). The difference could probably be explained by the small number of Chinese subjects used in the present study (20 subjects) and in the study by Cao’s group (10 subjects) (31) as compared to the studies done in Chinese subjects in China (9) as well as in Singapore (17) where their sample sizes were 185 and 92 subjects respectively.

The high prevalence for *P. intermedia* in this study is also consistent with many other studies (18,19,23,32,33). The results of this study also conform with the finding by Teanpaisan et al (34) who reported that the incidence of *P. intermedia* was no greater in disease than in health. However, reports of a much lower prevalence for *P. intermedia* have also been published (21,25).

In this study, *A. actinomycetemcomitans* and *P. intermedia* were found together in only 5.56% of the total infected subjects (healthy and diseased). This is consistent with the results by Hamlet and colleagues (25) where co-infection by *A. actinomycetemcomitans* and *P. intermedia* were found in only 4% of the infected subjects. The existence of antagonistic interactions whereby one microorganism (*P. intermedia*) can inhibit the second microorganism (*A. actinomycetemcomitans*) may have given rise to there being a protective oral flora (35,36).

The effect of mixed microbial infections that may enhance pathogenicity of individual species also needs to be considered. Slots & Genco (37) have stated that pure cultures of *Bacteroides* like *P. intermedia* generally do not cause an infectious lesion but combinations of *P. intermedia* and *P. gingivalis* may have a synergistic effect and cause such a lesion. Socransky et al (3) have also stated that *P. intermedia*, which is in the orange complex of the model of bacterial complexes of the dental biofilm associated with periodontal disease, acts as a bridge between the early colonizers and the red complex species (*P. gingivalis, T. denticola* and *T. forsythus*). Therefore in this study, there is a possibility that the diseased patients may have had an increased presence of *P. gingivalis*, which may not have been present in the healthy subjects, and this synergistic combination may be the cause for disease.

This study demonstrated that *A. actinomycetemcomitans* had a significant association with CAL and not with other clinical parameters. *P. intermedia* however was not associated with any of the clinical parameters. Other than the association that *A. actinomycetemcomitans* had on CAL, there was insufficient evidence to show similar findings that were seen in earlier studies (3,38) where they found that elevated levels of species like *A. actinomycetemcomitans, P. intermedia, P. gingivalis, T. forsythus* and *T. denticola* were correlated with increasing PPD and CAL. In contrast, studies by Cullinan et al (35) and Timmerman et al (8) showed that *A. actinomycetemcomitans* and *P. intermedia* had no significant effects on PPD and CAL.

**CONCLUSION**

In this study none of the pathogens were associated with the race and periodontal disease status of the subjects and only *A. actinomycetemcomitans* had a significant association with CAL. The findings in this study have to be taken with caution due to the small sample population. We recommend that a future study be conducted which involves a larger randomly selected sample population. This will reflect the true prevalence of these bacteria in the Malaysian population at large.

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