Prevalence of β-lactamase-producing bacteria in human periodontitis


Background and Objective: Beta-lactam antibiotics prescribed in periodontal therapy are vulnerable to degradation by bacterial β-lactamases. This study evaluated the occurrence of β-lactamase-positive subgingival bacteria in chronic periodontitis subjects of USA origin, and assessed their in vitro resistance to metronidazole at a breakpoint concentration of 4 μg/mL.

Material and Methods: Subgingival plaque specimens from deep periodontal pockets with bleeding on probing were removed from 564 adults with severe chronic periodontitis before treatment. The samples were transported in VMGA III and then plated onto: (i) nonselective enriched Brucella blood agar (EBBA) and incubated anaerobically for 7 d; and (ii) selective trypticase soy-bacitracin-vancomycin (TSBV) and incubated for 3 d in air + 5% CO₂. At the end of the incubation periods, the bacterial test species were identified and quantified. Specimen dilutions were also plated onto EBBA plates supplemented with 2 μg/mL of amoxicillin, a combination of 2 μg/mL of amoxicillin plus 2 μg/mL of the β-lactamase inhibitor clavulanic acid, or 4 μg/mL of metronidazole, followed by anaerobic incubation for 7 d. Bacterial test species presumptively positive for β-lactamase production were identified by growth on EBBA primary isolation plates supplemented with amoxicillin alone and no growth on EBBA primary isolation plates containing both amoxicillin plus clavulanic acid. A subset of such isolates was subjected to nitrocefin-based chromogenic disk testing to confirm the presence of β-lactamase activity. In vitro resistance to 4 μg/mL of metronidazole was noted when growth of test species occurred on metronidazole-supplemented EBBA culture plates.

Results: Two-hundred and ninety-four (52.1%) of the study subjects yielded β-lactamase-producing subgingival bacterial test species, with Prevotella intermedia/nigrescens, Fusobacterium nucleatum and other Prevotella species most frequently identified as β-lactamase-producing organisms. Of the β-lactamase-producing bacterial test species strains recovered, 98.9% were susceptible in vitro to metronidazole at 4 μg/mL.

Conclusion: The occurrence of β-lactamase-positive subgingival bacterial species in more than half of the subjects with severe chronic periodontitis raises questions about the therapeutic potential of single-drug regimens with β-lactam antibiotics in periodontal therapy. The in vitro effectiveness of metronidazole against nearly all recovered β-lactamase-producing subgingival bacterial species further supports clinical periodontitis treatment strategies involving the combination of systemic amoxicillin plus metronidazole.
Beta-lactamase production represents a major virulence factor by which pathogenic bacteria evade the broad-spectrum antimicrobial effects of β-lactam antibiotics and perpetuate human infections, including those in orofacial tissues (1). Bacterial β-lactamases rapidly hydrolyze amide bonds within the four-membered ring forming the foundational structure of β-lactam antibiotics, leaving them pharmacologically inactive as antimicrobial agents that disrupt bacterial cell-wall peptidoglycan biosynthesis (1).

Beta-lactamase activity has been detected in subgingival sites of subjects with chronic periodontitis at levels capable of inactivating β-lactam antibiotics passing into periodontal pockets through gingival crevicular fluid exudate (2). Subgingival β-lactamase has been significantly correlated with increasing periodontal probing depth measurements (2), recent treatment with systemic penicillin drugs (3), and carriage of β-lactamase-encoding genes by microbial species in subgingival plaque biofilms (4). Studies of patients with chronic periodontitis in the USA (3–5), The Netherlands (6,7), Spain (7), Norway (8), France (9,10) and the UK (11) have reported a 53.2–100% occurrence in subjects for subgingival β-lactamase-producing bacteria, with higher prevalence rates found in localities with greater over-the-counter access and consumption of systemic antimicrobial agents (7). However, these findings are limited by their inclusion of relatively few study subjects (12–47 patients with periodontitis per study), who were mostly dental-school patients and/or from localized geographic regions that may not necessarily be representative of more diverse community populations. For example, data from the USA on the occurrence of subgingival β-lactamase-producing bacterial species in patients with chronic periodontitis is presently derived from a total of 42 dental-school patients in Connecticut and 25 in Florida (3,5).

As a result, there is a need to assess subgingival β-lactamase-producing bacteria in larger-sized subject groups that are geographically distributed beyond a single city or dental-school patient population. The aim of the present study was to evaluate the occurrence of β-lactamase-positive subgingival bacteria in 564 geographically distributed subjects in the USA with chronic periodontitis and to assess their in vitro resistance to metronidazole at a breakpoint concentration of 4 μg/mL.

Material and methods

Subjects

A total of 564 adults (270 men and 294 women; age range, 33–91 years; mean age ± standard deviation = 49.1 ± 11.7 years), diagnosed with severe chronic periodontitis (12) by periodontists in private dental practices in the USA, were included in the present study as their subgingival plaque samples were consecutively received for microbiological analysis by the Oral Microbiology Testing Service (OMTS) Laboratory at Temple University School of Dentistry, Philadelphia (PA, USA). Three-hundred and fifty-four (62.8%) of the study subjects originated from Maryland (n = 174), Pennsylvania (n = 91), New Jersey (n = 51), Delaware (n = 25), Virginia (n = 7) and the District of Columbia (n = 6) in the mid-Atlantic region of the USA, with all others from Connecticut (n = 48), Florida (n = 35), Illinois (n = 20), 11 other states in the eastern USA (n = 47) and Texas (n = 60). Persons identified with aggressive periodontitis, or with antibiotic use in the past 6 mo, were excluded. Approval for the study was provided by the Temple University Human Subjects Protections Institutional Review Board.

Microbial sampling and transport

Subgingival plaque specimens were obtained by the diagnosing periodontists, who followed a standardized sampling protocol, before treatment from three to five deep (≥ 6 mm) periodontal pockets in each subject that exhibited bleeding on probing during the initial diagnostic evaluation. After isolation with cotton rolls, and removal of saliva and supragingival deposits, one or two sterile, absorbent, paper points (Johnson & Johnson, East Windsor, NJ, USA) were advanced into each selected periodontal site for approximately 10 s. Upon removal, all paper points per study subject were pooled in a glass vial containing six to eight small glass beads and 2.0 mL of anaerobically prepared and stored VMGA III transport medium (13), which possesses a high preservation capability for oral microorganisms during postsampling transit to the laboratory (13,14). The subgingival samples were then transported within 24 h to the OMTS Laboratory, which is licensed for high-complexity bacteriological analysis by the Pennsylvania Department of Health. The OMTS Laboratory is also federally certified by the United States Department of Health and Human Services to be in compliance with Clinical Laboratory Improvement Amendments-mandated proficiency testing, quality control, patient test management, personnel requirements and quality assurance standards required of clinical laboratories engaged in diagnostic testing of human specimens in the USA (15). All laboratory procedures were performed by personnel who were blinded to the clinical status of the study subjects and their inclusion in the present analysis.

Microbial culture and incubation

At the OMTS Laboratory, the specimens vials were warmed to 35°C to liquefy the VMGA III transport medium, and sampled microorganisms were mechanically dispersed from the paper points with a Vortex mixer, which was used at the maximal setting for 45 s.
Serial, 10-fold dilutions of the dispersed bacteria were prepared in Möller’s VMG I anaerobic dispersion solution, comprised of preruced, anaerobically sterilized, 0.25% tryptose, 0.25% thionate E peptone and 0.5% NaCl (13). Then, 0.1-mL dilution aliquots were spread, with a sterile bent glass rod, onto nonselcetive enriched Brucella blood agar (EBBA) primary isolation plates (16), composed of 4.3% Brucella agar supplemented with 0.3% bacto-agar, 5% defibrinated sheep blood, 0.2% hemol- yzed sheep red blood cells, 0.0005% hemin and 0.00005% menadione, and onto selective trypticase soy–bacitracin–vancomycin (TSBV) agar (17). The inoculated EBBA plates were incubated at 35°C for 7 d in a Coy anaero- bic chamber (Coy Laboratory Products, Ann Arbor, MI, USA) containing 85% N2, 10% H2 and 5% CO2, and the TSBV plates were incubated at 35°C for 3 d in air + 5% CO2.

Test species identification

Total anaerobic viable counts, and counts of the test species Porphyromonas gingivalis, Prevotella interme- dia/nigrescens, other Prevotella species (including Prevotella melaninogenica and nonpigmented Prevotella species), Fusobacterium nucleatum, Parvimonas micra, Capnocytophaga species, Streptococcus constellatus, Centipeda peri- odontii, Enterococcus faecalis and staphylococci, were made on nonse- lective EBBA primary isolation plates using a ring-light magnifying loupe, presumptive phenotypic methods previ- ously described (18–21) and the RapID ANA II (Innovative Diagnostic Systems, Atlanta, GA, USA) micro- method kit system for selected isolates. Aggregatibacter actinomycetemcomi- tans, gram-negative enteric rods/pseu- domonas and Candida species were quantitated on selective TSBV agar, as previously described (16,17). The propor- tional recovery of each test species was ascertained in each subject by cal- culating the percentage of test species colony-forming units relative to total subgingival anaerobic viable counts, as determined on nonselctive EBBA pri- mary isolation plates.

Detection of β-lactamase-producing organisms

Additional 0.1 mL aliquots of sub- gingival sample dilutions were inocula- ted onto EBBA primary isolation plates supplemented with either 2 μg/mL of amoxicillin, which was previ- ously established as a susceptibility breakpoint for amoxicillin-resistant β-lactamase-producing strains (22), or a combination of 2 μg/mL of amoxicillin plus 2 μg/mL of the β-lactamase-inhibitor, clavulanic acid (23), followed by anaerobic incuba- tion. Direct colony suspensions (equivalent to a 0.5 McFarland standard) of pure A. actinomycetemcomi- tans isolates from selective TSBV were subcultured onto these media as their identification is frequently obscured within mixed bacterial popu- lations on nonselcetive EBBA primary isolation plates (17).

A surface-overlay technique was used to add clavulanic acid, at a concentra- tion of 2 μg/mL, to amoxicillin-containing EBBA primary isolation plates (24). Using a sterile glass rod, 0.1 mL of a fresh 400-μg/mL solution of clavulanic acid, prepared by dis- solving 4.2 mg of a 95.3% pure lithium clavulanate powder (provided by SmithKline Beecham, Collegeville, PA, USA) into 10 mL of sterile 0.1 M phosphate buffer (pH 6.0), was evenly spread over the surfaces of EBBA plates supplemented with 20 mL of amoxicillin. The plates were held at room temperature for 30 min to allow surface drying and subsurface drug diffusion, and then inoculated with subgingival plaque specimens.

Test species positive for β-lactamase production were presumptively identified by growth on EBBA primary isolation plates supplemented with amoxicillin alone and by no growth on EBBA primary isolation plates containing both amoxicillin and clavulanic acid (6,7). A subset of 50 such presumptively identified isolates was subjected to a nitrocefin-based qualitative chromogenic disk assay (BBL Cefinase; BD Diagnostic Sys- tems, Sparks, MD, USA), following the manufacturer’s instructions, to confirm the presence of β-lactamase activity.

In vitro antibiotic-resistance testing

Aliquots (0.1 mL) of subgingival sam- ple dilutions were also inoculated onto EBBA primary isolation plates supplemented with metronidazole at a susceptibility breakpoint concentra- tion of 4 μg/mL (22), and incubated anaerobically for 7 d, in order to assess in vitro resistance to metronida- zole among β-lactamase-producing clinical isolates. In vitro resistance to metronidazole was defined as test species growth on metronidazole- supplemented EBBA primary isolation plates (16,20,25,26). Bacteroides thetaiotaomicron ATCC 29741, Clostridium perfringens ATCC 13124 and a multiantibiotic-resistant clinical periodontal isolate of F. nucleatum were employed as positive and negative quality controls for the antibiotic resistance testing. All antimicrobials were obtained as pure powder from Sigma-Aldrich (St Louis, MO, USA).

Data analysis

Descriptive analyses were used to cal- culate mean subject age and standard deviation values, the occurrence and proportional cultivable recovery of test species in subjects, the occurrence and proportional recovery of β-lactamase-producing organisms in sub- jects, and the occurrence in subjects of in vitro metronidazole drug resis- tance among the subgingival clinical isolates. Data analysis was performed using the SAS 9.2 for Windows (SAS Institute, Inc., Cary, NC, USA) statisti- cal software package.

Results

A subset of 50 test species, presum- ptively identified as β-lactamase positive by their growth on amoxicillin-supple- mented EBBA primary isolation plates and by no growth on amoxicillin plus clavulanic acid-supplemented EBBA primary isolation plates, were all con- firmed as β-lactamase-producing organ- isms using the nitrocefin chromogenic disk assay (data not shown).

A total of 294 (52.1%) study sub- jects yielded β-lactamase-producing subgingival test species. P. intermedia/
Aggregatibacter, Porphyromonas gingivalis

197 (34.9) 0 0 0

Enteric rods/pseudomonads 40 (7.1) 2 (0.4/5.0) 1.7

/C6 495 (87.8) 4 (0.7/0.8) 9.9

Parvimonas micra

three (98.9%) of the 357

Species

periodontitis

Staphylococcus

species 4 (0.7) 0 0 0

Enterococcus faecalis

5 (0.9) 0 0 0

Centipeda periodontii

4 (0.7) 0 0 0

Streptococcus constellatus

267 (47.3) 0 0 0

Prevotella intermedia/nigrescens

449 (79.6) 229 (40.6/51.0) 9.6 ± 0.8

Prevotella melaninogenica and other

Prevotella species were most fre-

cently identified as β-lactamase- 

positive species, with 51.0% of

all study subjects with cultivable

P. intermedia/nigrescens, 24.6% with

F. nucleatum and 66.2% with other

Prevotella species exhibiting β-lactam-

ase-producing strains of these species

(Table 1). Beta-lactamase production

was also found among 0.8% of P. mi-

cra species, 2.0% of Capnocytophaga

species and 5.0% of gram-negative

enteric rods/pseudomonads in culture-

positive subjects (Table 1).

A single β-lactamase-producing

subgingival test species was recovered

from 236 (80.3%) subjects, whereas

54 (18.4%) and four (1.3%) subjects

each yielded two and three different

β-lactamase-producing test species,

respectively (Fig. 1).

Subgingival proportions of β-lac-

tamase-positive P. intermedia/nigres-

cens, F. nucleatum and P. micra

averaged 6.7–9.9%, whereas mean sub-
gingival recovery levels of <1%

were found for β-lactamase-positive

isolates of other Prevotella species

and for Capnocytophaga species

(Table 1). Three-hundred and fifty-

three (98.9%) of the 357 β-lactamase-

producing subject test species recov-

ered (Table 1) were susceptible in vi-

tro to 4 μg/mL of metronidazole,

except for one P. intermedia/nigres-

cens strain, one F. nucleatum strain

and gram-negative enteric rods/pseu-

donads in two subjects.

All recovered strains of P. gingo-

valis, A. actinomycetemcomitans, S. con-

tellatus, C. periodontii, E. faecalis,

and Staphylococcus species, failed to
demonstrate presumptive β-lactamase

activity (Table 1).

Discussion

These findings confirm and extend pre-

vious studies revealing subgingival

β-lactamase-producing bacteria to be

present in a majority of patients

with chronic periodontitis (3–11). A

strength of the present study is that

it assessed the largest group, to date,
of subjects with chronic periodontitis

in vi tro –lactamase-producing bacteria

(n = 564); similar, previous, investi-
gations have collectively exami-

ned a total of only 255 subjects with

chronic periodontitis (3,5–11). The

present study data also evaluated a pri-

vate dental practice-based conveni-

cence sample of subjects with chronic peri-

odontitis originating from a wider geo-

graphic region than in previous reports

from the USA on subgingival β-lactam-

ase activity (2,3,5), which may per-

mit the findings to be more generalizable
to a broader range of USA community

population groups.

Owing to the instability of clavulan-
ic acid in agar-dilution plates stored

for more than 3 d (24), a surface-

overlay technique was used in this study
to add clavulanic acid to amoxicillin-

containing EBBA primary isolation

plates immediately before in vitro test-
ing. As clavulanic acid binds irrever-
sibly to β-lactamases and prevents

inactivation of β-lactam antibiotics

(23), microbial growth patterns on

these plates were compared with those

on EBBA plates supplemented with

amoxicillin only as a presumptive

method for detecting β-lactamase-

positive subgingival bacterial species.

This approach was validated with con-
firmatory nitrocefin chromogenic disk
testing for β-lactamase activity from

presumptively detected subgingival

bacterial species in previous studies

(6,7), as well as on a subset of such sub-

ject isolates in the present study.

In this study, 52.1% of subjects

with untreated chronic periodontitis

were beta-lactamase-positive.

Table 1. Occurrence and proportional subgingival recovery of β-lactamase-positive test species in 564 adults with severe chronic periodontitis

<table>
<thead>
<tr>
<th>Species</th>
<th>Culture-positive subjects n (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Subjects with β-lactamase-positive isolates&lt;sup&gt;b&lt;/sup&gt; n (%)&lt;sup&gt;c&lt;/sup&gt;&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Recovery of β-lactamase-positive isolates (mean percentage ± standard error)</th>
<th>Range %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevotella intermedia/nigrescens</td>
<td>449 (79.6)</td>
<td>229 (40.6/51.0)</td>
<td>9.6 ± 0.8</td>
<td>0.001–62.1</td>
</tr>
<tr>
<td>Fusobacterium nucleatum</td>
<td>293 (52.0)</td>
<td>72 (12.8/24.6)</td>
<td>6.7 ± 0.9</td>
<td>0.001–29.4</td>
</tr>
<tr>
<td>Other Prevotella species&lt;sup&gt;e&lt;/sup&gt;</td>
<td>68 (12.1)</td>
<td>45 (8.0/66.2)</td>
<td>0.6 ± 0.5</td>
<td>0.001–24.2</td>
</tr>
<tr>
<td>Capnocytophaga species</td>
<td>251 (44.5)</td>
<td>5 (0.9/2.0)</td>
<td>0.3 ± 0.1</td>
<td>0.002–0.6</td>
</tr>
<tr>
<td>Parvimonas micra</td>
<td>495 (87.8)</td>
<td>4 (0.7/0.8)</td>
<td>9.9 ± 3.9</td>
<td>1.5–19.7</td>
</tr>
<tr>
<td>Enteric rods/pseudomonads</td>
<td>40 (7.1)</td>
<td>2 (0.4/5.0)</td>
<td>1.7 ± 0.8</td>
<td>0.9–2.5</td>
</tr>
<tr>
<td>Porphyromonas gingivalis</td>
<td>197 (34.9)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Aggregatibacter</td>
<td>85 (15.1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>actinomycetemcomitans</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Streptococcus constellatus</td>
<td>267 (47.3)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Centipeda periodontii</td>
<td>4 (0.7)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>5 (0.9)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Staphylococcus species</td>
<td>4 (0.7)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Includes Prevotella melaninogenica and nonpigmented Prevotella species.

<sup>b</sup>Among all study subjects.

<sup>c</sup>Species growth on amoxicillin-supplemented enriched Brucella blood agar (EBBA) primary isolation plates, with no growth on similar plates with a clavulanic acid overlay.

<sup>d</sup>Among species-positive study subjects.
yielded β-lactamase-producing subgingival bacteria, which is at the lower end of the prevalence rates of 53.2–100% reported in previous studies (3,5–11). This may be because of differences in periodontal disease severity, treatment status and recent systemic antibiotic usage among the evaluated subjects with chronic periodontitis and the microbiological methods employed. For example, some previous studies evaluated only subjects with ‘refractory’ chronic periodontitis, responding poorly to conventional mechanical periodontal therapy (5,8).

As systemic antimicrobial therapy is frequently employed in such patients (27), which is positively correlated with increased subgingival β-lactamase activity (3), it is not surprising that a greater occurrence of β-lactamase-producing subgingival bacteria would be reported for patients with ‘refractory’ periodontitis compared with patients with untreated chronic periodontitis and without antibiotic use within the past 6 mo, as in the present study. In this regard, the 52.1% occurrence of subgingival β-lactamase-producing bacteria in the present study is similar to the 48% rate documented among 21 patients with chronic periodontitis not exposed to antibiotic therapy over the previous 12 mo (3). Moreover, a higher occurrence of subgingival β-lactamase would also be expected among subjects with chronic periodontitis from countries, such as in southern Europe, with greater antibiotic over-the-counter access and consumption rates than found in the USA (28).

P. intermedia/nigrescens, F. nucleatum and other Prevotella species were the most frequently identified β-lactamase-positive species in the present study, consistent with previous investigations (3,5–10). Subgingival recovery of cultivable enzyme-producing strains of P. intermedia/nigrescens and F. nucleatum averaged 9.6% and 6.7%, respectively, of total anaerobic viable counts in β-lactamase-positive subjects, whereas other Prevotella species occurred in lower proportions, averaging <1%. P. micra, Capnocytophaga species and gram-negative enteric rods/pseudomonads were also found to be β-lactamase positive in some study subjects. Interestingly, all 197 strains of P. gingivalis found in subjects in the present study were β-lactamase negative, in agreement with most previous studies of subgingival isolates (6,9,29). In contrast, 7.7% of 26 subgingival P. gingivalis strains were reported to be β-lactamase-positive in one study (30), and 25.5% of 51 strains in another study (31) were found in vitro to be resistant to amoxicillin, but susceptible to amoxicillin/clavulanic acid, indicative of β-lactamase production. Additional β-lactamase-positive microbial species and subjects would probably be identified using more sensitive methods, such as molecular analysis of bacterial 16S ribosomal RNA gene sequences (5,8) or β-lactamase-encoding genes (4,10,32,33), than the culture-based procedures employed in the present study.

This study also did not classify β-lactamase phenotypes, test for β-lactamase groups resistant to clavulanic acid or quantitate the strength of subgingival β-lactamase activity present in β-lactamase-positive subjects (1,2). Additionally, the private practice periodontists who diagnosed the study subjects were not calibrated in their assessments, although support for their diagnosis of severe chronic periodontitis was evidenced by their identification for microbiological sampling of three or more periodontal sites per subject with probing depths of ≥6 mm, which strongly correlates (94.1% positive predictive value) with the presence of severe periodontal attachment loss in adults (34).

The presence of β-lactamase activity in periodontal pockets poses potentially important therapeutic implications relative to the control of mixed populations of subgingival periodontal bacterial pathogens in dental plaque biofilms. Enzymatic degradation of β-lactam antibiotics may permit subgingival perpetuation of β-lactamase-producing microbial species and other organisms in the subgingival microbiota that otherwise would be suppressed by β-lactam antibiotics (1). An increasing prevalence of β-lactamase activity among oral bacterial isolates has been reported (35), which is largely thought to be the result of plasmid and transposon-mediated conjugal transfer and dissemination of β-lactamase-encoding genes among microorganisms surviving following selective pressure from β-lactam antibiotics (1). Beta-lactamases may predispose systemic penicillin monotherapy, unprotected by β-lactamase inhibitors, to oral cavity treatment failures, such as recalcitrant orofacial infections (36,37) and periodontal surgical flap necrosis (38), as well as enhance periodontal abscess risk in untreated periodontitis subjects (39,40).

Penicillin and amoxicillin are among the systemic antibiotics most frequently prescribed by periodontists (41). However, relatively little research attention has been given to their adjunctive use in periodontal therapy in the absence of β-lactamase inhibitors (42,43), with systemic phenoxymethyl penicillin failing to provide
significant treatment benefits in a clinical trial of patients with aggressive periodontitis (44). It is noteworthy that subgingival bacterial isolates resistant to 2 μg/mL of amoxicillin were found to show a transient increase from a pretreatment baseline of 0.5% to 35% (a 70-fold increase), over the course of an adjunctive 14-d system. In that study, *P. intermedia/nigrescens* and *P. melaninogenica*, often identified as β-lactamase-producing species in the present and previous studies (3,–10), were found to be among the most prevalent amoxicillin-resistant organisms (45). The extent to which bacterial β-lactamase activity contributed to these in vivo microbiological shifts was not addressed in the study and remains to be delineated in future investigations.

Finally, the present study found 98.9% of recovered subgingival β-lactamase-producing bacteria to be susceptible in *vitro* to 4 μg/mL of metronidazole, an observation consistent with previous reports (3,–5,8). As a result, metronidazole suppression of β-lactamase-positive microbial species may, in part, help to protect concurrently administered amoxicillin from *in vivo* degradation by bacterial β-lactamases, better enabling pharmacologically active amoxicillin to reach penicillin-binding proteins on bacterial cell membranes to exert antimicrobial effects (1), and contributing to the documented clinical and microbiological benefits of systemic amoxicillin plus metronidazole in periodontal therapy (46–49). However, this may not occur in patients from certain geographic regions, such as Columbia, where marked *in vitro* metronidazole resistance may be found among β-lactamase-positive subgingival isolates of *P. gingivalis, P. intermedia/nigrescens, P. melaninogenica* and *F. nucleatum* (31). In conclusion, the occurrence of β-lactamase-positive subgingival bacterial species in more than one-half of subjects with severe chronic periodontitis raises questions about the therapeutic potential of single-drug regimens with β-lactam anti-biotics in periodontal therapy. The *in vitro* effectiveness of metronidazole against nearly all recovered β-lactamase-producing subgingival bacterial species further supports clinical periodontitis treatment strategies involving the combination of systemic amoxicillin plus metronidazole.

Acknowledgements

The authors thank Diane Feik for her laboratory expertise and assistance, and Drs Linda A. Miller and James A. Poupard of SmithKline Beecham for their guidance with the clavulanic acid surface-overlay technique used in this study. Support for this research was provided, in part, by funds from the Paul H. Keyes Professorship in Periodontology held by Thomas E. Rams at Temple University School of Dentistry. No conflicts of interest, including financial, were reported by any of the authors relative to this study.

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